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Developing and implementing a method for analytical purposes

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CHALMERS UNIVERSITY OF TECHNOLOGY
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Cover: Illustration of a river used for transportation of logs in Sweden during the 20th century.

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Isolation and characterisation of residual lignin in wood and pulp
A method development study with the aim to isolate and characterise residual lignin in wood and pulp using the green solvent Cyrene
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Abstract

This study aims to develop and evaluate a method for isolating and characterizing residual lignin from wood and pulp using enzymatic treatments and a novel extraction approach with the biomass derived solvent Cyrene. The research focuses on optimizing parameters such as enzyme concentration, treatment duration, and temperature to enhance lignin extraction efficiency. The characterization techniques NMR and GPC are employed to analyze the isolated lignin's structure and composition.

Results indicate that the combination of repeated enzymatic treatment and Cyrene extraction is effective in producing high-purity lignin comparable to that obtained historically using dioxane. There are however some structural differences that need further research.

Keywords: Lignin, Hardwood, Enzymes, HSQC, Cyrene, Ball-milling, Isolation, Method, Degradation.

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Sofia Bosson and Herman Miller, Gothenburg, May 2024

List of Acronyms

Below is the list of acronyms that have been used throughout this thesis listed in alphabetical order:

B_f	The estimated occurence of a linkage per C9-unit
B_I	The integration of a cross-peak representing a lignin linkages
C_9-unit	A combined term for the lignin base units (H-, G-, and S-unit).
CLAF	Crude Lignin Acidified Fraction
CLF	Crude Lignin Fraction
DMSO	DiMetylSulfOxid
G-unit	Guaiacyl
GPC	Gel Permeation Chromatography
HPLS	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Coherence spectroscopy
H-unit	p-Coumaryl alcohol
IC_9	The value representing the amount of C9-units in a NMR sample
IG_2	The integration of the cross-peak signal from the second aromatic carbon in guaiacyl.
$IGS_{2,6}$	The integration of the cross-peak signal from the second and sixth aromatic carbons in syringyl.
KL	Kraft Lignin
L	Loss of weight
LCC	Lignin-Carbohydrate-Complex
NMR	Nuclear Magnetic Resonance
OSL	OrganoSolve Lignin
MWL	Milled Wood Lignin
PEG	PolyEthylene Glycol
R	Residual wood
RPM	Rotations Per Minute
S-unit	Syringyl
SL	Soda Lignin
TAPPI	Technical Association of the Pulp and Paper Industry
W	Waste fraction
MWEL	Milled Wood Enzyme Lignin
CEL	Cellulase Enzyme Lignin
BRL	Brown Rot Lignin

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1
Introduction

Wood, a simple and at the same time very complex materials that grows beside us. The journey to utilise the possibilities within this material has been an ongoing project for humans ever since we learnt how to use tools. In recent years, focus has been directed towards using wood derived chemicals and materials to substitute oil based ones in order to lower our carbon footprint [1]. The unknown scale of environmental change caused by the release of greenhouse gases drives a desire to locate and utilize alternative renewable sources for the services and products that society desires to maintain and develop our lifestyle [2].

Figure 1.1: An example of how wood could be transported using rivers in Sweden during 20th century.

Throughout history, humans have used wood for protection, as a heat- and energy source as well as a material source when developing tools, ships, and houses. As time has passed and societies developed so has the knowledge of wood utilization, but with the growth of both the human population and the need for these bio-based chemicals there is a necessity to maximize the yield of the chemicals and products derived from this bio-resource.

The pulp and paper industry is a sector that has the potential to be one of the major players in both learning and teaching us how to maximize the yield of wood products. To achieve this the sector is investing in research and development, one part of this journey is to map and understand the chemistry and material streams related to the pulp and paper processes. Through gaining this knowledge, the industry will have an increased ability to meet the growing demand for bio-based products while decreasing waste flows, energy requirements, and emissions.

1.0.1 The Kraft process

Until the 19th century, the majority of paper was made by hand from cotton rag, but around the 1840s Germany began experimenting if it was possible to substitute the rag with fibres derived from trees [3]. This led to the development of the sulfate- also known as Kraft- and sulfite method for the extraction of cellulose fibers in the 1870s. These method traveled quickly to the Swedish sawmills since they offered a solution to the dilemma of large waste flows of wood considered to be of low quality.

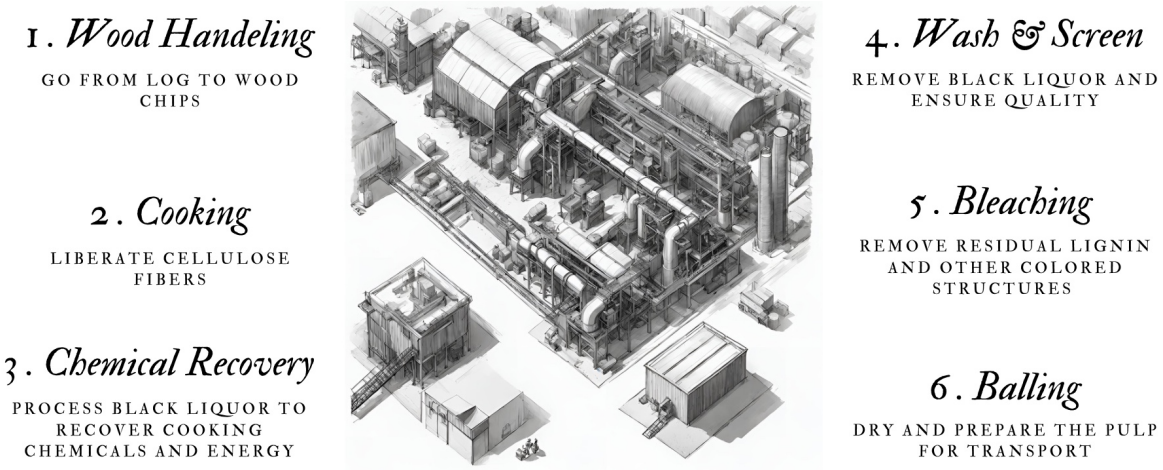

Figure 1.2: Summary of the steps in the Kraft process.

Today, the most common practice used for wood separation is the Kraft process, the main steps of this process when going from wood trunks to pulp can be seen in Figure 1.2 [4]. Even though the Kraft process is a well established process there is still a need to understand the complete mechanisms of the reactions and transport phenomena occurring in the cooking of wood when lignin is made soluble in order to liberate cellulose fibers. During the delignification lignin is transported from the wood into the cooking liquor. To understand these transport and reaction phenomena one approach is to isolate the residual lignin from the solid material at different stages of the cooking and apply analytical methods to characterize the structure and changes of the residual lignin through the cooking. To isolate lignin from a solid material a number of analytical methods are available, one weakness all methods

share is that the isolation procedure modifies the lignin structure. Another observed challenge of using these methods is that they have primary been used on softwood or uncooked wood. Because of this, there is still a need to continue the development of additional analytical methods for determining the molecular weight and structure of the residual lignin in solid material.

In Figure 1.3 the four steps of the cooking in the Kraft process are presented, the cooking can either be done in a continuous manner or batch wise. The cooking is initiated by steaming the wood chips, this is done in order to preheat the chips as well as to remove air from within the wood structure. This is necessary since any air left in the wood structure will prevent the cooking liquor known as white liquor from efficiently permeating into the chips. Once the air is removed, wood is mixed with white liquor in order to impregnate the wood and start the delignification process. During this step, the lignin and hemicellulose structure in the wood is largely fragmented and dissolved into the cooking liquor leaving a majority of cellulose fibers in a solid state.

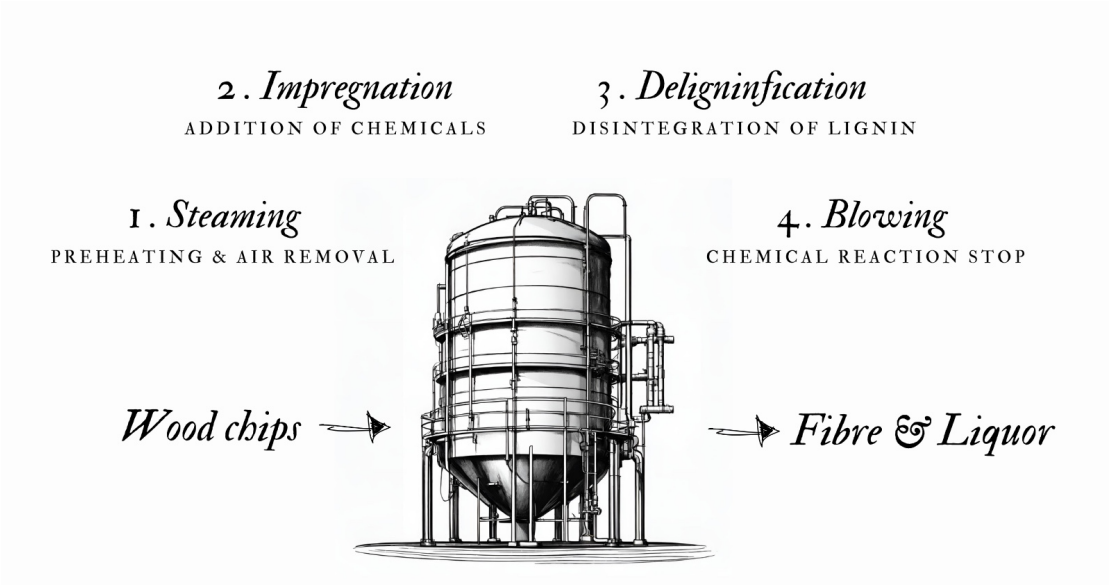

Figure 1.3: An overview of the treatment steps in the digester of the Kraft process.

Depending on the type of wood, hardwood or softwood, the time needed for the delignification step varies, it has been shown that hardwood delignifies faster than softwood because of the different lignin content and structure of the woods [5]. After delignification, the temperature is lowered to stop the chemical degradation of lignin and carbohydrates. The mixture of cooking liquor containing dissolved lignin fractions, carbohydrates, extractive, and the remaining fibre structure exits the digester and is washed resulting in a cellulose fiber fraction and a liquid fraction called weak black liquor.

Black liquor is a mixture of both inorganic and organic materials. To recover as much of the chemicals and energy as possible black liquor enters a chemical recovery plant.

In this plant, the black liquor is concentrated and combusted to generate steam for the process and recover the cooking chemicals needed in the digester.

1.0.2 Aim

The aim of this project is to evaluate and develop and implement a method for extraction and characterization of residual lignin from wood and partly delignified wood for analytical purposes. It is important to design the extraction method so that the lignin structure remains unmodified or goes through predictable structural changes. The selected method should be able to characterize the extracted lignin in terms of molecular weight and structural motifs.

2
Theory

In this chapter will explain the ultra and molecular structure of wood as well as the theory behind enzymatic digestion and lignin dissolution will be presented together with a complete presentation of the lignin molecule.

2.1 Wood structure

A tree can belong to one of two groups, hardwood trees or softwood trees. The difference between these lies both in their outer morphology, as seen in Figure 2.1, but also within the structural and compositional complexity of the tree [5]. Hardwood is genetically more evolved with a complex morphology and large structural variations between species while softwood has a simpler morphology and little variability in structure between species.

Figure 2.1: Illustration of two trees, a softwood and a hardwood tree.

Depending on the objective one can look at a tree on different levels and observe different structures as well as functions. This thesis will move between two levels, the ultrastructure and the molecular structure in order to create a context as well as a wider understanding of the subject discussed.

To understand the source of lignin, knowledge of the ultrastructure of wood and its cell wall structure is needed. A common way of describing this structure is by dividing the cell wall into different layers as seen in Figure 2.2 [5].

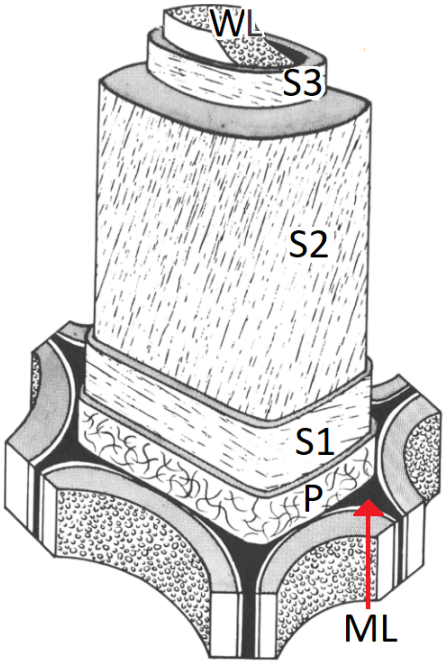

Figure 2.2: The different layers of the cell at the ultrastructure. ML = Middle Lamella, P = Primary cell wall, The second cell wall is divided into three layers (S1, S2, and S3), and WL = the Warty Layer. Adapted from the work of H. Höglund [5].

The outside of the cell is surrounded by a section called the middle lamella (ML) which is not part of the cell wall but rater an intercellular space filled with lignin glueing neighbouring cell together. The first layer of the actual cell is the primary cell wall (P), and then the secondary cell wall (S) which is further divided into three layers (S1, S2, and S3), and last layer of the cell wall is the warty layer (W).

The content in the layers consists of four main molecular structures, cellulose, hemicellulose, lignin, and extractive [5]. The highest lignin content is found in the ML [6]. The lignin concentration will thereafter go down in each consecutive following layer [7]. Figure 2.3 illustrates the change in chemical composition within the cell wall from the ML to the S3 layer.

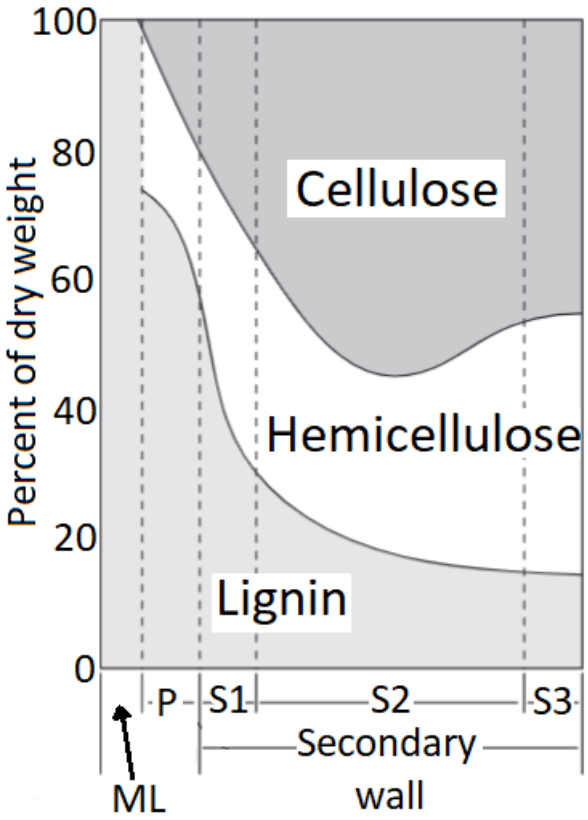

Figure 2.3: A visualisation of how the chemical content changes within the cell wall. Adapted from the work of W. A. Côté [7].

The cellulose found in these layers is organised in macrofibrils. A macrofibril is in fact a bundle of smaller microfibrils which consist of a number of cellulose chains [4]. These chains are assembled into sheets that stack on top of each other due to intramolecular bonds [8]. The bonds holding together this arrangement are hydrogen bonds and van der Waals bonds. This organisation of the cellulose chains is possible due their homopolymer structure with extensive possibilities for intra-and intermolecular interactions. Cellulose always consist of only D-glucopyranose ring units no matter the source. These units arraign themselves in the $^4C_1$-chair conformation which is the lowest energy conformation and also allows for the previously mentioned intra- and intermolercular interactions. A polymer is formed when these monomers connect with each other in a straight chain with β-1,4-glycosidic bonds with every second monomer turned 180°.

As previously mentioned, bundles of microfibrils assemble to form a macrofibril. These bundles are created by the previously mentioned intramolecular forces between cellulose surfaces. Hemicelluloses and lignin are arranged between cellulose fibrils. They will further stabilise the structure as seen in figure 2.4

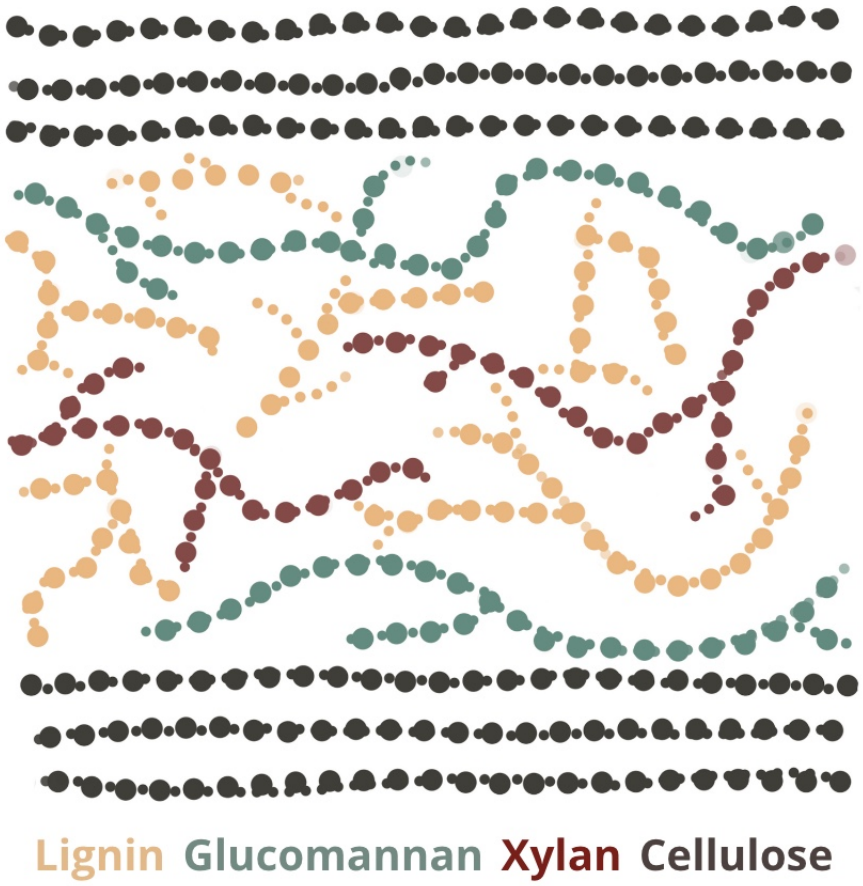

Figure 2.4: A representation of lignin and hemicellulose is located between two macrofibrils providing more stability.

The arraignment of macrofibrils in the cell walls varies depending on where in the tree they are located as it is important to provide favourable mechanical properties to the fiber. This arrangement is described by a microfibrillar angle, i.e. orientation of cellulose fibrils with the respect to the fiber axis [8]. The P layer consists of an irregular network of macrofibrils whilst the S1, S2, and S3 have an organised arraignment of their macrofibrils [7]. They are almost completely parallel to each other within the same layer. The S1 layer has a microfibrial angle of 50-70°. S2 has two different microfibrial angles, 5-10°for late wood, and 20-30°for earlywood. The inner layer of the secondary wall, S3, has a microfibrial angle of 50-90°.

2.2 Lignin

All processes that extract lignin will modify its structure to a high degree. This has caused a lack of knowledge regarding native lignin structure. This section will give a general account of lignin's role in the wood, where it can be located in the wood, How the lignin structure varies depending on its location in the cell wall and plant in general, and a description of the anticipated structure of lignin.

2.2.1 Lignin structure

Lignin is regarded as the most complex molecular structure found in wood and at the same time the least understood one [9]. The complexity of the lignin structure originates from the fact that it is not a true polymer. It has properties of polymers in the sense that it consists of repeating amounts of base units, phenyl propane. However, the linkages between these base units do not follow a predictable pattern and create a random mesh of ether and carbon-carbon linkages [4]. The base units in question are called monolignols which are illustrated in Figure 2.5.

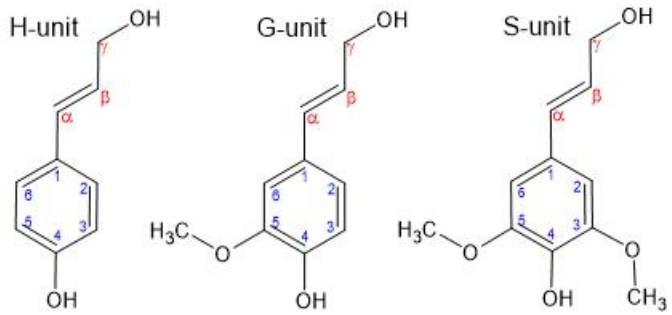

Figure 2.5: *p*-Coumaryl alcohol (H-unit), Coniferyl alcohol (G-unit), and Synapyl alcohol (S-unit).

The frequency of different base units found in lignin can be used to differentiate which type of lignocellulotic material it is derived from. H-units are mainly found in grasses which otherwise contain an even mixture of all base units, softwoods contain predominately G-units, and hardwood has an amount of G-units similar to grass but the main component is the S-unit [10]. Softwood lignin has been shown to contain similar structures between species but that is not the case for hardwoods [11]. The main structural difference amongst hardwood lignin is the ratio between G- and S-units depending on from which species it originates. The expected syringyl to guaiacyl ratio found in hardwoods like birch should be around 1.85 [12].

The distribution of different monolignols within the cell wall is generally regarded to be uneven [13]. The lignin synthesis begins in the cell corners of the ML and moves inwards towards the secondary cell wall. The monolignols synthesized during this process differ depending on where in the cell wall it occurs. The early synthesis in the ML results in a higher concentration of guaiacyl whereas the later formation in the secondary cell wall mostly results in the formation of syringyl.

The following section will describe some common lignin bonds based on the work of Katahira, Elder, and Beckham [14]. See Figure 2.6 for a graphic representation of the bonds.

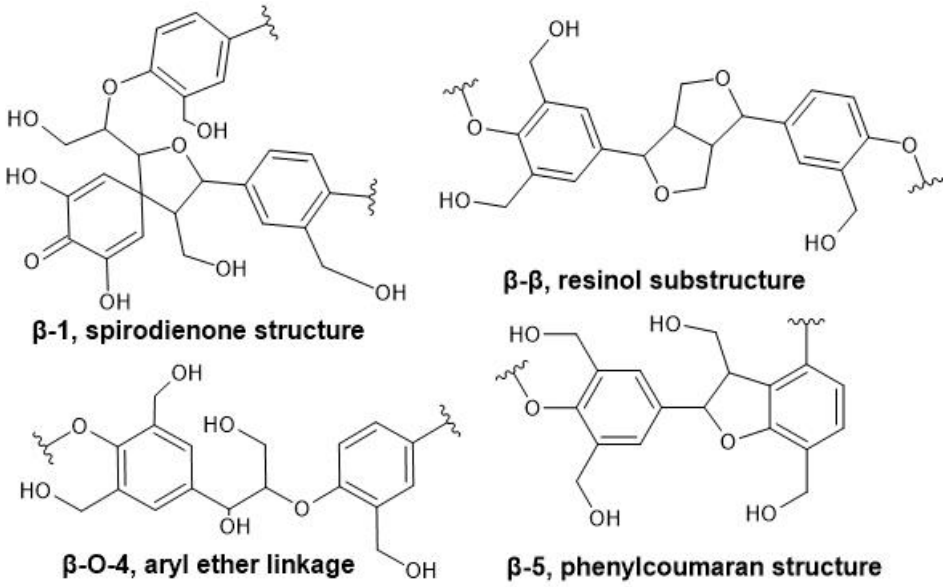

Figure 2.6: Lignin linkages involving the β carbon.

The most common linkage between two base units within the lignin macromolecule is the aryl-ether (β-O-4) linkage. This bond is linked to 40-60 % of all C9 units found in both softwood and hardwood lignin. Another common bond between base units is the $\beta-\beta$ bond. The formation of this bond creates a resinol unit. It is characterised by the formation of two 5-member-rings ($\alpha_A-\beta_A-\beta_B-\gamma_B-O-\alpha_A$ and $\alpha_B-\beta_B-\beta_A-\gamma_A-O-\alpha_B$). The subscript distinguishes if the carbon in the bond is from the unit A or B. The linkage mentioned above is found in both hard- and softwoods but in higher amounts in the hardwoods. The resinol unit is not the only common ring linkage in lignin. Another 5-member-ring found in lignin is the phenylcoumaran structure which is formed by the $\beta-5$ bond ($\alpha_A-\beta_A-5_B-4_B-O-\alpha_A$) and is reported to have a frequency of 5 to 7 % in hardwoods. A more complicated connection is the spirodienone structure. It involves 3 base units (A, B, and C), two are connected with a 5-member-ring and the last with the $\beta-O-4$ bond. Unit A is connected to B with an $\beta-1$ connection, B is also connected to A with a $\alpha-O-\alpha$ bond which forms the ring ($1_A-\beta_B-\alpha_B-O-\alpha_B-1_A$), the last connection is a β_A-O-4_C bond between unit A and C. Table 2.1 compares the occurrence of these bonds in aspen bassed on the work of Wang et al. and birch based on the work of Katahira, Elder, and Beckham [15], [14].

Table 2.1: Different content of lignin linkages per 100 C9-unit found in wood [14], [15].

Bond	Occurrence
$\beta-O-4$	64.3 %
$\beta-\beta$	13.7 %
$\beta-5$	4.6 %
$\beta-1$	2.777 %

a)Aspen [15]

Bond	Occurrence
$\beta-O-4$	60 %
$\beta-\beta$	3-5 %
$\beta-5$	5 %
$\beta-1$	3-4 %

b) Birch [14]

Another common linkage is the ester bond that connects end groups to the base units in the polymer chain [14]. These end groups are formed from incomplete monolignol biosynthesis resulting in products containing acyl groups. They often connect to the main polymer at the gamma position of a base unit. It has been documented that up to 10 % of the C9 units found in native aspen lignin contain the γ-p-hydroxybenzoate substituents, see Figure 2.7, as an end group.

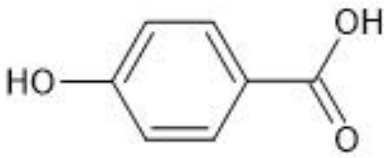

Figure 2.7: The end group p-hydroxybenzoate.

Lignin is not constructed exclusively by forming bonds between monolignols, there are also covalent bonds between lignin and polysaccharides which form lignin-carbohydrate-complexes (LCC) [14]. Some of the linkages in LCC are benzyl ether, γ-ester, conjugate γ-ester, and phenyl glycoside bond. The formation of covalent linkages between hemicellulose and lignin is more likely than that between cellulose and lignin. This is because lignin has a higher reactivity and physical contact with hemicellulose than it has with cellulose [16].

The frequency of different linkages found in the lignin samples was derived by comparing the integration of linkage cross-peaks to the combined cross-peaks of syringyl and guaiacyl in 2D NMR spectra[15]. The combination of syringyl and guaiacyl gave a representation for the amount of C9-units (IC_9) in the sample. Equation 2.1 was used to estimate the integration of the C9-units-

$$IC_9 = 0.5IS_{2,6} + IG_2 \tag{2.1}$$

were the signals from C2 (see Fig. 2.5) in guaiacyl (IG_2) and C2 and C6 (see Fig. 2.5) in syringyl ($IS_{2,6}$) were used. The reason for only using half of the signals from syringyl is because its C2 and C6 is seen as identical to the NMR which results in a multiplication of two for the syringyl signals while C2 in guaiacyl is unique and thus only has a multiplication of one for its signal. The frequency of a linkage (B_f) is derived by dividing the integration of the signal from a specific bond (B_I) with the IC_9 value obtained in eq. 2.1 as seen in Equation 2.2. [15].

$$B_f = \frac{B_I}{IC_9} \tag{2.2}$$

2.3 Isolation of lignin

To isolate native lignin one approach is to degrade the surrounding structures, cellulose, and hemicellulose, using enzymes to increase accessibility of lignin for a subsequent extraction. In addition to the enzymatic treatment, mild acidolysis during

the extraction step can also be performed to further liberate the lignin from the surrounding carbohydrates by breaking the bonds between the lignin and the remaining carbohydrates.

2.3.1 Enzymes

When looking at biochemical reactions the catalyst involved is often denoted as an enzyme. An enzyme is a protein that lowers the activation energy required for a reaction to take place, just as a catalyst [17]. This reaction occurs when the substrate attaches to the enzyme at its active site and is later released as a product as seen in Figure 2.8.

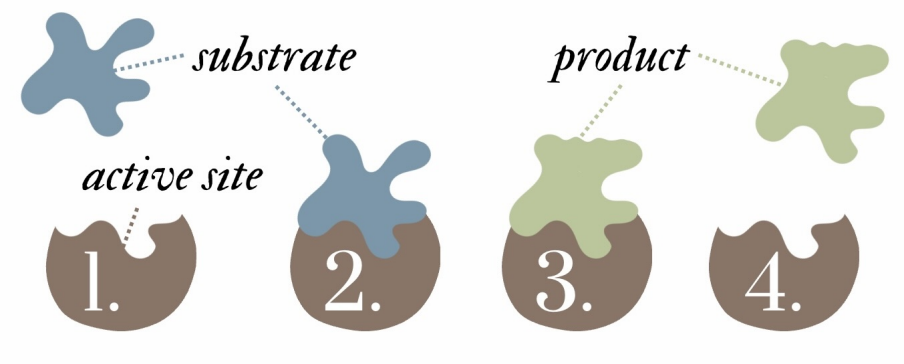

Figure 2.8: A substrate arriving atq the reaction site of an enzyme and being catalyzed to a product.

The efficiency of an enzyme can be affected both positively and negatively by environmental factors such as temperature and pH [18]. When looking for enzymes to degrade wood one can try to copy the mechanisms of nature. Fungi has shown to be an excellent provider for several enzymes that degrades wood components such as cellulose, hemicellulose, and lignin.

A major group of diverse enzymes called cellulases are key players in the process of degrading insoluble cellulose into soluble sugars. Cellulases are found closely related to organisms that degrade wood, as an example cellulases can be derived from Trichoderma which is a genus of fungi in the family Hypocreaceae [19]. In an article, Wilson summarizes thoroughly the different strategies and stages used by cellulolytic microorganisms when degrading cellulose in plant walls. The first step is the secretion of a synergistic set of free cellulases, then the production of multienzyme complexes called cellulosomes, and finally an unknown mechanism that does not require processive cellulases [20]. Today three types of cellulases are known, endoglucanases, exocellulases, and processive endoglucanases.

Cellulase and hemicellulase cocktails can be used to isolate lignin from wood samples by soaking the sample in an enzyme mixture, the enzymes will degrade cellulose and hemicelluloses leaving a solution of soluble sugars and a solid insoluble lignin.

Enzyme inhibitors refer to molecules that lower the catalytic activity of the enzyme. This occurs due to the inhibitor preventing the interaction of enzyme and substrate by blocking the enzyme's active site [21]. The blockade occurs due to the inhibitor changing the amino acids of the enzyme, the activity is mainly inhibited by reversible, irreversible inhibition, covalent, and non-covalent binding. The inhibiting substance can be added to the reaction to regulate the enzyme but also be an undesired product of the reaction.

2.3.2 Acidolysis

Acidolysis is carried out by exposing the sample to acid. The free protons found in acidic conditions can act as a catalyst and cleave the ether bonds discussed in the previous chapter. The strength of these bonds differ and it is generally accepted that the carbon-carbon bonds are stronger than the ether and ester bonds [22]. There are two main pathways according to which the cleavage of the $\beta - O - 4$ bond can take place under acid conditions [23]. One of the pathway that can occur results in an end product containing a three-carbon-chain connecting to an aryl group. That pathway is the cause of the formation of Hibbert ketones and can be seen in Figure 2.9.

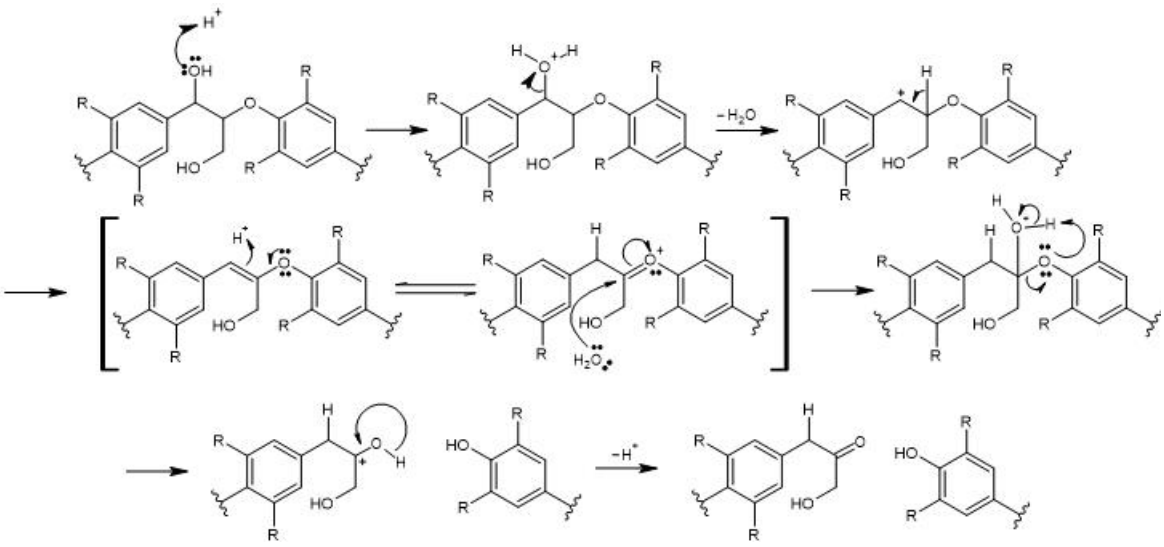

Figure 2.9: Reaction mechanism for the formation of a Hibbert ketone.

The cleavage of $\beta-O-4$ will start with the protonation of the hydroxyl group on the α carbon followed by an elimination of the newly formed oxonium ion. The reason for this starting location is that the aromatic ring next to the α carbon will stabilize the carbocation. As previously stated, this is just on of the pathway that can occur during acidolysis, the other pathway will form aldehydes and formaldehydes as products that then undergo condensation reactions and form new carbon-carbon bonds like $\beta - \beta$ and $\beta - 5$. Finaly, the formation of Hibbert ketones together with an increase of $\beta - \beta$ and $\beta - 5$ has been reported as a consequence of acidolytic cleavage of the $\beta - O - 4$ bond [16].

2.3.3 Reported methods for lignin isolation

Milled Wood Lignin (MWL) is a well established method based on mechanical degrading wood into a fine powder. Dioxane is used as a natural solvent to extract the lignin from the wood powder but also to increase the purity of lignin by dissolution and precipitation. The extracted lignin is often considered appropriate for further lignin studies where the native lignin is of interest even though this procedure causes certain structural changes to the lignin [24].

Milled Wood Enzyme Lignin (MWEL) is similar to MWL, the sample is ball milled and then treated with an enzymatic step in order to solubilize the remaining carbohydrates. MWEL has been reported to be a relatively good representation of native lignin but the method is challenged by a reduced solubility of MWEL in common lignin solvents and a carbohydrate residual of 10 to 12 %. Another alternative to MWL is cellulase enzyme lignin (CEL), similar to MWEL, enzymes are used to solubilize carbohydrates but when precipitating the lignin, CEL uses fractioning based on the solubility of lignin in the solvent and water. To increase the yield of lignin a mild acidolysis step can be introduced between the enzymatic treatment and the lignin extraction, this method is called Enzymatic Mild Acidolysis Lignin (EMAL) [25]. Using EMAL results in a more extensive modification of the structure but a higher and purer yield compared to MWL, MWEL, and CEL. A common solvent for lignin in these methods is dioxane[24].

Klason lignin is an example of another well established method where polysaccharides are hydrolyzed by sulfuric acid and form water-soluble sugars that are easy to separate from the remaining insoluble lignin. This method provides a relatively good measurement of the lignin content in a sample but the remaining lignin is highly condensed and altered, Klason lignin is therefore not representative of the native lignin but is useful for approximate quantification of lignin [24].

Brown Rot Lignin (BRL) is obtained by allowing a brown rot fungus to degrade the wood until 60-70 wt% of the weight is lost. To isolate lignin from the remaining wood and fungi an extraction or purification step is needed. This can be done by allowing the wood to dry and mill it, BRL can then be solubilized and precipitated using a suitable solvent [24]. There is a limited number of studies using BRL but one study done by Jin et al. has reported brown rotted wood to have a distribution of insoluble Klason lignin, hemicellulose and cellulose of 89.5 wt%, 1.6 wt% and 8.7 wt% based on the dry weight of the wood before extraction, the total lignin yield after an extraction step with organic solvents and NaOH was measured to 30.4 wt% [26].

An overview of lignin yields obtained by using different extraction and isolation methods can be seen in Table 2.2. The methods presented in the table are a selection of methods that extract a lignin fraction anticipated to be representative of the native lignin [24],[26], [27], [28], [29].

Method	Yield
Milled wood lignin	15-20 wt% [24], [25], [27]
Milled wood enzyme lignin	8-35 wt% [24]
Cellulase enzyme lignin	15-40 wt% [24], [25], [28]
Brown rot lignin	30-40 wt% [24], [26]
Enzymatic mild acidolysis lignin	25-83 wt% [25], [29]

Table 2.2: An overview of observed lignin yields for different isolation methods in earlier studies.

The values presented in Table 2.2 are a compilation of different studies and are reported in ranges to consider different reporting methods and specifics between the studies.

2.4 Dissolution of lignin

A few organic compounds have been found to act as relatively efficient solvents for lignin. Dioxane, pyridine, and DMSO are some of these solvents but, as the knowledge about these chemicals has increased as well as the awareness about the safety issues when handling some of them, a suitable alternative solvent is needed to replace them in future studies with lignin.

2.4.1 Acetone as a solvent

A more lenient option for a solvent of lignin is aqueous acetone which offers a safe, non-toxic, and environmentally sound solvent [30]. The highest solubility of lignin using aqueous acetone occurs at a concentration between 60-80 v% and any deviation from this will drastically decrease the solubility of lignin. It has been shown that lignin fractions of low molar mass dissolve in lower concentrations of acetone and as the molar mass increases so does the required acetone concentration for dissolution. Using aqueous acetone as a solvent has proven to result in high purity lignin fractions when precipitated and analyzed.

Depending on the purpose, aqueous acetone as a solvent of lignin can be sufficient. However, one should be aware that lignin fractions of a high molar mass will not dissolve using aqueous acetone thus limiting the applications of using acetone as a solvent for lignin.

2.4.2 Cyrene as a solvent

Dihydrolevoclucosenone, also known by its product name Cyrene, is a relatively new compound derived from cellulose. Cyrene is a bio-degradable, non-mutagenic, and non-toxic solvent that can, with water, completely dissolve technical lignin and therefore offers an alternative to previously used lignin solvents [31], [32]. Pure Cyrene has been shown to dissolve lignin poorly. However, if water is added, the efficiency of Cyrene as a solvent for lignin has been proven to increase to be a suitable

substitution for dioxane. The reason for the improved solubility with the addition of water is due to the formation of geminal diol and the increase of hydrogen bonding capacity which enables a better dissolution. The formation of the diol can be seen in Figure 2.10

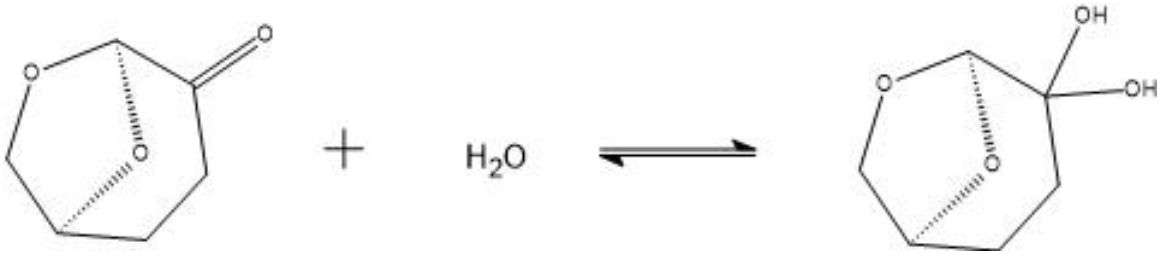

Figure 2.10: The formation of the geminal diol by the addition of water to Cyrene.

As seen in Figure 2.11, the dissolution ability of the Cyrene-water solution can be altered by changing the proportion of Cyrene to water. By doing this, different fractions of lignin can be extracted to facilitate further lignin characterization.

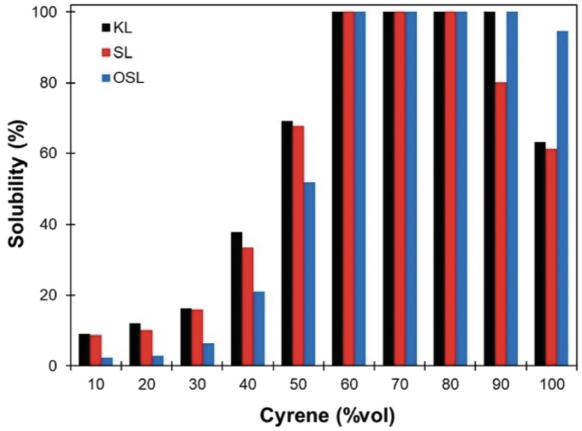

Figure 2.11: Solubility of three different technical lignins, Kraft Lignin, Soda Lignin, and OrganoSolv Lignin (KL, SL, and OSL) in Cyrene-water mixtures at a concentration of 50 g/L, depending on the proportion of Cyrene to water. Adapted from the work of Duval and Avérous [32].

2.5 Lignin analytics

There are several methods to characterise the lignin structure in the extracted samples. This report relied on HSQC and GPC to give a semi-quantitative representation of the isolated samples' linkages as well as their molecular size relative to each other.

2.5.1 ^{1}H ^{13}C HSQC NMR

HSQC (heteronuclear single quantum coherence) is an NMR method that combines the one-dimensional spectra from ^{1}H-NMR and the spectra from ^{13}C-NMR resulting in a highly detailed 2D spectra [33]. The one-dimensional spectrum from the ^{13}C-NMR is assigned to one axis and the ^{1}H-NMR spectrum to the other [34]. The intersection of these two spectrums creates cross-peaks that are used to identify signals from different structural motives. A limitation of HSQC is that ^{13}C signals from carbons not associated with any hydrogen will not create any cross-peaks and therefore not show up on the 2D spectra. In addition, the method is primarily qualitative as the mutual enhancement of ^{13}C and ^{1}H signals varies among the structural motives.

2.5.2 GPC

Gel permeation chromatography (GPC) is an analytical method used to determine the molecular weight of a polymer sample based on its retention time during passage through a porous packed column [35]. The column is packed with particles that contain pores of various diameters. The space of the column that is not occupied by the partials is filled with a solvent. The same solvent must then be used to dissolve the sample which is to be run in the GPC. When the dissolved sample is run through the column, larger polymers will avoid the smaller pores and pass quickly through the column while the smaller polymers will enter even the smaller pores and thus have a longer retention time in the column.

3
Methods

This chapter describes the methodology used to derive the method for lignin extraction and characterisation. The workflows and design of experiments can be found in this chapter while the resulting method to isolate lignin is found in the results.

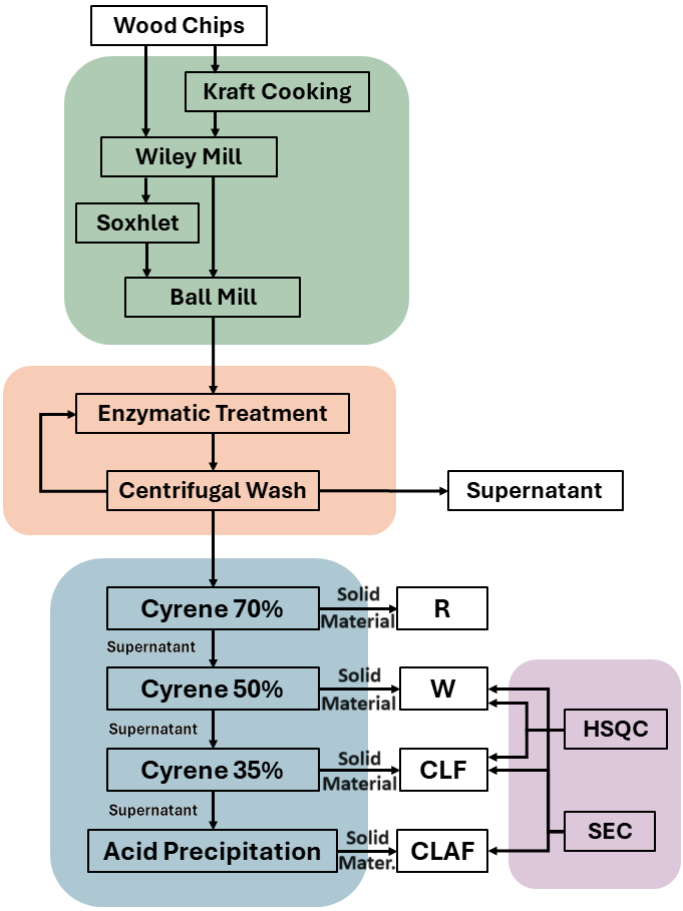

Figure 3.1: The method consists of four main parts, the pre-treatment of the wood seen in green, the enzymatic degradation step seen in orange, the Cyrene extraction of crude lignin seen in blue, and finally the analytical step in purple. The white boxes indicate raw material, as in the case of the wood chips, or an obtained product. R is the insoluble residue after the Cyrene extraction, W is a waste fraction believed to contain mostly hemicellulose, CLF is the crude lignin fraction which contains the most promising lignin fractions, and CLAF is the crud lignin acidified fraction containing the smallest fractions of lignin.

3.1 Preparation of samples.

Aspen and birch chips were treated in three ways to prepare a range of varying wood structures for investigation of lignin isolation.

3.1.1 Extractive removal

Two samples, aspen and birch with a size of 20 mesh were obtained from Wiley milling and treated with acetone in a soxhlet apparatus to remove the extractives from the wood. The extraction was performed according to the TAPPI standard "Solvent extractives of wood and pulp" (T 204 cm-97) [36]. The setup consisted of two soxhlet apparatuses with their condensers connected in series. The extraction flasks were heated to make the acetone evaporate up into the soxhlet apparatus. The extractions were always run in parallel with one aspen sample and one birch sample. The extractions either used a 4 g sample and 300 ml acetone or a 2 g sample and 150 ml acetone. Each extraction was aimed to be 24 cycles with a cycle period of 10 minutes.

Each sample was loaded in a paper thimble and left to dry in an oven at 100°C for 1 hour. The combined weight of the dried thimble and sample was measured before it was placed in the soxhlet apparatus. The thimble and the sample were then removed after approximately 24 cycles, placed in a glass beaker, and left to air dry in a fume hood overnight. Thereafter the thimble with wood was placed in a 105°C oven, after one hour the weight was recorded and compared to the pre-extracted weight. Any difference in weight was assumed to be because of lost extractive. The leftover acetone from the extraction was collected in pre-weighed containers and left in a fume hood for two days to allow the acetone to evaporate. The containers were then weighed to give an additional estimation of the amount of extracted extractive.

3.1.2 Kraft cooking

Four 50 g sets of wood chips were prepared for Kraft cooking using autoclaves and following Appendix 2, two of aspen and two of birch. The autoclaves were placed on a rotational axis in a PolyEthylene Glycol, PEG, bath which held a temperature of 160°C. After 30 minutes one tube of aspen and one tube of birch were recovered from the autoclave. The chips were washed from black liquor and placed in a plastic bucket filled with distilled water to remove the remaining black liquor. After 90 minutes the remaining two tubes were removed from the autoclave and the same washing procedure was repeated. After one night the water was changed, this was repeated until the water remained colorless after one night of soaking with the chips. The water was then removed and the chips were placed in an oven set to 105°C overnight or until dry.

3.1.3 Milling

The treated wood was placed in a ball mill in batches between 2 to 4 g together with 50 ceramic balls. The wood was ground for 10 minutes followed by a five minute pause to keep the temperature in the mill down, this was repeated 19 times. To ensure that the particle size of the grounded wood was smaller than 20 μm microscopic pictures were taken. The grounded wood was transferred to a container with a spoon for storage until needed.

3.2 Enzymatic treatment

To find a suitable method for lignin recovery different parameters related to enzymatic wood digestion were examined. This was done by setting up potential ranges as seen in Table 3.2 for the parameters where the optimal setting was believed to exist based on literature study. The parameters studied were temperature, enzyme to wood ratio, and the effect of using a mixture of different enzymes to isolate lignin. To develop the method an iterative approach was applied, therefore between each treatment, the results of the previous treatment were analyzed in order to find possible successful and unsuccessful settings.

To achieve a controlled temperature for the enzymatic treatment as seen in Figure 3.2 several water baths were installed with a thermometer controlled hotplate stirrer. The setup made it possible to control the temperature as well as the stirring during the treatment.

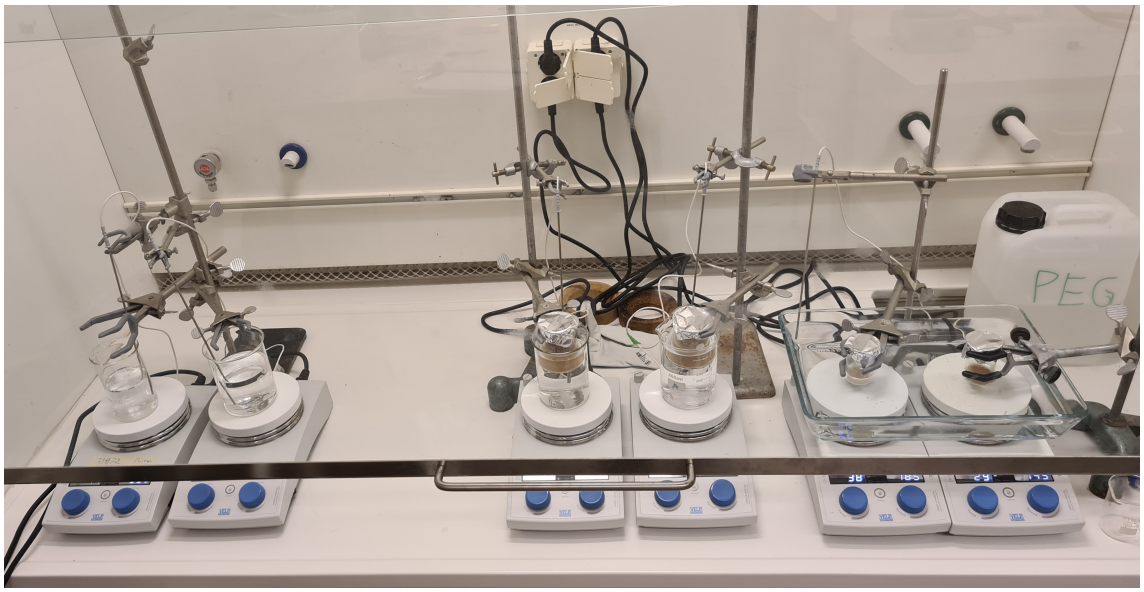

Figure 3.2: The experimental setup for enzymatic digestion where the treatment temperature was controlled by using a water bath and heating plates.

When starting an enzymatic treatment the amount of enzyme to wood weight was decided together with the proportion of each enzyme. The enzymes used were cel-

lulase from Trichoderma species with an activity of 3-10 U/mg and hemicellulase from *Aspergus niger* with an activity of 3 U/mg. For calculations, the activity of cellulase enzyme was assumed to be 6 U/mg. Another enzyme mixture called "life" consisting of the hemicellulases beta glucanases and xylanases was also tested but the activity of the enzymes were unknown. The amount of buffer was set to 10 ml per g of dry wood. This recipe was based on either the previous experimental results or inspired by design parameters in Table 3.1. The wood was weighed to a four digit precision and placed in a beaker. Enzymes and the buffer solution were added according to the experimental recipe. A magnet was placed in the beaker to stir the mixture throughout the treatment period and the beaker was covered with alumina foil. The beaker was placed in the water bath and left for the set treatment time.

Table 3.1: Summary of the investigated parameter intervals for temperature, enzyme dosage, time, number of treatments, and enzyme mixtures.

Parameter	Lower value	Upper value
Temperature	20 °C	50 °C
Enzyme dosage	100 U	4000 U
Cellulase:Hemicellulase	1:0	0:1
Treatment time	1h	48h
Number of treatments	1	4

When the treatment time was reached the wood and enzyme mix was transferred to a 50 ml falcon tube and washed by repetitive centrifuging. The supernatant was separated from the pellet and saved in a separate container for further treatment and analysis. The pellet was dispersed in deionized water and the same procedure was repeated two times. The recovered supernatant was placed in a fridge for further observations and mass balances. Figure 3.3 shows an example of saved supernatantes.

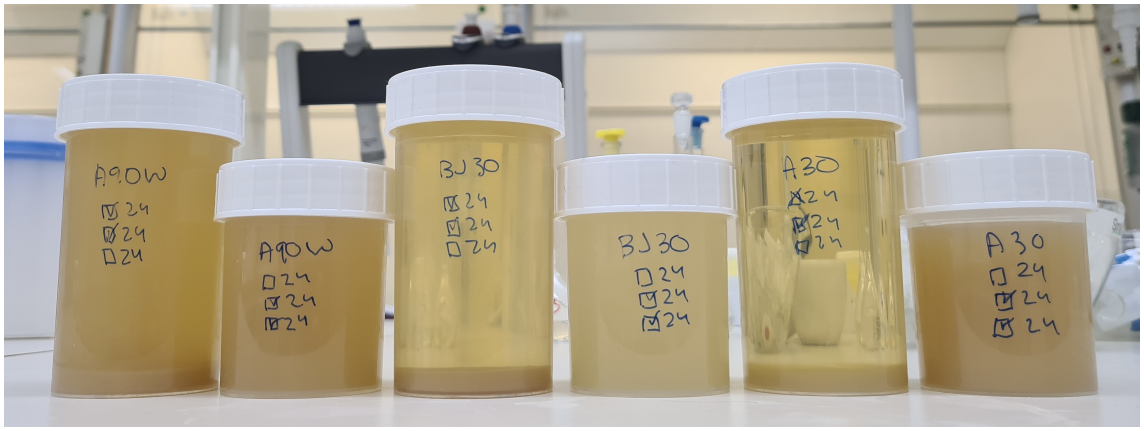

Figure 3.3: The combined supernatants from the washing of the enzyme treated wood. The supernatant is a mixture of buffer and deionized water.

The remaining pellet was either freeze dried for storage and analysis or purified in a later treatment step.

If the supernatant in the container was homogeneous a 1 wt% H_2SO_4 was added to the supernatant until a pH between 2 and 3 was reached. The solution was left at room temperature for two hours and then placed in a freezer overnight. This was done to precipitate any lignin in the supernatant. If the supernatant had begun to settle and two phases had formed the clear upper phase was separated to another container and the same procedure with 1 wt% H_2SO_4 was performed. The lower cloudy phase was freeze dried and saved for further analysis.

To quickly verify the effect of an enzymatic treatment the freeze dried material was weighed and compared to the starting weight of the wood. If the weight had decreased by more than 30 wt% the isolation procedure continued to the next step and if not the material was recovered for another enzymatic treatment with another design of the treatment.

The development of the enzymatic treatment method followed an iterative procedure where the results of the previous attempts were used to improve the treatment conditions for the following treatment design.

3.3 Cyrene purification

The method used for extracting the pure lignin from the material obtained after the enzymatic treatment was derived from a study about the solubility of technical lignin made by Duval and Avérous [32].

1 ml of 70 v% aqueous Cyrene per 50 mg of dry weight in sample material were mixed in a round bottom flask. In some cases, a few drops of 1 wt% H_2SO_4 were added to reach a pH between 2 and 3. The flask was then placed in an oil or water bath at 100 °C for at least 16 hours. The insoluble residue, the R fraction, left after the heat treatment was separated from the solution via centrifugation in a 50 ml falcon tube at 4 700 rpm for 5 minutes. The supernatant was decanted to a new falcon tube and the remaining pellet was thoroughly washed with deionized water. Three more fractions were obtained by precipitating them out from the supernatant. This was done by dilution, first to 50 v% Cyrenen and then to 35 v% Cyrene. This resulted in the precipitation of the fractions named W (Waste) and CLF (Crude Lignin Fraction) respectively. The precipitate was collected between each dilution step in the same manner as before, by centrifugation at 4 700 rpm for 5 minutes. The collected pellet was then washed thoroughly with deionized water. The washing step consisted of re-suspending the pellet in deionized water, centrifuging it, and then discarding the supernatant. This was done 3-4 times or until the supernatant was clear and the strong smell of Cyrene was almost gone. The falcon tubes containing the final pellet were pre-weighed such that the final weight of the pellet could easily be measured after freeze-drying.

A final fraction named CLAF (Crud Lignin Acidified Fraction) was obtained by precipitating the remaining amount of lignin found in the Cyrene supernatant from the 35 v% dilution. This precipitation was performed by acidifying the solution to a pH of 2-2.5. The solution was then left to rest for two hours before being placed in the freezer. The CLAF fraction was collected, washed, and dried in the same manner as the previously mentioned ones.

3.4 Lignin analysis and characterisation

3.4.1 Quantification of lignin according to the Klason method

The lignin content of the samples was evaluated with the Klason method at various points in the process. This method is only suitable for quantitative analysis due to the harsh chemical treatment of the sample [37]. The goal is to form an insoluble lignin residue by treating the lignocellulosic sample with 72 wt% H_2SO_4 which causes acid hydrolysis of the non-lignin content of the sample. The following procedure was used in this study.

3 ml of 72 wt% sulphuric acid was added to 200 mg of oven dried sample. The sample mixture was then placed in a vacuum for 15 minutes after being thoroughly mixed. A water bath at 30 °C was then used to further treat the sample. This was done for 1 h with stirring of the sample every 20 minutes. 84 g deionized water per 200 mg sample was added after the water bath. The beaker containing the sample was then covered and placed in an autoclave for 1 h at 125 °C. The sample was left untouched until the autoclave reached 90 °C after terminating the heat treatment. The insoluble lignin residue in the sample was isolated by filtrating the sample through a pre-weighed glass filter. The filter was then dried in an oven before a final weighing. The amount of isolated lignin was then compared to the amount of starting material to determine the lignin content of the sample.

3.4.2 HSQC analysis

The following method and NMR sequence used was provided by Liyang Liu. 30-40 mg of the freeze dried sample from the extracted lignin was dissolved in 1.5 ml DMSO-d6. The solution was then transferred to 5 mm NMR tubes. The tubes were then delivered to the NMR Centrume where the 2D HSQC NMR spectra were acquired using a Bruker AVANCE III HD Console equipped with a 5 mm TXO cryoprobe. A standard pulse program (hsqcetgpsisp2.3) was used with the following parameters: a 20.03 ppm sweep width in F2 (^{1}H) centered at 4.699 ppm acquiring 3072 data points, and a 200 ppm sweep width centered at 100 ppm in F1(^{13}C) acquiring 512 data points. The acquisition included 24 number of scans, a 1.0 s relaxation delay, and a total acquisition time of approximately 4 hours.

3.4.3 GPC analysis

Gel permeation chromatography (GPC) was used to compare the sizes of the extracted lignin fractions. The solvent used for this analysis was a solution of DMSO and lithium bromide with 0.872 g of LiBr per liter DMSO.

10 to 20 mg of the sample was dissolved in 1 ml of the aforementioned solvent. The samples were left to dissolve over night with the occasional re-agitations. 20 μm filters were used to remove any unwanted solids remaining in the solution before the samples were loaded into the GPC machine.

4
Results and Discussion

In this chapter, the final protocol for lignin isolation and characterisation is presented together with the results and observations related to the establishment of the protocol.

4.1 Working with the wood

The developed method suggests going from a solid material to a lignin powder through partial degradation of the starting material, solubilization, and precipitation of the lignin fractions. In this section, the difficulties of working with the material and important observations are summarized for the different stages of the method and presented in Table 4.1.

Table 4.1: Observations made about the material throughout the procedure.

Method step	**Observations**
Wiley milling	Large loss of material due to inefficient mill.
Ball milling	The cooked wood samples were much easier to recover, with almost no material loss when transferring between containers.
	The uncooked wood became sticky and a loss of 5 wt% of the loaded material was normal.
Freeze drying	Easy to handle.
Enzymatic treatment	When transferring the material to another container this was easiest done by dissolving the wood in water or the buffer and washing out all of the wood to the new container.
Cyrene extraction	A good yield of lignin was to be expected if the solution obtained a dark colour after the 16 hours of treatment.
	A general mass loss of 20 to 50 wt% is to be expected of a successful lignin extraction.
	Cyrene has a strong scent which is still quite noticeable in the obtained fraction even after three washes with deionized water.

4.2 Enzymatic treatment

In Table 4.2 the Klason lignin content of the starting material for the experiments is shown. Klason measurements were made on two replicates for each sample and a mean value of the replicates is presented in the table. The different experiments conducted assumed that the values given are a true representation of the lignin fraction in the starting sample, but the conclusions are made with the knowledge that the true values may have small deviations from the results seen in Table 4.2 due to the method being sensitive to incorrect weight measurements and potential loss of material in moments involving transferring the insoluble lignin. The initial lignin content of aspen and birch in Table 4.2 is lower than expected when comparing to literature values where the lignin content of both species should be closer 20% [4]. This could possibly be a consequence of the soxhlet extraction that preceded any further treatment of wood.

Table 4.2: Klason lignin percentage in starting wood for the enzymatic treatments where 30 and 90 represent the number of minutes the wood has been cooked.

Raw material	Klason Lignin
Aspen	15.46 %
Birch	17.11 %
Aspen 30	14.99 %
Aspen 90	4.66 %
Birch 30	17.53 %
Birch 90	4.69 %

The success of the lignin extraction using enzymatic degradation ranges between 1 wt% and 61 wt% of all the available lignin, the enzymatic treatments that resulted in the highest and lowest fraction of extracted lignin are presented in Table 4.3 and 4.4, the full dataset is found in Appendix 3.

To represent the washes of the sample and indicate any changes made between the washes, one sample can contain several rows of data in the tables. If any settings are changed this results in a new row where the new parameters are shown, if no changes are made between a wash this is indicated in the time column. An example: 12h:3 represents a sample treated for 12 hours three times with washes between each 12 hour session and no parameter changes between these sessions. The extracted lignin in Table 4.3 and 4.4 is reported as crude lignin (C.Lignin) and represents the weight of the lignin derived after the purification compared to the starting weight. The lignin is denoted as crude because of a reaming carbohydrate fraction in the extracted lignin. The percentage of extracted lignin determined the success of an experiment and the settings used.

Table 4.3: The enzymatic treatments which resulted in the largest fraction of crude lignin (C.Lignin) after purification using Cyrene. The results are derived using the starting weight of the sample, the Klason percentage of lignin in that sample and the final weight of the crude lignin derived from the sample to calculate the percentage of the extracted C.Lignin.

Wood	Weight	Cellulase	Hemicellulase	Temperature	Time	C.Lignin
Birch 30$_2$	3.0103 g	133 U/g	199 U/g	50°C	48h:1	
		149 U/g	50 U/g	37°C	24h:1	**61 %**
Birch 30$_3$	4.0048 g	125 U/g	50 U/g	40°C	24h:2	
		85 U/g	50 U/g	40°C	24h:1	**50 %**
Aspen$_3$	1.0679 g	187 U/g	118 U/g	37°C	3h:1	
		94 U/g	81 U/g	37°C	24h:1	**47 %**
Aspen$_2$	1.0239 g	293 U/g	315 U/g	37°C	20h:1	
		24 U/g	52 U/g	37°C	40h:1	**38 %**
Birch 90$_1$	4.0258 g	96 U/g	56 U/g	37°C	24h:1	
		96 U/g	99 U/g	37°C	24h:3	**36 %**

Table 4.4: The enzymatic treatments which resulted in the lowest fraction of of crude lignin (C.Lignin) after purification using Cyrene. The results are derived using the starting weight of the sample, the Klason percentage of lignin in that sample and the final weight of the C.lignin derived from the sample to calculate the percentage of the extracted C.Lignin.

Wood	Weight	Cellulase	Hemicellulase	Temperature	Time	C.Lignin
Aspen 90$_1$	4.0013 g	150 U/g	45 U/g	37°C	24h:2	
		75 U/g	25 U/g	37°C	24h:2	**1 %**
Aspen$_5$	2.0039 g	0 U/g	-	37°C	24h:2	**2 %**
Birch$_1$	1.0002 g	0 U/g	-	20°C	1h:1	**2 %**
Aspen$_1$	1.0016 g	99 U/g	170 U/g	37°C	43h:1	**4 %**
Birch$_4$	2.0050 g	0 U/g	-	37°C	24h:2	**5 %**

The numbers seen in Table 4.3 indicate that the method of using enzymatic treatment followed by a Cyrene extraction for lignin isolation has the potential to isolate a significant amount of the available lignin in both cooked and uncooked wood for analytical purposes. A majority of the experiments have resulted in an extraction of lignin between 30-50 wt% with a purity of 83 wt%.

In three samples seen in Table 4.4 the charge of the added hemicellulase is unknown, the hemicellulases enzyme consists of a mixture of beta glucanases and xylanases, this enzyme mixture is denoted as "life". The experiments using these enzymes constantly resulted in low lignin yields, which is reasonable since the enzymes only

target the hemicellulose structures of the wood. The result is expected but can offer guidance for future experiments when choosing suitable enzymes for this purpous. To only degrade the hemicellulose structure might increase the porosity of the wood sample thereby making the lignin more accessible for the Cyrene to dissolve the lignin but this was found ineffective.

When looking for correlations on how to predict the success of a treatment Figure 4.1 offers the best guidance. Weight losses under 30 wt% and above 90 wt% of the starting material can immediately be discarded as failed. If the weight loss is low the enzymatic step can be repeated.

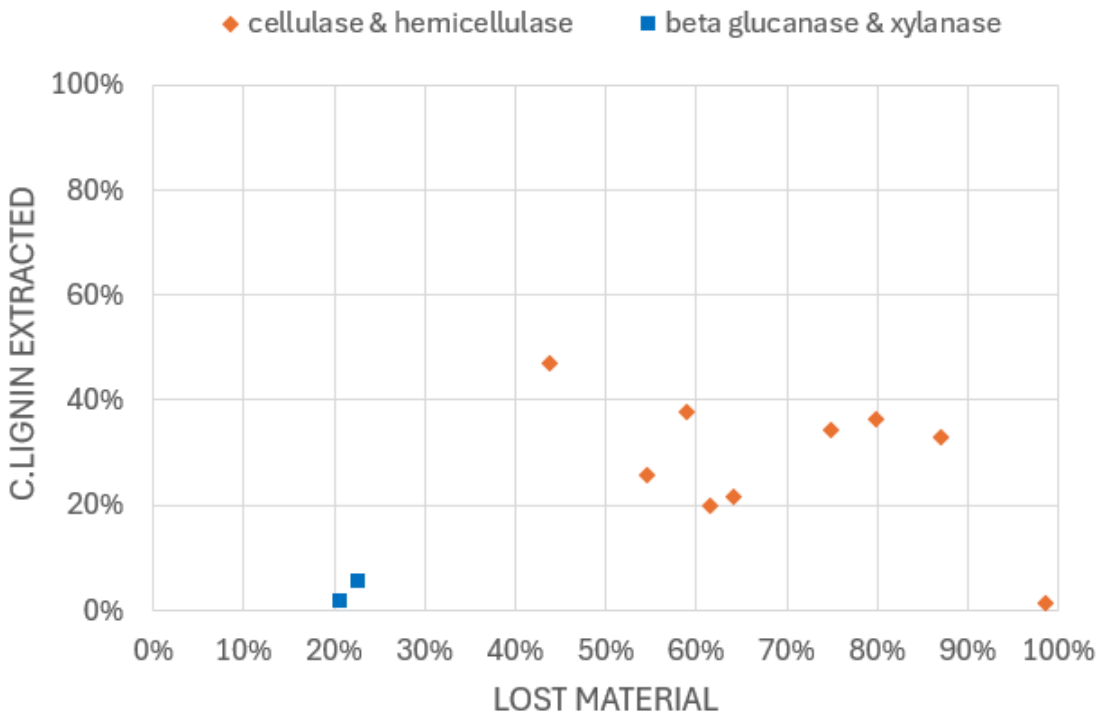

Figure 4.1: The extracted C.lignin, calculated as a weight percentage of the available lignin in the starting material for the enzymatic treatment, plotted against the lost material after the enzymatic treatment.

Figure 4.2 illustrates how the highest yield was achieved when the samples were treated two to three times by the enzymatic treatment. The wide spread of the data points in the plot showing the total hours of exposure to the enzymes for the sample indicates that the method has a wide acceptance for the treatment time. Comparing Figure 4.2 to Table 4.3 indicates that a total treatment time of around 60 hours with two or three washes will result in a good lignin yield.

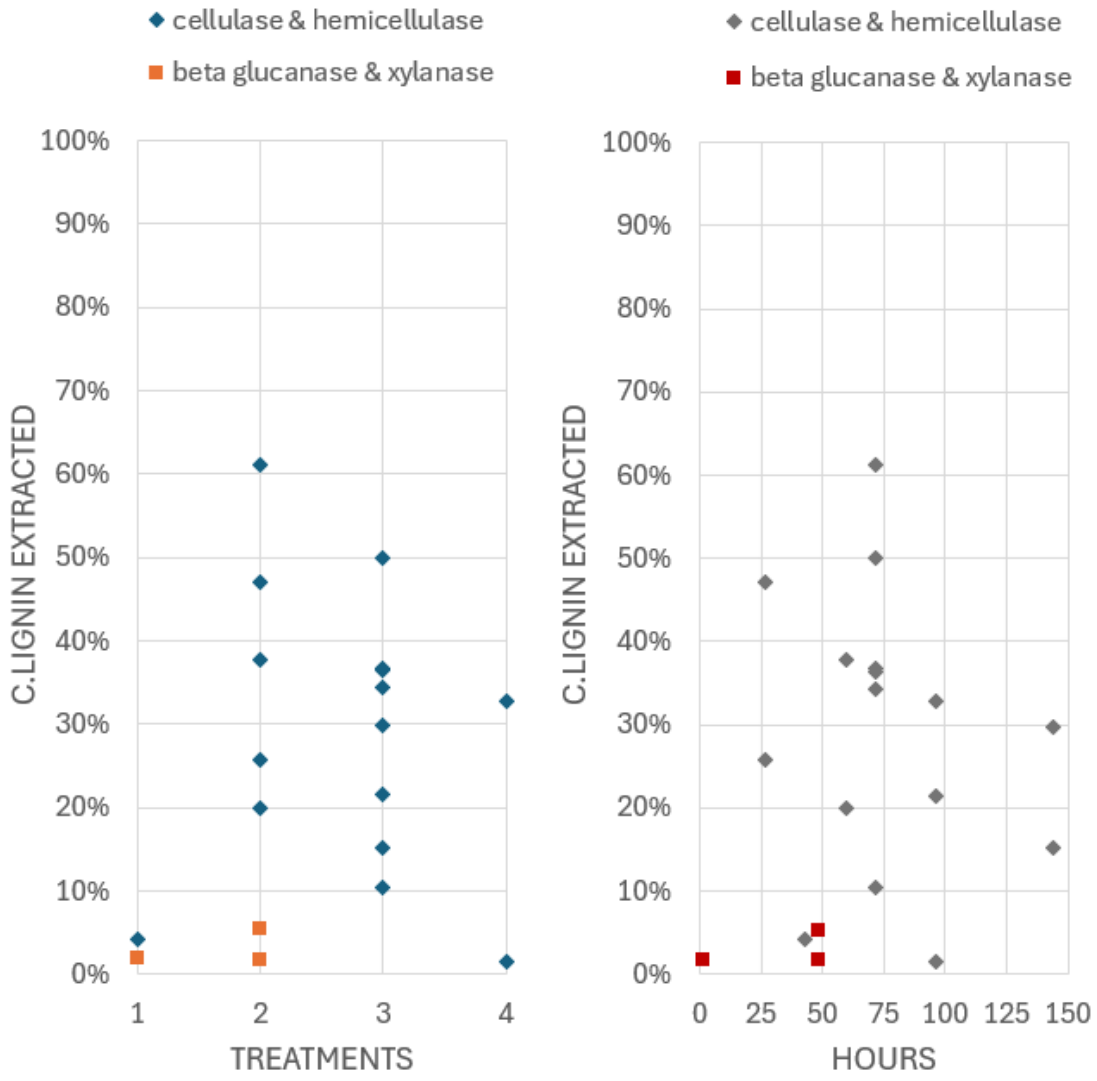

Figure 4.2: The extracted C.lignin calculated as a wt% of estimated available lignin plotted as a function of number of treatments (left) and as a function of the total amount of treatment hours (right).

Figure 4.3 suggests that if the temperature is increased from the recommended 37 ° C for the treatment it could cause a beneficial outcome. However, treatments performed below the recommended temperature of 37° C might decrease the amount of extracted material.

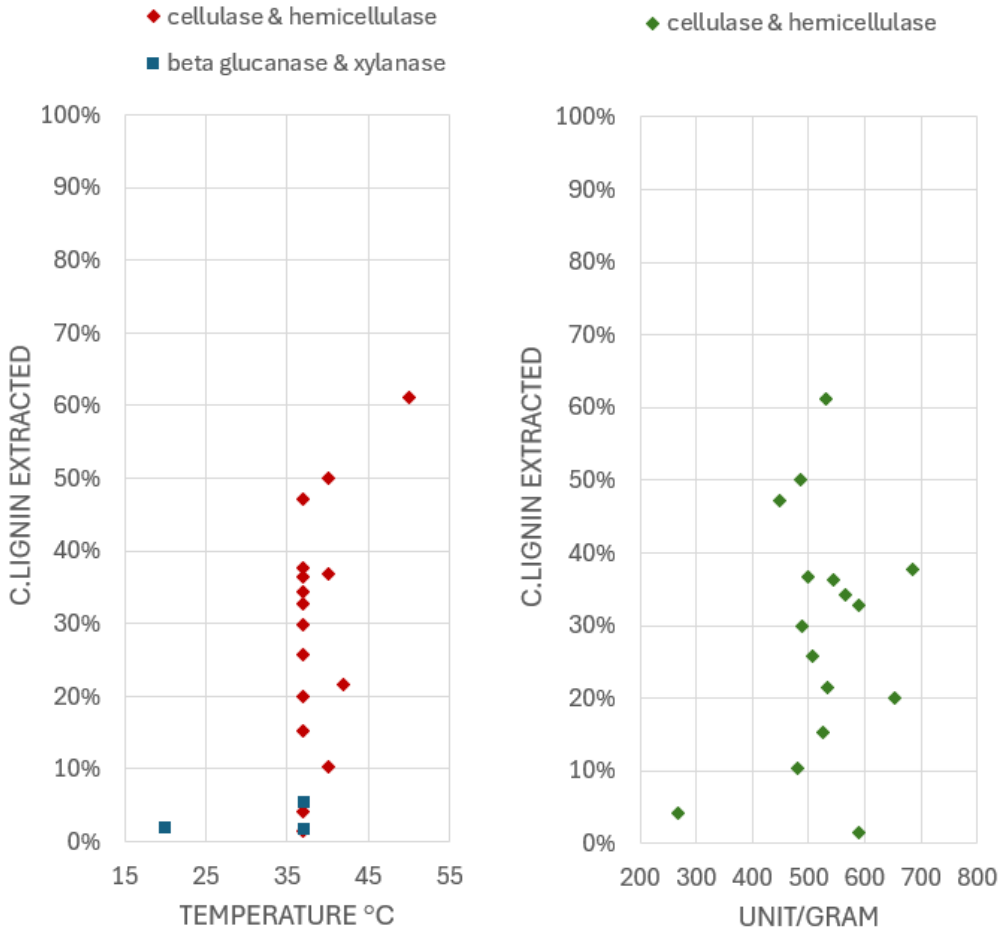

Figure 4.3: The extracted C.Lignin calculated as a wt% of estimated available lignin plotted as a function of temperature during the enzymatic treatments (left) and as a function of the amount of enzymes (right).

Looking at the wide spread of data points in Figure 4.2 and 4.3 it can be concluded that the enzymatic treatment has a wide operational range for its settings. The method is not dependent on one specific temperature, time, number of treatments or enzyme dosage. This creates a lot of liberty for the researcher wanting to adapt the method for a specific schedule or other surrounding factors. For economic and safety purposes it is recommended to use a lower enzyme dosage since no advantage has been observed with very high concentrations of enzymes.

4.2.1 Evaluation of the investigated parameters

The presented procedure for enzymatic lignin extraction will mediate isolation of lignin which can be used for analytical purposes, but there is still room to continue the development of this procedure. In Table 4.5, the intervals for the parameters presented in Table 3.1 have been narrowed but the range is still too wide for the method development to be considered completed.

Table 4.5: Summary of the identified parameter intervals for temperature, enzyme dosage, time, number of treatments, and enzyme mixtures.

Parameter	Lower value	Upper value
Temperature	37 °C	50 °C
Enzyme dosage	50 U/g	300 U/g
Cellulase:Hemicellulase	0.9:0.1	0.5:0.5
Treatment time	3h	48h
Number of treatments	2	3

The success of the lignin extraction was observed to be dependent on repeating the enzymatic treatment with a wash of the sample between each treatment. The number of treatments needed was determined to be a minimum of two and a maximum of three. Treating the sample one time was associated with a poor lignin extraction and treating the sample four times did not increase the yield significantly compared to two and three washes. The importance of washing between the treatments is believed to be because of an inhibitory effect of the product from the enzymatic degradation of cellulose and hemicellulose, which requires further analytical work for verification. Considering the significant increase of extracted lignin when introducing a washing step inhibition is a probable cause.

The obtained results made it possible to narrow the range of the suggested treatment hours. Experiments examining a lower range of one to three hours resulted in a small change in lignin content. There was no significant increase of extracted lignin when treating the sample at 48 hours intervals compared to 24 hours but one longer treatment combined with a shorter treatment time proved to be efficient for an increased lignin yield. When combining a longer and shorter treatment time the results suggest that the order is irrelevant, this result is discarded as a coincidence because of too few data points to support the pattern.

The amount of enzyme needed was concluded to be between 50 U/g and 300 U/g of ball milled wood. The experiments showed no clear relationship between the total dosage of enzymes and the amount of extracted lignin in the different wood samples. The ratio between cellulase to hemicellulase is believed to have a impact on the result and be an important factor when finding the optimal treatment parameters for lignin extraction. In this thesis, no clear relationships were observed.

The temperature was concluded to have a weak influence on the extraction results compared to the other parameters. However as discussed above the treatment temperature should not be below 37 °C unless it can be motivated by practical reasons such as a more flexible setup.

4.3 Cyrene extraction

The mass balance of all the Cyrene extractions can be seen in Table A.1 in Appendix 1. A general trend is that most of the material precipitated out from the solution

was obtained in CLF and CLAF (Crude Lignin Fraction and Crude Lignin Acidified Fraction). The Klason lignin content of CLF can be seen in Table 4.6.

Table 4.6: Lignin content in the CLF fractions. The numbers after the tree species indicated the amount of time in minutes that the sample has been exposed to a delignification process and the subscript is to differentiate different samples from each other that has undergone the same delignification treatment but have had different enzymatic treatments.

Raw material	Klason Lignin
$\text{Birch30}_{3(CLF)}$	87 wt%
$\text{Aspen30}_{1(CLF)}$	80 wt%
$\text{Birch30}_{2(CLF)}$	86 wt%
$\text{Birch30}_{2(CLF)}$	81 wt%
$\text{Birch30}_{1(CLF)}$	82 wt%

The general small amount of material obtained in the W, waste, fraction makes it hard to analyze. However, one HSQC analysis was performed on a CLF fraction which can be seen in Figure 4.4.

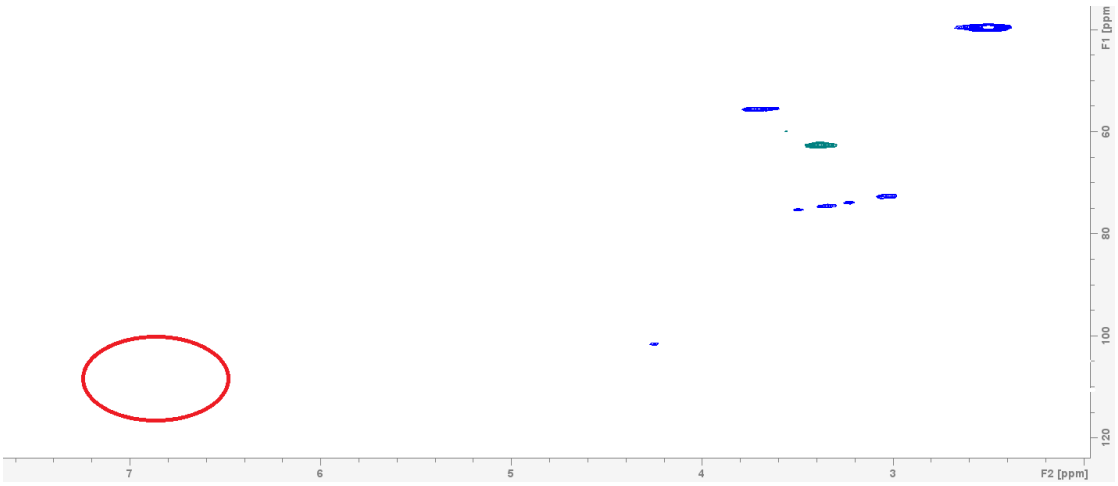

Figure 4.4: 2D-NMR spectra of a W fraction. The red oval indicates where the signals from the aromatic rings in the S and G units should be if there were any in the W fraction.

Figure 4.4 shows a complete lack of signals in the area for the S-unit (104/6.7) and the G-unit (111/7.0). Weak signals could be seen if the amplitude was increased indicating that some lignin was present in the sample. The signals seen in Figure 4.4 belong mainly to carbohydrates, this could indicate that Cyrene has some ability to dissolve carbohydrates. The result from the NMR in Figure 4.4 combined with the small yield of lignin obtained from the W lead to the decision to view this as a waste fraction. The dilution to 50 v% to obtain this fraction was seen as a cleaning step to remove non-lignin product that was precipitated out from the Cyrene solution.

NMR analysis of an additional five fractions was performed to analyze the structure of the fractions. All of these analyses used samples from the CLF fraction. The result can be seen in Table 4.7.

Table 4.7: Lignin linkages based on HSQC NMR (per 100 C9-unit). The numbers after the tree species indicated the amount of time in minutes that the sample has been exposed to a delignification process. If there is no such number, that indicates that the sample has been treated with the soxhlet extraction instead. The subscript is to differentiate different samples from each other that has undergone the same delignification or extractive removal treatment but have had different enzymatic treatments.

	$\beta-O-4$	$\beta-\beta$	$\beta-5$	$\beta-1$	Hibbert ketone
Aspen_5	59.1	12.9	2.4	0.38	0.39
Aspen30_1	56.3	17.4	3.5	0	0.36
Birch_2	45.8	8.66	3.1	0.93	0
Birch30_1	62.2	11.7	1.5	0.66	0
Birch90_1	33.7	17.6	2.1	0.41	0.47

The result from Birch_2 is unexpected, it should have the highest amount of $\beta-O-4$ bonds since it is uncooked. This deviation and the fact that there are no repetitions causes the results from that sample to be questionable. However, the amount of $\beta-O-4$ in the other samples agrees with previously reported data. The fact that there seems to be a correlation between cooking time and the amount of $\beta-O-4$ was expected. The amount of $\beta-O-4$ seems to decrease as a response to the cooking. The small decrease between Aspen_5 and Aspen30_1 compared to Birch30_1 and Birch90_1 is also expected. The cooking vessel has just reached the cooking temperature after 30 minutes. This means that the reactions for the samples cooked for 30 minutes are carried out at lower temperatures which is unfavorable for the delignification reaction since the reaction rate for breaking the $\beta-O-4$ is positively dependent on the temperature. However, the sample cooked for 90 minutes has an hour to react under favorable conditions for $\beta-O-4$ breakages.

The amount of the $\beta-\beta$ bonds found in the resinol substructure is around three times larger than in other reported studies. The presence of H_2SO_4 in the Cyrene extraction step could cause a favourable environment for condensation reaction if the pH is too low. That could explain the relative high occurring of $\beta-\beta$ and $\beta-5$ bonds. The amount of $\beta-5$ and $\beta-1$ are, however, relatively low compared to other studies which speaks against the case of condensation caused by low pH.

The use of Cyrene as the solvent used in the extraction step makes cross-referencing with other studies difficult due to the limited use of Cyrene in the past. Cyrene's HSQC spectra should be studied to determine if any overlaps of peaks could distort the result of the NMR. The composition of the lignin polymer for instance differed from previously reported findings regarding the syringyl and guaiasyl ratio. The ratios obtained from this study can be seen in Table 4.8.

Table 4.8: Syringyl and guaiacyl ratio based on HSQC NMR. The numbers after the tree species indicated the amount of time in minutes that the sample has been exposed to a delignification process. If there is no such number, that indicates that the sample has been treated with the soxhlet extraction instead. The subscript is to differentiate different samples from each other that has undergone the same delignification or extractive removal treatment but have had different enzymatic treatments.

	$S:G_{Data}$	$S:G_{Normalized}$
$Aspen_5$	54:22	2.45
$Aspen30_1$	51:17	3.00
$Birch_2$	53:22	2.41
$Birch30_1$	53:12	4.42
$Birch90_1$	54:20	2.70

This variation could originate from the fact that the different monolignols are not dispersed evenly throughout the cell wall. Guaiacyl is mostly found in the middle lamella and syringyl is mostly located in the secondary cell wall. These results could indicate that there has been an uneven extraction from the different parts of the cell wall.

GPC was also performed to evaluate the relative size of the obtained fractions. It is important to look at the relationship between these fractions and not the measurable polymer size due to column issues shifting all the retetion times.

The result from the GPC done on all obtained lignin fractions from $Birch30_2$, which can be seen in Figure 4.5, indicates that there is a relationship between the molecular size of the fraction and its solubility, which is expected. The W fraction is the first fraction precipitated out after diluting the Cyrene supernatant to 50 v%. This fraction has the broadest range of molecular weights but also the highest and most insoluble ones which makes the peaks more prevalent to the right hand side. The next dilution down to 35 v% will precipitate out most of the lignin fractions of relatively high molecular, the CLF. This can be seen since the curve is located more to the right in relation to the CLAF chromatogram which is obtained from the last fraction. The last fraction that is obtained is CLAF. The lignin found in this fraction is precipitated out by acidifying the remaining Cyrene solution. This fraction will contain the smallest lignin molecules with probably the highest solubility. This can clearly be seen since its chromatogram is distinctly shifted to the left compared to the W and CLF chromatograms.

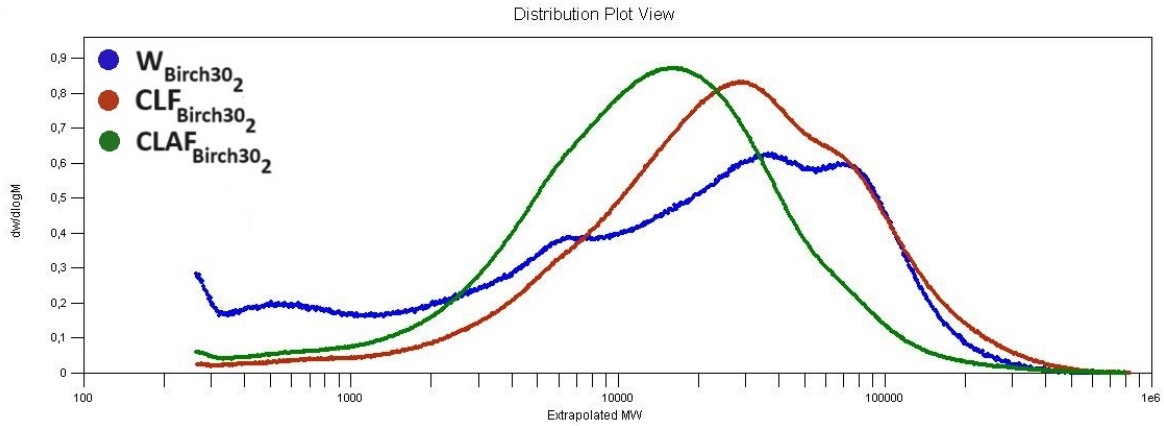

Figure 4.5: The molecular weight distribution curves of all precipitated lignin fractions from $Birch30_2$.

Figure 4.6 and 4.7 compare molecular weight distributions for two different CLF fractions from aspen and birch, respectively. Each Figure represents the data from two samples, the enzymatic treatment and the Cyrene extraction were performed independently.

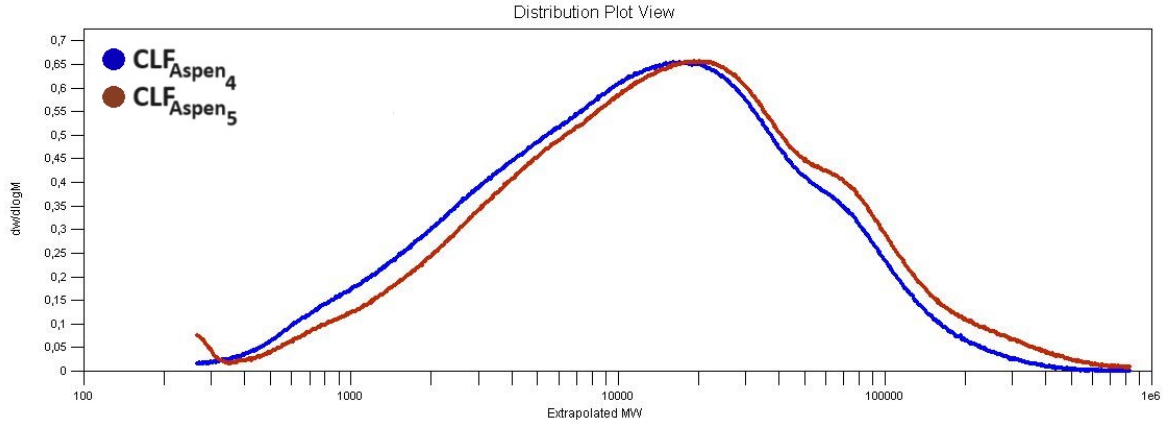

Figure 4.6: The molecular weight distribution curves of the CLF fraction from two different aspen samples, namely $Aspen_4$ and namely $Aspen_5$.

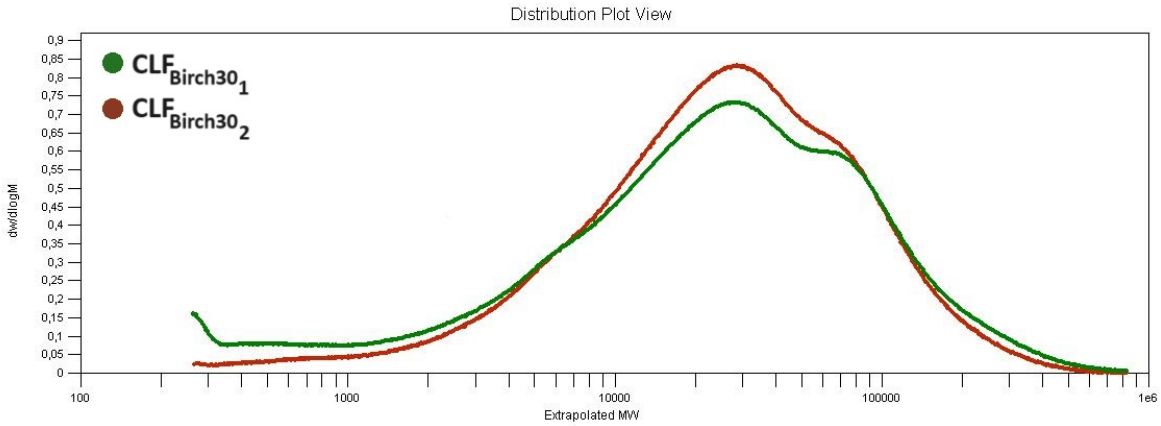

Figure 4.7: The molecular weight distribution curves of the CLF fraction from two different aspen samples, namely Birch30$_1$ and namely Birch30$_2$.

However, the wood used in each graph has had similar pretreatments. All samples were balled milled, the samples in 4.6 had their extractives removed via soxhlet treatment, and the samples in 4.7 were treated with conditions alike to the Kraft process for 30 minutes. The results indicate that the precipitation obtained from the same base material will have a similar distribution regarding molecular size within the CLF fraction. It can be noted that even though the enzymatic treatment between the samples in Figure 4.6 and 4.7 varied from each other, it did not cause the extracted fractions to vary when it came to their molecular size. With other words, the fact that the carbohydrates in the different samples were broken down differently seems not to affect the molecular weight distribution within the CLF fraction.

Figure 4.8 indicates a slight difference between two CLAF fractions obtained from Birch30.

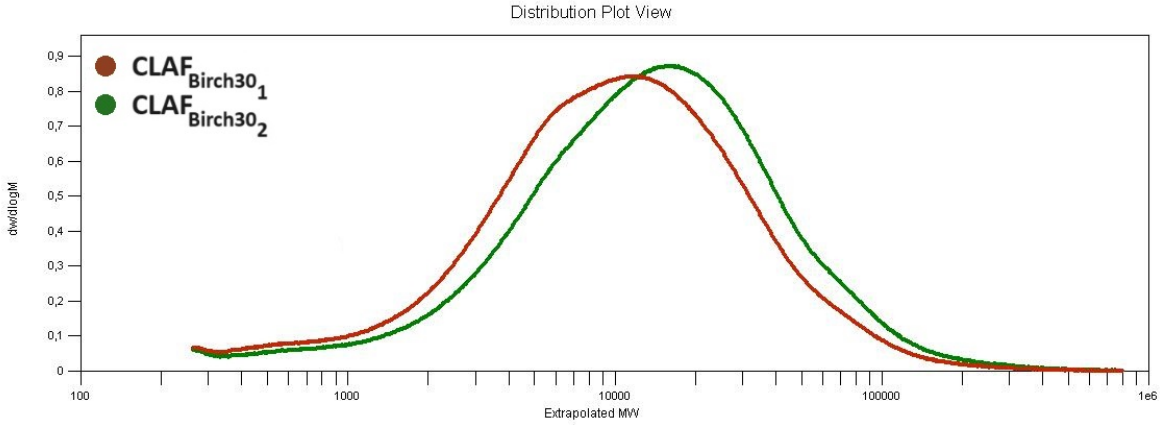

Figure 4.8: The size distribution of the CLAF fraction from two different samples.

This slight difference in distributions which was not seen in the CLF fractions previously discussed might stem from the fact that the CLAF fraction is the last precipitation containing the remaining lignin. The CLF fraction is a middle fraction, obtained from a sample that has had all larger lignin molecules, that are insoluble

at 50 v% Cyrene, removed in a previous precipitation step. The precipitation performed to obtain CLF lowers the Cyrene concentration to 35 v%. These sharp upper and lower Cyrene concentrations, 50 v% and 35 v%, could provide clear limits which results in a more uniform molecular size within the fraction. CLAF on the other hand contains all the size fractions that will precipitate from the solution when the Cyrene concentration falls below 35 v%, which will reslut in a much broader range of molecular weights.

4.4 A method for isolation and characterization of residual lignin

From the result presented above and additional observation during the collection of these data, a method was derived that could extract residual lignin and also characterise it. With this method, one can expect a lignin yield between 30-50 wt% of the available lignin in the sample with a purity of 83 wt%. The method is derived for lignin isolation in birch and aspen but is believed to work on other tree species as well.

4.4.1 Pretreatment of the wood material

Depending on the starting size of wood or pulp, different milling steps are needed to prepare the material for enzymatic treatment. This procedure assumes the starting material is around the size of a wood chip.

A desired amount of wood should be milled to a size of 20 mesh (0.85 mm). If using unprocessed wood, the extractives were removed using soxhlet extraction with acetone as solvent. The extractive free sample, due to either procced in a similar manor as in the Kraft process or by soxhlet extraction, was then placed in a ball mill and a program of 10 minutes milling followed by 5 minutes rest is repeated 19 times. The 5 minute rest is important to prevent a too extensive heating of the sample.

This should result in a sample size of 20 μm. A large loss of the starting material can occur in the Wiley mill and 10 wt% can be assumed to be lost after ball milling when transferring material between containers.

4.4.2 Enzymatic treatment

The amount of starting ball milled material (BM wood) needed for the enzymatic treatment in order to extract the desired amount of lignin is calculated using Eq. 4.1.

$$BM_{wood} = \frac{lignin_{wanted}}{0.4 * lignin_{wood}} \tag{4.1}$$

This amount is calculated using the $lignin_{wanted}$ which is the amount of lignin the method should result in, the lignin content in the sample denoted as $lignin_{wood}$ and assuming 40 % of the available lignin will be extracted.

The wood is placed in a beaker with 150 U/g of cellulase, 50 U/g of hemicellulase and 10 ml/g 5M acetate buffer at pH 5 with a magnetic stirring bar. The beaker is covered with alumina foil and placed in a water bath set to 37 °C and 300 rpm stirring for 24 hours.

After 24 hours the beaker is removed from the water bath and the content is transferred to a falcon tube for washing. The solid material is washed by centrifuging at 3500 rpm for 5 minutes, the supernatant decanted and saved in a separate container. The pellet is re-suspended by adding 20 ml of deionized water to the falcon tube. The centrifuging, decanting and re-suspending is repeated two times but after the last centrifuging the pellet is transferred back to the beaker instead of re-suspending it in the falcon tube. The buffer solution is used to recover all of the material from the falcon tube. The enzymatic treatment and washing are done three times and the remaining material is freeze-dried in a -40 °C vacuum of 0.02 mbar for two days.

4.4.3 Purification

The enzymatically treated wood obtained in the previous step is still rich in carbohydrates, around 70 wt%. The material thus needs further treatments to obtain purer lignin products. This will be done by chemical extraction with the use of Cyrene as the dissolving agent.

1 ml of 70 v% Cyrene was used for every 50 mg of material during the Cyrene extraction. The material and the Cyrene solution were mixed in a round bottom flask together with a magnetic stirrer. One drop (ca 36.5 μl) of 1 wt% H_2SO_4 was added for every 2 ml Cyrene used. This would result in a pH of around 2-2.5. The flask was placed in an oil bath set at 100 °C. The extraction took place over 16h under constant stirring. A reflux condenser was mounted on top of the round bottom flask to maintain a constant Cyrene concentration. The solution was transferred to a falcon tube after terminating the heat treatment. An additional 70 v% Cyrene was used to rinse out the round bottom flask.

The different fractions of crude lignin were obtained via centrifugation and dilution. The treatment steps in Table 4.9 were used to precipitate out the fractions. Four pellets were obtained from the precipitations. The first pellet was the residual wood fraction (R), the second pellet was a waste fraction (W), and the last pellet was the crude lignin fraction (CLF). All pellets were washed and freeze-dried in the same manner as before.

Table 4.9: Centrifugation = 4700 rpm x 5 min. Wash = re-suspend the pellet three times and centrifuge in between in order to remove the supernatant/wash water

1	Centrifugation	Dacant the supernatant to a new falcon tube and save the pellet	Dilute the supernatant to a concentration of 50 v% Cyrene with H_2O
2	Centrifugation	Dacant the supernatant to a new falcon tube and save the pellet	Dilute the supernatant to a concentration of 35 v% Cyrene with H_2O
3	Centrifugation	Dacant the supernatant to a container and save the pellet	Wash the residual and the two pellets thoroughly with H_2O

The last fraction of lignin, CLAF, was obtained by acidifying the saved supernatant to a pH of 2-2.5. It was left to rest for two hours and then put in the freezer, this was done to precipitate out any remaining lignin. The sample was then thawed and the centrifuge was used as before to collect the precipitated fraction.

4.4.3.1 Characterization

HSQC and GPC were used to determine the overall structure of the fractions. The samples were prepared in a similar manner for both analyses. 30 mg of a fraction was dissolved in ca 1.5 ml DMSO-d6 for the HSQC and 10 mg of a fraction in 1 ml DMSO/LiBr solution for the GPC. This was preferably done the night before to make sure that the sample would be completely dissolved before it was time to run the analysis.

5
Concluding remarks

The use of ball milling and enzymatic treatment to expose the lignin together with the use of Cyrene as a solvent for lignin was found to be suitable for the extraction and characterization of residual lignin from both wood and partly dignified wood as it is today. To extract 100 mg of lignin, with a purity of around 80 wt%, 1 to 4 g of wood is needed depending on the initial lignin content of the wood. The suggested method appears to be robust and quite unaffected by variations in the treatment conditions, such as temperature and enzyme dosage. The use of the relatively new solvent Cyrene was proven to be an adequate replacement for dioxane which has been banned from the use in universities due to safety reasons. The purity of the obtained lignin, when using Cyrene, is comparable to that of using dioxane and it is suspected that it could be increased with further treatment. Also, the yield itself is comparable to studies with dioxane that reports a lower yield.

The extraction and purification seem to be gentle enough to extract lignin without causing harm to the existing structure based on the relatively high occurrence of $\beta - O - 4$ bonds. There are however some unexpected results when it comes to the composition of the monolignols and more specifically the high occurrence of syringyl compared to guaiacyl. That together with the high frequency of $\beta - \beta$ bonds could suggest that a more thorough structural investigation of the obtained lignin is required than what could be performed within the scope of this project. Further studies are needed to determine whether these deviations from the priviously reported data data are justified or if they could be caused due to the use of Cyrene or the grinding conditions.

The use of precipitation through water dilution to obtain different lignin fractions seems to be a promising step in obtaining fractions of uniform sizes. But it seems that this uniformity was only obtained when a fraction has clear upper and lower precipitation limits, like the CLF fraction.

The experiments have proved the importance of washing the sample during the enzymatic treatment and adding new enzymes. This is speculated to be due to the inhibition of the enzymes by the treatment products. The products, especially the low-molecular ones, could act as inert analogs for the substrate and attach to the active site of the enzyme. This would block fresh substrate from reaching the active site and thus significantly reduce the efficiency of the enzymatic treatment. To maintain high efficiency during this step, it was found that washing the sample and providing fresh enzymes was the most crucial step in this method to obtain a

good lignin yield. What needs to be done in future work to increase the effectiveness of the enzymatic treatment is to identify the optimal treatment time for each step before the concentration of the aforementioned analogs starts to affect the reaction rate as well as the optimal amount and ratio of cellulases to hemicellulases.

6
Future work

To continue the development of a trustworthy time- and material efficient method it is suggested to continue the development of the method presented in this report. The future work consists of narrowing the range of the settings in order to find the optimal conditions for enzymatic lignin extraction and characterisation.

6.1 Enzymatic treatment

Treatment temperature

When treating the wood and pulp the temperature has been proven to be of importance but the exact temperature for the treatment has not been found.

Cellulase to hemicellulase

The optimal proportion of cellulases to hemicellulases used for treating wood and pulp is recommended to be establish. Depending on the tree and what pre-treatment it has been subjected to the fraction of cellulose, hemicellulose, and lignin has large variations within the wood and pulp. The enzymes used in this study target cellulose and hemicellulose separately. It would be interesting to find if the proportion of these enzymes significantly impacts the amount of lignin extracted when treating pulp of varying lignin fractions and if there is an optimal relationship between these enzymes. An alternative to investigating the proportions of enzymes is to investigate the efficiency and lignin yield using a brow rot fungi for degradation of cellulose and hemicellulose .

Treatment time

The optimal time one should treat the sample with enzymes should be identified and if this time varies depending on if it is the first, second, or third treatment.

6.2 Cyrene extraction

Extraction conditions

Only acid conditions were used when extracting lignin from wood that had been successfully treated with enzymes. It would be of interest to investigate if the Cyrene extraction could be performed on wood successfully treated with enzymes without applying acid conditions since it could cause structural changes in the obtained product.

Percipitation by cyrene dillution

Only 70 v% of aqueous Cyrene was used to extract lignin, it would be of interest to see if this truly is the optimum concentration to dissolve lignin found in wood. The precipitate obtained at 50 v% Cyrene has to be further investigated to determine if it can be used to recover hemicelluloses or if this fraction can be used as a cleaning step.

Analytics of the extracted lignin

The impact of Cyrene on the HSQC spectra needs to be evaluated to determine the peaks created by the solvent. This is important to evaluate how reliable the NMR result from the HSQC is when it comes to describing the ratio of monolignols and the bond frequency per C9 unit. Moreover, other complementary quantitative NMR methods should be employed.

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A

Appendix 1

Table A.1: Mass balances for different lignin extractions. A waste fraction, W, was obtained by diluting the Cyrene solution to 50 v%. A crude lignin fraction, CLF, was obtained by further diluting the solution to 35 v%. A crude lignin acidified fraction, CLAF, was obtained from acid precipitation of the 35 v% solution. The insoluble solids, R, separated from the original 70 v% solution and the estimated mass loss, L, completes the balance.

Sample	Weight [g]	W [w%]	CLF [w%]	CLAF [w%]	R [w%]	L [w%]
Birch$_1$	0.4507	0.60	0	0.11	90.79	8.50
Birch$_2$	0.2010	0.85	11.69	9.60	57.66	20.20
Aspen$_1$	0.3760	0	1.60	0.11	84.07	14.23
Birch$_6$	0.409	2.79	1.17	5.89	84.55	5.60
Aspen$_3$	0.58	6.34	1.88	5.19	28.12	58.47
Aspen$_2$	0.397	6.93	2.70	5.72	59.90	25.06
Birch$_5$	0.4038	1.76	1.24	5.70	60.28	31.03
Birch_30$_2$	1.0408	4.03	23.91	3.06	17.05	51.94
Birch_90$_1$	0.7606	3.08	5.67	0.28	70.19	20.79
Birch_30$_1$	0.9910	1.94	15.74	6.54	54.94	20.83
Aspen$_4$	1.5529	0.48	0.08	-0.20	69.90	29.74
Birch$_5$	1.5863	0.57	0.25	0.36	61.80	37.02
Aspen_90$_1$	0.0512	-1.18	5.66	1.37	28.13	66.60
Aspen_30$_1$	0.4902	1.06	33.35	5.67	25.07	34.84
Aspen$_5$	0.701	0.30	18.66	0.81	55.26	24.96
Birch$_7$	0.2474	1.05	10.51	1.82	40.14	46.48
Aspen$_4$	0.8054	1.47	9.39	1.56	73.23	14.35
Aspen 90.W$_1$	0.4838	1.94	2.09	0	26.97	66.00
Aspen_30$_2$	1.6327	2.95	17.33	0	27.42	52.30
Birch_30$_3$	1.3093	3.41	23.75	0	13.05	59.79

Table A.2: Treatment conditions from the Cyrene extraction. Wet means that the sample was treated directly after the enzymatic treatment without being freeze-dried. The moisture content of those samples was calculated to be around 80%

Sample	Heat source	Temp. [°C]	pH	Time [h]	Sample state
Birch$_1$	Water	100	2.63	16	Dry
Birch$_2$	Water	100	3.07	16.27	Dry
Aspen$_1$	Water	100	2.80	21.7	Dry
Birch$_6$	PEG	100	2.71	18.08	Dry
Aspen$_3$	PEG	100	2.02	18.08	Dry
Aspen$_2$	PEG	100	2.25	20.88	Dry
Birch$_5$	PEG	100	2.21	20.88	Dry
Birch_30$_2$	PEG	100	2.69	31.8	Wet
Birch_90$_1$	PEG	100	2.84	No data	Dry
Birch_30$_1$	PEG	100	2.81	6.54	Dry
Aspen$_4$	PEG	100	3.87	22.08	Dry
Birch$_5$	PEG	100	2.42	22.08	Dry
Aspen_90$_1$	PEG	100	2.20	17.12	Dry
Aspen_30$_1$	PEG	100	2.23	17.12	Dry
Aspen$_5$	PEG	100	2.31	16.48	Dry
Birch$_7$	PEG	100	2.28	16.48	Dry
Aspen$_4$	PEG	100	2.45	16.00	Dry
Aspen 90.W$_1$	PEG	100	No data	No data	Wet
Aspen_30$_2$	PEG	100	No data	No data	Wet
Birch_30$_3$	PEG	100	No data	No data	Wet

B

Appendix 2

B.1 Preparation of samples by kraft cooking

In Table B.1 the cooking conditions used to prepare a 30 and 90 minute cook of aspen and birch are shown.

Cooking conditions	
Liquid/Wood	20
HS^-	0.15 mol/kg liquor
OH^-	0.6 mol/kg liquor

Table B.1: Cooking conditions used when preparing the wood samples to simulate a 30 and 90 minute industrial cooking

1. Prepare the cooking liquor and charge the autoclave with wood shavings and white liquor.

2. Close the autoclave: place the gasket in the autoclave lid, place the lid over the autoclave vessel, and fasten the bolts.

3. Impregnate the wood shavings by pressurizing with nitrogen gas at 5 bar. Keep the pressure for two minutes and then release the excess by carefully opening the valve on the autoclave lid.

4. Place the autoclave in the polyethylene glycol (PEG) bath and start the time.

5. After 30 minutes, remove the 30 sample of each sample. Terminate the cook after 90 minutes and remove the remaining autoclaves. After the autoclaves are removed from the PEG, bath them in the cooling bath for a minimum of 10 minutes.

6. Separate the black liquor from the pulp by pouring the slurry into a Büchner funnel, and pour deionized water over the cooked shives to wash until clear.

C

Appendix 3

Table C.1 shows the first set of experiments. During this phase, the experiments aimed to increase the lignin fraction in the wood. To measure the effect of the enzymatic treatment the Klason lignin was measured.

Table C.1: Starting conditions and the increased lignin content as a weight percentage in the sample after the enzymatic treatment using hemicellulases.

Wood	Weight	Temperature	Time	K.Ligin t0	K.Ligin t1
Birch$_1$	1.002 g	20°C	1h:1	17 w%	**21 w%**
Birch$_2$	1.004 g	20°C	4h:1	17 w%	**24 w%**
Birch$_3$	1.001 g	20°C	48h:1	17 w%	**- w%**

In Table C.2 the experimental parameters on the extractive free wood are reported together with the extracted crude lignin.

Table C.2: Starting conditions and the resulted crude lignin (C.Lignin) extraction for the enzymatic treatment using a mixture of the enzymes cellulase and hemicellulases. The wight percentage here is that of the assumed available lignin in the starting material used in the enzymatic treatment.

Wood	Weight	Cellulase	Hemicellulase	Temperature	Time	C.Lignin
Aspen$_2$	1.0239 g	300 U	323 U	37°C	20h:1	
		25 U	53 U	37°C	40h:1	**37.7 w%**
Aspen$_3$	1.0679 g	200 U	126 U	37°C	3h:1	
		100 U	87 U	37°C	24:1	**47.1 w%**
Aspen$_4$	3.0060 g	400 U	600 U	42°C	48h:1	
		450 U	150 U	42°C	24h:1	**21.51 w%**
Aspen$_5$	3.0063 g	450 U	135 U	37°C	48h:2	
		225 U	70 U	37°C	48h:1	**29.81 w%**
Birch $_4$	1.0010 g	1650 U	1996 U	20°C	44h:1	**26.01%**
Birch$_5$	1.0289 g	300 U	320 U	37°C	20h:1	
		25 U	28 U	37°C	40h:1	**19.94 w%**
Birch$_6$	1.0134 g	200 U	106 U	37°C	3h:1	
		100 U	72 U	37°C	24:1	**25.71 w%**
Birch$_7$	1.2722 g	200 U	57 U	37°C	48h:2	
		100 U	50 U	37°C	48h:1	**15.21 w%**

In Table C.3 the experimental parameters on the 30 and 90 minute kraft wood are reported together with the extracted crude lignin.

Table C.3: Starting conditions and the resulted crude lignin (C.Lignin) extraction for the enzymatic treatment using a mixture of the enzymes cellulase and hemicellulase. The wight percentage here is that of the assumed available lignin in the starting material used in the enzymatic treatment.

Wood	**Weight**	**Cellulase**	**Hemicellulase**	**Temperature**	**Time**	**C.Lignin**
Aspen 30$_1$	4.0008 g	600 U	180 U	37°C	24h:2	
		300 U	100 U	37°C	24h:2	**32.78 w%**
***Aspen 30**$_2$	6.0122 g	800 U	300 U	40°C	24h:2	
		500 U	300 U	40°C	24h:1	**36.75 w%**
Birch 30$_1$	3.9968 g	388 U	290 U	37°C	24h:1	
		388 U	400 U	37°C	24:3	**34.28 w%**
Birch 30$_2$	3.0103 g	400 U	600 U	50°C	48h:1	
		450 U	150 U	37°C	24h:1	**61.17 w%**
***Birch 30**$_3$	4.0048 g	500 U	200 U	40°C	24h:2	
		340 U	200 U	40°C	24h:1	**50.68 w%**
Aspen 90$_1$	4.0013 g	600 U	180 U	37°C	24h:2	
		300 U	100 U	37°C	24h:2	**1.45 w%**
Birch 90$_1$	4.0258 g	388 U	226 U	37°C	24h:1	
		388 U	400 U	37°C	24h:3	**36.37 w%**
***Aspen 90.W**$_1$	4.0388 g	500 U	200 U	40°C	24h:2	
		340 U	200 U	40°C	24h:1	**10.36 w%**

* The yield does not include the CLAF fraction

D

Appendix 4

The values used to identify which linkages the cross-peak in the HSQC-NMRs belong to.

Source of the cross-peak	δ_C/δ_H
($C_{2,6}$) S-unit	103,8/6,71
($C_{2,6}$) S-unit Carbonyl C_α	106,2/7,23
(C_2) G-unit	110,9/6,98
(C_α) $\beta - O - 4$ normal and acetylated γ	71,8/4,86
(C_β) $\beta - O - 4$ normal to S-unit	85,9/4,12
(C_β) $\beta - O - 4$ normal to H/G-unit	83,9/4,26
(C_γ) B$\beta - O - 4$ normal	59,5-59,7/3,4-3,63
(C_β) $\beta - \beta$ resinol	53,5/3,06
(C_α) $\beta - \beta$ resinol	84,8/4,65
(C_γ) $\beta - \beta$ resinol	71,0/3,82
(C_γ) $\beta - \beta$ resinol	71,0/4,18
(Hk_γ) Hibbert	67,5/4,2
(C_γ) $\beta - 5$ and $\alpha - O - 4$ phenylcoumaran substructure	53,3/3,46
(C_α) $\beta - 1$ and $\alpha - O - \alpha$ spirodienone substructure	81,2/5,07
(C_β) $\beta - 1$ and $\alpha - O - \alpha$ spirodienone substructure	59,7/2,77
(C_β) $\beta - 5$ and $\alpha - O - 4$ phenylcoumaran substructure	53,3/3,46
(C_α) $\beta - 5$	87,7/5,5

Table D.1: The resonance signals used to identify the cross-peaks in HSQC NMR spectra.

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