

Antioxidant and Antimicrobial Properties of Lignin and Lignin-based Composites for Active Food Packaging Applications

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M.Sc.

Abla Al-Zagameem

aus Jordan, Al Tafila

Gutachter: apl. Prof. Dr. rer. nat. habil. Thomas Fischer

Gutachter: Prof. Dr. Margit Schulze

Gutachter: Prof. Dr. Judith Kreyenschmidt

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Dedication

My late Aunts: Nejmaeh Alzaghamiem who fostered my first step toward my future, and Sara Alzaghamiem as the first and greatest believer in me since I can ever remember.

*“Entrepreneurs don’t waste time/energy worrying about being better than someone else.
Focus on being the very best version of YOU.”*

– Abdulaziz Aljouf

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Summary

Due to the use of fossil fuel resources in every aspect of our life, many environmental problems have been exacerbated by these nonrenewable resources. Thus, recent research focus on the use of environmentally friendly materials from sustainable feedstock for future fuels, chemicals, fibers and polymers. Lignocellulosic biomass has become the raw material of choice for these new materials as cellulose and lignin are the most abundant biopolymers on earth. Lignin is a biomolecule that occurs in the plant cell wall, separating cellulose and hemicellulose. It is produced in large amounts as a side product of the pulping processes and burned to produce energy. Recently, research has focused on using lignin as a substitute material in many industrial applications since using lignin is environmentally-friendly, lignin is biodegradable and due to the polyphenolic nature of lignin, which has antioxidant and antimicrobial activities in addition to its role in supporting the mechanical properties of bio-based scaffolds and composites. However, the antimicrobial activity of lignin is not clear yet. Therefore, this research was designed to answer some questions in the puzzle by introducing new moieties that include lignin to investigate its activities and hence to orient lignin into the right designed applications.

The complex structure of lignin, composed of aromatic rings with hydroxy and methoxy functional groups, is responsible for this antioxidant potential. The application of technical lignins as natural antioxidants has not been implemented in the industrial sector, mainly due to the highly non-homogenous complex structure and high PD of lignin. Typically, purification and fractionation steps are necessary to enhance its stabilizing effect. Lignin is also a promising green agent against dangerous microorganisms. Its biocidal activity allows reducing the environmental problems related to the use of silver nanoparticles. Some studies have shown that lignin has an antimicrobial effect. The phenolic hydroxyl and methoxy groups contained in lignin have been reported to be biologically active and lignin extracts have shown considerable antimicrobial activity against gram-positive bacteria. In consequence, using lignin has the potential for the development of bio-based active food packaging sector.

The main purpose of this thesis is to apply lignins bioactivities (i.e. antioxidant activity and antimicrobial activity) feasibly in films for packaging purposes and to study the factors affecting those bioactivities. Therefore, the following questions are proposed:

- How much does the purity of lignin affect its structure and hence the bioactivity?
- What are the factors affecting the lignin structure/bioactivity?

- To what degree does the compatibility of lignin with cellulose, chitosan, and a mixture of both in the film moiety affect the bioactivity?
- What is the bioactive part of lignin as a powder and how is it incorporated in the cellulose/chitosan composites?

To answer these questions, Kraft lignins (KLs) (softwood-based) and Organosolv lignins (softwood-based, grass based) were isolated, purified, analyzed, investigated to their antioxidant and antimicrobial activities, polymerized with cellulose and chitosan in binary and ternary systems that were finally investigated to their antioxidant and antimicrobial activities. Structure-property was discussed to try finding some answers for the research questions.

In this thesis KL was extracted from black liquor via acidic precipitation and purified using selective extractions. The corresponding fractions were characterized by FT-IR, UV-Vis, ³¹P-NMR, HSQC, SEC, Pyrolysis GC/MS, TGA, DSC, and XRD. Significant improvement is confirmed via the analysis methods used. The KL fractions from the purification were tested to their antioxidant and antimicrobial activities together with Organosolv Lignins (beech wood (softwood-based), a spruce/pine mixture (softwood-based), and grass-based *Miscanthus x giganteus* (stem/leaf mixture) using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and the zone of inhibition methods respectively. The effects of purification, biomass source, and pulping process on lignin structure and bioactivity were investigated. The antioxidant activity (DPPH inhibition) of KL fractions exhibited higher values than the Organosolv Lignins (softwood-based, grass-based). Total phenol content (TPC) values of Organosolv Lignins (softwood-based, grass-based) were higher than those of the KL purification fractions. The first fraction of the purification of KL exhibited an antimicrobial activity against gram-positive and gram-negative bacteria as well as the softwood-based Organosolv Lignins. Grass-based Organosolv Lignins exhibited no antimicrobial activity. Storing the lignins in general reduced DPPH inhibition but enhanced TPC values and the antimicrobial activity. The previously mentioned lignins were added to hydroxypropylmethylcellulose (HPMC) film solutions and to other HPMC/chitosan film solutions in different percentages while HPMC and chitosan concentration remained constant. A DPPH assay was afterwards used to discuss the antiradical activity of HPMC/lignin and HPMC/chitosan/lignin films. The least addition of lignin (5%) to the HPMC and HPMC/chitosan films shows higher scavenging activity than higher additions. Lignin-containing films exhibited high antimicrobial activities against gram-positive and gram-negative bacteria at 35 °C and at low temperatures (0-7 °C). Lignin release has a positive effect on the antimicrobial activity of the studied films.

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List of Abbreviations and Symbols

AA	Antioxidant activity
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AIL	Acid insoluble lignin
ASL	Acid soluble lignin
ATR-IR	Attenuated total reflection infrared
BHA	2- <i>tert</i> -butyl-4-methoxyphenol/3- <i>tert</i> -butyl-4-methoxyphenol mixture
BHT	3,5-di- <i>tert</i> -butyl-4-hydroxytoluene
BL	Black liquor
CUPRAC	Cupric reduction antioxidant capacity
DHB	Dihydroxybenzoic acid
DMSO	Dimethyl sulfoxide
2D-NMR	Two dimensional nuclear magnetic resonance
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSC	Differential scanning calorimetry
DTG	Derivative thermogravimetric
EPR	Electron paramagnetic resonance
FC	Folin Ciocalteu
FRAP	Ferric reducing antioxidant power
FT-IR	Fourier transform infrared
GC	Gas chromatography
GPC	Gel permeation chromatography
G-unit	Guaiacyl unit
HAT	Hydrogen atom transfer
HPMC	Hydroxypropylmethylcellulose
HPLC	High performance liquid chromatography
H-unit	hydroxy-phenyl unit
KL	Kraft lignin
LC	Lignocellulosic biomass
LCC	Lignin-carbohydrate complex
LCF	Lignocellulosic feedstock
Mn	Number average molecular weight

Mw	Weight average molecular weight
NREL	National renewable energy laboratory
OMe	Methoxy
ORAC	Oxygen radical absorbance capacity
PEG	Polyethylene glycol
PD	Polydispersity (Mw/Mn)
PHB	Polyhydroxybutyrate
PLA	Poly(lactic acid)
PMMA	Poly(methyl methacrylate)
³¹ P-NMR	Phosphorus nuclear magnetic resonance
PS	Polystyrene
py-GC/MS	Pyrolysis gas chromatography/mass spectrometry
ROS	Reactive oxygen species
SEC	Size exclusion chromatography
SEM	Scanning electron microscopy
SET	Single electron transfer
S-unit	Syringyl unit
t	Time
TEAC	Trolox equivalent antioxidant capacity
TEM	Transmission electron microscopy
Tg	Glass transition temperature
TGA	Thermogravimetric analysis
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TPC	Total phenol content
TPTZ	2,4,6-tripyridyl-s-triazine
UV-Vis	Ultraviolet-visible
XRD	X-ray diffraction

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1. Introduction

1.1. Biorefinery and Lignocellulosic Feedstock

Environmental issues caused by the uncontrollable use of fossil energy require a renewable carbon neutral substitute. And thus, innovations in the chemical community have been demanded to provide the suitable substitutions. Therefore, the current industry must then face the challenge of converting industrial low value by-products into more valuable materials. In this sense new concepts have to be embedded in the current research for systematic development. All of these concepts are encompassed by the concept “biorefinery”. As defined by the International Energy Agency Bioenergy Task 42, biorefinery is the sustainable processing of biomass into a scope of profitable bio-based products (chemicals, materials) and bioenergy (power, fuels, and/or heat). Biorefinery systems that are frequently mentioned during research and development include lignocellulosic feedstock biorefineries, whole-crop biorefineries, green biorefineries, and two platform concept and syngas (Wenger and Stern, 2019).

The use of lignocellulosic feedstock (LCF) in biorefineries has been recognized as the most successful strategy to produce valuable products such as fuels, power, and chemicals. Lignocellulosic raw materials consist of cellulose, hemicellulose, and lignin (Figure 1.1). The main advantages of this strategy are the low costs of the raw materials (straw, reed, grass, wood, paper waste, etc.) and the utilization of natural polymers “made by nature” (via photosynthesis) and composed mainly of polysaccharides (Alzagameem *et al.*, 2018a). Lignocellulosic biomass is one of the feedstocks that can be clean and sustainable for the liquid fuels, biomaterials, and biochemicals production in future biorefineries. Cellulose, lignin, and hemicelluloses represent about 90% of the lignocellulosic biomass, in addition to small amount of protein, pectin and other minor ingredients (Wang *et al.*, 2019).

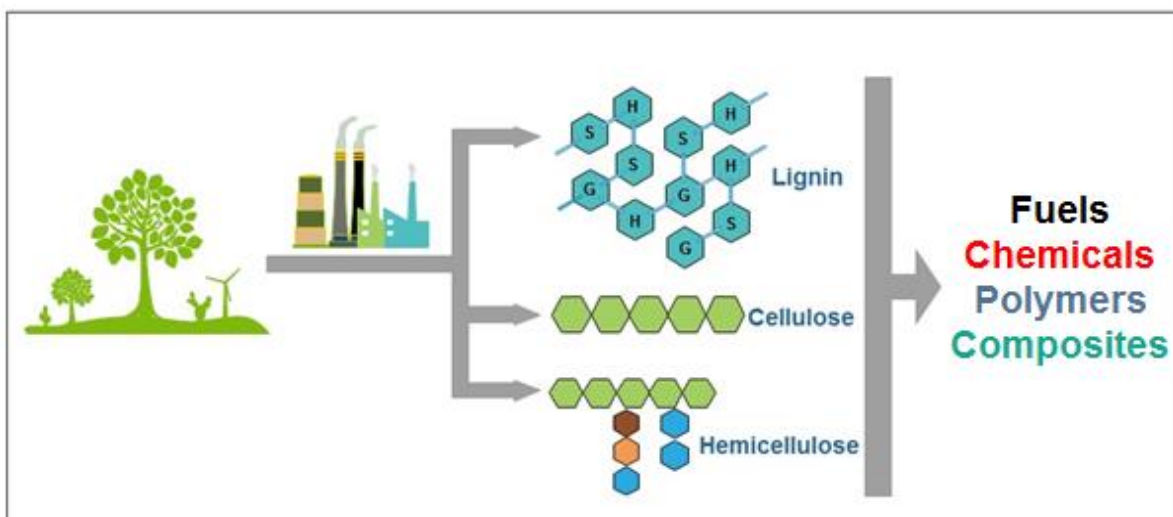


Figure 1. 1. Lignocellulosic feedstock biorefinery.

According to statistics, 170 million metric tons of lignocellulose are produced every year, whereas no more than 5% of the LCF components are used, mostly due to the significant recovery caused by lignin (Zhang *et al.*, 2019).

Today, lignocellulose-rich biomasses, including agrochemical waste, are processed all over the world in commercial mills, demonstration plants, and pilot scale facilities, to produce pulp, paper, lignin, and various LCF-derived chemicals (Table 1.1) (Alzagameem *et al.*, 2019).

Table 1. 1. Pilot plants and industrial production sites for LCF exploitation and valorization (Alzagameem *et al.*, 2019).

Company / Institution	Location	Production Scale	Feedstock and Products
Borregaard LignoTech	Sarpsborg, Norway	Industrial scale	World leader in lignin-based products (lignins and lignosulfonates and lignin-derived chemicals). In Fernandina Beach, FL, USA: Southern yellow pine-based lignin utilizing a coproduct of RYAM's sulfite pulping process.
Tembec / Rayonier Advanced Materials	Jacksonville, FL, USA	Industrial scale	Paper, pulp and lignin production
Domtar Corporation	Montreal, Canada	Industrial scale	LignoBoost plant in Plymouth. Pine-based BioChoice®
Asian Lignin Manufacturing Pvt. Ltd.	Chandigarh, India	Industrial scale	Paper, pulp and lignin production
Northway Lignin Chemical	Sturgeon Falls, ON, Canada	Industrial scale	Paper, pulp and lignin production
GreenValue SA	Orbe, Switzerland	Industrial scale	Sulfur-free lignin. Feedstock: wheat straw, flax, sugar cane. Aqueous alkaline extraction.
Domsjö Fabriker AB (world's 2nd largest producer of powder lignin). Domsjö is part of the Aditya Birla Group.	Örnsköldsvik, Sweden / Aditya Birla Headquarter Mumbai, India.	Industrial scale	Powder lignin. Domsjö is the world's 2nd largest producer of Lignin powder with its origin from sustainable Swedish forestry.

Changzhou Shanfeng Chemical Industry Co. Ltd.	Changzhou, Jiangsu, China	Industrial scale	Lignin polyether polyols
The Dallas Group of America	Whitehouse, NJ, USA	Industrial scale	Lignosulfonates
Nippon Paper Ind. Co. Ltd	Tokyo, Japan	Industrial scale	Lignosulfonates
Liquid Lignin Company, LLC	Clemson, South Carolina, U.S.	Industrial scale	Liquid Lignin Company develops and commercializes new lignin-based technologies.
Metsä Group	Espoo, Finland	Industrial scale	Forests and wood-based bioproducts
Fibria	Sao Paulo, Brasilia	Industrial scale	Forests and wood-based bioproducts. World's leader in Eucalyptus-derived pulp.
Lenzing AG	Lenzing, Austria	Industrial scale	Forests and wood-based bioproducts. European leader in pulp production.
Stora Enso	Helsinki, Finland	Industrial scale	LignoBoost plant at Sunila mill. Lineo™® (wood-based). Kraft pulping process of Nordic softwood, pine and spruce. The refined KL is available as a stable, free-flowing brown powder or a moist powder block. 50 000 tons of dry lignin per year.
Weyerhaeuser Company (in collaboration with Lignol Energy Corp./Fibria Cellulose SA)	Seattle, WA, USA	Industrial scale	Second generation biofuels and chemicals
GreenField	Boucherville, Quebec, Canada	Industrial scale	Biobased alcohols
Enchi Corp.	Lebanon, NH, USA	Industrial scale	Bioenergy and biofuels
Microbiogen	Lane Cove West, Australia	Industrial scale	Bioethanol and bioethanol producing yeast.
DuPont / VERBIO North America Corporation (VNA), Grand Rapids, Michigan, U.S.	Nevada, Iowa, U.S.	Industrial scale	Second generation biofuels and chemicals. World's largest cellulosic ethanol and biofuel facility (30 million gallons per year). Corn stover feedstock.
POET-DSM	Sioux Falls, SD, U.S.	Industrial scale	Second generation biofuels and chemicals
IOGEN Corp.	Ottawa, ON, Canada	Demonstration	Second generation biofuels and chemicals. Cellulosic Ethanol. Crop Residue Feedstock.
Fraunhofer Center for Chemical-Biotechnological Processes (CBP)	Leuna, Gemany	Pilot plant	Wood-based organosolv lignin: debarked beech wood (<i>Fagus sylvatica</i>) chips by ethanol–water-pulping in a batch process (70 kg dry biomass).
Bioprocess	Delft, The Netherlands	Pilot plant	Biomass hydrolysis and fermentation
BioCRACK	Schwechat, Austria	Pilot plant	Second generation biofuels

Biorefining is an important option for the implementation of innovative valorization of lignocellulosic material that has promoted extensive research on how to convert lignins into the target chemicals and fuel. Sources include LCF for the use of modern biotechnology, soft and hard wood, lignocellulose-rich grasses, and waste agroforestry. The market for biological products is expected to increase to 50 million € by 2030 at an annual average growth rate of 4% (Hassan *et al.*, 2019).

After cellulose, lignin is the second most plentiful biopolymer. It is produced as a waste from both lignocellulosic biorefinery and the paper-making industry. The current commercial utilization of lignin is mainly to burn the black liquor for heat recovery in spite of the recent classification of lignin as a value-added product. Utilization of lignin represents one of the most challenging issues for both the paper-making industry and lignocellulosic biorefinery. The most challenging obstacle for using lignin comes from its underlying inherent heterogeneity because of its multi-functional structure, different molecular weight, and polydispersity (PD) (Li *et al.*, 2019). The structure of this complex amorphous polyphenol molecule depends on the raw material, growing conditions of the plant, and extraction methods used (Gordobil *et al.*, 2015). The main role of lignin is to strengthen the plant and support it mechanically (Liu *et al.*, 2018). It is biosynthesized from three possible monomers of *p*-coumaryl, coniferyl, and sinapyl alcohols, which show *p*-hydroxyphenyl (H-units), guaiacyl (G-units) and syringyl (S-units) residues in lignin respectively, containing multiple hydroxyl and methoxy groups to generate various functional groups for lignin. Those monomers are mainly crosslinked via ether and carbon-carbon bonds (Figure 1.2) (Bergs *et al.*, 2019).

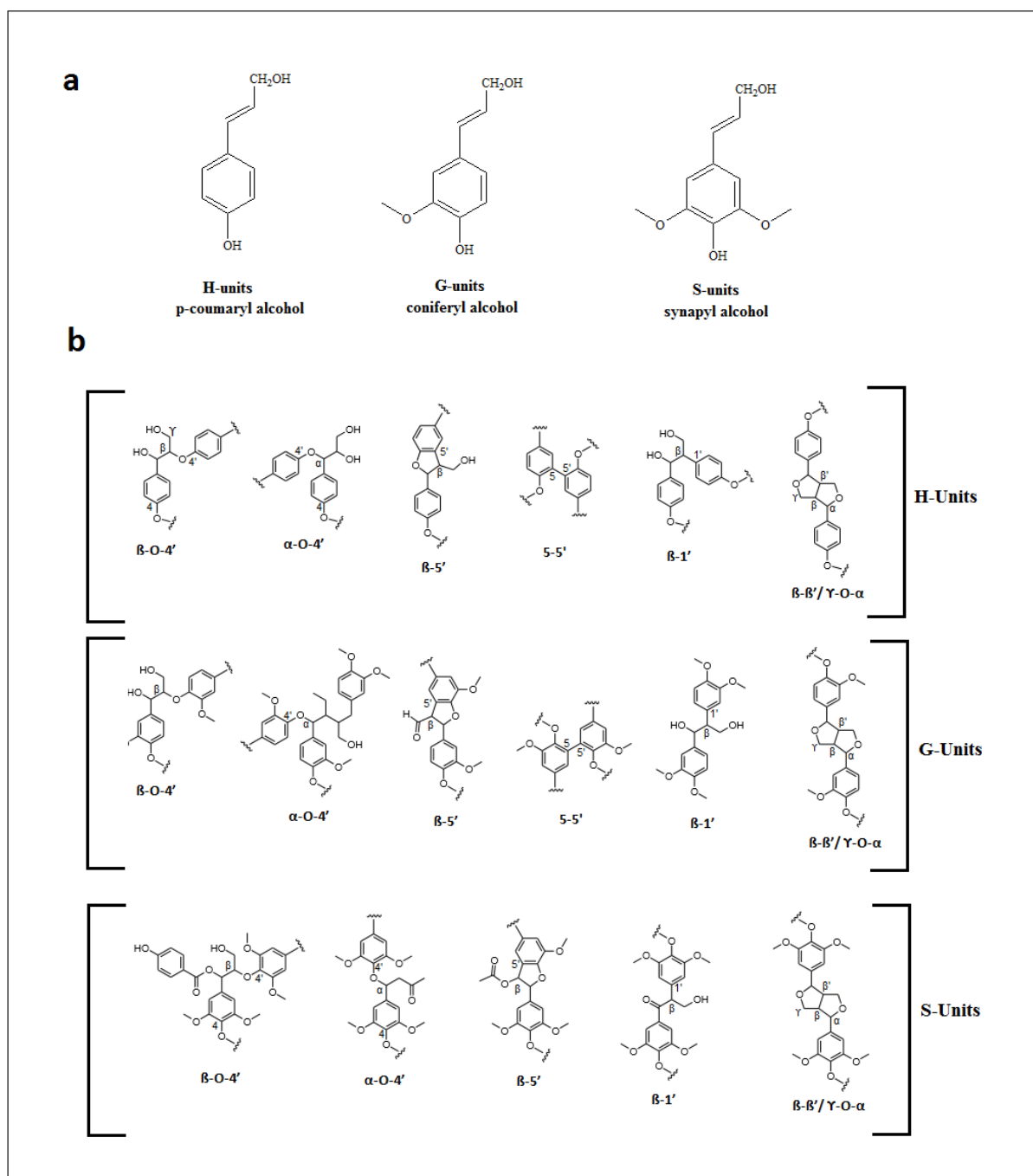


Figure 1. 2. (a) Lignin precursors (monolignols): H, G and S units derived from corresponding cinnamyl alcohols. (b) Most common monolignol linkages.

In the plant cell, lignin is associated with carbohydrates such as cellulose and hemicellulose. Lignin is extracted from other lignocellulosic parts via physical and/or chemical and biochemical treatments. The agricultural source, as well as the pulping process (delignification) and extraction procedures, influence the final structure, purity, and corresponding properties of the lignin produced. In common pulping processes, the lignocellulosic components are

separated via ester and ether linkage cleavage. The resultant technical lignin differs significantly from the lignin originally found in the plant after this step. Figure 1.3 shows the two main categories of extraction processes, sulfur and sulfur-free processes (Laurichesse and Avérous, 2014).

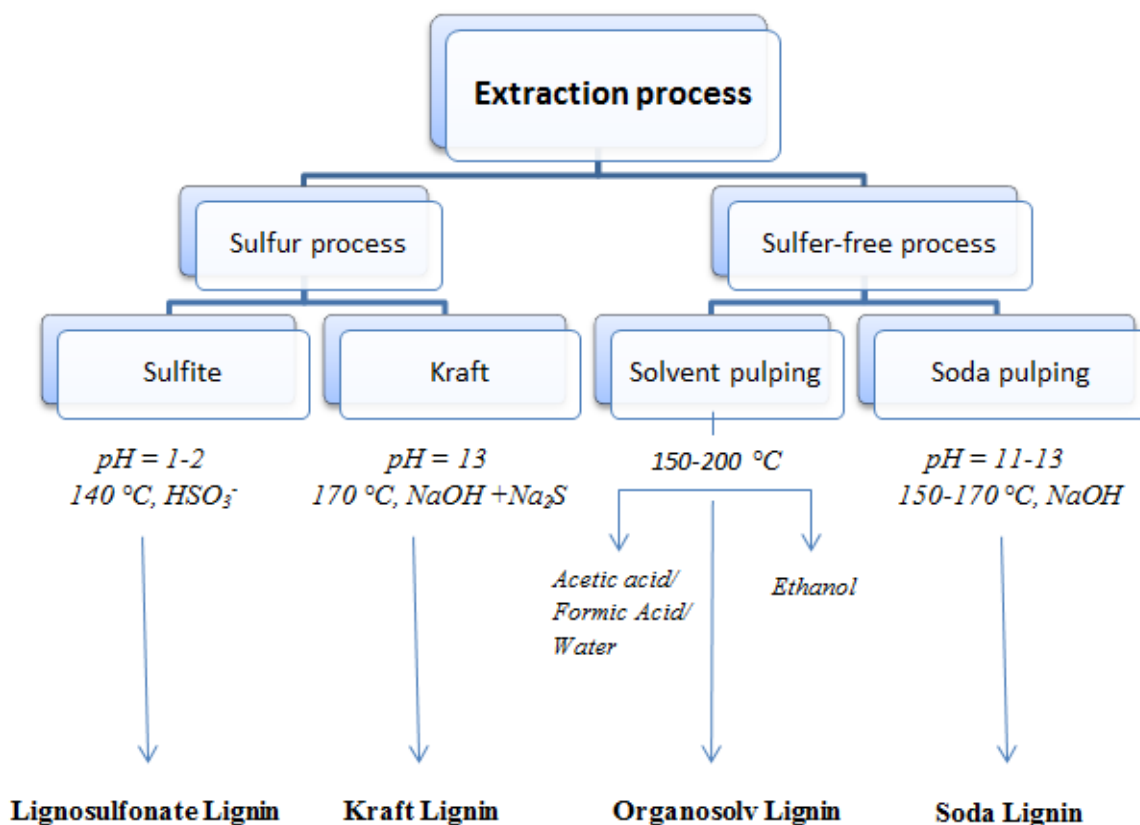


Figure 1. 3. Extraction processes of lignin from lignocellulose-rich feedstocks to produce technical lignins.

Kraft pulping is the most crucial process for the production of cellulose. It introduces numerous advantages to the industrial chemical extraction modification of lignin, particularly in fiber strength, which is one reason why it is the leading global process (Ribeiro *et al.*, 2019). The primary waste stream from this technique is black liquor, which is a strongly alkaline solution that contains various organic and inorganic components. KL comprises about 85% of the total lignin production in the world (Yang *et al.*, 2019). It is the predominating organic ingredient in black liquor. The structure of KL is different from native and other technical lignins. As a result of the cleavage of β -aryl bonds during the pulping process, KL contains a large amount of phenolic hydroxyl groups. Further, during the Kraft pulping process, some biphenyl and other condensed or re-polymerized structures are produced. Accordingly, the molecular weight

of KL obtained from black liquor has been reported within the range of 200–200,000 g/mol. The molecular weight of KL depends on the type of wood, isolation procedure used, and analysis method. Different methods have been suggested for the extraction of KL. The three main methods involve extraction based on solubility in organic solvents, selective acid precipitation at reduced pH values, and membrane ultrafiltration (Alzameem *et al.*, 2018a).

The Organosolv process is an intensively studied pretreatment for lignocellulosic biomass. Since lignocellulosic biomass contains valuable structural fractions, fractionation of its components is essential to optimally exploit the capability of each component and has become an important part of the scope of research. In Organosolv fractionation, lignin biopolymer was separated by the biomass treatment with an organic solvent at elevated temperatures. Lignin, as a polyphenolic network, is separated from the carbohydrate fractions of cellulose and hemicellulose (Dashtban *et al.*, 2010). The importance of the Organosolv process stems from its production of a cellulosic pulp showing good enzymatic digestibility as well as sulfur-free lignin fractions that can be used for material applications (Dababi *et al.*, 2016).

1.2. Lignin Extraction, Purification and Analysis

Lignin has been commonly extracted from biomass by dissolution in alkali media such as sodium hydroxide, ammonium hydroxide, and hydrogen peroxide. The alkaline treatment produces black liquor that contains carbohydrates and lignin produced by glycoside binding disorders in polysaccharides and lignin-biodegradable bonds (Ab Rahim *et al.*, 2018). KL has several unique features that distinguishes it from original and other technical lignins; it contains a large number of phenolic groups, due to the intensive cleavage of β -aryl bonds throughout Kraft pulping. Some biphenyl units add to other condensed structures as a result of the extreme cooking conditions (Lupoi *et al.*, 2015).

Black liquor still contains a noticeable amount of carbohydrate-derived substances, mostly aliphatic carboxylic acids due to the low selectivity of Kraft pulping (Alzameem *et al.*, 2018b). Lignin as a source of phenolic compounds is one of the most profitable candidates for diverse applications: it can be used in many fields due to the properties of dispersion, correlation, complexity, and emulsion stabilization (Witzler *et al.*, 2018). Nevertheless, lignin valorization is still a challenge due to its randomly linked monolignol units leading to a very

complex and irregular chemical structure. Thus, the upgrading of lignin-derived materials toward industrial applications is still limited to very few examples.

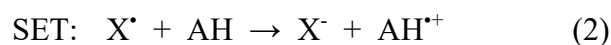
Lignin extraction from black liquor is a first step in transforming the regular Kraft pulp mill into a merged biorefinery and pulp mill. The precipitation of KL is sensitive to pH at low concentrations of salt even in the acidic range. It has been suggested that this is due to the carboxylic groups present on the KL molecules (Sewring *et al.*, 2019). There is an evident interest in understanding and studying methods to isolate the lignin from Kraft black liquor, since it is an environmentally friendly and economically interesting polymer. Considering that, some studies have investigated methods for the isolation of Kraft black liquor lignin, namely by using membranes, microorganisms, and chemicals. The most known methods are Lignoboost™ and Lignoforce Systems™. In Lignoboost™ the black liquor is precipitated by acidification with CO₂ or sulfuric acid followed by filtration. Then the lignin cake is re-slurred, re-filtrated, and washed, and results in a lignin with low ash content and high dry solids content. The peculiar characteristic of the Lignoboost™ method is the oxidation of the black liquor. The oxidation occurs before acid addition under controlled conditions (O₂ flow, temperature, and time). Comparatively to Lignoboost™, the second process (Lignoforce Systems™) utilizes the oxidation of malodorous sulfur compounds in black liquor to non-volatile species, which eliminates the problem of unpleasant smell. However, during the acidification process, there is emission of hydrogen sulfide and mercaptan, which are harmful compounds (Belo *et al.*, 2018).

Generally, the quality of a biomass requires reliable information about the chemical composition of the biomass to produce the best results. The structure of lignin is influenced by different factors: the biomass source and crop genotype/phenotype (i.e. soft and hard wood, grasses), the biomass age, the pulping process (e.g. Kraft, steam explosion, Organosolv), and the harvesting time (Alzameem *et al.*, 2019). Therefore, analytical methods are designed to elucidate a structural picture of lignin to broaden the scope of utilization. The most commonly used analytical methods for lignocellulosic biomass and products include gravimetry, chromatography (High performance liquid chromatography (HPLC), GC, size exclusion chromatography (SEC)), thermal analysis (Thermogravimetric Analysis (TGA), differential scanning calorimetry (DSC)), spectroscopy (Fourier Transform Infrared (FT-IR), Ultraviolet-visible (UV-Vis), Nuclear magnetic resonance (NMR)), mass spectrometry, and microscopy (TEM, SEM) (Lupoi *et al.*, 2015; Vas, 2015; Zhao *et al.*, 2017).

1.3. Antioxidant Activity, Antimicrobial Activity and Total Phenol Content

Recently, natural antioxidants have gained significant attention due to their abundance, biocompatibility, and ability to scavenge and neutralize free radicals to reduce damage to the human body, prevent the oxidation of oils and fats, and avoid the loss of nutrition in foods (Li *et al.*, 2018). Technical lignins contain several functional groups including phenolic hydroxyl, carbonyl, carboxyl, and methoxy groups. It has been reported that the phenolic hydroxyls and methoxy groups found in the lignin structure are bioactive. The antioxidant activity (AA) (i.e. efficiency to scavenge radicals) is one of the most studied properties in lignins in order to use it as natural antioxidants to substitute artificial or semi-artificial antioxidants in cosmetics, polymeric products, and pharmaceuticals (Espinoza-Acosta *et al.*, 2016). The AA of lignin has been previously confirmed (Alzagameem *et al.*, 2018b; Guo *et al.*, 2018; Iwasaki *et al.*, 2011; Liu *et al.*, 2015). And that makes it appropriate as an additive to prevent oxidation and photo- or bio-degradation in cosmetics, polymer blends, and pharmaceuticals (García *et al.*, 2017).

To specify the efficiency of an antioxidant, its capacity to scavenge radicals or its reducing potential are determined quantitatively. Mostly, AA assays can be divided into two mechanisms: the Hydrogen Atom Transfer (HAT) mechanism; where radicals are extinguished by the donation of hydrogen from the antioxidant, whereas the Single Electron Transfer (SET) mechanism uses the antioxidant's capability to transfer one electron for the reduction of any compound (Equations 1 and 2).



Where X^{\bullet} is the radical and AH the antioxidant, resulting in protonated radical XH and an antioxidant radical, or anion X^{-} and radical cation $\text{AH}^{\bullet+}$. Usually both of these mechanisms take place in parallel leading to very complicated reaction kinetics and multiple side reactions. Most of the relevant standards that specify the mechanism and effectiveness of antioxidants are the dissociation energy of the bond and the ionization energy. As a matter of fact, polyphenols such

as lignin have multiple activities. Thus, their AA depends on medium and corresponding solubility as well as testing substrate.

The Oxygen Radical Absorbance Capacity (ORAC) method depends on the reaction between the peroxy-radical ROO^\bullet and fluorescein. Values in the ORAC assay are often stated as Trolox equivalents with the use of a standard curve of different concentrations measurements of Trolox (Prior *et al.*, 2005). Ponomarenko and others investigated the fragmentation of hard and softwood LignoboostTM KLS, employing sequential extraction with organic solvents. They reported that all fractions exhibited good results in the ORAC assay reaching Trolox level or even better (Ponomarenko *et al.*, 2014). The Ferric Reducing Antioxidant Power (FRAP) assay measures the reduction of ferric 2,4,6-tripyridyl-s-triazine (TPTZ) to a colored product photometrically [Fe(III)/Fe(II)] (Benzie and Devaki, 2017). In this assay, antioxidant compounds with a redox potential less than 0.7 V can be observed. The Cupric Reduction Antioxidant Capacity (CUPRAC) assay is an alternative for the FRAP-assay (Özyürek *et al.*, 2011), in which a [Cu(II)/Cu(I)] reduction is used making this assay more selective than the FRAP-assay because of the lower redox potential. Hence, sugars (as potential residues of biomass pulping) are not determined by this assay. The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) is also based on a redox reaction of the ABTS radical cation. Results are expressed relative to Trolox (Cano and Arnao, 2017). Similarly, the DPPH assay is based on the redox reaction of the 2,2-diphenyl-1-picrylhydrazyl with an antioxidant leading to reduced color intensity which is directly proportional to the concentration of the antioxidant (Mishra *et al.*, 2012). The TPC of natural products has been measured by the Folin Ciocalteu (FC) assay or Total Phenolics assay for many years. This method is based on the reaction of the FC reagent (a mixture of phosphomolybdate and phosphotungstate) with an antioxidant. As a result, the color intensity of the solution changes proportionally to the antioxidant concentration. The results are expressed as Gallic Acid Equivalents or TPC where gallic acid is used as a reference compound (Sánchez-Rangel *et al.*, 2013).

If we compare these assays, the type of radicals is the most important factor to consider for *in vivo* environments, i.e. using lignin as an antioxidant additive in food, biomedicine, or cosmetics (Dizhbite *et al.*, 2004; Shivakumar and Yogendra Kumar, 2018). Here, the ORAC-assay is of the most interest as peroxy radicals ROO^\bullet are more relevant to *in vivo* conditions than DPPH^\bullet and $\text{ABTS}^{+\bullet}$ radicals, because of their size. Thus, the steric hindrance of DPPH^\bullet and $\text{ABTS}^{+\bullet}$ radicals influences the reaction kinetics. Additionally, values of the ORAC assay are unrelated to the reactivity rate in the antioxidant/substrate system. In contrast, the

estimation of FRAP, CUPRAC, ABTS, and DPPH assays requires a selected reaction end-point. However, this end-point usually does not represent the exact potential of the antioxidant. Table 1.2 summarizes the AA assays as described by Alzameem and others (Alzameem *et al.*, 2018b).

Table 1. 2. Antioxidant assays, the corresponding reaction mechanism, advantages and disadvantages.

Antioxidant Assay	Mechanism	Advantages	Disadvantages
ORAC (Oxygen Radical Absorbance Capacity)	Hydrogen Atom Transfer	<ul style="list-style-type: none"> • can be adapted to detect both hydrophilic and hydrophobic antioxidants by altering the radical source and solvent • ORAC values account for lag-time, initial rate and total extent of inhibition in a single value • automation is possible 	<ul style="list-style-type: none"> • fluorescence quenching is very sensitive, so any impurity has to be avoided • to achieve reproducible results, constant reaction conditions are required (temperature, pH, oxygen and reagent concentrations etc.) • detection requires fluorometer (fluorescence easy to be quenched) • analysis time about one hour • measurement is limited to peroxy-radicals as oxidants
FRAP (Ferric Reducing Antioxidant Power)	Single Electron Transfer	<ul style="list-style-type: none"> • simple, quick, inexpensive, robust, does not require special equipment • direct method to measure the combined activity of multiple, reductive antioxidants in a sample • automation is possible 	<ul style="list-style-type: none"> • no exact reaction time / reactivity is varying for different samples • thiol-containing antioxidants like glutathione are not detected
CUPRAC (Cupric Reduction Antioxidant Capacity)	Single Electron Transfer	<ul style="list-style-type: none"> • simple, quick, inexpensive, robust, does not require special equipment • all classes of antioxidants are detected, including thiols • applicable to both, hydrophilic and lipophilic antioxidants 	<ul style="list-style-type: none"> • no exact reaction time / reactivity is strongly varying for different samples
ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid))	mixture of HAT / SET	<ul style="list-style-type: none"> • simple, quick, wide pH-range, often used • soluble in aqueous and organic solvents and not affected by ionic strength -> applicable to a wide range of hydrophilic and lipophilic antioxidants • several wavelengths are available for photometric detection of the ABTS-radical 	<ul style="list-style-type: none"> • no exact reaction time / reactivity is strongly varying for different samples • the bulky ABTS-radical is not a good model for small, biologically more relevant radicals like HO• etc.
DPPH (2,2-diphenyl-1-picrylhydrazyl)	mixture of HAT / SET	<ul style="list-style-type: none"> • simple, quick, often used, no special equipment needed • DPPH-radical is commercially available; no <i>in situ</i>-generation necessary 	<ul style="list-style-type: none"> • no exact reaction time / reactivity is varying for different samples • DPPH-radical may have a poor reactivity with antioxidants due to its stability and steric hindrance
FC/TPC (Folin-Ciocalteu-Assay or Total-Phenolics-Assay)	mixture of HAT / SET	<ul style="list-style-type: none"> • simple, often used, does not require special equipment 	<ul style="list-style-type: none"> • no exact reaction time / reactivity is varying for different samples • interferences with other reductive substances may influence the results

So far, the AA of lignin has been studied mainly using two assays: DPPH and FC method. Dizhbite *et al.* studied the structure-property relationships in regard to the AA. They proposed that π -conjugation systems of lignins run as catalysts/activators during the interaction with DPPH radicals, whereas heterogeneity and PD reduce the antioxidant capacity critically. By the use of electron paramagnetic resonance (EPR) spectroscopy to identify paramagnetic polyconjugated clusters in lignins, Dizhbite *et al.* also proved that paramagnetic polyconjugated assemblies generated in a linear increment of antioxidant efficiency while aromatic-OH and OCH₃ groups have a lower effect (Dizhbite *et al.*, 2004). Santos *et al.* investigated the isolation and purification influence involving solvent effect by comparing alkaline solution versus water and organic solvents. They concluded that lignins that have a low percentage of phenols exhibited the highest abstraction of DPPH radicals (Santos *et al.*, 2014).

Aminzadeh *et al.* evaluated the AA by the ORAC assay using membrane filtration for LignoboostTM KL fractionation (weight average molecular weight (Mw) cut-off of 1 kDa). According to 2D-NMR, lignin oligomers with a high content of methoxy groups resulted. ³¹P-NMR spectroscopy showed a higher proportion of non-condensed phenolic-OH groups for samples of lower total content of phenolic-OH groups. Moreover, low Mw lignin fractions showed superior AA compared to non-fractionated LignoboostTM lignin in the ORAC assay and threefold higher inhibition of the substrate (fluorescein) than Trolox (Aminzadeh *et al.*, 2018).

Most obviously, structural elements influencing the AA are correlated to the antimicrobial capacity. Analogously to the AA, different types of lignins possess antimicrobial properties of varying degrees (Dong *et al.*, 2011; Guo *et al.*, 2018). Furthermore, lignin has been found to have a high degree of antimicrobial properties due to the large number of polyphenolic compounds that comprise the structure of lignin. These polyphenols can destroy the cell membranes, causing both gram-positive and negative bacteria to decay. While the exact mechanism for cell dissolution is not well understood, it is thought that the phenolic compounds in lignin can act as ionophores which are known to increase the ion permeability of the cell membrane causing cell death (Kalinowski and Shi, 2019). A variety of factors have been found in literature to affect the antimicrobial properties of lignin that include the concentration of lignin, the chemical structure of the lignin monomers/polymers being used, the origin of the

extracted lignin, and the type of microorganisms tested (Calvo-Flores *et al.*, 2015; Cazacu *et al.*, 2013).

Natsir and colleagues prepared natural pesticides by lignin photodegradation for antimicrobial activity on *Fusarium oxysporum* and *Xanthomonas* species. This study proposed that the antimicrobial activity of the photodegraded lignin might occur through: 1) the lipid devastation process on *Xanthomonas* species and *Fusarium oxysporum* plasma membrane, and 2) the existence of protein deformation by lignin decay compounds that degrade cell wall in order to be able to damage the cell walls of the microbe (Natsir *et al.*, 2018).

1.4. Lignin-based Composites

Plastics are extensively used in human life. Huge amounts of plastics are produced and used in different industrial sectors annually. Many plastics (fossil- and bio-based) are not biodegradable and therefore cause serious environmental issues. Besides, the unsustainability and considerable cost of fossil fuels require alternative and continual resources for our future. Hence, more effort has been put into developing bio-based plastics and biodegradable polymers in an aim to produce sustainable materials for different applications. Starch, polylactic acid (PLA) derived from corn, protein, and polyhydroxybutyrate (PHB) derived from microorganisms are the most effective biodegradable bio-based polymers present now in the bioplastic market (Yang *et al.*, 2019). Many applications recently focus on the ability of a material to biodegrade. Biodegradability supports the sustainability concept by reducing the waste impact of fossil-based polymers. Among those applications are food packaging and agricultural applications (Muñoz-Bonilla *et al.*, 2019). Nonetheless, these materials, in general, possess low mechanical resistance and are highly sensitive to environmental conditions. Therefore, intensive efforts have been employed to modify films/blends either based or containing bio-based materials (Rhim *et al.*, 2013).

In everyday life, food damage and degradation occurs mainly due to foodborne pathogens and other microorganisms, such as *Salmonella*, *Campylobacter*, *Escherichia coli*, *Yersinia enterocolitica*, and *Listeria monocytogenes*, which grow mainly on the surface of the food; there are also some microorganisms that grow in the volume of polymers throughout the pores and gels. Traditional methods for food preservation use drying, freezing, refrigeration,

fermentation, use of additives (such as organic acids and salts), and heat treatment to prevent microorganisms from growing and spoiling food. In order to preserve the microenvironment and offer a safe interaction between the food and the packaging materials, a relatively new concept called “active packaging” or “smart packaging” has been created. Active packaging extends the shelf life of food products (Huang *et al.*, 2019). Active packaging embraces AA in addition to antimicrobial activity.

Lignin, being the second most abundant biopolymer in the world and possessing antioxidant and antimicrobial activities, has played an essential role in the research development of the sustainable materials field. Recent research has focused on the use of lignin as a valuable additive in the preparation of thermoplastics or in packaging polymers through the development of sustainable materials using various types of matrices (Thakur *et al.*, 2018). The UV absorption properties and the free radical scavenging capacity of phenolic groups give lignin excellent antioxidant ability and can increase the oxidation and thermal stability of polymers in composites (Sadeghifar *et al.*, 2017). It has been reported that lignin has the potential to modify biopolymeric films and hence increase the shelf life of packaged oil products by reducing the oxidation rate in the package (Mohammad Zadeh *et al.*, 2019).

Cellulose is the most abundant natural polymer on earth (Nechyporchuk *et al.*, 2016). It is a linear polymer consisting of β -(1,4)-linked D-glucose monomers. Resulting from its chemical structure, cellulose is hydrophilic in nature, and is a highly crystalline, fibrous and insoluble material. Cellulose-based materials offer advantages such as “edibility, biocompatibility, barrier properties, aesthetic appearance, being non-toxic, non-polluting and having low cost” (Tajeddin, 2014). Numerous studies have been focused (and still do) on developing new films based on cellulose. Usually, cellulose films are combined with other polymers or plasticizers to improve or modify the physicochemical properties and expand its potential applications (Cazón *et al.*, 2019).

Among the many polysaccharidic biopolymers used for the preparation of edible films and coatings, hydroxypropylmethylcellulose (HPMC) has been the focus of much research due to its availability, edibility, good film-forming properties (resulting in transparent films with suitable mechanical performance), and excellent gas and grease barrier properties, as well as its ability to retain a large amount of active compounds (Rhimi *et al.*, 2018). In addition, they are biodegradable and offer very efficient oxygen, carbon dioxide, and lipid barriers (Imran *et al.*, 2010). However, the high water sensitivity of HPMC produces a loss of barrier properties

of the packaging or even solubilization into foods with high water content. This behavior limits its industrial application. Additionally, HPMC has poor mechanical resistance, low elasticity, and a rigid and breakable consistency (Sebti *et al.*, 2007), but is still suitable for edible coating purposes.

Chitosan is a bioactive, biodegradable, and non-toxic polymer from nature. Chitosan and chitin have been already used in the industry and health sectors. Chitosan consists of randomly distributed β -(1,4)-linked D-glucosamines (deacetylated unit) and N-acetyl-D-glucosamines (acetylated unit). Thus, it contains three functional groups: an amino group, a primary hydroxyl group, and a secondary hydroxyl group. The amino group in chitosan is positively-charged and is active towards negatively-charged groups (Kurniasih *et al.*, 2018). Deacetylation of amide groups ($R-NH-CO-CH_3$) induces the formation of ammonium groups which provides chitosan good film forming properties and an improved water solubility (Crouvisier-Urien *et al.*, 2016). It has been reported that chitosan and its derivatives possess some biological activities, such as an antimicrobial feature (Crouvisier-Urien *et al.*, 2016; Garrido-Maestu *et al.*, 2018; Muñoz-Bonilla *et al.*, 2019; Yang *et al.*, 2016b). Being able to form active, edible, or biodegradable films, chitosan films or coatings offer a moderate barrier against oxygen and good carbon dioxide barrier properties. They likely show oil and fat resistance and are selectively permeable to gases but have low water transmission resistance. This results from the strong hydrophilic character of polysaccharides, leading to an interaction with water molecules. Furthermore, chitosan coatings limit the contamination on the food surface. Compared to HPMC, chitosan films have better mechanical properties and show a higher tensile strength, Young's modulus, and elongation at break, but also a higher affinity to water resulting in higher water vapor permeability (Alzageem *et al.*, 2018a). Nevertheless, its applications have been restricted due to some drawbacks including low surface area, dissolution in highly acidic solutions, poor thermal and mechanical properties, and high cost (Yang *et al.*, 2016b).

Cellulose and lignin being the two major components in wood make them ideal components of bio-based nanocomposites. The main role of lignin as a strength, rigidity, and support provider to the plant cell walls would make it a suitable and logical candidate for the reinforcement of cellulosic nanocomposites (Farooq *et al.*, 2019). The same applies for food packaging films. Moreover, incorporation of lignin to cellulose and chitosan polymers enhances the mechanical properties and the water vapor permeability, since it is having multiple functionalities as reported by Wang *et al.* (Wang *et al.*, 2016).

1.5. Research Outline

Beside the introduction, this thesis contains three chapters: Chapter 2 covers the methods and materials used to retrieve the discussed results. This chapter is divided to three sections each describes the methodology of the topics covered in the introduction (Lignin Extraction, Purification and Analysis; Antioxidant Activity, Total Phenol Content and Antimicrobial Activity; Lignin-Based Composites).

Chapter 3 consists of the results and discussion. Similar to the outline of the introduction and the methods and materials, this chapter is divided to three sections. Each section introduces the results in subsections with their discussion. The first section covers the extraction of lignin and its purification using selective extraction with different organic solvents. The purification used during experimentation was controlled by thin layer chromatography (TLC). The produced fractions were analyzed and compared. In the second section, the antioxidant activity of the purification fractions was studied side by side with the total phenol content. The antimicrobial activity of the fractions was also studied. This section also discusses the effect of storage time, source and temperature on the structure and the bioactivities. The third section explores lignin-based films. Lignin was added to HPMC in different percentages. Also, chitosan was added to cover a wider scope of antimicrobial activity against gram positive and gram negative bacteria. The antioxidant and antimicrobial activities of the prepared films as well as the compatibility of lignin in these films were studied.

Chapter 4 is the conclusion of the entire thesis. It answers the research questions in regard to the empirical results found via experimentation. The future work has been stated as an extension to the scientific vision in this thesis. The discussed limitations were included with the suggested recommendations.

2. Methods and Materials

2.1. Lignin Extraction, Purification and Analysis

2.1.1. Isolation and Purification

Industrial black liquor (obtained from the pulping process of Spruce/Pine biomass) was acquired from Zellstoffwerk Blankenstein GmbH (Blankenstein, Germany). KL was extracted by acidic precipitation of black liquor using HCl and H₂SO₄ at varied pH, temperature, and time of stirring. The results of these precipitation conditions were examined for yield. The optimum conditions, for the highest yield, were using H₂SO₄ with stirring at room temperature (RT) and pH = 2 for 90–180 min to obtain the first fraction of lignin (L1).

Acidic precipitation (L1): 200 mL of black liquor was stirred at RT for 90 min, then 25% H₂SO₄ was added drop wise to the black liquor until the pH of the liquor reached 2. The KL then was filtered and washed with tap water, dried at RT, and then checked by TLC.

Soaking with diethyl ether (L2): 5g of L1 was soaked in 10 mL diethyl ether for 30 min, then was stirred for 5 min at RT and filtered. The precipitate was put in a fume hood for 30 min to dry and then checked by TLC

Selective extraction (L3): 20g of L1 was dissolved in 100 mL acetone, stirred for 15 min, and then filtered. The precipitate was washed with 50 mL acetone. The filtrate was concentrated to about 30 mL and diethyl ether was added until a precipitate appeared. The addition of diethyl ether was repeated until no more precipitate appeared. The lignin then was filtered and dried at RT and checked by TLC.

Double selective extraction (L4): dried L3 was dissolved in 100 mL ethanol, stirred for 15 min, and then filtered. The precipitate was washed with 50 mL ethanol. The filtrate was concentrated to about 30 mL and diethyl ether was added until a precipitate appeared. The lignin was filtered, dissolved in 50 mL ethanol, filtrated, and concentrated. Diethyl ether was added until a precipitate appeared. The addition of diethyl ether was repeated until no more precipitate appeared. The lignin was filtered, dried at RT, and then checked by TLC.

Thin Layer Chromatography (TLC): the fractions were dissolved in dimethyl sulfoxide (DMSO) and spotted on a silica sheet. A mixture of 10% n-hexane and 90% ethanol was used as the mobile phase with a drop of formic acid to avoid tailing.

Organosolv Lignins were prepared to be used for comparison studies according to an earlier published procedure (Hansen *et al.*, 2016b; Bergs *et al.*, 2019). The lignins were extracted from spruce/pine (L5), from beech wood (L6) and from *Miscanthus x giganteus* (stem/leaf mixture) (L7). The biomass was milled using a ball mill (Pulverisette 6, Fritsch, Idar-Oberstein, Germany) then sieved to a particle size <0.5 mm using a Modell AS 200 basic (Retsch, Haan, Germany). All samples were isolated without the use of catalysts. 100 g of (0.5 mm size) wood mill was dissolved in 450 mL (80% v/v) ethanol, placed in an autoclave (Parr reactor with a Parr 4848 Reactor Controller) at 700 °C for 3 h under continuous stirring, and then the autoclave was set to cool gradually for at least 3 h. The biomass is filtered with vacuum and washed 5 times with 50 mL ethanol (80% v/v). The filtrate was acidified with about 10 mL HCl until a precipitate was formed. The filtrate was measured, water was added (3 times the filtrate's volume), and then left to stand overnight. The mixture was centrifuged at 3500 rpm for 5 min and the precipitate was washed 3 times with distilled water, freeze-dried for 72 h, and finally collected.

2.1.2. FT-IR Analysis

FT-IR measurements were performed using KBr pellets on a Jasco FT-IR 410 (Tokyo, Japan) spectrometer. For this purpose, 3 mg of the dried sample and 300 mg of KBr were weighed into an agate mortar and then finely ground. The fine powder was pressed into pellets under vacuum for 2 min under a load of 10 tons by a hydraulic press. After a background measurement of the room air, the pellets were measured at a resolution of 4 cm⁻¹ and 30 scans in the range of 3800 to 500 cm⁻¹.

2.1.3. UV-Vis Analysis

A concentration of 50 $\mu\text{g/mL}$ in 0.1 M NaOH solution of lignin was analyzed by Hewlett-Packard (Waltham, MA, USA) 450 Diode Array Spectrometer. The UV-Vis absorption spectrum was measured over the range 210 to 500 nm.

2.1.4. 2D-NMR (HSQC) Analysis

NMR spectrometer Avance III 600 (Bruker, Karlsruhe, Germany) was used to measure the heteronuclear single quantum coherence (HSQC) spectra at 25 °C using deuterated DMSO (DMSO- d_6). 60 mg of lignin was dissolved in 0.5 mL of DMSO- d_6 for the measurements. Four scans and 16 prior dummy scans were run. 4000 points were recorded with a spectral width of 7211 Hz, a receiver gain of 2050, and a total acquisition time of 0.28 s. O1 was set to 5 ppm (^1H) and 80 ppm (^{13}C).

2.1.5. ^{31}P -NMR Analysis

20 mg of lignin samples were dissolved in 0.8 mL of anhydrous pyridine- d_5 and CDCl_3 (1.6:1.0, v/v) solvents with stirring. Then 100 μL of the relaxation agent chromium (III) acetylacetonate (5 mg/mL in anhydrous pyridine and deuterated chloroform 1.6:1.0, v/v) was added. Lastly, the phosphorylation reagent 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxapholane (0.1149 g) was added slowly. The mixture was stirred for 30 min using a magnetic stirrer. The measurement was carried out using a ^1H - ^{31}P decoupling experiment (Avance III 600, Bruker, Karlsruhe, Germany) with 32 scans and 2 prior dummy scans. 131,000 points were recorded with a spectral width of 12,175.324 Hz, a receiver gain of 2050, and a total acquisition time of 5.38 s. The evaluation took place via the integration of the surfaces, whereby the reference surface of the internal standard is set to one.

2.1.6. SEC Analysis

The weight average (M_w) and number average (M_n) molecular weights of the lignins, as well as their PD were determined by SEC (PSS SECurity2 GPC System, Mainz, Germany). Tetrahydrofuran (THF) was used as the mobile phase. 5 mg of lignin was weighed into a volumetric flask and dissolved in 5 mL THF. The samples were allowed to stand for about 30 min for better solubility. The samples which had not completely dissolved were freed of the undissolved particles before measurement by means of 0.45 μm PTFE microfilters. This prevents possible clogging of the sensitive column. The filtered sample was then transferred by means of a SEC cannula into the 100 μL sample loop injector and the measurement activated by means of manual switching. The injection volume is 20 μL . Polystyrene standards (PSS standards) were used for the calibration at different molecular weights. Samples were analyzed at 35 °C at a flow rate of 1 mL/min over a PSS SDV 8x50 mm guard column and two PSS SDV 8x300 mm Linear M styrene-divinylbenzene copolymer network columns. UV detection was recorded at 254 nm using an Agilent 1100 Series VWD detector. The calibration range of the PSS ReadyCal-Kit polystyrene standards used is 376-2,570,000 g/mol. The resulting logs were evaluated using the PSS Win SEC software. The software calculates the molecular weight distribution, the molecular weights (M_w and M_n) and the PD via calibration. Using the calibration line, the software can calculate the molecular weight distribution via the integral under the distribution.

2.1.7. Pyrolysis Gas Chromatography/Mass Spectrometry (py-GC/MS)

Approximately 1 mg of lignin sample was inserted without further preparation into the bore of the pyrolysis solids injector and then placed with the plunger on the quartz wool of the quartz tube of the furnace pyrolyzer Pyrojector IITM (SGE Analytical Science, Melbourne, Australia). The pyrolyzer was operated at constant temperature of 550 °C. The pressure of helium carrier gas at the inlet to the furnace was 95 kPa. The pyrolyzer was connected to a Trace 2000 gas chromatograph (ThermoQuest/CE Instruments, Milan, Italy) with a quadrupole mass spectrometer Voyager (ThermoQuest/Finnigan, MassLab Group, Manchester, UK) operated in electron impact ionization (EI) mode. The fused silica gas chromatography (GC) capillary column DB-5 ms with dimensions: 30 m long, 0.25 mm I.D., 0.25 μm film thickness

(J&W, Folsom, CA, USA) was used. The gas chromatographic conditions were as follows: programmed temperature of the capillary column from 60 °C (1 min hold) at 2.5 °C/min to 100 °C and then 10 °C/min to 280 °C (20 min hold at 280 °C). The temperature of the split/splitless injector was 250 °C and the split flow was 10 cm³/min. Helium, grade 5.0 (Westfalen AG, Muenster, Germany) was used as a carrier gas at constant pressure of 70 kPa during the whole analysis. The transfer line temperature was 280 °C. The MS EI ion source temperature was kept at 250 °C. The ionization occurred with a kinetic energy of the impacting electrons of 70 eV. The current emission of the rhenium filament was 150 μA. The MS detector voltage was 350 V. Mass spectra and reconstructed chromatograms (total ion current (TIC)) were obtained by automatic scanning in the mass range m/z 35–455 u. Pyrolysis–GC/MS data were processed with the Xcalibur software (ThermoQuest) and the NIST 05 mass spectral library.

2.1.8. Thermogravimetric Analysis

All lignin samples were independently obtained from the FH Aachen, Campus Jülich in the Laboratory of Applied Polymer Science. About 10 mg of dried lignin were transferred to a TGA crucible, the exact weight was determined after taring the internal TGA balance, a Netzsch TGA 209 F1, at RT. The samples were analyzed at a heating rate of 20 °C/min from RT to 800 °C as the final temperature. The nitrogen flow was 20 mL/min. The lignin mass was recorded continuously. First, preliminary tests were carried out to determine the optimum heating rate. The variation of the heating rate from 10 °C/min to 20 °C/min resulted in a shift of the decomposition profile to higher temperatures (about 5 °C). Since the curve was otherwise identical, a heating rate of 20 °C/min was chosen for all experiments. The raw data of the decay were evaluated with Excel; the thermogram was obtained with the mass plotted against the temperature. The first derivative of mass loss versus time was plotted versus temperature to obtain the derivative thermogravimetric (DTG) curve.

2.1.9. Differential Scanning Calorimetry

Glass transition temperatures (T_g) were determined using a Perkin Elmer 8000 differential scanning calorimeter. The previously dried lignin samples were placed in DSC aluminum

crucible, sealed and the lid pierced by means of a needle. The measurement was carried out with air as a reference sample. The choice of heating program could not be taken from the literature. Many preliminary tests were carried out here. The optimum heating program was as follows: heating occurred from RT to 120 °C at a heating rate of 20 °C/min and was held for 5 min. Subsequently, the temperature was quenched to -30 °C at a heating rate of 10 °C/min. This condition was maintained for 3 min under a nitrogen flow rate of 10 mL/min. Thereafter, the sample was again heated to temperatures of 160 °C and 226 °C, with a heating rate of 20 °C/min. Before being tested, the samples were extensively dried for 24 h in an oven at 50 °C.

2.1.10. X-Ray Diffraction

X-ray diffraction (XRD) patterns were obtained with a Bruker D2 PHASER X-ray diffractometer (Germany). Lignin samples were finely ground and were set in the sample holder and then analyzed. X-ray diffractograms were collected with 2θ , ranging from 10° to 65° using Theta/Theta geometry. A secondary monochromator with a CuK α rotation tube was used at 30 kV and 10 mA, with a scanning over 5407 (96 s/step).

2.2. Antioxidant Activity, Total Phenol Content and Antimicrobial Activity

2.2.1. Lignin Purity, Ash and Sugar Content via NREL Measurements

National Renewable Energy Laboratory (NREL) measurements were performed by the BIOPOS Research Institute (Teltow-Seehof, Germany). The chemical composition (% w/w) was measured in accordance to the standard analytical procedures issued by NREL. HPLC analysis was performed at a constant temperature of 90 °C using water as a mobile phase at a flow rate of 0.4 mL/min in a 300 × 7.8 mm column (Machery-Nagel, Germany). The NREL procedures described by Sluiter *et al.* were used to determine the structural carbohydrates in biomass and lignin samples (Sluiter *et al.*, 2008b, 2008a).

2.2.2. Antioxidant Activity

The AA determination was performed based on the use of the free radical DPPH using a spectrophotometer Jasco V-630. Lignin samples were dissolved in dioxane/water solution (90:10, v/v) with a concentration of 1 g/L. 0.1 mL of the lignin solution was mixed with 3.9 mL of a DPPH solution (6×10^{-5} M). The absorbance of the mixture was measured at 15 min and 30 min at a wavelength of 518 nm.

2.2.3. Total Phenol Content

A volume of 5 mL of 20% Na_2CO_3 solution was mixed with 2.5 mL of FC reagent and 0.5 mL of lignin solution (20 mg in 10 mL of DMSO). The mixture was stored for 30 min at 40 °C. Afterwards, the absorbance of the mixture was measured using a UV-Vis spectrophotometer (Jasco V-630) at 750 nm. The TPC was determined using a standard curve with gallic acid solutions.

2.2.4. Antimicrobial Activity of Lignins (Zone of Inhibition Test)

Inoculums of *Listeria monocytogenes* (DSM No 19094) (*L. monocytogenes*), *Staphylococcus aureus* (DSM No 799) (*S. aureus*), and *Escherichia coli* (DSM No 1576) (*E. coli*) were cultured by exposing a frozen culture to 10 mL of nutrient broth (Roth, Karlsruhe, Germany). Subsequently, the broth was incubated for 24 h at 37 °C. The inoculum was diluted in physiological saline solution with tryptone (Oxoid, Hampshire, UK) to a final concentration of 10^7 CFU/mL, then 1 mL of the suspension was diffused with a sterile spatula over a plate count agar plate (Roth, Karlsruhe, Germany). 0.1 g of lignin was dissolved in 1 mL DMSO and dried over a circular filter paper of 0.5 cm of diameter (platelet). Three of those platelets were placed on the agar plate. Three blank platelets were used as references and were placed on the same inoculated agar plate. The agar plates then were incubated at 37 °C for 24 h. A distinct inhibition zone (zone without microorganism growth) appeared illustrating the level of antimicrobial activity in the lignin samples.

2.3. Lignin-based Composites

2.3.1. Films Preparation

HPMC film was prepared following previously published procedure (Sebti *et al.*, 2007) by dissolving 3 parts of HPMC (3.00 g) in 200 parts of 0.01 mol/L HCl solution (200 g), 100 parts of absolute ethanol (100 g), and 10% (w/w HPMC) of Polyethylene glycol 400 (PEG 400). The solution was stirred for 15 min at RT. 25 mL of the prepared solution was cast onto glass. Bubbles were treated to avoid producing bubbly dried film. The solution was then dried at RT for about 36 h. The films were eventually peeled of carefully.

HPMC/lignin films were prepared by dissolving the following percentages separately: 5, 10, 15, 20, 25, and 30% (w/w HPMC) of the L1 in the smallest amount of DMSO with sonication, if needed, to guarantee 100% solubility. Afterwards, the lignin solution was added to the HPMC-film solution mentioned earlier with stirring for nearly 15 min at RT. 25 mL of the prepared solution was cast onto glass and dried at RT for 36 h after treating the bubbles formed by pouring the polymer solution into the glass. HPMC/Organosolv lignin (L5, L6, and L7) samples were prepared the same way. The films were then peeled of carefully.

HPMC/chitosan/lignin films solutions were prepared by dissolving 5% (w/w HPMC) of 85% deacetylated chitosan in absolute ethanol. The chitosan solution was added to the HPMC film forming solution. While stirring, 5, 10, 15, 20, 25 and 30% of solution was separately added (for L1, L5, L6, and L7), each to a HPMC/chitosan film forming solution, and stirred for 15 min at RT. Again, 25 mL of the polymer solution was poured onto glass and then dried at RT for 36 h after treating the bubbles. Lastly, the films were peeled of carefully.

2.3.2. Antioxidant Activity (DPPH Test) of the Films

The DPPH antioxidant activity test for the prepared films was performed following the method described by Yang *et al.*, with a minor modification (Yang *et al.*, 2016a). Films (0.1 g) were cut into small pieces and immersed in 2 mL of methanol for 24 h at RT then centrifuged for 2 min. The DPPH radical scavenging activity of the supernatant obtained was performed; part of the methanol extract (0.5 mL) was added to 0.5 mL of DPPH dissolved in methanol (50 mg/L).

The mixture was kept in the dark for 60 min at RT. The UV-Vis absorbance was detected at 517 nm using a Jasco V-630 UV-Vis spectrometer (Silver Spring, MD, USA). The mixture solution of DPPH methanol and methanol extracted from neat HPMC was used as a control. The DPPH antioxidant activity was calculated using Equation (3):

$$AA (\%) = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] * 100 \quad (3)$$

Where AA is the percent antioxidant activity, A_{control} is the absorbance of the control at $t = 0$, and A_{sample} is the absorbance of the sample at different incubation times.

2.3.3. Antimicrobial Activity of the Films

The antimicrobial activity of the samples was determined by a modified version of the Japanese Industrial Standard (JIS) Z 2801:2000 in order to test regular circumstances for spoiled products. The JIS compares the bacterial counts (*S. aureus*, *E. coli*) in saline solution on reference and on sample materials after a specific incubation temperature and time (at 35 °C, for 24 h) (Dohlen *et al.*, 2017; Hüwe *et al.*, 2018; Sebti *et al.*, 2007). Materials showing a calculated \log_{10} -reduction $\geq 2 \log_{10}$ units after incubation for 24 h are considered as powerful antimicrobial agents (JIS Z 2801:2000). The antimicrobial activity against *Brochothrix thermosphacta* (*B. thermosphacta*) and *Pseudomonas fluorescens* (*P. fluorescens*) is determined when \log_{10} -reduction $\geq 2 \log_{10}$ values are reached. The test was conducted at a constant temperature of 7 °C for 24 h (Braun C. *et al.*, 2017; Dohlen *et al.*, 2017; Hüwe *et al.*, 2018; Tamsyn Stanborough *et al.*, 2017).

3. Results and Discussion

3.1. Lignin Extraction, Purification and Analysis

3.1.1. FT-IR Analysis

Structural analysis to specify functionality in lignin is important to investigate the antioxidant capacity and related structure-property relationships. The crosslinking phenolic nature of lignin provides interesting activities for chemical and biological interactions with a large variety of additional functional groups like hydroxyl, carbonyl, methoxy, and carboxylic groups. An analysis of varied active groups connected to lignin is significant in a functional categorization of lignins, because it can alter the chemical and biophysical characteristics of the lignin. Such analysis can be accomplished by FT-IR analysis. FT-IR spectra of the purification fractions of KL show the vibrations typical for the G-unit. The intensity deviations are produced from the biomass, where G ring and C=O stretching are around 1260 cm^{-1} , and C–H out-of-plane vibrations in positions 2, 5, and 6 are around 850 cm^{-1} as shown in Figure 3.1 (B. Hansen *et al.*, 2016). The analysis is independent of the used sustainable resource.

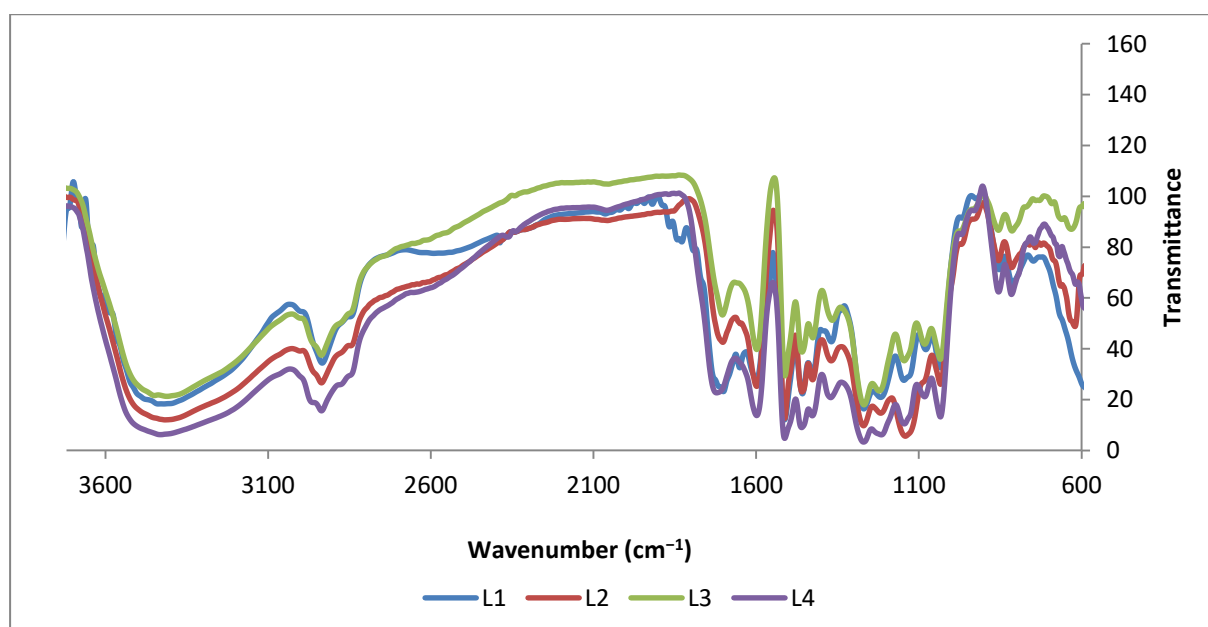


Figure 3. 1. FT-IR spectra of KL purification fractions: L1 (blue), L2 (red), L3 (green), and L4 (purple).

In Table 3.1, the signals of the KL fractions are in agreement with literature data (Vivekanand *et al.*, 2014). The broadest OH stretching peak is found in L4 since it has the fewest impurities. The broadness of this peak is decreased from L4 to L1. The spectra show fewer impurities and less noise: the signal/noise ratio from L1 to L4 proves the purification strategy. Aromatic and aliphatic-OH signals are detected at 3415 and 1265 cm^{-1} , respectively (Fițigău *et al.*, 2013; Vivekanand *et al.*, 2014). Besides literature data, a sharp signal of C–H out-of-plane in *m*-position of G-units was detected.

Table 3. 1. FT-IR functional group assignment of purification fractions. The most essential signals are listed with wavenumbers and signal assignment for the fractions: L1 to L4 in comparison with literature data of KL.

L1 (cm^{-1})	L2 (cm^{-1})	L3 (cm^{-1})	L4 (cm^{-1})	KL Lit. (Fițigău <i>et al.</i> , 2013) (cm^{-1})	Signal assignment
3396	3408	3414	3396	3415	O–H stretching
2931	2931	2926	2925	2935	C–H stretching
2834	2814	2834	2833	2843	tertiary C–H group
1695	1695	1700	1702	1660	carbonyl-carboxyl stretching
1577	1583	1593	1595	1505	aromatic/carbonyl stretching
1452	1449	1455	1459	1451	C–H deformation
1263	1262	1265	1262	1265	C–O stretching, aromatic (phenyl)
1028	1028	1026	1028	1029	C–O deformation (methoxy group)
810	810	807	807	814	C–H out-of-plane in <i>m</i> -position of guaiacyl units
848	848	848	848	-	C–H out-of-plane in <i>m</i> -position of guaiacyl units

3.1.2. UV-Vis Analysis

UV-V a spectrophotometric methodology best adapted for studying topochemistry, purity, and concentration of lignin. This method is performed for quantitative analysis and not for qualitative analysis since many compounds absorb UV-Vis light at the same wavelength. The absorbance level in UV-Vis spectra is directly proportional to the purity level of a compound i.e. lignin in this case. A lower absorbance is observed at higher pH value due to the co-

precipitation of the polysaccharide degradation product, wax and lipids (Ahuja *et al.*, 2017). The difference between the lignin fractions is apparently shown in Figure 3.2. Noticeable improvement was accomplished. L4 shows four distinct UV-Vis peaks (without any shoulders) due to π - π^* and n - π^* excitations of conjugated phenolic groups. As reported in the literature, most of the spectra of KL enclose at least two shoulders, as proved for the first three fractions (L1 to L3) of the purification procedure. The main absorption (usually around 280 nm) shifts to 215–222 nm due to the hypsochromic effect of NaOH.

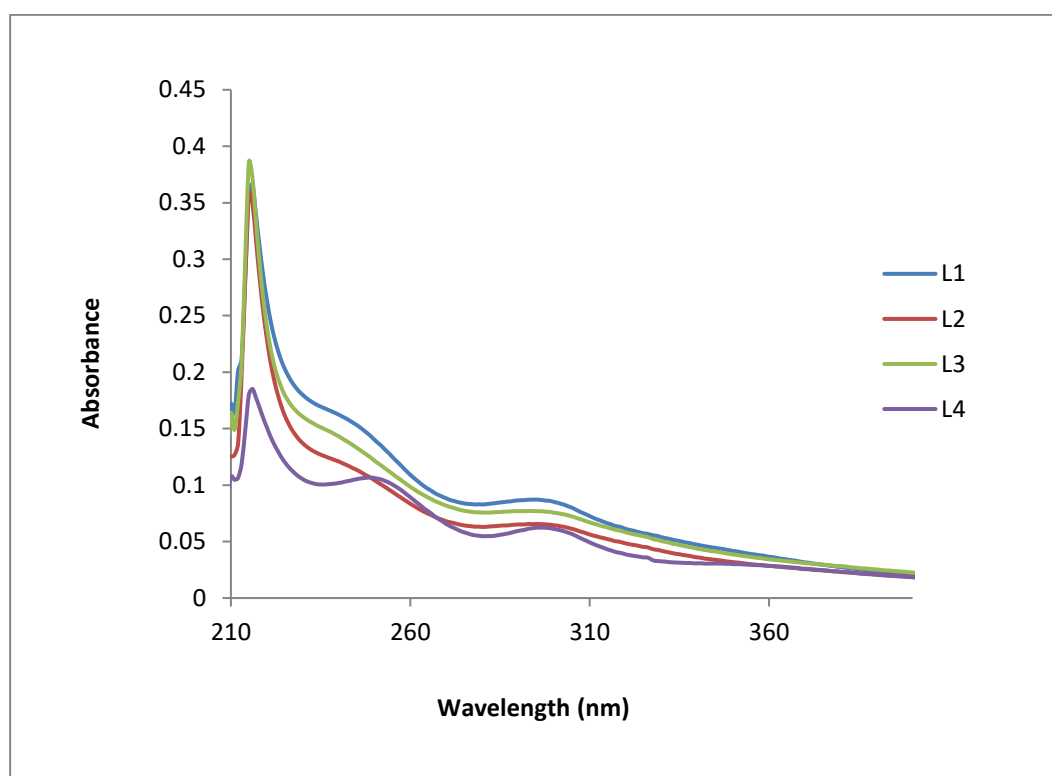


Figure 3. 2. UV-Vis light absorbance spectra of KL purification fractions in NaOH: L1 (blue), L2 (red), L3 (green), and L4 (purple).

Table 3.2 shows the two main UV-Vis absorption bands of lignin and compares them to literature data. The ester or ether bonds between ferulic acids, *p*-coumaric acids, and lignin were heavily cleaved during alkali treatment. The intensive absorbance at 279–280 nm relative to 316–320 nm displays a high content of G-units, analogous to that of other monocotyledons and is compatible with lignin rich of G-units (Azadi *et al.*, 2013; Vivekanand *et al.*, 2014).

Table 3. 2. UV-V absorption of lignin: main peaks and their characteristics.

λ Exp. (nm)	λ Lit. (nm)	Functional Group	Intensity	Excitation	Reference
215–222	279–280	Non-conjugated phenolic groups (G/S rich)	high	π - π^*	(Azadi <i>et al.</i> , 2013)
296–303	316–320	Conjugated phenolic groups (<i>p</i> -coumaric acid, ferulic acid)	low	n - π^*	(Vivekanand <i>et al.</i> , 2014)

3.1.3. 2D-NMR (HSQC)

NMR analysis (HSQC and ^{31}P) was used for structural illustration. HSQC is a two dimensional NMR (2D-NMR) that was performed to examine and indicate the monolignol content of lignins isolated from different sources. The indicated ratios of S-unit/G-unit/H-unit were in approximate concurrence with results acquired by py-GC/MS (Apak *et al.*, 2018; Schutyser *et al.*, 2018). For the investigated lignins, three regions of lignin structure were recognized by HSQC (Figure 3.3). The non-oxygenated aliphatic, oxygenated aliphatic side chain appear at $\delta\text{C}/\delta\text{H}$ 50.0-90.0/2.5-6.0, and the aromatic region where C-H correlations show signals at $\delta\text{C}/\delta\text{H}$ 100.0-135.0/5.5-8.5 confirmed by literature (Lupoi *et al.*, 2015). The following spectra, in Figure 3.3, exhibit intense signals at 56.0/3.7 corresponding to methoxyls and side chains in β -O-4-structures. Furthermore, a signal is seen at 62.0/3.2 which is attributed to the γ -C-H of gamma-acylated lignin units. A distinguished region is indicated at 110.0-120.0/6.4-7.0 which relates to C-H aromatic signals of G-units, whereas signals above 120.0 correlate to aromatic C-H signals of H-units.

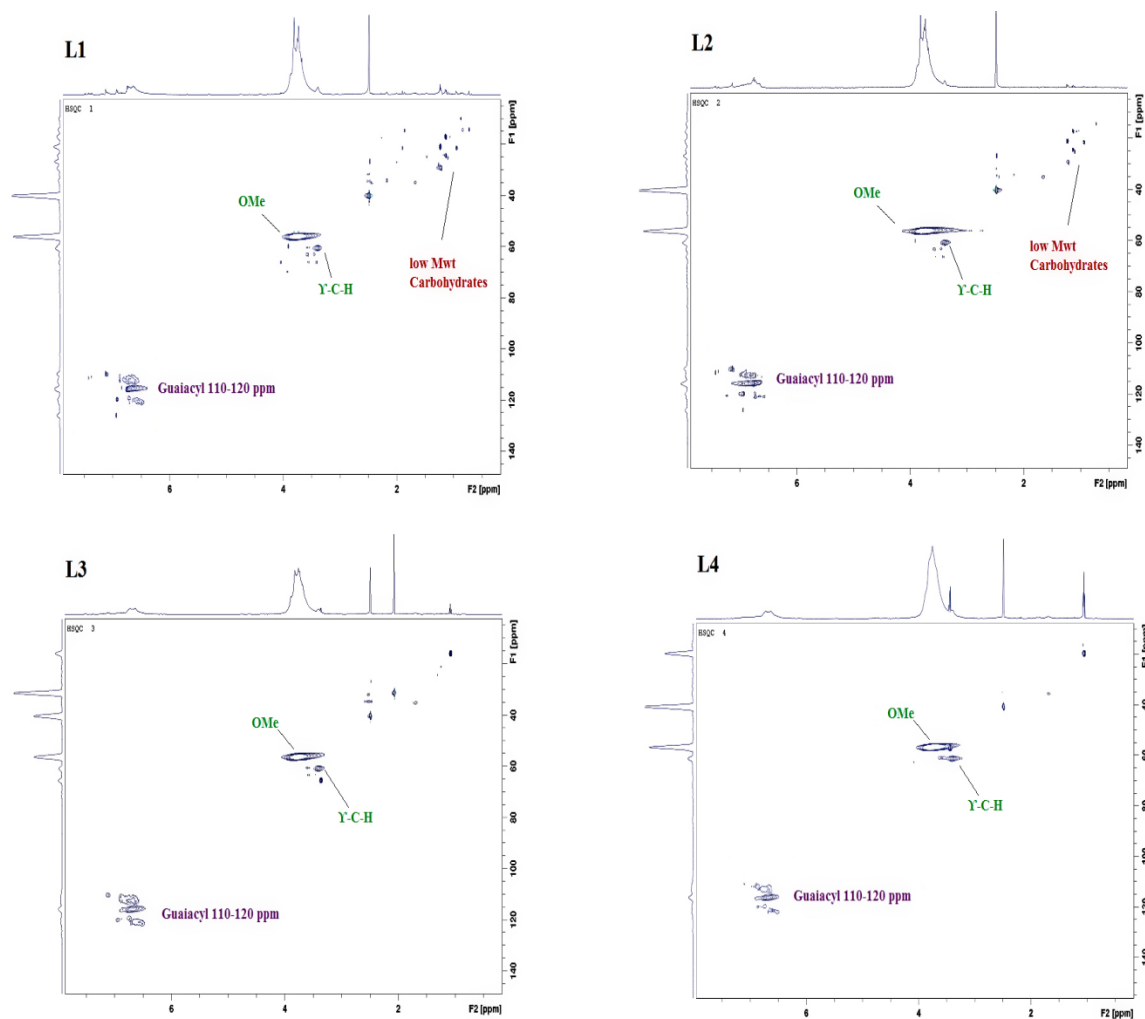


Figure 3. 3. HSQC spectra of KL purification fractions L1, L2, L3, and L4; assigning specifically methoxy (OMe) signals, γ -C-H signals, and G-unit signals.

3.1.4. ^{31}P -NMR Analysis

Phosphorous derivatization is a commonly used method to determine phenolic-OH groups (Gilca *et al.*, 2014). Still, the sensitivity of a ^{31}P -NMR experimentation is nearly 15 times less than that of a proton (^1H -NMR). The range of chemical shifts in ^{31}P -NMR is over 1000 ppm for a diversity of phosphorus compounds. The ^{31}P -NMR spectra in Figure 3.4 show four OH regions for KL fractions: aliphatic-OH between 151.0 and 144.7, condensed-OH between 144.0 and 141.5, G and dimethylated-OH between 141.5 and 137.5, and weak carboxylic acids peaks between 136.5 and 134.5. The lignin fractions were first phosphorylated with the use of 2-chloro-4,4,5,5-tetramethyl-1,2,3-dioxaphospholane then analyzed quantitatively by ^{31}P -NMR

spectroscopy. Endo-*N*-hydroxy-5-norbornene-2,3-dicarboximide was used as the internal standard in accordance with the procedure described by Sun *et al.* (S.-N. Sun *et al.*, 2014).

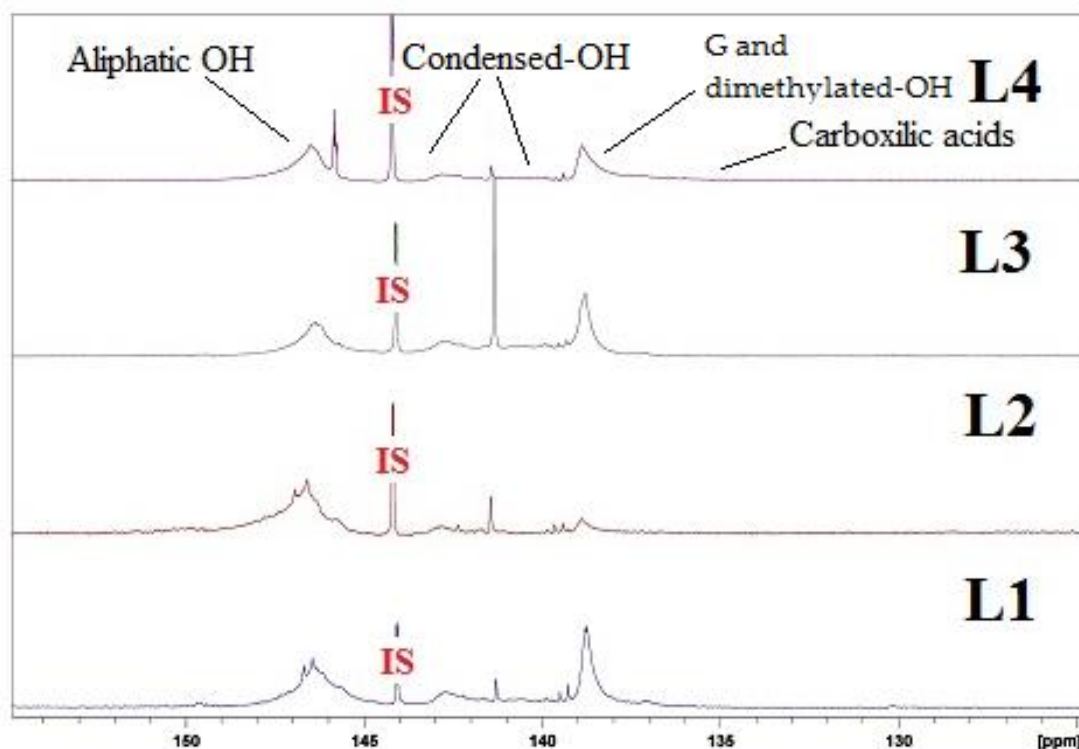


Figure 3. 4. ^{31}P -NMR spectra of Kraft lignin purification fractions: L1, L2, L3, and L4.

In Table 3.3 the aliphatic-OH peak is dominant, while in L3 both condensed and G and the dimethylated-OH peaks have almost comparable intensities with the aliphatic-OH peak. The ratio of G and dimethylated-OH to aliphatic-OH in L1 is nearly 0.7, while condensed-OH composes about half the aliphatic-OH, illustrating the attendance of some small fragments and/or carbohydrates in L1. Carboxylic acids do not exist in L2. Most likely, the fragments containing carboxylic acid were dissolved by the diethyl ether during soaking and disposed with the filtrate associated with some of the condensed-OH and aliphatic-OH-containing fragments. Selective extraction using acetone resulted in the loss of some aliphatic OH-containing fragments and hence the number of the aliphatic-OH decreased to roughly the half. Those fragments are potentially little fragments that dissolve in acetone and are disposed with the filtrate. In the double selective extraction, using ethanol, that produced L4; no significant change has been observed on the number of the Carboxylic acids-OH and the number of aliphatic-OH groups compared to L3. On the other hand, the number of condensed-OH has

become about fifth that of L3 and tenth of L1. The G and dimethylated-OH ratios of L4/L1 and L4/L3 were around 0.3 and 0.6 respectively. Coupling these results with more analytical data, i.e. UV-Vis, FT-IR, DSC, SEC, TGA, and XRD will confirm, by the end of this chapter, the effect of the purification process. L5, in comparison, showed S-OH rather than G-OH.

Table 3. 3. Number of OH in the lignins determined by ³¹P-NMR analysis.

	Aliphatic-OH	Condensed-OH	G and dimethylated-OH	Carboxylic acids-OH
L1	8.70	5.04	6.30	0.59
L2	8.52	0.28	0.71	0.00
L3	3.81	2.99	2.91	0.16
L4	3.17	0.48	1.79	0.15
L5*	3.02	0.21	0.18	0.02

* L5 contains S-OH: 0.01.

3.1.5. SEC Analysis

Studying the molecular mass distribution of technical lignin is essential for a better understanding of the reactivity and physicochemical properties of lignin. Many strategies have been used to determine the molecular weight distribution of lignin, primarily using SEC (also known gel permeation chromatography (GPC)), light scattering analyses, and viscosimetry. SEC is a versatile strategy used for measuring the molar mass distribution for lignin. The main disadvantage of the SEC studies is the usage of poly(methyl methacrylate) (PMMA) and polystyrene (PS) as standards due to the absence of suitable lignin standards. Both standards do not mirror the hydrodynamic volume of lignin. Nonetheless, universal calibration strategies would demand accurate concentration data, which are also hard to be detected because of the poor solubility of lignin. Monakhova and others developed a novel approach using SEC and HSQC data together with multifarious data analysis that facilitated determining the molecular weight and PD data for heparin (Monakhova *et al.*, 2018). Here, using PMMA, the molecular weight of the purification fractions ranged from 877 to 6117 g/mol, which agrees with literature values for analogous technical lignins (Gou *et al.*, 2018; Sulaeva *et al.*, 2017). The SEC results of the KL fractions (L1-L4) are shown in Figure 3.5.

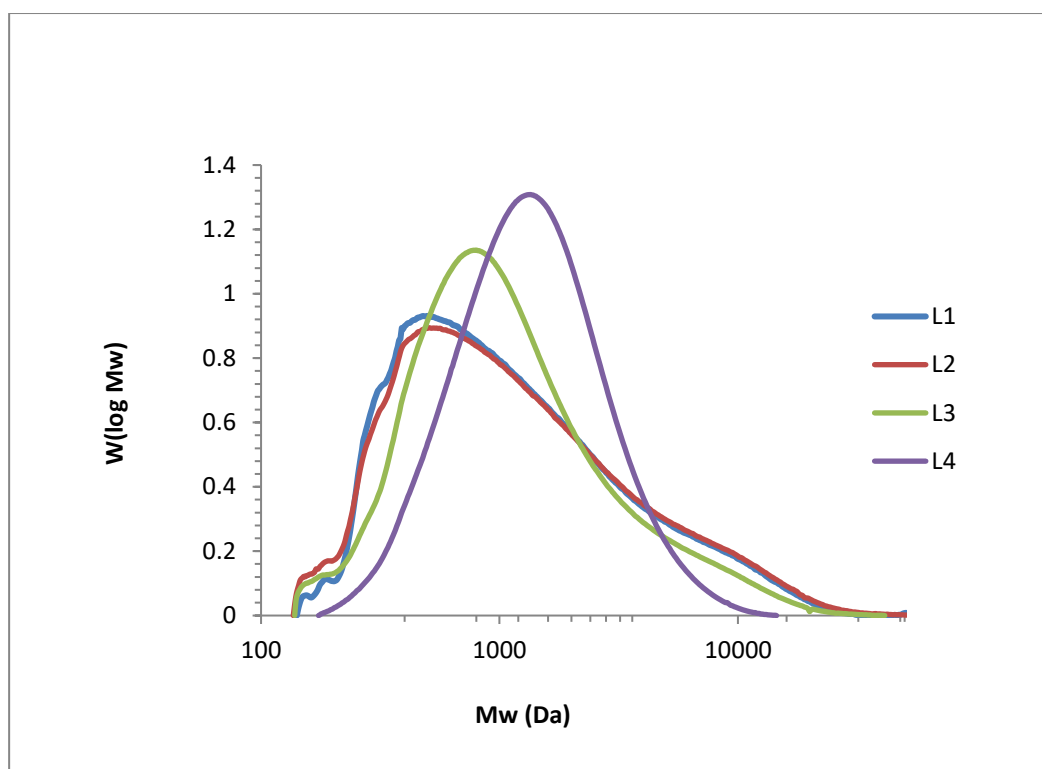


Figure 3. 5. SEC spectra of KL purification fractions: L1 (blue), L2 (red), L3 (green), and L4 (purple).

The values for the Mw, the Mn, and PD for the lignin purification fractions are listed in details in Table 3.4.

Table 3. 4. Mn, Mw, and PD of lignin fractions L1 to L4 obtained from the SEC analysis.

Fraction	Mn (g/mol)	Mw (g/mol)	PD
L1	720	2108	2.9
L2	706	2226	3.2
L3	757	1816	2.4
L4	1043	1690	1.6

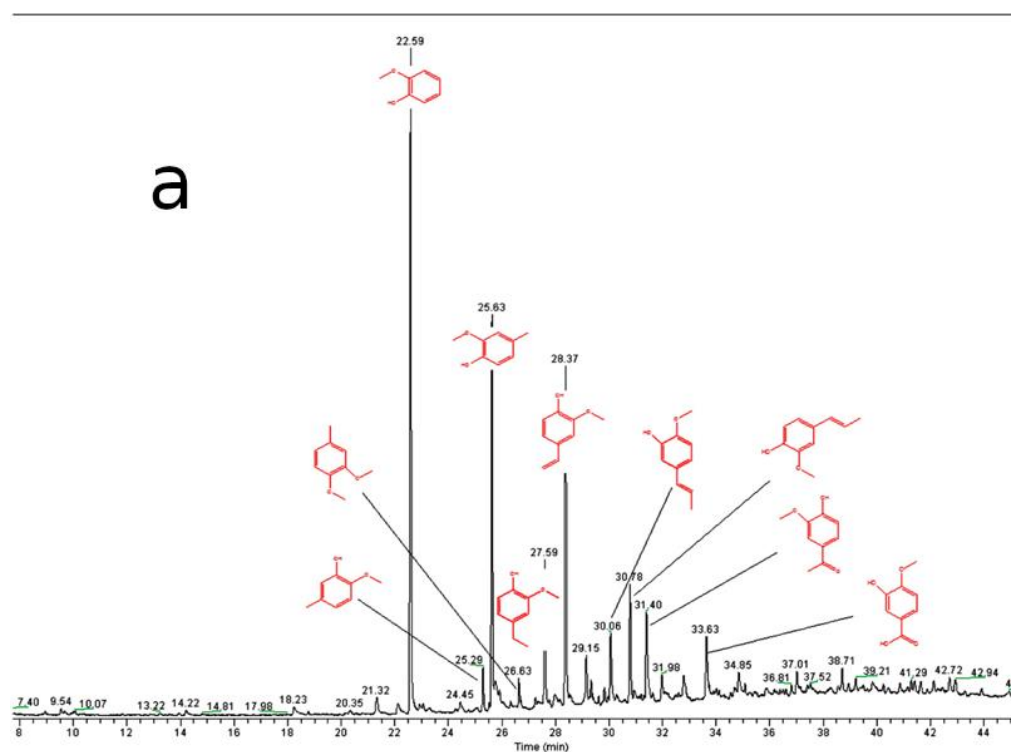
There is a clear state of correlation between purification and resulting SEC data for all fractions. In L4, the spectrum displays one sharp peak maximized at 1157 g/mol, while L1, L2, and L3 show fragments of low and high Mw.

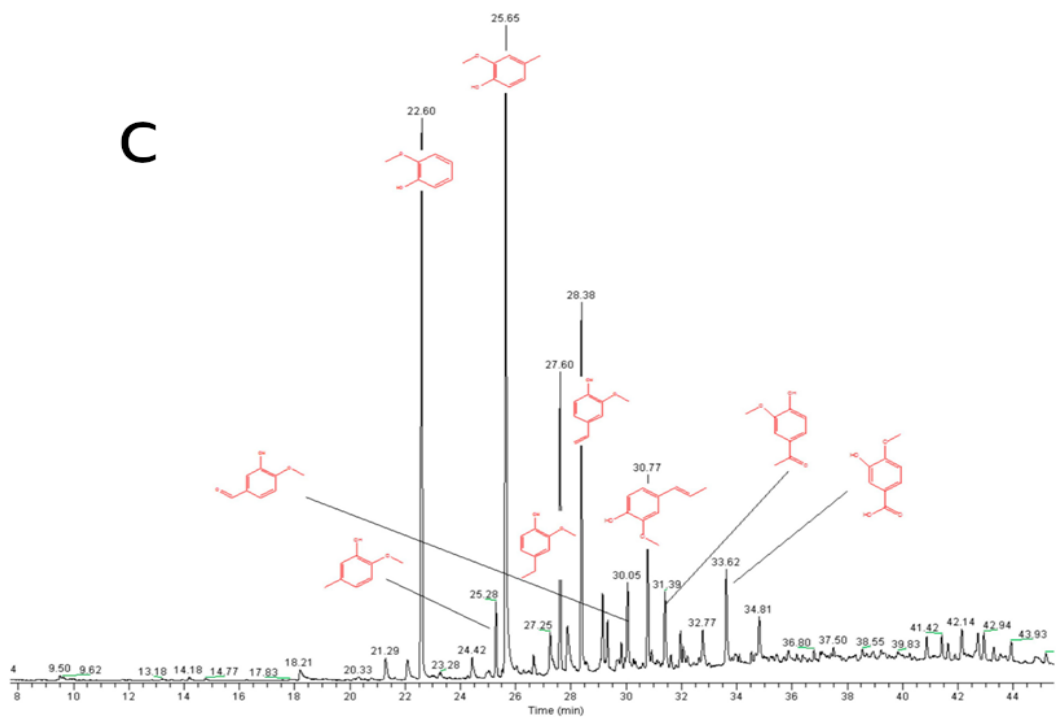
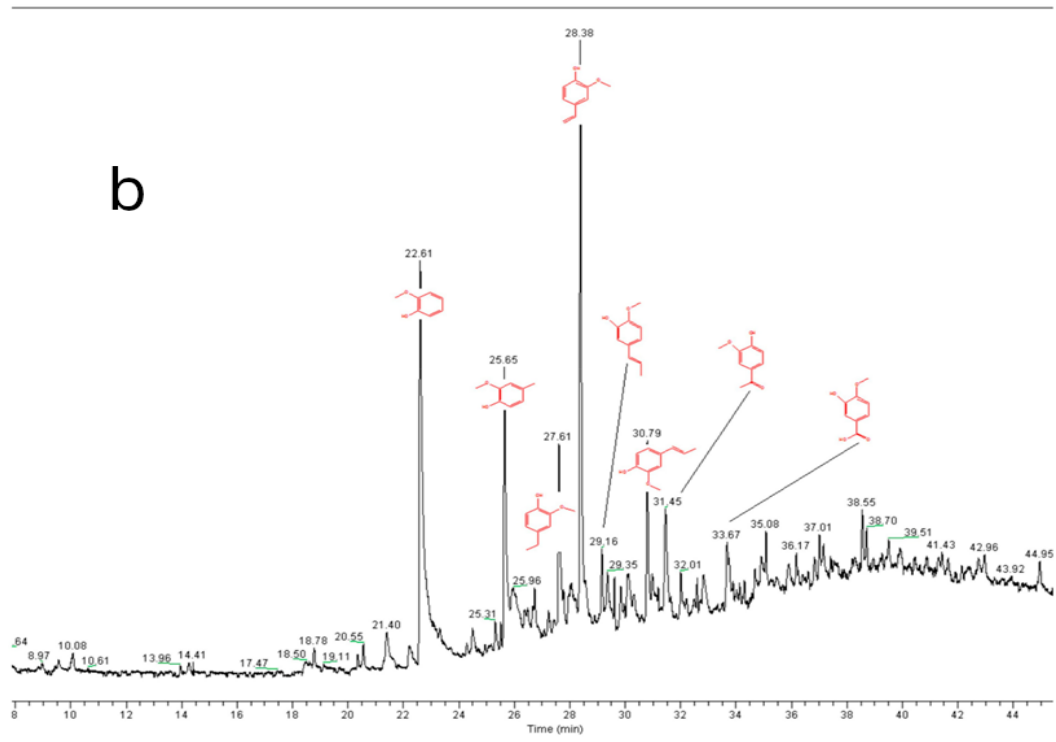
L1, L2, and L3 have maxima of 484, 466, and 878, respectively. The intensity increases as the impurities decrease. All four fractions (L1 to L4) have relatively low Mws in comparison with literature for KL (values are up to 6500 g/mol) (Humpert *et al.*, 2016).

3.1.6. Pyrolysis GC/MS

Lignin contains a diverse number of reactive functional groups due to its three dimensional amorphous nature attached by phenylpropane structural units via β -O-4 ether and C-C linkages, where methoxy is the most distinguished group. In accordance with the differences in the side chains, different structural fragments can be assembled: lilac lignin, guaiac wood-based lignin, and hydroxyphenyl lignin, resulting potentially in the formation of diverse phenolic compounds, like 4-methylguaiacol, 2-methoxyphenol, and 2-methoxy-4-vinylphenol, by means of propylene side chain cleavage. The splitting of the C-C bond in the guaiac wood-based lignin generates vanillin, whereas other products, such as ethers and alcohols, may be formed by the unstable fracture of the long straight side chain in the complex macromolecular structure of lignin.

Despite lignin having merely three basic structural units, the reactivity of the functional groups attached to the aromatic rings of each primary structural unit are varied. This leads to a highly complicated thermal decomposition (pyrolysis) of lignin (Zhao *et al.*, 2018). The py-GC/MS chromatograms for the fractions L1, L2, L3, and L4 are presented in Figure 3.6 a–d showing the assignment of the main signals.





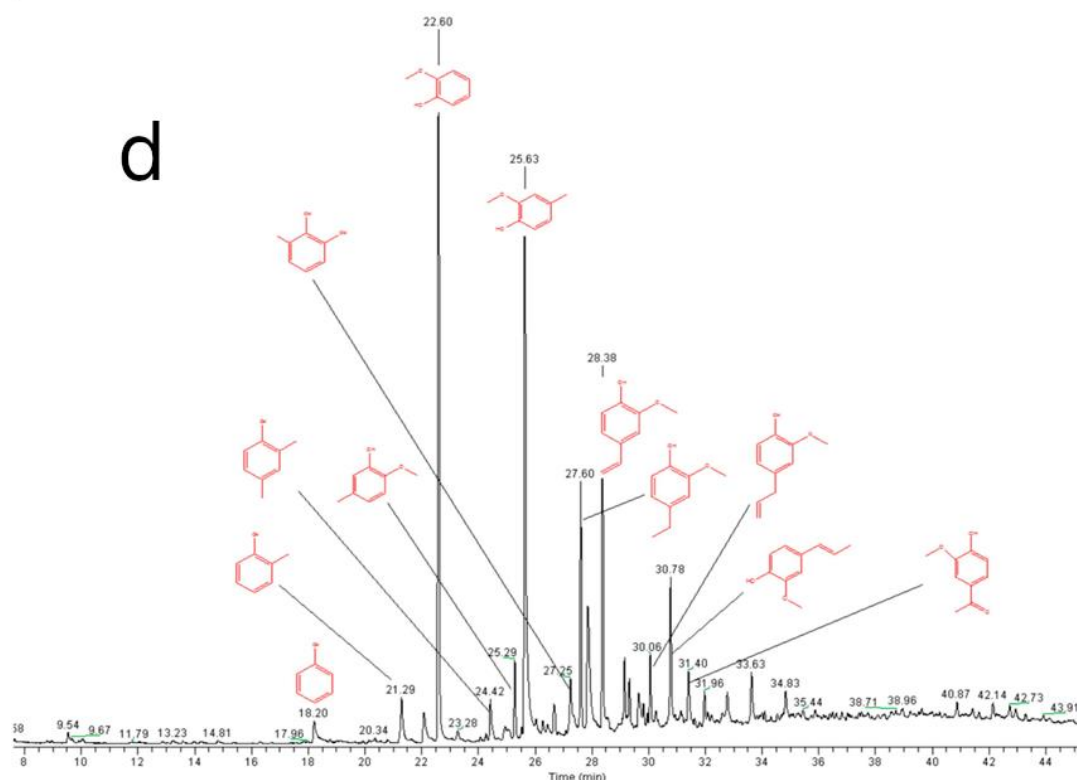


Figure 3. 6. Pyrograms of KL purification fractions: (a) L1, (b) L2, (c) L3, and (d) L4 (measured at 550 °C).

3.1.7. Thermogravimetric Analysis

Thermal stability and decomposition of organic polymers have been usually studied using TGA under nitrogen conditions. TGA is used to determine the mass loss of samples resulting from heat treatment, which shows the thermal stability and thermal decomposition of the compound. TGA of the lignin fractions was determined following a procedure used by Vallejos *et al.* (Vallejos *et al.*, 2011).

The results in Figure 3.7 show the complex decomposition of: L1 that is generated from five overlapping steps with the major maximum of mass loss rate at 60, 240, 380, 790, and 880 °C. Total mass loss reached 82.08 wt%; L2 decomposed through four steps at 60, 372, 780, and 880 °C with a total mass loss of 76.90 wt%; L3 decomposition took place in four steps: 60, 150, 395, and 899 °C. The total mass loss approached a 99.79 wt% and L4 decomposed at 60, 377, 810, and 899 °C. The total mass loss was 99.91 wt%. In TLC, spotting of the lignin fractions

at 25, 40, 60, and 90 °C; a new spot that appeared for the fractions at 60 and 90 °C can probably be appointed to a fragment in the mobile phase, not certainly evaporating water.

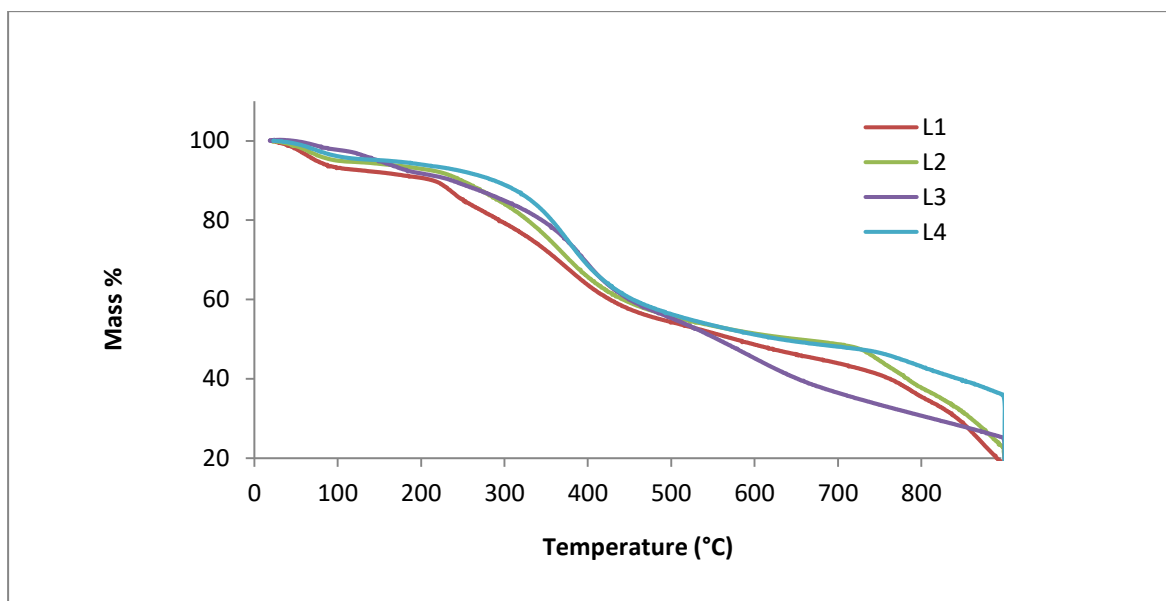


Figure 3. 7. TGA analysis of KL purification fractions: L1 (red), L2 (green), L3 (purple), and L4 (blue), measured from 0 to 900 °C.

The first weight loss (up to 259 °C) is assigned to the water content in the lignin and the liberation of volatile compounds, like carbon monoxide and carbon dioxide. The formation of vinyl guaiacol, methyl, and ethyl by-products is often detected between 230 and 260 °C together with the degradation of the propanoid side chains in lignin (Basma Hansen *et al.*, 2016; Santos *et al.*, 2014). Condensation processes followed the thermal treatment of lignin resulting in the formation of unsaturated C=C bonds between 160 and 270 °C. The main decomposition of lignin structure appeared between 260 and 478 °C. At temperatures less than 310 °C, a splitting of aryl ether links occurred because of its low thermal stability. Ultimately, the final step occurred above 478 °C, which included the formation of char residues (Ramezani and Sain, 2018).

3.1.8. Differential Scanning Calorimetry

Besides TGA, DSC is the most widespread approach used to study thermal behavior and Tg of polymers including lignin (Lupoi *et al.*, 2015; Constant *et al.*, 2016). Glass transition (a reversible phenomenon) is associated with the viscous behavior of the amorphous moieties.

The viscoelastic materials are stiff and glassy below T_g . This stiffness drops in the transition region; the material exhibits a rubber-like elasticity as a result of various parameters, such as chain tangles. Glass transitions are linked with local rotation or translational flow of molecular fragments at high temperatures. Thermal expansion enlarges the free volume of lignin. Particularly, softwood KLs exhibit higher T_g values than Organosolv Lignins obtained from hardwoods (Basma Hansen *et al.*, 2016). The T_g of lignin can be affected by the presence of low molecular weight contaminants among other factors: Mw, crosslinking, and thermal history. DSC measurements, as shown in Figure 3.8, illustrate that the T_g of L4 has the highest value of 185 °C, L2 comes second at 140 °C, 125 °C for L3, and L1 comes finally at 123 °C. According to literature reports, these T_g values are accompanied with hydrogen bonding between hydroxyl groups and to the aromatic nature of lignin (García.*et al.*, 2009)

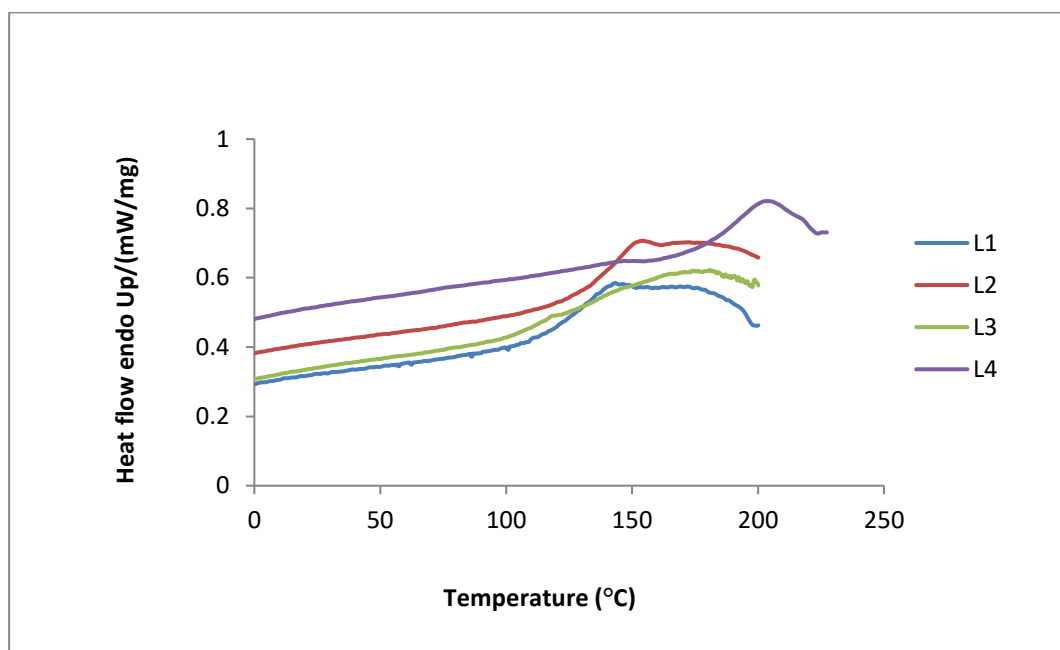


Figure 3. 8. DSC analysis of the purification fractions L1 in blue, L2 in red, L3 in green, and L4 in purple.

Thermal analysis displays that lignin fractions start to decompose at around 60 °C, followed by other decomposition phases at higher temperatures. Meaning that, the structural changes in the physical state of lignin occur as a result of partial decomposition. In 2011, Reda *et al.* recorded antioxidant stability data applying thermal analysis, verifying a preventive effect of lignin in edible vegetable oils at high temperatures (Reda *et al.*, 2011). DSC and TGA were

necessary to explain the thermal stability of the lignin purification fractions (L1 to L4). The purified lignins can be exploited at temperatures below 60 °C to avoid thermal decomposition.

3.1.9. X-Ray Diffraction

XRD studies were carried out to investigate the morphology of lignin in particular. The lignin fractions analyzed displayed a broad diffraction of amorphous halo with a maximum at around $2\theta = 20^\circ$ as seen in Figure 3.9. Fractions L1, L2, and L3 show some sharp peaks specifying certain crystallinity due to the presence of impurities and/or small crystalline molecules (e.g. salts used for the Kraft process) as investigated in the SEC results. In contrast, L4 illustrated an amorphous pattern free of sharp peaks. The intensity increased with the purity, from L1 to L4. The diffraction peaks broadened with decreasing the crystallite signal size. Once the size was sufficiently diminished, the crystalline diffraction peaks widened to the degree that they incorporated into one another, forming one broad diffraction peak (the blue halo) explaining the amorphous nature of lignin and demonstrating the effect of the purification process.

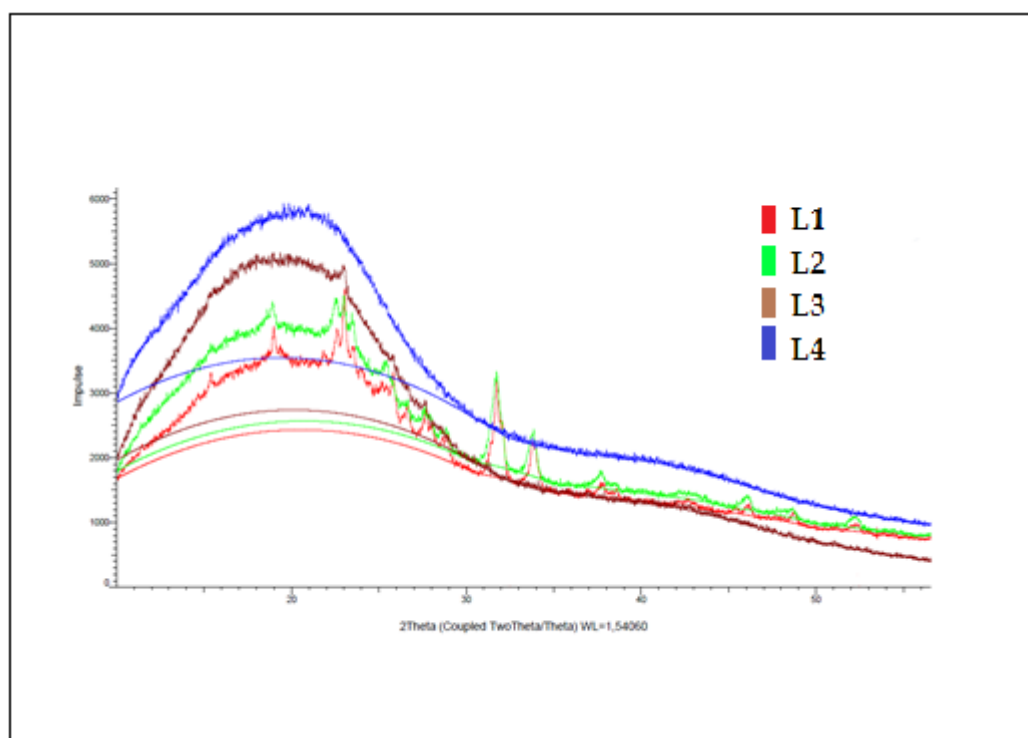


Figure 3. 9. XRD diffractogram of the purification fractions L1 in red, L2 in green, L3 in brown, and L4 in blue.

Similarly, Santos *et al.* precipitated lignins at varied pH conditions with the use of HCl and H₂SO₄, respectively. They used XRD to investigate the purity of lignin samples. The structure of lignin samples analyzed showed a variable composition in accordance with the acid and the pH value. In particular, a crystalline phase was noted in precipitated KLs appointed to sodium sulfate and sodium chloride salts genesis (Santos *et al.*, 2014).

3.2. Antioxidant Activity, Total Phenol Content and Antimicrobial Activity

3.2.1. Antioxidant Activity and Total Phenol Content

AA of the lignins was determined with the use of DPPH assay in accordance to studies described by Santos *et al.* (Santos *et al.*, 2014). Oxygen-containing free radicals (OH, RO, ROO, and O₂) have far higher reactivity than DPPH. The interaction rate of DPPH is not diffusion-controlled though. Rinaldi *et al.* reported good conformity of the values obtained by DPPH and ABTS assays (Rinaldi *et al.*, 2016). Dizhbite and others found that the free radical scavenger activity of lignin obtained from wood was higher when lignin consisted of non-etherified OH, phenolic groups, the double bond between the outermost carbon atoms on the side chain, hydroxyl groups, and *ortho*-methoxy groups (Dizhbite *et al.*, 2004). The TPC is used to screen the AA rapidly as the antioxidant efficiency of lignins is promoted by their hydroxyl groups. TPC is measured as chemical reducing capacity in respect to gallic acid. The TPC here was measured using the FC reagent. The DPPH inhibitions and TPCs of KLs are shown in Table 3.5 compared to Organosolv Lignins isolated from spruce and pine (L5), beech (L6) and *Miscanthus x giganteus* (L7).

Table 3. 5. DPPH inhibition and TPC values* of KL purification fractions (L1-L4) and Organosolv lignins: spruce/pine (L5), beech (L6) and *Miscanthus x giganteus* (L7).

	L1	L2	L3	L4	L5	L6	L7	Lit. (Santos <i>et al.</i> , 2014)
DPPH inhibition (%)	65.1 ± 3.7	66.8 ± 6.6	62.2 ± 9.5	68.2 ± 3.6	42.0 ± 1.9	64.0 ± 2.6	31.0 ± 1.0	54.76 ± 5.32

TPC (%)	30.0 ± 1.2	26.8 ± 0.5	33.5 ± 0.9	35.0 ± 1.0	34.1 ± 1.0	33.3 ± 1.6	34.2 ± 1.3	29.61 ± 1.61
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*Values are the mean ± SD of quartet experiments. Trolox DPPH inhibition = 98%.

KLs showed higher DPPH inhibition when compared to literature values of KL extracted under the same conditions. The ethanol extract (L4) has the highest value, while the acetone extract (L3) has the lowest. Organosolv Lignins vary in their activity by the biomass: beech > spruce/pine > *Miscanthus x giganteus* (softwood > grass). Differences in the antioxidant capacity of varied fractions were mostly ascribed to variations in their phenolic content and phenolic type that in turn relies upon the solvent used for the extraction (Singh *et al.*, 2014).

For the studied lignins, the DPPH inhibition was affected by the polarity of the extraction solvent. The DPPH inhibition of lignin fractions follows the trend: ethanol > diethyl ether > acetone. Here, the activity was influenced by two factors: the solvent polarity and the presence of fragments. The number of impurities in the first two fractions (L1 and L2) was higher than that in L3 and L4. Those impurities could consist of small molecular weight phenols (precursors) which show a certain AA. The second factor is the solvent used in the extraction, where the polarity of the solvent plays a role in obtaining more phenolics responsible for the AA.

Ponomarenko and colleagues investigated 50 different technical lignins of various botanical origins (e.g. annual plants, coniferous trees, and others) for their AA and spectroscopic analysis (i.e. UV-Vis). The studied lignins were fractionated by different techniques: Kraft process, hydrolysis, and fast pyrolysis. The AA was studied using DPPH and ABTS assays (Ponomarenko *et al.*, 2015). The authors used chemometric methods, such as multivariate regression analyses, to explain structure-property-relationships. As a result, they quantified the structure-related differences in the AA of lignins for the first time. The positive influence of conjugated structural fragments within the lignin was confirmed. This is also confirming the findings that show four distinct absorption bands (contrary to weak shoulders) for L4 (the purest fraction), possessing the strongest AA.

Aminzadeh *et al.* studied ^{31}P -NMR for lignin fractions isolated from membrane filtration, and showed that the low Mw samples had a higher percentage of non-condensed phenolic OH groups in spite of the lower total content of phenolic OH groups. The low Mw lignin fraction exhibited better AA than the non-fractionated LignoboostTM lignin in the kinetic ORAC assay and established three-fold stronger inhibition fluorescein than Trolox (Aminzadeh *et al.*, 2018).

L4, having the lowest molecular weight among the KL fractions confirm the findings of Aminzadeh *et al.* of being the most antioxidant of the purification fractions.

In the SEC analysis, the PD steadily decreases down to 1.6 for L4 as shown in Figure 3.5 and Table 3.4. Espinoza-Acosta *et al.* discussed, in their review article, fraction procedures (organic solvent fractionation, ultrafiltration, and differential precipitation) and their effect on the AA, underlining the positive influence of reduced PD (Espinoza-Acosta *et al.*, 2016). Also, Sadeghifar and Argyropoulos studied the relevance of the molecular weight and the AA for different lignin fractions. They confirmed that lignin fractions of lower Mw exhibited higher AA. Fractions of lowest PD (3.7 compared to 5.7 and 7.0) showed the highest activity (Sadeghifar and Argyropoulos, 2015). Those literature studies are in agreement with the SEC/AA findings in this work.

Cesari and others carried out five different antioxidant assays on eight phenolic surrogates of lignin depolymerization mainly present in lignin bio-oils: phenol, syringol, guaiacol, *o*-, *m*-, *p*-cresol, pyrocatechol, and vanillin. The resulted values were compared to reference molecules in the aim of classifying the phenolic compounds toward each antioxidant test (i.e. copper or iron chelating capacity, reducing power and response to the FC's reagent, and radical scavenging capacity). Comparison with reference particles allows the identification of four indicators to classify the compounds response to the reference for each studied test. All phenolic compounds studied exhibited an AA. Guaiacol, *o*-cresol, and *p*-cresol showed the best values of Trolox equivalent antioxidant capacity (TEAC). Additionally, these compounds show high reducing capacities, especially pyrocatechol, guaiacol, and syringol. Guaiacol was also found to be the most reactive molecule toward the FC's reagent (Cesari *et al.*, 2019). In light of those findings, and comparing the functionalities of the KL fractions (L1-L4), the HSQC analysis of all of them (Figure 3.3) shows the domination of the G-units in their structure at 110-120 ppm. G-units exhibit the same basicity of syringal groups, but the steric hindrance of the additional methoxy group in syringol inhibits the oxidation reaction and therefore, the AA of the lignins (L1-L4) was relatively high. L7 obtained from *Miscanthus x giganteus* is a stem/leaf mixture and has been studied thoroughly by Bergs and others. Based on the HSQC analysis, they concluded that nearly two-thirds of the G-units was found in leaves, while stem and mixture samples contain quite low G content. Instead, mixtures contain higher ratios of H and S units (Bergs *et al.*, 2019). Considering this with the DPPH inhibition in mind (Table 3.5) emphasizes the effect of the G content on the AA, where L7 shows the least DPPH inhibition and has lower G-units than H and S.

García *et al.* investigated the influence of processing parameters on lignin's radical scavenging activity using the ABTS assay. It was reported that higher amounts of lignin could accomplish the same level of AA like some well-known and powerful commercial antioxidants, as Trolox (García *et al.*, 2012). Similarly, Kaur and Uppal studied the efficiency of lignin obtained from sugarcane bagasse in the scavenging of DPPH radicals. They found that lignin possesses an AA higher than the activity of 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT), but it was less than the activity of 2-*tert*-butyl-4-methoxyphenol/3-*tert*-butyl-4-methoxyphenol mixture (BHA), and concluded that lignin obtained from sugarcane bagasse is a potential antioxidant in edible oils and fats (Kaur and Uppal, 2015). Sun *et al.* suggested that the antioxidant capacity of lignin is mainly due to the presence of methoxyl groups in the *ortho* position, which stabilizes the phenoxyl radicals via resonance beside hindering the radical procreation (Sun *et al.*, 2014). Conjugated double bonds via extended delocalization provide additional stabilization, which correlates to the highest values for the L4 fraction, displaying four clearly separated absorption signals in UV-Vis spectroscopy. Gadioli *et al.* compared lignin besides BHA and BHT with industrial Irganox 1010 as the primary stabilizer in polypropylene formulations. They showed a better antioxidant efficiency of lignin caused by its crosslinking macromolecule nature (Gadioli *et al.*, 2016). Hence, in terms of applications, lignin is a promising replacement to the currently being used antioxidants in polymers, foods, and cosmetics, though further research is necessary before these can be replaced with lignin.

KLs (L1-L4) showed TPC values between 26.8 and 35.0%; in contrast, Santos and colleagues reported maxima of 29.61% (Santos *et al.*, 2014). The TPC results show that the phenolic content increased with the purification of lignin starting from L1 to L4 (excluding L2 in which diethyl ether was used to soak L1 resulting in extraction of some of the impurities). L4, the ethanol extract with the highest purity, exhibited the highest TPC value. The TPC values were necessarily influenced by the solvent type used in the purification. This was reported in studies of polyphenol isolation from various plants, confirming a directly proportional affect between solvent polarity and the TPC value (Akowuah *et al.*, 2005; Singh *et al.*, 2014). Here, the results were in agreement with those of solvent extraction using ethanol, acetone, and diethyl ether. Diethyl ether, with the least polarity, had a negative influence on the TPC of the isolated lignin, whereas ethanol, having the highest polarity, had a positive influence. Organosolv Lignins (L5, L6, and L7) have, roughly, a higher TPC in comparison with KLs, indicating that the Organosolv process preserves a sensible amount of phenolics in the lignin structure. There is

no evident correlation between DPPH inhibition and TPC values, as reported in different studies, most likely because of the type of phenolics causing the AA (Felício *et al.*, 2003).

3.2.2. Antimicrobial Activity

The antibacterial activity of lignin purification fractions (L1-L4) was performed against both gram-positive (*S. aureus* and *L. monocytogenes*) and gram-negative bacteria (*E. coli*) to study the influence of the purification process on the antimicrobial efficiency. Unmodified lignins were found to be more effective against *Bacillus* species (gram-positive bacteria) than against *Klebsiella* species (gram-negative bacteria) (Kaur *et al.*, 2017). Rocca *et al.* studied the antibacterial activity of various lignins and nanocomposites that contain lignin with a high sugar content against *S. aureus* which, as a gram-positive species, has a thick peptidoglycan layer surrounding the cellular membrane of each cell and *E. coli* which as a gram-negative species has a thin peptidoglycan layer surrounding the cellular membrane of each cell. They found that the interaction between the nanocomposites and the bacterial cell wall can be controlled by the structure of lignin, which helps not only in controlling the stability of the particles but also in manipulating their selectivity toward various types of bacteria (Rocca *et al.*, 2018). The disk diffusion method was performed on the KL purification fractions (L1-L4). The fractions were dissolved, initially, in ethanol, acetone, and DMSO separately and then tested. The results show that the inhibition zone of L1 increased from ethanol to acetone to DMSO. L2, L3, and L4 show no activity at all using ethanol and acetone while L2 shows slight activity against *L. monocytogenes* when dissolved in DMSO. This is because of the solubility of lignin in each solvent (100% for DMSO, 60% for acetone, and 40% for ethanol) allowing for a smaller fraction to dissolve and enhancing the antibacterial effect.

Further investigations on lignin samples were performed using DMSO based on the influence of the solvent on the antibacterial activity. This is to guarantee that all active particles are dissolved and imported to the tested platelets. The results of the zone of inhibition method of the studied fractions are shown in Table 3.6 in comparison with two softwood-based Organosolv Lignins: obtained from spruce/pine (L5) and from beech (L6) in addition to a grass-derived Organosolv lignin: from *Miscanthus x giganteus* (L7).

The reference (DMSO platelet without lignin) does not have an inhibiting effect on the bacterial growth for each species tested. Instead, it is overgrown with colonies. *S. aureus* has a lower

antimicrobial capacity than *L. monocytogenes*. This is distinct in the L1, L2, L5, and L6 results, where the zones of inhibition around the platelets have appeared on the *L. monocytogenes* plates. The tests show, as well, that the first fraction of the purification (L1 to L4) has the highest activity. Organosolv Lignins isolated from softwood (L5, L6) have specific antibacterial activities whereas the lignin isolated from grass L7 has none. L6 (the beech-based) particularly has higher activity than that isolated from spruce/pine (L5). It actually has the highest antibacterial activity among all the studied lignins. L1 (KL), in contrast, has higher activity than L5. Organosolv Lignins, similar to KLS, have higher antimicrobial activities against gram-positive bacteria than against gram-negative. In regard to *E. coli*, all studied lignins show no activity at all, where lignin didn't inhibit bacterial growth.

Table 3. 6. Zones of inhibition of lignins against *S. aureus* and *L. monocytogenes*, acquired by disk diffusion method.

Lignin platelets	Bacteria	
	<i>S. aureus</i>	<i>L. monocytogenes</i>
Reference (DMSO)	— (1x contaminated with yeast)	—
L1	+ (max 1-2 mm)	++ (max 5 mm)
L2	—	+ (max 1 mm)
L3	—	+ (max 1 mm), slight growth in inhibition zone
L4	—	—
L5	+ (max 1 mm)	+ (max 1-2 mm)
L6	++ (max 2-3 mm)	++ (max 7 mm)
L7	—	—

*— = no inhibition, overgrowth of platelets; - = no inhibition; + = inhibition; ++ = strong inhibition; max = maximum: measured at the greatest distance from the platelet.

*All lignins showed no activity against *E. coli*.

The antimicrobial mechanism by which lignin inhibits bacterial growth may include the production of localized heat and different reactive oxygen species (ROS) after light irradiation. The adhesion of lignin to the bacterial membrane could involve the sugar content of the lignin (i.e. sugars can be attracted to the peptidoglycan layer present in the bacterial cell wall). Besides, the nanocomposites attached appear to increase the activity against *S. aureus* while it remains harmless to *E. coli*. This shows good selectivity towards *S. aureus* (Rocca *et al.*, 2018).

Black liquor is produced during Kraft pulping. It contains soluble KL that is extracted from the wood tissue besides degraded products from galactoglucomannans, polymeric and residual forms of xylan, and inorganic cooking chemicals (Sewring *et al.*, 2019). NREL results of the lignins (L1 to L4) are shown in Table 3.7 and indicate that L1 has the highest acid-soluble lignin content and the lowest acid-insoluble lignin content. Glucan content (polysaccharide) is the highest for L1. Also, only L1 and L2 have arabinan content. Xylan content for both L1 and L2 fractions also is far higher than that for L3 and L4. Here, the acid-soluble lignin content seems to have the main role in the antibacterial activity where L1 is more active than L2 and thus seems to relate to the antibacterial activity results. This agrees with the finding of Rocca *et al.* (Rocca *et al.*, 2018). KL isolated using acidic precipitation at pH=2 (PH2SK) by Klein *et al.* has almost the same carbohydrates values of L2 (Klein *et al.*, 2018).

Table 3. 7. Compositional analysis according to NREL procedure of lignin fractions L1 to L4.

Fraction	AIL [%]	ASL [%]	Total Lignin [%]	Ash [%]	Glucan [%]	Xylan [%]	Galactan [%]	Arabinan [%]	Rhamnan [%]	Mannan [%]	Sum [%]
L1	86.86	15.14	102.00	1.01	0.45	0.59	0.00	0.04	0.00	0.00	104.09
L2	93.37	9.76	103.13	0.88	0.31	0.76	0.00	0.06	0.00	0.00	105.15
L3	91.43	9.46	100.89	0.24	0.26	0.04	0.00	0.00	0.00	0.00	101.42
L4	95.17	2.42	97.59	0.40	0.36	0.07	0.00	0.00	0.00	0.00	98.42
PH2SK*	86.24	13.28	99.52	0.85	0.26	0.85	0.00	0.07	0.00	0.00	101.55

AIL = acid insoluble lignin; ASL = acid soluble lignin.

* PH2SK was extracted by Klein *et al.* (Klein *et al.*, 2018).

Low molecular weight species are found at chemical shift $\delta C/\delta H$ 10.0–37.0/2.5–0.7 as shown in the HSQC spectrum of purification fractions (Figure 3.3). With purification, the number of those species decreased (from L1 to L4). It refers apparently to the carbohydrate content. Here the NREL results indicate the presence of certain carbohydrates in KLs, but it is not necessarily all carbohydrates present in those fractions.

Additionally, the combination of HSQC and SEC evaluations can be used to specify Mw of biopolymers with the use of multivariate data analysis (principal component analysis) to mimic Mw data (Monakhova *et al.*, 2018). The ^{31}P -NMR analysis, on the other hand, shows the presence of aliphatic-OH content in all the purification fractions (section 3.1.4) confirming data reported for lignins obtained from other sources and isolated using different methods (Gilca *et al.*, 2014; Argyropoulos, 1994; Sun *et al.*, 2014).

The number of the OH in the lignin fractions (Table 3.3) was obtained from the ^{31}P -NMR analysis showing that the aliphatic-OH number of L1 is the highest followed by L2, L3, and L4. The number of the carboxylic acids-OH in L1 is the highest and is possibly responsible for establishing the antibacterial activity. Usually, the antibacterial activity is related to the origin of the lignin. It is particularly caused by the presence of phenolic species and different oxygen-containing functional groups (methoxyl and epoxy groups) in its structure (Alzagameem *et al.*, 2019; Kaur *et al.*, 2017; Lupoi *et al.*, 2015). PH2SK was extracted by Klein *et al.* (Klein *et al.*, 2018). The number of aliphatic-OH, condensed-OH, G and dimethylated-OH, and carboxylic acids-OH in PH2SK is: 7.32, 7.11, 9.73 and 3.10, respectively. PH2SK was extracted by heating the black liquor to 50-60 °C. Phenolic carboxylic acids-containing fragments could have been produced due to the heating. This is demonstrated by the high phenolic carboxylic acids-OH content. Primary antimicrobial activity tests show PH2SK having activity against gram-positive bacteria (*S. aureus*) which is also in agreement with the NREL results.

In light of the NREL results and in agreement with Huang *et al.* who concluded that the lignin-carbohydrate complexes preparation containing higher carbohydrate character contains lower AA (Huang *et al.*, 2018), it simply confirms the higher activity of L4 over L1 and L2 (Table 3.5).

3.2.3. Source, Storage, and Temperature Effects

Different method of extraction and purification of KL from black liquor leads to differences in properties such as antioxidant and antimicrobial activity. Studied lignins were extracted from three different batches of black liquor. The lignins were compiled corresponding to the former extractions in three groups, each of which represents a single source. The analytical characterization of those three sets of lignin fractions exhibited slight variations in the structure of lignin. The performed DPPH inhibition and TPC testing of the three sets (Figure 3.10) showed deviations without distinct correlations or trends. This could be due to the differences in the age of the biomass used for the pulping, the harvesting time of the biomass, and/or the percentage of spruce to pine in the biomass pulp.

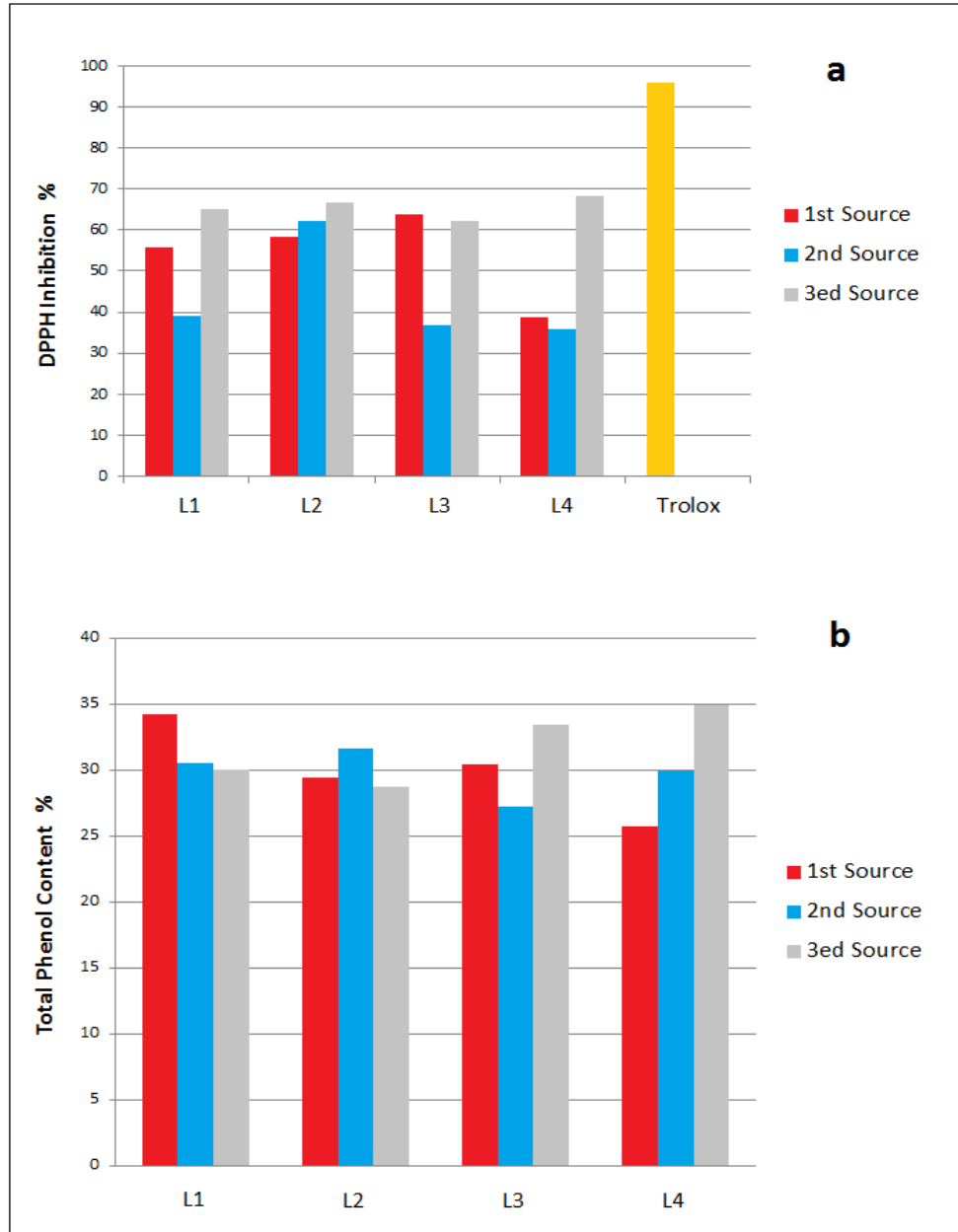


Figure 3. 10. Source influence on (a) DPPH inhibition and (b) Total Phenol Content.

The storage effect on the antioxidant efficiency and the TPC of the KL purification fractions was also studied. The fractions were tested for DPPH inhibition and TPC directly after purification, stored for six months, and DPPH inhibition and TPC were tested again. In Figure 3.11 the DPPH inhibitions of the fractions decreased for all fractions with storage. In contrast, the TPC values increased, probably due to hydroxyl formation with time. Those hydroxyls did not clearly contribute to the AA.

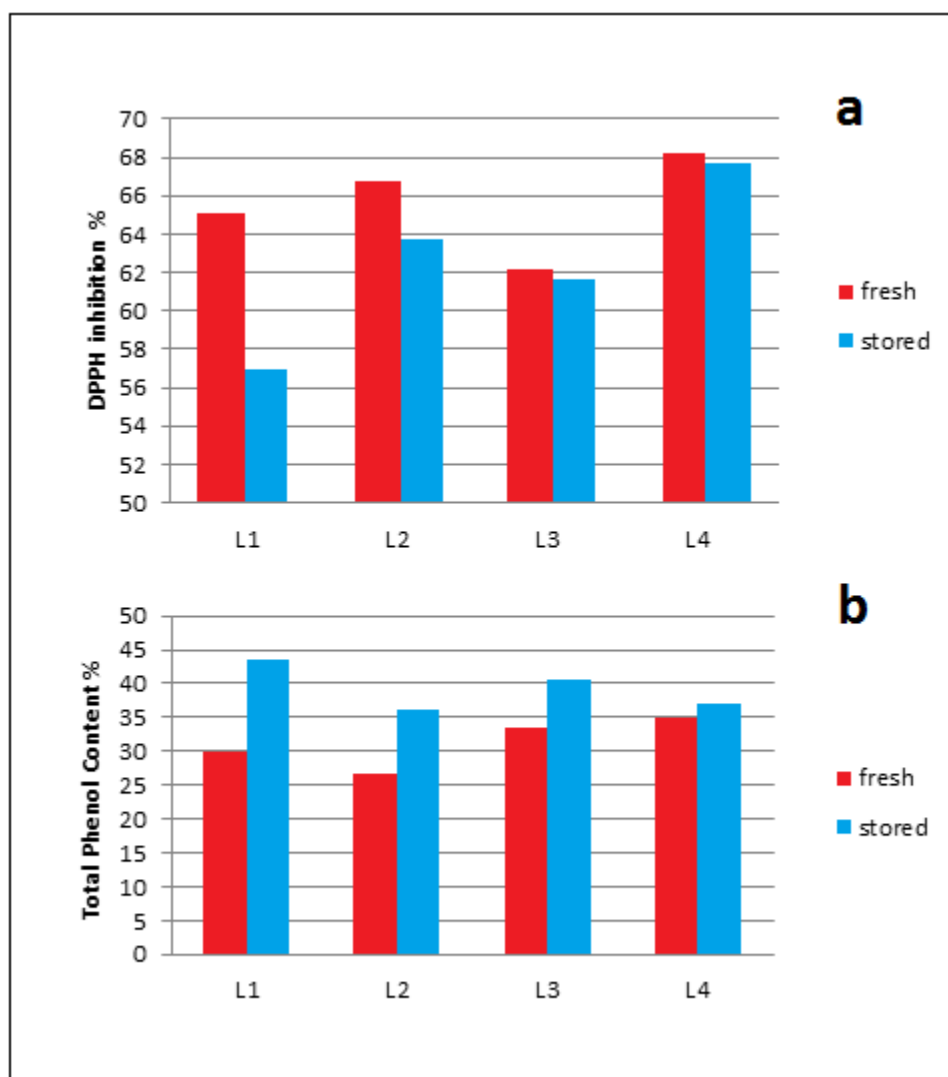


Figure 3. 11. The effect of storage of the purification fractions (fresh and stored samples) on: (a) DPPH inhibition; (b) Total Phenol Content.

Degradation processes of the macromolecular lignin structure were not only affected by storage, but also by temperature and UV-Vis irradiation. Storing lignin purification fractions for 45 days at RT caused structural changes that were monitored by TLC. The solvent system used for the study was 90% ethanol and 10% *n*-hexane. L1, for example, showed three spots: one dense spot at the baseline, which was the lignin spot; a second spot (small and light), located in the middle of the TLC sheet; and a third spot (large) traveling with the mobile phase. After 45 days of storage, TLC was repeated for the fractions. The spot in the middle for L1 looked denser and bigger than for fresh L1. This means that there were new fragment(s) in the lignin caused by degradation due to storage. SEC analysis of both stored and fresh extracts showed this fragmentation clearly (Figure 3.12a). New peaks at smaller molecular weights (45

g/mol and 73 g/mol) appeared for the stored sample. The fragmentation was neither caused by thermolysis since storage was performed at 25 °C nor by any chemical interaction. Samples were stored in aluminum foil-coated vials to prevent light from contributing to photolytic degradation, which otherwise would occur as observed and studied in detail for various lignins (Do *et al.*, 2017; Felício *et al.*, 2003).

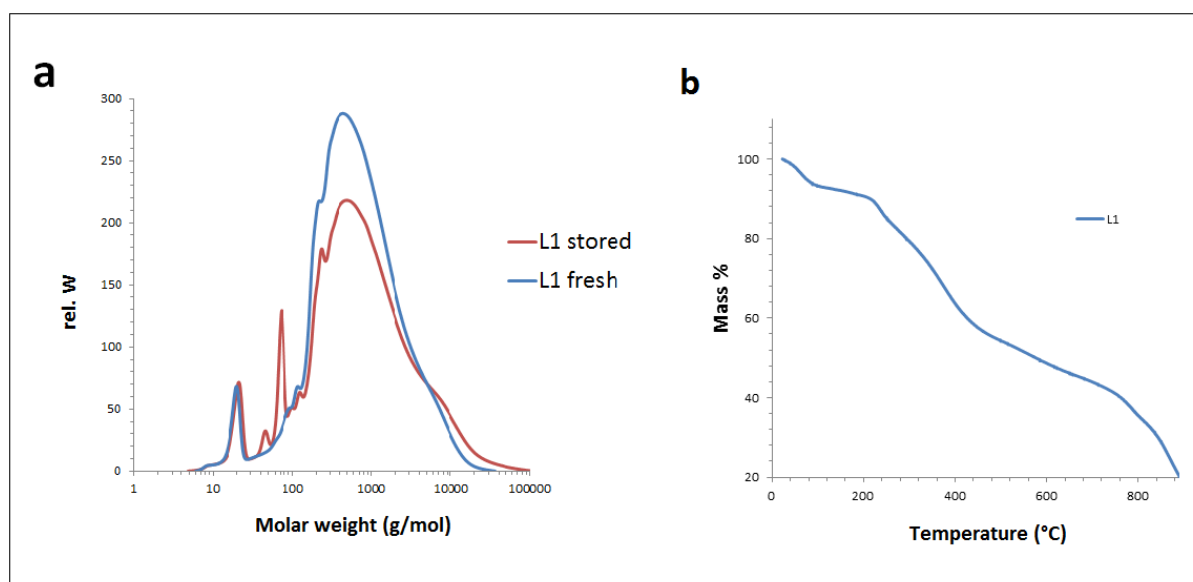


Figure 3. 12. (a) Storage effect on lignin structure via SEC analysis of freshly isolated (blue) versus stored (red) KL (sample L1). (b) Temperature effect on the structure of lignin via TGA of KL (sample L1).

The samples were dried at different temperatures: 25, 40, 60, 70, and 90 °C. One spot of L4 appeared in TLC at 25 °C and at 40 °C, while a new spot traveling with the mobile phase appeared after heating L4 to 60, 70, and 90 °C beside the lignin spot. The TLC sheet of L1 showed a denser and larger second spot in the middle of the sheet in comparison with the dried L1 at 25 and 40 °C. The TGA curve of L1, measured following Vallejos *et al.* procedure (Vallejos *et al.*, 2011), showed the temperatures for lignin decomposition were mainly 60, 380, and 880 °C. The temperatures of degradation for L4 were 60, 377, and 899 °C. Figure 3.12b shows the TGA curve of L1. Partial decomposition due to thermal impact explains the temperature effect.

Storage effect on the antibacterial activity of L1 was also studied, where a fresh sample of L1 was tested quantitatively against *S. aureus*. The fresh sample of L1 on the concentration of 0.1 g/mL gave a log₁₀ reduction of 4.4, and 2.1 at the concentration of 0.001 g/mL, after storing

L1 for six months, it showed better activity of \log_{10} reduction 4.5 at 0.1 g/mL and remained the same at 0.001 g/mL. Here the Storage effect (both positive and negative) was also verified by the analysis of both fresh and stored fractions, the DPPH inhibitions, and the TPCs of the KL purification fractions.

3.2.4. Lignin-Based Composites

3.2.4.1. Preparation of Lignin-Based Composites

Film preparation was based on the antioxidant and antimicrobial activities of the lignins. Hence, the polymerization of HPMC/lignin films was limited to the chosen bioactive lignins: L1, L5, and L6 samples to be then tested against gram-positive and gram-negative bacteria. The AA of the lignins was determined using the DPPH assay (section 3.2.1) as described by Rinaldi and others (Rinaldi *et al.*, 2016). All of the lignin samples exhibited a particular activity that ranged from high (68.2% of L4) to low (31.0% of L7) as shown in Table 3.5. Subsequently, HPMC/lignin films were prepared by adding the lignins: L1, L5, L6 and L7. The antioxidant efficiency of the prepared films was then studied.

PEG was added to the HPMC and lignin during polymerization to impact strength and improve the flexibility. It behaves as a bio-compatibilizer with the polymer. As aforementioned above, the antioxidant and the antibacterial activities of the lignins (L1, L5, L6 and L7) were the bases of the polymers preparation. The produced HPMC-film (reference) is transparent and water soluble. The addition of lignin decreased water solubility and changed the color of the films from a light honey to a denser brownish color (Figure 3.13). The brittleness appears at the addition of 25% of L1 and L7, at 20% of L5 and L6. Lignin release starts at 30% L1 and 15% L5, while L7 films didn't exhibit any release. L6 has lignin release at 20% and 25% but has none at 30%. Primary antimicrobial tests on the films against gram-positive bacteria prove the activity of the films against *S. aureus*. The films show no activity against *E. coli* (gram-negative), and therefore. 85% deacetylated chitosan (in 5% (w/w HPMC)) was included in the polymerization to produce HPMC/chitosan/lignin film solutions. Lignin was added to the HPMC and chitosan on the basis of the best, worst and intermediate superficial properties of the HPMC/Lignin combination (based on lignin release and brittleness). Lignin release in

HPMC/chitosan/lignin films started at lower percentages (at 5% in L1 films, at 5% and 10% in L5 films, and at 15% in L6 films) whereas the release of chitosan appeared at 20% and 30% for L1 and at 15% and 25% for L6, except for HPMC/chitosan/L5 films that showed no chitosan release. Lignin and/or chitosan release results from the compatibility level of the three components all together in the film moiety besides the added lignin type/origin. The mechanical properties and water solubility of the HPMC/lignin films were improved with the addition of lignin when compared to the HPMC film. Softwood-based Organosolv Lignins appeared to release at lower lignin percentages in comparison with KL. Grass-based Organosolv lignin-films showed no lignin release. The HPMC/chitosan/lignin films are more complicated because chitosan and lignin are non-thermodynamically compatible polymers, since chitosan is hydrophilic and soluble in aqueous phase, while lignin is hydrophobic and soluble in organic phase. Because of this poor compatibility, the physical incorporation of lignin into chitosan matrix leads to a heterogeneous system formation in which lignin aggregates are distributed within the polymer network (Crouvisier-Urien *et al.*, 2016); the lignin release appeared at lower lignin percentages whereas the chitosan started to release at higher lignin percentages. The released lignin/chitosan is apparently non-reacted species that failed the competition to bond in the film cluster and so left the film moiety. Chitosan “won” the competition at low lignin concentrations in L1 and L5 films and bonded successfully to the film leaving the rest of the lignin out of the film structure. At high lignin percentages, the lignin “won” the competition bonding completely with the film cluster in L1 and L6 films which resulted in leaving the rest of the chitosan unbounded to the polymer structure. This may be due to the increased number of the phenolic-OHs introduced by the lignin at higher concentrations.

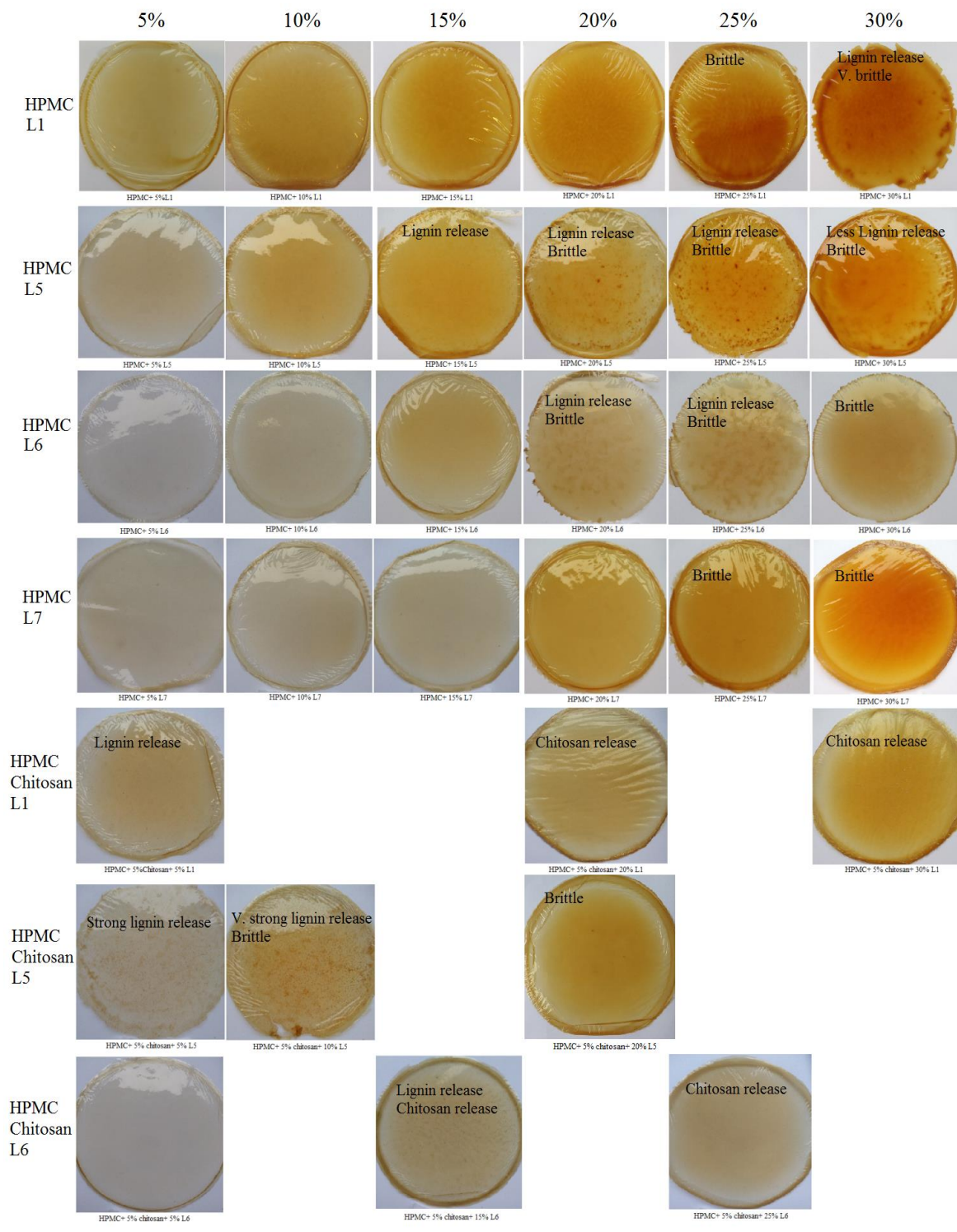


Figure 3. 13. HPMC/lignin and HPMC/chitosan/lignin composites.

3.2.4.2. Antioxidant Activity of Lignin-Based Films

Oxygen radicals exist in natural manner in the surrounding atmosphere. They can be also produced from thermal processes or by irradiation of packaging and food. Those radicals initiate the lipid chain oxidation in food. Therefore, the elimination of these radicals, from the headspace and the bulk of the food, is appealing to preserve food for longer. In polyphenols, the radical scavenging efficiency depends on the rate of hydrogen atom removal from the phenyl and also on the resulting radical's stability. Strategies employed in active packaging generally include: (1) the design of the active compound releasing systems (2) the systems of scavenging undesired compounds. The first strategy focuses on the generation of substances of low molecular weight inside the packaging material and on boosting their migration into the food products. In the second strategy, the radical scavengers are efficient upon contact where the release of active compounds is not a requirement. Yang *et al.* demonstrated this strategy by supporting packaging films with essential oils that scavenged hydroxyl radicals in the gaseous phase (Yang *et al.*, 2016a). The mobile hydrogen atom in phenols (ArO-H) plays the main role in capability of monomeric phenolic compounds to eliminate free radicals (R[•]). The process is based on the following reaction: $\text{ArO-H} + \text{R}^{\bullet} \rightarrow \text{ArO}^{\bullet} + \text{R-H}$. Lignin is usually associated with carbohydrate polymers in plant cell walls. During the isolation and purification of lignin, non-phenolic carbohydrate impurities remain strongly associated with it. Their presence can decrease the active phenolic-OH groups' concentration by increasing the O-H bond dissociation enthalpy and therefore affect their reactivity negatively. The phenoxy radicals in some types of lignin can participate in the reaction with oxidant radicals as secondary radical scavengers (Rajalakshmi A. *et al.*, 2013). Figure 3.14 shows the AA of the HPMC-based films. The films were investigated depending on the percentage of the lignin added to yield a better understanding of the influence of lignin addition on both the antioxidant and antimicrobial activities; the 5% addition (the lowest) and the lignin addition producing both the best and the worst films for each lignin sample were hence studied.

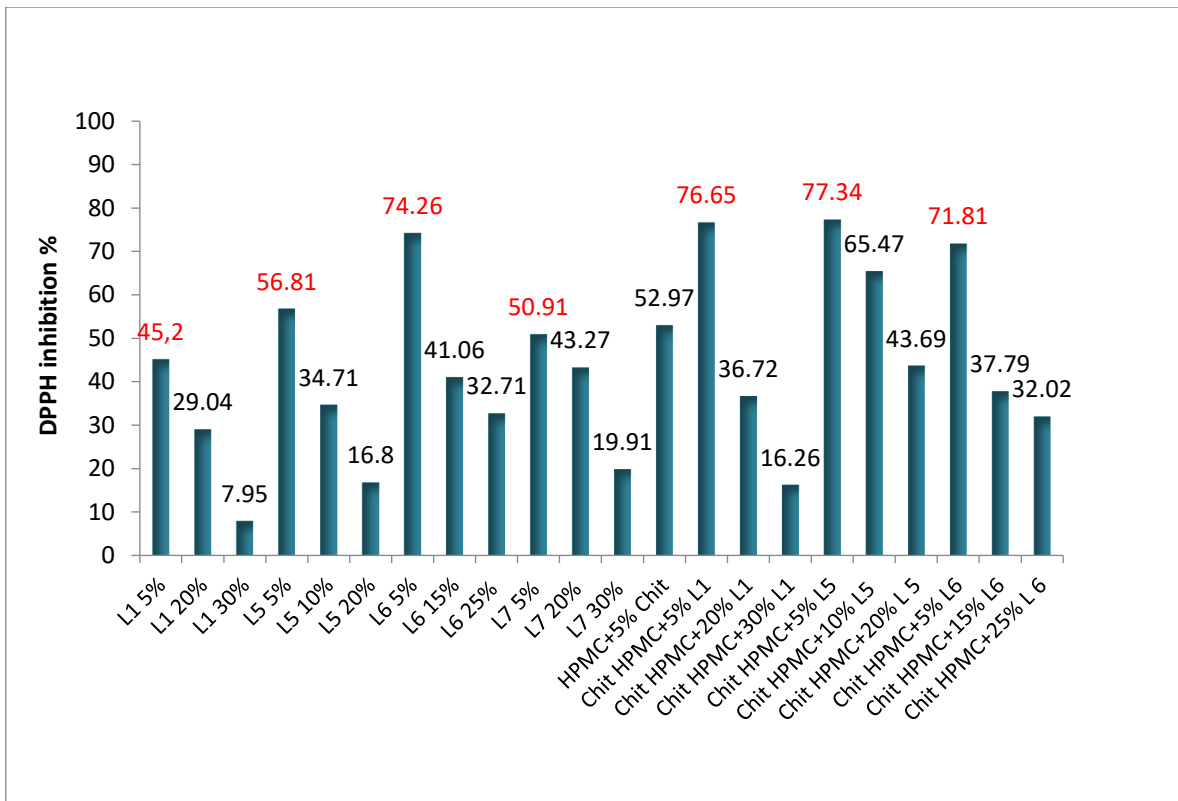


Figure 3. 14. DPPH inhibitions of HPMC/lignin and HPMC/chitosan/lignin films. The measurements were performed in triplicate. The red data labels show the highest activity for each lignin type.

The lowest addition of the lignin showed the highest AA while the highest addition of lignin showed the lowest AA. The UV-Vis absorption here increases going from the lowest to the highest additions of lignin and the AA decreased. During the polymerization, ether or ester bonds might be formed between lignin and HPMC equivalent to the covalent bonds, mainly ether bond that might present between lignin and cellulose (Jiang *et al.*, 2016; Zhang *et al.*, 2015). At the lowest addition of lignin was added, some phenolic-OHs were left free causing the AA. However, not every phenolic-OH group participates in the radical scavenging activity (Rajalakshmi A. *et al.*, 2013). At the highest addition, the lignin connectivity to HPMC increases because of the high concentration of lignin. Here, the phenolic-OHs are converted to ether or ester bonds or even self-react; this leads to the reduction of the number of active phenolic-OHs and decreases the AA eventually. AA for HPMC/chitosan/lignin/ films is higher than that for HPMC/Lignin films in general due to the activity of neat chitosan, which is basically caused by the hydroxyl and the amine groups found in its structure (Shen *et al.*, 2010), this activity in fact adds to the HPMC/Lignin activity. Also, the reaction between lignin,

HPMC, and chitosan on the α -position of the phenolic group in lignin is likable, in which an electron donating group (such as $-\text{CH}_3$ or $-\text{CH}_2-$) enhance the AA and an electron withdrawing group (like $-\text{CO}$) reduces it by increasing the polarization of the $-\text{OH}$ bond and as a result complicating its hemolytic dissociation (Dizhbite, 2004; Mansouri and Salvadó, 2006). The type of the lignin added also affects the AA value. Based on the lignin type, the AA of the films follows the following trend: Organosolv softwood-based lignin > Organosolv grass-based lignin > Kraft softwood-based lignin. The methoxyl content in Organosolv Lignins, in general, is higher than that in KLs that may behave as a secondary antioxidant (Mansouri and Salvadó, 2006). The carbohydrates content is higher in KL than Organosolv, resulting in a decreased AA value due to the mentioned negative influence of the non-phenolic carbohydrates on the number of active phenolic-OHs and hence the AA.

3.2.4.3. Antimicrobial Activity of HPMC/Lignin and HPMC/chitosan/lignin Composites

The films were investigated concerning their antimicrobial activity against *S. aureus* and *E. coli* for 24 h at 35 °C in pure culture. As seen in Dohlen *et al.*, the films reaching a \log_{10} -reduction $\geq 2 \log_{10}$ were also tested against *B. thermosphacta* (a gram-positive bacteria and one of the most abundant spoilage organisms of fresh and preserved fish, meats, and fish products, because of its resistance to low pH and high salt conditions, its capability to breed at refrigeration temperatures (4 °C), and its production of organoleptically undesired compounds (T. Stanborough *et al.*, 2017)) and *P. fluorescens* (a gram-negative bacteria that causes a particular spoilage problem in refrigerated (0-7 °C) dairy products and meat). A \log_{10} -reduction $\geq 2 \log_{10}$ allows the adaption of temperature and inoculated solution. The test was performed for 24 h at a constant temperature of 7 °C (Dohlen *et al.*, 2016). The antimicrobial activities of the investigated films against *S. aureus* and *E. coli* are shown in Figure 3.15 and against *B. thermosphacta* and *P. fluorescens* are shown in Figure 3.16.

There is a correlation between the results in Figure 3.15 and the zone of inhibition test results (Table 3.6); the activity of the lignin films against gram-positive bacteria is higher than that against gram-negative bacteria. Regarding *S. aureus*, Organosolv lignin films in general are more active than KLs films. This could be because the carbohydrates in the KL might have participated in the reaction with the HPMC entering the film structure which leaves the film

without the sugar content that could have participated in the antimicrobial activity of the neat lignin. The activity of the lignin films in general increased with increasing the lignin concentration in the film which introduces more active functional groups (aliphatic-OH, CO, Carboxylic acids) into the film structure. In case of *E. coli*, the KL films showed no activity at decreased concentrations. Only the 30% addition exhibited an activity which is explained by the introduction of higher number of active functional groups into the film structure, in addition to the potential positive influence of the lignin release. HPMC/L5 films exhibited high activity at higher percentages, this also could be enhanced by the released lignins; the 5% addition has the lowest activity due to the concentration factor. The 25% HPMC/L6 film is the only active one among HPMC/L6 films. The activity was improved with the addition of chitosan to the HPMC/lignin since chitosan is reported to possess a significant activity against both gram-positive and gram-negative bacteria. Its activity is clearly gained from the amino group and the OH functionality present in its structure (Garrido-Maestu *et al.*, 2018; Kurniasih *et al.*, 2018).

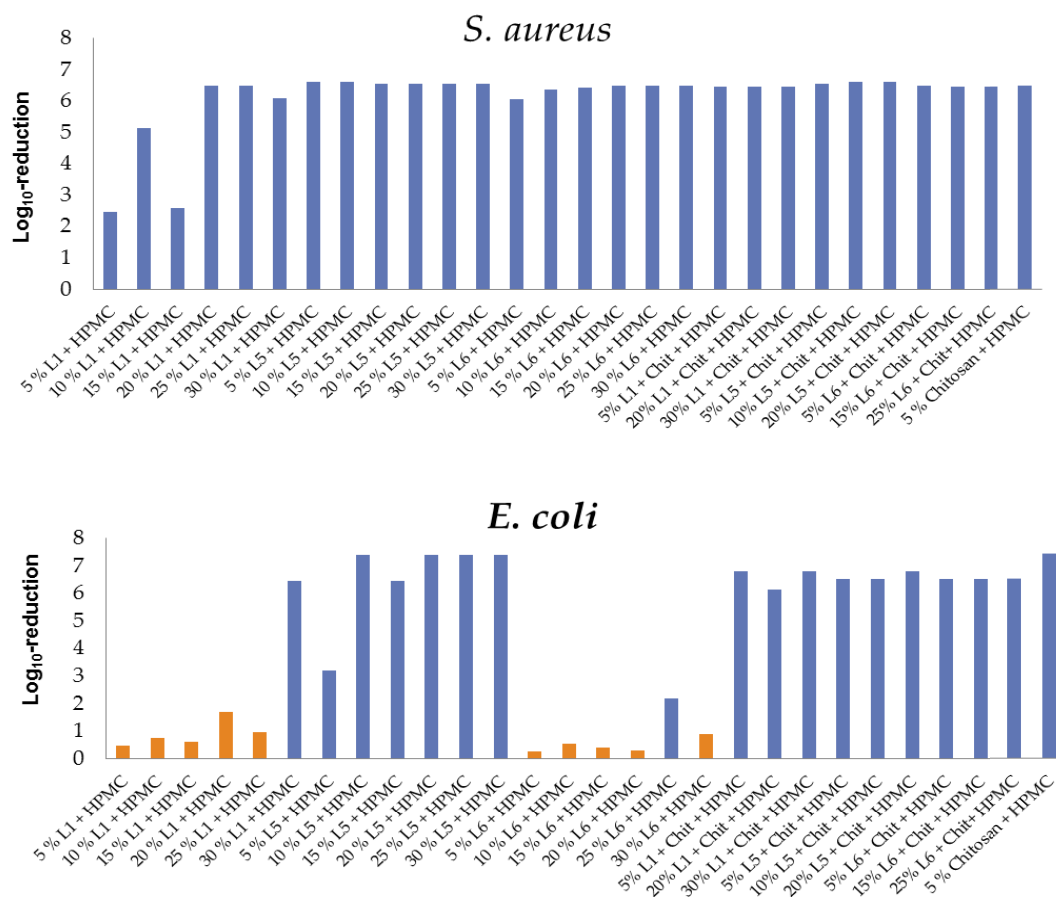


Figure 3. 15. Antibacterial activity of HPMC/lignin and HPMC/chitosan/lignin films against *S. aureus* and *E. coli*. All films are active against *S. aureus*. Orange columns present the non-active films

against *E. coli*. Chitosan incorporation increased the activity of these films. L5 films showed highest activities against both bacterial species.

Chitosan release did not have a significant influence on the antimicrobial activity. The results show similarity between the activities of all of the studied films while they vary for the HPMC/Lignin films. In other words, the chitosan influence on the activity of the corresponding films dominates over the lignin influence. This is distinct in the HPMC/chitosan activities in comparison with the HPMC/chitosan/lignin activities, excluding the minor lignin negative influence on the activity of the films where more amino or OH groups of chitosan and/or lignin are bounded to the HPMC which block the antimicrobial capability for some of those functionalities.

The films antibacterial activity against spoilage bacteria growing at low temperatures (0-7 °C): *B. thermosphacta* (gram-positive) and *P. fluorescens* (gram-negative) was also tested. Only the highest addition of all lignins in HPMC/lignin films is active against *B. thermosphacta* as shown in Figure 3.16. While only the 5% addition in the HPMC/chitosan/lignin films is active. The activities are close to each other and show no effect of the lignin type.

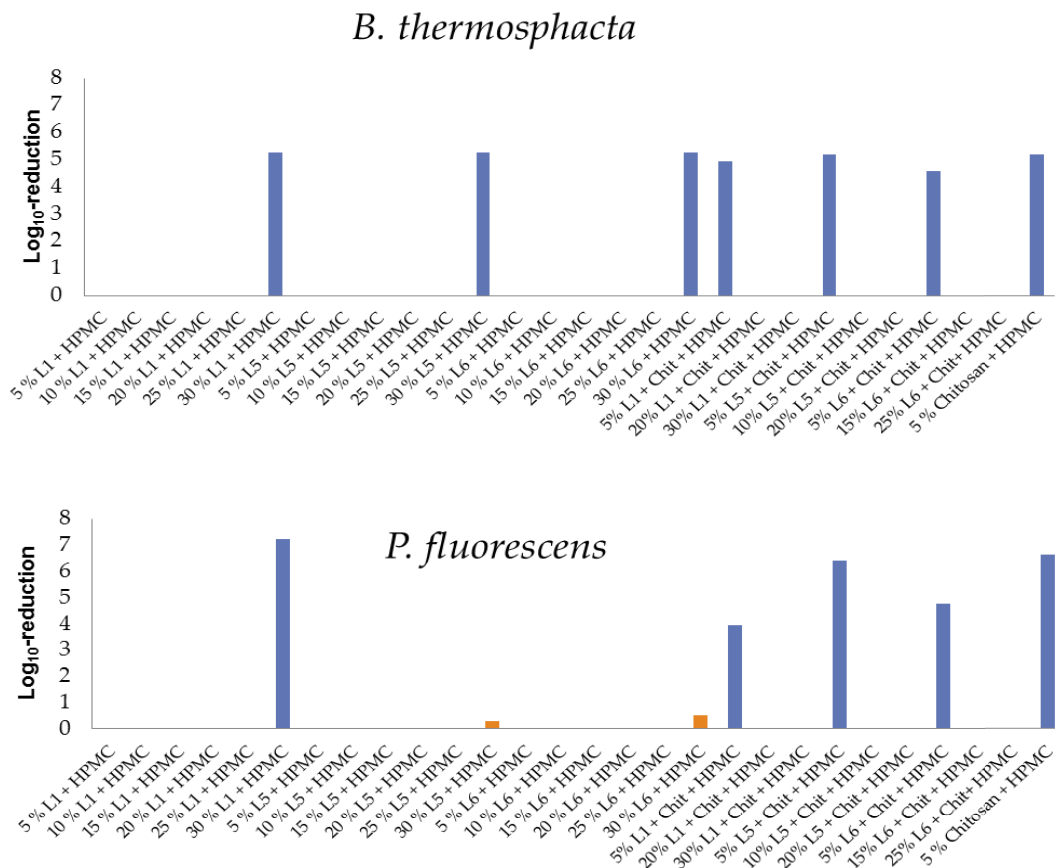


Figure 3. 16. Antibacterial activity of HPMC/lignin and HPMC/chitosan/lignin films against *B. thermosphacta* and *P. fluorescens*. Orange columns present the non-active films against *P. fluorescens*.

The addition of 30% of L1 to the HPMC/lignin films produced the only active film against *P. fluorescens*. L5 and L6 showed no activities. In HPMC/chitosan/lignin films, the 5% addition of L5 has the highest activity. Oddly, the activity of HPMC/L1 film against *B. thermosphacta* is lower than that against *P. fluorescens*; this possibly due to the lignin release.

Garrido-Maestu and others studied chitosan-derived nanoparticles in aims of enhancing the antimicrobial activity against foodborne pathogen *E. coli*. The best condition of chitosan nanoparticles generation with antimicrobial activity is the use of low molecular weight chitosan 75-85% deacetylated with sodium sulfate as a crosslinker at a final concentration of 0.4-0.6%. Sodium sulfate was simply added drop wise with constant stirring and sonication during the preparation of the chitosan nanoparticles (Garrido-Maestu *et al.*, 2018). The crystalline phase appearing in the XRD analysis most probably could be assigned to sodium sulfate and sodium chloride salts formation as reported by Santos *et al.* (Santos *et al.*, 2014). Similarly, in the XRD analysis of the KL fractions L1 to L4 (section 3.1.9); crystalline peaks appear in L1 diffractogram and with smaller intensity in L2. After searching the library of a matching XRD diffractogram, it showed that those peaks are assigned to sodium sulfate as in Figure 3.17. Since the bioactivity of lignin and lignin-containing composites is still not fully understood, a chance of crosslinking with sodium sulfate could have contributed in the collective bioactivity of the cluster.

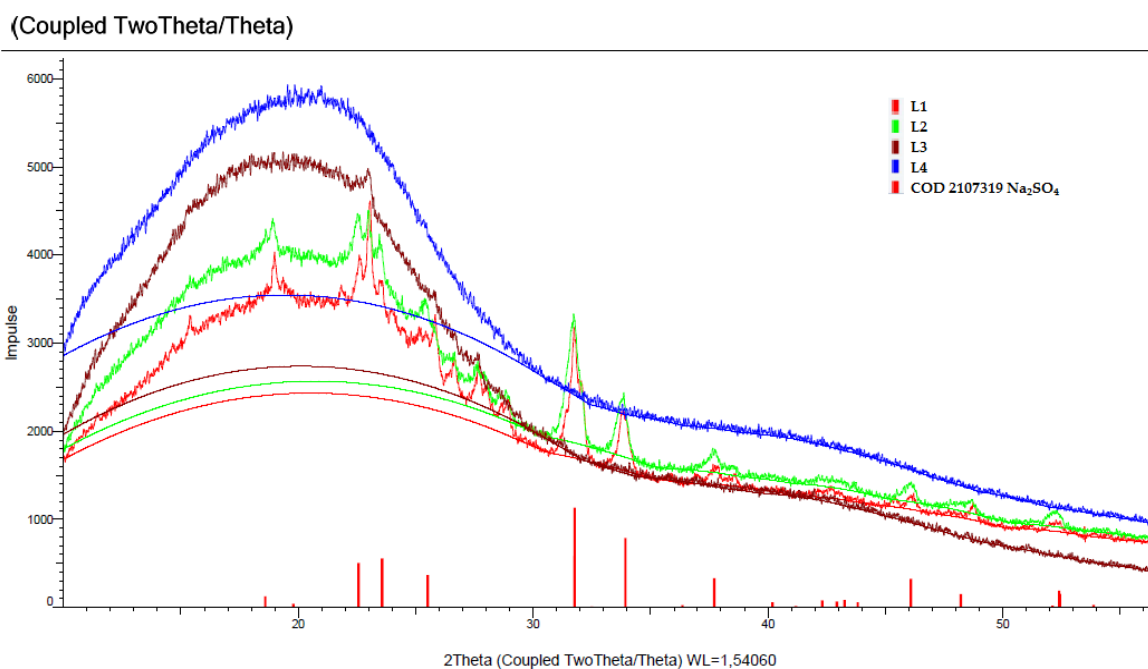


Figure 3. 17. XRD diffractogram of lignin fractions: L1-L4 and the crystalline assignment of the sodium sulfate present in L1 and L2.

4. Conclusion and Outlook

This thesis has been set out to investigate the concept of sustainable materials, specifically lignin, to develop innovative bio-based components and additives for food packaging applications. In detail, it investigated the purification of KL with simple classical organic separation methods and the effect of the purity degree on the structure, AA, TPC, and the antimicrobial capacity. The purification fractions (L1-L4) were compared with Organosolv Lignins obtained from the same biomass (L5) and from other biomasses (L6, L7) to develop a more detailed understanding of the structure-property relationships in both lignins and lignin-based polymers. The study has also sought to know whether unmodified lignin can be a good candidate for active bio-based films formation, particularly with its excess production, as a waste, in pulp and paper industries. The general theoretical literature on this subject and specifically in the context of lignin is wide-spread and bifurcated. Every minute a new scientific question arises in the field of lignin development and its incorporation in future-industrialized materials. This study sought to answer, to some extent, the following questions:

1. How much does the purity of lignin affect its structure and hence the bioactivity?
2. What are the factors affecting the lignin structure and related bioactivity?

3. For what degree does the compatibility of lignin with cellulose, chitosan, and cellulose/chitosan in the film moiety affect the bioactivity?
4. What is indeed the active part of lignin as a powder and as incorporated in the cellulose/chitosan composites?

The main empirical findings are section specific and were summarized within the respective empirical section (Lignin Extraction, Purification and Analysis). This section will synthesize the empirical findings to answer partially the first question.

1. How much does the purity of lignin affect its structure and hence the bioactivity?

The purity of a compound/material is governed by the presence of a compound/material in a whole. It is considered 100% if the whole contains only this compound/material and 0% if it does not contain any of the material. The percentages in-between necessarily mean that the entire behavior of the mixture is influenced by the presence of impurities. With lignin, it is hard to specify the structure because of its complexity. During pretreatments and extractions, lignin could either degrade or be associated with other fragments or compounds that are not necessarily derived from its structure. Those are what we refer as impurities. So far, all the literature approaches of lignin characterization methods were not 100% successful in visualizing its entire structure, simply because the structure of lignin is not fixed for all biomasses, and even for different batches of the same biomass. The analysis of an extracted lignin without any kind of purification will also involve the pretreatment's fragments and/or lignin's fragments. Hence, the proposed structure will be diluted. In this work, all the analysis methods used for lignin characterization show clearly the effect of the impurities on the proposed structure. The purification fractions were clearly distinguished through some analytical methods: UV-Vis, SEC, HSQC and XRD. The accomplished purity was not 100%, but it was the best to have using simple and available purification methods.

The bioactivity of the lignins was investigated in the respective section: Antioxidant activity, Total Phenol Content, and Antimicrobial Activity. The bioactivity concept here includes both the AA and the antimicrobial activity. As the purity of the lignins affected the structure, it affected the bioactivities. Lignin has found to be an antioxidant and antimicrobial agent due to the presence of (substituted) phenols, aliphatic hydroxyl (OH), alkoxy (RO; R being an alkyl group), and/or carboxyl (ROO; R being an alkyl group) in its structure and the effect of the carbohydrate content. Those functional groups are highly present in both, the purified lignin fractions, and the impurities structures. Logically, the purification will lead to the absence of

some of those functionalities since it excludes the impurities. This is clear in the antimicrobial activity of the purification fractions, where the antimicrobial activity decreased as one goes from the impure lignin to the purest.

For the studied lignins, there were several factors affecting the AA: the solvent used for the purification, the Mw of the lignin, the type of the residues present in its structure, and the PD. The results of the purification combined with the corresponding AA demonstrate the effect of the purification. They also have no correlation with the TPC results. Meaning, the phenolics type matters rather than its number.

2. What are the factors affecting the lignin structure and related bioactivity?

The answer of the second question lies in section 3.2 (Antioxidant activity, Total Phenol Content, and Antimicrobial Activity). All the analytical methods carried out on the studied lignins demonstrate the effect of the: (1) biomass type (the batches of the same biomass represented by harvesting time, the age of the plant and the storage time), (2) the temperature applied on the biomass and/or the extracted lignin; (3) the solvent used in: the pretreatment, the analytical and biological methods, and (4) the purity of lignin.

The structure-property-relationship has been established clearly by empirical results of the TPC, antioxidative and antimicrobial activities. Qualitatively, NMR analysis emphasizes the effect of the mentioned factors.

Subsection 3.2.4 (Lignin-Based Composites) synthesizes the empirical findings to answer the third research question:

3. For what degree does lignins compatibility with cellulose, chitosan and cellulose/chitosan in the film moiety affect the bioactivity?

The release of lignin in the studied films was found to affect both the antioxidant and antimicrobial activities. Incorporating the functional groups within a polymer moiety affects the carried property via changing the type of the functionality and/or the surrounding functionalities. As clearly known, the property of a monomer differs from the property of its corresponding polymer. Likewise, the property of a compatible composite differs from the property of incompatible one due to the effect of the non-bonded species. Here lignin and chitosan each has its proven activity. Hence, the release of any of those two components in the polymer moiety must have an effect. The release shown in subsection 3.2.4, combined with the

antimicrobial activity, is a potential interpretation in this regard. For all studied samples, the effect of the release on the antimicrobial activity was positive. Most obviously, the presence of available functionalities from the released components adds to the activity of the whole polymer resulting in higher antimicrobial activity. In contrast, the AA is not affected by the release. There was a constant trend for the AA of the films increasing from the lowest addition to the highest. It didn't change even with the release in some of the films due to the incompatibility. It is safe then to conclude that the responsible parts for both the AA and the antimicrobial activity are not certainly the same since the results show no correlation between the compatibility-activity-relationship.

As to answer the research fourth question:

4. What is indeed the active part of lignin as a powder and as incorporated in the cellulose/chitosan composites?

Results obtained in this study cannot answer this question sufficiently. More investigation must be done. It is connected directly to the same discussed concept: the structure-property relationship. So far, the detailed mechanisms regarding the bioactivity effects are not understood, in particular the observed differences between the different bacteria. The mechanism naturally is based on the structure which is not 100% clear for lignin. The solvent use, the corresponding biomass of the lignins, and the carbohydrate content all have effects on the antimicrobial activity. The same goes for when it is incorporated in the cellulose/chitosan composites. In this regard, every possible analysis for lignin must be done to fill the gaps in its structure puzzle. Accordingly, the scale of this debate must be extended to explore the complete lignin structure. Deeper investigations using ATR FT-IR and Raman spectroscopy and py-GC/MS in addition to thorough elemental analysis are required to visualize the films' functional groups composition in order to understand the mechanism of antimicrobial activities of lignin-based films. Future research must include the effect of sulfur on the antimicrobial activity since it has been emphasized that sodium sulfate present in the first purification fraction. The fact that sulfur is essential in the Kraft pulping process requires more attention in this regard.

Also, chemometrics for modeling SEC, IR, and Raman data will be applied on the purification fractions and lignin-based films to provide additional insights in the structure of lignin building blocks and the effect of the purification on the structure and the resulting properties in addition to the active functionalities in the lignin-based polymers.

The complexity of the structure of lignin is considered the main limitation to the understanding of its benefits when it comes to biorefineries. In extension, the use of HPMC in this investigation has its drawbacks. The solubility of HPMC in water is the main issue when it comes to food packaging. Therefore, other cellulose derivatives and/or biopolymers should be studied using the same approach to expand the research in this field and to avoid as much scientific and practical disadvantages.

In spite of what has been reported regarding the obstacles of exploiting lignin in LCF biorefinery concepts, it has been validated due to its sustainability, availability, and promising properties that could qualitatively exceed the fossil resources.

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List of Publications

Original paper (*peer-reviewed*)

1. **Alzameem, A.**, Khaldi-Hansen, B., Büchner, D., Larkins, M., Kamm, B., Witzleben, S., Schulze, M. (2018) Lignocellulosic Biomass as Source for Lignin-Based Environmentally Benign Antioxidants. *Molecules* 23, 2664. <https://doi.org/10.3390/molecules23102664>
2. **Alzameem, A.**, Klein, SE., Bergs, M., Do, XT., Korte I., Dohlen, S., Hüwe, C, Kreyenschmidt, J., Kamm, B., Larkins, M., Schulze, M. (2019) Antimicrobial Activity of Lignin and Lignin-Derived Cellulose and Chitosan Composites Against Selected Pathogenic and Spoilage Microorganisms. *Polymers* 11, 670. <https://doi.org/10.3390/polym11040670>
3. Klein, SE., **Alzameem, A.**, Korte, I., Kreyenschmidt, J., Schulze, M. (2019) Antimicrobial Activity of Lignin-derived Polyurethane. *Coatings* 9(8), 494. doi: 10.3390/coatings9080494 .

Review articles (*peer-reviewed*)

4. Witzler, M., **Alzameem, A.**, Bergs, M., El Khaldi-Hansen, B., Klein, SK., Hielscher, D., Kamm, B., Kreyenschmidt, J., Tobiasch, E., Schulze, M. (2018) Lignin-based Biomaterials for Tissue Engineering and Drug Release. *Molecules* 23, 1885. doi:10.3390/molecules23081885
5. **Alzameem, A.**, Bergs, M., Do, XT, Klein, SE., Rumpf, J., Larkins, M., Monakhova, Y., Pude, R., Schulze, M. (2019) Low-Input Crops as Lignocellulosic Feedstock for Second Generation Biorefineries and the Potential of Chemometrics in Biomass Quality Control. *Appl. Sci.* 9(11), 2252. doi:10.3390/app9112252

Book chapter

6. **Alzameem A**, El Khaldi-Hansen B, Kamm B, Schulze M. (2018) Lignocellulosic biomass for energy, biofuels, biomaterials, and chemicals, In: *Biomass and Green Chemistry*, pp. 95-133. Springer. doi/10.1007/978-3-319-66736-2

Conference contributions

1. Rumpf J, Do XT, Burger R, **Alzagameem A**, Bergs M, Monakhova Y, Pude R, Rehahn M, Schulze M. Composition of Lignins Isolated from Low-Input Crops using Chemometric Data Analysis: A Comparative Study. ACS Spring Meeting, March 2020, Philadelphia, USA (invited lecture MS).
2. **Alzagameem A**, Kamm B, Kreyenschmidt J, Schulze M. Antimicrobial Activity of Lignin and Lignin-Derived Cellulose and Chitosan Composites Against Selected Pathogenic and Spoilage Microorganisms. GDCh-Wissenschaftsforum wifo2019, Aachen, Germany, September 15-18, 2019 (oral presentation AA).
3. **Alzagameem A**, El Khaldi-Hansen B, Dreier T, Braun C, Dohlen S, Kreyenschmidt J, Larkins M, Kamm B, Schulze M. Antioxidant Capacity of Kraft Lignin versus Miscanthus-Derived Organosolv Lignin. Jahrestagung der GDCh Fachgruppe „Nachhaltige Chemie“, Aachen, Germany, September 15-17, 2018 (poster).
4. **Alzagameem A**, El Khaldi-Hansen B, Dreier T, Schulze M, Braun C, Kreyenschmidt J, Kamm B. Lignocellulose-rich biomass: renewable source for sustainable lignin-based antioxidants to be used in packaging and biomedicine. European Polymer Federation Congress, Lyon, France, July 2-7th, 2017 (poster).
5. **Alzagameem A**, Kamm B, El Khaldi-Hansen B, Dreier T, Schulze M, Braun C, Kreyenschmidt J. Environmentally Benign Antioxidants: Lignin-based Materials for Food Packaging Applications, GDCh-Wissenschaftsforum, Berlin, Germany, September 10-14, 2017 (poster).
6. **Alzagameem A**, Kreyenschmidt J, Schulze M. Antimicrobial Activity of Lignin-Based Hydrogels for Drug-Release in Dental Bone Regeneration. Euro Dentistry Congress, Dublin, Ireland, September 20/21, 2017 (poster).
7. **Alzagameem A**, Kreyenschmidt J, Schulze M. Sustainable Packaging Materials Developed on Lignin-based Antioxidants, 6th International Conference Cold-Chain-Management, Bonn, Germany, June 6-7th, 2016 (poster).
8. **Alzagameem A**, Kreyenschmidt J, Schulze M. Lignin – a Natural Antioxidant Isolated via Selective Extraction from Lignocellulose Feedstocks to be Used in Packaging Applications. Industrial Bio 2016, Berlin, Germany, July 28-29th 2016 (poster).

9. **Alzageem A**, Kreyenschmidt J, Schulze M. Lignocellulose-rich biomass: renewable source for sustainable lignin-based antioxidants to be used in packaging and biomedicine. European Wood Panel Symposium, Hamburg, Germany, October 5th, 2016 (poster).
10. **Alzageem A**, Hansen B, Kamm B, Kusch P, Schulze M, Witzleben S. Structure Analysis of Beech-Wood Lignins and their Application as Polymer Components. GDCh-Wissenschaftsforum Chemie, Dresden, Germany, September 1-3, 2015 (poster).
11. **Alzageem A**, Do T, Klein SE, Schulze M, Witzleben S. Synthesis of Lignin-based Polyurethanes. European Polymer Federation Congress, Dresden, Germany, June 21-24, 2015 (oral presentation MS).