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INNOVATIVE SUSTAINABLE WOOD PRESERVATIVES FROM PULP AND PAPER
INDUSTRY BYPRODUCT

By

Raisa Carmen Andeme Ela

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In Chemical Engineering

MICHIGAN TECHNOLOGICAL UNIVERSITY

2020

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This dissertation has been approved in partial fulfillment of the requirements for the Degree of DOCTOR OF PHILOSOPHY in Chemical Engineering.

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Preface

The idea of producing a novel wood preservative from kraft lignin was formulated by my advisor Dr. Rebecca G. Ong; along whom I formulated my specific research goals and designed my experiments.

The material presented in the Abstract and in Chapters 1 and 2 has been published in a manuscript entitled *Understanding the Effects of Precipitation Process Variables on Hardwood Lignin Characteristics and Recovery from Black Liquor*, by Raisa Carmen Andeme Ela, Lauren Spahn, Niloofar SafaiRobert Charles Ferrier, Jr., and Rebecca G. Ong. This work is reproduced with permission from ACS Sustain. Chem. Eng. 2020, 8 (37), 13997-14005, DOI 10.1021/acssuschemeng.0c03692. Copyright 2020 American Chemical Society. Permission can be found in Appendix B. Lauren Spahn assisted in conducting lignin precipitation experiments, measuring the carbohydrate and ash contents. Dr. Ferrier and his student Niloofar Safaie, measured the molecular weight of lignin.

The material presented in the Abstract and in Chapters 1 and 3 has been published in a manuscript entitled *Double-Shell Lignin Nanocapsules Are a Stable Vehicle for Fungicide Encapsulation and Release*, by Raisa Carmen Andeme Ela, Momoko Tajiri, Nick K. Newberry, Patricia A. Heiden, and Rebecca G. Ong. This work is reproduced with permission from ACS Sustain. Chem. Eng. 2020, 8 (46), 17299-17306, DOI 10.1021/acssuschemeng.0c06686. Copyright 2020 American Chemical Society. Publishing agreement can be found in Appendix B. Dr. Heiden contributed in the design of experiments, Momoko Tajiri took the UV measurements, and Nick K. Newberry, the surface area and pore distribution data.

The material presented in the Abstract and in Chapters 1 and 4 has been submitted for publication in a manuscript entitled *Lignin-Propiconazole Nanocapsules Are an Effective Bio-based Wood Preservative*, by Raisa Carmen Andeme Ela, Tara L. Bal, Xinfeng Xie, and Rebecca G. Ong. This work is reproduced with permission from ACS Sustainable Chemistry and Engineering. Copyright 2020 American Chemical Society. Publishing agreement can be found in Appendix B. Dr. Bal assisted with the design and coordination of the wood degradation tests, and Dr. Xie offered scientific input in the design of the same.

This dissertation engenders a practical solution to the critical problem of sustainable preservation of wood. I hope you relish exploring through a piece of work that captivated my heart as soon as I looked closely at it.

Acknowledgements

I would like to express my sincere admiration and gratitude to my advisor Dr. G. Rebecca Ong. Thank you for granting me the opportunity to pursue a research degree in your laboratory at a time I would deem myself inexperienced and a complete amateur. Under your guidance and coordination I learnt patience, the importance of team work, and the importance of quality and reproducibility in research. I admire your drive, diligence, and how resourceful you are in various fields.

Thank you Dr. Patricia Heiden, for putting your laboratory and your resources at my disposition throughout the duration of my degree.

A special thanks to Dr. Pinaki Mukherjee for unreservedly helping capture all the transmission electron microscopy data I enthusiastically but sometimes unrealistically requested.

Thank you, Dr. Edward Laitila for your patience while assisting me with fluorescence x-ray spectroscopy and x-ray diffraction spectroscopy measurements. You went above and beyond investigating the literature to make sure I was employing the correct methodology.

A special gratitude to Dr. Erik Keranen, and Mr. Joe Eskola for the technical assistance they provided while I conducted the wood decay experiments.

Thank you Dr. Tara Bal, for your incredible patience and skill at sparking my interest and passion for a field I was indifferent to. I admire how you document the design and execution of experiments in a such an organized and clear manner.

My sincere gratitude to all my committee members for their commitment to improve the quality of my work and my performance.

I am truly grateful to my office mates, Meenaa and Sarvada, and all my lab mates, for making the experience so friendly and familial.

To the Department of Chemical Engineering, and Michigan Tech, thank you for the full financial support for the entire duration of my degree. Thank you for the resources that aim at developing our professional, emotional and social skills.

To my father, Mr. Pedro Ela Nguema Buna, thank you very much for your unwavering support in my education. To my fiancé, Mr. Allan M. Kambindama, my daughter, Ana Isabel, and the rest of my family, thank you very much for the unconditional love and support, without which I would not have accomplished this goal.

Above all, I thank GOD ALMIGHTY, the opportunity to live this dream, the landscape and the people in the upper peninsula have been nothing short of amazing.

List of Abbreviations

BL: Black liquor

BET: Brunauer, Emmett and Teller

CCA: Chromated Copper Arsenate

CTAB: Cetyl trimethyl ammonium bromide

DS: Double-shelled, hollow lignin nanocapsule

DS+PCZ: Double-shelled, hollow lignin nanocapsule encapsulating propiconazole

EtOH: Ethanol

FTIR: Fourier transform infrared spectroscopy

KL: Kraft lignin

p -: Para

³¹P-NMR: Phosphorus³¹ nuclear magnetic resonance

PCZ: Propiconazole

SDS: Sodium dodecyl sulfate

SS: Single-shelled, hollow lignin nanocapsule

SS+PCZ: Single-shelled, hollow lignin nanocapsule encapsulating propiconazole

TEM: Transmission electron microscopy

TMG: Trimethyl glycine betaine

THF: Tetrahydrofuran

UV: ultraviolet

XPS: X-ray photoelectron spectroscopy

Abstract

In the modern forest industry, the need for bio-based, renewable, and environmentally-benign wood preservatives is increasing. Preservatives are used to prevent or limit decay and there has been an increasing interest in developing wood preservatives from renewable materials. To support the need for bio-based, environmentally-friendly preservatives, this work employed kraft lignin as a raw material to produce a novel bio-based wood preservative.

For the first project, a statistical design of experiments approach in Minitab® was used to study how fractionation process variables during acid precipitation of lignin influence the lignin mass yield and characteristics. After precipitating lignin from black liquor, we found a positive correlation between temperature, mass yield, molecular weight and dark coloration. For $\text{pH} < 4$, condensation of molecular fragments and sorption onto larger chains seem to drive the precipitation process. The optimum precipitation conditions were found to be $\text{pH} 1.7$, $85\text{ }^{\circ}\text{C}$, 2.5 hours.

In the second study, the recovered kraft lignin was dissolved without further modification in tetrahydrofuran and inserted dropwise into a water/ethanol solution containing sacrificial surfactant templates, to produce innovative, monodispersed, hollow, double-shell nanocapsules via two-step self-assembly. The nanocapsules were loaded with propiconazole and preserved in water to investigate the release mechanism of the system. The nanocapsules had an average hydrodynamic diameter of $242 \pm 33\text{ nm}$, and encapsulated propiconazole at an efficiency of $56 \pm 2\%$.

In the third project, the water-dispersible, biologically-activated preservative system, which consisted of the double-shelled nanocapsule encapsulating propiconazole, was tested for its synergistic biocidal efficacy at combating *Gleophyllum trabeum* decay in southern yellow pine wood. Following three months of soil-jar incubation, only $20.1 \pm 2.1\%$ weight loss was observed from vacuum-pressure impregnation compared to $42.1 \pm 9.5\%$ from control blocks with no preservative, $43.8 \pm 7.1\%$ from blocks vacuum-pressure treated with propiconazole, and $16.4 \pm 3.8\%$ from blocks vacuum-pressure treated with a standard inorganic preservative, chromated copper arsenate (CCA).

In conclusion, the novel preservative system is a viable bio-based fungicide. The double-shell nanocapsule could replace non-biodegradable actives in preservatives employing propiconazole as a co-biocide. Furthermore, this novel synthetic methodology will support designing lignin nanoparticles for different uses.

1 Introduction

1.1 Motivation

As the rate of urbanization and overall population numbers rise, so does the need for renewable, sustainable, and environmentally benign materials [1]. Wood is a renewable material that can have a long service-life when protected adequately. Some alternatives to wood are concrete in construction, fossil fuels for energy, and steel and aluminum for utensils. However, these have greater production costs, higher negative environmental impact, and increased dependency on foreign raw material sources [2]. Environmental awareness has caused the demand for wood, and bio-based products to rise.

One of the downsides of using wood as a raw material is its susceptibility to microbial, fungal, and insect degradation, particularly when exposed to moisture. Wood decay results in loss of mechanical strength and biomass, roughening of surface texture, alterations in wood permeability, the loss of electrical resistance, insulating capacity, and color [3]. These limitations resulting from degradation comprise the use of wood in various applications. Basidiomycete fungi are the predominant wood degrading organisms and account for the most severe economic losses [4]. Brown rot fungi are responsible for the largest amount of wood degradation, predominantly in regions with moderate climate [5]. The brown rot fungi, *Gloeophyllum trabeum*, and the dry rot fungi, *Serpula lacrymans*, are the most prevalent fungal species degrading wood in residential areas [4]. This could be due to the significant use of softwood frames in housing.

Wood preservatives are employed to extend the service life of wood, and bolster its resistance against degradation. The majority of traditional wood preservatives are made of heavy and non-biodegradable metals such as copper, and toxic and poorly biodegradable chemicals, such as hydrocarbon derived creosote [6]. Some of these conventional preservatives have been banned for use in residential areas, such as, chromated copper arsenate (CCA) in Denmark and Indonesia, as it poses a significant health hazard [7]. Natural and bio-degradable preservatives, such as those derived from plant-seed oil and waxes, can serve as an effective preservative on the surface of the wood [8]. There is an ample variety of bio-based preservatives, including essential oils, resins from bark, and biopolymers [8]. However, their main disadvantage has been regarded as their mild susceptibility to leaching [8]. A few ways researchers have been able to resolve the issue of leaching is by enzymatic polymerization of wood extractives with the lignin contained in the wood [9] and, by employing nanoparticles [10]. However, there is still a need for low-cost, effective, non-hazardous, biologically and environmentally friendly preservatives that easily penetrate the wood [11].

In this work, kraft lignin was precipitated from hardwood black liquor by altering various process variables; temperature, pH and residence time, with the objective of understanding the effect of process variables on the properties and characteristics of the recovered lignin, as this is of academic and industrial relevance. Afterwards, the precipitated kraft lignin was

employed to produce an inventive mesoporous, monodispersed double-shelled, hollow nanocapsule. And the common foliar fungicide, propiconazole was encapsulated into the nanocapsules. Propiconazole is a wood co-fungicide utilized in above ground applications and is typically paired with another wood preservative, such as copper azole type c (CA-C). Propiconazole has modest solubility in water which restricts its treatability concentration in water-born systems. Lastly, the efficacy of this novel, double-shelled kraft lignin-propiconazole nanocapsule against *Gloeophyllum trabeum* in southern yellow pine wood blocks was evaluated through a standard industrial testing method for a period of twelve weeks.

1.2 Wood physical and chemical structure

Wood is made up largely of the polymers cellulose (40-50 %), hemicellulose (25-40 %), lignin (20-35%), as well as extractives (~5%) [12, 13]. Cellulose is the most abundant polymer in the biosphere composed of D-glucose monomers polymerized through β -1,4-glycosidic bonds [14], and these represent the skeleton of the plant cell wall. Hemicellulose is a polysaccharide consisting of links of glucose, xylose, mannose, arabinose and galactose monomers. Hemicellulose also forms part of the cell wall matrix interwoven with cellulose, with lignin filling in the gaps between the two carbohydrate polymers [12]. The three main parts of wood are bark, sapwood, and heartwood (**Figure 1.1**). Sapwood is the living part of the wood, although it may also contain dead cells. It usually has a lighter color, and its main function is the storage of food, and the transportation of water and sap [15]. It is found surrounding the heartwood, which is the dead part of the wood. Bark is the outer covering of the tree, its inner layer consists of living cells which main role is to transport nutrients from the leaves to growing parts of the tree, while the outer layer is made up of dead and dried cells that grant protection against biological attack, and serve as a non-permeable layer that prevents water and nutrients from leaching out of the tree [15, 16]. The other parts of wood include cambium, which is a layer in the inner bark, and the pith, a core of tissue at the center of the trunk [15]. Another feature are the wood rays, which are found in some species of wood, are oriented radially, and connect layers between the pith and the bark, facilitating the transfer of nutrients [15]. For wood decay studies, sapwood is usually employed. However, sapwood has little biological decay resistance, unlike heartwood which contains fungitoxic compounds formed from sugars, starch and extractives stored in dying sapwood [17]. Heartwood is also less permeable, and its treatability and the depth of penetration of preservatives is lower [18].

Figure 1.1. Schematic of the cross section of a tree trunk, displaying the bark, sapwood, heartwood, the pith, growth rings and the wood rays. Image created in BioRender.com

The concentric bands in the wood are known as growth rings and represent seasonal growth, with lighter bands representing faster growth in spring and early summer, and darker bands representing slower growth in late summer and fall. Generally, earlywood is comprised of cells with large cavities and thin cell walls, while the cells in latewood have smaller cavities and thicker cell walls [15]. The basic wood cells are tracheids, parenchyma, vessel members and fibres [19]. Wood cells are surrounded by a cell wall that is comprised of two layers, the primary cell wall and the secondary cell wall (**Figure 1.2**). The primary layer consists primarily of cellulose, hemicellulose, and pectin [20]. The secondary cell wall is divided into three sections, S1, S2 and S3, and the composition and structure of the polymers present in these sub-layers differ [21], including a difference in the direction of the cellulose microfibrils [22]. Typically, S1 contains the highest concentration of lignin, S2 the highest amount of cellulose and hemicellulose, and S3 contains the least amount of lignin [23, 24]. The difference in lignin content makes the secondary cell wall less flexible, and less permeable to water than the primary cell wall, and this makes it more resistant to degradation [25]. The middle lamella is the layer connecting adjacent cells and contains the highest concentration of lignin [23].

Figure 1.2. Schematic of the structure of the woody plant cell wall, and the primary components in the secondary wall. The enlarged portion represents a simplified cross-section through the secondary cell wall, with lignin filling in the gaps between the sugars.
Image created with BioRender.com

1.2.1 Cellulose

Cellulose is a linear polymer comprised of 500-15,000 D-glucose units, and the compact cellulose fibrils result from hydrogen bonding between the cellulose chains [23]. The structure of cellulose can consist of well-organized fibrils, making up the crystalline regions, and not so organized networks, denominated as amorphous regions, with the crystalline regions being less susceptible to biodegradation [26]. Nonetheless, there is a lot of debate as to whether there are distinct crystalline and amorphous regions in cellulose [27]. However, there are defects that can form that are more susceptible to enzymatic attack [28].

1.2.2 Hemicellulose

In softwoods, the major hemicellulose is *O*-acetylated galactoglucomannan, and in hardwoods is xylan. Xylan is linked together by β -1,4 glycosidic bonds [29], and it links to other pentose sugars by bonds such as, α -1,2 bonds [23]. Xylan makes up 7-30 % of the dry weight of hardwoods [30]. The backbone chain of galactoglucomannan is composed of D-mannopyranosyl and D-glucopyranosyl linked by β -1,4 glycosidic bonds, and they make up 15-125% of the dry weight of softwoods [31, 32]. In the plant cell wall, hemicellulose is linked to cellulose by hydrogen bond, and to lignin by covalent bonds forming lignin-carbohydrate complexes (LCC) [33], and by ionic interactions [34].

1.2.3 Lignin

Lignin is a heterogenous, three-dimensional, amorphous polymer found in woody and vascular tissues. It accounts for 30% of the non-fossil renewable carbon in the biosphere [35]. There is no definite chemical structure for lignin, but the monolignol monomers coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol (**Figure 1.3**) in the form of phenylpropanoids guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) are cross-linked together by ethers (~two-thirds of all bonds, of which the most common is the β -O-4), and C-C bonds [36-39] (**Figure 1.4**). The functions of lignin in wood include providing rigidity and mechanical strength to the cell, and facilitating water transportation. Other properties include antibacterial and antifungal resistance, resistance against oxidative stress [40], ultraviolet rays absorption, and stiffness. Furthermore, the heterogenous and cross-linked nature of the lignin polymer further increases its natural resistance against biological degradation [41].

Figure 1.3. Phenyl-propanoid units that radically polymerize to form the lignin polymer.
Created with ChemDraw Professional.

Figure 1.4. Typical ether and carbon-carbon linkages between lignin monomers. Image created in ChemDraw Professional.

The two major classifications of wood are hardwood and softwood species. Hardwoods are usually denser, contain more hemicellulose, more natural oils, and are more expensive to process [42]. The structure of lignin also varies between these two groups, with softwood

lignin composed mainly of guaiacyl monomers which can readily undergo crosslinking [43]. This results in a higher molecular weight, highly branched polymer [42]. Hardwood consists of syringyl (predominantly) and guaiacyl monomers linked irregularly, which results in a more planar and lower molecular weight polymer due to the greater proportion of β -aryl ether bonds, for which the methoxyl groups are not able to undergo crosslinking [35, 44]. Both types of wood can be used interchangeably for the same purposes, with preference given based on the specifications for the finished product. In the pulp and paper industry, hardwood pulp gives rise to a softer paper which is more adequate for printing, while softwood pulp is more convenient when producing hard paper for packaging applications [45]. Similarly, in construction applications, softwoods can be used on the windows, and hardwoods for flooring.

1.2.4 Elemental composition of wood

The elemental composition of wood varies between species, but all are dominated with organic components, with an average composition of 50% carbon, 42% oxygen, 6% hydrogen, 1% nitrogen, and the remaining 1% composed of calcium, potassium, sodium, manganese, and magnesium, and other minor elements [46]. An energy dispersive x-ray spectroscopy (EDS) analysis on southern yellow pine (*Pinus ssp.*) sapwood revealed the significant presence of carbon, oxygen, aluminum, magnesium, silicon, phosphorus, iron, nitrogen, chlorine and sulfur (**Figure 1.5**). Hasanen and Huttunen analyzed the elemental composition in pine (*Pinus sylvestris* L.) wood rings and their average concentration in xylem cells were, 4.2 mg/kg aluminum, 65 mg/kg manganese, 8.4 mg/kg zinc, 390 mg/kg potassium, 2.2 mg/kg rubidium, 930 mg/kg calcium, 131 mg/kg magnesium, 38 mg/kg phosphorus, 56 mg/kg sulfur, 0.65 mg/kg nitrogen, 4.3 mg/kg boron and 0.03 mg/kg molybdenum [47]. Similarly, dos Santos Viana *et al.* measured the elemental composition in maritime pine (*Pinus pinaster* Aiton), and reported 46.5-48.6 % carbon, 42.1 to 46.4 % oxygen, 0.13-0.74 % nitrogen, 0.029-0.86 % sulfur, and 6.7-6.9 % hydrogen [48].

Figure 1.5. Elemental analysis of southern yellow pine sapwood sample. A high angle annular dark-field (HAADF) image was produced to map the elemental distribution of the sample in a scanning transmission electron microscope (STEM). The sample was collected by scraping the surface of a wood block. Wood block provided by Wood Protection Group.

1.3 Wood preservation

1.3.1 Wood degradation

Naturally durable wood is the primary choice in construction applications [49]. Nonetheless, natural reserves of durable hardwood are diminishing around the world, which requires the utilization of less durable wood, which are more vulnerable to biological degradation. Wood tends to age naturally, however weathering, fire, UV-radiation, and biological agents expedite the process. Fungi are the primary biological species causing wood decay. These organisms attack both dead and living wood, inflicting diseases in the latter, such as heart rot. The largest group of fungi responsible for wood degradation are the basidiomycetes, and the three major types of fungi in this category are brown rot, white rot, and soft rot [50]; with brown and white rot being the most ubiquitous. Ascomycetes can also employ a similar enzymatic behavior to that of basidiomycetes for resource allocation and lignin degradation [51, 52]. Appropriate conditions are needed for basidiomycete fungal growth and subsequent degradation of wood. These include a pH between 3 and 6, temperatures between 0-45 °C, and availability of water, oxygen, and nutrients in the wood [49]. Because of this, the fungal hyphae tend to grow in the cell lumens, where all of these conditions can be met and there is space for growth. The hyphae then migrate across the cell network through pits in the cell wall. Not all fungi cause degradation, Mildew fungi stain the wood under humid conditions without degrading it [53].

1.3.1.1 Fungal hyphae

The inception of fungal hyphae could be sexual or asexual reproduction. In the case of brown rot fungi, which reproduce by sexual haploid cells; under warm and humid environments, the cells fuse to form circular spores [54]. The spores are released from the hyphae tips (basidium), they absorb water and nutrients to eventually germinate into hypha (tubular cell) [55]. These hyphae are able to grow by expanding at their tips (apical zone) in branches. During elongation, new cells are formed. The hyphae can come into contact with other hyphae, from the parent material or developed from other spores, and fuse to form a filamentous network. The hyphae network is called mycelium [56]. The hyphae are responsible for absorbing water and nutrients from the environment, and releasing chemicals and enzymes into the environment. Hyphae have an average size of 4-6 μm [56], and the diameter has been reported to be less than 0.5 μm [57]. As such, they are able to

penetrate the meso- and macro pores in wood (80-58000 nm) [58, 59]. They may also penetrate through boreholes formed after oxidation, and extracellular enzymatic depolymerization [57].

1.3.1.2 White rot decay

White rot make up more than 90% of all known fungi species in North America [49] and have a preference for digesting hardwood [60]. They selectively degrade the lignin through oxidative cleavage of the side chains and aromatic rings, and mineralize these to carbon dioxide [61]. In the initial stages of decay, the cellulose can be found unaltered [57]. Wood decayed by white rot is characterized by a white or yellow finished color, with a moist, soft, and spongy surface. White rot fungi release peroxidases and laccases enzymes which are ligninolytic enzymes that act to break down the lignin structure. They also produce cellulase enzymes [13]. Peroxidases are oxidized by hydrogen peroxide to form two-electron deficient component that oxidizes the phenols, methoxy, and the aliphatic hydroxyl groups [62].

1.3.1.3 Brown rot decay

Brown rot fungi selectively feed on the carbohydrates of dead wood, and preferably target softwoods due to the easier deconstruction of glucomannan (predominant hemicellulose in softwoods) compared to xylan (the main hemicellulose in hardwoods) [49]. In the initial stages of degradation, they oxidize cellulose through a non-enzymatic Fenton reaction that uses iron as a catalyst, and hydrogen peroxide produced by the fungi using chemicals in the wood [63]. The dissolved sugars are absorbed by the hyphae. Possible initiators of the Fenton reaction include, cellobiose dehydrogenase [64], and iron-binding catechols [65] which reduce Fe^{3+} . **Equations 1-5** show the chemical reactions resulting from a hydroquinone driven Fenton chemistry during *Gloeophyllum trabeum* oxidation. Q represents quinone, H_2Q hydroquinone, HQ semiquinone, and OOH hydroperoxyl radical [66].

Wood degraded by brown rot has a brown color finish, a dry and brittle surface, and it is sometimes called cubic rot due to the way the wood breaks apart along planar surfaces.

Brown rot can modify lignin by enzymatic demethylation of the side chains, which involves catechol oxidation [67]. Studies by Filley *et al.* [67] demonstrated that the content of aromatic dihydroxy groups increased after spruce degradation by brown rot fungi *Postia placenta* and *Gloeophyllum trabeum*, due to the demethylation of the 3-methoxyl carbon. The hyphae of brown rot develops primarily in the cell lumen, and significantly degrade the secondary (S2) wall [13]. For this, it is believed, enzymes are not involved in the initial stage of degradation as they are too large to penetrate the pores of the S3 layer which can be as small as 2 nm [68]. Moreover, in-vitro studies have demonstrated that the enzymes produced by brown rot had little effect on cellulose [13].

1.3.1.4 Lignin degradation

As mentioned earlier, white rot fungi are the most effective at degrading lignin. They achieve this by an oxidation mechanism, employing a collection of extracellular enzymes known as the lignin modifying enzymes [69], with phenol oxidase (laccase) and heme peroxidases [69] being the most effective of these enzymes. Typical ligninolytic enzymes include lignin peroxidase and manganese peroxidase [61]. Non-phenolic lignin compounds may also be oxidized as a result of hydroxyl radical producing systems [70]. The oxidation pathway proceeds from the syringyl units, to the guaiacyl units, and lastly, to the p-hydroxyphenyl units. This results in first, breaking the C-C linkages, followed by the β -O-4 linkages, and lastly the aromatic rings [49, 71]. Brown rots can partially modify lignin by de-methylation of the side chains [72]. **Figure 1.6** below shows typical lignin biodegradation reactions. Reaction 1.6 is the propyl side chain oxidation, characteristic in white rot decay, and reactions 1.7 and 1.8 are ring hydroxylation and demethylation respectively, characteristic in brown rot decay [73].

Figure 1.6. Characteristic lignin fungal degradation reactions. Reaction 6, propyl side chain oxidation (white rot), reaction 7, ring hydroxylation (brown rot), and reaction 8, ring side chain demethylation (brown rot) [73]. R represents points of incorporation into the lignin polymer. Image created in ChemDraw Professional.

1.3.2 Contemporary wood preservatives

Chemical preservatives are employed to bolster the resistance of wood species against biological degradation. Other forms of protecting wood include treating it with biopolymers from industrial waste and byproducts, wood acetylation, and heat treatments. The three major categories of chemical wood preservatives are water-borne, oil-borne, and light organic solvent-borne [74]. Water-borne preservatives have the advantages of being odorless and the treated product can easily post-processed, such as by painting. However, they have major disadvantages. They are easily leached from the wood, they cause wood swelling, and they are highly toxic to humans and the environment [74]. Examples of these preservatives are chromated copper arsenate (CCA), which dominated the market from 1970s until 2004 [74], but it has now been banned for residential use, and copper azoles.

Oil-borne preservatives are highly effective, moreover they are water repellent, which would inhibit fungal growth. They are also long lasting and inexpensive. However, upon exposure they are toxic to the environment. They are also associated with unpleasant odors, and post-processing of the treated product is challenging. Examples of oil-borne preservatives include creosote, pentachlorophenol, and copper naphthenate [74]. Light organic solvent-borne preservatives consist of the same chemicals utilized in water and oil-borne groups but instead are dissolved in an organic solvent that evaporates upon treatment, leaving the preservative chemicals embedded in the wood. These are slightly less toxic, but they are very expensive and there are still some environmental concerns with the type of organic solvents utilized.

These major three preservative categories fall under two major categories, older wood preservatives (previously registered before November 1, 1984), and the newer preservatives. In 2008, all of the registered older preservatives were re-evaluated for health and environmental hazards by the U.S. environmental protection agency (EPA), and amongst these, only chromated arsenicals, pentachlorophenols, and creosote were re-approved [75], however, chromated arsenicals and creosote have since been discontinued for residential usage.

Other types of newer preservatives include borates (naturally occurring minerals, such as, disodium octoborate tetrahydrate, and polymeric betaine) [74], carbamates (carba3-Iodoprop-2-yn-1-yl butylcarbamate (IPBC)), trichloromethyl sulfenyl fungicides (captan), and triazoles (fluconazole and propiconazole).

In this work, we have decided to work with propiconazole, and chromated copper arsenate as a comparative standard. Propiconazole is an EPA registered triazole fungicide, approved as of 1981. It is employed as a co-biocide for surface applications of lumber and timber, however, it provides no protection against insects. Propiconazole is also used in the pressure and surface treatments of sidings, plywood, millwork, shingles and shakes [75]. It is used as a co-fungicide in the commercial preservative copper azole type c (CA-c), and permethrin. CA-c uses propiconazole at a level of about 0.01-0.06 lb/ft³. Propiconazole is also utilized as an agricultural fungicide [74]. Its mechanism of action against fungi is by binding to the 12- α demethylase enzyme, preventing it from demethylating an ergosterol precursor. Ergosterol is a vital fungal cell membrane component, without which cellular growth cannot occur.

Chromated copper arsenate is made from chromium trioxide (chromic acid, CrO₃), arsenic pentoxide (As₂O₅) and copper oxide (CuO) [76], and it is utilized in aqueous solutions at concentrations between 0.6-6.0 % (w/w) [77]. Copper provides the primary protection against fungal decay, arsenic grants primary protection against insects, and chromium binds the chemicals to the cellulose and lignin by means of chemical complexes [78].

1.4 Industrial Lignin

Figure 1.7. Schematic of the kraft pulping process, displaying the separation of the pulp, the black liquor end use, and the recovery of process chemicals. Process flow diagram created in CHEMCAD and edited with BioRender.com

The primary source of industrial lignin is the kraft (sulfate) process [79]. Other sources include, organosolv pulping, an industrial technique that utilizes an organic solvent and an acid or alkali catalyst to separate the cellulose fibers from lignin and hemicellulose [80], and the sulfite process, which employs sulfur dioxide and sulfide ions [81].

In the kraft process (**Figure 1.7**), wood chips are mixed with steam and white liquor, a mixture of sodium sulfide and sodium hydroxide at elevated temperatures and pressures in either a batch, or continuous digesting process. This mixture depolymerizes the wood, and solubilizes up to 93% of the lignin and up to 71% of the hemicellulose [82]. These constitute the black liquor byproduct, which has a pH of ~14, and of which lignin makes up approximately 45% of the dry solid content after the evaporators [38]. During the kraft process, the molecular weight of lignin decreases as a result of the degradation of the ether linkages in between the phenylpropanoid monomers [38]. Furthermore, the hydrosulfide (HS^-) and mercaptide ions (CH_3S^-) may bond to the lignin methoxyl groups to form methyl mercaptide and dimethyl sulfide [83].

More than 99% of the generated kraft lignin is incinerated in the recovery boiler, to produce steam that is used to drive the process or produce electricity, and recover the white liquor chemicals. While some combustion of lignin is necessary for the energy balance of the plant, this is a low-value application, and precludes the commercial exploitation of kraft lignin for higher value applications [84]. Studies have demonstrated that extracting ~25%

of the lignin from the black liquor, which contributes two-thirds of the total heating value of black liquor [82], is beneficial for pulping [85, 86], by reducing the heat load to the recovery boiler, preventing overheating [45]. Additionally, it was recently reported that fractioning the lignin from black liquor can open avenues for the recovery of carboxylic acids and inorganic components [82].

The earliest kraft pulping mills were reported to discard the environmentally unfriendly black liquor into water streams. This occurred until the invention of the recovery boiler in the 1930s [35]. Nowadays, kraft mills combust the majority of all the black liquor they produce in order to recover pulping chemicals and generate energy. In the U.S., the energy produced from black liquor incineration accounts for 28.5 terawatt hours of electricity per year [87]. It is known that up to 25% of the lignin can be removed to avoid boiler overheating, but recently, it was reported that 40% lignin removal is the limit for efficient recovery boiler performance [88]. Black liquor comprises the alkali soluble wood components (**Table 1.1**), with hemicellulose often attached to lignin by LCC. The black liquor also contains aliphatic carboxylic acids [82], and the spent inorganic cooking chemicals. It is important to note that the compositions listed in **Table 1.1** are not fixed, these vary depending on the wood species, delignification conditions, and pulping process [38, 82]. At elevated pH, the lignin molecules are ionized by deprotonation, and this allows them to remain soluble in the black liquor due to electrostatic repulsions [89, 90]. These anionic charges arise from the hydroxyl groups in the molecule [91].

Table 1.1. Typical dry solids composition of mixed wood kraft black liquors derived from North American wood species [92].

Component	Weight (%)
Lignin	30-45
Carbohydrate degradation products	
Hydroxyl acids	25-35
Acetic acid	5
Formic acid	3
Extractives	3-5
Methanol	1
Sulphur	3-5
Sodium	15-20

1.4.1.1 Technologies for kraft lignin recovery from black liquor

The primary factors affecting lignin recovery from black liquor are solubility and molecular weight [93]. The three most explored techniques for the separation of lignin from black liquor are acid-precipitation, which is based on the solubility of lignin, membrane separation, based on the differences in molecular weight, and electrolysis, based on the conductivity of the black liquor [94]. The most efficient process is assigned to the one that results in the highest recovery yield, and purity of product at the lowest cost. Moreover, the process needs to be easy to implement [95]. To date, the most efficient method catering to all these requirements is acid-precipitation [38, 82], and for this method, the most common acids utilized are carbon dioxide and sulfuric acid. Organic acids such as, acetic acid [96], formic acid and citric acid [97] have been used to precipitate lignin, however, these acids have been reported to alter the chemical structure of lignin. Similarly, the inorganic acid, hydrochloric acid, was reported to incorporate impurities to the lignin product [98]. Important characteristics of kraft lignin that determines its valorization, are the ash and metal content. For applications with a high impact in society such as carbon fibers, a sodium content of less than 200 ppm is desired [96].

Kraft lignin recovery by ultrafiltration membranes

Lignin recovered using ultrafiltration membranes has a high purity [99]. This process usually employs membranes of various molecular weight cut-offs, depending on the desired molecular weight of the recovered product, and can be composed of various materials such as polysulfone and cellulose acetate [100]. Membrane separation is generally utilized for weak black liquors that have a strong ionic content [93]. It was reported that low molecular weight lignin recovered from membrane contained a high amount of sulfur [93]. One setback of this method is that there is a restriction on how high the temperature of the process can be, to prevent the formation of agglomerates [93]. Some studies have even utilized acidification as a post-recovery treatment to increase the purity of the product [101]

Kraft lignin recovery by electrolysis

This method yields a lignin of high purity, along with producing hydrogen, which can be used as a fuel [102]. These electrochemical techniques employ electrodes made from materials such as platinum and nickel, to oxidize the lignin contained in the black liquor [102]. This method can be used in conjunction with membrane technology and acidification [103].

Kraft lignin recovery by acidification

Kraft lignin (**Figure 1.8**) recovery by acidification has been performed since the 1800s [38]. The first patent was registered in 1872. This process involved the spraying of carbon dioxide gas into black liquor at a high temperature. This served as a purification step to recycle the white liquor back to the plant [104]. A similar procedure was later patented for the recovery of kraft lignin by conducting acidification at elevated temperatures, with a subsequent high temperature filtration step [105].

Figure 1.8. Plausible simplified structure of a kraft lignin chain displaying various hydroxyl groups. R represents points of incorporation into the lignin polymer. Image created with ChemDraw Professional.

Many researchers have investigated the complex mechanisms involved in lignin precipitation and the effect process variables have on the resultant properties. The results reported indicate that higher pressures result in a higher yield, and, shorten the duration of the recovery process [45, 106]. Alen *et al.* [106] studied the effect of the initial black liquor solid content on the product yield. They concluded that maximum yields for a softwood black liquor are obtained for a solid content between 27-30%, and for hardwood between 30-35%. They also concluded that highly dilute mixtures prolong the recovery process. Furthermore, they reported that the maximum yield was achieved for an initial solid content of 42% for hardwood black liquor, but this resulted in a filter cake consisting of finer particles which could have reduced filtration efficiency and total recovery. This same product also contained a higher sodium content [45].

As mentioned earlier, the only acids widely considered viable for this application are carbon dioxide and sulfuric acid, as other acids, including mineral and organic generally add new components to the recovered lignin, either as impurities or attachments in the main molecule [37, 82]. The carbon dioxide carbocation is highly effective up to a pH range of 8-9, with maximum lignin recovery yields reported between 65-75%, and improved filterability of the lignin [45]. Recently, a yield of 87% from hardwood black liquor was

obtained by solely acidifying with carbon dioxide at 80 °C to a pH of 7 [98], however the process time was not reported. On the other hand, acidification with sulfuric acid alone only results in significant precipitation for pH lower than 7.7 [107], and results in a gel-like product at pH 2-3 that is difficult to wash and filter [45]. During acidification by sulfuric acid, the dissociated ions liberate and dissolve most of the sodium and sulfur attached to lignin, with a fraction of sulfur is removed as evolved hydrogen sulfide gas [82]. The remaining sulfur, which accounts for 2-3% of solid kraft lignin is found attached to the lower molecular weight lignin [93].

Other variables that affect the yield, quality, and purity of the recovered lignin are chemical composition of the black liquor [108], the speed of the process, the ionic content of the black liquor, the molecular weight of lignin, and process temperature [85]. With increasing ionic strength, the charges on the lignin molecules become balanced thus reducing the electrostatic repulsion between the molecules. This enhances chain aggregation and subsequent precipitation.

It is known that as the pH of black liquor is lowered, protonation of the ionized hydroxyl groups on the molecule occurs causing hydrophobic and Van de Waals forces to dominate, resulting in molecule aggregation and subsequent precipitation [82]. Similarly, it has been demonstrated that the presence of specific anions bonded to the sodium ion (Na^+) may induce kraft lignin precipitation at different rates, in a phenomenon known as the salting-out effect [109]. Nonetheless, there are significant discrepancies regarding the effect of temperature on the process [110]. Zhu et al. [37, 38] studied the precipitation of kraft lignin from softwood and mixed softwood and hardwood black liquors between 45-75 °C, and final pH 9.5-11. They attributed the largest contribution to yield to the drop in pH, but also reported a slight decrease in precipitation yield with increasing temperature. Evstigneev [111] reported similar results. They explained that the solubility of lignin is directly proportional to temperature, as the pKa is lower at higher temperatures. Nonetheless, determining the pKa-solubility relationship of black liquor is challenging as it may vary depending on the concentration of functional groups, which in turn vary at different pH values [112] i.e. Phenolic groups (pKa 9.4–10.8) may experience exothermic dissociation, and carboxyl groups (pKa $\approx$ 4.4) endothermic dissociation [37]. In contrast, other studies have reported a higher yield with elevated temperatures for both hardwood and softwood species [98]. It is unclear whether this is due to an improvement in filterability of the precipitated lignin at higher temperatures. Both Howel and Thring [98] and Loufti et al. [45] reported that above 80 °C, the precipitate forms a ‘tar-like’ substance.

Helander *et al.* [93] reported that the separation of low molecular weight lignin could not be achieved at elevated temperature due to the formation of fairly large lignin agglomerates, while at room temperature, finer particles of a low molecular weight lignin were obtained [93]. Researchers have also studied how acid-precipitation process variables affect the physical and chemical properties of the isolated lignin. It is known that as the pH is lowered lower molecular weight fragments are recovered. However, the molecular

weight of the precipitated lignin may increase due to the lignin fragments in the black liquor condensing [82].

As stated before, as the pH of black liquor is lowered, protonation of the ionized phenolic groups on the lignin molecule occurs causing Van der Waals attractive forces to dominate, which results in a decrease in the electrostatic repulsion between the lignin molecules [38, 82, 113] leading them to aggregate and precipitate. Another plausible phenomenon states that hydrogen bonding occurs between phenolic groups and phenolic-ether linkages between pH of 8-13, which could promote molecule aggregation [114]. The phenomenon based on electrostatics could be confirmed through zeta (ζ)-potential measurements [115], and the intensity of hydrogen bonds by atomic force microscopy (AFM) according to recently published direct methods [116] and Fourier transfer infrared spectroscopy (FTIR). Recently, a modified Poisson-Boltzmann modelling approach was utilized to study the width of the electric double layer, and the ionic charge distribution of lignin molecules in black liquor. The results suggested a very unstable and heterogeneous charge distribution around the lignin molecules [117]. All of these findings suggest that the heterogeneous chemical structure of the lignin in combination with the experimental variables and the concentration of the lignin in the black liquor will affect the response to pH, making it a highly complex system.

Mixing is required during acidification to ensure an even distribution of chemicals, and uniform precipitation. It has been reported that high speed accelerates the acidification process, and produces smaller particles. However, finer particles pose difficulties during filtration [36]. Moreover, moderate mixing speed has also been reported to retarded collision between the lignin molecules [98]. As such, a laminar/transition flow would be appropriate, but because this is difficult to obtain on a stir plate, researcher have recommended mixing kept just below turbulent flow regime [36].

1.4.1.2 The effect of molecular weight on kraft lignin precipitation

During precipitation, the larger lignin molecules serve as adsorption sites for smaller fragments, and thus increase the overall yield [114], however, the particular phenomenon by which this occurs is unclear. During precipitation, researchers who reported an increase in the yield with temperature, have also reported an increase in phenolic and methoxyl groups with increased yield [37]. However, those reporting lower yields with increased temperature reported that the molecular weight of the separated lignin decreased with increasing yield [37]. However, it is also possible that sulfuric acid can catalyze condensation of the lignin molecules in solution [118], and one would expect the molecular weight to increase with decreasing pH. One plausible explanation for this dichotomy could relate to the chemical structure of the precipitated lignin, as the ratio of different phenylpropanoid units varies across softwood and hardwood species. However, Zhu and Thandelier [37] backed their results by stating that larger lignin molecules tend to be

attracted to the hydrogen ions in solution, increasing their solubility, thus at much lower pH the molecular weight of the precipitated lignin will be low [113].

1.4.1.3 LignoBoost™ process

The most efficient acidification technology is the two-stage commercialized process known as LignoBoost™ (**Figure 1.9**) [36, 82, 119]. The first LignoBoost industrial operation in the United States was conducted by Domtar in early 2013 [38]. LignoBoost™ was developed from a collaboration between the company Innventia, and the Chalmers University in Sweden [119]. The general process uses an aqueous black liquor mixture at 40% solids content. The black liquor is then acidified with carbon dioxide down to a pH of 9.5 and filtered. The filter cake is then re-dispersed and acidified with sulfuric acid to a pH of 2.5, the resulting slurry is filtered, and the filter cake is washed with water at pH 2.5. The temperature is kept constant at all steps in the process. This method produces a high lignin yield with low inorganic and carbohydrate content. The maximum experimental yield reported in the literature for hardwood black liquor is 91% at a final pH of 2 [82]. Ziesig *et al.* [120, 121] reported that reducing the pH to 1.5 could reduce the ash content of the precipitated lignin, as at this pH the ionic solubility is maximized.

Another technology, LignoForce™, is a variation on the LignoBoost™ technology. This procedure oxidizes the black liquor prior to carrying out the LignoBoost™ method in order to convert all the reduced sulfur compounds to non-volatile species [83]. In this process, the filterability of the product is improved, and hydrogen sulfide and related emissions are reduced in the acidification process.

The sequential liquid lignin recovery and purification method (SLRP™) is another variation of the LignoBoost™ process [122]. This process conducts the carbonation step at elevated temperature and pressure, producing a liquid lignin product [122]. Furthermore, they have incorporated a vent-gas recycle system which redirects the carbon dioxide formed from the bicarbonate backward reaction, and the totally-reduced sulfur compounds formed during acidification such as, hydrogen sulfide, back into the process to pre-acidify the black liquor [122].

Figure 1.9. Schematic of the LignoBoost process. Black liquor acidification with carbon dioxide and sulfuric acid. Process flow diagram created in CHEMCAD and edited in BioRender.com.

The amount of inorganics, residual sugars, functional groups (e.g. phenolic and methoxyl groups), color, and molecular weight of the recovered kraft lignin strongly affect its physical and chemical properties, and hence its applicability. Because of this, it is important to optimize precipitation to cater to final product specifications. It is equally imperative to develop a deep understanding of the mechanisms behind the solubility, colloidal stability and precipitation of lignin in black liquor, and how is affected by the process variables. However, the work published on precipitation mechanisms is very scant and superficial [123]. Furthermore, most studies on lignin precipitation have been conducted either on softwood or mixed softwood and hardwood lignin, but there are few studies on pure hardwood kraft lignin obtained through the LignoBoost process [123]

1.4.1.4 The color of kraft lignin

Crucial characteristics that affect the use of lignin are the color and the odor. For example, in the cosmetics industry, lignin of a very light color would be more appealing [124]. Despite this, these properties have received very little attention in research studies [91, 124], and producers and researchers have ventured into costly and tedious approaches to bleach out the color of lignin [125]. A color can be defined based on a combination of its hue, its saturation, and its luminous intensity [126]. For this, understanding how process variables affect the ability of lignin to absorb or reflect light, and relating color to physical and chemical properties is of great interest academically, and industrially.

1.4.2 Proposed uses for kraft lignin based on scientific reports

Kraft lignin is an attractive raw material for producing high-performance, high-value biomaterials, as it represents a major source organic aromatic compounds, and is a chemically active organic polymer containing carboxylic, phenolic, aliphatic hydroxyl, and methoxy groups (**Table 1.2**) [93, 127]. Current industrial uses of kraft lignin include combustion for fuel and as a raw material in the manufacture of pesticides and herbicides after chemical modification [128]. Various high-value, carbon-neutral, lignin-based products have also been proposed, and the global revenue of lignin-based materials is projected to increase at a rate of 5.3% per year [129]. The major advances have been focused on polymers, adhesives, and resins [130]; carbon fiber technology [131]; hydrogels [132]; and nanoparticles [124, 133, 134]. Lignin nanoparticles have applications in biomedical, environmental, electronic, personal care products, and thermoplastics [124, 133-135]. Nonetheless, widespread industrial use of lignin as a raw material has not yet materialized due to the natural heterogeneity, and inherent chemical and physical variability, which depend on the source of the lignin, and the delignification and purification processes [136].

Table 1.2. Typical functional groups found in kraft lignin quantified per 100 C₆C₃ units (reproduced from [35]).

Functional group	Softwood species	Hardwood species
Methoxy	92-97	139-158
Phenolic hydroxyl	15-30	10-15
Benzyl alcohol	30-14-	40-50
Carbonyl	10-15	-

1.4.2.1 Studies on kraft lignin as an anti-fungal wood preservative

Kraft lignin is a potential renewable wood preservative [137-141]. Technical lignin has been evaluated as a potential anti-fungal wood preservative [142], and satisfactory results have been reported, indicating that technical lignin hampers fungal degradation in wood [138-140, 143]. Particularly, kraft lignin is highly hydrophobic, which makes its application in a neutral and acidic water-borne wood preservatives challenging [144]. Researchers have reported utilizing aqueous solutions of lignin [138], while others have employing the black liquor directly as a preservative, without any mention of pH adjustments [137]. However, the latter practice is less environmentally-friendly, as black liquor has proven to be toxic to aquatic life [145]. Additionally, as fungi are inactive under

alkaline conditions [49], this raises the question of whether the lignin itself is inhibitory or it was the pH used in their experiments.

Satisfactory reports on the anti-fungal properties of lignin include work by Fernández-Costas *et al.* They grafted kraft lignin of unspecified origin with a laccase enzyme to form covalent bonds on the lignin, and observed 50% growth inhibition for various white and brown rot species grown in malt extract agar petri dish cultures [141]. Dos Santos *et al.* [140] evaluated the biocidal efficacy of eucalyptus organosolv lignin at a 1% concentration in a water/ketone solution, their results showed higher decay resistance to both white-rot and brown-rot fungi for the treated samples in comparison to the untreated samples. The lignin utilized was extracted directly from the wood by submerging the wood at high temperatures and pressures in an ethanol-water solution. This was followed by acid precipitation of the mixture with 72% sulfuric acid. The prepared 1% lignin solution was impregnated into the wood by vacuum-pressure treatment. The results were satisfactory, however, the weight losses in the control samples were very low which cast some doubt on the validity of their conclusions [142]. Chirkova *et al.* investigated the antifungal efficacy of aqueous solutions (0.5-1%) of alkali lignin, kraft lignin, sulfonated lignin and enzymatic hydrolysis lignin. These solutions were impregnated by vacuum-pressure into pine wood. They reported 0% weight loss at the end of sixteen weeks for the 0.5 % kraft lignin solution against *Gloeophyllum trabeum*, as opposed to 36 ± 4.5 % weight loss in the control block [139]. However, they made no mention of the pH of their solutions.

The anti-radical activity of lignin is related to its anti-microbial activity, a higher anti-radical activity results in a higher anti-microbial activity [146]. Lourencon *et al.* observed a higher ratio of syringyl to guaiacyl, and higher anti-oxidant activity for hardwood kraft lignin samples recovered at lower pH [147], and the anti-radical properties of lignin have been reported to be enhanced in nanoparticle form [148]. Klein *et al.* reported higher anti-oxidant activity for kraft lignin in comparison to organosolv lignin [149]. Furthermore, Ponomarenko *et al.* reported radical scavenging activity for both softwood and hardwood kraft lignin, and they also observed a decrease in anti-radical activity for highly polydispersed samples [150]. Pina *et al.* suggested that a high molecular weight could reduce the anti-oxidant activity of kraft lignin [151].

1.5 Lignin nanoparticles

Producing nanoparticles from industrial lignin is one way to valorize this material [152]. One of the properties of kraft lignin that hinder its industrial exploration is its inherent hydrophobicity, which causes it to sediment in aqueous systems. Lignin nanoparticles are able to overcome this challenge, by forming nanoparticles in water that are stabilized by electrostatic repulsions between the ionized surface hydroxyl groups [136, 153]. Lignin nanoparticles have applications in biomedical engineering, environmental processes, electronic devices, personal care products, and thermoplastics [124, 133-135]. However, the physical and chemical properties of the final particle depend on the type of lignin precursor, synthesis route, and post-synthesis modifications.

Hollow lignin spheres have been produced from unmodified kraft lignin through a solvent exchange process [154]. This is a significant advancement, as most methods have relied on co-polymerization and unsustainable approaches to chemically modify the lignin precursor prior to nanoparticle synthesis [136, 153]. The production of hollow particles from unmodified kraft lignin was achieved by dissolving lignin in tetrahydrofuran and slowly inserting water into the system. The particles ranged in size, 200-600 nm, and they were reported to be stable in water for forty-five days at room temperature [154]. Nonetheless, the specific mechanism responsible for the formation of the hollow structure was not described sufficiently, as solid spheres have also been produced from kraft lignin using the same approach [133, 136]. All papers have attributed the nanoparticle formation mechanism primarily to π - π stacking between the aromatic rings. Thus, a deeper understanding of the phenomenon that promotes cavity formation is needed. Li *et al.* reported perforated kraft lignin nanocapsules [155] that were obtained by adding water to a lignin in ethanol solution. This method produced fine particles. Nonetheless, the shells easily disintegrated, the core of some particles fused together upon ethanol removal, and the particles were reported to flatten upon drying.

Various studies have studied the suitability of lignin nanoparticles for drug-delivery [156], however, only Zhou *et al.* [157] investigated the mechanisms driving encapsulation and release, in this case of a hydrophilic drug, from lignin nanoparticles. As such, there is significant room for additional research. Producing uniform and stable hollow structures from unmodified kraft lignin could pave the way towards its commercial utilization in drug-delivery applications. One way to achieve a high yield of regular structures is by employing surfactant templates [158]. Li *et al.* [159] prepared an alkali lignin-anionic surfactant nanoparticle complex, and reported it to be effective for the encapsulation of a hydrophobic compound, but released it at a fast rate. Yiamawas *et al.* [160] used a nonionic surfactant to envelope a kraft lignin-based nanoparticle encapsulating a UV-active cargo. Both methods modified the lignin precursor to promote the molecular assembly.

1.5.1 Self-assembly of lignin

Nanoparticles are structures with a single dimension of less than 100 nm, and fine particles are structures with dimension between 100-2500 nm. The most effective method that has been reported for lignin nanoparticle synthesis is solvent displacement. This involves the self-assembly of molecules into static micelles or reverse micelles in a solvent/non-solvent system [1, 136, 161]. Micelle configurations (polar groups at the peripheral) in polar solvents and anti-micelle configurations (non-polar groups at the peripheral) in non-polar solvents. In a self-assembly process, the molecules spontaneously arrange themselves into stable, well-shaped, ordered aggregates. This spontaneous arrangement is carried out by the internal forces and interactions occurring in the system that result in an equilibrium. The arrangement can occur at a macro, micro, and nano molecular levels [42]. Nonetheless, self-assembly configurations are reversible, they are kept in place by non-bonding interacting forces, such as, ion-dipole, hydrogen bonding, π - π interactions, and dispersion

forces, with strong interactions diminishing the thermal molecular motion of the lignin molecules, hence being more favorable [42].

The nature of lignin affects its self-assembly characteristics. Hardwood lignin is expected to form more uniform structures than softwood lignin because it is less branched, has a lower molecular weight, and is less capable of self-association in a solution in comparison to hardwood [42]. The inorganics present in kraft lignin are considered impurities, however, some degree of sulfonation is desired, as this may minimize the Gibbs free energy at the molecular surface, minimize self-aggregation, and enhance Van der Waals interactions and lignin-ionic interactions needed for nanoparticle formation [162]. When the lignin molecules self-aggregate in solution they form cylindrical blocks composed of 4-10 molecules. These represent the base on which nucleation occurs, giving rise to larger isotropic particles that adopt different, well-defined shapes as a function of the overall effect of all the process conditions [42]. It has been reported that at lower temperatures, self-association of lignin molecules occurs, hence higher temperatures are preferred for uniform lignin nanoparticle synthesis [163], however, it was also reported that too high temperatures could result in oxidation of the hydroxyl groups and affect the overall charge at the surface of the nanoparticle [164]. Thus, the mechanisms behind lignin nanoparticle formation are reported to be kinetically controlled nucleation growth [165], mass diffusion [166], the π - π stacking of aromatic rings, hydrogen bonding, and Van der Waals interactions [133].

Pi-stacking interactions occur between an electron-rich aromatic ring and an electron-deficient aromatic ring [152]. These non-covalent interactions can be of two types, J type and H type., which are defined by the orientation of the aromatic rings relative to each other. When the aromatic rings are connected in a head to tail pattern, the angle between the molecular axis connecting the molecules and the resultant dipole moment is less than 54.7° , this causes a bathochromic (red) shift in the absorption curve of these molecules, and the molecules are said to be experiencing J-aggregation [152]. When the orientation is head to head (parallel), the angle then becomes larger than 54.7° , the absorption curve of the molecules experiences a hypsochromic (blue) shift, and the molecules are said to experience H-aggregation [167].

Other variables that affect lignin nanoparticle formation are pH of the system, and the mixing speed. Most researchers have reported faster speeds as being more suitable for mono-dispersed particles, with the minimum speed reported 300 rpm, and the maximum 1000 rpm [168]. The mixing speed affects the particle nucleation and growth. Mixing is required to achieve homogenous heating of the system, as temperature affects the properties of the final particle. Similarly, mixing speed determines the Reynolds number. At different flows, laminar, transient, and turbulent, flow patterns vary, which can in-turn affect molecular collisions, and thus influence the size, and shape the particles adopt. The flow in a cylindrical flask is rotational motion, which can be modelled as flow around a circular cylinder. Reynold's numbers should be chosen to avoid the system in a region where the vortex street is fully developed, which gives rise to flow separations and

asymmetric forces [169]. As such, appropriate speed needs to be chosen in accordance to the process equipment and variables, to avoid the formation of various thermal boundary layers which could occur when separated fluid hits the walls of the cylinder, and this could increase the polydispersity index.

1.5.1.1 Esterification of lignin with maleic anhydride

Functional groups in the lignin molecule grant it some degree of polarity, and these include the hydroxyl and carboxyl groups [170, 171]. Esterification of lignin molecules is a modification process that has been employed to decrease the polarity, and thus increase the solubility of the polymer in organic solvents [172, 173]. Furthermore, esterification is a cross-linking process, which adds structural stability to the nanoparticle under conditions where the ester bonds are stable. However, cross-linking may also reduce the electrostatic stability due to the replacement of hydroxyl groups as the particle becomes less polar [174]. Another benefit of crosslinking using esters is that these are a fairly easy bonds to break biologically, which could enable a targeted release of bio-actives from the nanoparticles [175]. **Figure 1.10** shows the typical chemical route for crosslinking lignin with maleic anhydride [172], the reaction has been reported to occur predominantly in the aliphatic hydroxyl groups [176]. Nonetheless, the carboxylic acid groups incorporated are also able to react with other lignin hydroxyls.

Figure 1.10. Esterification reaction of lignin with maleic anhydride, at a phenolic and aliphatic hydroxyl group. Image created with ChemDraw Professional.

1.5.2 Synthetic methodologies for lignin nanoparticle synthesis

The most common method reported for lignin nanoparticle synthesis are bottom-up methods, such as solvent precipitation [1], solvent exchange [153], dialysis [144],

sonication [177], water-in-oil micro-emulsion polymerization [178], and copolymerization with other monomers (e.g. styrene [179], and silver [132]). The physical and chemical properties of the final particle such as morphology, hydrophobicity, surface charge, surface functional groups, and stability are dependent on the type of lignin precursor, synthesis route, and nanoparticle post-synthesis modifications [166]. One example of a post-synthesis chemical modification is coating the final nanoparticle with polyelectrolytes in order to reverse and homogenize the surface change and increase stability at extreme pH conditions [166]. Most synthesis methods reported are unreliable for practical applicability, and/or unsustainable [1, 136]. From publications on lignin nanoparticle synthesis, the most popular organic solvents used to dissolve the lignin precursor are ethylene glycol, for which the driving force is hydrogen bonding, as concluded by Yang *et al.* [180], tetrahydrofuran (THF) [144], ethanol [155], and methanol [133]. The most thermodynamically compatible solvent depends on the lignin molecular weight and the amount of hydroxyl groups and methoxy groups present in the lignin. More hydrophobic lignin dissolves better in THF, followed by methanol; while more hydrophilic lignin dissolves better in ethylene glycol. It has also been reported that phenolic hydroxyl groups have the largest contribution to the solubility of lignin [136]. When considering the Hildebrand theory, it is expected that the closer the solubility parameters of the solute and the solvent, the better the solubility [42], however, researchers have observed that the solubility of lignin cannot be estimated solely based on the Hildebrand theory [181]. The Hildebrand theory could be used to match polymers to an organic solvent, in a system where no hydrogen bonding occurs. However, the great variability encountered within the lignin molecule would preclude making single value ascertainments. As such, the solubility of lignin in a polar or non-polar solvent will greatly depend on the chemical properties of the specific material, which again vary greatly within the same material. Sameni *et al.* could not predict the solubility of lignin, but concluded that lower molecular weight lignin dissolved better in organic solvents, while a high aliphatic hydroxyl content reduced the solubility of the polymer in organic solvents [181]. Nonetheless, THF has been reported as a compatible solvent across lignin of different grades, and has also been reported to yield structurally-uniform nanoparticles [136, 153, 182].

1.5.2.1 Solvent displacement

Solvent displacement (**Figure 1.11**) is a two-step method to produce polymeric nanoparticles [183]. In the first step, the polymer is dissolved in an organic solvent that is miscible/partially-soluble in water. The solution is then inserted into a stirred water recipient that may or may not contain templates/stabilizer (surfactant). The self-assembly occurs as the polymer, which is not miscible with water, surrounds the solvent emulsion droplets, while simultaneously interacting with the stabilizer. In the second step, the solvent diffuses into the water, or can be evaporated from the system. Because of the incompatibility of the particles with water, this can cause them to precipitate [184]. Stable dispersion of the nanoparticles in water is possible but depends on the electrostatic charges on the surface of the particles [144, 185]. Solvent displacement and emulsification can also be used to encapsulate a hydrophobic drug directly within the nanoparticles, by dissolving

both the drug and the polymer in the organic solvent prior to inserting the solvent into water [186, 187]. However, addition of the drug after the nanoparticle has been formed has also been practiced [157, 182].

Figure 1.11. Schematic of two-step solvent displacement approach of nanoparticle synthesis. The enlarged portion represents the second step when the solvent can be removed through solvent exchange or precipitation. Created in BioRender.com

1.5.2.1.1 The role of surfactants in the solvent displacement method

Surfactants are molecules that lower the surface tension between two phases. Surfactants self-assemble into micelle configurations (polar groups at the peripheral) in polar solvents and anti-micelle configurations (non-polar groups at the peripheral) in non-polar solvents. Additionally, the shape and size of the resultant particle depends on the concentration of surfactant in the system and the process conditions [188]. Surfactants can be cationic, anionic, possess both anionic and cationic groups, in which case they are known as zwitterionic, and nonionic (**Figure 1.12**) [158]. Norgren and Mackin [189] reported that cationic surfactants promoted the aggregation and subsequent precipitation of softwood kraft lignin. It is also known that surfactants aid in separating the lignin from fatty acid soaps [82], this could imply that utilizing them in nanoparticle production results in a purer product formation.

When the system is made up of both a solvent and an anti-solvent, an interface will be created, a surfactant double layer develops at the solvent/non-solvent interface in the most stable conformation [190]. When a dissolved polymer is inserted to self-assemble in a ternary system (solvent, non-solvent and surfactant), the surfactant stabilizes the interface,

thus acts as a template on which the polymeric nanoparticles develop. By reducing the surface tension at the interfaces, the surfactants ensure that the formed nanoparticles do not aggregate, this can give rise to smaller-sized and dispersed particles. Nonetheless, self-assembly configurations are reversible, they are kept in place by non-bonding interacting forces, such as, ion-dipole, hydrogen bonding, π - π interactions, and dispersion forces [42]. Furthermore, surfactants can be used to promote a high yield of monodispersed, porous nanoparticles [158]. The size, concentration, and the charge on the hydrophilic head, as well as other process variables such as temperature, affect the shape and size the surfactants adopt, and their interactions with the polymer [191].

Figure 1.12. Representation of the amphiphilic nature of surfactants. Displaying a cationic surfactant (cetyltrimonium bromide, CTAB), an anionic surfactant (sodium dodecyl sulfate, SDS), amphoteric surfactant (trimethyl glycine betaine, TMG), and a nonionic surfactant (monolaurin). Image created in ChemDraw Professional.

1.6 Objectives

The overall goal of this project was to understand how various process variables affect the properties and recovery of kraft lignin by acidification, and to use this kraft lignin to make nanocapsules for use as a wood preservative. The specific goals are as follows:

Chapter 2: Statistically optimize lignin fractionation from hardwood black liquor for maximum yield and characteristics.

- Determine the effect of process conditions on mass yield and molecular weight, and phenolic hydroxyl group, residual sugar, and total inorganic contents.

- b. Investigate the specific mechanisms driving lignin solubility as related to the process parameters.
- c. Develop a correlation between kraft lignin color, experimental variables, and chemical properties.

Publication: Andeme Ela, R. C.; Spahn, L.; Safaie, N.; Ferrier, R. C.; Ong, R. G. Understanding the effect of precipitation process variables on hardwood lignin characteristics and recovery from black liquor. ACS Sustain. Chem. Eng. 2020, 8 (37), 13997-14005, DOI 10.1021/acssuschemeng.0c03692.

Chapter 3: Synthesize single- and double-shell, lignin nanocapsules from kraft lignin and encapsulate a secondary biocidal compound.

- a. Investigate the controllable properties of the empty nanocapsules (shape, size, zeta potential and their stability over variations in time, pre-cursor concentration, and pH).
- b. Quantify the loading efficiency of the fungicide, propiconazole, into the nanoparticle and study the release profiles.

Publication: Andeme Ela, R. C.; Tajiri, M.; Newberry, N. K.; Heiden, P.A.; Ong, R. G. Double-shell lignin nanocapsules are a stable vehicle for fungicide encapsulation and release. ACS Sustain. Chem. Eng. 2020, 8 (46), 17299-17306, DOI 10.1021/acssuschemeng.0c06686.

Chapter 4: Determine the effectiveness of lignin nanocapsules at preventing fungal degradation in southern yellow pine compared to the conventional preservative, copper chromium arsenate (CCA).

- a. Quantify the extent of preservative impregnation into the wood blocks by pressure and dip treatment methods.
- b. Assess the depth of penetration of the most effective lignin-based preservative into the wood blocks.
- c. Evaluate the effectiveness of the preservatives at preventing brown rot degradation by *Gloeophyllum trabeum*.

Andeme Ela, R. C.; Bal, T. L.; Xie, X.; Ong, R. G. Lignin-Propiconazole Nanocapsules Are an Effective Bio-based Wood Preservative. Submitted for publication.

2 Lignin purification

2.1 Introduction

The primary goal of this project is to understand the effect and mechanisms of various process variables, such as temperature and pH, during hardwood kraft black liquor acidification on the properties and characteristics of the recovered lignin. The second goal was to optimize the precipitation process for mass yield, using a Box-Behnken response surface statistical methodology, and determine correlations between the process conditions (temperature, pH, and residence time), chemical properties (molecular weight, hydroxyl groups content, ash and residual sugar content), and the physical properties (color and texture) of precipitated kraft lignin.

Box-Behnken response surface methodology. The most appropriate statistical experimental design is selected based on the goal of the project and the number of experimental variables. Response surface methods permit the study of the interactions and quadratic effects between process variables, and allow for the optimization of a specified output through the combination of process factors. Response surface methods can also help create a model that is less sensitive to experimental errors [192]. Amongst the various response surface designs, the Box-Behnken design has the lowest number of experimental runs when the process only has three factors. This methodology requires at least three factors, and each factor is distributed into three levels, -1, 0, +1. This design does not operate on a factorial design, the combination of variables are taken from the midpoints and at the center of the process levels [193]. The geometry of the Box-Behnken design is represented by a sphere fitted in the process variables box, in a manner such that the edges of the sphere extend outwardly intersecting the midpoints at the faces of the box. In our process, we investigated three process variables, the design had three blocks with two factors varied through the possible combinations of low and high per block, and three center point replicates.

A group of articles studying the effect of temperature on the LignoBoost process reported an increase in mass yield with a decrease in temperature [37], while another group reported an increase in mass yield with an increase in temperature [98]. As these articles relied solely on the mass yield to determine the effect of temperature, there was no investigation into how temperature affected the mechanisms driving lignin precipitation from solution, we evaluated the electrokinetic properties of black liquor as a function of pH and at two temperatures, to investigate the colloidal stability in the solution by means of dynamic light scattering techniques, the hydrodynamic size and the zeta-potential. Studies have shown that the lignin molecule is highly ionized in alkaline black liquor, nonetheless, it is only partially soluble in the alkaline black liquor [45, 85, 92, 106]. This implies that we should be able to measure zeta-potential magnitude that implies stability, that is, equal or greater than 30 [59], and as the ions get protonated, the zeta-potential should decrease. Similarly, the hydrodynamic diameter is expected to be low at high pH, because the colloids are electrostatically stable [165], but as the acidification proceeds, these are expected to

aggregate as a result of electrostatic instability. Precipitation temperature was varied to determine the effect on zeta-potential and hydrodynamic size with temperature. Furthermore, the electrokinetic changes observed as a result of temperature were paired with surface analysis x-ray photoelectron spectroscopy (XPS) to understand the chemical changes that occur at the surface of the colloid as the molecules are protonated.

Work in the literature studying the variability in the color of lignin is very scant [124, 194-196]. There is still a lot of room to expand these studies, particularly, relating the color of lignin to its chemical and physical properties [124, 196]. In this work, we have measured the color of lignin precipitated at various process conditions by mean of the CIE L*a*b color system, and made correlations between the darkness (distance of the measured color from the white color) and its molecular weight, hydroxyl group content, and the granularity of the particles as analyzed in ImageJ® from binary images.

For the purpose of developing a novel wood antifungal preservative, our model, along with the correlation chart will aid in selecting the most appropriate processing conditions to generate a lignin sample with the most desirable characteristics; lowest molecular weight, lowest polydispersity, lowest ash and residual sugar content, and the highest phenolic hydroxyl group content, to be used in the preparation of lignin-based nanocapsules.

2.2 Experimental

Figure 2.1. Schematic of overall lignin recovery process and characterization. The black liquor was subjected to acidification at various temperatures and residence times. The mechanisms driving colloidal stability in the black liquor was be studied by means of

dynamic light scattering techniques, and FTIR. The precipitated lignin was further analyzed for molecular weight, color, and bulk and surface functional groups using ^{31}P -NMR and XPS. Image Created with BioRender.com

Materials

The mixed-hardwood, strong black liquor was obtained from Verso (Quinnesec, MI) from the black liquor stream leaving the evaporators. The samples were collected on the same day.

Black liquor characterization

The moisture content of the black liquor was measured using an electronic moisture analyzer (MA35, Sartorius, Inc.) The pH of the solution/slurry was measured with silver/silver chloride (Ag/AgCl) pH electrodes (Orion refillable, Thermo Scientific) calibrated for pH 4.0, 7.0, and 10. The ash content was determined by weighing the mass remaining after dry oxidation at 575 °C relative to the 105 °C oven-dry weight of the sample (Sartorius AZ302 M-power) [197]. The klason lignin and the acid-soluble lignin were measured according to a published method [37, 38]. The acid-soluble lignin (ASL) was measured with a UV-Vis-NIR spectrophotometer (Shimadzu UV-3600 Plus) at a wavelength of 280 nm with a quartz cuvette, and the concentration calculated according to **Equation 2.1** [198]. The adsorption constant (ϵ) $110 \text{ dm}^3\text{g}^{-1}\text{cm}^{-1}$ [37, 98], oven-dry weight (ODW), and a cell pathlength of 1 cm were utilized. The UV-Vis measurement was performed within 2 hours from filtration. The hydrolysate was diluted until a valid absorbance value (0.3-0.8) was obtained [98].

$$\text{ASL (\%)} = \frac{\text{UV}_{\text{abs}} \times \text{Volume}_{\text{filtrate}} \times \text{Dilution}}{\epsilon \times \text{ODW}_{\text{sample}} \times \text{Pathlength}} \times 100 \quad (2.1)$$

The functional groups in the black liquor supernatant at different pH was studied by Fourier transform infrared spectrophotometer with 20 scans on a MIRacle A Diamond prism-ZnSe support element (SHIMADZU, IRTracer-100). The concentration of sodium was measured by X-ray fluorescence spectrometer (Xenometrix EX-6600 EDS), and the analysis was performed from calibration standards. The kinematic behavior of the lignin colloids in black liquor was analyzed as a function of temperature and pH with a Malvern Nano-ZS zetasizer at a refractive index of 1.61 [199].

Lignin fractionation

The fractionation and characterization were conducted as displayed in **Figure 2.1** above. Under the fume hood, 100 ml of a 38.5% solid content black liquor solution was placed in a water bath at the desired temperature and atmospheric pressure. The slurry was acidified under continual stirring with carbon dioxide gas at 80 kPa for 1 hour, followed by dropwise addition of 72% sulfuric acid until the desired final pH was reached. Lignin was allowed to precipitate for the designated time, then the lignin slurry was vacuum filtered through

20-25 μm porous paper (Whatman, grade 4). The filter cake was re-dispersed in a solution at the same pH as the final slurry, and continually rinsed with ultrapure water. The filtrate was either vacuum freeze-dried (FreeZone 4.5L, 77500/77510, Labconco) for 18 hours at a temperature of $-40\text{ }^{\circ}\text{C}$, or furnace dried at $105\text{ }^{\circ}\text{C}$ for 12 hours (Furnace, 6000, Thermolyne). The drying method was kept consistent for samples used in comparative studies, as the drying method can affect the oxidation state of the molecule [124].

Kraft lignin characterization

The mass yield was calculated based on the klason lignin according to **Equation 2.2**, where $Klason_{BL}$ represents the klason lignin originally in the black liquor, and $Klason_{KL}$ the klason lignin recovered. The residual sugars in the lignin were hydrolyzed according to standard procedures [198], followed by analysis of the moieties in a high performance liquid chromatography (HPLC) instrument (1100 series, RID and DAD, Agilent Technologies) operating with a HPX-87 P-Column (300 x 7.8 mm). This system uses ultra-filtered water as the mobile phase, and 9 μm lead particles packed in column as the stationary phase. The column employs polystyrene divinylbenzene cross-linker at a rate of 8%. Measurements were performed at $23\text{ }^{\circ}\text{C}$. The analysis was performed by creating four-point calibration standards for glucose, xylose, mannose, arabinose, and galactose (0.5-24 mg/ml).

$$Yield (\%) = \frac{Klason_{KL}}{Klason_{BL}} \times 100 \quad (2.2)$$

Lignin samples were acetylated prior to measurement according to a published procedure [200], and the details can be found in the supplemental document. The number averaged molecular weight (M_w) of the acetylated kraft lignin was measured by size exclusion chromatography (Malvern OMNISEC) operating with two Viscotek columns (300x8.0 mm, T3000 and T4000) at $35\text{ }^{\circ}\text{C}$, with tetrahydrofuran as the mobile phase. The absolute number averaged molecular weight was measured by triple detection from light scattering (LS), viscosity and refractive index (RI). The relative number averaged molecular weight was measured by polystyrene standards (exclusion limit 400kDa).

The hydroxyl groups were measured via ^{31}P -NMR (Unity Inova, Varian, 400 MHz) at $25\text{ }^{\circ}\text{C}$. The sample preparation and analytical method were adopted from Argyropoulos [201]. The details of the process can be found in the supplemental document.

The surface elemental environment of the precipitated lignin was studied with X-ray photoelectron spectrometer (PHI 5800) using a magnesium (Mg) beam. The analysis was performed in the XPS Casa software.

Design of experiments

Statistical design of experiments permits the optimization of variables towards a target output and elucidates the interactions between process variables [202]. In this work, a Box-Behnken (response surface) design of experiments approach in Minitab® was employed to study the effect of process variables on product yield. These in turn were related to the functional groups and molecular weights of the samples. The central design point was conducted in triplicate, summing a total of 15 points in the model. The analyses were conducted at a confidence interval of 95%, and a significance level (α) of 0.05.

Color analysis

To quantify the variability in color at various precipitation conditions, a color reader (CR-10 Plus, Konica Minolta) was employed to measure precisely in CIE Lab color system, the ΔE (color difference), ΔL (difference in lightness), Δa (proportion of green (-) and red (+)), and Δb (proportion of blue (-) and yellow (+)) of each sample against a white color target. The color reader is equipped with the standard illuminant D65, a 10° observer and specular component (SCI), and a Xenon lamp light source to ensure stable reading. For a quantitative comparison, the brown index (BI) was calculated according to **Equation 2.3**. A higher brown index pertains to a lighter color [124], and delta E (ΔE) was employed to compare the difference in visual perception between samples.

$$BI = \left[\frac{(a+1.75L) \times a}{5.645L+a-3.012b} - 0.31 \right] \div 0.17 \quad (2.3)$$

The surface plots of the samples were studied from images captured in a light-box imaging system (EL Logic 200) under white light Epi-illumination and analyzed in ImageJ® in an attempt to relate the precipitate granularity to color. Similarly, the color was related to the hydroxyl groups and the molecular weight of the sample by correlation matrix.

2.3 Results and Discussion

Some important properties of the black liquor are shown in **Table 2.1**. The high ash and acid soluble lignin content are expected for hardwood black liquor [37], as well as a small amount of residual sugars [203].

The titration curve (**Figure 2.2**) gives an indication of the pKa strength of the black liquor, and reveals regions of buffer behavior [37]. We observe a region of constant pH along the curve (pH 8.5), and a corresponding change in apparent slope at pH 5.5. The buffering region indicates insignificant lignin protonation. Furthermore, in this buffer zone, basic byproducts could be formed, such as the sodium sulfide and the bicarbonate buffer systems [204], thus counteracting the effect of acidification. The steep declines preceding the buffer region, and the change in apparent slope suggest significant protonation and precipitation of molecules. The steep declines between pH 14-8, with equivalence point at pH 11, and pH 8.5-6.5 with equivalence point at pH 7 could be attributed to the protonation of large and medium size chains respectively, while the sluggish decline between pH 5.5 and 2.5

could represent the protonation of smaller chains, or a reduced concentration of molecules after significant precipitation, in-turn, retarding molecular aggregation.

Table 2.1. Characteristics of the black liquor.

Parameter	Value
pH	13-14
Concentration (g/ml)	1.25 ± 0.07
Moisture content (g/kg liquid)	252.5 ± 20.7
Klason lignin (g/kg liquid)	290.6 ± 6.1
Acid-soluble lignin (g/kg liquid)	37.9 ± 3.2
Ash (g/kg liquid)	394.3 ± 15.1
Sodium (Na)	155.7 ± 9.3
Hemicelluloses (g/kg liquid) ^s	21.2 ± 0.5
Xylose	17.1 ± 0.1
Galactose	0.65 ± 0.02
Glucose	2.0 ± 0.7
Mannose	1.5 ± 0.2

^s Individual sugars were quantified after acid hydrolysis and represent soluble monomers and oligomers.

At increased hydrophobicity, the dissociation of sodium phenolates reduces [82] and this, along with the undissolved solutes, might resist the change in pH.

Knowledge of the electrokinetic properties of kraft lignin is of great importance [205]. The hydrodynamic data (**Figure 2.3A**) suggests that between pH 8-14 the average molecular aggregation is significantly small, indicating that the molecules are highly ionized. This colloidal stability is confirmed by the highly negative zeta (ζ)-potentials (**Figure 2.3B**), indicating the presence of electrostatic repulsion between molecules. Highly dispersible colloids are considered to have ζ -potentials greater than +30 mV or lower than -30 mV, and charges between -10 and +10 mV are considered neutral [59].

Between pH 8-14 the hydrodynamic size increases slightly with an increase in temperature, similarly, the ζ -potential becomes less negative with an increase in temperature, these results suggest electrical instability as a result of the protonation of molecules, thus a positive correlation between a higher temperature, a lower pH, and the recovery yield can be inferred for this region. At a pH of 8 and 70 °C, there is a sharp decrease in the ζ -potential. Responsive basic compounds could contribute to the sudden negative charge, which would be apparent at a high temperature because the added heat accelerates acidification, thus shifting the buffer towards reactants [206]. This phenomenon could also be responsible for the sudden drop in charge at pH 4. Below pH 6, the hydrodynamic size reduces with temperature, this could occur because at lower pH and higher temperatures, the sugars attached to the lignin molecule are degraded faster (**Figure A1.1 B**), and at higher temperatures there are more inorganics associated with the lignin (**Figure A1.2**). There may also be more inter- and intramolecular hydrogen bonds in the system, as revealed by the presence of carboxyl dimers, revealing carboxylic groups are hydrogen bonded to each other, and a broader peak with decreasing pH in the region 3000 to 3500 cm^{-1} (**Figure 2.3C**), and hydrogen bonds are known to be less stable at higher temperatures. The ζ -potential in this region continues to decrease with temperature, except at pH 2 when the surface charge on the molecules is almost neutral, and at this point hydrophobic forces dominate. It could also be possible that the high salt content in the solution is collapsing the electric double layers [207]. Surina et al. [205] measured the ζ -potential of non-wood lignin in black liquor at a pH of 6.6 ± 0.1 by lowering the pH with 72% sulfuric acid. They found it to be -18.42 ± 0.53 mV. This value agrees with what was obtained in this study at pH of 6, and with the decrease in charge observed in the region pH 6-8 (**Figure 2.3B**).

At pH 6 there is accelerated precipitation of protonated large and medium molecular weight molecules, on which smaller molecules may adsorb [165]. This corresponds to the change in slope previously mentioned (**Figure 2.2**). This phenomenon is evident from the change in modality of the curves, from 4-5 at pH 6, to 2-3 at pH 4, to 1 at pH 2 (**Figure 2.4**). The increased aggregation observed below pH 6, and the reduction in ζ -potential between pH 4-6, coincide with the augmentation in hydroxyl groups with reduced pH (**Table 2.2**), implying stronger ionic and molecular hydrogen bonding interactions at pH 4, consequently making the molecules more stable in solution. These observations agree with the observations by Serwing et al. [123], they reported that there was no significant increase in mass yield between pH 4-6.

Figure 2.2. Titration curve of the black liquor with 72% sulfuric acid at room temperature. *Edited with BioRender.com*

Figure 2.3. A) Hydrodynamic size and B) ζ -potential of lignin molecules in black liquor as a function of pH. The error bars represent the standard deviation from three replicates. The blue curve represents measurements taken at 25 °C, and the red curve at 70 °C. C) FTIR spectra of the black liquor as a function of pH at 70 °C. Spectra are spaced vertically for ease of interpretation and do not indicate absolute differences in transmittance between samples.

Howell and Thring [98] also observed some stagnation in yield between pH 6-7. These hydrogen bonds are believed to occur predominantly in the carboxylic groups [170], and the carboxyl dimer peak in the supernatant (**Figure 2.3C**) is broader at pH 4 and pH 2. Similarly, other studies have reported that hydrogen bonding occurs between phenolic groups and phenolic-ether linkages [114], and these are shown to be pronounced in kraft lignin precipitated at pH 4 (**Figure 2.4H**). Nonetheless, the unimodal peak observed at pH 2 (**Figure 2.3C**) could have other underlying reasons besides hydrogen bonding.

Figure 2.4. Precipitated lignin hydrodynamic size (A-C) and external functional groups from C1s XPS (D-F) and O1s XPS (G-I) at pH 6 (A, D & G), pH 4 (B, E, & H), and pH 2 (C, F, & I). The blue curves for hydrodynamic size (A-C) are measurements taken at 25 °C, and the red 70 °C. Lignin for all XPS spectra was precipitated at 70 °C.

The polydispersity index ($\bar{D}$) increased as a function of pH, reaching its peak at pH 6 in the 70 °C sample, and pH 4 in the 25 °C sample (**Table A1.1**), which would be expected if the molecules are drawn together by hydrogen bonding, and there is minimal protonation of molecules (**Figure 2.3B**), however, subsequently the $\bar{D}$ decreased significantly. These could be an indication of a condensation reaction or an acid catalyzed symmetrical etherification between the smaller molecular fragments [118], towards an equilibrium particle size range (**Figure 2.4C**), this could be true in our system if we notice the conjugated alkene peak (**Figure 2.3C**) becomes broader at pH 2, and the alkene peak at $\sim 800\text{ cm}^{-1}$ became stronger with decreasing pH. Dos Santos et al. [140] also reported an increase in C=C bonds at a pH of 2. Moreover, the increase in the ester bond environment peak (O=C-O-C/C-O-H) with reduced pH (**Figure 2.4 G-I**) could indicate esterification reactions. This is supported by the reduction in the carboxyl hydroxyl peak with pH, and the narrowing of the overall hydroxyl stretch with pH (**Figure 2.3C**), as carboxylic acids and alcohols could be partaking in acid-catalyzed esterification. Similarly, for polymers in solution, the scattering intensity and the molecular weight are related by a square-proportionality (**Figure 2.4 A-C**) [208]. To corroborate this, an inverse behavior should be observed for the recovered lignin. The Mw decreases with decreased pH while the $\bar{D}$ increases (**Table 2.2**). The equilibrium size range theory is plausible, as Norgren et al.

[113] reported, the larger lignin molecules tend to be attracted to the hydrogen ions in solution, and they observed lower Mw for precipitates at low pH. Furthermore, it is known that lignin fragments are prone to condensation reactions [209]. The reduction in aliphatic hydroxyl groups with temperature could also support this claim (**Table 2.2**). Moreover, the concentration of aliphatic hydroxyl groups increases with residence time, and the concentration of carboxyl groups decreases. This trend could be due to esterification reactions, and the sorption of molecular fragments onto larger chains as revealed by the increase in $\bar{D}$ with residence time (**Table 2.2**). Similarly, the Mw increases and the $\bar{D}$ decreases as the pH increases (**Table 2.2**).

Temperature, pH, and residence time significantly affect the mass yield of lignin (**Table 2.2**), of which pH has the largest impact (**Figure A1.4**). To assess the adequacy of fit for a model the first step is to study the residual plots (**Figure A1.5**). The versus fit indicates randomly distributed data, and the absence of a trend in the versus order signifies that the errors are independent. The bimodality of the histogram may indicate a fourth,

Table 2.2. Hydroxyl group content of precipitated lignin measured by ^{31}P -NMR, and absolute number averaged molecular weight (Mw) and polydispersity ($\bar{D}$) determined from SEC.

Temp (°C)	pH	Residence time (h)	Aliphatic OH (mmol/g lignin)	Sinapyl OH (mmol/g lignin)	Coniferyl OH (mmol/g lignin)	<i>p</i> -Coumaryl OH (mmol/g lignin)	Carboxyl OH (mmol/g lignin)	Total OH (mmol/g lignin)	phenolic	Mw [†] (kDa)	$\bar{D}$ [†]
85	1.7	1.5	5.63 ± 0.13	3.73 ± 0.18	0.75 ± 0.18	0.16 ± 0.02	0.69 ± 0.22	4.64 ± 0.02		53.26 ± 0.63	4.70 ± 0.44
85	2.4	1.5	4.06 ± 0.36	2.95 ± 0.23	0.47 ± 0.01	0.10 ± 0.04	0.84 ± 0.35	3.52 ± 0.18		65.17 ± 1.23	4.50 ± 0.57
85	4.2	1.5	3.09 ± 0.22	2.21 ± 0.42	1.08 ± 0.11	0.30 ± 0.08	0.94 ± 0.26	3.60 ± 0.45		68.61 ± 1.99	3.31 ± 0.05
65	4.2	1	3.67 ± 0.22	1.86 ± 0.11	0.55 ± 0.15	0.21 ± 0.03	0.70 ± 0.04	2.62 ± 0.29		46.86 ± 1.61	4.16 ± 0.16
70	4.2	1	3.21 ± 0.49	1.89 ± 0.23	0.93 ± 0.03	0.21 ± 0.02	0.61 ± 0.30	3.04 ± 0.28		50.61 ± 0.01	4.21 ± 1.17
85	4.2	1	2.96 ± 0.02	2.61 ± 0.13	1.50 ± 0.57	0.41 ± 0.07	0.81 ± 0.08	4.52 ± 0.78		60.73 ± 0.57	3.03 ± 1.34
65	4.2	0.5	3.56 ± 0.54	2.13 ± 0.56	0.75 ± 0.25	0.33 ± 0.02	0.74 ± 0.03	3.21 ± 0.79		77.45 ± 0.28	2.24 ± 0.05
65	4.2	1	3.67 ± 0.22	1.86 ± 0.11	0.55 ± 0.15	0.21 ± 0.03	0.70 ± 0.04	2.62 ± 0.29		46.86 ± 1.86	4.16 ± 0.16
65	4.2	1.5	4.24 ± 0.05	1.82 ± 0.54	0.39 ± 0.05	0.15 ± 0.03	0.61 ± 0.18	2.35 ± 0.53		15.06 ± 0.36	5.36 ± 0.06

The error represents the standard deviation from experimental replicates.

[†] The absolute molecular weight determined from SEC with LS, RI, and viscosity triple detection system.

unknown variable also affects the mass yield, perhaps the ionic content [37]. The probability plot has observations distributed proportionally on each side, and the highest residual is slightly higher than 3, of which pure error is the biggest contributor (**Table 2.3**). As such, the model can be considered fit. The model (**Equation 2.4**) has an R^2 value of 95.58%, R^2 (adj) of 94.06%, R^2 (pred) of 94.06%, and a standard deviation of 1.76.

$$\text{Mass yield (\%)} = 65.95 - 3.86pH + 0.11T + 4.55t \quad (2.4)$$

The slight decrease from R^2 to R^2 (adj) is an indication that the simultaneous effect of the variables makes it more difficult to predict the outcome, which is expected for a system as complex as black liquor. The comparable value of R^2 (pred) to R^2 indicates that the model is not over-fit, and its predictive ability expands beyond the range of parameters explored in this work.

The probability (p-value) of the model is below the significance level, as such, we can reject the null hypothesis in favor of the model. The lack of fit P-value is significantly higher than the significance level, so we accept the null hypothesis that there is no lack of fit. Additionally, the degrees of freedom of the pure error is less than that of the lack of fit, which reinforces the adequacy of the model. Similarly, the low model p-value ensures that the model indeed addresses the variations in the response. The values from the F-test can also be used to assign significance. The highest variable F-value obtained was for pH, significantly higher than the rest, which again confirms that pH is the primary factor driving the precipitation process, this is confirmed by the pareto chart (**Figure A1.4**), which displays the relative importance of the different factors.

The mass yield tended to increase with temperature (**Figure A1.6**), as confirmed by the reduction in ζ -potential with temperature (**Figure 2.3B**), and the increase in Mw at 85 °C which could be due to condensation reactions, as such, the drop in agglomerate size at a higher temperature (**Figure 2A**) can be attributed to fewer hydrogen bonds. The effects of temperature and pH on the mass yield are only significantly dependent on time for residence time intervals longer than 2 hours (**Figure A1.6**) and this effect is more pronounced at higher pH, when the rate of precipitation is lower. Similarly, it could also be inferred from this plot that at higher temperatures the precipitation proceeded faster.

Table 2.3. Summary of the analysis of variance for the response surface model.

Source	Degrees of freedom (DF)	F-value	P-value ^a
Model	3	103.50	0.000
pH	1	270.18	0.000

Temperature (T)	1	10.31	0.008
Time (t)	1	30.02	0.000
Error	11	-	-
Lack of fit	9	0.38	0.872
Pure error	2	-	-
^a $\infty = 0.05$			

For precipitation at a constant pH of 4.2 and residence time of 1 hour, Mw results suggest an increase with temperature, from 65 °C to 85 °C (**Table 2.2**). A plausible explanation for this could be reduced solubility of larger chains at higher temperatures due to protonation, leading to a shift in the composition of precipitated lignin toward higher molecular weights [37, 93], and an increase in condensation of lignin fragments (**Figure 2.4 D-I**) with increasing temperature [210], leading to higher molecular weight products. Similarly, the Mw increased with increasing pH at 85 °C and 1.5 hours residence time (**Table 2.2**). This trend agrees with previous results [38, 123], and could be due larger molecules being more unstable and prone to hydrophobic aggregations and subsequent precipitation. Our results show that the Mw decreases with residence time, but the Đ increases (**Table 2.2**) These results complement the trend in the titration curve (Figure 1), and the data in Figure 3 A-I, indicating that increased time allows for prolonged unspecific condensations of molecular fragments, and the sorption of smaller molecules onto larger chains, resulting in a lower Mw and higher Đ. The hydrodynamic radii were also employed to determine the corresponding molecular weight of the polymers in solution, these ranged from ~1,000-500,000 kDa, which is significantly larger than the measured Mw (**Table 2.2**), indicating that the lignin chains in black liquor exist as electrically stable globules. The relative Mw computed in our study based on polystyrene standards (Table S2) are in close proximity to what has been reported for hardwood kraft lignin analyzed through conventional methods [83, 124]. The absolute Mw and corresponding Đ reported in this study are within the range of what has been reported previously for hardwood kraft lignin [211, 212].

P-NMR analysis revealed that the temperature and pH of the acidification process significantly affect the hydroxyl content of the lignin (**Table 2.2**). The concentration of sinapyl hydroxyls increase with decreasing pH and residence time, and increasing temperature. These are the main monomers in the hardwood chain and would be ubiquitous at conditions that yield high Mw. Moreover, the concentration of carboxyl hydroxyls increased with increasing pH, and this agrees with the elemental environment analysis (**Figure 2.4 D-I**).

The total amount of hemicellulose moieties decreased with temperature, and was significantly lower at the highest temperature 85 °C (**Figure A1.1**). The xylose moiety was detected in the highest concentration, which is what is expected for hardwood [86]. The total amount of sugars was observed to decrease with decreasing pH (**Figure A1.1**). Also, interesting to note, arabinose was only detected for one sample, the precipitation experiment that is allowed to proceed the longest (pH 4.2, 65 °C, 2.5 hours). We also observed that the amount of xylose is lower as the mass yield increases. Moreover, the presence of xylose is associated with more β -O-4 bonds [33], and thus a higher molecular weight. This agrees with the Mw trend as a function of pH (**Table 2.2**).

Assuming adequate washing, the inorganic content decreases significantly as a function of pH, particularly at a lower temperature (**Figure A1.2**). Howell and Thing [98], reported that the sodium content in the kraft lignin present as Na₂O reduced by ~5% after two washes with sulfuric acid. Ziesig et al. [120] concluded that at lower pH the inorganic ions detach from lignin, moreover, at low pH volatile, totally reduced sulfur species form hydrogen sulfide (H₂S), methyl mercaptan (CH₃SH), and dimethyl sulfide (CH₃SCH₃) [83]. The inorganic content increases slightly with temperature, although the values are fairly similar at 70 °C and 85 °C. This agrees with what Haz et al. [213] observed.

The color of the kraft lignin varied at different experimental conditions (**Figure A1.3 A-F**, **Table S3**), it was observed that at higher temperatures, the product becomes very compact and sticky (**Figure A1.3 E**), confirming what has been reported by other authors [36, 85, 98], this characteristic in-turn, makes the sample difficult to process [98]. The amount of xylose decreased with increasing temperature, and this could be one factor inducing the closely-packed precipitate (**Figure A1.3 D-F**) as it has been discerned that the absence of xylose promotes particle nucleation growth during kraft lignin acidification [123]. It is known for organic molecules, that the more double bonds a molecule contains, and the more functional groups attached to it, the more light it can absorb [214]. This is because in double bonds, the gap between the ground and the excited state is small. As such, a tiny amount of energy would be required to excite the valence electrons. This gap is even smaller for conjugated systems, which will absorb the lowest energies of light, and visibly reflect a dark color. The color of lignin became lighter (dark brown to light brown/yellowish) as the pH decreased, however, below pH 4, the samples turned to a greenish-yellowish color, and the outer surfaces exposed to the slurry had a very dark color. The darkest surface was obtained at pH 1.7 and 85 °C, which supports our theory that below pH 4 condensation reactions might take place. Furthermore, at the same temperature and residence time of 1.5 hours, this sample contains the highest number of hydroxyl groups (**Table 2.2**). Moreover, the color of kraft lignin is darker for higher pH precipitations, which is expected as our Mw values increased with pH. Zhang et al. [124] reported bulk density as their primary factor affecting the color of lignin. The more loosely packed a sample was, the lighter it was in color. Our particle surface analysis results demonstrate that the particles become finer and tightly-packed as the temperature is increased (**Figure A1.3 G-L**), this can be observed from a reduction in the width of the peaks. The closely-packed particles could promote more pi-pi (Π - Π) molecular interactions, and chain entanglement [110], thus increasing the absorption capacity of the

sample. The chromophore moieties in kraft lignin are expected to adsorb onto larger molecules, or partake in condensation reactions, as such a darker color can be expected for samples recovered at elevated pH, elevated temperature, and pH <4 [91, 214]. Some of the chromophores and leucochromophores associated with lignin contain phenolic groups, such as, coniferaldehyde [91]. This has been confirmed in our studies as the coniferyl hydroxyl group increases with increasing pH and temperature, and decreases with decreasing residence time (**Table 2.2, Figure 2.5**). The brown index (BI) has a strong negative correlation with respect to Mw (**Figure 2.5**). Moreover, the highest Mw chains would contain the most unsaturated bonds, thus reflect a darker color. The difference in visual perception (ΔE) has a positive correlation with Mw and a moderate correlation with sinapyl and carboxyl hydroxyl groups, and a negative correlation with BI, indicating that for dark samples the color change is evident, while differentiating the color of separate dark samples visually becomes challenging.

Figure 2.5. Pearson correlation matrix depicting the relationship between process variables, color, molecular weight and hydroxyl groups. Colors coefficient shows with continuous gradient. Chart created in Displayr.com

2.4 Conclusions

The effects of process variables, namely, temperature, pH and residence time on the recovery, and physical and chemical properties of hardwood kraft lignin through the

LignoBoost® process have been investigated in this work. In conclusion, our results suggest that the primary factor influencing lignin recovery through acidification is the pH of the solution, followed by the residence time, and ultimately temperature, all of which have a positive correlation to the recovery yield. Hydrodynamic and ζ -potential data revealed that kraft lignin exists as globules in black liquor and these are electrically stable above pH 6. Similarly, a higher temperature resulted in accelerated protonation of the lignin molecules in solution, thus making the system less stable. As the pH is lowered, the concentration of aliphatic hydroxyls in the purified lignin increases, the molecular weight decreases, and the polydispersity of the molecules increases. The same effect is observed as the residence time increases. This suggests that lower molecular weight samples, which in turn will be lighter in color, are recovered at these conditions. This could be due to molecular fragment sorption onto larger chains. The molecular weight increased with increasing temperature, and the molecules became increasingly tightly-packed. Thus, the samples recovered at higher temperatures were darker. Similarly, below pH 4, the concentration of alkene and ester bonds increased with decreasing pH, suggesting condensation and acid-catalyzed esterification reactions occur.

3 Lignin nanocapsule synthesis

3.1 Introduction

Porous nanomaterials have gained recognition for applications in medicine [158], electronics, environmental remediation and biotechnology [215], amongst others. The porosity of nanomaterials can be classified into three categories, microporous, when the pore diameter is less than 2 nm, mesoporous, when the diameter of the pores lies between 2 and 50 nm, and macroporous, when the diameter of the pores are greater than 50 nm [216]. The size of the pores affects the chemical properties of the material [217]. Mesoporous nanomaterials offer the most versatility in terms of encapsulating a wide range of molecules, and providing slow release kinetics [215]. Uniform mesopores in a nanomaterial can be achieved by employing surfactants [158, 215]. Furthermore, colloidal nanoparticles made from polymers, for use in drug encapsulation, should be able to adsorb drug molecules on their surface or internalize the molecules within their matrix, by adsorption, dispersion or encapsulation [185].

Previously reported hollow particles produced from kraft lignin were stable in water for up to 45 days [154], however the hollow space sealed upon solvent removal, and the particles flattened upon drying [155]. For wood decay testing, the particles need to be stable for at least 6 weeks, and should be able to resist drying, as the treated wood blocks are dried prior to inoculation. Furthermore, for drug encapsulation, a stable hollow space is needed. For this, a novel nanoparticle was produced that was structurally and electrostatically stable for 8 months, and retained its structural integrity upon solvent removal, and after drying.

The possibility of encapsulating propiconazole into novel mesoporous nanocapsules is of great significance. If the nanocapsules themselves displayed antifungal activity, the nanocapsules could replace non-biodegradable materials, such as copper, in applications where propiconazole is used as a co-biocide. Similarly, the nanocapsules could serve as a stable carrier of propiconazole, resulting in a greater loading than if wood were absorbing propiconazole directly from an aqueous solution. This could also result in a more uniform distribution of the preservative in the wood blocks. Furthermore, it is possible for the nanocapsules to release their contents in a controlled, and stimuli-activated manner. Crosslinking the nanocapsules with maleic anhydride would decrease the porosity of the nanocapsules, reducing the amount of drug that diffuses out, while introducing ester linkages that could be subject to enzymatic cleavage, triggering greater release of the bio-active cargo in the presence of fungal enzymes.

In this work, we utilized the kraft lignin precipitated in **Chapter 2** at 70 °C, pH 4 and total residence time of 2 hours, and pre-existing knowledge in the literature to synthesize a novel kraft lignin nanoparticle. This lignin sample was chosen over the condition that gave the highest mass yield at 85 °C, pH 1.7 and total residence time of 2.5 hours, because it had a lower polydispersity (**Table 2.2**) and produced more structurally-homogenous particles.

The high aliphatic hydroxyl content of the optimum sample increased its polarity [181], which could have caused it to be partially-dissolved in tetrahydrofuran (THF), and affected the colloidal stability in water.

In this project, an inventive mesoporous, uniform, double-shelled, hollow particle was produced from unmodified kraft lignin. The mechanisms inducing particle morphology and stability in water were studied. We encapsulated the common foliar fungicide, propiconazole, which has modest solubility in water into the mesoporous double-shell nanocapsule, with the expectation that the amphiphilic nature of lignin would enhance the uptake and retention of the propiconazole within the nanocapsules. The drug encapsulation and retention mechanisms for this system were also investigated.

3.2 Experimental

Figure 3.1. Schematic of overall nanocapsule system synthesis process and characterization. The nanocapsules are produced by solvent displacement, with a kraft lignin/THF solution, and an aqueous solution composed of water and ethanol containing sacrificial surfactant templates. The surfactant-free nanocapsules are loaded with propiconazole and the release profiles were studied via UV-spectroscopy. Image Created with BioRender.com.

Materials. The kraft lignin utilized in this study was precipitated from a mixed-hardwood black liquor obtained from Verso (Quinnesec, MI) through the LignoBoost® process to a final pH of 4.2 at 70 °C. The lignin was vacuum freeze-dried (FreeZone 4.5L, 77500/77510, Labconco) for 18 hours at a temperature of -40 °C [218]. The characterization methodology can be found in the supplemental document. All reagents were utilized as purchased. The ethanol (EtOH) utilized was 95% concentration, and the water ultrapure.

Nanocapsule synthesis. The nanocapsule system and release studies characterization were conducted as displayed in **Figure 3.1** above. Kraft lignin was dissolved in tetrahydrofuran (THF) (1.2 g: 35 mL) under continuous stirring for two hours at room temperature. Approximately 0.15 g remained undissolved and was separated from the liquid by decanting. A water/EtOH solution (2:1, v/v) was prepared and 220 mL was placed in a water bath at 67 ± 3 °C on a hot plate rotating at medium-low speed. To this mixture, 0.8 g of either the cationic surfactant cetrimonium bromide (CTAB) or anionic surfactant sodium dodecyl sulfate (SDS) was added. The surfactant was allowed to disperse for 20 minutes, then 20 mL of the lignin/THF solution was added dropwise with a 1 mL pipette within an interval of 20 minutes. The self-assembly was allowed to proceed under stirring for 30 minutes, then the dispersion was centrifuged (5810R, Eppendorf) for one hour at 5 °C and 1200 rpm. The supernatant was discarded, and the pellet was sonicated in a warm bath at 69 °C (M1800H, Branson) for 30 minutes with a 5% (v/v) EtOH water solution containing 1% (v/v) sulfuric acid at pH 6 to remove the surfactant [158]. This washing step was repeated twice. The nanocapsules were subsequently cross-linked in a dilute aqueous mixture containing 20 mg maleic anhydride and 200 μ L dibutyl tin dilaurate as a catalyst. The esterification was allowed to proceed for 15 minutes at room temperature. Then, the nanocapsules were washed once with EtOH, and twice with water to remove all traces of the reagents by means of centrifugation and resuspension through sonication. To create the second shell, the process was repeated on the single shell particles with 15 mL lignin/THF solution. The nanocapsules were kept suspended in distilled water at a pH of 7.

Nanocapsule characterization. The morphologies of the nanostructures were studied using transmission electron microscopy (FEI 200kV Titan Themis) operating at 60 kV. The sample preparation was done by dripping 10 μ L of the aqueous nanocapsule suspension onto a copper grid, the drop was allowed to disperse out for 2 minutes, and the excess water was removed by absorbing it from the sides of the grid using filter paper, the grids were left to dry in a desiccator for 4 days. The particles were not stained. Images collected and shown are representative after observing different areas throughout the grid.

The mean hydrodynamic particle size and zeta (ζ)-potential of the nanocapsules were measured at 25 °C by a zetasizer (Malvern Nano-ZS) at a refractive index of 1.61 [199]. The spatial distribution and relative proportions of the elements were studied by energy dispersive X-ray spectrometry (EDS), and high angle annular dark-field (HAADF) imaging techniques (STEM, FEI 200kV Titan Themis).

The samples (precipitated kraft lignin and the nanocapsules) were oven-dried at 105 °C for 3.5 hours prior to surface area measurement. These were utilized to determine the specific

surface area and the porosity of each. The dried samples were degassed (at 100 °C, 1 mm mercury (Hg) for 4 hours), and the measurements were performed by nitrogen (N₂) and helium (He) sorption respectively, through the 5-point Brunauer–Emmett–Teller (BET) method (ASAP 2020, Micrometrics).

The functional groups of the recovered kraft lignin, the empty single-shell before cross-linking, the cross-linked single-shell, and the final double-shell nanocapsule were evaluated using Fourier transform infrared spectroscopy on MIRacle A Diamond prism-ZnSe support element with 65 scans (SHIMADZU, IRTracer-100).

The average number of molecules assembled per particle (N_{ass}) was computed from **Equation 3.1**,

$$N_{ass} = \frac{M_{wp}}{M_{wm}} \quad (3.1)$$

$$\rho = \frac{M_{np}}{N_A 4\pi \left(\frac{R_h^3}{3}\right)} \quad (3.2)$$

where M_{wp} represent the molecular weight of the particle estimated from the shell hydrodynamic radius and M_{wm} represents the molecular weight of KL, and the particle density (ρ) from **Equation 3.2** [155], where N_A represents Avogadro's number, and R_h the hydrodynamic radius of the shell.

Fungicide encapsulation and release. The nanocapsules were loaded according to a published method with some modifications [157, 182]. Briefly, the lignin nanocapsules were incubated in a 50 mL centrifuge tube with a 0.01 % (v/v) propiconazole solution in water, at a ratio of 10:1 (w/w) nanocapsules to propiconazole on a mass basis. The tube was kept in the dark for 20 hours on an orbital shaker at ambient conditions. The mixture was centrifuged, and the loaded nanocapsule pellet washed once, and re-dispersed in water at a concentration of 20 mg/ml. The percentage of the mass attributed to the drug per nanocapsule could be determined by **Equation 3.4**, and **Equation 3.5** permitted the total entrapment efficiency of propiconazole in the total nanocapsule mass. The maximum absorbance for propiconazole was taken at a wavelength of 223 nm. Measurements were conducted through UV spectrophotometry using a 1 cm quartz cuvette, and the calculation performed by a calibration curve (UV-Vis-NIR spectrophotometer, Shimadzu UV-3600 Plus).

$$\text{Drug loading (\%)} = \frac{\text{weight of drug in nanocapsules}}{\text{weight of nanocapsules}} \times 100\% \quad (3.4)$$

$$\text{Entrapment efficiency (\%)} = \frac{\text{weight of drug in nanocapsules}}{\text{weight of drug fed initially}} \times 100\% \quad (3.5)$$

The drug release studies were performed by placing the loaded drug dispersion in a 6-8 kDa dialysis tube and placing it in a parafilm-sealed 500 mL glass beaker containing 150

mL of water rotating on an orbital shaker at ambient conditions. A red-fluorescence label (Cy5) was added to propiconazole via a click reaction. The details are given in the supplemental document. The specific location of Cy5-labelled propiconazole within the nanocapsule was visualized by confocal fluorescence microscopy at an excitation of 488 nm for lignin and 635 nm for propiconazole at a single confocal point. The objective lens numerical aperture was 0.9, and the magnification 40X. (Olympus FluoView, FV1000). The sample preparation was done by simply dripping the loaded nanocapsule solution on a microscope dish. To confirm the loading amount of the Cy5-labelled propiconazole into the nanocapsules, an energy dispersive x-ray spectroscopy was performed on the empty double-shell, and the loaded double-shell with the regular and the tagged preservatives, to track the relative elemental composition (STEM, FEI 200kV Titan Themis).

3.3 Results and Discussion

Kraft lignin. The first objective of this work was to make sure that the key properties of the kraft were comparable to those reported in the literature. The number averaged molecular weight (M_w) of the kraft lignin is relatively high (**Table 3.1**), as such, the chains are not expected to solubilize in ethanol, which would act as an anti-solvent [155, 219]. Moreover, the presence of hydroxyl groups induces a charge on the surface of the nanocapsule at pH values outside the pK_a of lignin (**Figure 3.2B**), thus stabilizing the structures [136, 153]. Similarly, the significant presence of sodium ion (Na^+) (**Table 3.1**) will contribute to particle stability by promoting ion- π interactions [152]. The aliphatic and phenolic hydroxyl content agrees with what has been previously reported for kraft lignin (1.65-3.28 mmol/g, and 1.58-3.58 mmol/g respectively) [134, 220, 221]. Similarly, the ratio of carbon to oxygen atoms (C:O) coincides with previous reports (2.4-3.1) [213, 222]. The physical and chemical properties suggest that the performance of our lignin should not differ from that of other kraft lignin.

Double-shell nanocapsule. The nanocapsule synthesis method reported herein yielded monodispersed, double-shelled particles, which were stable in water in the pH range 4-7 (**Figure 3.1**, **Figure 3.2 A-B**). These particles are considered fine particles with an average hydrodynamic size of 242 ± 33 nm at pH 7 (**Figure 3.2**). The self-assembly of the kraft lignin molecules was induced by solvent evaporation. It is well known that ethanol and water are mutually soluble, nonetheless, previous work on kraft lignin nanoparticles proved that in a water-ethanol system, a considerable portion of ethanol was entrapped in the hydrophobic core of the nanocapsules [155, 223, 224]. Moreover, ethanol has been reported to enhance the homogeneity of the resultant nanoparticles [223]. This leads us to a hypothesis, in a water-ethanol system, the ethanol provides specific sites for hydrophobic segregation, thus producing globules of uniform size and shape at an optimum precursor concentration. Amphiphilic molecules adopt the conformation (self-assembly) that costs them less energy with respect to the system conditions [188]. The surfactant and the lignin polymer assembled into a spherical shape (**Figure 3.1 A-D**). These properties are influenced by the synthetic methodology.

Table 3.1. Chemical properties measured for the kraft lignin utilized in this study.

Property	Value
Mw (kDa)	2.57 ± 0.06
Polydispersity ($\bar{M}$)	4.63 ± 0.13
Aliphatic OH (mmol/g lignin)	3.61 ± 0.11
Sinapyl OH (mmol/g lignin)	1.91 ± 0.77
Coniferyl OH (mmol/g lignin)	1.03 ± 0.06
<i>p</i> -Coumaryl OH (mmol/g lignin)	0.25 ± 0.03
Carboxyl OH (mmol/g lignin)	0.56 ± 0.16
Ash (%)	4.72 ± 0.34
C:O (%)	2.90 ± 0.07
Na (g/kg)	52.5 ± 3.13
Hemicelluloses (%)	1.68 ± 0.16
Xylose	1.34 ± 0.05
Glucose	0.082 ± 0.002
Galactose	0.048 ± 0.004
Mannose	0.13 ± 0.01

Synthetic methodology. The sacrificial surfactant sodium dodecyl sulfate (SDS), produced nanocapsules of a wide size range (**Figure 3.1A**), which could be due to the negative head of the surfactant electrostatically repulsing the negatively ionized lignin molecules. The sulfur content in the nanocapsules produced by SDS was monitored through energy dispersive spectroscopy (EDS), and this was utilized to assess the effectiveness of the surfactant removal method (**Table A2.1**). On the other hand, the cetrimonium bromide (CTAB) surfactant, which has a positively charged head group, yielded monodispersed nanocapsules (**Figure 3.1 B-C**). We observed that when the lignin precursor concentration was increased from 0.03 g/mL to 0.05 g/mL, under the same experimental conditions, the average hydrodynamic diameter of the double-shell nanocapsule increased as well, from 242 ± 33 nm to 489 ± 25 . This occurs as a result of enhanced nucleation-growth mechanism of the particles due to excess lignin molecules [136].

After successful removal of the surfactant (**Table A2.1**), the particles were cross-linked (**Figure 3.1G**) with maleic anhydride, which forms ester cross-links between aliphatic hydroxyl groups [176]. Crosslinking with maleic anhydride retains most of the antiradical and antioxidative properties of lignin, which stem primarily from the phenolic hydroxyl groups [225], and enhances cargo retention for drug-delivery applications [226]. Moreover, cross-linking makes the particles less hydrophilic while remaining dispersed in water. Crosslinking typically results in some volume reduction due to loss of chain ends, and this could partially contribute to the shrinking of the particles after maleylation and second shell addition (**Figure 3.1 G-H**). The shrinking could also occur as a result of adding additional weight to a coreless shell, as such, the double-shell may also contribute to the observed shrinkage. The shrinking phenomenon as a result of acetylation was also observed by Chen *et al.* [227]. Crosslinking enhances the structural integrity, and assists in generating a second shell by modestly increasing the hydrophobicity of the first shell. This is observed from a slight reduction in the ζ -potential after cross-linking (**Figure 3.1 F and G**). The increased hydrophobicity would promote surfactant attachment to the existing shell and facilitate generation of a second hollow shell [227, 228].

The two shells were successfully produced (**Figure 3.1 H-J**) and the white-black under-focus/over-focus microscope technique revealed the two detected shells are at different heights (**Figure 3.1I**). Moreover, **Figure 3.1H** suggests that the two shells are separated by a distance of ~ 6 nm, the first shell has a thickness of ~ 5 nm, and the second shell of ~ 7 nm.

Nanocapsule stability properties. For ζ -potentials between -10 mV and 10 mV, particles are considered neutral [59]. The nanocapsules become neutrally charged below pH 4, and they aggregate as a result of hydrophobic interactions (**Figure 3.2 A-B**). Above pH 7, the

molecules become significantly ionized and the nanocapsules start expanding (**Figure 3.2 A-B**). Furthermore, at elevated pH, ester bonds can undergo hydrolysis, and this could also contribute to nanocapsule expansion.

The nanocapsules have a unimodal size distribution, with a minute peak of 0.2% intensity between 0.5-0.8 μm (**Figure 3.2C**). The sizes reported by TEM are lower than the hydrodynamic sizes. This discrepancy results as the hydrodynamic size includes the water molecules that interact at the electric double layer. The particles were observed to be stable in water at pH 7 for a period of eight months (**Figure 3.2 D-E**). Nonetheless, the ζ -potential trend reveals the particles become slightly protonated over time. This could be because even though the nanocapsules are well-dispersed they may experience flocculation at high concentrations [229].

Mechanisms of formation. To study the nanocapsule formation mechanisms, the spatial distribution of the elements on the surface of the particles, and their mutual orientations were analyzed by EDS and HAADF. Changes in the functional groups and their conformations were evaluated by FTIR and UV spectra. The results showed that the ration of carbon to oxygen (C:O) is higher at the periphery of the hollow space across the surface of the first shell (**Figure A2.1 A-B**). This suggests a more hydrophobic core. The potassium ion (K^+), sodium ion (Na^+) and sulfur (S^{2-}) appear to be randomly distributed. Moreover, the presence of Na^+ in the second shell (**Figure A2.1 D**) suggests ion- π interactions could be occurring.

Figure 3.2. A) Single-shell encapsulating SDS. B) Single-shell encapsulating CTAB. C) Enlarged image of single-shell encapsulating CTAB. D) A close-up of the surfactant-core single-shell. E) Surfactant-free single-shell. F) Lateral view of surfactant-free single-shell. G) Cross-linked single-shell. H) Double-shell nanocapsule, I) White/black underfocus-overfocus revealing the two shells at different heights. J) Interior of hollow structure of double-shell nanocapsule. The copper grid is the mesh-like structure in the background. The red arrows point at the two shells.

Figure 3.3. A) Hydrodynamic size of the double-shell nanocapsule as a function of pH of solvent (water). B) ζ -potential of the nanocapsules as a function of pH of solvent (water). C) Size distribution of the nanocapsules at a solvent (water) pH of 7. D) Hydrodynamic size of the nanocapsule as a function of time in water at pH 7 for a period of 8 months. E) ζ -potential of the nanocapsules as a function of time in water at pH 7 for a period of 8 months. The letters represent the results from Tukey simultaneous tests for the difference of means on the hydrodynamic size as a function of pH. Analysis performed in Minitab. averages that do not share a letter are significantly different.

Table 3.2. Properties and characteristics of kraft lignin, single-shell nanocapsules, and double-shell nanocapsules.

	Specific Surface Area [SA _p] (cm ² /g)	Average Pore Volume [V _p] (mm ³ /g)	Average Pore Width [W _p] (nm)	Entrapment efficiency (%)	Average Number of Lignin Molecules Assembled [N _{ass}]	Nanocapsule Density [ρ] (g/cm ³)	Hydrodynamic radius [R _h] (nm)
Kraft lignin	650	0.1	7.1	-	-	-	-
Single shell	1,590	1.0	26.2	34.7 ± 0.9	9.4 × 10 ³	0.7	22.9 ± 0.3
Double shell	2,700	0.9	13.2	56.1 ± 2.1	9.0 × 10 ⁴	0.8	48.4 ± 6.7

Figure 3.4. UV spectra of kraft lignin and double-shell nanocapsule. Image edited with BioRender.com.

The ultraviolet spectrum of kraft lignin and the double-shell nanocapsules revealed a bathochromic (red) shift in the nanocapsule curve (**Figure 3.4**). This shift is typical of a J-aggregation, and it has been interpreted as revealing π - π interactions between benzene rings [154]. J-aggregation is a head-to-tail orientation between interacting rings. The hyperchromic shift and the adsorption peak at 232 nm in the double-shell spectrum could be attributed to the increased presence of carboxyl groups from the esterification, thus increasing the absorptivity [174, 230].

Fungicide encapsulation. Lignin nanostructures can be employed in applications than exploit the inherent properties of lignin, such as UV-protection [196, 231]. In this work, the inherent ability of lignin to act as radical scavenger is being exploited [232]. This property is enhanced in nanomaterials compared to the raw molecule [148]. In this work, this property is used for the purpose of creating a dual action, anti-fungal system. Nanomaterials possess the quality of high surface area to volume ratio [158], an interesting trait for optimum cargo loading [134]. Surface area provides available space that cargo may occupy. The specific surface area (SA_p) of kraft lignin increased by 2.5 times in the single shell and 4.2 times in the double-shell (**Table 3.2**). Similarly, the average pore dimensions also increased, however, the average pore volume (V_p) of the single-shell is 16.9% higher than that of the double-shell, and the average pore width (W_p) was 12.95 nm larger in the single-shell. There is a reported trade-off between SA_p and V_p [158], the larger the surface area, the smaller the pore volume. Moreover, the pore distribution in the second shell, as well as cross-linking may have contributed to the reduction of W_p . Surface area is the primary factor aiding cargo loading, as such, the entrapment efficiency of the fungicide propiconazole in the double-shell is 21.4 % higher than in the single-shell, and the drug loading of the double-shell was calculated to be 5.6 %. Moreover, the larger pore width

(W_p) in the single-shell may have reduced cargo entrapment by facilitating cargo diffusion into the storage medium [227]. Moreover, the double-shell possesses a double barrier which would further reduce diffusion. Propiconazole has modest solubility in water and would result in a preference to settle in the hydrophobic core of the nanocapsule in an aqueous media and remain there. Moreover, slowing down the diffusion out of the nanoparticles. The density (ρ) of the double-shell is less than that of water, as such, the particles are expected to float in the storage media. The number of molecules (N_{ass}) forming the second compartment is $\sim 8.5\times$ larger than the number of molecules forming the first shell, which could contribute to particle elongation in the double-shell (**Figure 3.1H**).

According to the FTIR spectra, the hydroxyl peak is broader in nanocapsule conformations (**Figure A2.2**). This could be due to the stacking of the molecules in nanocapsule form resulting in inter, and intramolecular hydrogen bonding [177]. The FTIR spectra reveals that crosslinking increases the concentration of carbonyl hydroxyl groups, carbonyl (C=O), alkene (C=C) and ester (C-O) groups, indicating that the esterification has taken place. All the nanocapsule spectra show the presence of intermolecular hydrogen bonding, which is inferred from the broad hydroxyl stretch in the region $3350\text{--}3200\text{ cm}^{-1}$, and which would contribute to the particle stability in water. THF absorption peaks are seen at $3125\text{--}3165\text{ cm}^{-1}$. None of our spectra have additional peaks in this region, so this confirms the absence of THF in the particles [153].

Figure 3.5. A) Confocal fluorescence microscopic image of double-shell encapsulating propiconazole, taken six-days post-incubation. B) Release profile of propiconazole from double-shell. C) Pore thickness distribution in single-shell and double-shell.

Based on confocal imaging, and the increase in nanocapsule nitrogen content, the double-shell nanocapsules successfully encapsulated propiconazole (**Figure 3.5A**, **Table A2.2**), as a result, the hydrodynamic radius (R_h) of the double-shell increased from 120.9 ± 16.65 nm (including hollow), to 212.3 ± 8.5 nm, but the ζ -potential decreased slightly from -38.65 ± 0.64 mV to -27.1 ± 1.5 mV. The double-shell nanocapsules displayed slow release of propiconazole (**Figure 4B**), retaining 15.7 % of the initially loaded drug by the end of the second week. This sustained release of active cargo will prolong the active effect of the fungicide [233]. Furthermore, the size and polydispersity of a particle contribute to the stability of the cargo release, and the double-shell particles are considered monodispersed (polydispersity less than 0.7) [234], and fall within the nanoparticle-fine particle range. The single-shell and double-shell nanocapsules possessed similar pore thickness (~ 0.35 - 0.5 nm), while the double-shell had an extra pore thickness dimension at 0.61 nm, which could be attributed to the second shell (**Figure 3.5C**).

Mechanisms of fungicide encapsulation. The mechanism of propiconazole encapsulation into the double-shell nanocapsules was investigated by UV spectroscopy (**Figure 3.6**). It is expected that the propiconazole molecules settling in the pores of the nanocapsule will sterically hinder intermolecular hydrogen bonding [235]. This would result in a slight decrease in electrostatic stability (**Figure 3.5A**).

Figure 3.6. UV spectra of double-shell with and without propiconazole.

The UV-spectra of the double-shell nanocapsules with and without propiconazole (**Figure 3.6**) reveals a hypochromic shift. This could be due to molecular stacking between the double-shell and propiconazole, or due to the submerged hydroxyl groups, which resulted

in a slight decrease in absorptivity. This phenomenon could also be responsible for the decreased electrostatic stability of double-shell nanocapsules upon loading propiconazole. As such, it can be concluded that diffusion, and hydrophobic interactions drive the encapsulation process.

3.4 Conclusions

We have synthesized unprecedented double-compartment nanocapsules from unmodified kraft lignin. These structures are monodispersed and stable for periods of up to 8 months in water at neutral pH, as indicated by stable hydrodynamic size. Similarly, the ζ -potential, remained strongly negative for the same duration. The increased carbon to oxygen ratio at the periphery of the hollow cavity reveals the hydrophobic nature of the hollow cores. The mechanisms driving the nanocapsule formation are believed to be hydrophobic interactions, nucleation-growth, π - π stacking of benzene rings, plausibly ion- π stacking, and intermolecular and intramolecular hydrogen bonding. Upon successful encapsulation of propiconazole into the double-shell capsule by diffusion and hydrophobic interactions, the nanocapsules displayed slow release kinetics preserving ~16 % of the initially loaded drug at the end of two weeks. This novel synthetic methodology will support designing diverse lignin nanoparticles for different uses.

4 Wood decay studies

4.1 Introduction

In this project we evaluate and compare the anti-fungal efficacy of the various lignin-based materials produced in **Chapter 2** and **Chapter 3**, namely kraft lignin, the empty single-shell nanocapsule, the propiconazole loaded single-shell nanocapsule, the empty double-shell nanocapsule, and the propiconazole-loaded double-shell nanocapsule. The efficacy of these were compared to aqueous solutions of propiconazole and chromated copper arsenate at comparable retained concentration. These studies were conducted according to the American Wood Protection Association wood decay testing standard under controlled laboratory conditions [236]. The comparable concentrations in the woodblocks treated with propiconazole encapsulated into nanocapsules, and water-dissolved propiconazole will enable us to discriminate the antifungal contributions from each component in the nanocapsule-propiconazole combined system. To determine if the observed efficacy for the lignin-based preservatives was significant in comparison to that of the untreated control block, and in relation to each other, a Tukey's pairwise comparison of means statistical analysis was performed. We also evaluated the concentration dependence of the tests using Kirby-Bauer disk diffusion experiments. The disk diffusion tests enabled us to determine the sensitivity of *G. trabeum* to each of the preservatives, and assign a category of inhibition to individual preservatives based on the zone of inhibition formed. Furthermore, these tests enabled us to elucidate whether the inhibition efficacy was concentration dependent and if the inhibition observed in the wood block tests were limited by preservative uptake.

Previous studies evaluating the anti-fungal properties of kraft black liquor and lignin made no mention of the pH of their solutions, nor of incorporating alkaline controls in the system [137, 139]. They reported satisfactory results, nonetheless, it would be important to evaluate kraft lignin at pH 7 to determine if the antifungal effect is solely due to the properties of lignin, or the high pH had an effect in inactivating the fungi.

In this work, double-shelled nanocapsules were produced from kraft lignin to overcome the hydrophobicity of the raw material and to serve as a carrier for the organic antifungal, propiconazole. The lignin nanocapsules were highly dispersible and stable in water [237], and by containing and controlling the release of propiconazole, could increase the amount of fungicide that penetrates the wood. Southern yellow pine was pressure and dip treated using empty and propiconazole-loaded lignin nanocapsules and the loading and penetration of the nanocapsules into the wood were evaluated. The efficacy of the preservatives at combating fungal degradation by *G. trabeum* was evaluated compared to that of the conventional preservative, chromated copper arsenate, and propiconazole without encapsulation. The specific mechanisms of action of the lignin-propiconazole system were also investigated.

4.2 Experimental

Materials. Kraft lignin (KL) was recovered from hardwood black liquor according to our previous work [218]. The black liquor was precipitated to a final pH of 4.2 at 70 °C. Analytical grade propiconazole (PCZ) was purchased from Sigma Aldrich, and 14.3% propiconazole from Keystones Pest Solutions. Southern yellow pine (SYP, *Pinus spp.*) sapwood blocks (19 x 19 x 19 mm), feeder strips, chromated copper arsenate (CCA) and the fungus *Gloeophyllum trabeum* (ATCC 11539) were supplied by the Michigan Technological University Wood Protection Group. Virgin topsoil from the forest was employed in the standard soil-jar studies and the water was distilled. Free-radical 2,2-diphenyl-1-picrylhydrazyl (95% DPPH) was purchased from Fisher Alfa Aesar.

Lignin-propiconazole nanocapsule synthesis. The method for preparing loaded nanocapsules is as reported in our previous work [237]. Briefly, 1 g of kraft lignin was dissolved in 35 mL of tetrahydrofuran. This solution was inserted dropwise into a water/ethanol solution containing sacrificial surfactant templates. The surfactant was washed off, and hollow, single-shelled (SS) nanocapsules were formed. These were crosslinked with maleic anhydride, and the synthesis procedure was repeated on the SS nanocapsules to create a second shell. The hollow, double-shelled lignin nanocapsules (DS) and the SS were loaded with propiconazole (DS+PCZ and SS+PCZ) by diffusion.

Wood impregnation. Preservative treatments of 0.53 mg/mL for KL, SS, DS, CCA, SS+PCZ, DS+PCZ, and 0.04 mg/mL for PCZ were prepared. These concentrations were chosen based on the recommended retention of CCA for above-ground applications, and the concentration of propiconazole in CA-C. Preservative impregnation was implemented by two methods: dip-diffusion and vacuum impregnation. For each treatment method, ten carefully selected wood blocks were immersed in 1 L of treatment solution. For dip-diffusion, samples were left in the solution for 5 minutes. For vacuum impregnation, the samples were placed in a small-scale cylinder at 25 inch Hg vacuum for 30 minutes, followed by pressure treatment at 110 psi for one hour. Treatment retention was measured according to **Equation 4.1**, where G represents the weight (g) of the treating solution absorbed, C the grams of preservative in 100 g of treating solution, and V the volume of the block before treatment in mL [236].

$$\text{Retention} \left(\frac{\text{mg}}{\text{ml}} \right) = \frac{G \times C}{V} \times 10 \quad (4.1)$$

The treatability was measured according to **Equation 4.2**, where Retention_a represents the amount of treatment that was impregnated into the wood, and Retention_t represents the original amount of preservative in the solution.

$$\text{Treatability (\%)} = \frac{\text{Retention}_a}{\text{Retention}_t} \times 100 \quad (4.2)$$

Five treated blocks per treatment type were selected based on similar actual retention to proceed with the inoculation. The wood blocks were oven-dried at 40 °C for 24 hours, wrapped in aluminum foil and steam-sterilized for 30 minutes prior to inoculation.

Soil-jar preparation. The fungal inoculum was grown for 10 days before inoculation on a 2% malt extract agar plate. Culture bottles were filled with 100 g of topsoil and 30 mL water and then, the day before inoculation, were autoclaved at 120 °C, 15 psi for 45 minutes.

Inoculation. A single wood block was inserted into a soil-jar with 1 cm long inoculum agar squeezed between the feeder strip and the woodblock, following the standardized protocol for brown rot soil-block test [236]. The prepared culture bottles were incubated for 12 weeks at 27 °C and 80% relative humidity, according to the American Wood Protection Association (AWPA) standards [236].

Wood characterization. After 12 weeks of incubation, the mycelium and soil surrounding the decayed wood blocks were carefully brushed off. The blocks were dried at 40 °C for 24 hours, and the extent of degradation was evaluated by weight loss percentage. The elemental environment on the surface of the wood blocks before and after decay was studied using a X-ray fluorescence spectrophotometer (Xenometrix EX-6600 EDS). The functional group change in the wood blocks before and after decay were studied by Fourier transform infrared spectrophotometer (SHIMADZU, IRTracer-100). The nanocapsules were observed inside the wood tissue using transmission electron microscopy (FEI 200kV Titan Themis) operating at 60 kV. The specimen was prepared by scraping wood into water, and then pipetting 10 µL of the wood suspension onto a copper grid. The drop was left to disperse for 2 minutes, and the excess water removed by absorption with filter paper. The grids were dried in a desiccator for 2 days at ambient conditions. To determine cellulose crystallinity of the wood blocks before and after decay, the crystallinity index (C_rI) was measured based on the procedure developed by Segal et al. [238]. Diffraction data was collected from 5° to 40° 2θ, with a step size of 0.05° and a 10 second count time, using a Scintag XDS-2000 θ/θ diffractometer equipped with a copper target x-ray tube (ThermoARL Dearborn, MI). The x-ray optics consisted of 1 and 2 mm beam slits, 0.5 and 0.3 mm receiving slits with a graphite crystal monochromator. C_rI (**Equation 4.3**) is based on the intensity area sum of cellulose I and cellulose II (I_{200}) diffraction area peaks located at ~22.5° and ~21° 2θ respectively minus the amorphous area (I_{am}) with a centroid of the peak located at ~18° [238]. Analysis was performed using Jade diffraction software (MDI Livermore, CA).

$$C_rI (\%) = \left(\frac{I_{200} - I_{am}}{I_{200}} \right) \times 100 \quad (4.3)$$

Kirby-Bauer disk diffusion test. Solutions at various concentration categories were prepared for each of the test preservatives (kraft lignin, empty double-shell nanocapsule, double-shell nanocapsule + propiconazole, and propiconazole) (**Table 4.1**). The negative inhibition control solution was deionized water, and the positive inhibition control was an 800 mg/L solution of chromated copper arsenate. *Gloeophyllum trabeum* (ATCC 11539)

was grown on agar plates (150 x 15 mm) containing 2% malt extract for 9 days at 27 °C and 80% relative humidity. The plates were kept inside sterile plastic bags to increase the humidity. Antimicrobial filter disks (6 mm, grade AA, Whatman™) were dipped into the various preservative solutions (**Table 4.1**) for 5 minutes, and the impregnated disks were dried for 24 hours at 40 °C. The dried treated disks were autoclaved using a wrapped cycle at 135 °C for 10 minutes (Sterilemax 1277, Thermo Scientific). On the ninth day of fungal inoculation, the treated disks were added to the agar plates at 60 ° from each other and 3 cm from the wall of the plate. Samples in the same concentration category (**Table 4.1**) were run on the same agar plate in triplicate. Following six days of incubation post addition of the filter disks, the diameter of the zone of inhibition was measured, and level of inhibition was assigned based on an ASTM procedure [239].

Anti-radical activity test. The antioxidant activity of the various preservatives assayed according to a modified standard method [240]. A solution of the free-radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol (40 µg/ml) was prepared, and the absorbance maxima measured using a UV-Vis-NIR spectrophotometer (Shimadzu UV-3600 Plus) at 517 nm against methanol. To 3.8 mL aliquots of this solution, 200 µl of solutions of kraft lignin, single-shell nanocapsule, double-shell nanocapsule and propiconazole in methanol at concentrations of 0.05, 0.075 and 0.1 mg/ml were added. The mixture was allowed to react in the dark at room temperature for 45 minutes and the absorbance was measured to assess the free radical scavenging activity (S_{ac}) according to **Equation 4.4** where $Absorbance_{control}$ represents the absorbance of the DPPH solution with 200 µl of methanol. The samples were prepared freshly before analysis.

$$S_{ac} (\%) = \left(\frac{Absorbance_{control} - Absorbance_{sample}}{Absorbance_{control}} \right) \times 100 \quad (4.4)$$

Table 4.1. Concentrations of treatments used in the disk-diffusion study.

Category	Highest concentration in wood block* A	Lowest concentration in wood block* B	Highest concentration C	Lowest concentration D
Treatments	Concentration (mg/L)			
Kraft lignin	420	200	1800	100
Empty double-shell nanocapsule	470	400	1800	100
Double-shell nanocapsule + propiconazole	310 (20 ^p)	60 (3 ^p)	1800 (100 ^p)	100 (10 ^p)
Propiconazole	30	2	100	10

* Highest or lowest concentration in wood block by either dip diffusion or vacuum impregnation.
^p Propiconazole concentration (mg/ml treatment solution), based on the amount encapsulated in the wood preservatives and the total nanocapsule retention.

Statistical Analysis. The weight losses recorded for the various preservative treatments for both impregnation methods (dip and vacuum) were input into Minitab with five experimental replicates per sample. A one-way analysis of variance (ANOVA) was conducted to analyze the Tukey simultaneous tests for the difference of means using a two-

sided confidence interval of 95%. A general linear model analysis of variance (ANOVA) was also generated at a significance level of 0.05 if the mode of preservative impregnation had an effect on the decay.

4.3 Results and Discussion

Table 4.2. Treatability and retention of the various treatments into the wood block by dip-diffusion and pressure impregnation. Average and standard deviation for each treatment and method is based on 3 replicate wood blocks.

Treatment	Retention (mg/ml)		Treatability (%)	
	<u>Dip</u>	<u>Vacuum</u>	<u>Dip</u>	<u>Vacuum</u>
Single shell (empty)	7.3 ± 0.1	3.6 ± 0.4	95.1 ± 2.2	47.2 ± 6.8
Double shell (empty)	7.2 ± 0.1	6.1 ± 0.1	93.7 ± 2.2	78.3 ± 1.1
Single shell + propiconazole	5.9 ± 0.3	2.9 ± 0.4	77.4 ± 7.5	37.7 ± 10.2
	^P (0.21 ± 0.01)	^P (0.14 ± 0.01)		
Double shell + propiconazole	1.0 ± 0.2	4.7 ± 0.3	12.1 ± 4.4	61.6 ± 4.7
	^P (0.06 ± 0.01)	^P (0.26 ± 0.02)		
Kraft lignin	6.5 ± 0.2	2.5 ± 0.6	84.3 ± 4.7	33.0 ± 9.3
Propiconazole	0.3 ± 0.1	0.2 ± 0.1	62.5 ± 1.8	37.5 ± 1.1
Chromated copper arsenate (CCA)	2.3 ± 0.5	6.5 ± 0.1	30.2 ± 6.7	84.1 ± 0.4

^P Propiconazole concentration (mg/ml treatment solution), based on the amount encapsulated in the wood preservatives and the total nanocapsule retention.

The anti-fungal nanocapsule systems are comprised of particles that are well-dispersed and stable in water (DS+PCZ, 424.6 ± 17.1 nm, -27.1 ± 1.5 mV) [237]. These properties are important to ensure the nanocapsules do not settle and impede treatment. Furthermore, the nanocapsules are small enough to allow for penetration into the macro and mesopores in wood (80-58000 nm diameter) [58, 59]. The results on treatability and retention (**Table 4.2**) suggest that pressure impregnation is a more effective treatment for the double-shell + propiconazole and CCA. This is the industrial mode of treatment for CCA, and so the results align with what would be expected. On the contrary, dip-diffusion resulted in higher retention for empty double shell capsules and propiconazole, the latter of which has been approved by EPA to be impregnated in wood by both surface and pressure methods [75]. We believe the explanation as to which mode of treatment is more appropriate could lie in

the inherent resistance to flow and solubility of the treatments [241]. In dip-treatment, the flow is driven by capillary action, whereas in pressure treatment, flow is driven by a pressure gradient. The treatability and retention results suggest that KL, SS, DS, SS+PCZ and DS+PCZ could be impregnated into the wood blocks. Moreover, DS and DS+PCZ have uniform distribution for both treatments, and KL, SS and SS+PCZ have uniform distribution with dip-diffusion treatment; treatability and retention are important characteristics that ensure the efficacy of the preservative formulation [242, 243].

At the end of the three-month inoculation period, the weight loss in the wood blocks was measured, and physical deterioration captured (**Figure 4.1** and **Figure 4.2**). *Gloeophyllum trabeum* prefers to feed on carbohydrates, primarily cellulose, but simultaneously modifies the lignin by oxidation and demethylation [4]. The result is a degraded cell wall accompanied by significant losses in weight and strength (**Figure 4.1** and **Figure 4.2**). In this study, significant wood block degradation was characterized by obvious visual compression of the wood structure and brown discoloration, as is typical of brown rot (**Figure 4.1**). The average weight loss for all treatments, with the exception of the pressure treatment DS+PCZ and CCA, were not statistically different compared to the control block (**Figure 4.2**). This means that the DS+PCZ represents the most efficacious lignin-based treatment explored in this work. The success of the DS+PCZ formulation is attributed to the ability of the nanocapsules to increase the treatability and retention of propiconazole in the wood, as propiconazole on its own displayed poor treatability and consequently poor preservative effect. From this data it is unclear whether the double-shell nanocapsules themselves possess antifungal activity that enhances the activity of the propiconazole or if they simply serve as a carrier. Although the DS+PCZ wood blocks were darker at the end of the incubation period compared to the CCA blocks (**Figure 4.1**), this does not seem to be due to the effect of fungal degradation, but may be due to oxidation of the lignin nanocapsules over time, as immediately following treatment, the samples all looked similar in color to the original woodblock (**Figure 4.1A**).

Figure 4.1. Extent of SYP wood appearance degradation. A) Original state of the wood block before decay. B) Control block after decay. C) Pressure-treated DS block after decay. D) pressure-treated DS+PCZ block after decay. E) Pressure-treated PCZ block after decay. F) Pressure-treated CCA block after decay. The DS and PCZ treated blocks sections in the center appear to be less degraded, probably as a result of non-uniform distribution of the treatment.

Figure 4.2. Weight loss of the control and treated wood blocks after decay. The letters represent the results from one-way ANOVA Tukey simultaneous tests for the difference of means in Minitab at a two-sided confidence interval of 95%. Bars that do not share a letter are significantly different.

From our results, for each preservative type, the greater the treatability, the lower the weight loss, with the exception of DS, SS+PCZ, and PCZ. Although dip-diffusion resulted in a higher treatability, the vacuum-pressure treated blocks performed better on an average basis. It was observed after treatment that KL and SS appeared as stains on the surface (grain side) of the wood block. We believe that although treatability by dip diffusion was high for these treatments, poor depth of penetration into the wood structure may be one reason for poor performance. Additionally, the lignin itself may have had insufficient antifungal activity to act as a sole preservative.

A general linear model analysis of variance (ANOVA) was generated to analyze statistically if the treatment type and preservative type have a direct correlation with the extent of degradation. Our results at a significance level of 0.05 confirm that treatment type (dip-diffusion vs. vacuum-pressure) has no statistically significant effect on the degradation, while the type of preservative does (KL, DS, DS+PCZ, SS, CCA). The efficacy of the most effective lignin-based treatment (vacuum-pressure treated DS+PCZ) is statistically comparable to that of vacuum-pressure treated CCA.

The specific location DS+PCZ settles in the wood block was visualized by TEM at 1-2 mm below the surface (**Figure 4.3**). It can be observed that DS+PCZ settled within the cell lumen. The fungi access the the cell wall contents through the cell lumen, and the localization of the fungicide in the lumen will provide greater opportunity for contact with the fungi [244]. Also, being situated in the cell lumen hamper the diffusion of enzymes and chemicals from the fungus into the cell and, likewise, the transfer of water and nutrients

from the cell to the fungus. The depth of penetration of the anti-fungal system into the wood block was estimated based on the sodium and sulfur content across the wood block. The block originally contained an undetectable amount of sodium, and $\sim 17 \text{ mg/cm}^2$ sulfur (Table 4.3), but as there is some sodium and sulfur associated with the lignin nanocapsules from the kraft lignin precursor (Table A2.2.3), this can be used to indicate the presence of nanocapsules within the wood. As observed in Table 4.3, sodium was not detected in the virgin wood block. However, upon DS+PCZ incorporation, sodium was observed up to a depth of $\sim 5 \text{ mm}$ into the surface of the wood block (Table 4.3), indicating nanocapsules had also penetrated to that depth. Additionally, the sulfur, some of which is covalently bound to kraft lignin, was $\sim 3 \text{ mg/cm}^2$ higher at 4.5 mm and $\sim 5 \text{ mg/cm}^2$ higher at the surface, compared to the center of the wood block. This again indicates that the nanocapsules penetrated at least to 4.5 mm in depth into the wood block.

Figure 4.3. A) Location of DS+PCZ inside the SYP wood block approximately 1-2 mm below the wood surface. B) Enlarged image of region circled on A.

Table 4.3. Elemental concentrations of control block and sections within the pressure-treated double-shell + propiconazole (DS+PCZ) block before decay.

Elemental concentrations before decay (mg/cm^2)					
<u>Sections across DS+PCZ treated block</u>					<u>Control block</u>
<i>Element</i>	0 mm ^S	4.5 mm	7.5 mm	8.5 mm	1 mm
Na	1.4 ± 0.1	0.9 ± 0.1	*	*	*
Al	50.3 ± 3.2	49.7 ± 3.0	49.6 ± 3.2	50.5 ± 3.2	47.1 ± 4.1
S	25.9 ± 1.5	20.5 ± 1.2	17.5 ± 1.3	17.2 ± 1.2	17.3 ± 1.1
K	6.7 ± 1.8	8.1 ± 1.5	5.5 ± 1.7	5.9 ± 1.6	6.1 ± 1.8
Ca	15.7 ± 2.1	20.7 ± 1.9	27.3 ± 2.1	25.8 ± 2.0	29.5 ± 2.5

* Not detected in significant amounts

^s Surface/face of wood block

Table 4.4. Crystallinity index of the control and pressure-treated wood blocks before and after decay. The error represents the standard deviation from three technical replicates.

Treatment	CrI (%) ^a	
	<u>Before decay</u>	<u>After decay</u>
Control	71.8 ± 5.0	66.5 ± 13.7
DS	75.9 ± 7.1	67.1 ± 22.5
DS+PCZ	82.1 ± 11.5	55.6 ± 15.7
PCZ	68.4 ± 19.4	58.9 ± 14.5
CCA	64.7 ± 4.1	59.2 ± 16.1

^a All means are statistically identical per Tukey's comparison in Minitab.

Cellulose constitutes 40-45% of the total dry weight of wood. It is formed by D-glucose monomers that are linked together by β -1,4-glycosidic bonds [14]. The cellulose matrix contains both, crystalline regions where the individual polymer chains are linked by highly complex bonding patterns, and amorphous regions [245]. Thus, the crystallinity index is expected to decrease after decay, particularly in the presence of fungicides [14]. The crystallinity index (CrI) of the wood blocks was evaluated before and after decay in the control block, the most effective treatments (CCA and DS+PCZ), and their constituents (DS and PCZ). The CrI decreased in the decayed control and pressure-treated wood blocks (**Table 4.4**). The biggest decrease of 26.5% was observed for the DS+PCZ treatment, followed by 19.5% in the DS treatment. Nonetheless, only 5.3% crystallinity loss was observed for the control block. However, as determined by Tukey's comparison of means analysis, all the averages are statistically equal, as such, there is no actual difference in the CrI change between the control and vacuum-pressure treated blocks. And the change in crystallinity index appears to have no relationship with the amount of fungal degradation.

Gloeophyllum trabeum depolymerizes carbohydrates through an oxidative system employing Fenton's reagents ($\text{Fe}^{+2} + \text{H}_2\text{O}_2$) [244]. We observed an increase in the content of iron (Fe) in the wood block after decay (**Table 4.5**), and with the exception of the CCA-treated block, the manganese (Mn) content also increased for the remaining blocks after decay (**Table 4.5**). Manganese is known to participate in the degradation of lignin [246]. Other papers have previously reported an augmentation in the concentration of these two elements in the wood block after fungal decay. These are transported by fungi from the soil into the wood block [247].

Table 4.5. Transition state metals concentration change in wood blocks before and after decay.

Elemental concentration Before Decay (mg/cm ²)					
<u>Element</u>	<u>Control</u>	<u>DS</u>	<u>DS+PCZ</u>	<u>PCZ</u>	<u>CCA</u>
Mn	*	*	1.3 ± 0.3	4.8 ± 2.4	6.6 ± 0.8
Fe	*	*	*	4.4 ± 2.8	1.1 ± 0.6
Elemental concentration After Decay (mg/cm ²)					
Mn	6.8 ± 2.1	8.8 ± 1.2	7.1 ± 0.4	6.3 ± 1.5	3.9 ± 0.4
Fe	7.6 ± 2.5	*	7.2 ± 0.4	6.4 ± 1.8	3.6 ± 0.4
* Not detected in significant amounts					

Kirby-Bauer disk diffusion tests

This method makes use of filter paper disks that had been soaked in specific preservative solutions, dried and placed on an agar petri-dish. During this experiment, the anti-microbial agent diffuses into the agar around the treated disk. If the agent is inhibitory to the microorganism, this results in a ring around the disc where no growth is observed, whose size is dependent on the level of inhibition and rate of diffusion of the chemical to the agar. In this work, four concentrations per lignin-based preservative treatment were evaluated, to determine an approximate minimum inhibitory concentration for the effective preservatives. Following nine days after fungal incubation, the mycelium was expanding uniformly in a circular pattern (**Figure A3.2.1**), at this stage, the treated disks were added. Following six day after adding the treatment-impregnated disks, the disks treated with propiconazole and those treated with the double-shells encapsulating propiconazole had inhibition zones (**Figure 4.4**) for all concentration levels, except for propiconazole at the lowest concentration (B = 2 mg/L propiconazole) (**Table 4.6**). The fact that the double-shell + propiconazole showed a large zone for almost the same concentration (B = 3 mg/L propiconazole) indicates that either this concentration represents a critical threshold in antifungal activity, or the nanocapsules provide some synergistic activity at low propiconazole concentrations. It is possible that the negative performance of CCA and the lignin-based preservatives (without propiconazole) (**Table 4.6**) was due to the fact that these compounds were not able to diffuse into the agar, as this is the mechanism required for the assay. Moreover, the presence of mycellium in the direct vicinity, and on top of the CCA treated disk suggests no significant anti-fungal activity. Nonetheless, the mycelia growth in some regions on the agar plate, predominantly around the CCA treated disk was fluffier, this effect is associated with a difference in the absorbed sugars. This could result from a stress mechanism as a results of the activity of the treatments.

Figure 4.4. State of agar dishes 16 days post fungi inoculation / 7 days after inserting the impregnated disks.

Table 4.6. Diameter of *G. trabeum* inhibition zone based on disc diffusion experiments

Category	Highest concentration in wood block*	Lowest concentration in wood block*	Highest concentration	Lowest concentration
Treatments	A	B	C	D
Kraft lignin	ND	ND	ND	ND
Empty double-shell nanocapsule	ND	ND	ND	ND
Double-shell nanocapsule + propiconazole	18 ± 8 mm	21 ± 4 mm	18 ± 8 mm	23 ± 3 mm
Propiconazole	14 ± 6 mm	ND	17 ± 8 mm	17 ± 2 mm

* Highest or lowest concentration in wood block by either dip diffusion or vacuum impregnation.
^P Propiconazole concentration (mg/ml treatment solution), based on the amount encapsulated in the wood preservatives and the total nanocapsule retention.

Antioxidant activity tests

It has been reported that lignin nanocapsules would have enhanced radical scavenging activity compared to the raw lignin molecule [148]. The radical scavenging activity (Figure 4.5) gives an indication of the anti-oxidative capacity of propiconazole, and the various individual lignin-based treatments. The reactivity of DPPH explored in this work, is lower than that of oxygen containing free radicals, such as the hydroxyl radical ($\cdot\text{OH}$), nonetheless, it has been used as a standard to evaluate the antioxidant properties of lignin [248, 249].

Figure 4.5. Radical scavenging assay of kraft lignin, single-shell nanocapsules, double-shell nanocapsules and propiconazole at three different concentrations.

The anti-radical activity results (**Figure 4.5**) suggest that propiconazole, kraft lignin, the single-shell nanocapsule, and the double-shell nanocapsule all have anti-radical activity. At the lowest concentration of 0.05 mg/ml, kraft lignin and propiconazole displayed equal anti-radical activity, similarly, the single-shell and the double-shell nanocapsules also displayed similar anti-radical activity, which was higher than that of kraft lignin and propiconazole. At a treatment concentration 0.075 mg/ml, the anti-radical activity increased for all the treatments, in the increasing order of propiconazole, kraft lignin, single-shell nanocapsule and double-shell nanocapsule; nonetheless, at a treatment concentration of 0.1 mg/ml the anti-radical activity of propiconazole, kraft lignin and the single-shell did not increase, suggesting that above 0.07 mg/ml the anti-radical activity is not concentration dependent. On the contrary, the anti-radical activity of the double-shell nanocapsule continued to increase at 0.1 mg/ml, and the augmentation in anti-radical activity at 0.075 mg/ml and 0.1 mg/ml was much higher than that of kraft lignin and the single-shell nanocapsule. The increased performance of the double-shell at 0.075 and 0.1 mg/ml could be attributed to the incorporation of an alkene group, a carbonyl group and a hydroxyl group during cross-linking, this enhanced effect appears to be relevant at concentrations higher than 0.075 mg/ml, which would facilitate the transfer H-atoms, and aid in stabilizing the lignin radicals formed [248]. These results suggest that at the maximum concentration levels impregnated into the wood blocks, propiconazole (~0.003 mg/ml), kraft lignin (~0.44 mg/ml), the single-shell and the double-shell (~0.49 mg/ml), the radical neutralization activities are expected to be lower than 14% for propiconazole ~27% for kraft lignin, ~38% for the single-shell, and higher than 73% for the double-shell nanocapsule.

The anti-oxidant activity of lignin against DPPH occurs via electron, and H-atom transfer [250]. Other studies on lignin anti-radical activity have indicated that greater concentrations of phenolic groups, methoxy groups aliphatic hydroxyl groups, and pi-conjugation [150, 248, 251-253], lead to greater anti-radical activity. This is relevant because high anti-radical activity of lignin has been linked to high anti-bacterial activity [248], and so could also be correlated with high anti-fungal activity. The anti-radical

activity of propiconazole stems from its triazole group, which employ protons in their structure to neutralize free radicals [254].

The radical scavenging activity of phenolic compounds depends on two factors, the transfer of H-atoms to the free radical and the stability of the new radical formed, and the stability of the new radical formed will be enhanced by a conjugation with an adjacent group [248]. Nonetheless, conjugation to a carbonyl group forming part of the propanoid side chain will result in an unstable group and thus, decrease the overall anti-radical activity [248], while the presence of carboxylic groups attached to the benzene ring increases the polarity, retards homolytic cleavage, and increases the anti-radical activity [248]. The presence of these groups will increase the electron withdrawing induction effect, thus facilitate the transfer of electrons to the free radicals.

The anti-radical activities found in this study for kraft lignin were compared to values reported in the literature. Bhat *et al.* precipitated lignin from palm oil black liquor using sulfuric acid, they observed anti-radical activities against DPPH of approximately 62 and 65% at 0.05 and 0.1 mg/ml respectively using dimethyl sulfoxide as a solvent; and anti-radical activity of approximately 80 and 78% at 0.05 and 0.1 mg/ml respectively using 2-methoxy ethanol as a solvent [255]. Meliana and Setiawan studied the effect of precipitation pH and treatment concentration on the anti-radical activity of palm fruit lignin from bioethanol byproduct, they found an anti-radical activity against DPPH of 39.3 and 54.9 % for lignin precipitated at pH 2, and 14.4 and 15.1 % for lignin precipitated at pH 12 using methanol as the solvent [256]. Gordobil *et al.* reported anti-radical activities of approximately 68% and 52% for softwood (precipitated at pH 2) and hardwood kraft lignin at a concentration of 0.037 mg/ml in methanol [257]. It appears that the anti-radical activity is higher for lignin samples precipitated at lower pH, and this could be due to an increased concentration of phenolic groups, greater conjugation and the occurrence of esterification reactions at lower pH, as found in studies in Chapter 2.

4.4 Conclusions

The desire for wood and bio-based products is continually increasing as the world shifts into a more sustainable lifestyle. Nonetheless, developing more environmentally friendly wood preservatives is still a challenge, particularly due to the increased public awareness of sustainability. In this study, novel water-borne lignin-based wood preservatives were formulated, particularly single-shelled and double-shelled lignin nanocapsules, either empty, or encapsulating the fungicide propiconazole. These were tested for their efficacy at combating *Gloeophyllum trabeum* decay in southern yellow pine in comparison to standard preservatives, chromated copper arsenate, propiconazole dissolved in water, and the raw material used to manufacture the nanocapsules, kraft lignin. Statistical analysis of our results showed that only vacuum-pressure treated chromium copper arsenate and double-shelled lignin nanocapsule encapsulating propiconazole were effective at controlling fungal degradation compared to no preservative. The weight loss from the chromium copper arsenate treated wood blocks were 3.6% lower than the nanocapsule treated wood blocks. The double-shelled lignin nanocapsule plus propiconazole had a

retention of 4.8 mg/ml, with propiconazole constituting only 5.6% of the preservative dry mass, compared to 6.5 mg/ml retention of chromated copper arsenate, both via pressure impregnation treatment. Moreover, propiconazole has poor solubility in water, and the water-dispersible nanocapsules served as a viable vehicle to transport the drug into the wood block. Kraft lignin did not show any anti-fungal effect compared to the control. It is possible that at a higher retention concentration its activity could be enhanced. The Kirby-Bauer disk diffusion test results demonstrate that the anti-fungal activity of propiconazole is concentration dependent, and that the double-shell nanocapsule serves as a viable vehicle to prolong the active phase of the preservative and may contribute some antifungal activity at low propiconazole concentrations. The anti-oxidant test results suggest that the double-shell nanocapsule has enhanced anti-radical activity compared to kraft lignin and the single-shell for concentrations above 0.05 mg/ml. This could also signify that the superior anti-radical properties of the double-shell nanocapsule could contribute to the anti-fungal activity of propiconazole in the combined system. This work is further aimed at promoting research in the field of lignin nanomaterials for the purpose of wood preservation.

5 Conclusions and Future Work

Summary of Results

Chapter 2. The effects of process variables, namely, temperature, pH and residence time on the recovery, and physical and chemical properties of hardwood kraft lignin through the LignoBoost® process have been investigated in this work. In conclusion, our results suggest that the primary factor influencing lignin recovery through acidification is the pH of the solution, followed by the residence time, and ultimately temperature, all of which have a positive correlation to the recovery yield. Hydrodynamic and ζ -potential data revealed that kraft lignin exists as globules in black liquor and these are electrostatically stable above pH 6. Similarly, a higher temperature resulted in accelerated protonation of the lignin molecules in the solution. As the pH is lowered, the concentration of aliphatic hydroxyls in the purified lignin increases, the molecular weight decreases, and the polydispersity of the molecules increases. The same effect is observed as the residence time increases. This suggests that lower molecular weight samples are recovered at these conditions. This could be due to molecular fragment sorption onto larger chains. The molecular weight increased with increasing temperature, and the molecules became increasingly tightly-packed. Thus, the samples recovered at higher temperatures were darker. Similarly, below pH 4, the presence of alkene groups in the black liquor, and ester bonds in the recovered lignin increased with decreasing pH, suggesting condensation and acid-catalyzed esterification reactions occur at very low pH (<4).

Chapter 3. We have synthesized novel double-compartment nanocapsules from unmodified kraft lignin. These structures are monodispersed and stable for periods of up to 8 months in water at neutral pH, as indicated by stable hydrodynamic size. Similarly, the ζ -potential, remained strongly negative for the same duration. The increased carbon to oxygen ratio at the periphery of the hollow cavity reveals the hydrophobic nature of the hollow cores. The mechanisms driving the initial nanocapsule formation have been determined to be hydrophobic interactions, nucleation-growth, π - π stackings of benzene rings, plausibly ion- π stackings, intermolecular and intramolecular hydrogen bonding. Upon successful encapsulation of propiconazole into the double-shell capsule by diffusion, hydrophobic interactions and π - π stacking, the nanocapsules displayed slow release kinetics preserving ~16 % of the initially loaded drug at the end of two weeks. This novel synthetic methodology will support designing diverse lignin nanoparticles for different applications.

Chapter 4. The desire for wood and bio-based products is continually increasing as the world shifts into a more sustainable lifestyle. Nonetheless, developing more environmentally friendly wood preservatives is still a challenge, particularly due to the increased public awareness of sustainability. In this study, novel water-borne lignin-based wood preservatives were formulated, particularly single-shelled and double-shelled lignin nanocapsules, either empty, or encapsulating the fungicide propiconazole. These were tested for their efficacy at combating *Gloeophyllum trabeum* decay in southern yellow pine in comparison to standard preservatives, chromated copper arsenate, propiconazole

dissolved in water, and the raw material used to manufacture the nanocapsules, kraft lignin. Statistical analysis of our results showed that only vacuum-pressure treated chromium copper arsenate and double-shelled lignin nanocapsule encapsulating propiconazole were effective at controlling fungal degradation compared to no preservative. The weight loss from the chromium copper arsenate treated wood blocks were 3.6% lower than the nanocapsule treated wood blocks. Following pressure impregnation, the double-shelled lignin nanocapsule plus propiconazole was retained at 4.8 mg/ml, with propiconazole constituting only 5.6% of the preservative dry mass, compared to 6.5 mg/ml retention of chromated copper arsenate. Moreover, propiconazole has low solubility in water, its anti-fungal activity is concentration dependent, and the water-dispersible nanocapsules served as a viable vehicle to transport the drug into the wood block. Kraft lignin molecule did not show any anti-fungal effect at pH 7 between concentrations of 0.1 and 1.8 mg/ml as revealed by Kirby-Bauer disk diffusion tests. However, there are indications that the double-shell nanocapsules may contribute some antifungal activity at the low propiconazole concentrations in the wood blocks, and this could be related to their high anti-radical activity.

Possible future studies

The results of **Chapter 2** demonstrate that the concentration of hydroxyl groups in the precipitated lignin increases with decreasing pH. Moreover, it was observed that the concentration of ester groups increased in the same pattern. Various literature articles have suggested condensation reactions are occurring [258, 259]. However, it would be beneficial to investigate whether the observed chemical changes occur due to carbohydrate degradation, or as a result of decreased solubility or condensation of the short chain fragments. This could be investigated by running precipitation experiments at room temperature and with a dilute acid (<6 %), at these conditions, carbohydrate hydrolysis is not expected to occur [260].

The results of **Chapter 3** invite for further work on this nanocapsule synthetic methodology. This could involve a robust study on the effect of various process conditions on the physical and chemical properties of the resultant nanoparticles, such as the shapes, sizes, and surface properties. This would optimize the synthetic methodology to generate nanocapsules and particles targeted for specific applications. Moreover, validating the method for other types of industrial lignin with similar chemical properties would also be important.

The results of **Chapter 4**, invite for larger scale wood degradation tests and field studies, and also, evaluating the efficacy of the lignin-based preservatives against other types of fungi.

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A Supplemental Information

A.1 Understanding the effect of precipitation process variables on hardwood lignin characteristics and recovery from black liquor

A.1.1 Experimental

Lignin acetylation procedure

In a typical experiment, 100 mg of oven-dry lignin was mixed with 5 ml of a pyridine/acetic anhydride solution (1:1, v/v). The reaction was allowed to proceed at room temperature for 18 hours. The reaction was quenched with 40 ml of a 50% aqueous methanol solution. The samples were washed sequentially with 99.5% methanol (X1), distilled water (X2), and 95% ethanol (X3). The samples were oven dried at 105 °C.

Hydroxyl group determination

Under continuous stirring, 40 mg of oven-dried lignin was dissolved in 500 µl of an anhydrous pyridine/deuterated chloroform solution (1.6:1, v/v). Upon complete dissolution, 100 µl of cyclohexanol was added to the solution as an internal standard followed by 50 µl of chromium (III) acetylacetone was added as a relaxation reagent. Then, 100 µl of 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane was added as a phosphitylation reagent, and the reaction was allowed to proceed for 25 minutes. 600 µl of the mixture was transferred to a 5 mm NMR tube, and diluted in deuterated chloroform. The NMR analysis was carried with a relaxation delay of 5 s and 512 scans.

The hydroxyl content was calculated based on the ratio of the internal standard peak area to integrated areas at the following spectral regions: aliphatic hydroxyls (148-146 ppm), cyclohexanol (144.3-144.9 ppm), syringyl phenolic hydroxyls (143.4 ppm), guaiacyl phenolic hydroxyls (139.5 ppm), *p*-hydroxyphenyl hydroxyls (138 ppm), carboxyl hydroxyls (135.9-134.2 ppm).

A.1.2 Results and Discussion

Table A1.2.1. Polydispersity index of the hydrodynamic sizes of the lignin polymer in black liquor at various pH. The error represents the standard deviation from the experimental runs.

Temperature (°C)	pH 14	pH 10	pH 8	pH 6	pH 4	pH 2
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25	0.53 ± 0.12	0.63 ± 0.20	0.62 ± 0.19	0.66 ± 0.09	0.92 ± 0.12	0.56 ± 0.30
70	0.38 ± 0.12	0.38 ± 0.08	0.65 ± 0.14	0.65 ± 0.10	0.46 ± 0.22	0.36 ± 0.05

Figure A1.2.1. Sugar content of precipitated kraft lignin as a function of pH and temperature. A (50 °C, 1 hr) B (pH 4.2, 1 hr).

Figure A1.2.2. Inorganic content of precipitated kraft lignin for residence time 1 hour.

Figure A1.2.3. Precipitated lignin color variation images (A-L) and surface analysis plots (G-L). Precipitation conditions are the same in upper and lower images: A & G (50 °C, pH 4, 1 hr, air-dried), B & H (65 °C, pH 4, 0.5 hr, air-dried), C & I (70 °C, pH 4, 1 hr, vacuum-dried), D & J (70 °C, pH 6, 1 hr, vacuum-dried), E & K (85 °C, pH 1.7, 1.5 hr), F & L (50 °C, pH 1.7, 1.5 hr).

Figure A1.2.4. Pareto chart of standardized effects for mass yield at $\alpha = 0.05$.

Figure A1.2.5 Residual plots for the mass yield fitted quadratic model. A) normal probability plot, B) Versus fit, C) residual histogram, D) versus order.

Figure A1.2.6. Contour plots depicting the interacting effect of process variables on the mass yield. The hold values are 67.5 °C pH 4.35 and 1.5 hours.

Table A1.2.2. Raw data, hydroxyl content, molecular weight (M_w), and quantitative color values for precipitated lignin at various experimental conditions.

<i>Batch 1</i>												
Temperature (°C)	pH	Residence time (h)	Aliphatic OH (mmol/g)	Sinapyl OH (mmol/g)	Coniferyl OH (mmol/g)	<i>p</i> -Coumaryl OH (mmol/g)	Carboxyl OH (mmol/g)	Total OH (mmol/g)	M_w^S (kDa)	$\bar{D}^S$	Brown index (BI)	ΔE (ΔE)
85	1.7	1.5	5.53 ± 0.26	3.85 ± 0.63	0.62 ± 0.15	0.15 ± 0.06	0.85 ± 0.23	11 ± 0.82	2.63 ± 0.06	4.41	13.51 ± 4.91	76.42 ± 2.71
85	2.4	1.5	4.32 ± 0.27	2.78 ± 1.24	0.48 ± 0.47	0.13 ± 0.02	1.10 ± 0.33	8.81 ± 1.86	3.29 ± 0.08	3.23	17.42 ± 7.23	77.22 ± 3.41
85	4.2	1.5	2.93 ± 0.35	1.91 ± 0.15	1.01 ± 0.05	0.36 ± 0.17	0.76 ± 0.48	6.61 ± 0.45	3.93 ± 0.05	2.24	24.41 ± 8.32	71.71 ± 2.18
65	4.2	1	3.52 ± 0.39	1.93 ± 0.03	0.65 ± 0.03	0.23 ± 0.07	0.73 ± 0.13	7.07 ± 0.43	2.34 ± 0.09	3.67	23.44 ± 1.82	78.46 ± 1.34
70	4.2	1	3.20 ± 0.70	1.73 ± 0.63	0.91 ± 0.78	0.20 ± 0.12	0.82 ± 0.46	6.86 ± 2.22	2.52 ± 0.11	4.54	27.52 ± 0.91	75.94 ± 1.12
85	4.2	1	2.97 ± 0.42	2.52 ± 0.49	1.10 ± 0.08	0.36 ± 0.13	0.75 ± 0.33	6.96 ± 0.91	6.87 ± 0.11	2.62	25.94 ± 5.87	72.838 ± 2.34
65	4.2	0.5	3.94 ± 0.55	1.73 ± 0.56	0.58 ± 0.29	0.34 ± 0.24	0.76 ± 0.33	7.35 ± 0.97	4.22 ± 0.15	1.40	23.72 ± 1.63	75.64 ± 1.72
65	4.2	1.5	4.26 ± 1.02	2.20 ± 0.81	0.35 ± 0.16	0.17 ± 0.13	0.74 ± 0.52	7.72 ± 2.40	1.48 ± 0.15	4.61	43.74 ± 0.92	73.50 ± 0.81
<i>Batch 2</i>												
85	1.7	1.5	5.72 ± 0.17	3.60 ± 0.40	0.87 ± 0.11	0.18 ± 0.13	0.54 ± 0.23	10.91 ± 0.39	2.67	-	14.82 ± 6.62	77.34 ± 3.51
85	2.4	1.5	3.81 ± 0.41	3.11 ± 0.86	0.47 ± 0.02	0.07 ± 0.03	0.58 ± 0.05	8.04 ± 0.50	3.21	-	16.24 ± 6.52	77.72 ± 3.91
85	4.2	1.5	3.25 ± 0.21	2.51 ± 0.63	1.16 ± 0.69	0.24 ± 0.07	1.12 ± 0.16	8.29 ± 1.33	3.98	-	29.2 ± 11.4	72.3 ± 3.1
65	4.2	1	3.83 ± 0.09	1.78 ± 0.14	0.44 ± 0.05	0.19 ± 0.08	0.67 ± 0.21	6.91 ± 0.38	2.30	-	23.64 ± 1.53	76.92 ± 0.91
70	4.2	1	3.22 ± 0.13	2.06 ± 0.12	0.95 ± 0.86	0.23 ± 0.20	0.40 ± 0.16	6.86 ± 1.53	2.61	-	24.81 ± 3.52	76.13 ± 3.34
85	4.2	1	2.94 ± 0.27	2.71 ± 0.29	1.91 ± 0.40	0.46 ± 0.10	0.86 ± 0.21	8.88 ± 0.52	2.92 ± 0.03	-	27.64 ± 11.22	73.83 ± 3.54

65	4.2	0.5	3.18 ± 0.67	2.53 ± 0.94	0.93 ± 0.3	0.31 ± 0.08	0.72 ± 0.23	7.67 ± 1.28	4.2	-	21.22 ± 1.34	77.7 ± 2.41
65	4.2	1.5	4.18 ± 0.93	1.44 ± 0.42	0.42 ± 0.06	0.12 ± 0.01	0.48 ± 0.26	6.64 ± 1.37	1.41	-	34.54 ± 7.83	75.1 ± 1.53
<p>The error represents the standard deviation from technical triplicates.</p> <p>^s The M_w was determined from polystyrene standards.</p>												

A.2 Double-shell lignin nanocapsules are a stable vehicle for fungicide encapsulation and release

A.2.1 Experimental

Red fluorescence label addition to propiconazole

In a typical experiment, 10 mg of propiconazole was dissolved in 100 mL ultra-filtered water. To it, 5 mg of ammonium tetrachlorocuprate (II) dihydrate, 1 mg of 95% Cy5-alkyne, and 0.1 mL of a sodium acetylide suspension in xylene were added. The reaction was conducted at ambient conditions [261].

A.2.2 Results and Discussion

Table A2.2.3. Evidence of removal of surfactant using elemental concentrations of sulfur and sodium determined by EDS.

	Atomic fraction (%)	
	S	Na
Kraft lignin	0.5 ± 0.2	1.8 ± 0.7
Single shell + SDS	1.1 ± 0.4	0.2 ± 0.1
Single shell	0.2 ± 0.1	0.2 ± 0.1
Double shell + SDS	1.3 ± 0.5	0.8 ± 0.3
Double shell	0.5 ± 0.1	0.1 ± 0.0

Table A2.2.4. Concentration changes of the elemental composition of nanocapsules at different stages of formation. Increased nitrogen concentration correlates to increased propiconazole encapsulation.

	Atomic fraction (%)
--	---------------------

	C	O	N	S	Na
Kraft lignin	93.1 ± 2.2	4.1 ± 1.4	0	0.5 ± 0.2	1.8 ± 0.7
Single shell	94.1 ± 2.0	4.5 ± 0.1	0	0.2 ± 0.1	0.4 ± 0.2
Double shell	93.3 ± 0.4	5.5 ± 1.1	0.7 ± 0.2	0.5 ± 0.1	0.1 ± 0.0
Double shell + propiconazole	91.2 ± 2.7	5.6 ± 1.1	2.5 ± 0.5	0.3 ± 0.1	0.4 ± 0.1

Figure A2.2.1. A) HAADF spectra and corresponding B) EDS plot of the single-shell. The units represent milli Counts (mCounts) and kilo Counts (kCounts). *The black dashed lines show transitions from one nanoparticle domain to another.* C) Elemental distribution on the surface of the single-shell. D) Elemental distribution on the surface of the double-shell.

Figure A2.2.2 FTIR spectra of kraft lignin, non-crosslinked single shell, cross-linked single shell, and cross-linked double-shell nanocapsules. The spectra have been stacked vertically for clarity.

A.3 Lignin-Propiconazole Nanocapsules Are an Effective Bio-based Wood Preservative

A.3.1 Results and Discussion

Figure A3.1.1. Fungal growth 9 days after inoculation, and 16 days post inoculation. Before the treated disks were inserted the mycelium expanded in a uniform circular pattern, after the treated disks were added the growth pattern became irregular in the regions containing the PCZ and DS+PCZ treated disks.

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B.1 Understanding the effect of precipitation process variables on hardwood lignin characteristics and recovery from black liquor

Understanding the Effect of Precipitation Process Variables on Hardwood Lignin Characteristics and Recovery from Black Liquor

Author: Raisa Carmen Andeme Ela, Lauren Spahn, Niloofer Safaie, et al

Publication: ACS Sustainable Chemistry & Engineering

Publisher: American Chemical Society

Date: Sep 1, 2020

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B.2 Double-shell lignin nanocapsules are a stable vehicle for fungicide encapsulation and release

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Manuscript ID: sc-2020-06686f
Received: 10-Sep-2020

Name of ACS Publication: ACS Sustainable Chemistry & Engineering

Manuscript Title: Double-Shell Lignin Nanocapsules Are a Stable Vehicle for Fungicide Encapsulation and Release

Corresponding Author's Name and Address:

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B.3 Lignin-propiconazole nanocapsules are an effective bio-based wood preservative

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