ÅBO AKADEMI UNIVERSITY
FACULTY OF SCIENCE AND ENGINEERING

Fractionation and Characterisation of Enzymatic Hydrolysis Lignin

Master's thesis

by

Minette Kvikant

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Carried out at the Laboratory of Natural Materials Technology at Åbo Akademi University under the supervision of Professor Chunlin Xu, Docent Anna Sundberg, and MSc Luyao Wang at Åbo Akademi University

Abstract

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Supervisors

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Keywords

Biomass, Lignocellulosic Biomass, Biorefinery, Fractionation, Lignin, Lignin Fractionation, Acidic Fractionation, Solvent Fractionation

Lignin is one of the major components in lignocellulosic biomass and the most abundant aromatic building block in nature. Technical lignins extracted from either pulping or biorefining processes provide a variety of complex and polydisperse phenolic polymers, harbouring great potential as a value-added product from the bio-based industry. Lignin has for long been associated with being a challenging material to work with due to its heterogeneous structure. This heterogeneity and wide distribution of molar mass restrict the high value-added applications. However, the valorisation of lignin has come a long way due to the incentive to produce more sustainable options in the hope of a more circular bio-economy. As a result, several strategies have been developed that produce well-defined lignin with narrow $Đ_M$ through fractionation. In this thesis, enzymatic hydrolysis lignin was fractionated using different methods, and the fractions were characterised to understand how different fractionation methods can tailor properties of lignin.

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Minette Kvikant

Abbreviations

AIL	Acid-insoluble lignin
ASL	Acid-soluble lignin
$Đ_M$	Molar mass dispersity
EHL	Enzymatic hydrolysis lignin
EtOH	Ethanol
GC	Gas chromatography
GPC	Gel permeation chromatography
G unit	Guaiacyl unit
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
H unit	*p*-hydroxyphenyl unit
LS	Light scattering
MTBE	Methyl tert-butyl ether
Mn	Number-average molar mass
Mw	Weight-average molar mass
$MALS_{(IR)}$	Multiangle light scattering detector in the infrared-range
N_2	Nitrogen
NaOH	Sodium hydroxide
Na_2S	Sodium sulphide
Res.	Residue
RI	Refractive index
SO_2	Sulphur dioxide
Sol.	Soluble
S unit	Syringyl unit
Tg	Glass transition temperature
TMCS	Trimethylchlorosilane
TMDP	Tetramethyl-1,3,2-dioxaphospholane
UV-vis	Ultraviolet-visible
Wt%	Weight percentage

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1 Introduction

A global increase in population has resulted in limited resources on the planet. Society's dependence on non-renewable petroleum-based products for fuel and chemicals increases energy costs and environmental concerns. To move away from this state, sustainable technologies that support the resource-efficient utilisation of renewable resources should be developed. An example is converting renewable biomass into clean fuels and high value-added chemicals to supplement or gradually replace the petroleum-based industry (Oh, Pang, & Chua, 2010; Steen et al., 2010). The pulp and papermaking industry and carbohydrate-based biorefineries produce vast amounts of lignin and lignin-rich wastes, which challenges the economic feasibility of those biomass fractionation processes. The technologies for converting carbohydrates to value-added products, such as pulp, textile fibres, monomeric sugar precursors, and bioethanol (biofuel), are well-established. However, the methodologies for lignin valorisation are significantly less developed, and technical lignins are almost always burnt for energy. Developing a sustainable biorefinery process relies on the optimal use of all three major lignocellulose components: cellulose, hemicellulose, and lignin. Although challenging, developing reliable, affordable and economically viable applications for valorisation of biomass by utilising lignin can be a starting point for creating a future circular bioeconomy (Baumberger, 2002; Sherwood, 2020).

Lignin is nature's most abundant aromatic building block, making it a cheap alternative to petroleum-based aromatic compounds. Lately, much attention has been paid to the functional groups in lignin with coveted complexity for value-added applications. However, the heterogeneous structure of lignin makes utilisation of lignin a great obstacle to overcome, which results in the lignin not being used to its full potential. Instead, the lignin is most commonly burned as a low-grade fuel while simultaneously polluting the environment. A solution to this bottleneck would be to utilise lignin as a partial replacement for fossil-based resources by recognising the advantages of lignin, such as accessibility and affordability. These properties can make up for the deficiencies in product performance due to the obstacles mentioned above. The lignin must undergo a value-added fractionation process, creating a more homogeneous and stable product. Several studies have shown that fractionating the technical lignin makes it possible to

control and, therefore, to tailor the lignin properties, such as molar mass and solubility, creating a more homogenous product suitable for future valorisation (Jiang et al., 2020; Pang et al., 2020; Sadeghifar & Ragauskas, 2020; J. Xu et al., 2020).

The goal of this master's thesis was to fractionate and purify technical lignin, resulting in relatively homogeneous lignin fractions. This is a requirement to be able to reveal the lignin structure-property correlation for further modification. Furthermore, this thesis determines the effects of different fractionation methods on the properties of the lignin fractions. Fractions with optimal properties would then be further utilised in value-added applications. The lignin was fractionated by two methods to achieve this goal, and the lignin fractions were characterised using a comprehensive structural analysis. A flow chart of the thesis work can be observed in
Figure 1**.**

The goals of the thesis were the following:

- ✓ To select suitable fractionation methods
- ✓ To achieve proper purification of lignin
- ✓ To determine the composition in each lignin fraction
- ✓ To compare molar mass of each lignin fraction
- ✓ To quantify the hydroxyl groups in each lignin fraction
- ✓ To establish the thermal properties of each lignin fraction
- ✓ To determine the most suitable lignin for further modification or compatibilisation

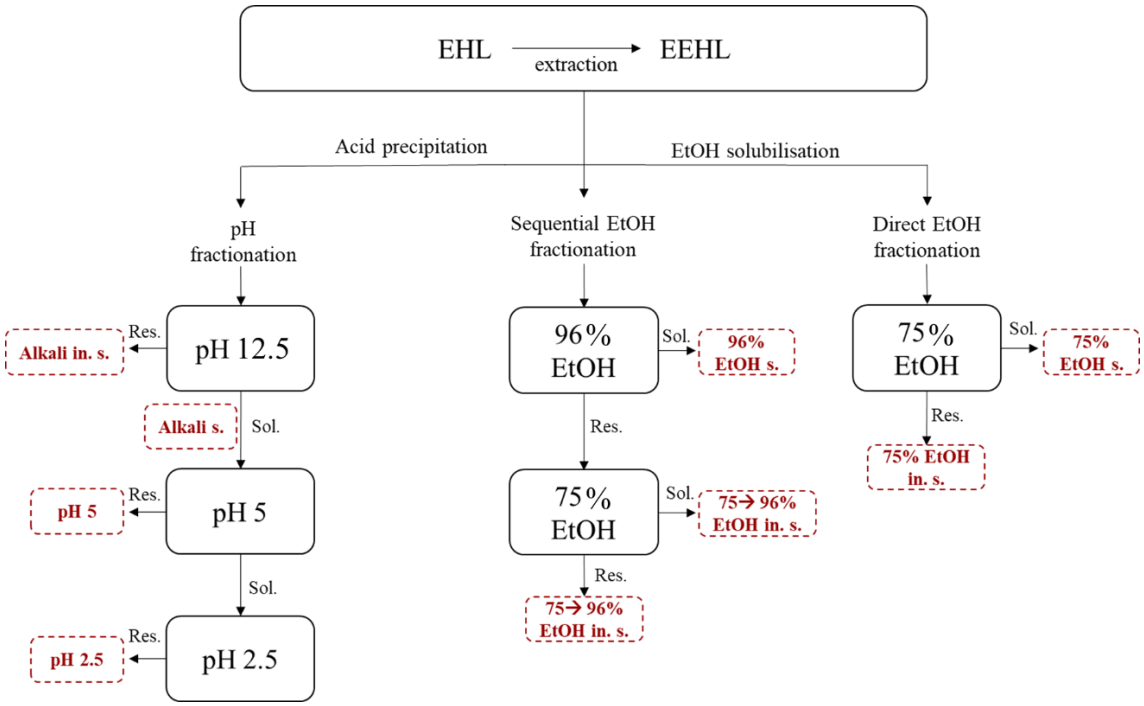

Figure 1 Flow chart of fractionation of enzymatic hydrolysis lignin (EHL). The fractions which were isolated and characterised are marked in red. The extracted enzymatic hydrolysis lignin is abbreviated as EEHL, and the soluble fractions and residues are abbreviated as Sol. and Res., respectively.

2 Theory

2.1 Lignocellulosic biomass

Lignocellulosic biomass is the main fraction in most plant-based materials, such as wood, perennial energy crop, cereal straws and bagasse (Wyman, 1994). It consists mainly of cellulose (40%−60%), hemicelluloses (20%−40%), and lignin (10%−25%). Their ratio varies depending on the origin of the plant (Liew et al., 2018). Together, the components form a matrix that creates the cell wall in plants, as shown in Figure 2. Additionally, lignocellulosic biomass contains small amounts of pectins, extractives, and inorganic components (Brandt, Gräsvik, Hallett, & Welton, 2013). The abundance of lignocellulosic biomass makes this the most economical and highly renewable natural resource in the world (Qian, 2014).

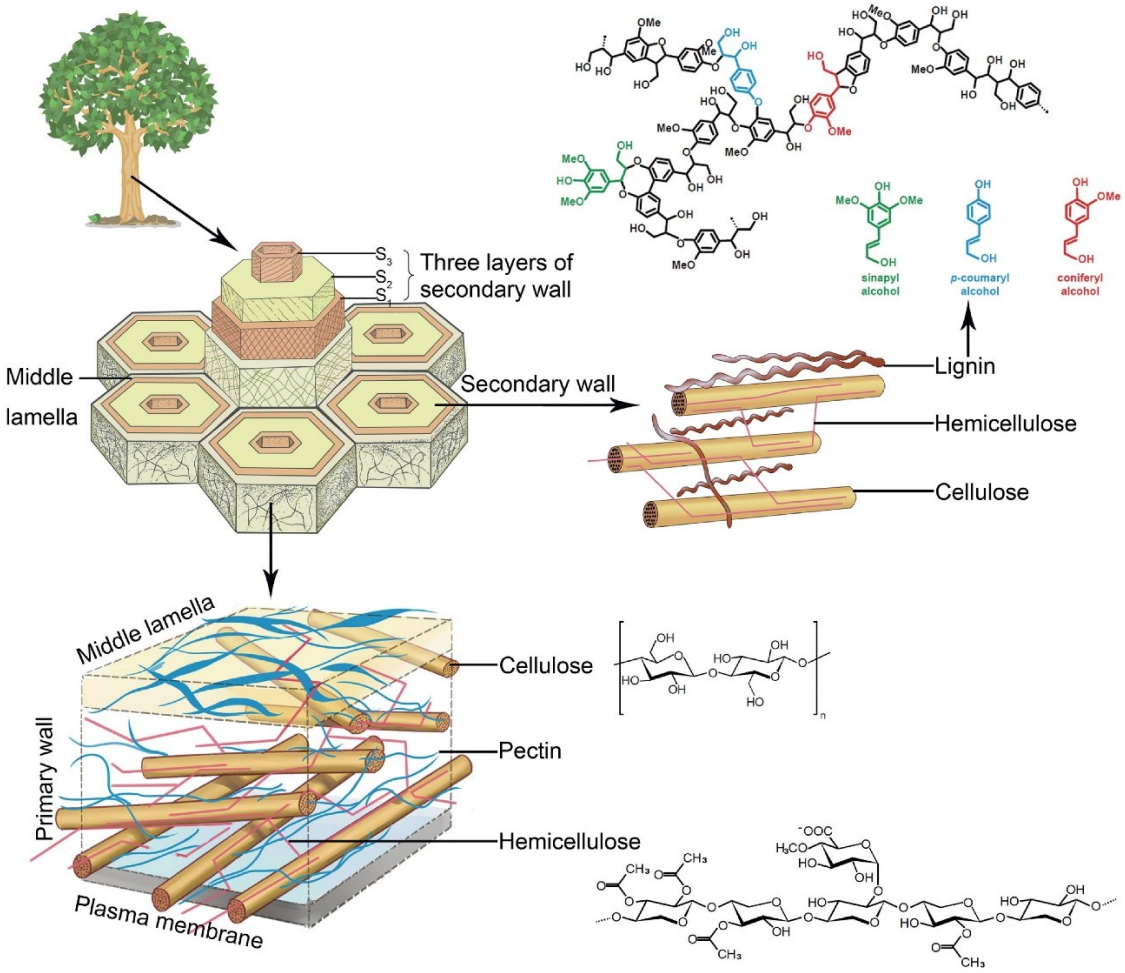

Figure 2 Illustration of components in lignocellulosic Biomass (Zhao et al., 2019).

2.1.1 Components in lignocellulosic biomass

Lignin

The purpose of lignin is to stiffen and fortify secondary cell walls, which are especially important in forming cell walls found in wood and bark (Argyropoulos & Menachem, 1998). The lignin works as a naturally occurring composite material, filling the matrix's space and acting as a glue, keeping the plant cells together, as illustrated in Figure 2. Furthermore, lignin also plays an essential role in protecting biomass against invasion by pathogens and insects and hindering enzymatic hydrolysis (Schoenherr, Ebrahimi, & Czermak, 2018; Zhao, Zhang, & Liu, 2012).

Lignin comprises mainly three phenylpropanoid units: the monolignols of coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol, observed in Figure 3 (Z. Sun, Fridrich, de Santi, Elangovan, & Barta, 2018). These monolignols can be seen as precursors and are usually mentioned as phenylpropane or C9 units linked through a hydroxyl group to the C4, and substitutions with one or two methoxyl groups are present at the C3 and C5. If a ring from the monolignol is unmethoxylated or has one or two methoxyl groups, they create *p*-hydroxyphenyl (H-unit), guaiacyl (G-unit) and syringyl (S-unit), respectively (Abdelaziz et al., 2016). The linkages between the monolignols consist of carbon-carbon and ether bonds, resulting in a complex three-dimensional network with a highly irregular structure. Approximately >50% of the linkages between the monolignols are β-O-4 ether bonds. Due to this aromatic and highly branched structure, the lignin resists degradation.

The composition of lignin varies vastly between different biomass species. The lignin content of hardwoods is usually in the range of 17–25%, consisting of G and S units, while softwood has a bit higher content, between 25% and 35%, and consists of G units and a small portion of H units (Lourenço & Pereira, 2017). Non-woods generally have a lower lignin content, within the 9–20% range, and are characterised by a high proportion of H units. The properties of lignocellulosic materials demand an understanding of lignin composition and macromolecular structure and work as a base for vital issues in producing lignin-based products within biomass biorefineries.

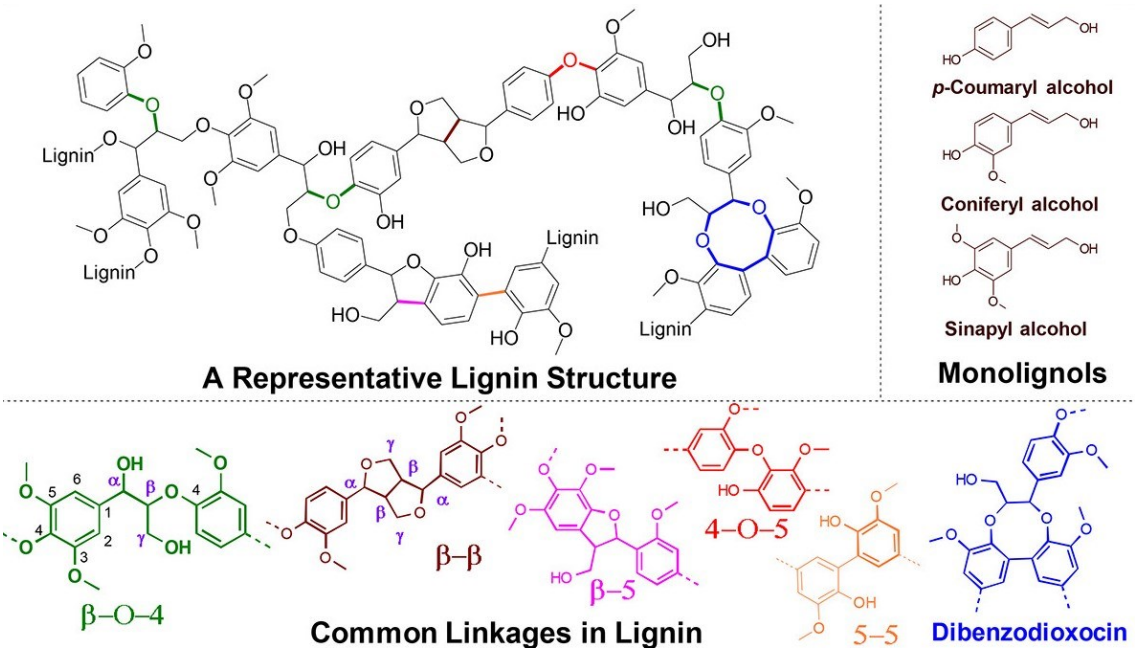

Figure 3 A representative lignin structure with common lignin subunits and linkages. Modified from Sun et al. (2018).

Cellulose

Cellulose gives the cell wall protection and stability. It is a linear homopolysaccharide consisting of β-1,4-glycosidic bond linked units of D-anhydroglucopyranose sugar units, seen in Figure 4 (Zhou & Wu, 2012). The hydroxyl groups in the glucose chain are polar, which means that hydrogen bonds are easily formed between the hydrogen and oxygen molecules, both between different cellulose polymers and within the polymer itself, resulting in crystalline and amorphous regions within the microfibrils (Börjesson & Westman, 2015). The crystallinity provides the polymer with mechanical properties. However, high crystallinity and an abundance of hydrogen bonds in the cellulose fibres make cellulose insoluble in water and most conventional organic solvents. The solubility of cellulose is a significant factor when considering alternatives for cellulose isolation and lignin purification. The low cost of producing cellulose and its coveted properties, such as biodegradability and low toxicity, makes it an excellent polymer for paper and packaging, the food industry, cosmetic industry, pharmaceuticals, and textiles (Wertz, Deleu, Coppée, & Richel, 2018).

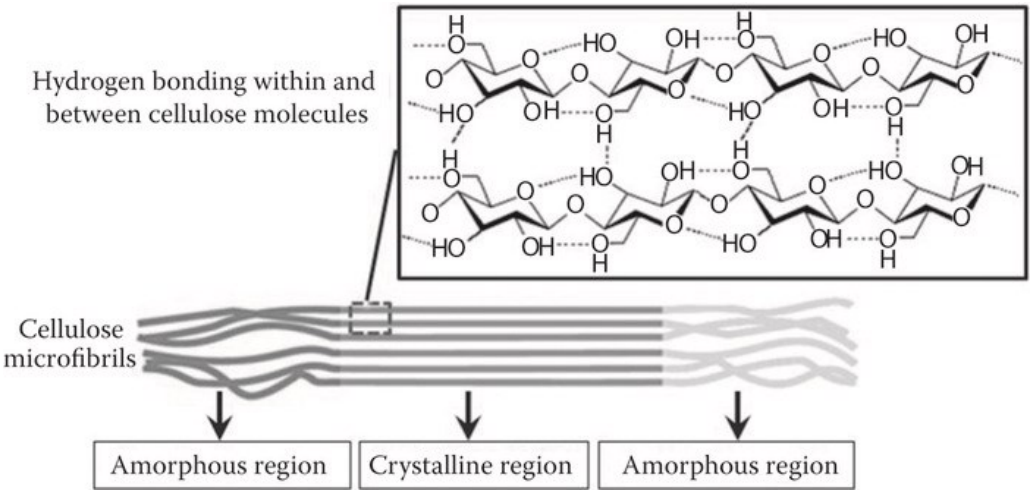

Figure 4 Cellulose molecular chains within the crystalline and amorphous regions of cellulose microfibrils (Zhou & Wu, 2012).

Hemicelluloses

Hemicelluloses are different saccharide polymers that interact through hydrogen bonds with cellulose and have covalent bonds to lignin, strengthening the cell wall. Unlike cellulose, hemicelluloses contain multiple types of sugar units with various glycosidic bonds in one molecule. Thus, hemicelluloses appear in amorphous form. Furthermore, the backbone of hemicelluloses is either linked by β-(1,4)-glycosidic bonds or sometimes by β-(1,3)-glycosidic bonds. These are usually branched homo- or heteropolysaccharides composed of pentoses (e.g., xylose, rhamnose, and arabinose), hexoses (e.g., glucose, mannose, and galactose), and uronic acids (e.g., 4-*O*-methyl glucuronic, glucuronic, and galacturonic acids) (Tanczos, Schwarzinger, Schmidt, & Balla, 2003).

Almost all the monosaccharides in hemicellulose have the D configuration, except arabinose, which occurs in L-configuration. The composition of hemicellulose in lignocellulosic biomass varies depending on the source (Su, 2012). Hemicelluloses from softwood are usually found in the 25–35% range and are rich in glucomannans (e.g., galacatoglucomannans, seen in Figure 5a). The hemicellulose content usually varies between 18% and 25% in hardwood and contains mostly xylans (e.g., arabinoglucoronxylan, seen in Figure 5b). Hemicellulose acts as a condensation polymer, removing a water molecule from every linkage when forming glycosidic bonds (Bajpai, 2018a). The hemicelluloses can be hydrolysed to oligomeric and monomeric saccharides to be used as value-added chemicals and promote the commercial production of

lignocellulosic ethanol by converting the hemicelluloses into fermentable sugars fuels (Jing, Guo, Xia, Liu, & Wang, 2019).

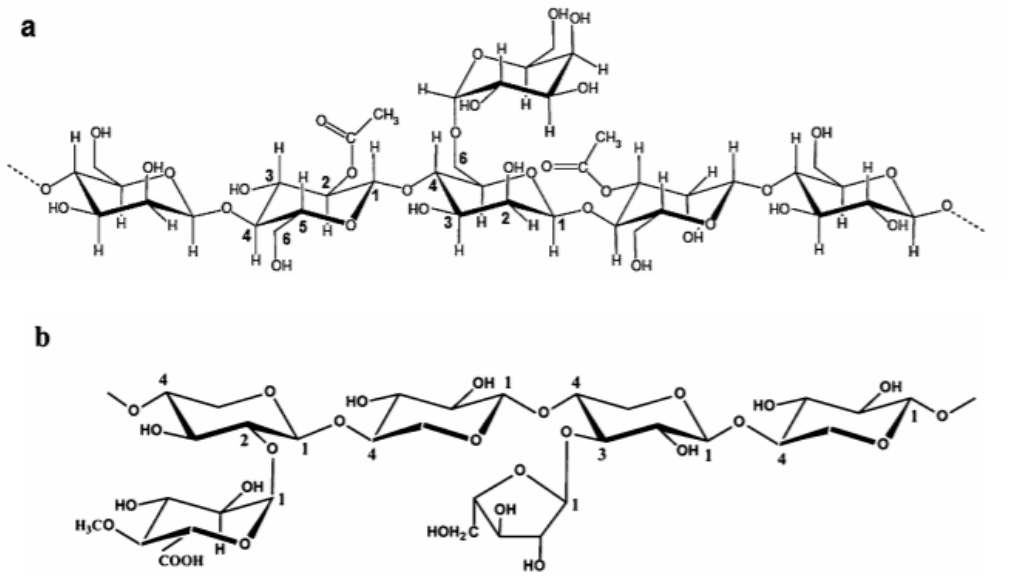

Figure 5 Chemical structures of the dominant hemicelluloses in softwood, a) Galacatoglucomannans and hardwood, b) Arabinoglucoronxylans (Su, 2012).

Extractives

Extractives can be defined as the non-structural components in biomass, such as terpenes, fats, waxes, phenolic compounds and lignans, which are illustrated in Figure 6 (Zimbardi & Viola, 2001). Depending on the solubility, the extractives can be divided into lipophilic and hydrophilic extractives (Willför, Smeds, & Holmbom, 2006). Common lipophilic extractives are waxes, fats, fatty acids, terpenoids, and steryl esters. These can cause disturbance during analysis of biomass. Therefore, removing the lipophilic extractives is usually required to avoid disturbance, which can be done using nonpolar organic solvents (e.g., hexane, methyl tert-butyl ether, or toluene). Examples of hydrophilic extractives are stilbenes, lignans, flavonoids, tannins and simple phenolics. These are extracted by more polar solvents, e.g., methanol, acetone or ethanol (Vek, Oven, & Poljanšek, 2016).

Extractives have several different roles in biomass, giving the plant colour and smell, and can also function as an additional nutrient, plant hormones and regulate the plant cell's moisture content. Furthermore, extractives can also bind together with metals to act as catalysts for biosynthesis. Nevertheless, the primary role of extractives is to work as a

chemical and physical defence against bacteria, fungi, and insects (Thygesen et al., 2020; Nisula, 2018.). Removing the non-structural material from biomass before analysis is critical in characterising biomass due to the interference extractives usually cause during the analytical steps. Removing the extractive is usually done in a two-step extraction process (e.g., organic solvent and water) to remove lipophilic and hydrophilic components. Components such as inorganic material, non-structural sugars, and nitrogenous material are usually removed with water extraction, while other components such as waxes, oil, or other minor components require organic solvents to be extracted from the biomass (Sluiter et al., 2008).

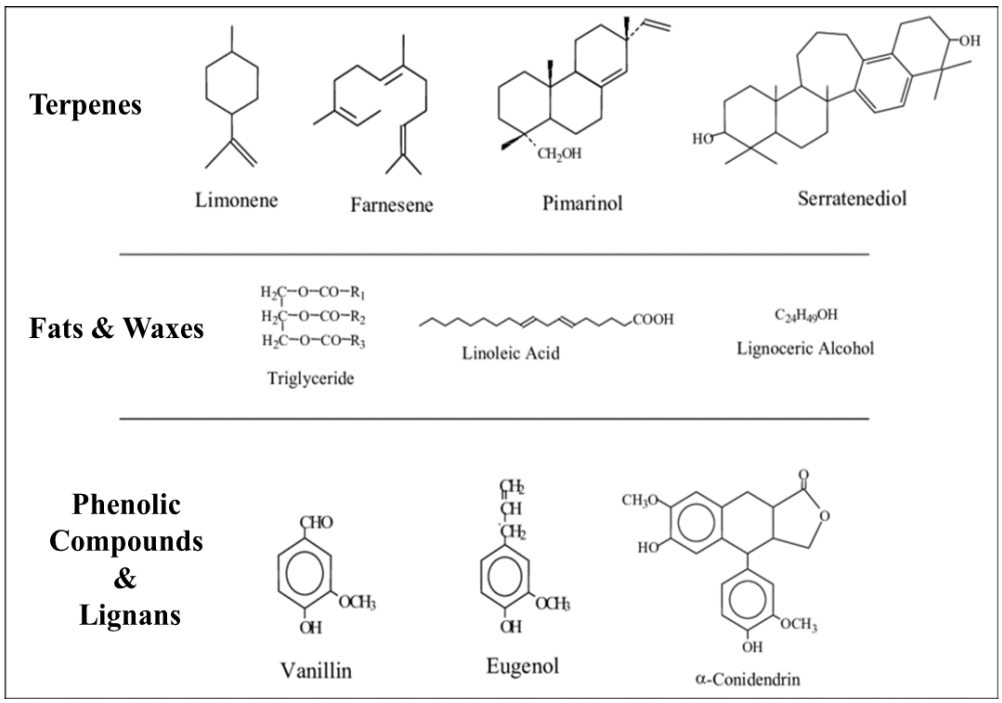

Figure 6 Chemical structure of common extractives (Zimbardi & Viola, 2001).

2.2 Lignin from pulp industry

Lignin from the pulp industry, also known as technical lignin, can be isolated through various chemical, biochemical, and physical treatments depending on the desired end product. Depending on the methods, the chemical structure and purity can be altered. Common technical lignin includes lignosulfonates, kraft lignin, organosolv lignin, and soda lignin (C. Xu & Ferdosian, 2017). Due to the varying nature of biomass, there is no universal pretreatment for all technical lignins. Most treatments still have their drawbacks and advantages.

The pulp and paper industry is among the largest industries in the world (Bajpai, 2014). The pulping process mostly separates the cellulose and hemicelluloses from lignin in biomass. It also removes extractives, which results in a pure fibre pulp that can be used to produce paper and board products. Two different kinds of pulping processes can be used: mechanical and chemical pulping (Nuortila-Jokinen, Mänttfäri, & Nyström, 2003)

Mechanical pulping is a pulping process that separates fibres without the addition of chemicals (Young, Kundrot, & Tillman, 2003). This results in a high yield pulp. However, lignin is retained in the pulp, which results in fibres with high lignin content and consequently lowers the fibres' strength and brightness. Thus, it is not possible to obtain technical lignin from mechanical pulping

Chemical pulping is the most common process today due to the high purity cellulose fibres it produces. This chemical pulping can be divided into two sections: sulphur-based processes and sulphur-free processes, illustrated in Figure 7. The most common method of chemical pulping is kraft pulping, which is a type of sulphur-based process. Other common chemical pulping methods include sulphite- and soda pulping. These chemical processes have for a long time seen lignin as a waste product. Due to its complexity, it has been challenging to use the lignin for other purposes than energy recovery. This creates additional pollution in the environment. This could be avoided by utilising the technical lignin, creating a debottleneck process (Sadeghifar & Ragauskas, 2020), and utilising a lignin first approach (Renders, van den Bosch, Koelewijn, Schutyser, & Sels, 2017).

2.2.1 Sulphur-containing pulping

Sulphur-containing lignins are derived from either kraft pulping or sulphite pulping. Kraft pulping is the most common pulping process. The extracted lignin from kraft process accounts for about 85% of the total lignin production in the world. The kraft process uses a mixture of chemicals, including sodium hydroxide (NaOH) and sodium sulphide (Na_2S), to process the biomass at high temperature (145–170 °C), which dissolves 90–95% of the lignin into black liquor, which can be acidified to recover the lignins. This pulping process fractionates the lignin macromolecules, which decreases molar mass of lignin. Little progress has been made in the industrialisation of kraft lignin, which results

in the black liquor usually being utilised as a low-value energy source in the form of combustion for heat recovery (Chen, 2015). However, several industry parties have gathered in the late 1990s to develop the Lignoboost technology (Tomani, 2010). This created a giant leap in industrialisation by exploiting the energy surplus of a modern kraft pulp mill by extracting lignin from the black liquor, creating new opportunities for economic development. This process differs from traditional lignin precipitation by re-dispersing and acidifying the filter cake after filtration instead of washing lignin immediately, as in conventional approaches. This helps the lignin from clogging the filter cake in the filter medium, creating the primary source of alkali lignin in the market after water-soluble lignosulfonate.

The sulphite pulping process uses an aqueous sulphur dioxide (SO_2) and an aqueous solution of base, usually calcium, sodium, magnesium, or ammonium (Laurichesse & Avérous, 2014). This separates the lignin from the cellulose in a non-precipitated form producing lignosulfonates. The brown liquor of the sulphite pulping can later be precipitated out though alkali conditions to recover the lignins.

2.2.2 Sulphur-free pulping

Sulphur-free lignins are of particular interest due to the structure mainly remaining the same throughout the extraction process. Sulphur-free lignins can be divided into two main categories, organosolv lignin, which originates from solvent pulping and soda lignin, which comes from soda pulping (Shrotri, Kobayashi, & Fukuoka, 2017).

Solvent pulping employs organic solvents, such as acetic acid, formic acid, ethanol, and organic peroxide to dissolve the lignin. This method can easily separate the lignin from the hemicellulose stream and cellulose fraction, resulting in high-quality lignin. However, there are some drawbacks, such as the cost of chemicals and energy consumption compared to other leading pretreatment processes. On the other hand, depending on the raw material, soda pulping extracts lignin by hydrolytic cleavage of the native lignin, which can result in relatively unmodified lignin chemically, with high silicate and nitrogen contents compared to the other lignin types (Laurichesse & Avérous, 2014).

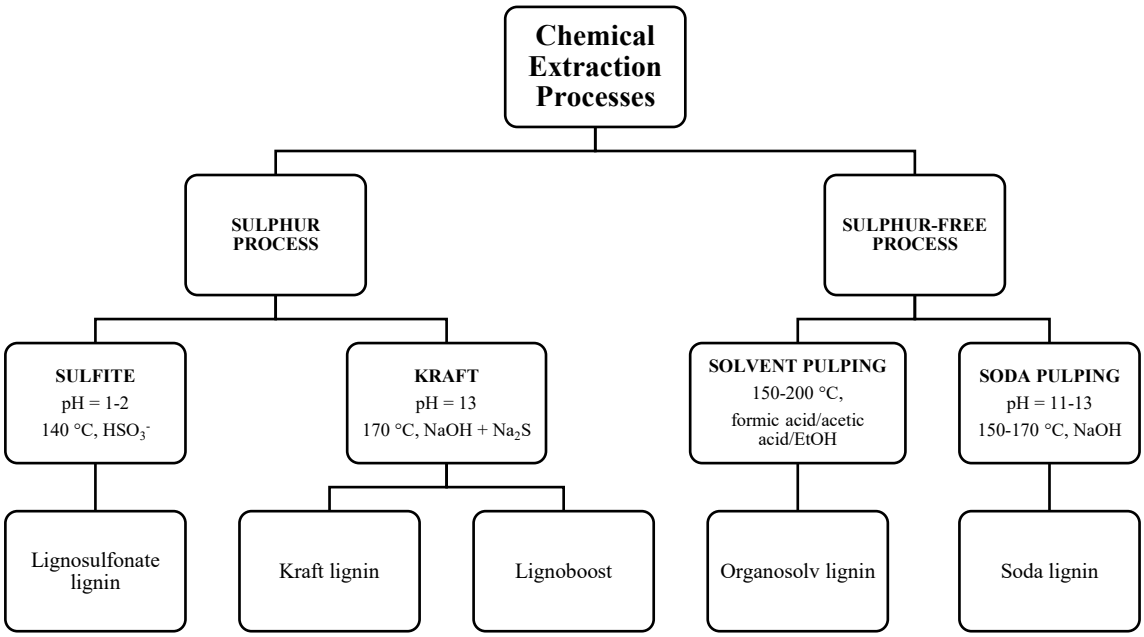

Figure 7 Various chemical processes and their corresponding technical lignins. Modified from Laurichesse & Avérous (2014).

2.3 Lignin from biorefineries

Biorefining can be defined as a sustainable processing of biomass into a spectrum of marketable products and energy. Biorefineries cover an extensive range of combined technologies to achieve sustainable biomass transformation and utilisation as valuable building blocks for biofuels, energy, platform chemicals, and various materials (Morais & Bogel-Lukasik, 2013).

A biorefinery that uses lignocellulosic biomass can be integrated into a two-step process. With suitable technology, the first step focuses on fractionating the biomass into the three main components, cellulose, hemicellulose, and lignin. The second step refines the individual fractions, creating value-added products that can substitute products derived from the fossil industry (Rajesh Banu et al., 2021). The Cellunolix® process of St1 will be presented as an example of a biorefinery utilising timber-industrial waste, such as sawdust and recycled wood as a raw material to obtain value-added chemicals.

Cellunolix® biorefinery concept

St1 Biofuels Oy is focusing on ethanol production technologies that utilize sustainable sources, such as food industry residues, household and grocery biowaste, which are under the concept of Etanolix® and Bionolix® biorefinery production. The Cellunolix® ethanol biorefinery plant built in Kajaani in 2017 was the first of its kind to demonstrate the possibility of producing advanced ethanol from substantial potential source-lignocellulosic sawdust (Borrega, Pihlajaniemi, Liitiä, Wikström, & Tamminen, 2021). In addition to ethanol, lignin, vinasse, turpentine, furfural, carbon dioxide and biogas are produced as by-products of the process. The refining process starts by thermochemically pre-treating the raw material before hydrolysing the raw material with enzymes (Figure 8). The enzymatic hydrolysis process starts by transferring enzymes from the bulk aqueous phase to the surface of the cellulose. Afterwards, the enzymes are absorbed, and the enzyme-substrate complexes are formed. Then, the cellulose is hydrolysed with cellulase, which works as a catalyst by hydrolysing the β-1,4 glycosidic linkages in cellulose. Lastly, the hydrolysis products from the surface of the cellulosic particles are transferred to the bulk aqueous phase. The cellodextrins and cellobiose are then hydrolysed into glucose in the aqueous phase, resulting in sugars that can be fermented and later distilled and dehydrated to produce ethanol (Fan, 2014; Wertz et al., 2018).

The hydrolysis step generates a significant amount of lignin, which could be further integrated into polymers. However, the Cellunolix® lignin is still considered to have a condensed structure with low reactivity (Borrega et al., 2021). Utilisation of the lignin demands fractionation and purifications steps due to its heterogeneous complex structure, which is unsuitable for advanced polymer applications. This hurdle has resulted in the lignin commonly being used in electricity and heat production.

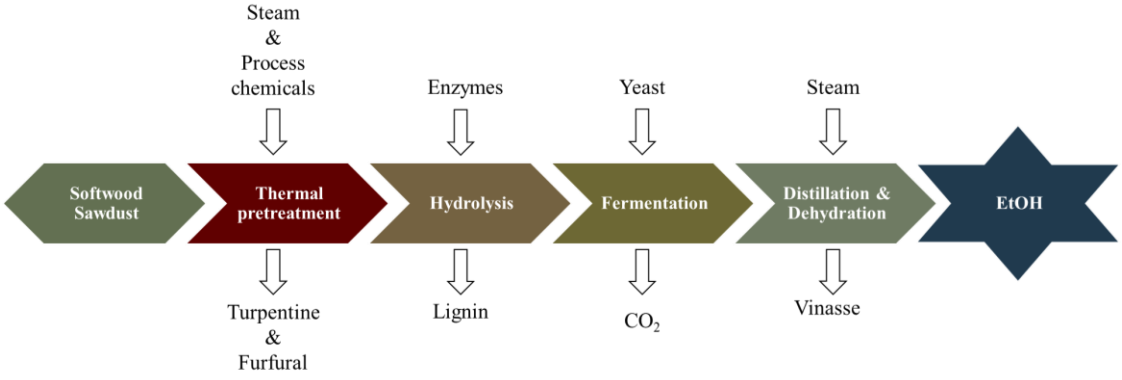

Figure 8 Cellunolix® biorefinery process modified from (Borrega et al., 2021).

2.4 Lignin fractionation for narrow molar mass distribution

Technical lignin derived from the traditional pulping and biorefinery process (biofuel production) provides a class of heterogeneous phenolic polymers. The high degree of variability and diversity of technical lignins creates problems when engineering lignin into a value-added material. In addition, the different interunit bonds of the lignin produce an unpredictable sequence, which results in an undefined primary structure. Combining this unpredictable structure with broad molar mass dispersity makes the reactivity of the lignin challenging to predict and tune (Gigli & Crestini, 2020).

Studies have concluded that molar mass and distribution impact physicochemical properties, obtaining lignin fractions with different characteristics, promoting industrial utilisation (Gigli & Crestini, 2020; Huang, He, Narron, Wang, & Yong, 2017; R. Sun, Tomkinson, & Bolton, 1999). Preparation of lignin with narrower molar mass dispersity and lower structural complexity through lignin fractionation method is one of the primary solution to valorise lignin. Gradual acid precipitation and solvent fractionation are two standard methods for lignin fractionation, which are illustrated in Figure 9.

2.4.1 Lignin fractionation using acids

Sequential lignin fractionation by pH-controlled precipitation from high to low pH is one of the primary developed method. The lignin forms colloidal structures at high pH due to the negative charge on the surface of the colloid. When acid comes into contact with the base, hydronium ions (H^+) neutralise the charge on the colloid surface, and the lignin is precipitated and separated using centrifugation or filtration (Pang, Wang, Sun, Sui, & Si, 2021). Several studies have suggested that high molar mass lignin with small apparent pKa usually precipitates first due to lower solubility and weaker acid groups. However, lignin with low molar mass has a higher content of negative groups (e.g. phenolic OH and COOH), creating a higher negative charge, which results in higher colloidal stability. This makes gradient pH fractionation an excellent way to achieve more homogeneous lignin fractions (Norgren, Edlund, Wågberg, Lindström, & Annergren, 2001; G. Wang & Chen, 2013).

2.4.2 Lignin fractionation using organic solvents

Solvent fractionation utilises the different solubility of lignin fragments in organic solvents to yield more homogenous fractions (Jääskeläinen, Liitiä, Mikkelson, &

Tamminen, 2017). Several studies have tested different kinds of organic solvent for this use, such as ethanol, acetone, methanol and hexane (Ajao, Jeaidi, Benali, Abdelaziz, & Hulteberg, 2019; Meng et al., 2019). The organic solvents can be combined with other solvents with different polarities, which may change the solubility of the lignin fractions. The organic solvent can also be combined with a moderate amount of water, which has been shown as a highly efficient plasticiser to enhance the solubility of lignin, consequently increasing the yield of lignin fractions (Liang, Liu, Fu, & Chang, 2016; Y.-Y. Wang, Li, Wyman, Cai, & Ragauskas, 2018). This creates an environmentally friendly and cost-effective alternative to using several organic solvents. In addition, lignin dissolution can be adjusted by changing the solvent to water ratio depending on the solvent's capability (Boeriu et al., 2014; S. N. Sun, Li, Yuan, Xu, & Sun, 2012).

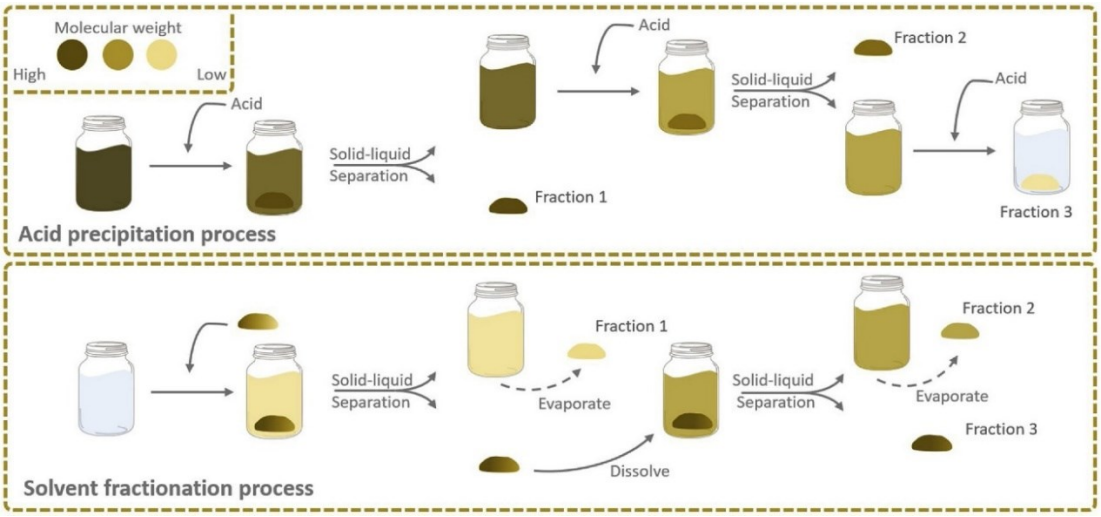

Figure 9 Two common types of lignin fractionation processes resulting in different molar masses. Modified from Pang et al. (2021).

2.5 Driving force towards the valorisation of lignin

Lignin is often classified as a low-value product and is an underutilised feedstock in the growing biofuels industry, even though lignin is a primary component of lignocellulosic biomass. The highly irregular structure of lignin results in heterogeneous chemical features, such as molar mass characteristics, functional group distributions, constituent aromatic units, and interunit bonding patterns. Furthermore, technical lignin usually contains a high amount of impurities, such as extractives, ash, and carbohydrates. These factors usually result in the lignin being dumped or burned as a low-grade fuel, which should be seen as a waste of resources and sustains severe environmental pollution (Gadhave, Mahanwar, & Gadekar, 2018).

The industrial valorisation of technical lignin requires a high purity to be able to produce high-value platform chemicals. Developing an effective method for obtaining low heterogeneity lignin fractions and taking into account the lignins' impurities, composition, molar mass distribution, hydroxyl content, and content of lignin-relevant aromatic units is essential for the realisation of sustainable resource management. By utilising innovative fractionation techniques, lignin can be used to produce a range of value-added chemicals, processed into many products, such as binders, dispersants, phenols, plastic composites, resins, surfactants, and vanillin (Bajpai, 2018b). Several studies have concluded that fractionating lignin using methods such as gradient pH fractionation, solvent fractionation by single/mixture solvent, and a sequential cascade of multiple solvents could result in high purity and polysaccharide-free lignin fractions, which can be utilised as high-value products (Balakshin et al., 2021; Cao et al., 2018; Liu et al., 2021). Recycling lignin to replace petroleum-based resources can alleviate the situation of over-reliance on petrochemicals and reduce environmental pollution. This awareness has also created an incentive to produce methods to isolate purer lignin from the paper- and pulp industry, for example, the lignin-first process (Renders et al., 2017).

2.5.1 Lignin-based thermoplastic materials

Thermoplastics are elastomers that combine the properties of thermo-plasticity and rubber-like behaviour (Holden, 1987). When processed and melted, the thermoplastics keep the same chemical structure and immediately develop rubber-like properties on cooling. The process is rapid and reversible from a processible melt to a solid rubber-like object that differs from the slower process of conventional rubbers, which must be vulcanised to give valuable properties. The advantage of thermoplastic is processing flexibility, which provides them with significant commercial applications. However, thermoplastics are lacking in some end-use properties, such as solvent resistance or upper service temperature. This usually means that thermoplastic elastomers are applied in areas where these factors are less critical, such as footwear, wire insulations, or adhesives.

Lignin-containing polymers can be synthesised by blending rigid lignin molecules with flexible polymers, such as polyethene, ethylene-vinyl acetate or polypropylene. The molecules are either subsequently crosslinked or copolymerised, which results in copolymers of different morphologies. However, the highly polydisperse lignin fractions

are a potential hindrance to synthesising recyclable high-performance lignin copolymers. The high crosslink density and the lignin segments' low molar mass result in thermosets and brittle materials, i.e., barely recyclable (Tomonori Saito et al., 2012). Due to the amorphous properties of lignin, lignin molecules typically has a *Tg* in the range of 90 to 170 °C depending on the plant origin and extraction method. Chao Wang's et al. paper, "Lignin-Based Thermoplastic Materials", summarises some reported glass transition temperature (*Tg*) versus molar mass of kraft lignin. However, it needs to be noted that variations in plant species, extraction and separation approaches, moisture and ash contents, and measurement methods may exist among the literature values (C. Wang, Kelley, & Venditti, 2016).

3 Experimental

3.1 Materials and methods

3.1.1 Enzymatic hydrolysis lignin (EHL)

The lignin used in this study was enzymatic hydrolysis lignin (EHL), which was provided by St1 (Kajaani, Finland). The lignin was obtained as a by-product during bio-ethanol production from pine (*Pinus sylvesteris*) and spruce (*Picea abies*) sawdust with enzyme hydrolysis and yeast fermentation, known as the St1 Cellunolix® process. The EHL was received in a wet cake form, which was freeze-dried before fractionation and characterisation.

3.1.2 Extracted EHL (EEHL)

The extraction was done by washing the EHL with an acid solution (pH 2.5, adjusted by 2.5 M hydrochloric acid (HCl)) and filtered on a 0.2 µm nylon membrane filter to remove any potential salts and water-soluble sugars from the EHL. The washing process was repeated three times before drying the EHL in a freeze drier. Afterwards, methyl tert-butyl ether (MTBE) was used to remove the extractives by mixing the EHL with MTBE under constant shaking using an analogue shaker for at least 72 h. The MTBE phase was then concentrated by using rotary evaporator to recycle the MTBE solution. Subsequently, the MTBE concentrate was dried under N_2 flow and further dried under vacuum desiccator at 40 °C for 12 h to obtain the extracted EHL (EEHL).

3.1.3 Fractionation of EHL and EEHL

The EHL and EEHL were fractionated by gradient acid precipitation or EtOH/H_2O solvent extraction, resulting in ten fractions from each starting material. The fractions and abbreviations can be seen in Table 1.

Table 1 Lignin fractions and abbreviations.

1	EHL/EEHL (freeze-dried)	EHL/EEHL
2	Alkali soluble	Alkali s.
3	pH 5 fractions	pH 5
4	pH 2.5 fractions	pH 2.5
5	75% direct EtOH soluble	75% EtOH s.
6	75% direct EtOH insoluble	75% EtOH in.s.
7	96% sequence EtOH soluble	96% EtOH s.
8	96→75% sequence EtOH soluble	96→75% EtOH s.
9	96→75% sequence EtOH insoluble	96→75% EtOH in.s.

Gradient pH fractionation

The first step of the fractionation process started with a screening to determine which pH levels should be chosen for the gradient fractionation. This screening consisted of mixing the freeze-dried EHL/EEHL in a sodium hydroxyde (NaOH) solution (pH 10, adjusted by 6 M NaOH(aq))Then, 6 M NaOH(aq) was added until a pH of 12.5 was achieved. The suspension was heated to 90 °C while stirring for 2 h to ensure that lignin was fully soluble in the NaOH solution. Afterwards, the mixture was cooled down to ambient temperature before centrifuging and filtrating on a 0.2 µm nylon membrane filter to excludeinsoluble fractions, e.g., carbohydrates that were not entirely removed by the enzymatic hydrolysis process. Then the filtrate composed of the alkali-soluble mixturewas divided into seven parts. The pH of the solutionwas separately adjusted to pH 9, 7, 6, 5, 4, 3, and 2.5 with 2 M HCl(aq) to precipitate lignin from the alkaline solution as seen in Figure 10. The screening experiment concluded that most of the lignin was precipitated at pH 5 and the lowest pH of 2.5. Therefore, the gradient fractionation was continued using these two pHs.

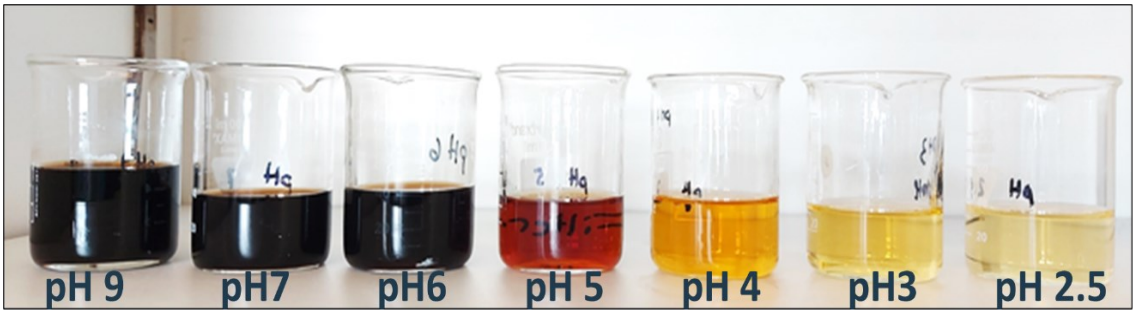

Figure 10 The filtrates collected from pH screening, consisting of pH 9, 7, 6, 5, 4, 3, and 2.5 filtrates.

The gradient pH fractionation started by dissolving EHL/EEHL in NaOH solution at pH 12.5 (90 °C, 2 h). Then the pH of the alkali-soluble solution was directly adjusted to pH 5 with 2 M HCl(aq) to precipitate part of the lignin. The mixture was then centrifuged at 10 000 rpm for 10 min, and then the sediments were mixed with acid water (pH ≤2.5), re-centrifuged, and finally filtrated on a 0.25 µm nylon membrane filter and washed again with acidic water. The filter cake was then freeze-dried.

The pH of the filtrate was further adjusted from pH 5 to 2.5 to precipitate and isolate the remaining lignin using the same procedure as above. The procedure is illustrated in Figure 11.

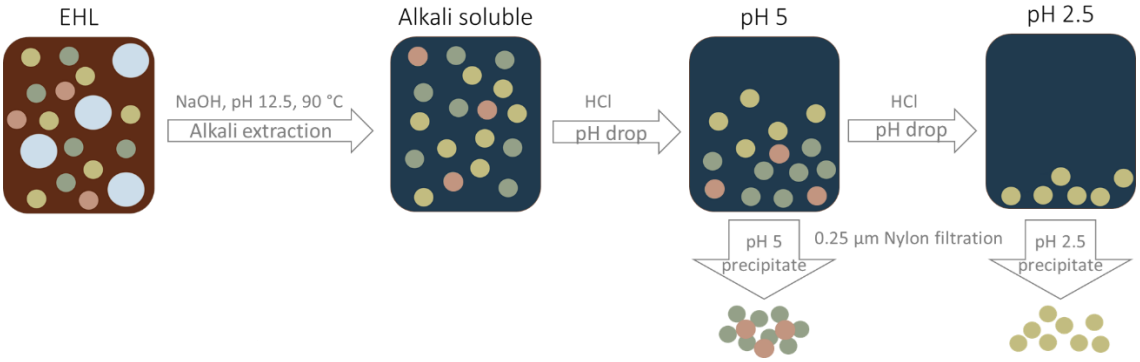

Figure 11 Process scheme of gradient pH fractionation, resulting in a pH 5 and a pH 2.5 soluble fraction.

Solvent fractionation

The second lignin fractionation process involved using technical ethanol (EtOH) to extract lignin sequentially. Firstly, freeze-dried EHL/EEHL was mixed with 96% EtOH at a constant room temperature for 4 h (Figure 12). The soluble and insoluble fractions were separated by filtration. The soluble fraction was recovered by evaporating the EtOH under reduced pressure in a rotary evaporator. The insoluble fraction was further dissolved in 75% EtOH in water, and the separation process was repeated. All fractions were further dried in a vacuum oven at 40 °C for 12 h.

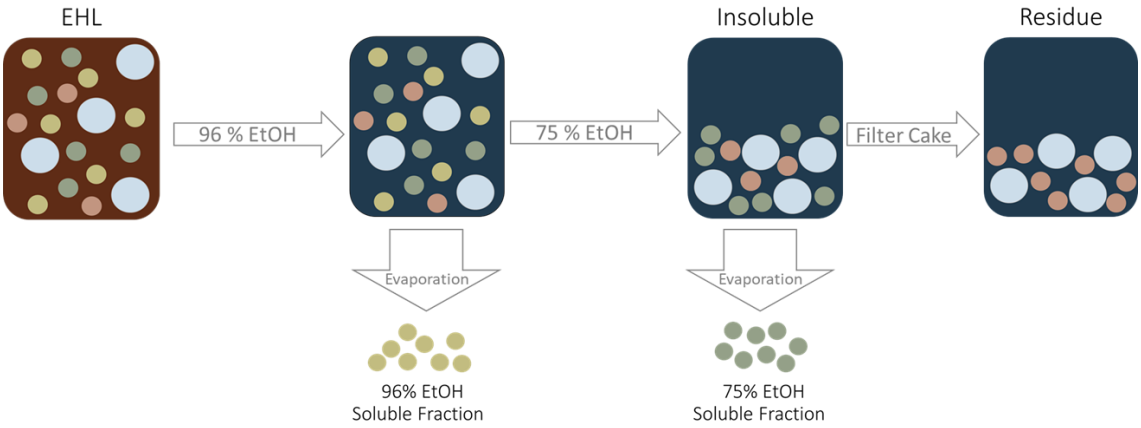

Figure 12 Process scheme of sequential EtOH fractionation, resulting in two different soluble fractions from 96% and 75% ETOH, respectively.

To be able to study the impact of sequential EtOH fractionation on the properties of lignin fractions, a direct solvent fractionation using 75% EtOH in water was used according to the same procedure as above. The direct fractionation procedure is illustrated in Figure 13.

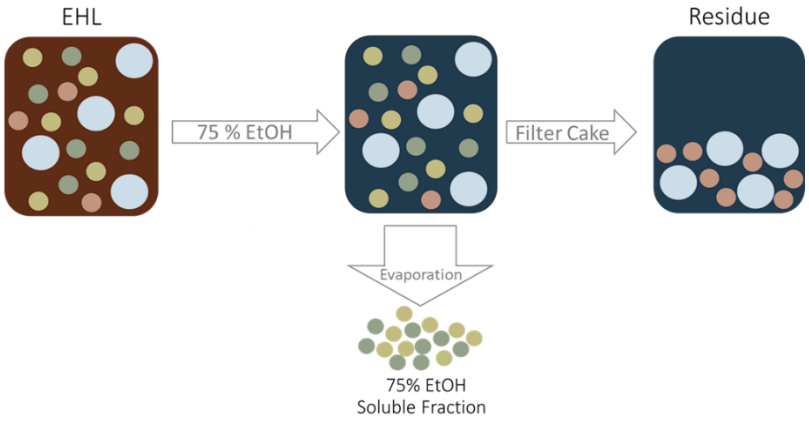

Figure 13 Process scheme of direct 75% EtOH fractionation, providing one soluble fraction at 75% EtOH.

3.2 Characterisation

3.2.1 Molar mass determination

Determination of weight-average (*M*w) and number-average (*M*n) molar mass, as well as molar mass dispersity ($Đ_M$) of the EHL and EEHL fractions, were performed on a gel permeation chromatography (GPC) system equipped with a differential refractive index (RI) concentration detector and a multiangle light scattering detector in the infrared range ($MALS_{(IR)}$). The instrumental setup and the measurement with GPC/RI/$MALS_{(IR)}$ were carried out following the conditions described in detail by Wang et al. (2021). The freeze-dried sample was dissolved in DMSO/LiBr (0.05 M) to 10 mg/mL, filtered on a 0.2 µm Nylon syringe filter and analysed with a series of connected columns (Jordi Gel Glucose Mixed-bed guard column (10 × 50 mm, I.D. × L) and a Jordi Gel GBR Mixed-bed column (10 × 250 mm, I.D. × L)) using DMSO/LiBr (0.05 M) as eluent. The GPC system was operated under the following conditions: eluent flow rate of 0.5 mL/min; 100 µL injection volume, 60 °C column temperature. The dn/dc value of 0.15 mL/g was used for molar mass moments calculation. Data evaluations were done using ASTRA software, version 7.3.2.

3.2.2 Lignin content

The lignin content was determined using the standardised Klason method and an ultraviolet-visible (UV-vis) spectrophotometry analysis to quantify the acid-insoluble (AIL) and the acid-soluble (ASL) lignin, respectively (Maekawa, Ichizawa, & Koshijima, 2006; Schwanninger & Hinterstoisser, 2002). Briefly, the AIL was determined by hydrolysing approximately 100 mg samples with 1.5 mL 72% sulphuric acid (H_2SO_4) in an ice bath. The lignin samples were stirred frequently for 2 h to ensure that the samples were fully impregnated. Afterwards, distilled water was added to dilute the mixture into a 3% concentration of H_2SO_4, and the mixture was then boiled for 4 h. The samples were then left at ambient temperature for at least 8 h. The samples were filtrated on a glass filter (grade 3 porosity) and washed with hot water before drying in an oven at 105 °C for 12 h. The acid-insoluble lignin was then calculated by the difference between initial sample weight and weight of residue, described in Equation 1 (Fagerstedt et al., 2015; Innventia; Korpinen et al., 2014).

Equation 1 $$AIL\ (wt\%) = \frac{m}{M} \cdot 100$$

where

m = The residue after drying, in g

M = Oven-dry weight of sample (i.e., as 100% dry matter), in g

The ASL remaining in the filtrate from the Klason analysis was later used to determine the ALS content according to the TAPPI UM 250 standard. In short, the ASL content was determined by UV-vis, which is measuring the absorbance difference between the Klason filtrate and 3 wt% H_2SO_4 reference solution at 205 nm. The ASL content was calculated using Equation 2.

Equation 2 $$ASL\ (wt\%) = \frac{A \cdot D \cdot V}{a \cdot b \cdot M} \cdot 100$$

where

A = Absorption difference at 205 nm

D = Dilution factor

V = The volume (L) of the filtrate, 0.0571 L

a = 110 L/g cm, extinction coefficient of lignin at 205 nm according to TAPPI UM 250)

b = cuvette path length (cm), 1 cm

M = Weight of sample (as 100% dry matter), in g.

3.2.3 Carbohydrate content

The carbohydrate content of the lignin fractions was determined by performing both acid methanolysis and acid hydrolysis, followed by gas chromatography (GC) according to the procedure described by Sundberg et al. (1996). The acid methanolysis approach determines the sugar units in hemicelluloses and pectins as well as in monosaccharides and oligosaccharides, while acid hydrolysis gives the total sugar content.

Acid methanolysis

To determine the chemical composition as well as the content of non-cellulosic carbohydrates in EHL and EEHL fractions, acid methanolysis and GC analysis were performed following the procedures described by Sundberg et al. (1996). Briefly, approximately 10 mg (± 0.1 mg) of sample was methanolysed into monosaccharides using 2 mL 2 M HCl in anhydrous methanol solution at 105 °C for 4 h. A carbohydrate

calibration solution containing an equal amount of neutral monosaccharide (e.g., xylose, galactose, mannose, rhamnose, arabinose and glucose) and uronic acid (e.g., galacturonic acid and glucuronic acid) was also subjected to the same methanolysis process. The mixture was afterwards cooled down to ambient temperature and neutralised with pyridine. An internal standard of 1 mL of 0.1 mg/mL resorcinol was added before the samples were dried by N_2 flow in a 50 °C water bath. Lastly, the samples were trimethylsilylated by adding 150 µL pyridine, 150 µL hexamethyldisilazane, and 70 µL trimethylchlorosilane (TMCS). The trimethysilylated-derivatives of sugars and uronic acids were analysed with a GC-FID (PerkinElmer AutoSystemXL) equipped with an Agilent HP-1 (0.20 mm × 25 m, I.D. × L, 0.11 µm film thickness) and HP-5 (0.20 mm × 25 m, I.D. × L, 0.11 µm film thickness) capillary columns. The non-cellulosic carbohydrates were quantified against resorcinol using the correction factor (Equation 3, using arabinose as an example) determined by each monosaccharides and uronic acids from the calibration sample.

Equation 3

$$correction\ factor = \left(\frac{Area_{Arabinos}}{Area_{Recorcinol}}\right)^{-1}$$

Lastly, an anhydrosugar coefficient for pentoses (0.88), hexoses (0.90), and uronic acid (0.91) were used to calculate the carbohydrate content as polysaccharides. All analyses were done at least as duplicates.

Acid hydrolysis

The carbohydrate content from cellulose was determined by subjecting approximately 10 mg of freeze-dried sample to acid hydrolysis. This procedure consisted of adding 0.2 mL of 72% H_2SO_4 into each sample and placing the samples in a vacuum oven, letting the pressure go to zero and then back to normal to ensure that the H_2SO_4 penetrated the samples, then adding 0.5 mL distilled water in each test tube, waiting 4 h and adding an additional 6 mL of distilled water in each test tube and leaving the samples at ambient temperature for at least 8 h. This procedure used glass balls in the vials and was kept under constant shaking using an analogue shaker during the addition of water and the waiting period. Afterwards, the samples went through an autoclavation for 90 min at 125 °C and were then cooled down to ambient temperature before adding barium carbonate to neutralise the samples. An internal standard of 1 mL of 1 mg/mL sorbitol was added

before the samples were centrifuged. Then 0.5 mL of supernatant was mixed with approximately 8 mL of pure acetone and dried before performing the trimethylsilylation and GC-FID analysis with the same procedure as described in the methanolysis section. In order to estimate the correctional factor, the same procedures were performed in a separate vial with glucose.

3.2.4 Extractive content analysis

The extractive content in the lignin samples was determined by GC-FID after extraction with MTBE at acidic conditions (Örså & Holmbom, 1994). Approximately 20 mg was used for the EHL samples and 40 mg for the EEHL samples. Each sample was mixed with 4 mL of HCl solution at pH 2.5. To ensure that all the samples had pH under 3, one drop of bromocresol green as pH indicator was added, and if needed, 0.05 M of H_2SO_4 was added to the samples for pH adjustment. Then, 2 mL of MTBE solution containing internal standards of heneicosanoic acid, betulinol, cholesteryl heptadecanate and 1,3-dipalmitoyl-2-oleoylglycerol with the concentration of 0.02 mg/mL was added. The samples were shaken intensely for 1 minute before centrifugation to isolate the upper phase containing the extractives. The extraction process was repeated two more times using pure MTBE solvent. Afterwards, the MTBE phase was evaporated using N_2. The samples were then silylated using 80 µL bis(trimethylsilyl)trifluoroacetamide, 20 µL pyridine and 20 µL TMCS. The samples were kept in an oven at 70 °C for 45 minutes to ensure proper silylation. Afterwards, the samples were analysed using GC-MS (Agilent G1530A, Agilent MSD 5973) to identify the extractives and GC-FID (PerkinElmer, Clarus 500) on an Agilent J&W HP-1/SIMDIST capillary column (0.53 mm × 6 m, I.D. × L, 0.15 µm film thickness) to quantify the extractives.

3.2.1 Ash content analysis

The ash content was determined gravimetrically according to Tappi T211 (1993) (Sluiter et al., 2008). Approximately 0.25 g of EHL/EEHL was placed in a muffle furnace set to 525 ± 25 °C and left in the oven until constant weight. The ash weight percentage was then calculated using Equation 4.

Equation 4

$$Ash\ (wt\%) = \frac{Weight_{\ Container\ plus\ ash} - Weight_{\ Container}}{Oven\ dry\ weight} \cdot 100$$

3.2.1 Hydroxyl groups quantification

The hydroxyl groups in the samples were analysed by quantitative ^{31}P NMR (298 K) with a Bruker AVANCE III 500 MHz spectrometer operating at 500.13 MHz for ^{1}H, 125.77 MHz for ^{13}C, and 202.46 MHz for ^{31}P, using the *zgig* pulse program.

The samples were prepared by drying the samples in a vacuum oven at 40 °C for 12 h to ensure that the dry weight was accurately known for the quantification. Afterwards, 20 mg of the samples were phosphorylated with 100 µL of 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane in (TMDP) 550 µL of anhydrous pyridine and deuterated chloroform (1.6:1, v/v) solution containing an internal standard of 12 µmol of endo-N-hydroxy-5-norbornene-2,3-dicarboximide and 1.04 g/L of $Cr(acac)_3$ as a relaxation reagent. The acquisition parameters had an interpulse delay of 10 s, an acquisition time of 2.0 s, and a number of scans of 64. A signal of Cl-TMDP-water phosphorylation product at 132.2 ppm was used to calibrate the ^{31}P NMR spectra and the standard peak for calculating the integral area of the internal at 152.4-151.2 ppm was set to 0.6 mmol/g.

3.2.2 Thermal properties

Glass transition temperature (*Tg*)

The *Tg* of each sample was determined using a differential scanning calorimetry (DSC) with a TA instrument DSC250. The temperature profile was calibrated based on indium standards. Each lignin fraction was dried at 40 °C under reduced pressure for 12 h before being precisely weighted into a Tzero pan (T 140829, Switzerland) and sealed with a Tzero lid (T 140826, Switzerland). The temperature protocol started from 40 °C and was ramped up 10 °C/min to 120 °C and kept isothermally for 2 minutes. Then the temperature was taken from 0 to 200 °C with a rate of 20 °C/min under N_2 atmosphere (50 mL/min). This created a ramp curve, where the midpoint of the inflexion in the second heating trace is reported as *Tg*.

4 Results and discussion

4.1 Extraction results

The EHL contained a noticeable amount of non-lignin substances that originated from the wood itself, as well as some residual inorganic salts, making it unsuitable for most industrial uses. To promote understanding of the lignin structure-application performance correlation, it is vital to identify the components in commercial, technical lignin.

The extractive analysis results of the EHL and EEHL are shown in Figure 14. The unfractionated EHL contained 1.2 wt% of extractives, which decreased to 0.4 wt% in the EEHL. The most significant change occurred in the content of free fatty acids, the phenolic related extractives and resins acids. On the other hand, the smallest difference was observed in the triterpenoids. The lower content of extractive compounds in EEHL indicated that the extractives removal using MTBE was successful, which resulted in a purified lignin fraction.

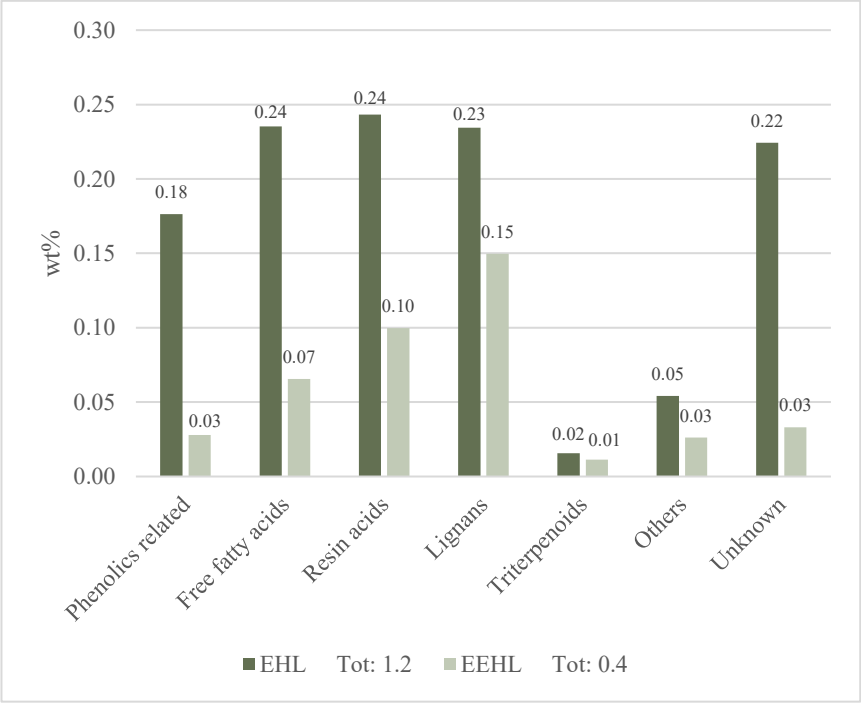

Figure 14 Extractive content in EHL (1.2 wt% of extractives) and EEHL. (0.4 wt% of extractives).

4.2 Fraction analysis results

4.2.1 Yield of EHL and EEHL fractions

A compatible yield from the fractionation process is vital for industries to utilise the process step. The yields of EHL and the EEHL fractions are based on the initial EHL and EEHL, respectively.

EHL fractions

The yields of the EHL fractions are illustrated in Figure 15. The pH fractions of the EHL started with 30% from the alkali-soluble fraction due to 70% of the lignin being alkali-insoluble. Then 17% of the lignin was obtained from the pH 5 precipitation, and the remaining lignin precipitated out at pH 2.5 had a yield of 11%. Due to the small yield of the pH 2.5 fraction, there was not enough material to go through all characterisations. As expected, the yield of lignin depended on the precipitation pH, which resulted in a gradual decrease while the lignin was precipitated out in each step.

The yield of the sequential EtOH fractions decreased along with the sequence, starting with a yield of 13% from the 96% EtOH soluble fraction. The remaining residue was redissolved in 75% EtOH, which resulted in a yield of 5%. In comparison, the direct 75% EtOH soluble fractionation had a slightly higher yield of 20%, resulting from a less homogenous fractionation.

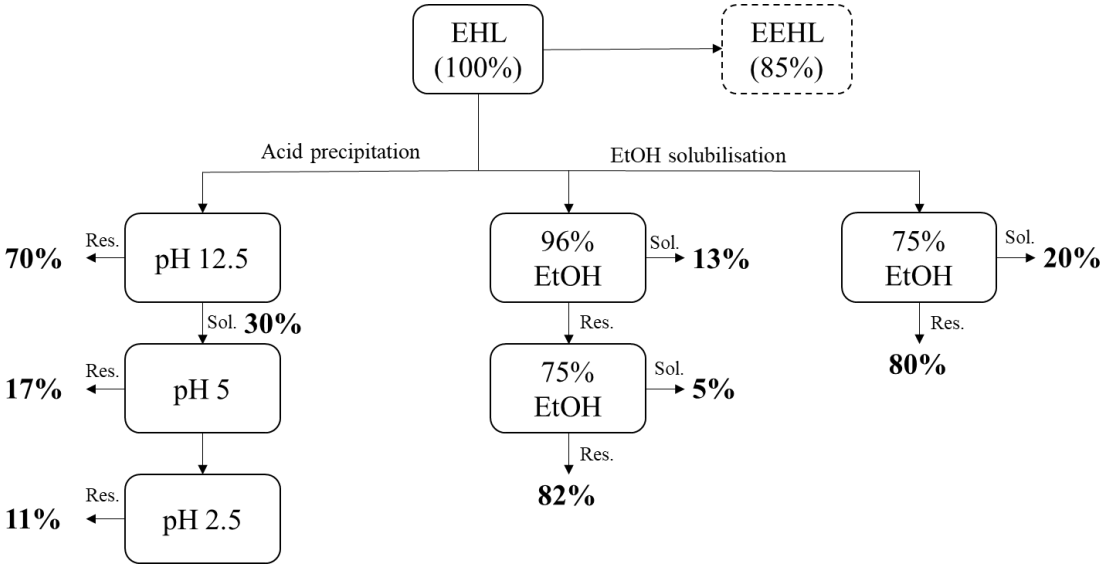

Figure 15 EHL fraction yields.

EEHL fractions

The yield of EEHL fractions is illustrated in Figure 16. The MTBE extraction resulted in 85% of the EHL being recovered due to the removal of extractives and other soluble compounds. This resulted in higher yields in the EEHL fractions. About 40% of the EEHL was recovered in the alkali solution. From this, 23% was obtained in the pH 5 fraction were obtained. The EEHL EtOH fractions had generally higher yields than the EHL fractions, except the sequential 96% EtOH fraction, which had slightly lower yield at 9%. This could be due to the 96% EtOH not being as efficient in dissolving the more homogenous EEHL than the EHL. The direct 75% EtOH soluble fraction had the highest soluble yield at 30%.

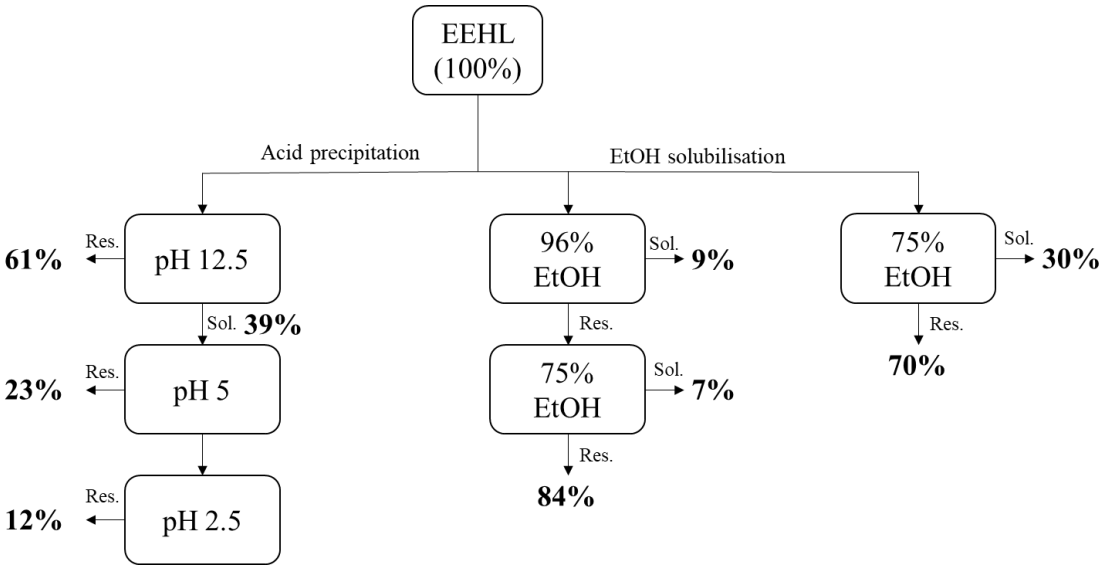

Figure 16 EEHL fraction yields.

4.2.2 Molar mass analysis

Valorisation of technical lignin demands lignin with a homogeneous feature in terms of molar mass dispersity ($Đ_M$). Previous research has concluded that the bulk nature of technical lignins is too heterogeneous in structure, too impure, and too complex for utilisation in advanced applications as a polymer (Pang et al., 2021). Therefore, the EHL/EEHL fractions should display a change in weight-average molar mass (*Mw*), number-average number-average molar mass and $Đ_M$ (*Mw*/*Mn*), and along with a decrease in impurities, such as extractives, ash, and carbohydrates and other heterogeneous chemical features in terms of functional group distributions, aromatic units, and interunit bonding. Determining the molar mass characteristics of the lignin is still a significant challenge due to the absence of reliable calibration standards. However,

GPC/RI/MALS$_{(IR)}$ has been proven to be a valuable tool to monitor the absolute molar masses of lignin (L. Wang et al., 2021).

EHL fractions

Due to the dissociation of phenol and carboxyl groups of lignin, EHL/EEHL will be dissolved in alkaline conditions. When HCl comes into contact with the alkali solution, H^+ neutralises the charge on the colloid surface, and the lignin is precipitated (Pang et al., 2021; G. Wang & Chen, 2013).

The *Mw*, *Mn* and $Đ_M$ of the EHL fractions are presented in Table 2. The *Mw*, *Mn* and $Đ_M$ were higher in alkali-soluble fraction than in the EHL. The alkali-soluble fraction had a significant higher *Mw* (30 000 g/mol) than the (12 000 g/mol) EHL, while the pH 5 fractions *Mw* (32 000 g/mol) was slightly higher than the alkali soluble but lower than the pH 2.5, which had the highest *Mw* of all fractions (54 000 g/mol). The *Mn* of the EHL (9 900 g/mol) also got higher throughout the gradient pH fractionation. The alkali-soluble fraction (29 000 g/mol) had a bit lower value compared to the pH 5 fraction (31 000 g/mol) and the pH 2.5 fraction again showed the highest value (54 000 g/mol). The $Đ_M$ of the EHL started at 1.2 and decreased to 1 in all pH fractions.

It was found that the EtOH 75% soluble and 96% soluble fractions had a much lower *Mw* (7 700 g/mol and 4 800 g/mol, respectively) than the initial EHL (12 000 g/mol) and pH fractions (Table 2). The $Đ_M$ of the 75% EtOH soluble ($Đ_M$ = 1.4) and the 96% EtOH fraction ($Đ_M$ = 1.3) was slightly higher than the EHL ($Đ_M$ = 1.2). It has been demonstrated that fractionation with EtOH results in a fraction with more carbohydrates and extractives, thus lowering the molar mass (Ni & Hu, 1995). The EtOH-insoluble fractions were not fully soluble in the DMSO/LiBr eluent and thus were not adequately analysed.

The EtOH sequential fractionation used water to dilute the solvent while simultaneously increasing the solubility of EtOH to lignin. The 75% EtOH soluble fraction had a bit higher *Mw* (7 700 g/mol) and *Mn* (5 600 g/mol) compared to the 96% EtOH soluble fraction (4 300 and 3 100 g/mol, respectively). Furthermore, the sequential 96% to 75% EtOH soluble fraction had significantly higher *Mw* and *Mn* values (14 000 and 11 000 g/mol, respectively), while the $Đ_M$ (1.3) was slightly lower compared to the direct 75%

EtOH soluble fraction (1.4). This could result from the lower value *Mw* and *Mn* of the lignin being separated during the 96% EtOH sequential fractionation step.

Table 2 Molar mass characteristics of EHL fractions, including initial unfractionated EHL.

EHL fractions	Mw [g/mol]	Mn [g/mol]	$Đ_M$
Unfractionated EHL	12 000	9 900	1.2
pH fractions			
Alkali s	30 000	29 000	1.0
pH 5	32 000	31 000	1.0
pH 2.5	54 000	54 000	1.0
EtOH fractions			
75% EtOH s	7 700	5 600	1.4
75% EtOH in.s	11 000	7 900	1.5
96% EtOH s	4 800	3 600	1.3
96→75% EtOH s	14 000	11 000	1.3
96→75% EtOH in.s	16 000	14 000	1.2

The alkali-soluble, pH 5 and pH 2.5 fractions showed two distinct peaks in the GPC chromatograms (Figure 17), the first peak appeared at 15-25 min and the latter appeared at around 25-35 min. However, due to aggregation, the light scatter (LS) signal created a significant exclusion peak where the refractive index (RI) signal representing the concentration was not observed in the first peak, making the peak at around 30 min more reliable for molar mass calculation. All RI values were similar for all fractions, while the LS values mostly varied.

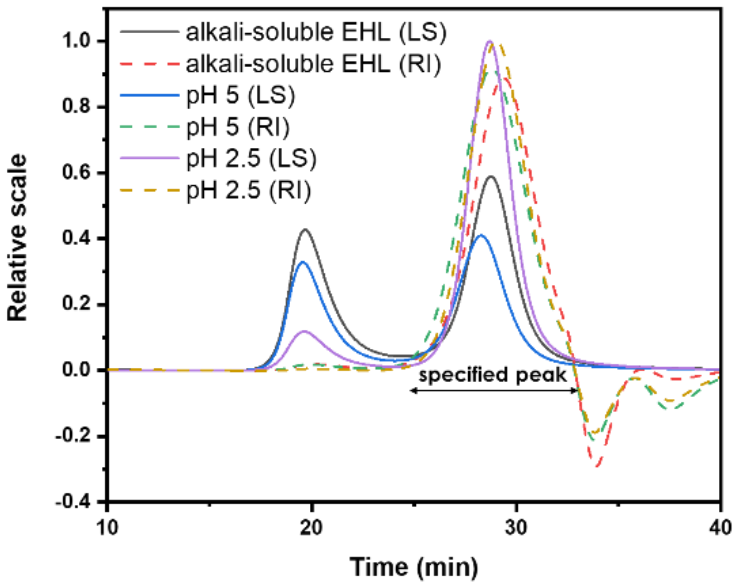

Figure 17 Molar mass distribution of alkali-soluble and pH fractions.

The GPC/RI/MALS curves of the EtOH soluble fractions have a singlet profile (Figure 18), which is the most significant difference compared with the GPC/RI/MALS profile of pH fractions. The LS values of the soluble 75% EtOH soluble fraction was similar to the EHL. However, compared to the EHL, the RI value was higher in the EtOH soluble fraction. The sequential EtOH fractionation slightly changed the LS compared to the unfractionated EHL. However, the RI profiles were more similar than the direct 75% EtOH fractionation.

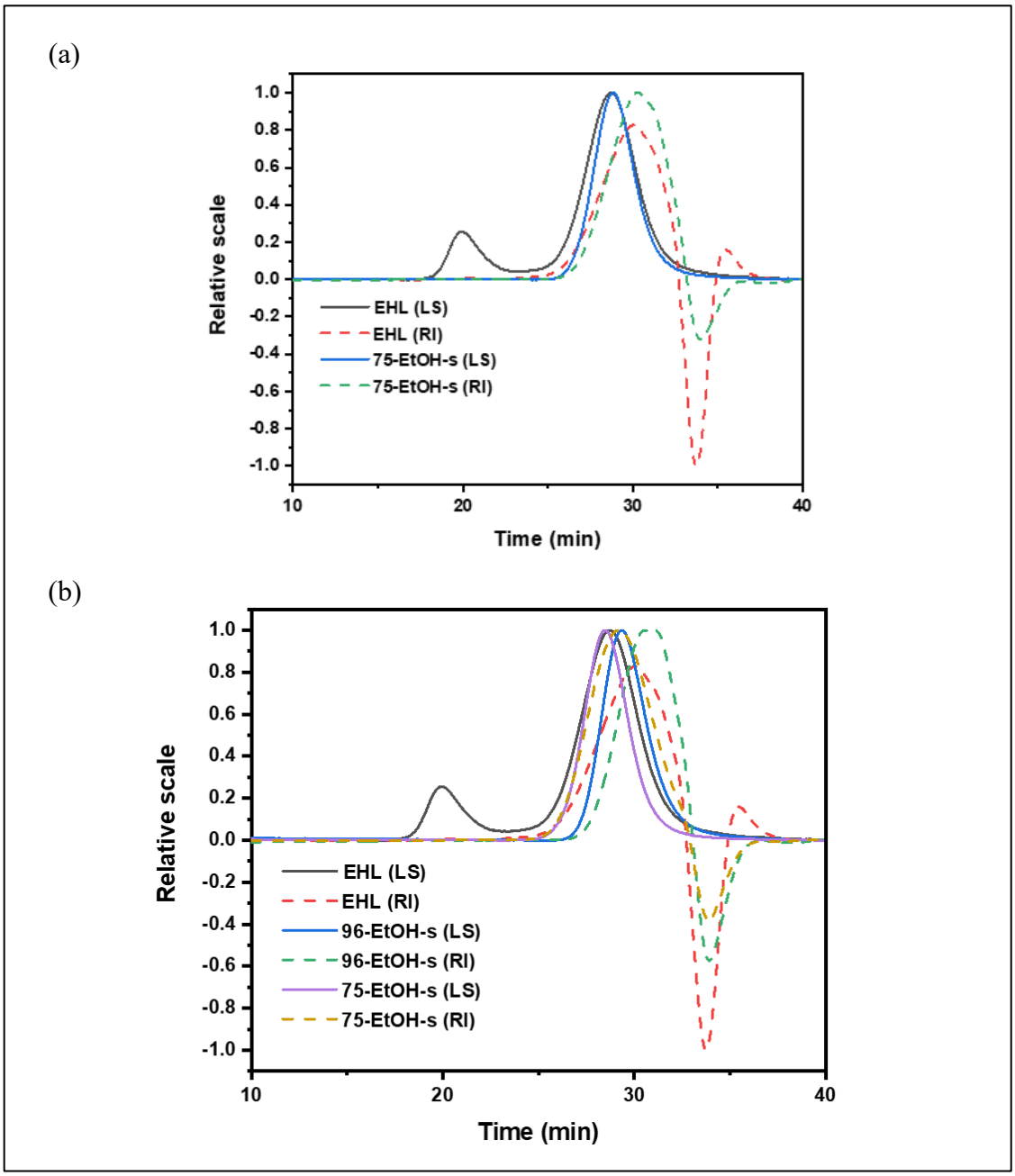

Figure 18. Molar mass distribution of 75% EtOH fractions (a) and sequential 96 to 75% EtOH fractions (b).

EEHL fractions

The molar mass results of the EEHL fractions can be seen in Table 3. There was an apparent decrease in molar mass in the unfractionated EEHL (11 000 g/mol) compared to the unfractionated EHL (12 000 g/mol), concluding that the MTBE extraction could

remove impurities. All EEHL fractions showed similar molar mass changes as the EHL fractions did during fractionation.

The alkali-soluble fraction had a higher *Mw* (24 000 g/mol compared to 11 000 g/mol) and a slightly lower $Đ_M$ (1.1) than the unfractionated EEHL ($Đ_M$ = 1.2). The pH 5 fraction had the same *Mw* as the alkali soluble fraction, however, the $Đ_M$ (1.3) was slightly higher in the pH 5. The pH 2.5 had the highest *Mw* of all EEHL fractions (31 000 g/mol) and its $Đ_M$ (1.1) decreased compared to pH 5 fraction.

The EtOH fractions again showed a lower molar mass than the pH fractions, similar to the EHL fractions. Similar to the EHL fractions, the EEHL 75% EtOH soluble fraction had a slightly higher *Mw* (6 300 g/mol) and *Mn* (4 800 g/mol) than the 96% EtOH fraction (4 400 and 3 500 g/mol, respectively). Likewise, the sequential 96% to 75% EtOH fraction also had higher *Mw* and *Mn* values (9 600 and 8 000 g/mol, respectively) than 75% EtOH soluble fraction. This could also be explained by the lower values of *Mw* and *Mn* of the lignin being separated during the 96% EtOH sequential fractionation step.

Table 3 Molar mass characteristics of EEHL fractions, including initial unfractionated EEHL.

EEHL fractions	Mw [g/mol]	Mn [g/mol]	ĐM
Unfractionated EEHL	11 000	9 300	1.2
pH fractions			
Alkali s.	24 000	21 000	1.1
pH 5	24 000	18 000	1.3
pH 2.5	31 000	27 000	1.1
EtOH fractions			
75% EtOH s.	6 300	4 800	1.3
75% EtOH in.s.	15 000	13 000	1.2
96% EtOH s.	4 400	3 500	1.3
96→75% EtOH s.	9 600	8 000	1.2
96→75% EtOH in.s.	19 000	18 000	1.1

The GPC/RI/MALS curves of the EEHL alkali-soluble, pH 5 and pH 2.5 fraction are illustrated in Figure 19. They have the same double-peak profile as the pH fractions from EHL

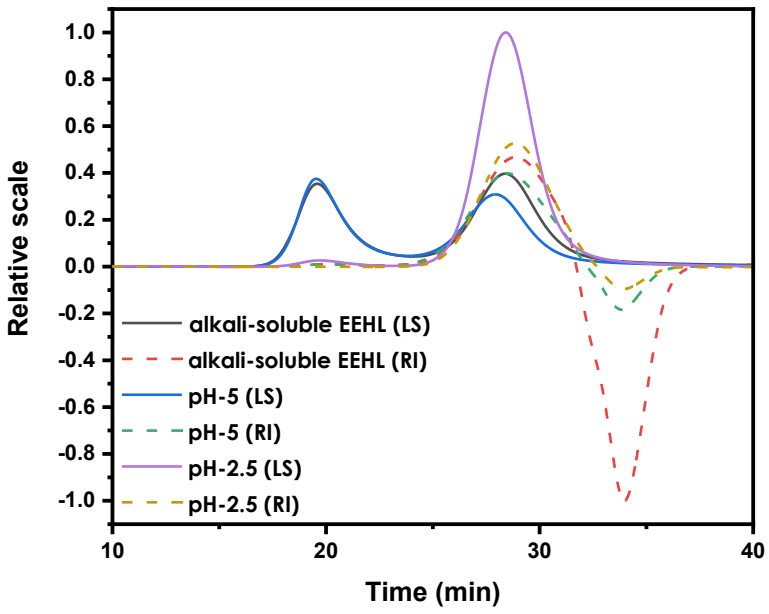

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Figure 19 Molar mass distribution of EEHL alkali-soluble and pH fractions.

The $Đ_M$ of the EEHL EtOH fractions had a more noticeable change compared to the pH fractions, as seen in Figure 20. The LS profile of EEHL 75% EtOH soluble fractions had the slightest change, while all the other fractions decreased in LS and RI values. The sequential EtOH fractionation had the most significant change, compared to the EHL fractions, with a considerable decrease in LS and RI in all sequential fractions.

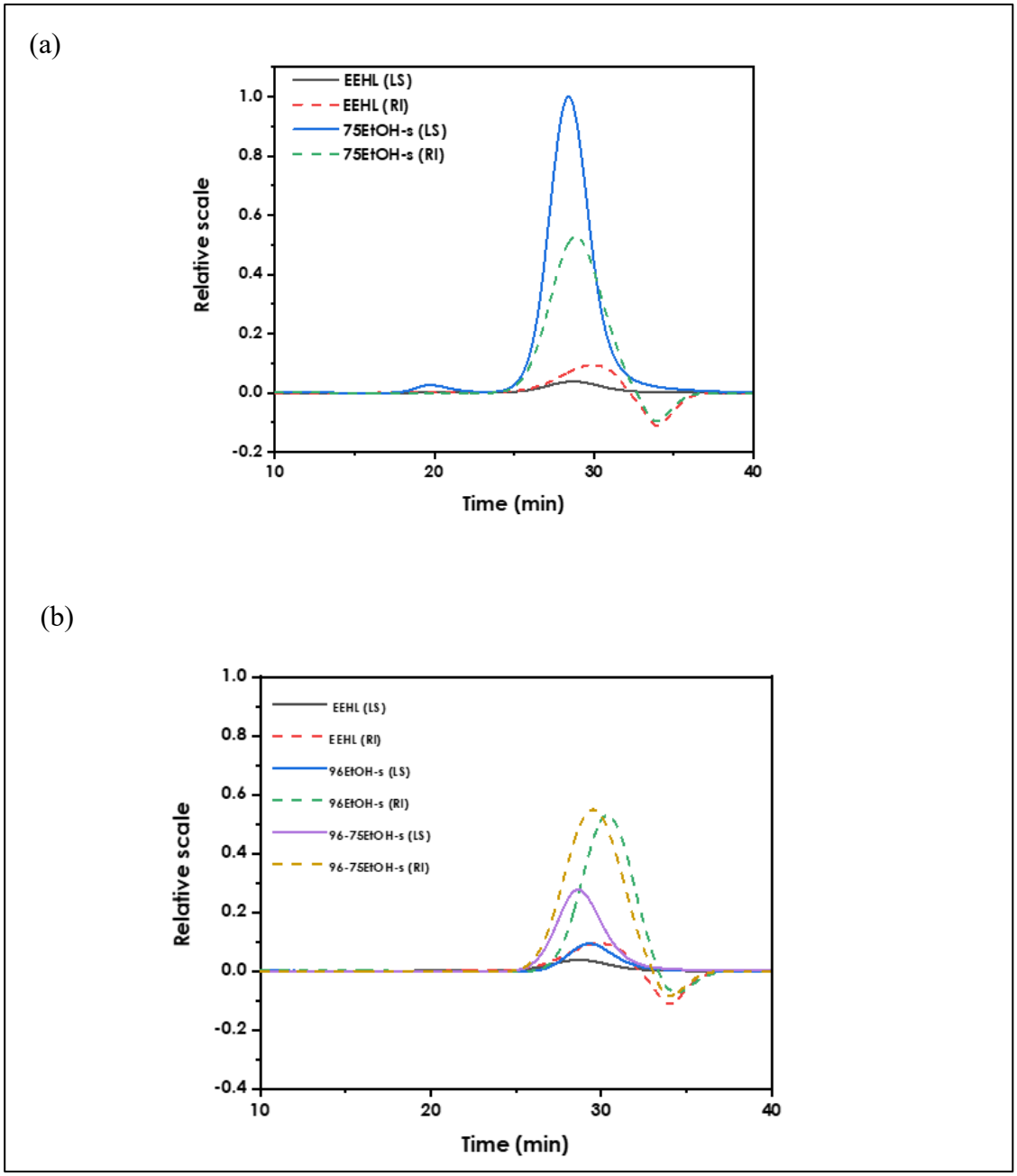

Figure 20 Molar mass distribution of 75% EtOH fractions (a) and sequential 96 to 75% EtOH fractions (b)

4.2.3 Compositional analysis

EHL fractions

The compositional analysis of the EHL fractions is shown in Figure 21. The EHL had a relatively high lignin content (77 wt%). However, it displayed quite heterogeneous properties with a noticeable amount of carbohydrates (14 wt%) as well as extractives (1.2 wt%) and inorganic compounds (0.2 wt%). The lignin content (92 wt%) and extractive content (3.2 wt%) were noticeably higher in the alkali-soluble fraction, while the carbohydrate content was significantly lower than that of the initial EHL. This indicates that most of the carbohydrates were removed during the alkali fractionation step, whereas the extractives were also dissolved in the alkaline solution.

As is a result of the acid precipitation process, which partially removes the dissolved inorganic materials and polysaccharides during the alkali-extraction (R. Sun et al., 1999), both pH 5 and pH 2.5 fractions had high lignin contents, at 97 and 96 wt%, respectively. Furthermore, it concurs with the decrease of carbohydrate along with the fractionation steps.

The ash content of the fractions was all at 0.2 wt%. However, the pH 5 fraction has a higher ash content than the other fractions (0.7 wt%). This could be a result of an insufficient acid wash during the pH fractionation process. Many high-value products derived from lignin require ash content below 0.1 wt%, putting most of these fractions above the limit. (Roberts, Khan, & Spontak, 1996).

The EtOH fractions had relatively lower lignin content compared to the pH fractions. The EtOH insoluble fraction showed high content in carbohydrates, demonstrating its ability to purify lignin by EtOH/H_2O fractionation. The 96% to 75% sequential EtOH fraction showed among the highest lignin content, 96.3 wt%. In comparison, the direct 75% EtOH soluble fraction and the 96% EtOH soluble fraction had a similar lignin content, around 84 wt%, with a low carbohydrate content. The sequential method seems to be more effective in removing most of the carbohydrates compared to the direct EtOH fractionation. All EtOH fractions had relatively low and similar ash content, except the 96% EtOH soluble fraction, which had 0.8 wt%. This indicates that the lack of additional water could disadvantage the salt removal from the unfractionated EHL. The EtOH-

soluble fractions had much higher extractives content compared to their insoluble counterparts due to the high solubility of extractives in EtOH.

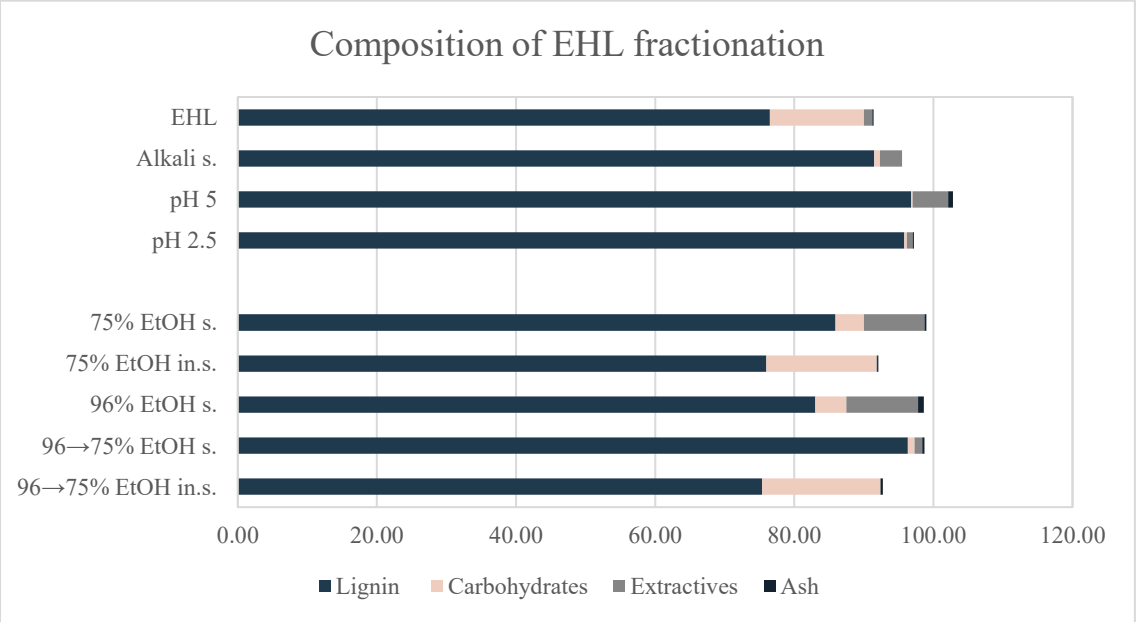

Figure 21 Compositional results of lignin fractions from EHL. The results are given in wt%.

EEHL fractions

The compositional analysis of the EEHL fractions is shown in Figure 22. As concluded in the purification results, the EEHL became more homogeneous, with higher lignin content (85 wt%), lower carbohydrate (13 wt%), and extractives content (0.4 wt%), compared to the EHL. The pH 5 fraction showed a lower lignin content (81.7 wt%) than the EEHL. The pH 2.5 fraction, on the other hand, displayed the highest lignin content (91.9 wt%). The alkali extraction to EEHL resulted in a similar change as the EHL, with an apparent increase in lignin content (89.8 wt%) and decreased in carbohydrate content.

The EOH fractions had a generally higher lignin content, except for the 75% EtOH insoluble fraction, which slightly decreased (74 wt%) compared to the EHL EtOH-soluble fractions. The EtOH-soluble fractions from EEHL had significant lower extractives content than those from EHL, most likely due to the sufficient purification of lignin by MTBE extraction.

The extractive content of the EEHL fractions showed a decrease compared to the EHL fraction. Furthermore, throughout the fractionation processes, the extractive content showed similar changes as the EHL fractions. The most significant change could be seen in the pH 5 fraction, which decreased from 5.1 wt% to 0.28 wt%. The ash content in the EEHL fractions was also generally lower than the EHL fractions, making the lignin fraction from MTBE extracted lignin better for high-value products compared to the EHL fractions.

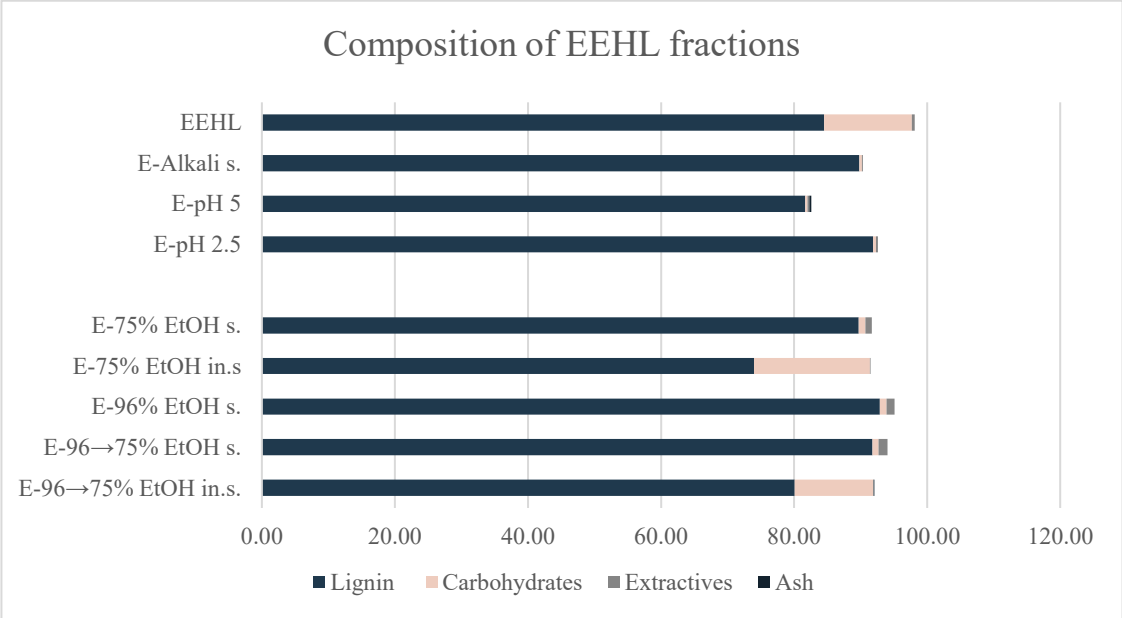

Figure 22 Compositional results of lignin fraction from EEHL. The results are given in wt%.

4.2.4 Hydroxyl groups quantification

EHL fractions

The quantitative ^{31}P NMR analysis result of the lignin samples is shown in Table 4. None of the insoluble fractions could go through the ^{31}P NMR analyses due to the problem with dissolving in the NMR solvent ($CDCl_3$/pyridine). Similarly, it should be pointed out that the hydroxyl quantification of initial EHL and EEHL are not reliable due to the solubility problem in the NMR solvent, resulting in presumably lower values. The total of hydroxyl groups stayed quite similar at around 4 mmol/g throughout the gradual acid precipitation fractionation. However, there was a composition change, as the content of aliphatic-OH groups decreased, while the carboxyl acids increased throughout the fractionation. On the

other hand, the EtOH fractionation increased the total hydroxyl content in the EtOH-soluble fraction, compared to the pH fractions from EEHL. (Pavia et al., 2007).

Table 4 Hydroxyl group distribution of EHL fractions. Presented in mmol/g.

EHL fractions	aliphatic-OH	phenolic-OH	carboxylic acid	total -OH
Unfractionated EHL	3.7	1.0	0.2	4.9
pH fractions				
Alkali s	1.4	2.3	0.6	4.3
pH 5	1.2	2.3	0.7	4.2
pH 2.5	1.1	1.9	0.9	3.9
EtOH fractions				
75% EtOH s	3.2	2.4	0.6	6.3
96% EtOH s	3.3	2.2	0.6	6.1
96→75% EtOH s	1.2	2.3	0.2	3.7

EEHL fractions

The ^{31}P NMR analysis results of EEHL fractions are shown in Table 5. Like the EHL fractions, the hydroxyl groups stayed around 4 mmol/g throughout the gradual acid precipitation fractionation and was a bit higher in the EtOH fractions, around 5 mmol/g. The gradual acid precipitation fractionation resulted a similar amount of aliphatic-OH and carboxylic acid groups as pH fractions from EHL. In contrast, the total phenolic-OH was slightly higher in pH fractions from EEHL. In EtOH soluble fractions from EEHL, the highest content of phenolic-OH was found in the 96% EtOH fraction (3.7 mmol/g), while the lowest appeared in the sequential 96% to 75% EtOH soluble fraction at 3.2 mmol/g. The higher content of aliphatic-OH groups in EtOH-soluble fractions from EHL, compared with the counterparts from EEHL could be ascribed to the interference of extractives and carbohydrates, which is consistent with the compositional analysis. Furthermore, the EtOH-soluble fractions from EEHL containing higher amount of phenolic-OH than the counterparts from EHL.

Table 5 Hydroxyl group distribution of EEHL fractions. Presented in mmol/g.

EEHL fractions	aliphatic-OH	phenolic-OH	carboxylic acid	total -OH
Unfractionated EEHL	3.5	0.7	0.1	4.3
pH fractions				
Alkali s.	1.3	2.6	0.5	4.4
pH 5	1.3	2.8	0.6	4.7
pH 2.5	1.2	2.4	0.8	4.4
EtOH fractions				
75% EtOH s.	1.5	3.5	0.4	5.4
96% EtOH s.	1.5	3.7	0.4	5.6
96→75% EtOH s.	1.6	3.2	0.3	5.0

4.2.5 Thermal properties

The Tg of the EHL fractions was analysed to determine the thermal properties. A low Tg is beneficial for blending lignin with thermal plastics. The Tg results of the EHL fraction are presented in Figure 23. The unfractionated EHL/EEHL and pH fractions showed no signs of Tg in the temperature range of 0 to 200 °C, while the alkali-soluble fraction had a high Tg value, such as 132 °C and 152°C for the alkali-soluble fractions from EHL and EEHL, respectively. This may be due to the use of HCl in the acid precipitation process, which affects the thermal properties of the obtained lignin fractions. All EtOH-soluble fractions showed Tg, and the lowest value of 57 °C appeared in the 96% EtOH-soluble fraction from EHL. However, the Tg value of the 96% EtOH-soluble fraction from EEHL was 89 °C. Similarly, the Tg value of EEHL fractions were generally higher than that of EHL counterparts. A reason for this could be that the EtOH may extract out the extractives remained in the EHL, lowering the Tg of lignin fractions. It is common for Tg to decrease along with molar mass (Laurichesse & Avérous, 2014). As expected, the Tg values of EtOH-soluble fractions from EHL and EEHL decreased with the decreasing molar mass of lignin.

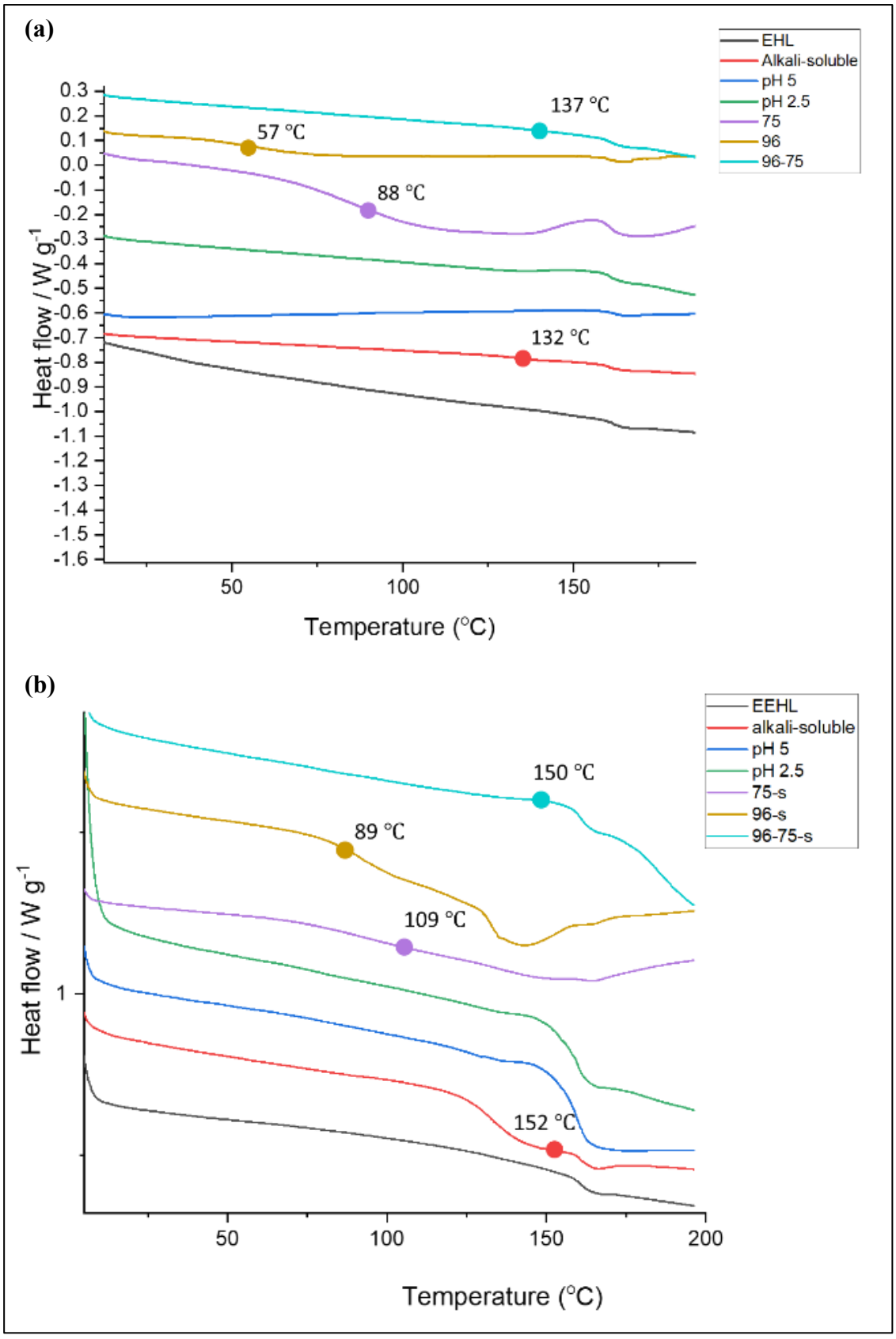

Figure 23 DCS curves of a) EHL fractions and b) EEHL fractions.

5 Conclusions

The EHL obtained from the Cellunolix® biorefinery process requires purification and fractionation to obtain more homogeneous lignin with a higher reactivity suitable for further modification. An MTBE-extraction and two different types of fractionation methods were tested to obtain lignin fractions with defined properties. The EHL and EEHL, as well as all the fractions obtained, were characterised, and compared by determining the composition, molar mass, hydroxyl groups distributions and thermal properties. The goal was to determine which fractionation method produced the most suitable fraction that could be further used for modification or compatibilisation.

The gradient pH fractionation started in alkali condition. In addition to lignin, it also dissolved inorganic impurities and part of polysaccharides in the solutions, and water-soluble compounds were removed during the acid precipitation process. This made the gradient fractionation an overall more efficient method to purify the lignin, which was evidenced by the pH 5 fraction from EHL having the highest lignin content at 96 wt%. Furthermore, the pH fractions also had higher yields (20–40%) compared to the EtOH fractions (10–20%). However, only the alkali-soluble fraction from the pH fractions displayed a Tg during the thermal analysis.

On the other hand, the EtOH fractionation was not successful in purifying the fractions, and the EtOH-soluble fractions contained more impurities than the pH fractions due to the direct fraction of EHL, which did not properly remove extractives, carbohydrates and salts from the EHL. If the EtOH fractions were used in further studies to study the property-application relationship properly, it would most likely require a purification process in combination with acid water washing and MTBE extraction to remove some of the carbohydrates and extractives. Due to the presence of extractives, the EtOH-soluble fractions from EHL generally obtained lower molar mass values higher $Đ_M$, and lower Tg values. As expected, fractionating the EEHL with EtOH resulted in a further increase in Tg. The 96% to 75% sequential fractionation purified the EHL most efficiently

compared to the other EtOH fractions. However, compared to the other EtOH-soluble fractions, the 96% to 75% sequential fractionation had a very low yield. The fraction with the overall lowest *Mw* and $Đ_M$ was the EEHL 96% EtOH soluble fraction.

The MTBE extraction was successful in removing the extractives and purifying the initial EHL. Both fractionation methods showed promising results for potential value-added products. All of the fractions had different pros and cons. However, based on yield, molar mass, hydroxyl groups and Tg analysis, the alkali-soluble, pH 5, and 75% EtOH soluble fractions from EHL/EEHL are suitable candidates for further modification or compatibilisation with polymers.

6 Summary in Swedish - Svensk sammanfattning

Fraktionering och karakterisering av enzymatiskt hydrolyslignin

Lignin är naturens mest förekommande aromatiska polymer, vilket gör det till ett billigt alternativ till petroleumbaserade aromatiska föreningar. Tack vare ligninets funktionella grupper och eftertraktade komplexitet bidrar ligninet med utmärkt potential för mervärdestillämpningar. Dock finns det flera hinder för att isolera ett homogent lignin med hög reaktivitet på grund av ligninets heterogena struktur, vilket resulterar i att ligninet inte används till sin fulla potential och i stället förbränns som energi inom pappersindustrin och bioraffinaderier. Flera studier har visat att fraktionering av ligninet gör det möjligt att kontrollera och därför skräddarsy ligninets egenskaper, såsom molmassa och löslighet, vilket skapar en mer homogen produkt lämplig för framtida mervärdestillämpningar (Jiang et al., 2020; Pang et al., 2020; Sadeghifar & Ragauskas, 2020; Xu et al., 2020). Ligninets fördelar, såsom tillgänglighet och låga priser, ger ligninet mycket potential som råvara för delvis ersättning av fossilbaserade resurser.

Ligninets egenskap- och applikationsrelation är kritiskt att definiera för att kunna utnyttja ligninet för ytterligare modifikation eller kompatibilisering, vilket kräver ett homogent lignin med hög reaktivitet. Målet med detta diplomarbete var att fraktionera och rena tekniskt lignin, för att isolera relativt homogena ligninfraktioner. Dessutom avgör denna avhandling hur olika fraktioneringsmetoder påverkar egenskaperna hos ligninfraktionerna. Två olika fraktionsmetoder (gradient-pH-fraktionering och lösningsmedelsfraktionering) analyserades och karakteriserades med hjälp av en omfattande strukturanalys. De fraktionerna med optimala egenskaper skulle sedan användas som kandidater för ytterligare tillämpningar.

Enzymatiskt hydrolyslignin (EHL) tillhandahölls av St1. Ligninet är en biprodukt vid produktion av bioetanol från barrträd med enzymhydrolys och jästjäsning, känd som St1 Cellunolix®-processen. Detta EHL kräver rening och fraktionering för att erhålla mer homogent lignin som är lämplig för ytterligare modifiering. En MTBE-extraktion och två olika typer av fraktioneringsmetoder testades för att erhålla en renad EHL. Både EHL och EEHL, såväl som alla erhållna fraktioner, karakteriserades och jämfördes genom att bestämma sammansättning, molmassa, hydroxylgrupper och termiska egenskaper. Målet

var att bestämma vilken fraktioneringsmetod som gav den mest lämpliga fraktionen som kunde användas vidare för modifiering eller kompatibilisering.

Cellunolix® EHL var i en våtkaksform, som frystorkades före fraktionering och karakterisering. Extraktion av ligninet utfördes genom att tvätta EHL med en syralösning av 2,5 M saltsyra (HCl) och filtreras på ett 25 µm nylonfilter för att avlägsna eventuella salter från EHL. Tvättprocessen upprepades tre gånger före torkning av EHL i en frystork. Efteråt användes metyl-tert-butyleter (MTBE) för att avlägsna extraktivämnen genom att blanda EHL med MTBE under konstant skakning med en analog skakapparat i minst 72 timmar. MTBE-fasen filtrerades senare ut och torkades i en vakuumugn vid 40 °C i 12 timmar för att erhålla den extraherade EHL (EEHL). Både EHL och EEHL fraktionerades genom gradient-pH-fraktionering och lösningsmedelsfraktionering, vilket resulterade i tio fraktioner från varje utgångsmaterial. Fraktionerna och förkortningarna kan ses i tabell 1.

Det första steget i pH fraktioneringsprocessen började med en gallring för att bestämma vilka pH-nivåer som bör väljas för gradientfraktioneringen. Gallringen utföres genom att blanda den frystorkade EHL/EEHL i en natriumhydroxidlösning (NaOH) med en koncentration av 6 M. Ytterligare NaOH tillsattes tills ett pH av 12,5 uppnåddes. Suspensionen upphettades till 90 °C under omrörning i 2 timmar för att säkerställa att lignin var helt lösligt i NaOH-lösningen. Efteråt kyldes blandningen ned till omgivningstemperatur innan den centrifugerades och filtrerades på ett 25 µm nylonfilter för att utesluta fällningar, så som kolhydrater som inte helt avlägsnades genom den enzymatiska hydrolysmetoden. Därefter delades alkalilösningen i sju delar och provernas pH justerades till pH 9, 7, 6, 5, 4, 3 och 2,5 med HCl (2 M) för att fälla ut lignin från alkaliblandningen, se figur 11. Från denna gallring drogs slutsatsen att det mesta av ligninet fälldes ut vid pH 5 och det lägsta mängden vid pH 2,5. Gradientfraktioneringen fortsattes med användning av dessa två pH-värden.

Gradient pH-fraktioneringen startade genom att lösa EHL/EEHL i NaOH-lösning vid pH 12,5 (90 °C, 2 timmar). Därefter justerades provets pH direkt till pH 5 med HCl för att fälla ut en del av ligninet från alkaliblandningen. Blandningen centrifugerades sedan vid 10 000 varv/min under 10 minuter, blandades med surt vatten (pH $\leqslant$2,5) och åter

centrifugerades och filtrerades slutligen på ett 0,25 µm nylonfilter och tvättades igen med surt vatten. Filterkakan frystorkades sedan. Filtratets pH justerades ytterligare från pH 5 till 2,5 för att fälla ut och isolera det återstående ligninet med användning av samma procedur som ovan. Proceduren illustreras i figur 12.

Den andra ligninfraktioneringsprocessen utnyttjade teknisk etanol (EtOH) för att extrahera lignin sekventiellt. Först blandades frystorkad EHL/EEHL med 96 % EtOH vid konstant rumstemperatur i 4 timmar. De lösliga och olösliga fraktionerna separerades genom filtrering. Den lösliga fraktionen utvanns genom att indunsta EtOH under reducerat tryck i en rotationsindunstare. Den olösliga fraktionen löstes ytterligare i 75 % EtOH i vatten, och separationsprocessen upprepades. Alla fraktioner torkades sedan i en vakuumugn vid 40°C under 12 timmar.

För att kunna jämföra den sekventiella effekten av EtOH-fraktioneringen användes en direkt lösningsmedelsfraktionering med 75 % EtOH i vatten enligt samma procedur som ovan. Proceduren för direkt lösningsfraktionering illustreras i figur 14.

Gradient pH-fraktioneringen startade under alkaliska förhållanden, vilket löste de oorganiska materialen och polysackariderna i lösningarna och avlägsnades under sura utfällningsprocessen. Detta gjorde gradientfraktioneringen till en över lag mer effektiv metod för att rena EHL. Detta resulterade i relativt homogena fraktioner, varav pH 5 fraktionen från EHL visade högsta ligninhalten vid 96 viktprocent. Dessutom hade pH-fraktionerna också högre utbyten (20–40 %) jämfört med EtOH-fraktionerna (10–20 %). Dock uppvisade endast den alkalilösliga fraktionen en glasomvandlingstemperatur (*Tg*) under den termiska analysen.

Å andra sidan var EtOH-fraktioneringen inte framgångsrik i att rena fraktionen och innehöll fler föroreningar än pH-fraktionerna på grund av den direkta fraktionen av EHL, som inte korrekt avlägsnade kolhydrater eller salter från EHL. Om EtOH-fraktionerna användes i ytterligare studier för att korrekt studera egenskaps-tillämpningsförhållandet, skulle det med största sannolikhet kräva en extraktion med ett mer polärt lösningsmedel, såsom MTBE-extraktion, för att avlägsna en del av extraktivämnena och kolhydraterna. Emellertid erhöll EtOH-fraktioneringen från EHL lägre molmassa (*Mw*) och högre molmassadispersitet ($Đ_M$). Dessutom visade EtOH-fraktionen lägre *Tg*-värden än EHL.

Vidare resulterade EtOH-fraktionering av EEHL ytterligare lägre *Tg*-värden. Därtill förbrukade EtOH-fraktioneringen mindre energi och var lättare att använda och en snabbare process. Av EtOH-fraktionerna renade 96 % till 75 % sekventiell fraktionen bäst EHL. Dock hade denna fraktion väldigt lågt utbyte och högre *Mw* och $Đ_M$ jämfört med de andra lösliga EtOH-fraktionerna. Fraktionen med den totala lägsta *Mw* och $Đ_M$ var den EEHL 96% EtOH-lösliga fraktionen.

MTBE-extraktionen var framgångsrik för att minska mängden extraktivämnen och rena EHL. De erhållna fraktionerna hade olika för- och nackdelar och det konstaterades att båda fraktioneringsmetoderna tillsammans med MTBE-extraktionen visade lovande resultat för vidare mervärdestillämpningar av EHL. Baserat på utbyte, molmassa, hydroxylgrupper och *Tg*-analys var EEHL fraktioner som fraktionerats via pH 5 fraktionering, samt de lösliga fraktionerna från alkalilösningen och 75 % den direkta EtOH lämpliga kandidater för ytterligare modifiering eller kompatibilisering med polymerer.

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