

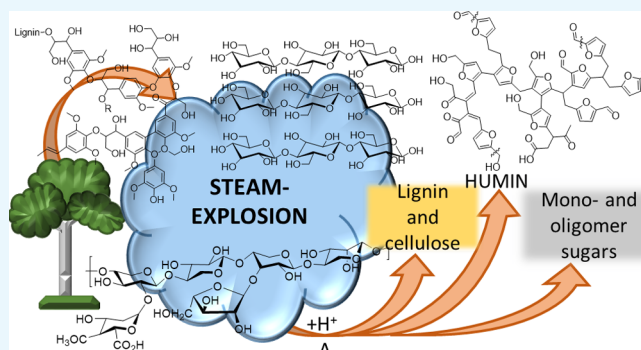
Characterization of Pseudo-Lignin from Steam Exploded Birch

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S Supporting Information

ABSTRACT: There is a growing interest in a more wholesome utilization of biomass as the need for greener chemistry and non-mineral oil-based products increases. Lignin is the largest renewable resource for aromatic chemicals, which is found in all types of lignocellulosic biomass. Steam-explosion of lignocellulosic biomass is a useful pretreatment technique to make the polymeric material more available for processing. However, this heat-based pretreatment is known to result in the formation of pseudo-lignin, a lignin-like polymer made from carbohydrate degradation products. In this work, we have analyzed steam-exploded birch with a varying severity factor (3.1–5.0) by pyrolysis–gas chromatography–mass spectrometry, 2D-NMR, and Fourier transform infrared spectroscopy. The main results reveal a consumption of acetic acid at higher temperatures, with the increase of furan components in the pyrolyzate. The IR and NMR spectral data support these results, and there is a reason to believe that the conditions for humin formation are accomplished under steam explosion. Pseudo-lignin seems to be a humin-like compound.



INTRODUCTION

Total utilization of lignocellulosic biomass and conversion into a range of valuable products is a central goal in biorefining and green chemistry. This renewable material has the potential to replace depleting fossil resources for the production of energy, chemicals, and fuels.^{1–3} Lignin is the most abundant renewable aromatic polymer on the planet and is found in all types of lignocellulosic biomass. However, in the pulp and paper industry and the emerging cellulosic ethanol industry, lignin is considered to be a waste product and is currently mostly burned to generate process heat.^{4–8} For biochemical production of ethanol, a pretreatment of the lignocellulosic biomass is needed to make the carbohydrates accessible for enzymes. Steam explosion (SE) is a treatment with a high industrial potential, as it has a low cost for energy and does not need addition of other chemicals than steam, thereby producing no extra waste.^{9–11} SE is both a chemical and physical treatment. It hydrolyses the hemicellulose into soluble monomers and oligomers and makes the cellulose fibers much more accessible for further degradation with enzymes.^{12–14} The lignin and cellulose part is not expected to be significantly altered during SE, but as several other authors have noticed, the β -O-4 bonds in lignin are degraded with increasing pretreatment severity.¹⁵ For SE, the severity factor can be calculated as $\log R_0$ ¹⁶ that includes both temperature (T °C) and residence time (rt) as variables

$$\log R_0 = \log(\text{rt} \times e^{T-100/14.75})$$

Interestingly, several studies show an increase of Klason lignin (KL) content after SE.^{17,18} This increase has been attributed to the formation of pseudo-lignin during pretreat-

ment.¹⁹ Recently, several studies have attempted to identify the structure of pseudo-lignin, as it has been shown to hamper the enzymatic hydrolysis of cellulose.^{17–23} The general pseudo-lignin definition is broad and diffuse and the most accepted one states that it is “an aromatic material that yields a positive Klason lignin value that is not derived from native lignin.”¹⁹ The KL analysis is however only a crude method, as it just measures the increase of nonhydrolysable residues after acid hydrolysis, and is therefore not a proper identification test.¹⁸

SE treatment of hardwoods releases acetic acid from the hemicellulose, which leads to pH drop and drives the autohydrolysis.^{24,25} The acetic acid is generated from the acetylated carbohydrates, mainly xylans, and it can accumulate to a pH of 3.²⁶ Both time and temperature in SE have an effect on the release of acetic acid. Li et al. showed that this relationship is almost linear under hydrolysis with increasing time and temperature.²⁷

With the combined effects of high temperature and pressure in addition to the low pH under SE, several side reactions can take place. The cleavage of the β -O-4 bonding pattern in lignin has been reported by Li et al.²⁸ at pH 3, but the cleavage also happens at a higher pH as reported by Yelle et al.²⁹ A study of Glasser and Wright³⁰ shows that the MW of lignin decreases during SE treatment at severities above $\log R_0$ 4.2.

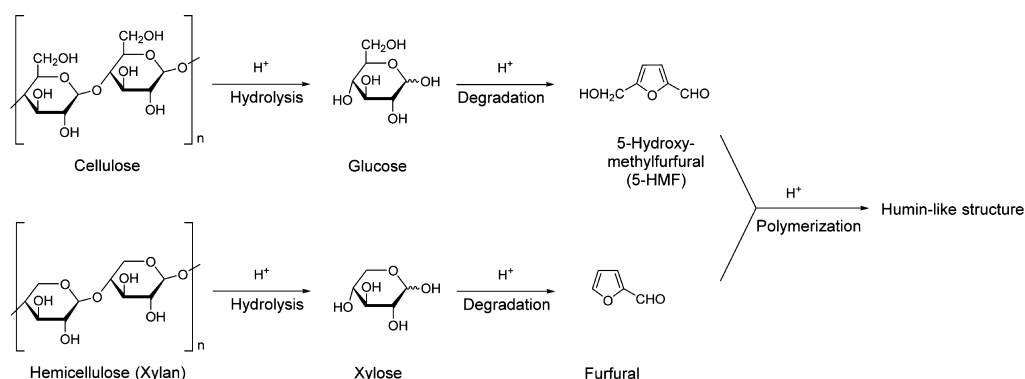
The formation of 5-hydroxymethylfurfural (5-HMF) from carbohydrates are reported by several groups, as a degradation

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Scheme 1. Overview of Reaction Pathway of Cellulose and Hemicellulose into Furan Components and Humin-Like Structures (Modified from Li et al.)²⁵



product of C6 sugars such as glucose in cellulose, [Scheme 1](#).^{22,25}

Additionally, there is formation of furfural from C5 sugars such as xylose from xylan. Generally, C6 sugars are more stable than C5 sugars during high-temperature pretreatments. These furan-like components can under certain conditions form a polymer structure called humin, [Figure 1](#). Humins are an

et al. reduced 5-HMF with Pd/C and formaldehyde and made a crystalline structure of 22.4 nm size.³⁷

The byproduct called pseudo-lignin apparent in the increase of KL content is formed under SE-treatment with identical conditions to what is needed for the formation of humins. The aim of this study was to investigate this unknown byproduct with pyrolysis–gas chromatography–mass spectrometry (GC–MS), 2D-NMR, and Fourier transform infrared spectroscopy (FT-IR) for characterization and identifications of similarities between pseudo-lignin and the humins.

RESULTS AND DISCUSSION

[Table 1](#) shows that the KL content (% KL) increased with the severity of the SE, from 22% in untreated birch to 40–42% at

Table 1. SE Conditions and KL Content from Birch Wood^a

severity factor	pretreatment condition	KL content (%)
	untreated	22
3.1	170 °C—10 min	23
3.4	180 °C—10 min	24
3.6	190 °C—10 min	25
3.9	200 °C—10 min	28
3.9	210 °C—5 min	30
4.2	210 °C—10 min	32
4.2	220 °C—5 min	32
4.4	210 °C—15 min	32
4.5	220 °C—10 min	37
4.5	230 °C—5 min	37
4.7	220 °C—15 min	42
4.8	230 °C—10 min	41
5.0	230 °C—15 min	40

^aKL analysis were carried out in triplicates.

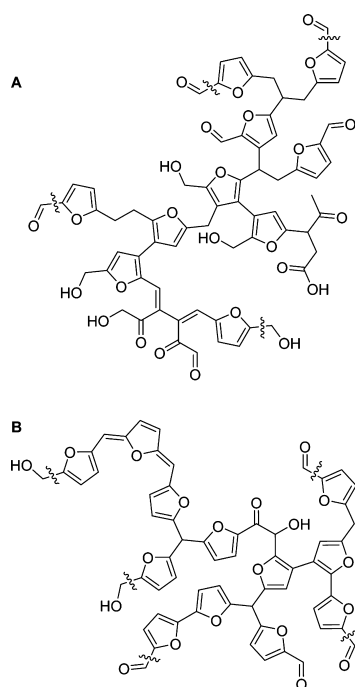


Figure 1. Humin structures derived from (A) glucose and (B) xylose (van Zandvoort et al.).³³

organic compound class which are insoluble in water at all pH's. The term is used in two related contexts, soil and carbohydrate chemistry. Humins from carbohydrates are produced under dehydration of sugars, the subsequent formaldehydes, and 5-HMF form further polymers.^{31,32} van Zandvoort et al. found that the furan and phenol compounds formed under acid-catalyzed dehydration of sugars are a part of the humin structure formation.³³ Patil et al. showed the direct formation of humins from glucose, fructose, and 5-HMF as a condensation product.^{34,35} In addition, Tuercke et al. did a microreactor synthesis of 5-HMF from dehydrating fructose, where it polymerized into a humin structure.³⁶ A different group, Date

the most severe pretreatment conditions. The estimated increase of KL as an effect of xylan loss has been calculated to be 25%; this work has been described by Vivekanand et al.¹⁸ In an effort to characterize this pseudo-lignin the samples were analyzed by fractionated pyrolysis and 2D-NMR.

2D-NMR Heteronuclear Single Quantum Coherence (HSQC). NMR experiments of the solvable fraction of untreated and severely pretreated ($\log R_0$ 4.7) birch before pyrolysis show the change in both aliphatic ([Figure 2](#)) and aromatic ([Figure 3](#)) regions. In the aliphatic area ([Figure 2](#)), from 2.6/45.0 to 5.3/115.0 ppm, the most noticeable difference is around the proton peaks of the β -O-4 bonding pattern. The

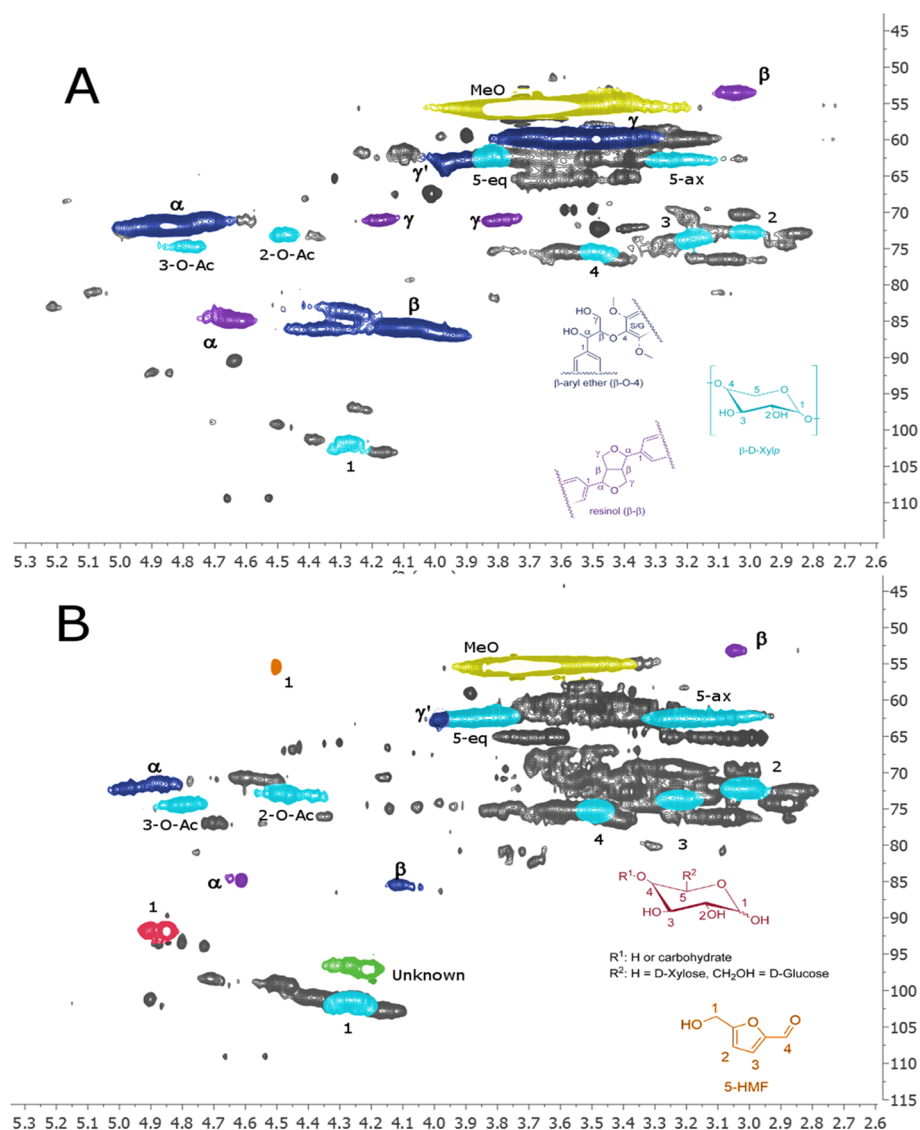


Figure 2. HSQC of lignocellulosic biomass at (A) untreated and (B) severity factor 4.7 (220 °C—15 min), focused on aliphatic region.

α -H shifts to the left and the signal is lower for the SE sample than untreated birch. The β -H signal has a similar pattern. In addition, the γ -H and γ -H' (α) disappears nearly completely after SE. Overall, this indicates that the β -O-4 bonding fragments with the pretreatment. Interestingly, this happens even at the lowest severity conditions in these pretreatment experiments, at 170 °C 10 min (Figure S1), even before the KL amount increases. This means that the changes in the NMR spectrum are not directly related to the accumulation of KL. Increasing the SE severity factor only results in limited alterations in the NMR spectra, while the KL content increases. Therefore, the byproduct formed under SE is not dissolvable in any of the three deuterated solvents used in this study.

The main change in the NMR spectra is the emergence of xylan peaks. These are visible in the spectrum (Figure 2) at 4.27/101, 3.88/3.17/63.0, 3.50/75.0, 3.25/74.0, and 3.0/72.5 ppm. The two peaks at 4.80/74.10 and 4.50/72.80 ppm are β -D-Xylp acetylated in position C-3 and C-2, respectively.

The peaks at 4.85/93.0 and 4.90/93.0 ppm are actually the anomeric carbon in D-xylose and D-glucose, respectively. These peaks are detectable in even the lowest SE-treatment, but the signal intensity is stronger at high severity factor such as 4.7 as

ppm. This means that carbohydrates are released, most likely from fragmentation of lignin–carbohydrate complexes, cellulose, and hemicellulose under SE as a result of depolymerization of lignocellulosic biomass.

In the aromatic region (Figure 3), there is also an immediate change in the NMR spectra at lowest SE. The G_2 and G_6 shifts disappear and only the G_5 and $S_{2,6}$ are left (Figure 3). With the increasing SE-treatment, there is a new area that becomes stronger in the aromatic region right below the $S_{2,6}$ region. An additional change in the aromatic region with the increasing SE-treatment is a shift in the $S_{2,6}$ peak. In the untreated version (Figure S2), there is an “iceberg” shape and with increasing treatment the top peak shifts toward the lower proton area. This small shift in $S_{2,6}$ corresponds to the difference in the enantiomer versions erythro and threo as shown by Schmid and Bardet et al.^{38,39} This is in compliance with hydrolysis in α -position in β -O-4, as the signals shift from racemic to thermodynamic stable enantiomer signals.

In the SE-treated spectra in both regions (Figures 2 and 3), there are peaks that correlate with the 5-HMF, at shift values, 4.5/56.5, 6.6/110.1, and 7.5/125.0. The peaks have a low

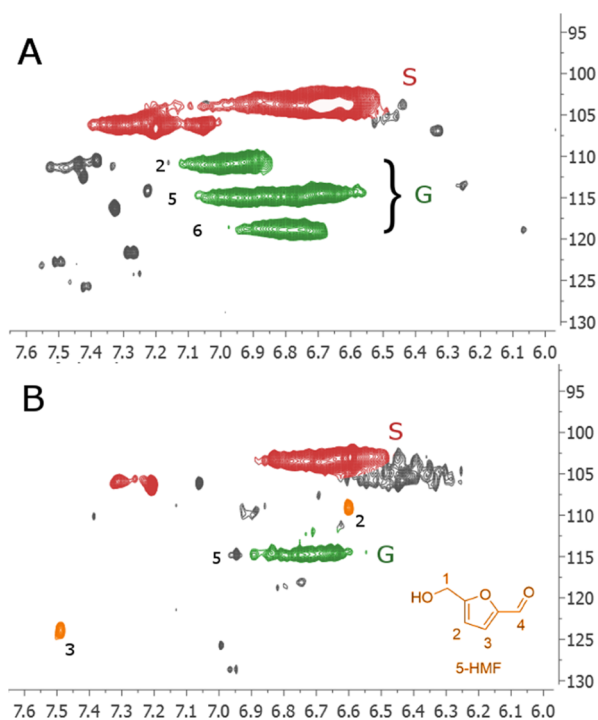


Figure 3. HSQC of lignocellulosic biomass at (A) untreated and (B) severity factor 4.7 (220 °C—15 min), focused on aromatic region.

intensity, meaning that there is a low concentration of 5-HMF dissolved in the sample.

Pyrolysis–GC–MS. The fractionated temperatures in the pyrolysis was determined based on previous results, Aarum et al. and Jurak,^{40,41} as the carbohydrates will be mostly valorized at 350 °C and then subsequently pyrolysis at 600 °C would yield the lignin fraction. This turned out to be a mostly correct assumption, as the 350 °C pyrogram contained small amounts of lignin components. The amount of lignin at 350 °C does increase with the SE-treatment, which is a result of the hydrolysis of the β -O-4 bond in lignin at higher severities.

All detected components after pyrolysis–GC–MS are described in Table S1. At 350 °C, the pyrolyzate is mainly composed of the most volatile products such as acetic acid (1), small furan-like rings (2, 3, 9, and 12), and some lignin structures (10, 15, 23, 24, and 25), see Figure 4.

The pyrolyzate at 350 °C has an increase of lignin products such as 15 (4-hydroxy-3-methoxybenzaldehyde, vanillin), 23 (4-[(*E*)-3-hydroxyprop-1-enyl]-2,6-dimethoxyphenol), and 24 (3-(4-hydroxy-3,5-dimethoxyphenyl)butan-2-one), with the increasing severity of the SE-treatment, Figure 4. The increase of volatile lignin components means that the polymer is more fragmented, which corresponds to the hydrolysis and cleavage of the β -O-4 bond as described in other works.^{28,30,42} In the pyrolyzate at 350 °C, there is a significant increase of 5-HMF 12, first detected (1.5%) with a severity factor of 3.9 at a temperature of 210 °C and a rt of 5 min. In untreated wood samples, this compound was not detected at all. The amount of 5-HMF thereafter increased to approximately 25% at a severity factor 4.5, 4.8, and 5.0 (230 °C, all rts). HMF is a typical dehydration product of C6 sugars.

The four main components that decrease at 350 °C are 1 (acetic acid), 4 (unknown m/z 114), 5 (unknown m/z 114), and 10 (unknown m/z 152). Components 4 and 5 have the same molecular mass, but display distinctly different

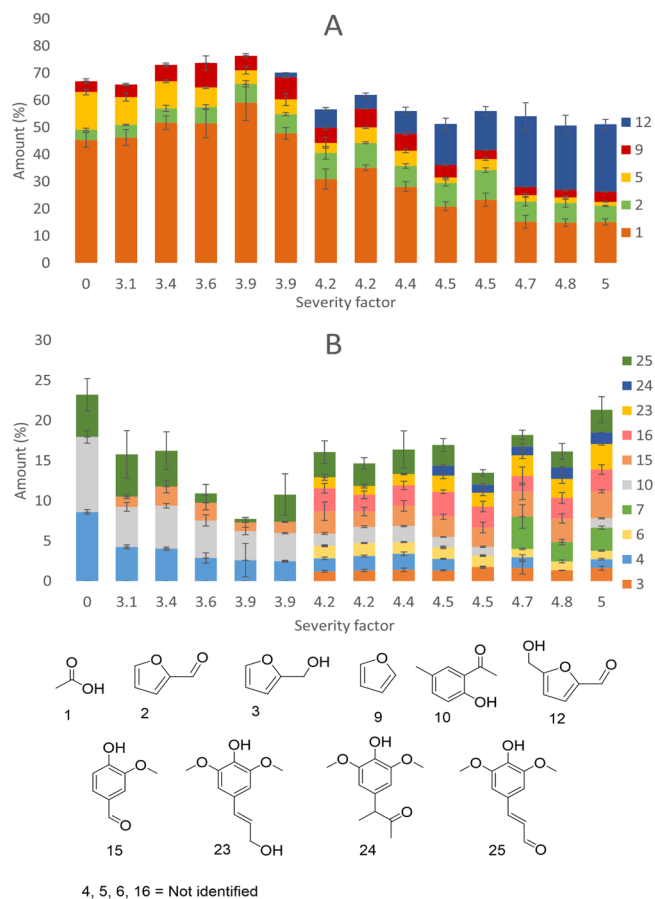


Figure 4. Amount and structure of several components in the pyrolyzate at 350 °C, as a function of severity factors of SE. The amounts are the average of normalization with standard deviation of three replicates. (A) Components of major amounts and (B) components of minor amounts.

fragmentation patterns in their mass spectra and are therefore evidently not the same compound.

The amount of acetic acid increases (45–59%) until a severity factor of 3.9 (210 °C, 5 min), from which point the amount decreases (59–15%). The increase and subsequent loss of 1 has been previously reported by Sunqvist et al., where they observed consumption of acetic acid by water extraction using a temperature around 200 °C.²⁶ Formation of 12 in the pyrolyzate coincides with the consumption of 1 at a severity factor of 3.9. This can explain that production of 12 is a building block in the formation of humins that also need acid, as shown in Figure 5 and Scheme 1.

Using the pyrolysis temperature (at 600 °C, shown in Figure 6), there is mainly a decrease of 1. The other lignin components that increase in amounts are 13 (2,6-dimethoxyphenol), 36 (2-methoxyphenol), 37 (2-methylphenol), 40 (2-methoxy-4-methylphenol), and 49 (2,6-dimethoxy-4-methylphenol). The three lignin components that are decreasing in amounts are 21 (3,5-dimethoxy-4-hydroxybenzaldehyde), 22 (unknown m/z 196), and 25 (3,5-dimethoxy-4-hydroxycinnamaldehyde).

In general, we have observed a trend of decreasing amounts of aldehydes and lignin's with shorter side chains in the C-4 position with the increasing severity factor. The β -O-4 bonding pattern can undergo hydrolysis at both hydroxyl groups on the side chain C-4. However, the α -hydroxyl gives the most stable

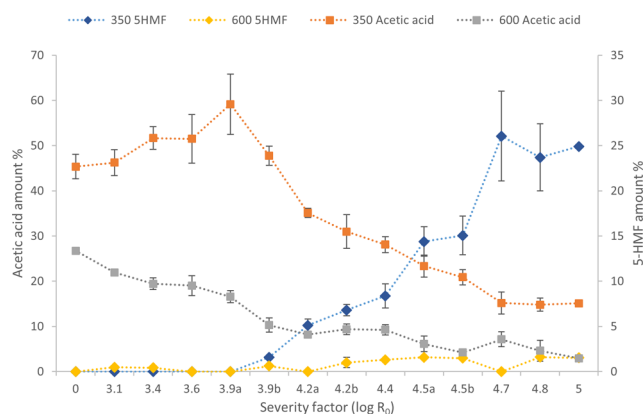


Figure 5. 5-HMF and acetic acid pyrolyzate content, (3.9a) 200 °C—10 min, (3.9b) 210 °C—5 min, (4.2a) 220 °C—5 min, (4.2b) 210 °C—10 min, (4.5a) 230 °C—5 min, (4.5b) 220 °C—10 min, 0 is untreated biomass).

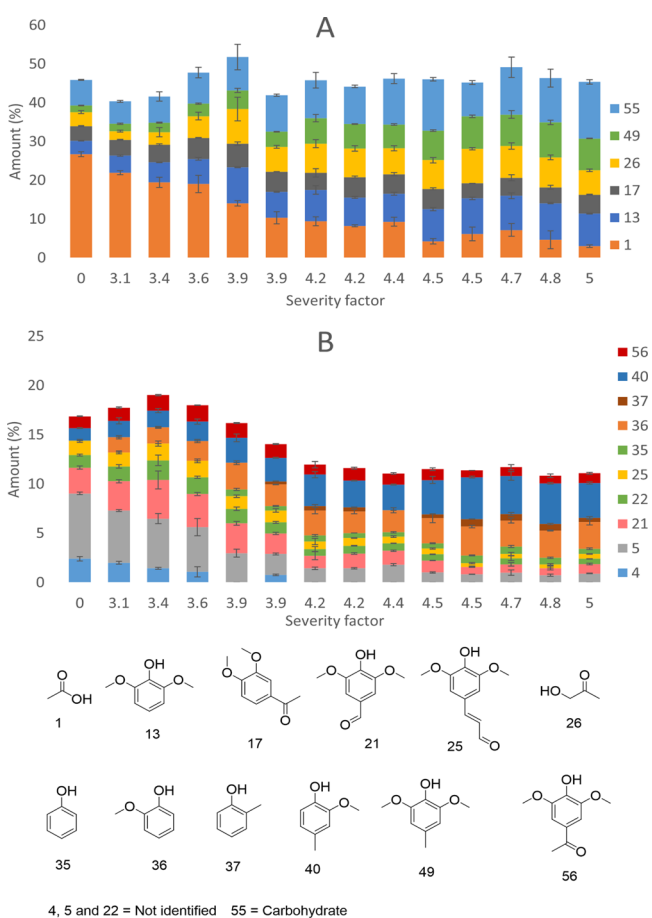


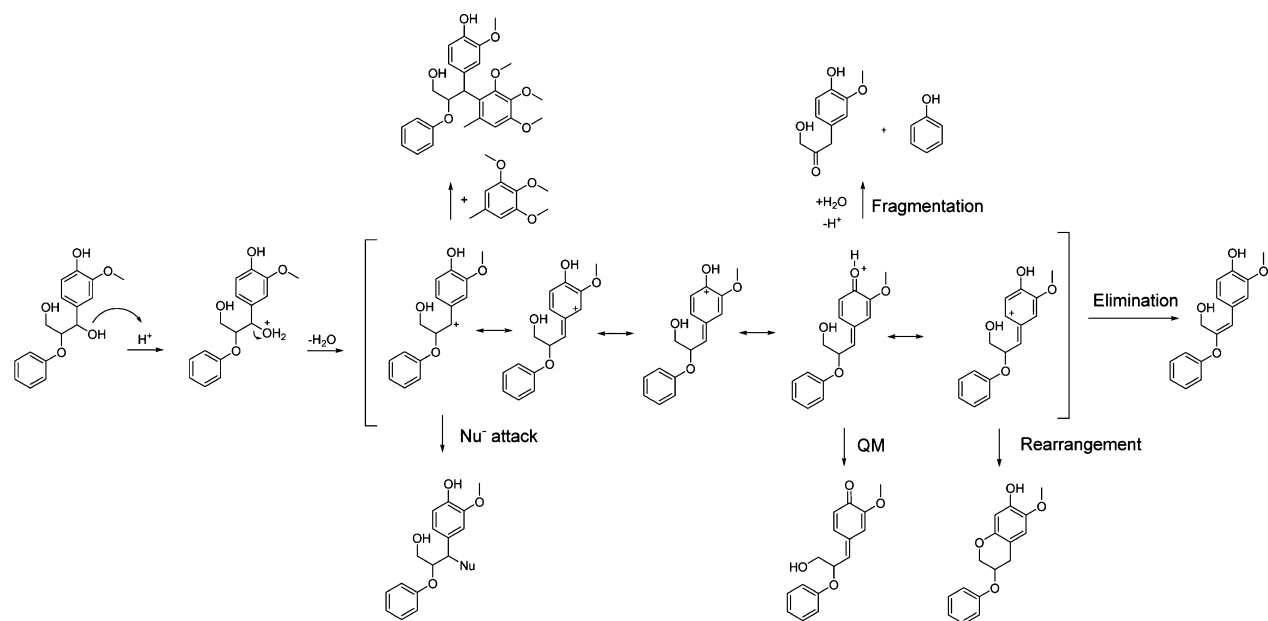
Figure 6. Amount and structure of several components in the pyrolyzate at 600 °C, at different severity factors of SE. The amounts are the average of a normalization with standard deviation of three replicates. (A) Components of major amounts and (B) components of minor amounts.

intermediate cation. This carbocation intermediate is subsequently open for nucleophilic attack, rearrangement, or both, Scheme 2. The changes seen in the pyrolyzate composition is mostly related to the α -carbon on the side chain, with a general decrease in number of oxygen atoms.

Because there are little or no changes in the phenyl content in the pyrolyzate, there is a reason to believe that pseudo-lignin does not contain this structure in a large amount. On the other hand, there is a significant increase of component 12 (5-HMF) and other furan-components in the pyrolyzate with the increase of the severity factor. 5-HMF is visible in the NMR, but the intensity of the peaks are low and do not correspond to the increase seen in the pyrolyzate. There are also several other components appearing with log R_0 3.9 in the pyrolyzate such as: 2 (furfural), 3 (2-furanmethanol), and 7 (2,5-furandicarboxaldehyde). These compounds are related to the pyrolytic cleavage of humins as shown by van Zandvoort et al.³³ We propose that the byproduct formed by SE, called pseudo-lignin, corresponds to humin because of the furan polymer structure that was found. According to van Zandvoort et al.³³ and Patil and Lund,³⁵ the formation of humins from carbohydrates takes place under harsh conditions, for example, high temperature and acidic pH. The acetic acid released is consumed in forming the humin polymer; therefore, after pyrolysis, the acid content drops and the depolymerized humin increases with furan-like components in the pyrolyzate.

To support that humins are formed under SE-treatment, FT-IR spectra were recorded (Figures S3 and S4). The untreated samples had IR transmittance at 1722, 1655, and 1594 cm^{-1} , but in the treated sample (severity factor 4.7 (220 °C, 15 min)), there is instead transmittance at 1711, 1605, and 1515 cm^{-1} . These results are in agreement to IR of humins as described by van Zandvoort et al.³³ Baccile et al.⁴⁶ did structural characterization of hydrothermal carbon spheres, revealing a structure and bonding pattern similar to the proposed humin structure (Figure 1). These carbon spheres were accumulated from carbohydrates and raw biomass with temperatures between 160 and 220 °C. The solid-state NMR of the insoluble part showed a clear furan-ring polymeric structure, very similar in structure as suggested by van Zandvoort et al.³³ Tuercke et al.³⁶ acid catalyzed (0.1 M HCl) dehydration of fructose into 5-HMF, but noticed that at temperatures from 200 °C the yield started to decline. They found that this was caused by the formation of humin and other insoluble polymeric byproducts.

To summarize, the changes in NMR spectra do not correlate with the increase in the KL content and therefore is not consistent with the structure of pseudo-lignin, but they confirm the release of several monomeric carbohydrates, some 5-HMF, and hydrolysis of lignin. The SE-temperature of 200–210 °C is a critical temperature for SE-treatment of biomass. This is the temperature where the inhibition of enzymes and formation of pseudo-lignin escalates. At the severity factor above 3.9 (210 °C), there is a decrease of acetic acid in the pyrolyzate. We believe the acetic acid is consumed as a result of polymerization with furan components to form humins. Several furan components, especially 5-HMF, are increasing in the pyrolyzate with SE-treatment. There are also small amounts of this that are visible in the liquid NMR fraction, but the intensity does not correspond to the amount in pyrolyzate. This strongly indicates that 5-HMF is bound in a polymeric molecule not solvable in NMR solvents. These furan-like components are known to be related to humin structures. The conditions under SE are conducive for the synthesis of these humins, with acid, heat, and water. Therefore, pseudo-lignin as the byproduct formed during the SE-treatment of birch seems to be a type of humin structure and not a more condensed lignin structure.

Scheme 2. Reaction Pathways under SE of Lignin that Might Take Place^a

^aThe first resonance structure is open for a nucleophilic attack, Li et al.⁴³ and Shimada et al.⁴⁴ The fragmentation reaction in the fourth resonance form is calculated to be exothermic by Sturgeon et al.⁴⁵ QM is the formation of the quinone methide-structure. The rearrangement in the fifth resonance would yield flavonoid-like structures as detected by Rasmussen et al.²¹

METHODS

Materials. Birch (*Betula pubescens*) stem wood without bark was pretreated with SE.¹⁸ The list of standards are listed in Supporting Information and is used for retention validation of components.⁴⁰ The hemicellulose 4-*O*-methyl-*D*-glucurono-*D*-xylan and cellulose (powder) were acquired from Sigma-Aldrich (Steinheim, Germany). The milling was done with a Retsch GmbH 100PM (Haan, Germany) instrument with zirconium balls (ZrO₂) at 350 rpm, for 12 h with 15 min on/off increments.

Steam Explosion. The SE was done on birch stem wood at several different temperatures (170–230 °C with a 10 °C increment) and resident times (rt, 5, 10, and 15 min), see Table 1. The pretreatment was performed at the SE facility, at Norwegian University of Life Sciences (NMBU) in Ås, designed by Cambi AS. Three hundred grams of dry matter of milled birch was added to preheated pressure chambers (10 min). SE at temperatures of 170–200 °C was only done with 10 min rt.¹⁸ These samples were dried and stored in room temperature, before applying the powder to the pyrolysis filament, with a micropipette designed for powder. The Klason-lignin analysis was carried out in triplicates.

Pyrolysis–GC–MS. The flash filament Pyrola 2000 pyrolyzer (Pyrol AB, Lund, Sweden) was coupled to a GC–MS (7890B-7000C triple quadrupole GC–MS instrument from Agilent technologies) to characterize the volatile pyrolyzate generated from fast pyrolysis of birch samples. The GCMS method and identification was done as previously described, with a capillary column (TraceGOLD TG-1710MS 60 m, ID 0.25 mm, and 0.25 μm film thickness, Thermo Fisher Scientific).⁴⁰ The total pyrolysis time is 2 s, with a heating time of 8 ms, which is injected on-line to the GC with a total run time of 76.4 min. The pyrolysis was a fractionated pyrolysis which is, according to IUPAC system, “pyrolysis in which the sample is pyrolyzed at different temperatures at different times to study a special fraction of the sample”⁴⁷ and in this case at

350 and 600 °C, before each GC-run. The amounts of each component are a normalization by maximum peak area and therefore only represent the change in the composition as a function of the SE-treatment. Each pyrolysis has been performed in three replicates, and the value shown is the average with corresponding standard deviation.

2D-NMR HSQC. For the preparation of NMR samples, three different deuterated solvents were tested, DMSO-*d*₆, DMF-*d*₄, and acetic acid-*d*₄. Generally, all of the different solvents resulted in similar spectra. NMR spectra with DMSO-*d*₆ as the solvent were chosen to present in this study because DMSO is known to be a good solvent for lignin materials. DMF-*d*₆ gave a somewhat better spectra resolution but was not used, as the gain was small. The NMR spectra were recorded on a Bruker Ascend 400 spectrometer (400 MHz) as previously described.⁴⁰ The SE-treated material was ball-milled after drying and dissolved in the deuterated solvent for 5 min. Then, they were filtered through glass wool, to remove any unsolved particles, directly into the NMR-tube.

Fourier Transform Infrared Spectroscopy. The IR spectra were recorded on an Agilent technologies FTIR 5500 (single reflection diamond attenuated total reflection-cell), with the solid powder after SE-treatment, drying, and milling. The resultant IR spectra consist of 32 co-added interferograms recorded at 8 cm⁻¹ resolution in the 4000–650 cm⁻¹ wavelength region.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b00381.

HSQC spectrum of birch biomass treated with steam-explosion at a severity factor of 3.1 (170 °C—10 min); topographical visualization of untreated and treated biomass (220 °C—5 min); FTIR-spectrum of untreated, treated biomass (220 °C, 15 min), cellulose, and

hemicellulose overlaid; FT-IR-spectra of untreated and steam-exploded at severity factor 4.7 (220 °C—15 min); HSQC of 5-HMF in DMSO-*d*₆; and all detected components from fractionated py-GC-MS at 350 and 600 °C (PDF)

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Notes

The authors declare no competing financial interest.

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