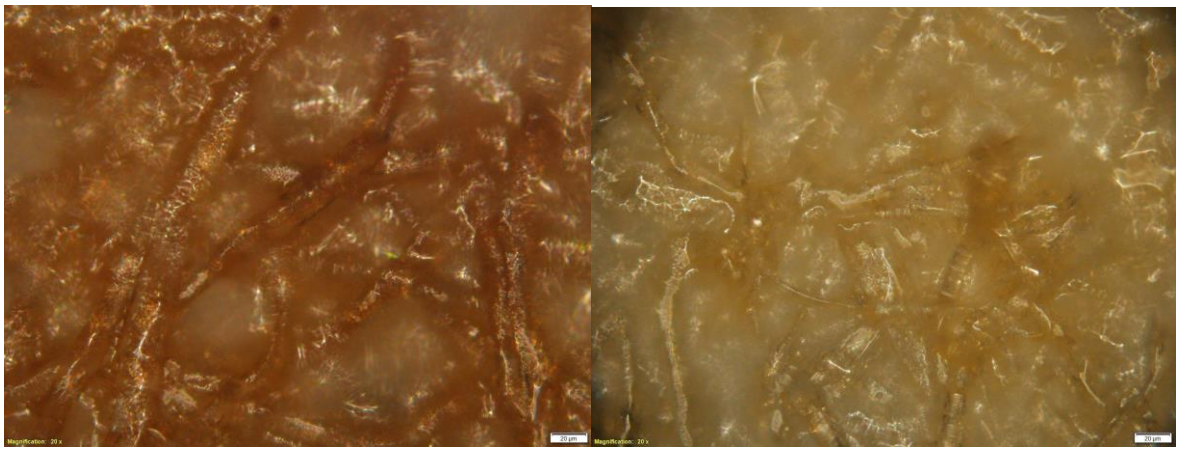
Investigation of Milled Wood Lignin Extraction Using Enzymatic Hydrolysis for Application in Hydrophobic Barriers in the Food Packaging Industry



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Abstract

The objective of this report is twofold: to optimise the extraction method of lignin from spruce altering a few process parameters and to investigate the use of lignin as a hydrophobic barrier in food applications. The process used was STEX (steam explosion) pretreatment followed by enzymatic hydrolysis and MWL (milled wood lignin) extraction using a mixture of dioxane-water. The lignin was then collected, dissolved in acetone and coated on filter papers using an airbrush. The extraction was examined with and without enzymatic hydrolysis and with STEX pretreatment for 5 or 15 minutes. NREL (National Renewable Energy Laboratory) procedure was used for the determination of lignin and structural carbohydrate content in the raw and pretreated material. HSQC 2D-NMR was used for a semiquantitative and qualitative analysis of the extracted lignin. For the hydrophobicity testing, a Cobb test was performed to assess the water absorption properties, and a drop test was conducted to measure the contact angle. Furthermore, the surface was observed more closely using an optical microscope.

The results showed that the lignin yield (g lignin extracted/g lignin in raw material) was approximately 25% for the 15-minute STEX material and 7-8% for the 5-minute STEX material, regardless of the addition of an enzymatic hydrolysis step. Furthermore, the HSQC 2D-NMR analysis revealed a cleavage of β-O-4 bonds and a higher presence of sugars in the 5-minute STEX samples compared to the 15-minute STEX samples. However, regardless of the STEX duration, the drop test showed an average contact angle of 102 to 110° for all samples. The Cobb test resulted in a Cobb value of 17g/m^2.

In conclusion, the findings suggest that the enzymatic hydrolysis did not significantly affect the yield or performance of the extracted lignin. Additionally, although the 5-minute STEX material had a slightly higher contact angle than the 15-minute STEX material, all samples showed promising hydrophobic properties suitable for hydrophobic barrier applications in the food industry.

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1, Introduction

The climate crisis and global warming are upon us and with that the increasing need to replace fossil based with renewable materials. One example is in the packaging industry where today plastic, typically made from fossil sources, is necessary for the use as a hydrophobic barrier in order to protect the products. A renewable material that shows potential for use as a hydrophobic barrier in these applications is lignin. Lignin is the second most abundant polymer found in nature, and can be recovered from most plant tissues such as wood or straw (Hamidreza Ghaffar, Fan 2014). The polymer with its aromatic structure shows hydrophobic properties if not too condensed (Lisý et al, 2022). Besides its chemical properties, an advantage of using lignin compared to other bioplastics is that the material is often left as an unexploited byproduct from many processes that use wood as starting material. Examples of this are in the pulp and paper industry, and in bioethanol production. In fact, about 95% of the lignin from these processes is today burned and used for energy production (A. Nasrullah et al, 2017). However, even though lignin does show potential as a hydrophobic material that could replace plastic in some applications, the challenge lies in extracting the molecule from the biomass efficiently without losing these properties. There are a variety of extraction methods available today and one that has shown potential for preserving the lignin's properties is the milled wood lignin, MWL, method. (Lin & Dence 1992)

1.1, Aim

The objective of this master thesis project is to optimise an extraction method of lignin for use as a hydrophobic barrier in food packaging applications. The project includes an investigation of the milled wood lignin, MWL, extraction method more closely by evaluating the effect of two different STEX (steam explosion) pretreatment severities as well as the addition of an enzymatic hydrolysis step before the MWL extraction. Parameters that will be evaluated are the process yield, the coating structure and the hydrophobicity of the coating.

1.2, Background

This section of the report will in more detail treat lignin, the chemical composition of wood as well as a more in-depth background of the extraction method investigated.

1.2.1, The Chemical Composition of Spruce

To understand the extraction of lignin from wood, in this case from Norway spruce, it is necessary to understand the chemical composition of the raw material. Spruce, as all woods and other lignocellulosic biomasses, consists of the three macromolecules cellulose, hemicellulose and lignin and the composition has been found to remain relatively constant throughout the length and thickness of the tree. Chemical analysis of Norway spruce has shown a composition of 39 to 43% cellulose, 35% hemicellulose and 24 to 26% lignin. (Čabalová et al, 2021). The lignin content in

softwoods, such as spruce or pine, is typically higher than that of hardwoods such as birch or oak. This makes it advantageous to use spruce in lignin studies (Rowell 2012).

To better understand the composition of spruce it is imperative to discuss the composition of the macromolecules on a smaller scale. Cellulose, with glucose as the smallest repeating unit, is a long linear polymer that, connected with other cellulose polymer chains, composes microfibrils. Each microfibril normally contains up to 40 individual cellulose polymers. To get a better view, please see the illustration in figure 1 below. The microfibrils in turn, are interconnected by hemicellulose, and compose larger fibres which provides structural support for the plant. Hemicellulose, with its branched structure, crosslinks between the cellulose microfibrils, thus supporting the material. Lignin is typically present in the voids found between the hemicellulose and cellulose. (Jayasekara & Ratnayake 2019) While, as previously mentioned, cellulose is a linear polymer composed only by glucose, hemicellulose is branched and composed by the sugars arabinose, galactose, xylose and mannose (Norlailiza & Mohd Rafein 2019).

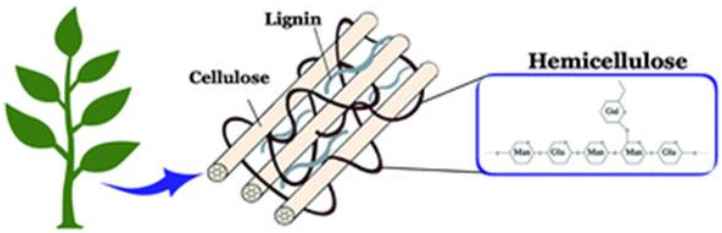

Figure 1 – *An illustration of the microfibrils in wood. Please note that the figure is not an exact reflection of reality but rather a helpful illustration. (Naidjonoka et al, 2020)*

1.2.2, Lignin

As previously mentioned, lignin is one of the most abundant polymers in nature and a promising source for different applications such as in hydrophobic barriers in the packaging industry, but also as feedstock for the production of bulk chemicals or glue (Södra, 2023). There are many types of lignin, categorised by the extraction method used as this will affect the properties of the extracted lignin. For instance, the pulp and paper industry yields sulphur lignins. These include both Kraft lignin and lignosulfonates, where lignosulfonates are characterised by being water soluble and Kraft lignin is characterised by its highly condensed structure which under alkaline conditions is soluble in water (Maitz et al, 2020). Kraft lignins typically also contain a higher concentration of phenolic group, suggestably as a result of the β-O-4 bond cleavage (the bond can be seen in figure 3 below). Sulphur-free lignins on the other hand can, for instance, originate from the bioethanol production process. These include alkaline lignin and organosolv lignins. Organosolv lignin is generally not soluble in water but in the organic solvent used in the extraction process. Examples of solvents typically used are acetone, ethanol and methanol (Liu et al., 2022). Alkaline lignins are generally obtained by pulping and contain a higher nitrogen and silicate content. (Nasrullah et al, 2017)

Looking at lignin on a smaller scale the polymers consist of three different repeating units or monomers linked in various types of bonds. The monolignins when connected in the lignin polymer form the different repeating units, in short called S, G, and H, and are linked together by different bonds as seen in figure 3. The monolignins and repeating units are presented in figure 2 below. (Lisý et al, 2022)

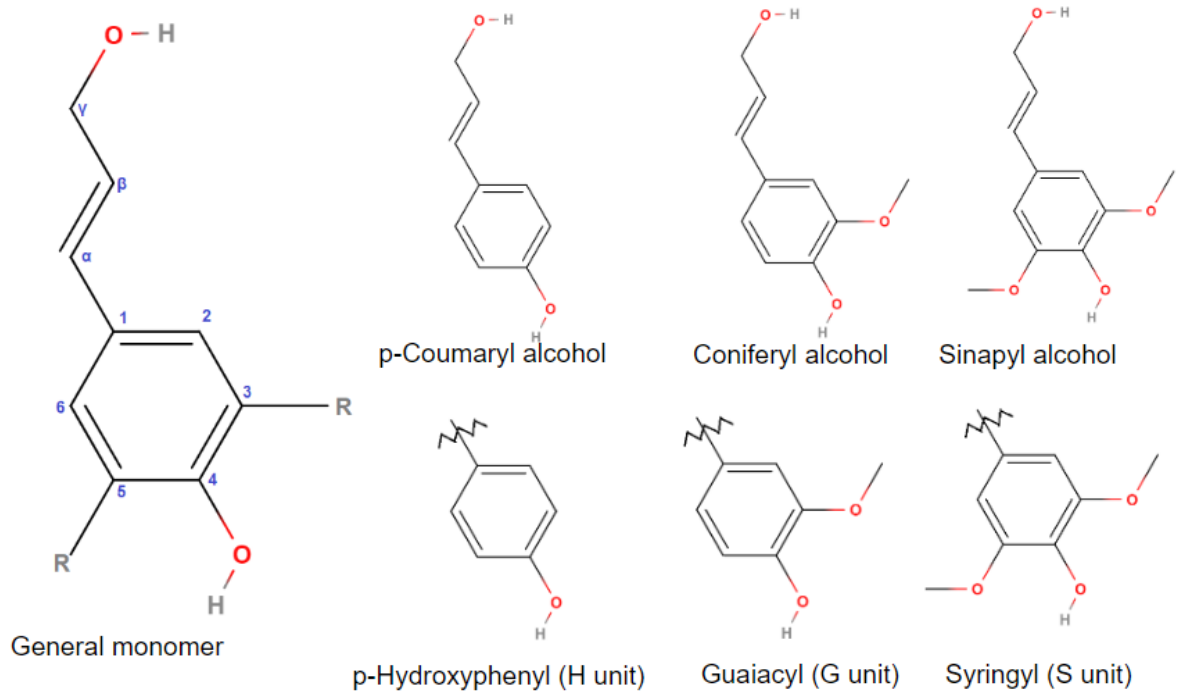

Figure 2 - *The three monolignins and the repeating units.*

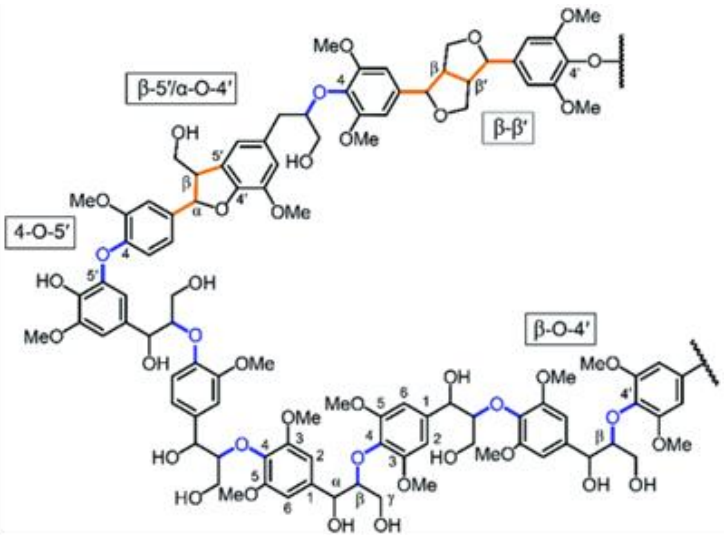

Figure 3 - *Representation of the chemical bonds present in lignin. Picture from (Talebi et al, 2019)*

Because lignin is a naturally occurring polymer the concentration of the different monomers is different depending on the source the lignin is taken from. Spruce does not contain any S units and softwoods in general tend to have a higher content of G units as well as more carbon-carbon and less β-O-4 bonds than hardwoods (Lisý et al, 2022)(Ahmad et al, 2020). Even though softwoods do have a lower β-O-4 bond frequency than hardwoods, the bond is, at 45-50%, the most abundant bond even in softwoods (Chakar & Ragauskas 2004). Moreover, softwoods generally have a higher concentration of Klason lignin, i.e the residue left after complete acid hydrolysis compared to hardwoods (Rowell 2012).

Regarding the hydrophobicity of lignin, it has been found that the number of functional groups capable of forming hydrogen bonds play an important role in decreasing the hydrophobicity of lignin. (Lisý et al, 2022) In the literature, it is suggested that the cleavage of the β-O-4 bond in lignin can lead to the formation of phenolic hydroxyl groups which, as hydrogen bond forming groups, could potentially decrease the hydrophobicity of the extracted lignin (Jääskeläinen et al, 2003)(Lisý et al, 2022). It is however also suggested that phenolic hydroxyl groups are stabilised by the valence electrons in the aromatic structure and not as likely to react with water as aliphatic hydroxyl groups (Lisý et al, 2022). The hydrophobicity of lignin could also be improved if the functional groups are modified, for instance by adding a long fatty acid (Lisý et al, 2022). While this provides interesting opportunities for future work it is out of scope for this project.

1.2.3, Extraction Processes

There is a wide range of extraction processes used to isolate lignin from their source in plant tissues which all have different advantages and disadvantages. As previously mentioned in the lignin subsection, the methods affect certain properties in the extracted product. Besides the ones already mentioned, factors such as the molecular weight, the purity and composition of the lignin obtained. (Zinovyev et al. 2018) (Ross and Mazza 2010) (Jääskeläinen et al. 2003) In more detail, Kraft Pulping followed by acidic hydrolysis has been shown to yield a pure lignin with low molecular weight. In a similar process, pulping followed by enzymatic hydrolysis has been shown to result in a higher molecular weight, however extracted lignin of lower purity (Jääskeläinen et al, 2003). The pretreatment method and its severity, i.e "harshness", has also shown to have a great impact on the final product obtained (Caputo et al, 2022).

The process investigated in this work is STEX pretreatment followed by the milled wood lignin method with or without the addition of an enzymatic hydrolysis step before the extraction. These process steps are presented in more detail in the following subsections.

1.2.3.1, STEX

In this work, the pretreatment used was steam explosion (STEX). STEX is a common and, because it does not utilise environmentally harmful chemicals, environmentally friendly pretreatment method which is often used for the pretreatment of lignocellulosic biomass (Delgado et al, 2021). A STEX pretreatment prior to the extraction works by breaking the intramolecular bonds in hemicellulose, and the intermolecular bonds between the hemicellulose, cellulose and lignin. Hence, the hemicellulose is solubilised and the fibres in the wood are separated, thus making the following lignin extraction more efficient. The severity of the STEX, i.e the temperature, the duration and/or the addition of a catalyst all play an important role in the physical properties of the extracted lignin. An increased severity will result in the depolymerisation of lignin, i.e a lower molecular weight product, and typically also a higher process yield. Spruce lignin contains a number of different bonds which have all been shown to be broken by STEX treatment, however to a different extent. The most abundant bond, the β-O-4 one, has in previous studies been shown to be more sensitive to STEX than for instance the β-β- or β-5 bonds are. A suggested reason for this has been that the lignin carbon-carbon bonds are stronger than the β-O-4 bond under acidic conditions. (Caputo et al, 2022). As a result of the efficient degradation of hemicellulose and opening of the fibres the extraction yield is also increased by an increased STEX severity. (Caputo et al, 2022).

1.2.3.2, Milled Wood Lignin, MWL

A well-established method when it comes to the extraction of lignin from wood is the milled wood lignin method that was developed by Björkman in the 1950s. The method falls under the organosolv methods and in short consists of milling the wood to a fine powder before extracting it using a dioxane-water solution. The MWL procedure has been found to keep the lignin polymers

intact and is therefore suitable to use as a reference point to compare with other extraction methods. (Lin & Dense 1992)

The extraction is more efficient at higher temperatures but because of the heat sensitive nature of lignin the temperature should be kept lower than the boiling point of the solvent. In the parameter study by Lin & Dense 1992 it was found that the optimum extraction temperature was 80°C. Moreover, it was found that if the extraction is divided into intervals where the solvent is replaced every 24 h, most of the lignin will be extracted during the first two 24h extractions. (Lin & Dense 1992)

1.2.3.3, Enzymatic Hydrolysis

The enzymatic hydrolysis method, utilises the enzymatic mixtures hemicellulase and cellulase to depolymerize the surrounding structures while not resulting in further cleavage of the lignin polymers. Enzymatic hydrolysis is not an extraction on its own, but used as a step in the process that lowers the hemicellulose and cellulose content in the biomass, thus increasing the lignin accessibility. This method is often followed by alkaline extraction in NaOH. For cellulose the enzymes work by hydrolysing the bonds between the glucose units (Jayasekara & Ratnayake 2019). A study of enzymatic hydrolysis extraction of lignin from kraft pulp showed promising results to yield a high molecular weight product however, it also contained impurities such as carbohydrates and protein from the enzymes. (Jääskeläinen et al. 2003)

1.2.4, Analysis

Several analysis methods have been used to evaluate the process in this project. These were as follows: NREL, HSQC 2D-NMR, Cobb test, Water Contact Angle and microscopy. A closer explanation on how the analysis methods were used will follow in section 2.5 in the next chapter. A previous approach for the detection of cracks or pinholes in similar coatings (spray coated lignin on paper) has been to use SEM, however this method proved unsuccessful as the surface appeared too rugged and dusty to get pictures of sufficient quality for analysis, which is why an optical microscope will be used instead.

2. Method

This section describes the method used for the laboratory part of this work. In summation the biomass, Norway spruce wood chips, was pretreated with STEX together with the supervisor at LTH. Following the STEX pretreatment, half of the biomass underwent enzymatic hydrolysis before the MWL extraction, while the remaining portion did not. After the extractions smaller fractions of the extracted lignin samples were analysed with HSQC 2D-NMR. For the coating tests, the lignin was weighed and applied to filter papers before being sent to the Tetra Pak laboratories for optical microscopy and hydrophobicity testing. The raw material and STEX treated material at different severities were analysed for lignin and carbohydrate content using the NREL

method. The process steps mentioned here are described more closely in following sections. A flow sheet of the process can be seen in figure 4 below.

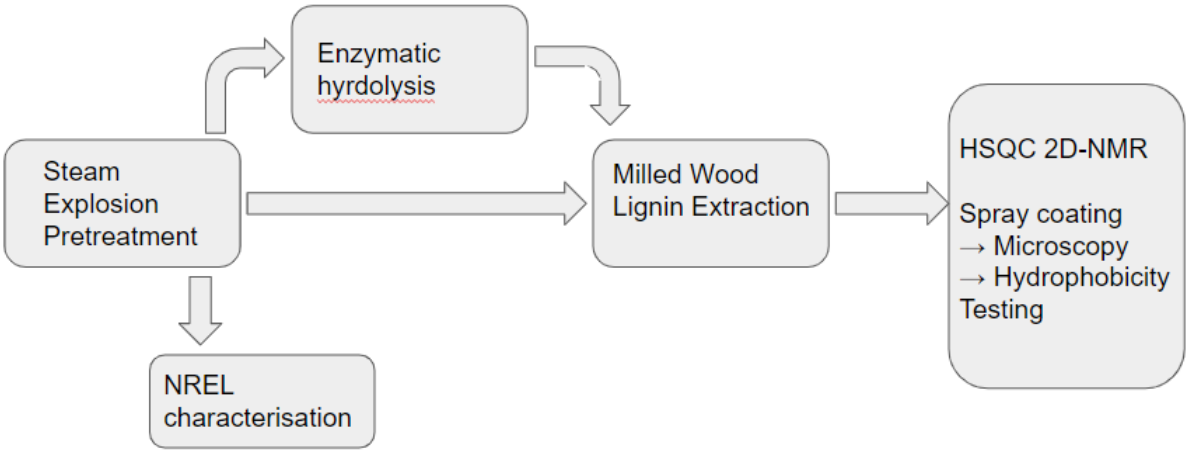

Figure 4 - *A simplified schematic view of the laboratory process.*

2.1, STEX/Pretreatment

1.5kg wood chips were milled through a 20mm mesh grid before being subjected to the steam explosion pretreatment. 750g was treated for 5 minutes at 210°C and the remaining 750g was treated for 15 minutes at 210°C. The biomass was then washed two times with 2L deionized water and put in an oven at 45°C to dry over the weekend. The dried biomass was milled through a 1mm mesh grid to a powder before being put back into the oven and dried again before the extraction steps.

2.2, Enzymatic Hydrolysis

40g of the milled and dried STEX pretreated wood was measured and put into a mixing vessel together with 760 ml of water. The pH was measured and 1M HCl was added to the mixture until a pH of 5.5 was reached. 6g of the enzyme mixture CTec2, activity 200 FPU/g, provided by Novozymes was then added to the mixture. The solution was mixed by hand and added to a fermenter with continuous stirring and a temperature at 50°C. The hydrolysis went on for 96 hours and samples from the hydrolysis were collected every 24 hours. These samples were put in the freezer until they were analysed for carbohydrate content using a Dionex ICS-3000 together with the samples and standards from the NREL analysis as explained later on in the report.

After the 96h, the mixture was collected from the fermentation vessel and the solids separated from the liquid/slurry using filter paper. The filter cake was washed twice with deionized water to remove enzymes and carbohydrates before being collected and put in an oven at 45°C to dry over the weekend.

2.3, MWL

First the dioxane-water solution was mixed by adding 50 ml of dioxane and 2 ml of deionized water to a hydrolysis flask using a volumetric pipette for the dioxane and an automatic pipette for the deionized water. The solution was then mixed carefully. 3g of the pretreated wood was added to a thimble that was put in a hydrolysis flask with the previously added dioxane water. The mixture was put in a water bath at 80°C for 24 hours. After 24 hours the flasks were removed from the water bath and the thimbles with the solid fraction were removed and placed to dry in a fume hood. The liquid fraction of the samples were put in a round flask that was placed in a one step distillation unit where the dioxane water was recovered. The solvent was then reused. The lignin in the bottom of the round flask was dissolved in acetone and collected in an aluminium tray.

2.4, Coating

For the coating, 0.5g of lignin was dissolved in 50 ml of acetone. The solution was then filtered through a 0.2µm syringe filter. The 12.5 cm diameter filter paper used was then pretreated by spraying 2 ml of absolute ethanol on the paper. The lignin-acetone solution was then sprayed onto the filter paper in layers of 5 ml. The filter paper was rotated 90° between each layer in order to obtain an even coating.

2.5, Analysis

The characterisation and analysis of the obtained mass was performed using several analysis methods more closely described in the following subsections. The NREL and HSQC 2D-NMR were performed in the laboratory at Kemicentrum and the Hydrophobicity testing was performed at the Tetra Pak testing laboratory.

2.5.1, NREL

The NREL, as described elsewhere in (Sluiter et al, 2008), was used for the determination of acid soluble lignin, ASL, acid insoluble lignin, AIL, ash content and carbohydrate analysis. Some changes were made to the procedure, primarily the crucibles and flasks from steps 10.1.1 and 10.1.2 in the procedure description were put in an oven at 105°C overnight instead of 575°C 4 h. Moreover, due to analytic equipment failure, the samples for the HPLC (High Performance Liquid Chromatography) analysis were kept in the freezer for 9 weeks for the raw material analysis and 3 weeks for the steam explosion pretreated material.

For the determination of carbohydrates in the samples a high-performance-anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (ICS-3000+, Thermo Fisher Scientific Inc,. Waltham, MA, USA) with the analytical column CarboPac PA1 (Thermo Fisher Scientific Inc., Waltham, MA, USA), was used. Deionized water was used as eluent at a flow rate of 1 ml/min with 100mM NaOH post-column addition at a flow rate of 0.5 ml/min. The calibration

standards employed were L-arabinose, D-galactose, D-glucose, D-xylose and D-mannose of analytic grade. The monosugar content was anhydro corrected with 0.88 for pentoses and 0.9 for hexoses to evaluate the hemicellulose content in the solution. The free monosaccharide contents of the samples was determined using the HPAEC-PAD method without the acid hydrolysis step and anhydro corrections.

The samples were prepared for the NREL analysis by weighing 5g of biomass in a previously weighed extraction thimble which was put in a Soxhlet apparatus. 150ml ethanol, 96%, was measured and put at the bottom of the device before starting the extraction which went on for 24 h. Multivapor flasks were weighed and put in an oven at 105°C overnight. The weight was then recorded again and the flasks put in a desiccator until use.

After the extraction had finished, the liquids were collected into the weighted multivapor flasks and the liquids were evaporated. The flasks were then put in an oven at 105°C overnight and weighted the following day. The solids from the extraction were collected and washed with 100 ml of fresh ethanol, 96%, on a previously weighed filter paper. The solids and filter papers were put in an oven at 105°C to dry overnight. This is the biomass used for the following NREL analysis.

2.5.2, HSQC 2D-NMR

To determine and quantify the structure of the extracted lignin (from the STEX pretreated material) a HSQC 2D-NMR (Heteronuclear Single Quantum Coherence Spectroscopy 2-Dimensional Nuclear Magnetic Resonance) analysis was performed in the following way. 80mg of lignin was measured and put in a 4 ml glass vial. To the vial, 0.6 ml DMSO-d_6 was added and the mixture was shaken by hand. The mixture was then analysed at room temperature with a Bruker Avance III HD 600 MHz spectrometer (Bruker BioSpin GmbH, Karlsruhe, Germany). For more detailed settings please see Appendix A.

2.5.3, Hydrophobicity Testing

The hydrophobicity of the lignin coated filter papers was analysed in the test laboratories at Tetra Pak Packaging Solutions. The surface structure was studied using a microscope (in scales 2x20 and 2x40) and the hydrophobicity was tested using Cobb's test. For the Cobb's test, the ISO–standard method was used with a duration time of 60 seconds. Furthermore, the surface tension was tested by dropping water and diiodo-methane onto the surface and studying the contact angle using the OWRK-method.

3. Results and Discussion

This section of the report is dedicated to presenting and discussing the results from the laboratory work performed in this project. In some of the results the experiments are referred to as sample 1-4, the samples and their respective extraction method are compiled in table 1 below.

Table 1 – *The samples and their respective extraction method.*

Sample	**Extraction method**
1	5 minutes STEX + MWL
2	5 minutes STEX + enzymatic hydrolysis + MWL
3(1)	15 minutes STEX + MWL
3(2)	15 minutes STEX + MWL
4	15 minutes STEX + enzymatic hydrolysis + MWL

3.1, NREL Results

The NREL analysis yielded results on the structural composition of the spruce used in the trials. The results are presented in this section. Figure 5 below shows the effect of the STEX pretreatment on the amount of ASL (acid soluble lignin) and AIL (acid insoluble lignin) in the biomass and figure 6 shows the amount of ethanol extractable material in the raw material and in the STEX pretreated material.

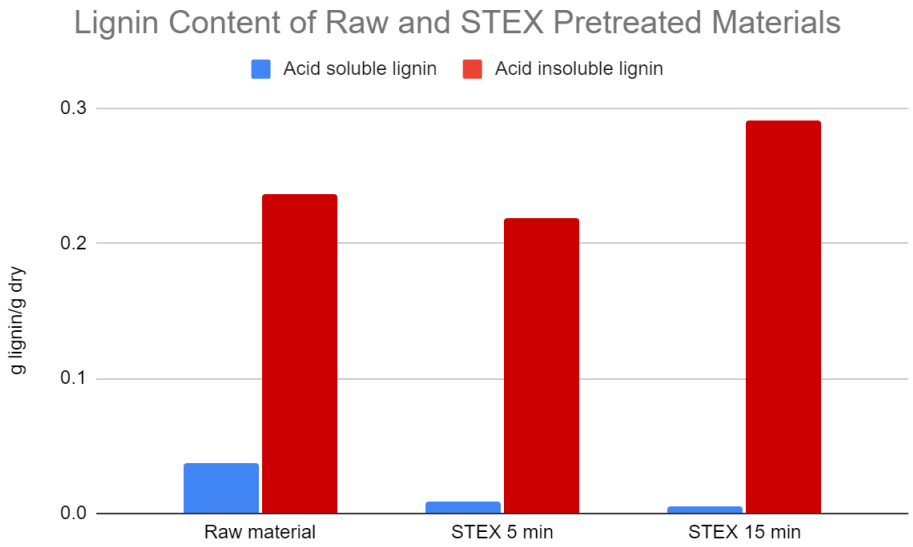

Figure 5 - *Concentration of ASL (acid soluble lignin) and AIL (acid insoluble lignin) in raw material compared to biomass pretreated with 5- and 15-minutes steam explosion at 210°C.*

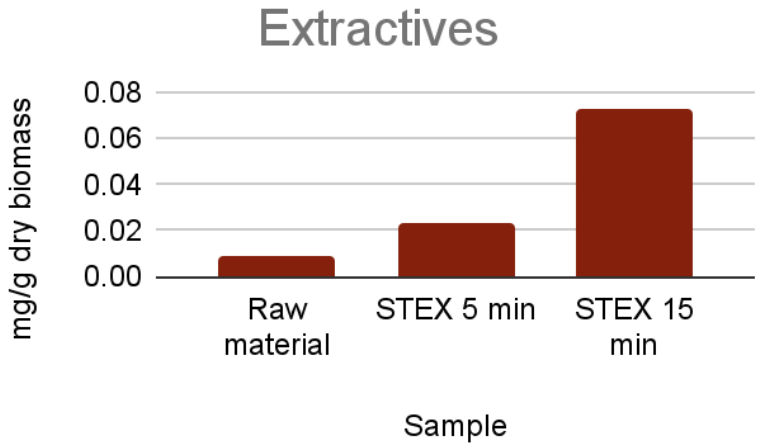

Figure 6 - *Mass yield of extractives from the ethanol extraction of the NREL analysis.*

As can be seen in figure 5 above, the ASL content decreases with the severity of the pretreatment while the AIL content, compared to in the raw material, is lower in the 5 minutes STEX and higher in the 15 minutes STEX material. ASL is composed of lower molecular weight phenolic compounds as a result of the degradation of acid insoluble lignin (Chen et al, 2019). The literature also states that a harsher pretreatment increases the decomposition of the lignin. From the results in figure 5, it can be concluded that, as it is not reasonable that more lignin is formed between the 5 and 15 minutes in the STEX, something else has happened that changes the composition of the biomass. Hence, the hypothesis is that the lignin degrades during the STEX, and so is a part of the hemicellulose and that during the 5 minutes STEX, the degradation of lignin is larger than the

degradation of hemicellulose, making the lignin content lower and the content of hemicellulose and cellulose higher. However, in the 15 minutes STEX material, more of the hemicellulose is degraded making the AIL content increase compared to the weight of the (pretreated) biomass. To summarise, the significant loss of mass affects the contents of a biomass and the contents cannot be compared between the different pretreatments without this in mind.

Furthermore, the increasing extractives content with increasing pretreatment severity revealed in figure 6 suggests that a larger amount of the biomass becomes soluble in ethanol. This indicates a further breakdown of the macromolecules and that not all solubles were excreted in the liquid phase after the STEX.

Moreover, the NREL analysis showed a total lignin content in the raw material of 0.27 g lignin/g dry biomass. For the pretreated materials the lignin contents were 0.22 g lignin/g dry biomass for the 5 minutes STEX material and 0.27 g lignin/g dry biomass for the 15 minutes STEX material. The calculations can be found in Appendix B. The fact that the lignin content is lower in the 5 minutes STEX material compared to the 15 minutes STEX material and the raw material further strengthens the hypothesis that a loss of mass changes the ratio of the macromolecules making the comparison in between the different pretreatment durations and the raw material difficult. Additionally, it complicates calculation of the yields. In order to compare the results, the yields were calculated using the lignin content in the raw material, however this will give higher yields for the 15 minutes STEX material relative to the 5 minutes STEX, which likely had a lower decrease of mass. Despite this source of error, the results obtained in figures 5 and 6 reveal large differences between the samples and can hence be considered qualitatively and it can be concluded that the amount of ASL decreases and that the extractives amount increases with an increased STEX severity.

From the NREL analysis, the carbohydrate content of the raw material as well as the STEX 5 and 15 minutes pretreated material was obtained. The results can be found in figure 7.

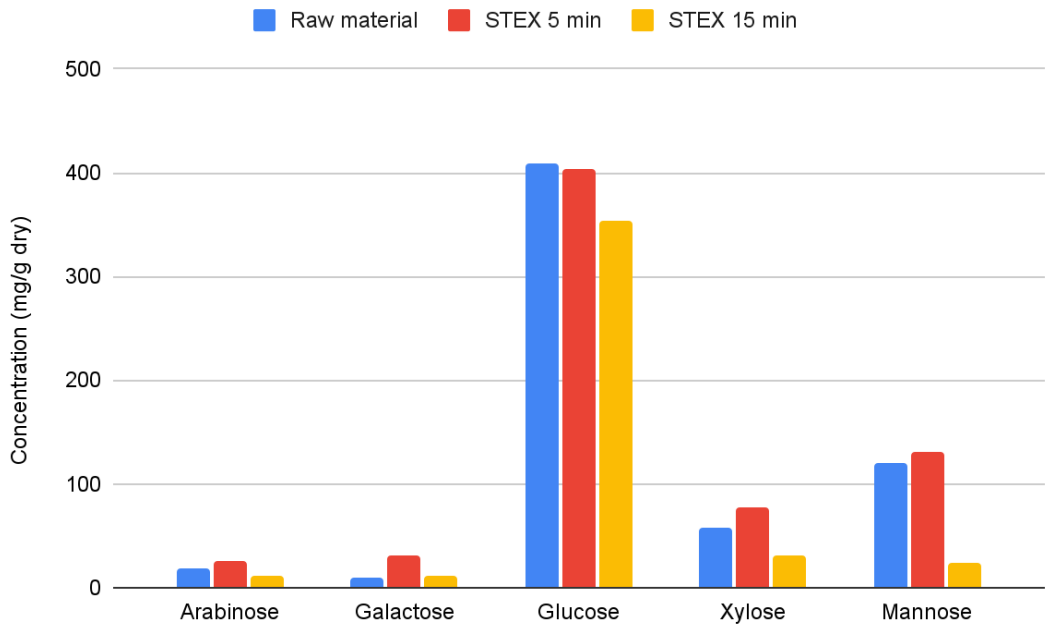

Figure 7 - *Carbohydrate content in the raw material, 5 and 15 minutes STEX pretreated material.*

The results in figure 7 reveal that the hemicellulose sugar concentrations in the 5 minutes STEX material was higher than in the raw material. An explanation for this result is, as mentioned before, that a loss of mass during the pretreatment changes the composition ratios and that for the 5 minutes STEX material, the degradation of lignin (and possibly other components) is higher than the degradation of hemicellulose, making the contents of hemicellulose sugars arabinose, mannose, xylose and galactose higher.

This hypothesis furthermore suggests a low level of hemicellulose hydrolysis in the 5 minutes STEX, which was anticipated when visually observing the solid product from the STEX. The biomass coming out of the steam explosion after 5 minutes STEX appeared almost unchanged while the 15 minutes STEX biomass was clearly affected by the severity of the treatment. See Appendix A for pictures of the biomasses after STEX. Comparing the 5 minutes STEX performed in this report with similar treatments done in the same vessel previously it is clear that the biomass was less affected than usual. This suggests that the equipment did not operate optimally, perhaps due to a build-up of dirt in the vessel leading to a lower than expected heat transfer.

As seen from figure 7, all sugar concentrations were lower in the STEX 15 minutes pretreated material than in the raw material. Especially the concentration of hemicellulose sugars (arabinose, galactose, xylose and mannose) is decimated. This suggests that a 15 minutes STEX pretreatment hydrolyses the hemicellulose and washes out most of the sugars into the liquid phase after the STEX.

As for the glucose content of the biomasses, figure 7 suggests that the glucose levels remain high throughout the STEX severity increase. If the glucose contents are put in relation to the amounts of AIL, contents of 1.5, 1.8, and 1.3 g glucose/g lignin are obtained. It is, due to the loss of mass in the STEX, difficult to say whether or not the cellulose was affected by the STEX pretreatment. Clear to say though is that there is still a significant amount of cellulose left, even after the 15 minutes STEX pretreatment.

3.2, Process Yields, Enzymatic Hydrolysis Data and MWL

This section presents the most important results from the enzymatic hydrolysis and MWL extractions. The process yields calculated are presented in table 2 below. For the calculation of the yields, see Appendix B.

Table 2 - *Lignin process yields (g lignin extracted/g lignins in raw material used) from MWL extraction of lignin from material with and without having been subjected to enzymatic hydrolysis prior to the MWL extraction. The yields were calculated using the lignin content of the biomass determined in the NREL analysis.*

Without enzymatic hydrolysis		**With enzymatic hydrolysis**	
Steam Explosion	Average Yield (wt%)	**Steam Explosion**	Average Yield (wt%)
5 min (sample 1)	9%	**5 min (sample 2)**	6%
15 min (sample 3(2))	24%	**15 min (sample 4)**	26%
Reference values from the literature			
Method:	Yield (wt%)	Source	
MWL (norway spruce) (no STEX)	20-30%	(Obst & Kirk, 1988)	

From table 2 it can be concluded that the process factor that most affected the yield is the severity of the steam explosion pretreatment performed. The enzymatic hydrolysis could have some impact on the yield as the data from the 15 minutes STEX pretreated material implies a slightly higher yield from extractions after enzymatic hydrolysis, however this was not the case for the 5 minutes STEX pretreated material which indicated that the hydrolysis makes no difference to the process yield. The low yields compared to a reference found in the literature could be explained by for instance the lack of stirring during the MWL extraction in this work.

One reason behind the apparent effect of pretreatment duration time could be that there was more time for heat transfer for the 15 minutes trial, thus making the steam explosion more efficient and separating the fibres and breaking down the hemicellulose more efficiently. It should be mentioned that the physical appearance of the 5 minutes STEX material was relatively unchanged compared to other equal treatments performed before in the same reactor which indicates a lower than usual heat transfer in the heating vessel. The result could furthermore be compared to the literature, where an extraction yield of 20.6% was found for MWL after 5 minutes STEX treatment at 210°C (Caputo et al., 2022). Pictures of the material obtained after the STEX can be found in Appendix A.

Figures 8 and 9 below presents the sugar concentration in the liquid phase of the enzymatic hydrolysis during the hydrolysis.

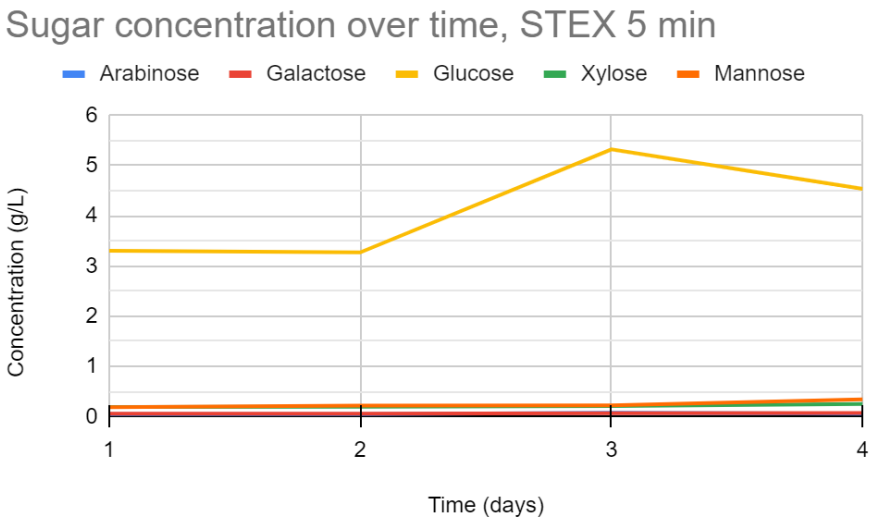

Figure 8 - *Sugar concentration in liquid fraction during the enzymatic hydrolysis for the 5 minutes STEX pretreated biomass.*

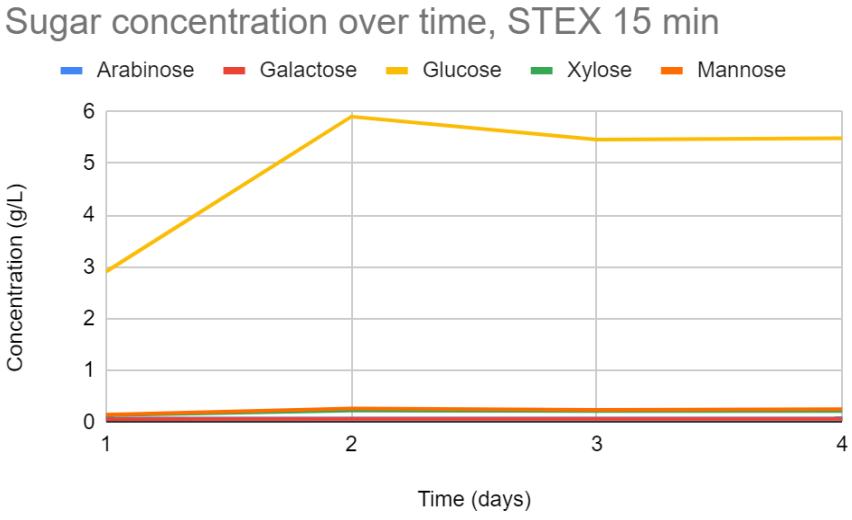

Figure 9 - *Sugar concentration in liquid fraction during the enzymatic hydrolysis for the 15 minutes STEX pretreated biomass.*

In figures 8 and 9, a low change in concentration of arabinose, galactose, xylose and mannose over time for both the 5 and 15 minutes STEX pretreated materials can be detected, while the increase of glucose is significantly higher. This indicates a low level of hemicellulose hydrolysis, probably due to already low levels after the hemicellulose degradation in the STEX, and a higher level of cellulose hydrolysis. This indicates that an enzymatic hydrolysis step breaks down the cellulose further which could potentially increase the total process yield. Revisiting the results in table 2 however, the hydrolysis of cellulose observed does not seem to increase the extraction yields, meaning that the enzymatic hydrolysis in this experiment did not affect the yields.

When looking closer at the glucose concentration over time a concentration peak can be found for both the 5 and 15 minutes STEX pretreated material. This could be because of measuring errors however more likely due to the degradation of released glucose over time, for instance there may be some microorganisms present in the solutions that consume the glucose. Since there is no further increase of glucose and the hydrolysis was done with an abundance of enzymes it could be suggested that the hydrolysis is done after 48-72 hours and that continuing the hydrolysis does not improve the results further.

One possible explanation for the difference in when the glucose peak appears for the two samples in figures 8 and 9, could be that the harsher pretreatment for the 15 minutes STEX sample in figure 9 makes the cellulose fibres more exposed, leading to easier access for the enzymes and a faster reaction time than in the 5 minutes STEX sample.

Regarding the 15 minutes STEX experiment it is worth mentioning that the fermenter initially used did not have a functioning heating system. The consequence was that the mixture was let to react at room temperature for the first 24 hours before being transferred to another reactor which did have heating. This could potentially contribute to a lower than optimal hydrolysis of cellulose. However, judging from the high increase in glucose concentration after 24 hours it seems like the hydrolysis was not in a greater way affected by this error. If the experiment was repeated with heating from the beginning it could be possible to see a higher hydrolysis of glucose.

3.3, HSQC 2D-NMR Results

The HSQC 2D-NMR performed in this project yielded one spectrum for each extraction method. These are shown in figures 10 to 13 below. Some of the peaks in each spectrum were identified, the abbreviations used in the figures 10 to 13 are compiled in table 3 below. Using the Topspin programme provided by Bruker the intensity of the intramolecular bonds β-O-4, β-β and β-5 were computed and compared to the frequency of aromatic rings. These results are compiled in table 4

further below. It should however be noted that HSQC 2D-NMR is a semiquantitative analytical method and that the results obtained in this experiment are not absolute.

Table 3 - *Peak explanations for HSQC 2D-NMR spectrum. Peaks are found left to right in the spectrum. Note that with exception of the C2 peak the peaks are carbons present in certain bonds/units/molecules and not the bonds/units themselves.*

Peak/Area	**Explanation**	**Chemical Shift (F2)**	**Chemical Shift (F1)**
1	Region for the peaks of carbon 2 in the aromatic ring		
B5	β-5 bond	5.46	87.17
B-O-4	Cα-Hα in β-O-4 linked to G	4.77	71.37
Xylose (terminal)	Xylose at the reducing end of a polymer chain	4.87	92.57
B-B	Bond between β-carbons in two Guaiacyl units	4.61	85.07
Mannose (terminal)	Mannose at the reducing end of a polymer chain	4.65	94.89
Methoxy	Methoxy groups	3.82	55.48

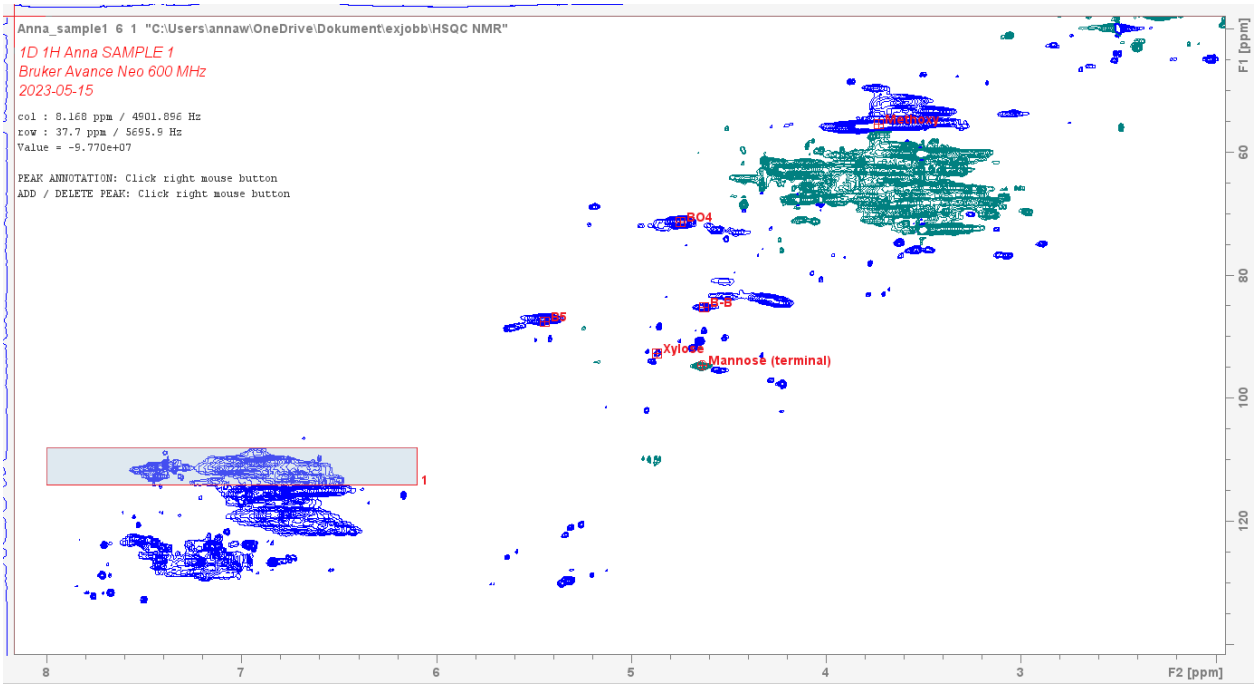

Figure 10 - *HSQC 2D-NMR spectra of lignin sample 1 extracted by 5 min STEX + MWL.*

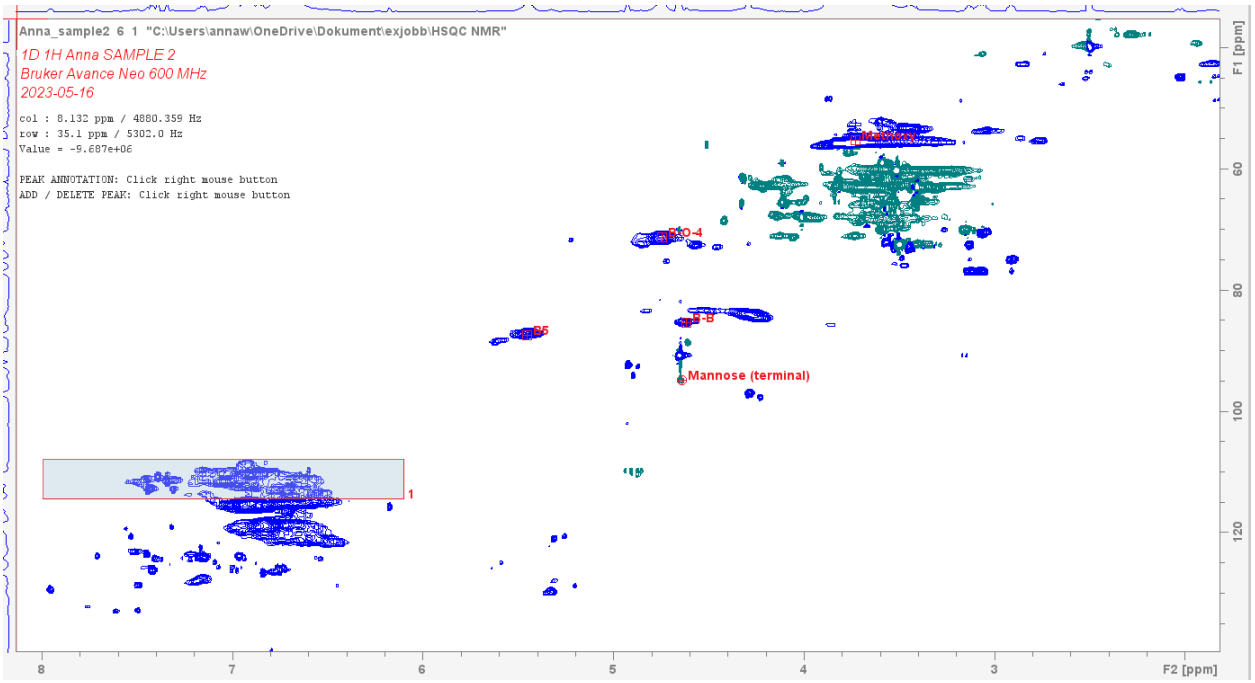

Figure 11 - *HSQC 2D-NMR spectra of lignin sample 2 extracted by 5 min STEX + enzymatic hydrolysis + MWL.*

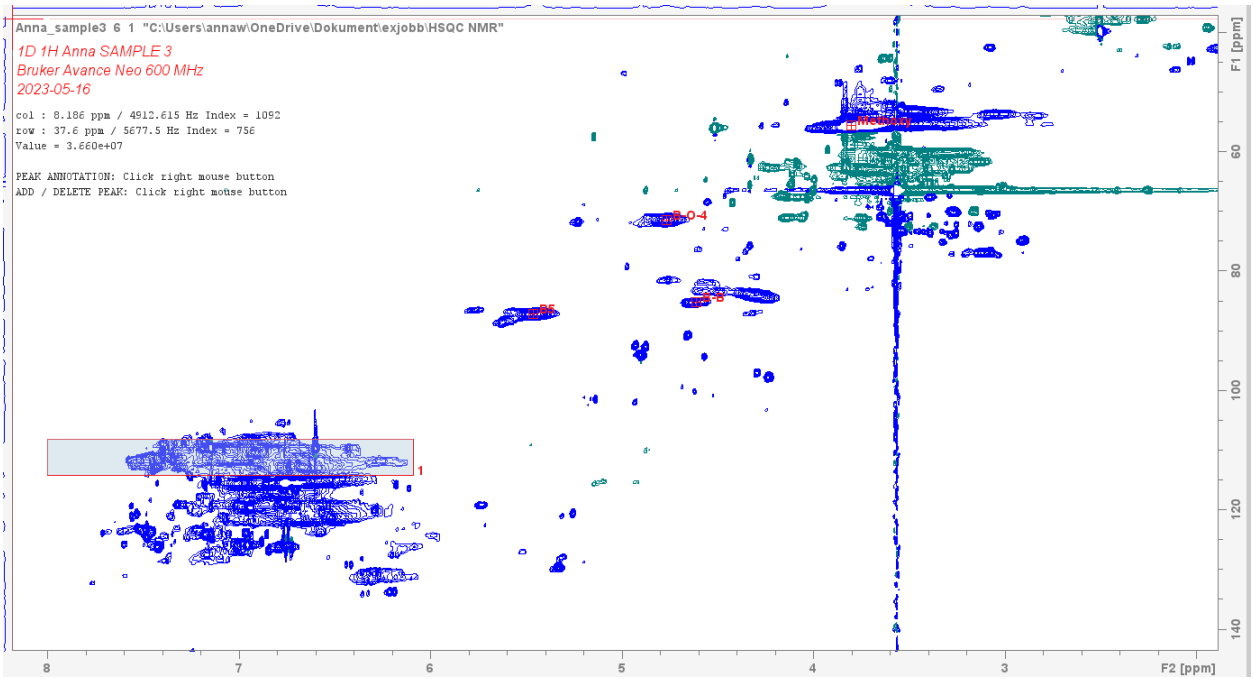

Figure 12 - *HSQC 2D-NMR spectra of lignin sample 3 extracted by 15 min STEX + MWL.*

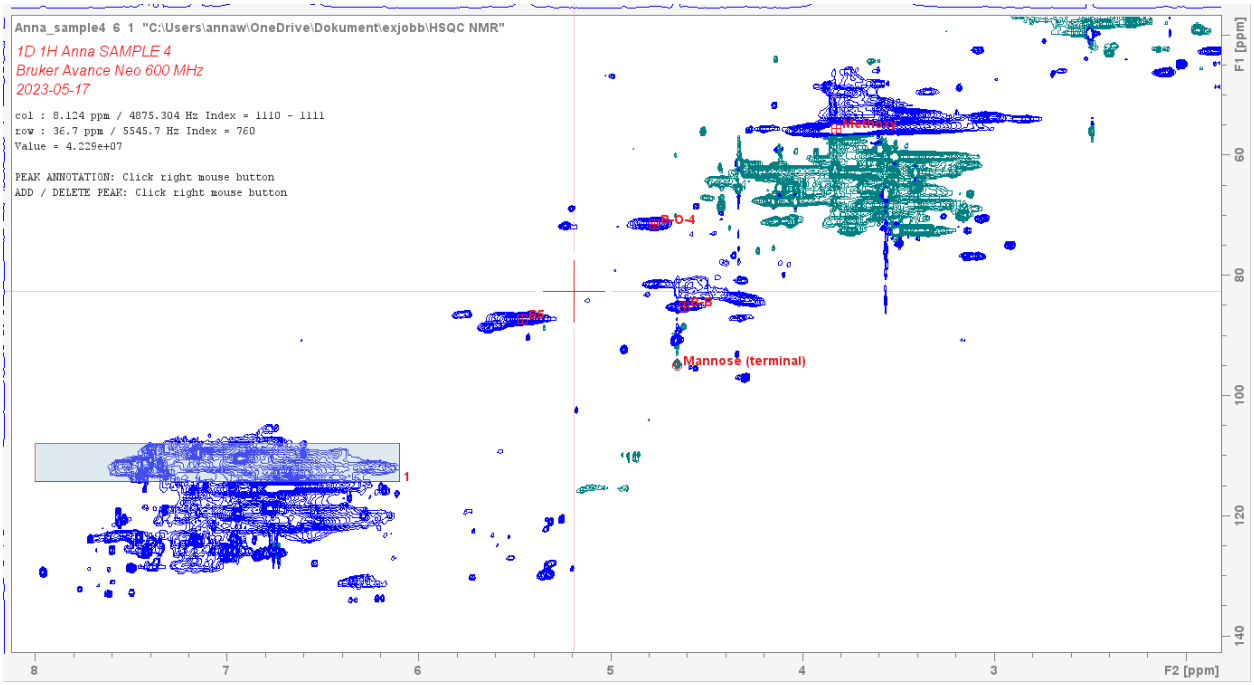

Figure 13 - *HSQC 2D-NMR spectra of lignin sample 4 extracted by 15 min STEX + enzymatic hydrolysis + MWL.*

The NMR spectrums in figures 10 to 13 show several qualities of the lignin obtained in the different extraction methods. First of all, the intramolecular bonds β-O-4, β-β, and β-5 are present in all samples. For a semiquantitative analysis of the intramolecular bond concentrations their respective peaks and the aromatic C2 carbon (area 1 in the figures) peaks were integrated. These results are presented and further discussed in table 4 below.

Besides the intramolecular bonds and G units present in the lignin, the NMR spectrums show signs of some sugars and aromatic methoxy groups present. In figure 10, clear peaks for both terminal xylose and terminal mannose were observed. Smaller peaks for terminal mannose were also detectable in figures 11 and 13 but not in 12. As the peaks are identified as carbons on sugars at the end of a polymer chain the peaks suggest either the presence of hemicellulose chains or that sugars have bonded into the lignin. Signals found in the region 62-63 ppm, 3.8-4.5 ppm suggest the possible presence of gamma-ester lignin carbohydrate complexes (LCC). Since the peaks for terminal xylose (and mannose in sample 3) disappear following the STEX severity, it could be assumed that less hemicellulose sugars are present overall due to a more severe pretreatment. However, the fact that figure 12 stands out without any detectable peaks for either mannose or xylose could be explained by the resonance decreasing the quality and credibility of the result.

Table 4 - *Semi-quantitative results on the percentages of intramolecular bonds in lignin in the different samples and in the literature (% bonds compared to number of aromatic units). The results for the samples tried in this work are derived from integration of the peaks in the HSQC 2D-NMR spectrum obtained.*

Without enzymatic hydrolysis				**With enzymatic hydrolysis**			
STEX duration	% β-O-4	% β-β	% β-5	**STEX duration**	% β-O-4	% β-β	% β-5
5 min (sample 1)	7.6	1.2	7.1	**5 min (sample 2)**	10.2	1.3	7.2
15 min (sample 3(2))	4.7	1.2	8.1	**15 min (sample 4)**	2.8	1.8	9.4
Values from the literature							
Pretreatment	% β-O-4		% β-β		% β-5		**Source**
STEX 210 °C 5 min	9.5		1.0		11.9		(Caputo et al, 2022)
Ballmilling	37		3		12		(Giummarella et al, 2016)

From the results presented in table 4 it can be concluded that there is an apparent cleavage of the β-O-4 bonds compared to values for lignin extracted only using the MWL method found in the

literature. Regarding the other intramolecular bonds, β-β and β-5, no apparent difference in β-β could be observed between the two STEX severities. For the β-5 bond however, there is a small but consistent increase in concentration following the STEX duration. This could be explained by the condensation of β-O-4 bonds into β-5. Overall though, it seems that the total bond concentration is decreased meaning that the harshness of the STEX decreases the polymer chain length. Comparing with the bond concentrations found in the literature it seems that the β-O-4 bond concentration was most affected by the STEX pretreatment even though a decrease in concentration of all bonds was observed.

When comparing the difference in intramolecular bond concentrations for the samples with and without enzymatic hydrolysis on the other hand, a small increase of 0.1 to 0.4% could be detected for the β-β bond when including the enzymatic hydrolysis step, however this change may be insignificant. For the β-5 bond an increase of 0.1 to 1.3% was observed when including the enzymatic hydrolysis step but due to the very small change, it may be regarded as insignificant as well. Regarding the β-O-4 bond, the results are ambiguous and no clear increase or decrease when including or excluding the enzymatic hydrolysis step from the process could be observed.

3.4, Hydrophobicity Testing

The weights of the lignin coatings applied to the filter papers are presented in table 5 below. From the results it can be concluded that the mass loss in the coating is large, up to 76%. This could be because a part of the weighted material did not dissolve in the acetone, leading to the solution being cloudy and having to be filtered through a 0.2μm syringe filter before being sprayed onto the paper. As the entire solution did not fit into the syringe, multiple syringes and filters had to be used and some of the lignin-acetone solution was therefore lost into the volume of the filters used. Moreover, the undissolved part of the mass was filtered away leading to a lower weight being applied in the end. The undissolved part could consist of sugars as the retentate in the filter appeared light in colour and mannose (and xylose) was detected in the HSQC 2D-NMR as discussed previously. For pictures of the residue in the solution see Appendix A.

Table 5 - *Coating masses for all samples prepared in the report.*

Sample	Extraction method	Lignin used (g)	Lignin applied (g)
1	5 min STEX + MWL	0.3681	0.0856
2	5 min STEX + enzymatic hydrolysis + MWL	0.3080	0.0882
3(1)	15 min STEX + MWL	0.4931	0.1924*
3(2)	15 min STEX + MWL	0.3315	0.0636
4	15 min STEX + enzymatic hydrolysis + MWL	0.3276	0.0881

**The filter paper was not weighed before so the number is an approximation based on the average filter paper weight and the weight of the filter paper and applied coating layer.*

As a part of the hydrophobicity analysis of the coated filter papers the coating was investigated more closely using a microscope. Pictures from the microscopy can be seen in figures 14 to 17. These pictures should be compared with that of the uncoated filter paper shown in figure 18.

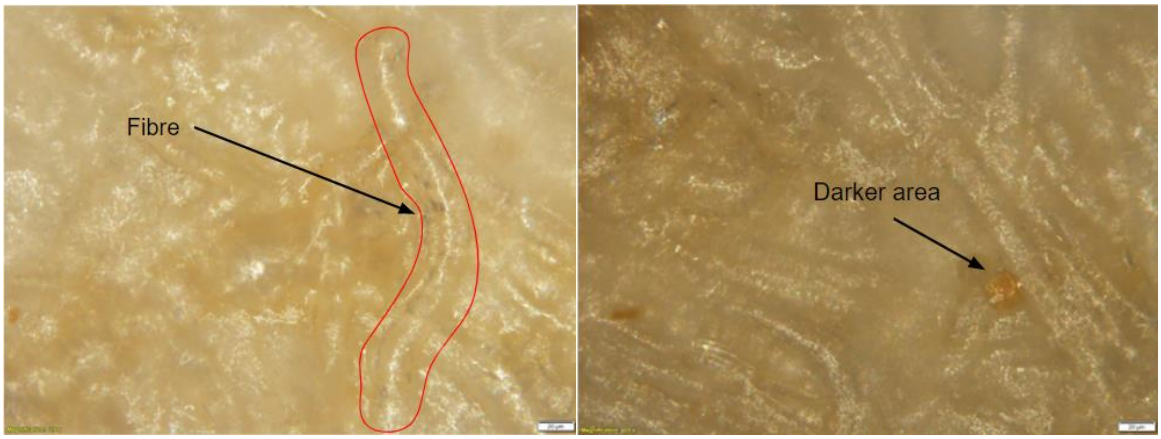

Figure 14 - *Close up pictures from microscopy of the filter paper coated with lignin extracted with: 5 min STEX + MWL (sample 1). The white square in the bottom right corner of the images is 20 µm long.*

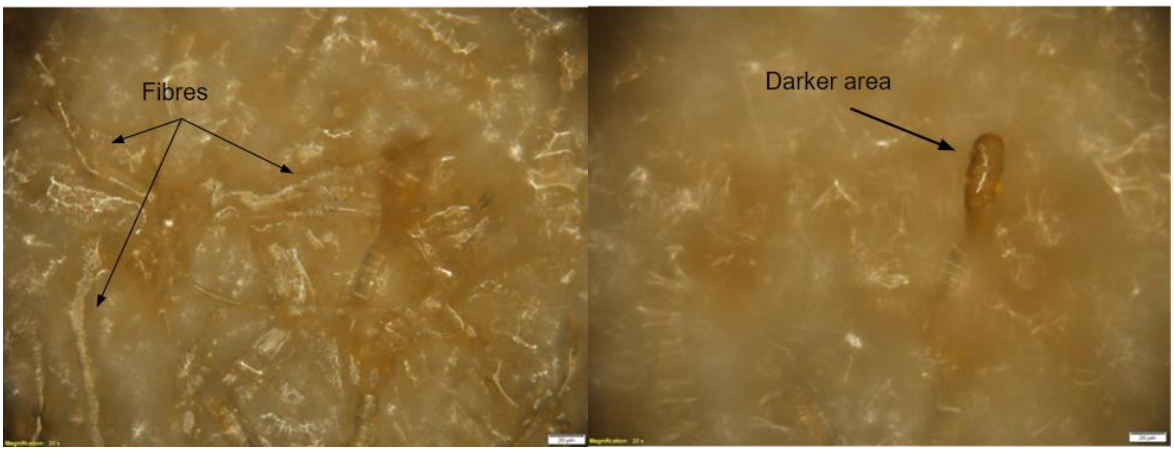

Figure 15 - *Close up pictures from microscopy of the filter paper coated with lignin extracted with: 5 min STEX + enzymatic hydrolysis + MWL (sample 2). The white square in the bottom right corner of the images is 20 µm long.*

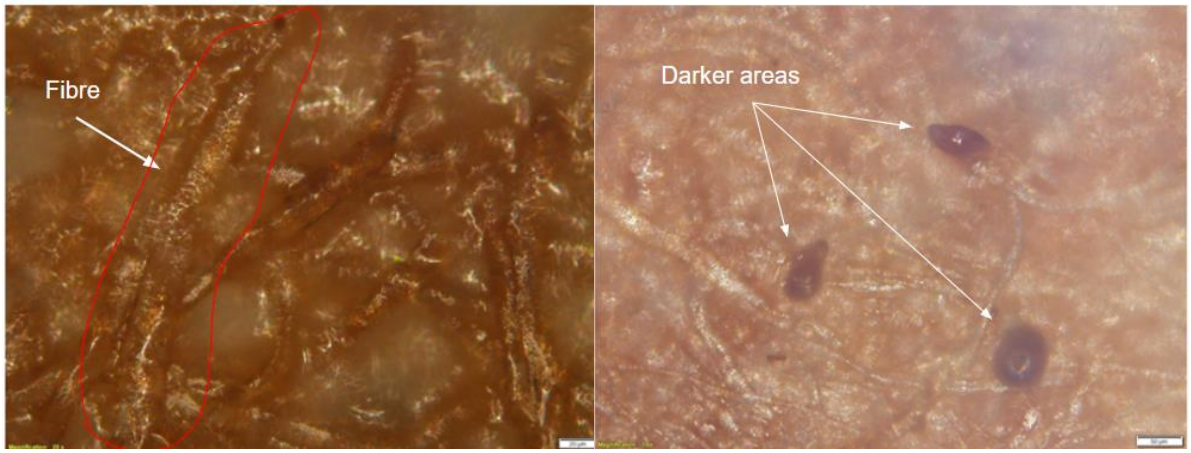

Figure 16 - *Close up pictures from microscopy of the filter paper coated with lignin extracted with: 15 min STEX + MWL (first photo is sample 3(2), second photo is sample 3(1)). The white square in the bottom right corner of the images is 20 µm long in the left image and 50 µm in the right image.*

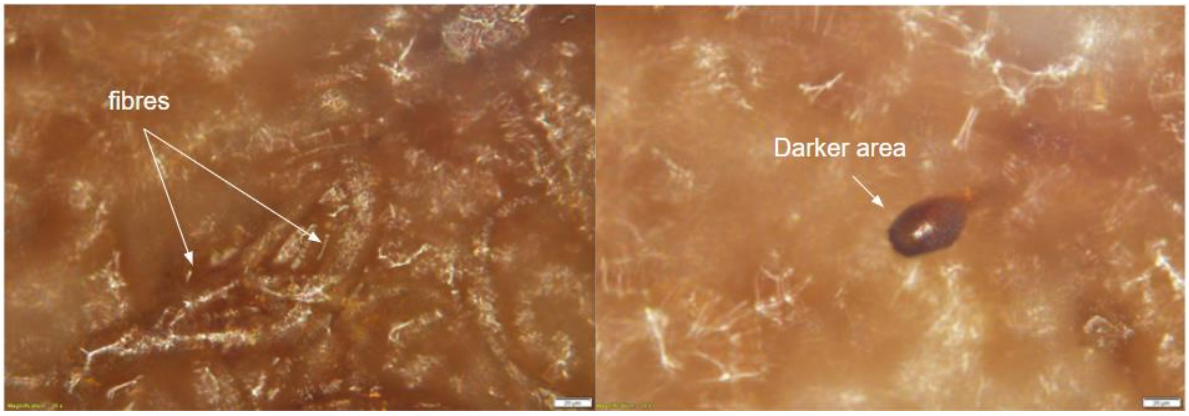

Figure 17 - *Close up pictures from microscopy of the filter paper coated with lignin extracted with 15 min STEX + enzymatic hydrolysis + MWL (sample 4). The white square in the bottom right corner of the images is 20 µm long.*

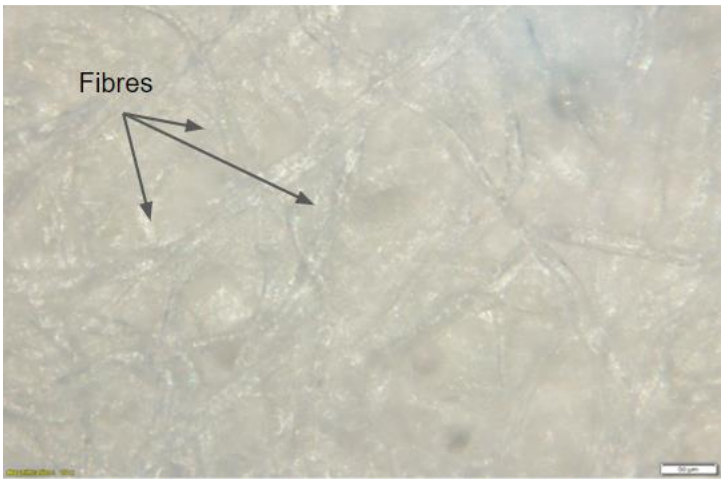

Figure 18 - *Picture from microscopy of uncoated filter paper. The white square in the bottom right corner of the image is 20 µm long.*

In the figures 14 to 18 above, a relationship between the severity of the pretreatment and the colour of the extracted lignin can be detected. The literature suggests that there is a correlation between a more condensed lignin and a darker colour of the material (Zhang et al, 2020), thus implying that the colour indicates a more condensed lignin in the 15 minutes STEX samples than the 5 minutes STEX ones. This result is further indicated by the increased concentration of the condensed bonds β-β and β-5 bonds relative to the uncondensed β-O-4 bond observed in the HSQC 2D-NMR spectrums, see table 4. The same article furthermore suggests that the colour darkening could be correlated with the aggregation of lignin, hence making this a possible explanation for the colour change observed here.

Furthermore, small darker areas were discovered in all the coatings. These could be because of uneven spraying or lignin aggregates in the lignin acetone solution sprayed onto the paper. The darker areas were of similar sizes in all of the samples. In the pictures it can also be seen that the surface is uneven and that the lignin coatings are not thick enough to cover the fibre structure of the paper which can be seen in figure 18. On the microscale no pinholes or cracks in the coating could be detected, it is however possible that they are visible on an even closer scale.

Moreover, it should be noted that in figures 14 to 17 the surface appears rugged, however it completely covers the filter paper with no pinholes to be observed on this scale. Nevertheless, pinholes might still be detected on a smaller scale.

Besides observing the coating through a microscope, a Cobb's test was performed on one of the lignin coatings and a droptest on all of the samples. The results from these tests are compiled in tables 5 and 6 below. For more detailed numbers and pictures of the droplets, please see Appendix A.

Table 6 - *Results from the Cobb test.*

Sample	**Absorption of water (g/m^2)**
Lignin coating (15 min STEX + MWL) (sample 3(1))	17
Filter paper	100*
Standard sized paper	20-25*

*Standard values of the industry, not tested in this work

From the results in table 6 it can be concluded that the addition of a lignin coating to a filter paper decreases the absorption of water per square metre dramatically. It should be noted that, as can be seen in table 5, the coating on the sample subjected to Cobb's test is slightly thicker than the other coatings. Moreover, the effect of the coating on the surface seems to be stronger than that of any sizing typically used in for instance a standard copy paper. For applications in the food packaging industry however, the value needs to be close to zero. It should furthermore be noted that this test should be performed on all the different coatings and different coating masses in order to adequately judge the performance and hydrophobic properties of lignin coatings on paper.

Table 7 - *Contact angles of water and diiodo-methane droplets at the lignin coating surface.*

Without enzymatic hydrolysis			**With enzymatic hydrolysis**		
Steam Explosion	Mean contact angle (water)	Mean contact angle (diiodo-methane)	**Steam Explosion**	Mean contact angle (water)	Mean contact angle (diiodo-methane)
5 min (sample 1)	110.48° +/- 5.96°	34.36° +/- 8.66°	**5 min (sample 2)**	103.77° +/- 5.93°	57.74° +/- 9.59°
15 min (sample 3(2))	102.42° +/- 7.89°	30.07° +/- 3.07°	**15 min (sample 4)**	107.41° +/- 6.00°	45.43° +/- 8.03°

Table 7 compiles the mean contact angles of water and diiodo-methane on the coated surfaces. Due to rapid absorption by the surface, no drop test could be performed on the reference uncoated filter paper. Thus, the results in table drop show an increased hydrophobicity of the surface with the lignin coating compared to without. Furthermore, it can be deduced that the mean contact angle appears slightly higher for the 5 minutes STEX coatings compared to the 15 minutes STEX ones. While this could be interpreted as an increase in hydrophobicity the difference is too small to be deemed as significant and more tests would have to be performed in order to confirm or reject this. Moreover, the difference between the surfaces treated and not treated with enzymatic hydrolysis

is small and inconsistent which makes it unlikely that the addition of an enzymatic hydrolysis step makes a difference in the performance of the lignin coating in packaging applications.

Important to note here is that the coating mass (see table 5 for all coating masses) is not equal for all the samples, and that sample 3(1) has a lower coating mass than the other which could explain the slightly smaller mean contact angle.

4. Conclusions

In conclusion, this work aimed to investigate and optimise the extraction method STEX followed by MWL with or without the addition of an enzymatic hydrolysis step for applications of lignin as a hydrophobic barrier in food applications. The overall conclusion to be drawn is that a lignin coating does have a hydrophobic effect on a strongly hydrophilic surface and therefore does show potential for replacing plastic as a hydrophobic barrier in the future. This is true for all extraction methods as all coatings showed a water droplet contact angle above 100°, however the 5 minutes STEX material (samples 1 and 2) showed a slightly higher value and was thereby potentially advantageous in hydrophobic barrier applications. Although, with the great difference in extraction yields between the 5 minutes STEX and 15 minutes STEX materials (9% compared to 24%), the small difference in hydrophobicity may be insignificant from an industrial point of view.

From the HSQC 2D-NMR, a condensation of the lignin was observed following the STEX duration as the concentration of β-O-4 decreased while the concentration of β-5 increased. The β-β concentration remained constant. Overall though, the total concentration of intramolecular bonds decreased. Hence, the observed effect of the pretreatment was a slightly more condensed lignin with fewer bonds, suggesting a net cleavage of β-O-4 and a lower molecular weight product.

It is possible that the cleavage of β-O-4 also yielded an increase in phenolic hydroxyl groups which could decrease the hydrophobicity. However, the hypothetical effect of the possible increase of these groups proved insignificant in this work, as no difference in hydrophobicity between the samples could be observed. Although, it should be noted that only one set of samples were tested and that a difference in hydrophobicity may have been detectable, if duplicates and triplicates of each experiment were made. Moreover, only one Cobb's test was performed due to a lack of material. Were the other samples to be tested with Cobb's test too there may have been a clearer answer to the question of how the condensation of lignin affects the hydrophobic properties of the coating.

Even though no pinholes or cracks could be seen in the surface structure analysis performed using an optical microscope, a Cobb value of 17 g/m^2 was measured, meaning that there was an absorption of water to the coated material. This could be explained by either the lignin not being hydrophobic enough or by the existence of pinholes or cracks on a smaller scale.

As for the enzymatic hydrolysis, the results are ambiguous, and it cannot be concluded that the addition of an enzymatic hydrolysis step increases either the extraction yield or the performance of the lignin coating. Although the sugar content analysis showed a degradation of especially cellulose, judging by the HSQC 2D-NMR results, this does not seem to have had a significant impact on the presence of hemicellulose sugars mannose and xylose in the extracted lignin.

5. Future Recommendations

For future work a few aspects would be of particular interest to investigate further. One of the biggest issues in this work was difficulty of extracting enough lignin for adequate testing. The most important reason for this is that the use of dioxane limits the volumes for all extractions, leading to many small extractions instead of a few large ones which would be more efficient. It would therefore be of interest to investigate if another solvent, acetone to mention one, could be used for the extraction step.

As already mentioned in the discussion part of this report it was not clear which one of the methods was best from a hydrophobicity point of view. For future work it would therefore be relevant to make a more careful investigation with more samples, duplicates and triplicates. This would improve the understanding of the effect of the steps in the extraction and hopefully provide a more detailed picture.

Another aspect to dig deeper into in future works is the role the layer thickness plays in the hydrophobicity. If the absorption of water during the Cobb's test could be explained by pinholes or cracks a thicker layer might solve the problem. Since lignin is brittle, it would furthermore be interesting to investigate if the addition of a plasticizer could improve the hydrophobic barrier properties of the coating.

In the literature, introducing modifications have been listed as something that could increase the hydrophobicity of lignin. The addition of a long fatty acid on carbon 3 or 5 in the lignin monomer would improve the hydrophobicity of the polymer.

Furthermore, additional research should be conducted to further explore the coating method employed. In this report the lignin was dissolved in acetone and then spray coated in layers onto the surface. The result was a hopefully evenly distributed surface, even though the microscopic evaluation revealed that the surface was also rugged and dusty. A smoother, glossy surface would have a smaller surface area and could therefore be advantageous in hydrophobic barrier applications. This may be achievable by for instance changing the coating method or adding a plasticiser.

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Appendix A

Appendix A compiles the most important pictures from the laboratory work together with the detailed HSQC NMR settings.

Table A1 - *The detailed HSQC NMR settings used in the analysis.*

Temperature	298.2°C
Pulse sequence	hsqcedetgpsisp2.3
Experiment	HSQC-EDITED
Probe	Z168362_0001(CP2.1 QCI 600S3 H&F/P/C-N-D05 Z XT)
Receiver gain	101
Relaxation delay	8 s
Acquisition time	0.1499 s
Spectrometer Frequence	(600.13,150.92) Hz
Spectral Width	(8196.7, 30211.5)
Lowest frequency	(-1101.3, -833.1) Hz
Nucleus	(1H, 13C)
Acquired size	(1229, 512)
Spectral size	(2048, 2048)
Digital resolution	(4.00, 14.75)

Figure A1 - Biomass after steam explosion. Samples left to right: STEX 5 min, STEX 15 min.

Figure A2 - Filter paper with lignin coatings. Samples left to right: STEX 5 min + enzymatic hydrolysis + MWL, STEX 15 min + MWL, STEX 15 min + enzymatic hydrolysis + MWL.

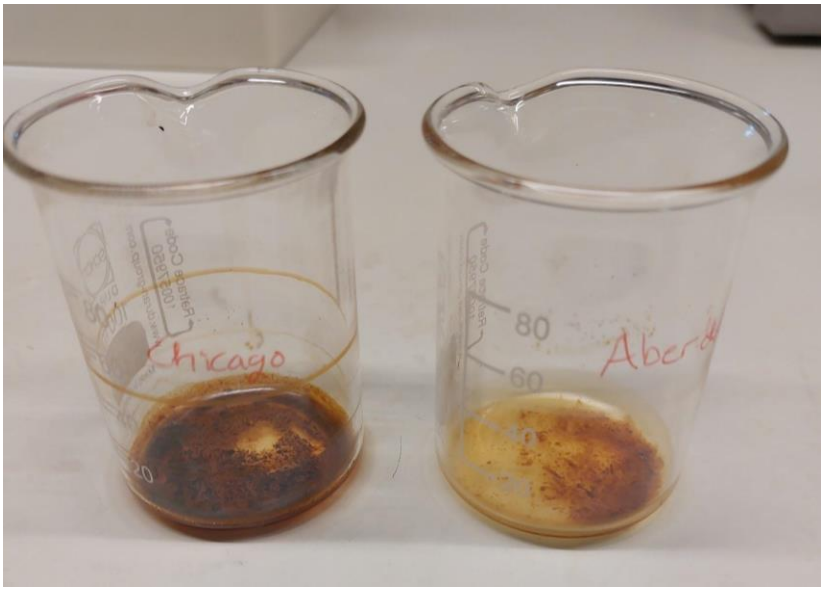

Figure A3 - Cloudy residue after dissolution of extracted lignin in acetone. Samples left to right: STEX 15 min + enzymatic hydrolysis + MWL, STEX 5 min + enzymatic hydrolysis + MWL.

Images from the drop test:

Figure A4 - Drop test on coating: 5 min STEX + MWL, left=diiodomethane, right=water

Figure A5 - Drop test on coating: 5 min STEX + enzymatic hydrolysis + MWL, left=diiodomethane, right=water

Figure A6 - Drop test on coating: 15 min STEX + MWL, diiodomethane droplet, water picture missing

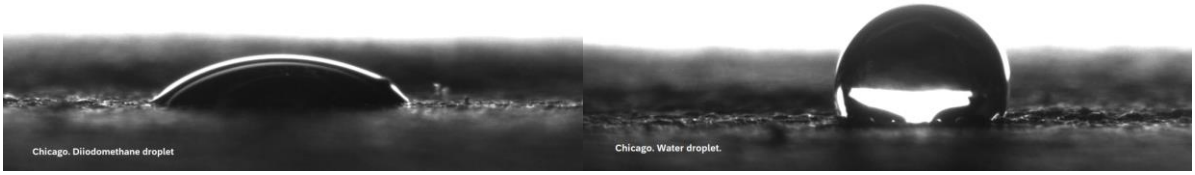

Figure A7 - Drop test on coating: 15 min STEX + enzymatic hydrolysis + MWL, left=diiodomethane, right=water

Appendix B

This appendix compiles the most important calculations made in this report.

Yield calculations:

The AIL (acid insoluble lignin) and ASL (acid soluble lignin) were determined to the equations found in the NREL procedure in https://www.nrel.gov/docs/gen/fy13/42618.pdf as referred to in the report. The total lignin content of the raw material was calculated using equation 1 and the yields were then calculated using equation 2 as shown below.

$$Lignin_{g/g\ dry\ raw\ material} = \frac{AIL + ASL}{Mass_{Dry}} \quad (1)$$

$$Yield\% = 100\% \times \frac{Lignin_{Extracted}}{Mass_{Biomass\ used\ (dry)}} \times Lignin_{g/g\ dry\ raw\ material} \quad (2)$$