Fundamental Delignification Chemistry of Laccase-Mediator Systems on High-Lignin-Content Kraft Pulps

A Dissertation Submitted by
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This Thesis is Dedicated to the Memory of my Grandfather

Marco Jean Zincovitch

1909-1987
It is your work in life that is the ultimate seduction

Pablo Picasso (1881-1973)
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<td>Alkaline extraction reinforced with peroxide and oxygen</td>
</tr>
<tr>
<td>ECF</td>
<td>Elemental chlorine free</td>
</tr>
<tr>
<td>HBT</td>
<td>1-Hydroxybenzotriazole</td>
</tr>
<tr>
<td>LMS</td>
<td>Laccase-mediator system</td>
</tr>
<tr>
<td>M5</td>
<td>Mediator system in the absence of laccase</td>
</tr>
<tr>
<td>NHA</td>
<td>N-acetyl-N-phenylhydroxylamine</td>
</tr>
<tr>
<td>VA</td>
<td>Violuric acid</td>
</tr>
<tr>
<td>LMS&lt;sub&gt;HRF&lt;/sub&gt;(E)</td>
<td>Laccase mediator treatment employing HBT followed by an alkaline extraction</td>
</tr>
<tr>
<td>LMS&lt;sub&gt;NHA&lt;/sub&gt;(E)</td>
<td>Laccase mediator treatment employing NHA followed by an alkaline extraction</td>
</tr>
<tr>
<td>LMS&lt;sub&gt;VA&lt;/sub&gt;(E)</td>
<td>Laccase mediator treatment employing VA followed by an alkaline extraction</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>O</td>
<td>Oxygen stage</td>
</tr>
<tr>
<td>OO</td>
<td>Double oxygen stage</td>
</tr>
<tr>
<td>SM</td>
<td>Starting material</td>
</tr>
<tr>
<td>TCF</td>
<td>Total chlorine free</td>
</tr>
<tr>
<td>TMP</td>
<td>Trimethylphosphite</td>
</tr>
</tbody>
</table>
1. ABSTRACT

Research efforts in laccase biobleaching technologies have been ongoing for almost a decade. Interest in this field was strengthened when it was discovered that laccase, a lignolytic enzyme, in the presence of a mediator could effectively delignify both softwood and hardwood kraft pulps with high selectivity. The two most studied mediators in laccase biobleaching are 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) and 1-hydroxybenzo[d]iazole (HBT). The chemistry of these laccase-mediator systems (LMS) has been studied primarily on low-kappa pulps (kappa no. < 35).

This research project, which was consequential in furthering the fundamental delignification chemistry of LMS on high-kappa kraft pulps, employed a new generation of N-hydroxy mediators, more specifically, N-acetyl-N-phenylhydroxylamine (NHA) and violuric acid (VA). The application of LMS toward high-kappa kraft pulps is a feasible technology for improving pulping and bleaching yields.

Biobleaching treatments employing HBT, NHA, and VA as mediators conclusively demonstrated that an LMS could effectively delignify high-lignin content kraft pulps with an initial kappa number greater than 70. In all LMS treatments, VA outperformed both NHA and HBT with respect to delignification. However, accompanying delignification was a loss in brightness, irrespective of which mediator was employed. Oxidative reinforcement of the alkaline extractions with oxygen, peroxide, and peroxide and oxygen was beneficial in countering this undesirable effect. These deleterious brightness trends were also observed when a Kraft pulp (kappa no. 33.8), originating from the same wood source as the high-kappa kraft pulp, was subjected to LMS using HBT and NHA as mediators.

A two-step central composite experimental design illustrated the efficiency of an LMSVA system; most of the delignification occurred under one hour. The delignification response of LMSVA treatments on high-kappa kraft pulps was comparable to oxygen delignification. Most noteworthy, however, LMSVA treatments outperformed oxygen delignification with respect to retention of end viscosity of pulps – another unique feature
of LMS. Furthermore, gravimetric pulp yield measurements subsequent to LMS treatments were greater than 90%.

Extensive $^{31}$p NMR characterization of residual lignins isolated from NHA- and VA-LMS treated kraft pulps revealed a substantial depletion of various lignin functional, especially phenolic lignin moieties. However, the mediators exhibited different selectivity toward lignin functional groups, despite the common N-OH moiety.

The brightness loss observed after LMS treatments was speculated to be due to the presence of quinonoid structures. This research presented some of the first spectroscopic data on the combined concentration of ortho- and para- quinones. Evidence for the formation of quinonoid structures during LMS was established. The trends also revealed a decrease in these compounds subsequent to alkaline extractions.

This project also investigated the kismet of residual lignins first isolated and then treated with laccase and LMS. Spectral analysis revealed that the residual lignin reacted with only laccase was significantly modified. The depletion of lignin functional groups followed similar trends to those observed in the lignins isolated from LMS treated kraft pulps. The presence of mediators did not lead to any further lignin transformation, suggesting that in solution, the role of the mediator is secondary.
2. INTRODUCTION

Over the last few decades, the North American pulp and paper industry has successfully refashioned its pulping and bleaching operations. Some of the most notable transformations include the implementation of oxygen delignification and elemental chlorine free (ECF) bleaching technologies. Indeed, the number of oxygen systems installed in the USA grew from zero in 1970 to more than a 100 in 1998. Similarly, world production of ECF pulp grew from 3.5 million tonnes in 1990 to more than 48.5 million tonnes in 1999 (1). U.S. production of ECF bleached pulps grew from 0.5 million tonnes in 1990 to more than 18.1 million tonnes in 1999 (1, 2).

The genesis of this hasty metamorphosis is linked to the marketplace and to the United States Environmental Protection Agency (EPA) Cluster Rule. The Cluster Rule is based partly on the premise that ECF bleaching is the best available technology for kraft and soda grade paper (2). As these environmental issues continue to be addressed, neoteric research opportunities are developing. Indeed, the North American pulp and paper industry of the future will need to adopt technologies that will not only be environmentally compatible but also reduce capital and operating costs while still producing high-quality products.

Because inexpensive fibers are anticipated to become less available, attention to efficient wood utilization has become increasingly important. Pulp producers will be challenged to address this resource issue to remain competitive in a global market.

One of the most promising approaches to improve the economics of kraft pulp production consists of increasing overall pulp yields. This can be achieved by halting the kraft cook at a relatively high kappa (~ 45) prior to reaching the terminal phase. The pulp is then subjected to a single or double oxygen stage before it is bleached. This approach has been shown to improve pulp yields from 2-4% (3). However, because of the capital and operating costs associated with this process, alternative methods such as biological systems, which could potentially either displace or complement oxygen delignification, are
warranted. This Herculean vision can only be achieved through fundamental research in collaboration with academia, industry and government.

The potential for using enzymatic treatments was first realized in the mid 1980s, when xylanases were introduced (4). Pretreatments of chemical pulps prior to their subsequent bleaching with chlorine, chlorine dioxide or hydrogen peroxide could lead to substantial savings on bleaching chemicals. However, the need for more efficient biobleaching systems led to the exploration of lignolytic enzymes, such as laccase.

Historically, the application of laccase for bleaching kraft pulps was hindered due to the minimal delignification response that was attainable. This inefficiency was attributed to the size of the enzyme and hence, to its inability to diffuse into the secondary wall of a pulp fiber to contact the residual lignin (5). More recently, this hurdle was circumvented with the aid of mediators. In a laccase-antioxidant-system (LMS), the laccase oxidizes the mediator. In turn, the oxidized mediator diffuses into the pulp fiber and oxidizes the lignin.

The first mediator, ABTS (2,2'-azinobis-(3-ethyl benzthiazoline), was introduced by Bourbonnais and Paice in 1990 (6). Their studies indicated that a laccase-ABTS system could selectively remove as much as 32% of the residual lignin in both softwood and hardwood kraft pulps. However, the implementation of this laccase-ABTS system on a mill scale is unlikely, in part due to the insufficient achievable delignification levels and the high cost of the mediator.

In 1993, a new class of mediators containing the N-OH moiety was introduced. Call found 1-hydroxybenztriazole (HBT) to be the most effective one (7). He reported that under mild conditions delignification levels as high as 60% for both softwood and hardwood kraft pulps in a single stage. These preliminary results launched a worldwide interest in understanding the chemistry of HBT. Although a laccase-HBT system has been found to effectively delignify kraft pulps, its commercialization is not feasible since in part, it is neither economical nor catalytic.

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In 1997, Amann reported $N$-acetyl-$N$-phenylhydroxylamine (NHA) and violuric acid (VA) as candidates that could potentially outperform HBT (8). However, the factors contributing to this enhanced delignification effect remain unknown. Further research into the delignification chemistry was therefore warranted. The goal of this research project was to explore the fundamental delignification chemistry of NHA and VA on high-lignin content kraft pulps by investigating lignin structural changes. The high selectivity of LMS could potentially be exploited in the future to address wood utilization practice issues.
3. LITERATURE REVIEW

The first three sections of the literature review are dedicated to reviewing some of the fundamentals on lignin, kraft pulping and residual lignin. The literature on enzymes, laccase and mediators follow.

3.1 Lignin

3.1.1 Lignin: an overview

Wood is essentially composed of carbohydrates, extractives, and lignin. The term lignin is derived from the Latin word "lignum," for wood (9, 10). In its native state as found in plants, lignin is referred to as protolignin. Lignin, a three-dimensional amorphous polymer consisting of methoxylated phenylpropane structures, is essential to the life of vascular plants. As such, the main functions of lignin are threefold. First, lignin plays an important role in the intricate transport of water and nutrients by decreasing the permeability of water across the cell walls (10). Second, it imparts rigidity and mechanical support to the cell wall to resist compression and bending (10). Third, lignin can protect the cell wall from microorganisms by halting the penetration of destructive enzymes into the cell wall (10).

3.1.2 Lignin precursors and biosynthesis

It is widely accepted that the biosynthesis of lignin stems from the polymerization of three types of phenylpropane units, also referred to as monolignols (9, 11). These units are coniferyl, sinapyl, and p-coumaryl alcohol. The three structures are depicted in Figure 1. Figure 2 illustrates the dehydrogenation of coniferyl alcohol and the resonance-stabilized phenox radical (9). The convention shown in Figure 1 to denote the carbon atoms in the side chain will hold throughout this paper.
Figure 1. The three building blocks of lignin (9).

The polymerization process is initiated by an enzyme-catalyzed oxidation of the monolignol phenolic hydroxyl groups to yield free radicals (9) (see Figure 2).

Figure 2. Dehydrogenation of coniferyl alcohol and the mesomeric radicals (9).

A monolignol free radical can then couple with another monolignol free radical to generate a dilignol. Subsequent nucleophilic attack by water, alcohols, or phenolic hydroxyl groups on the benzyl carbon of the quinone methide intermediate will restore the aromaticity of the benzene ring (9). The generated dilignols will then undergo further polymerization. An example of a radical coupling is shown in Figure 3 (9).
Figure 3. Radical coupling of coniferyl alcohol (2).
3.1.3 *Structural aspects of lignin*

The exact structure of protolignin is virtually unknown. Fortunately, improvements in methods for identifying degradation products and advancements in spectroscopic methods have enabled scientists to elucidate some of the structural features of lignin, such as the dominant linkages between the phenylpropane units and their abundance, as well as the frequency of some functional groups (9). Figure 4 depicts some of the common linkages found in softwood lignin. The abundance of these types of linkages is shown in Table 1. The dominant linkage in softwood lignin is the β-O-4 linkage. Recently, Karhunen et al. (12, 13) discovered a new linkage in softwood lignin, known as dibenzodioxocin (see Figure 4). Dibenzodioxocins are estimated to account for approximately 10-15% of 5-5 biphenyl structures (14). Table 2 illustrates the abundance of some of the functional groups found in softwood lignin and Figure 5 is a schematic representation of a softwood lignin proposed by Adler (15). This model was constructed based on the analysis of various linkages and functional groups. The phenylpropane units in lignin are joined both by ether (C-O-C) and C-C linkages. It is important to note that the model proposed by Adler does not depict the actual structure of lignin, but serves as a tool to visualize the linkages and functional groups that are believed to occur in lignin.

Covalent linkages between lignin and hemicellulose, also known as lignin-carbohydrate complexes (LCC’s), may also exist in the native wood (16, 17, 18, 19, 20).
Figure 4. The common linkages between phenylpropane units in lignin (9, 12-14).
Table 1. Proportions of different types of linkages connecting the phenylpropane units in softwood lignin (9, 14).

<table>
<thead>
<tr>
<th>Linkage Type</th>
<th>Dimer Structure</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-O-4</td>
<td>Phenylpropane β-aryl ether</td>
<td>50</td>
</tr>
<tr>
<td>β-5</td>
<td>Phenylcoumaran</td>
<td>9-12</td>
</tr>
<tr>
<td>5-5</td>
<td>Biphenyl</td>
<td>15-25</td>
</tr>
<tr>
<td>5-5/α-O-4</td>
<td>Dibenzodioxin</td>
<td>10-15</td>
</tr>
<tr>
<td>4-O-5</td>
<td>Diaryl ether</td>
<td>4</td>
</tr>
<tr>
<td>β-1</td>
<td>1,2-Diaryl propane</td>
<td>7</td>
</tr>
<tr>
<td>β-β</td>
<td>β-β-linked structures</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2. Functional groups in softwood lignin (per 100 C9 units) (9).

<table>
<thead>
<tr>
<th>Functional Group</th>
<th>Softwood Lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methoxyl</td>
<td>92-97</td>
</tr>
<tr>
<td>Phenolic Hydroxyl</td>
<td>15-30</td>
</tr>
<tr>
<td>Benzyl Alcohol</td>
<td>30-40</td>
</tr>
<tr>
<td>Carbonyl</td>
<td>10-15</td>
</tr>
</tbody>
</table>
Figure 5. Structural model of softwood lignin proposed by Adler (9, 15).
3.2 Kraft Pulping

3.2.1 Brief description

The objective of chemical pulping processes is to remove enough lignin to separate cellulosic fibers one from another, producing a pulp suitable for the manufacture of paper and other related products (21). The prevalent chemical pulping process in North America is the kraft process. In 1995, the production of kraft pulp in the United States was estimated to be greater than twenty million metric tons (air-dried basis) (22).

In a conventional kraft cook, an aqueous solution of sodium hydroxide and sodium sulfide, also known as white liquor, is reacted with the wood chips in a large pressure vessel, called a digester. The white liquor and the chips are heated to a temperature of about 170°C and are allowed to cook at that temperature for about two hours (21, 23). During this treatment, the hydroxide and hydrosulfide anions react with the lignin, causing the polymer to fragment into smaller water/alkali-soluble fragments.

The fragmentation of the lignin macromolecule proceeds through the cleavage of the linkages holding the phenylpropane units together, with a concomitant generation of free phenolic hydroxyl groups (24, 33). The presence of these hydroxyl groups increases the hydrophilicity of the lignin and the lignin fragments. As a result, the solubility of the lignin in the cooking liquor is increased (9). The carbon-carbon linkages, being more stable, tend to survive the pulping process.

Delignification in the kraft cook proceeds in three distinct phases (9, 23, 25): the initial phase, the bulk phase, and the final or residual phase. The three phases are shown in Figure 6 (25). The initial phase of delignification takes place up to a temperature of about 150°C and is controlled by diffusion (9, 31). The bulk phase includes a heating period where the temperature goes from about 150 to 170°C and then stays at 170°C for approximately 2 hours. The rate of delignification in the bulk phase is controlled by chemical reactions (9, 31). Most of the lignin is removed in this phase. The residual or final phase, in which the rate of delignification significantly decreases, begins when about
90% of the lignin has been removed and marks the end of the cook. The selectivity in this phase is poor, and further pulping could result in significant degradation of carbohydrates. The remaining or residual lignin, typically 4-5% (by weight) at the end of a conventional softwood kraft cook, is removed via bleaching techniques (23). It has been suggested that the poor selectivity in the residual delignification phase may be attributed to: (a) the low reactivity of the residual lignin towards the pulping chemicals, and hence, more resistance to delignification (26, 27); (b) the residual lignin being chemically linked to carbohydrates and, thus, resistant to delignification (28, 29, 30).

![Lignin content (% on wood) vs. reaction time for conventional kraft pulping](image)

**Figure 6.** Lignin content (% on wood) vs. reaction time for conventional kraft pulping (25).

### 3.2.2 Chemical reactions in kraft pulping

The types of lignin reactions that occur during kraft pulping can be classified under two main headings: degradation and condensation reactions. Degradation reactions are desirable, since they lead to the liberation of lignin fragments and also enhance their dissolution. Conversely, condensation reactions lead to the formation of alkali-stable linkages and are therefore less desirable (31-34).
3.2.2.1 Degradation Reactions

The prevalent degradation reactions during kraft pulping are the cleavage of α-aryl ether and β-aryl ether bonds (31, 32). However, the reactivity of such linkages is sensitive to the type of moiety (i.e., free or etherified phenolic group) present at the para position relative to the propane side chain (33, 34).

Phenolic Units

α-Aryl ether linkages in phenolic units are readily cleaved by the conversion of the phenolate unit into the corresponding quinone methide intermediates. This type of cleavage is illustrated in Figure 7.

![Figure 7. Alkaline cleavage of α-aryl ether bonds in phenolic arylpropane units (31-34).](image)

β-Aryl ether linkages in phenolic units can also be readily cleaved. The phenolate unit can be converted into the quinone methide intermediate if a suitable leaving group is present at the α-carbon. The reaction proceeds by the addition of the hydrosulfide ion, which yields a benzyl mercaptide structure. The mercaptide anion then attacks the β-carbon to form a thirane intermediate and eliminates the β-aroyloxy group (neighboring group participation). Finally, a loss of elemental sulfur generates a coniferyl alcohol-type structure.

The quinone methide intermediate can also be converted to an alkali-stable enol ether by the elimination of the terminal hydroxymethyl group as formaldehyde. Although this
type of reaction is more dominant in the absence of hydrogen sulfide anions. Enol ether structures have been detected in kraft lignins, thus suggesting that both pathways shown in Figure 8 can occur in kraft pulping (26, 35).

Figure 8. Sulfidolytic cleavage of β-aryl ether bonds in phenolic arylpropane units and conversion into enol-ether units (31-34).

The cleavage of β-aryl ether linkages in nonphenolic units (see Figure 9) involves the attack of an ionized hydroxyl group present on the α or γ carbon. The intramolecular attack of the alkoxide anion on the β-carbon generates an epoxide and eliminates the β-aroxy substituent. The subsequent attack of the nucleophiles found in the cooking liquor (hydrogen sulfide and sodium hydroxide anions) breaks the epoxide and generates diols and monothioglycols structures.

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3.2.2.2 Condensation reactions

The hydrogen sulfide and sodium hydroxide anions (external nucleophiles) have to compete with other nucleophiles (e.g., carbanions from phenolic structures) for quinone methide intermediates. The addition of the nucleophile to the quinone methide is reversible. The result of the competition will not only depend on the nucleophilicity of the species, but also on the ability of the addition product to undergo a fast, irreversible reaction. In structures containing good leaving groups (e.g., R = OAr) at the β-carbon, the neighboring group participation reactions, resulting in the cleavage of β-aryl ether linkages, will predominate over condensation reactions. When the substituent on the β-carbon is an alkyl or an aryl, condensation reactions may occur. Figure 10 illustrates the competitive addition nucleophiles. Condensation reactions proceed via Michael addition, where the quinone methide acts as an acceptor and the phenolate ion acts as the nucleophile. The addition of the nucleophile is followed by abstraction of a proton and rearomatization.
Quinone methides are not the only acceptors. Formaldehyde constitutes another type of acceptor (16). Figure 11 illustrates the reaction between formaldehyde and two phenolate units. The primary addition product, a hydroxybenzyl alcohol, generated from the addition of the phenolic ion and formaldehyde, is converted to an o-quinone methide. Another phenolic ion reacts with it to generate a diarylmethane structure.

Another reaction that occurs during kraft pulping is the cleavage of methyl aryl ether bonds (see Figure 12). Hydrosulfide ions, possessing a strong nucleophilic character, bring about this cleavage. Methyl mercaptan and a catechol are the products that are generated after demethylation. The methyl mercaptide ion may cleave another methyl aryl ether bond (methoxy group) to generate dimethyl sulfide, or it can become oxidized to yield dimethyl disulfide.
Figure 11. Alkali-promoted condensation reactions in phenolic units (31-34).

Figure 12. Demethylation by hydrosulfide and methyl mercaptide ions (32).
3.3 *On The Nature of The Residual Lignin in Kraft Pulps*

Numerous studies have been conducted to elucidate the nature of the residual lignin in kraft pulps (38-65). Some of the major conclusions that stem from these studies are that (a) the nature of the residual lignin falls between that of the lignin in wood and dissolved lignins and (b) changes in lignin functional groups are to a large extent a function of the degree of delignification.

3.3.1 *Phenolic hydroxyl content in residual lignin*

The phenolic hydroxyl content in the residual lignin is of great interest to researchers, since it is the major functional group that enhances the solubility of lignin in kraft pulping (37).

The content of these groups has been quantified. Gellerstedt et al. estimated that there are about 27 phenolic hydroxyl groups per 100 C9 units in the residual lignin of kraft pulps (*Pinus sylvestris*, kappa = 31.4). This value is between that of the wood lignin (−13 groups/100 C9 units) and the dissolved lignins (60–70 groups/100 C9 units) (38, 39). These values are, overall, in general agreement with data reported by Mansson (40) and others (41, 42).

Gellerstedt et al. also monitored, the content of phenolic hydroxyl groups was monitored during the course of the cook. The results are shown in Figure 13. The slight drop in phenolic content when the cook reaches its maximum temperature can be attributed to a decrease in the degree of cleavage of β-O-aryl ether linkages, which in turn is due to a decrease in sulfidity at that point in the cook (39). At about 170 minutes into the kraft cook, the content of phenolic hydroxyl groups seems to level off. The researchers attribute this observation to a decrease in the concentration of hydroxide anions and to a decrease in the amount of phenylpropane-β-O-aryl ether structures in the residual lignin (39).
Figure 13. Frequency of phenolic hydroxyl groups as a function of cooking time in the residual lignin in kraft pulps (open symbols) and corresponding dissolved lignin (filled symbols) (39).

Lai et al. have investigated the content of free phenolic hydroxyl groups in pine kraft pulps at various kappa numbers (10-40). The data shown in Figure 14 indicate that as the kappa number is lowered, the content of free phenolic hydroxyl groups increases (41).

Figure 14. The phenolic hydroxyl group content of residual lignin as related to the kappa number of unbleached pine kraft pulp (43).
Frooss et al. (44, 45) have also quantified the content of phenolic hydroxyl lignin groups in dissolved and residual lignins (viz. $^{31}$P NMR and $^1$H NMR) isolated from conventional kraft pulps prepared from the same source of wood (Pinus taeda) cooked to three different kappa numbers (kappa no. 28, 18.5, and 13). Essential observations were that the phenolic hydroxyl content increased as the kappa number decreased for both the dissolved and residual lignins (kappa no. 28 vs. kappa no. 13). In addition, they reported a higher content of these moieties in the dissolved than in the residual lignins. Table 3 summarizes their findings. Wang et al. (46) have reported a similar value for the total phenolic hydroxyl groups (1.80 mmol/g lignin) in residual lignin isolated from a conventional kraft pulp (Picea mariana, kappa no. 28.9). Similarly, Moe and Ragauskas (47) reported 2.2 mmol/g lignin as the total phenolic hydroxyl groups in residual lignin isolated from a conventional 23.3 kappa-kraft pulp (Picea abies).

<table>
<thead>
<tr>
<th>Pulp</th>
<th>Total Phenolic OH groups (mmol/g lignin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Residual Lignin</td>
</tr>
<tr>
<td>Conventional kraft, kappa no. = 28</td>
<td>2 0</td>
</tr>
<tr>
<td>Conventional kraft, kappa no. = 18</td>
<td>2 3</td>
</tr>
<tr>
<td>Conventional kraft, kappa no. = 13</td>
<td>2 2</td>
</tr>
</tbody>
</table>

### 3.3.2 Beta aryl ether and enol ether structures

Beta-aryl ether structures are the dominant structures in lignin. Furthermore, their cleavage during alkaline pulping constitutes one of the principal pathways by which the lignin macromolecule is depolymerized and fragmented. The fragmentation of such linkages leads to the generation of phenolic hydroxyl groups and, in turn, the lignin fragments are rendered water/alkali soluble.

Gellerstedt et al. examined the presence of beta-aryl ether and enol ethers in the residual lignin of pine kraft pulps, dissolved lignin, and dissolved lignins isolated from a flow-through cook (48, 49). The results are highlighted in Figure 15. The difference in the amounts of beta-O-aryl ether structures present in the pulp and the dissolved lignins reflects
the extent of β-O-aryl ether linkages needed to render the lignin water soluble (49). Towards the end of the cook, the three curves approach each other, suggesting that this type of cleavage has now decreased (49). Another important observation is that at the end of the cook, a substantial number of β-O-aryl ether structures remains in the pulp (49).

Figure 15. Content of noncondensed β-O-aryl ether structures in residual lignin in kraft pulp (open symbol), corresponding dissolved kraft lignin (+), and dissolved lignin (♦) from a flow-through cook as a function of time (49).

Figure 16 shows that the presence of enol ether structures reached a maximum when the cook attained its maximum temperature, for both the residual lignin and the dissolved lignins. This observation has been attributed to a possible shortage of hydrosulfide ions at that time in the cook (49). In addition, it could be related to the quinone methide (QM) chemistry, which is no longer dominating at this point of the cook (31). QM intermediates are precursors to vinyl ether structures. Another observation to note is that the content of such structures decreases slightly towards the end of the cook. Deamul and Bovee suggest that it is possible that such structures might become degraded after being exposed to the maximum temperature for a prolonged period of time (50). Although some enol
ethers might be degraded, their presence is still detected at the end of the cook. The formation of enol ether structures is not desirable, since enol ethers are, to a large extent, relatively stable under alkaline condition. Their formation could impede hydrolyzable alkyl aryl ether linkages from being cleaved during the cook (49).

![Figure 16. Content of noncondensed enol ether structures in residual lignin of kraft pulp (open symbols), corresponding dissolved kraft lignin (†), and dissolved lignin from a flow-through cook (∗) as a function of time (49).](image)

Gustavsson et al. (21) have also examined the content of β-O-aryl ether structures in residual lignins (from Pinus sylvestris kraft pulps) isolated at various cooking points. Their results, shown in Figure 17, support the trends observed by Gellerstedt (49). They observed a steep declivity in these structures until an H-factor of 3000, after which the decrease was much slower. Once again, experimental evidence illustrates that the content of such structures in kraft pulps is still considerable.

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Jiang and Argyropoulos (52) have quantified the amount of β-O-aryl ether structures in residual lignins and milled wood lignin (MWL) that originated from *Picea mariana*. As expected, the residual lignin isolated from the pulp (at 93.6% delignification) contained fewer β-O-aryl ether structures (0.62 mmol/g lignin) than the MWL (1.86 mmol/g lignin). Frooss at al. (44, 53) have determined the content of β-O-aryl ether structures in acetylated residual lignins isolated from three conventional kraft pulps cooked to various kappa numbers. Their results, shown in Figure 18, clearly demonstrate the relationship between the extent of delignification and the content of β-O-aryl ether structures. Similar observations about β-O-aryl ether structures in residual lignins have also been made by Pasco et al. (54).
3.3.3 Condensed lignin structures

The term condensed lignin structures refers to lignin groups that contain alkyl or aryl substituents at the C-5 and/or C-6 position of the aromatic ring as a result of reactions that occur during pulping. Lignin model compound studies, as well as studies on wood meal lignin, published by Gierer (36, 55) and others (56, 57), suggest that condensation reactions may take place under alkaline pulping conditions.

Despite these findings, direct evidence for the formation of condensation products in residual and dissolved lignins remains a debatable issue. This uncertainty stems from the well-known presence of carbon-carbon linkages in protolignin. Hence, the detection of condensed lignin structures in lignins does not necessarily imply that condensation reactions took place. In addition, an enrichment of such structures in lignins could simply be attributed to the depletion of aryl-ether linkages during pulping and not to condensation
reactions. Nonetheless, indirect evidence from several studies indicates that the formation of condensation is possible.

Argyropoulos (58) and Granata and Argyropoulos (59) quantified (via \(^{31}\)P NMR) the content of phenolic lignin structures condensed at C-5 of kraft lignins, originating from *Picea mariana*, isolated at various stages of a conventional kraft cook. Their results, shown in Table 4, indicate an increase in condensed moieties with increasing levels of delignification.

<table>
<thead>
<tr>
<th>Lignin Sample</th>
<th>Degree of Delignification in %</th>
<th>Condensed at C-5 lignin structures mmol/g lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MWL</td>
<td>-</td>
<td>0.36</td>
</tr>
<tr>
<td>Kraft lignin</td>
<td>16.5</td>
<td>0.87</td>
</tr>
<tr>
<td>Kraft lignin</td>
<td>22.1</td>
<td>0.91</td>
</tr>
<tr>
<td>Kraft lignin</td>
<td>39.1</td>
<td>1.31</td>
</tr>
</tbody>
</table>

Ragauskas et al. (60, 61) have investigated the content of C-5 substituted phenolic lignin structures in residual lignins isolated from conventional kraft pulps that originated from *Pinus taeda* and that were cooked to various kappa numbers. Overall, their data suggest an increasing accumulation of condensed structures with a decreasing kappa number.

Gellerstedt and Robert (62) provided supporting evidence for the formation of diphenylmethane (DPM) structures during kraft pulping. \(^{13}\)C NMR spectral analysis of kraft lignin obtained from a conventional cook (70\% delignification, wood source *Pinus sylvestris*) revealed a signal at \(\delta\) 29.7 ppm. This signal is assigned to the methylene carbon in DPM structures. They estimated the content of methylene carbons to be 0.63 per aromatic ring for the kraft lignin and that of the MWL to be 0.18 per aromatic ring. \(^{13}\)C
NMR analysis of kraft lignins, reported by Kringstad and Morck (63), also suggested the formation of condensation products.

By using $^{31}$P NMR, Froass and Ragauskas (64) were able to quantify primary hydroxyl groups and phenolic hydroxyl groups in biphenyl type structures in the residual lignin of pulps from a conventional kraft cook (kappa = 27.4), an interrupted EMCC (kappa = 66.6), and an EMCC cook (kappa = 17). The same source of wood, southern pine chips, was used for all three cooks.

Froass and Ragauskas' results are shown in Table 5. The decrease in primary alcohol groups is evident for the two residual lignins from well-cooked conditions when compared to the lignin in wood. This suggests the release of formaldehyde during the cook. However, the residual lignin from the interrupted cook still contained more primary alcohol groups than the other two residual lignins. The content of biphenyl/condensed type structures is higher for the three different cooks when compared to the wood lignin. Again, the residual lignin from the interrupted cook contains fewer of these structures than the other two lignins (64).

Table 5. Quantification of various hydroxyl groups via $^{31}$P NMR (64).

<table>
<thead>
<tr>
<th>Lignin</th>
<th>Aromatic OH (mmol/g lignin)</th>
<th>Primary OH mmol/g lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Guaiacyl</td>
<td>Biphenyl/ Cond</td>
</tr>
<tr>
<td>Wood</td>
<td>0.86</td>
<td>0.37</td>
</tr>
<tr>
<td>Interrupted EMCC</td>
<td>0.83</td>
<td>0.57</td>
</tr>
<tr>
<td>Conventional</td>
<td>0.87</td>
<td>0.63</td>
</tr>
<tr>
<td>EMCC</td>
<td>0.85</td>
<td>0.66</td>
</tr>
</tbody>
</table>

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3.3.4 Other structural features of the residual lignin

Gellerstedt reported that catechol structures are formed under kraft pulping conditions (24, 65, 66). Their formation is attributed to the well-known demethylation reaction of aromatic methoxy moieties (32). Catechols can then be further oxidized to o-quinones in the presence of oxygen (67). The presence of quinonoid structures is believed to be responsible, in part, for the color of kraft pulps (68). Iyama and Nakano (69) estimated softwood kraft lignin to contain 3-4 o-quinone structures per 100 C-9 units. Similarly, Furman (70) and Furchan and Lonsky (71) reported a value of 3 per 100 C9 for the content of o-quinones in softwood kraft lignin (Pinus taeda, kappa no. 39). Zawadzki (72) and Zawadzki et al. (73) reported a value of 1.6 per 100 C9 units for the combined content of o- and p-quinones in the residual lignin from Pinus taeda pulp, kappa no. 30. Clearly, these reported values indicate that the presence of quinonoid structures in residual and kraft lignins is low.

p-Hydroxyphenyl lignin structures are typically present in high concentrations in compression wood, which is found in the lower side of leaning trunks or branches of conifers (9, 74). The presence of p-hydroxyphenyl units structures has also been detected in normal wood in small concentrations. Smit et al. (75) have quantified the content of p-hydroxyphenyl structures in Pinus radiata normal wood, compression wood, MWL, and kraft lignin (from resultant black liquor of a 17.5-kappa kraft cook). Their results are summarized in Table 6.

### Table 6. p-Hydroxyphenyl (mmol/g lignin) in normal wood, compression wood, MWL, and kraft lignin. The wood source was Pinus radiata (75).

<table>
<thead>
<tr>
<th></th>
<th>Normal wood</th>
<th>Compression wood</th>
<th>MWL</th>
<th>Kraft lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.09</td>
<td>0.48</td>
<td>0.09</td>
<td>0.05</td>
</tr>
</tbody>
</table>

The carboxylic acid content in both residual and kraft lignin of unbleached kraft pulps has often been measured (26-28, 44, 47, 52, 59, 60, 76). Although these moieties are present in such furnishes, their concentration is low. Gellerstedt has estimated 2.1 and 1.5
carboxyl groups per 100 C9 units in residual and dissolved lignin (kraft cook to kappa no. 30), respectively (26).

3.4 Enzymes

3.4.1 Enzymes: a brief historical perspective

The term enzyme is derived from the Middle Greek word *enzymes* which means leavened. In accord with Webster's dictionary (77), today the word enzyme is defined as:

"any of various protein-like substances, formed in plants and animal cells, that act as organic catalysts in initiating or speeding up specific chemical reactions and that usually become inactive or unusable at high temperatures."

While the use of this word in the English language is first traced to 1881, the application of these catalysts predates the term enzyme. Indeed, the oldest known reference for the commercial application of enzymes dates to Ancient Babylon, 2100 B.C., and revolves around the use of microorganisms as catalysts for the production of wine (78). Ancient manuscripts referring to such processes were also found in early civilizations of Rome, Greece, Egypt, China, and India. The production of vinegar, viz. the conversion of alcohol to acetic acid for the storage of food and for medicinal applications, was quite common in ancient times. Ficin, an extract from fig trees, was exploited in antiquity for its enzymatic action in the production of cheese. The use of yeast for leavening bread was well known in ancient civilizations. The ancient inhabitants of the Pacific islands used the juice of papaya fruit in which the active enzyme is a protease, also known as papain, as a meat tenderizer and as a treatment for ringworm. By the beginning of the 18th century, the Europeans (British Navy) had begun exploring the islands and, consequently, were introduced to papain. Information on the properties of the papaya fruit, relayed back to Europe, may have initiated some of the first systematic investigations of digestive enzymes (78).
3.4.2 Enzymes: an overview

Virtually all enzymes are proteins and, in turn, proteins are made from amino acids. Enzymes are biological catalysts. Like all catalysts, they increase the rate of reactions, and they do so by lowering the activation energy without being changed during the overall reaction. Indeed, the rates of enzymatically catalyzed reactions are much greater than the corresponding uncatalyzed reactions (factor of $10^5$-$10^{12}$) (79). For example, the enzyme carbonic anhydrase catalyses the interconversion of bicarbonate ion and protons with carbon dioxide and water,

$$
\text{carbonic anhydrase} \quad \text{CO}_2 + \text{H}_2\text{O} \xleftrightarrow{} \text{HCO}_3^- + \text{H}^+
$$

In actively metabolizing cells, where the supply of CO$_2$ is high, the equilibrium shifts to the right and each molecule of the enzyme helps in the conversion of 600,000 molecules of CO$_2$ per second (80). In the absence of the catalyst, the reaction would be ten million times slower (80).

Some of the most notable characteristics of enzymatic-catalyzed processes are the reaction conditions under which they are carried out. Enzymatic reactions are frequently performed under mild temperatures (below 100°C), pressures (atmospheric), and pH (4-8). In contrast, chemically catalyzed reactions require high temperatures, pressures, and extreme pH settings.

Amazingly enough, not only do enzymes enhance the rate of reactions under mild conditions, but they are also highly specific. Indeed, an enzyme is selective both in the substance (substrate) with which it interacts and in the reaction which it catalyses (80, 81). One theory concerning the specificity of enzymes, the so-called "lock and key theory," was developed in 1894 by Emil Fischer (80, 81). An enzyme contains one or more active sites. At these sites the enzyme interacts with the substrate; the rest of the molecule maintains the three-dimensional integrity of the network. According to Fischer's theory, the active site is considered to have a rigid structure like a lock. The substrate molecule is assumed to have the complementary structure and acts as a key. Although this theory

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paved the way for many research endeavors in enzymology, it presents some discrepancies. For example, substrates of different shapes and sizes can react with the same enzyme. Accordingly, the enzyme’s specificity cannot be explained in terms of structural rigidity alone (80, 81). Another theory, the so-called “induced-fit theory,” has also been proposed. According to this theory, during the formation of the substrate-enzyme complex, the molecular groups of the substrate induce a change in the shape of the enzyme molecule so that a better fit can take place (80, 81).

Many enzymes possess nonprotein components, called cofactors, which carry out the catalytic function of the enzyme. Cofactors can simply be metal ions such as Cu²⁺, or small organic molecules, referred to as coenzymes. Many vitamins such as folic acid, riboflavin, and thiamine are coenzyme precursors. Some coenzymes are but transiently associated with the enzyme and others are covalently linked to the enzyme. The latter types of cofactors are referred to as prosthetic groups. Coenzymes are actively involved in the catalytic reaction of the enzyme and serve as intermediate carriers of functional groups in the conversion of substrate into products (80, 81).

The actual molar amount of the enzyme is most often unknown, so its amount is expressed in terms of the activity observed (81). Two units are frequently used, the International Unit, and the katal. One international unit (U) is defined as the amount of "enzyme that catalyses the formation of one micromole of product in one minute" (81). A katal is defined as the "amount of enzyme catalyzing the conversion of one mole of substrate to product in one second" (81). The molecular activity, or turnover number, k₅₀, measures the maximal catalytic activity of an enzyme. It is defined as the "number of substrate molecules converted into product per enzyme molecule per unit time when the enzyme is saturated with substrate" (81). It should be noted that the activity of an enzyme is sensitive to factors such as pH, temperature, and solution conditions (81). These factors need to be controlled in order for the activity of the enzyme to be optimized. The activity of many enzymes can be extended over a wide range of pH, temperature, and solution conditions (81).
The International Union of Biochemistry and Molecular Biology (IUBMB) has adopted a systematic protocol for the classification and nomenclature of enzymes. Enzymes are classified in accordance with the nature of chemical reactions they catalyze. There are six main classes of reactions that enzymes catalyze (see Table 7). Within these major classes there are subclasses and sub-subclasses. Each enzyme is assigned two names and a four-digit classification.

Table 7. Classification of enzymes according to reaction type (79).

<table>
<thead>
<tr>
<th>Classification</th>
<th>Type of reaction catalyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Oxidoreductase</td>
<td>Oxidation-reduction reactions</td>
</tr>
<tr>
<td>2. Transferases</td>
<td>Transfer of functional groups</td>
</tr>
<tr>
<td>3. Hydrolases</td>
<td>Hydrolysis reactions</td>
</tr>
<tr>
<td>4. Lyases</td>
<td>Group elimination to form double bonds</td>
</tr>
<tr>
<td>5. Isomerases</td>
<td>Isomerization</td>
</tr>
<tr>
<td>6. Ligases</td>
<td>Bond formation coupled with ATP hydrolysis</td>
</tr>
</tbody>
</table>

The enzyme laccase, which is a member of the oxidoreductase class is of special interest in this project. Laccase has a broad substrate specificity and is classified as (p-diphenol: dioxygen oxidoreductase, EC 1.10.3.2), where EC stands for the Enzyme Commission (82).

3.4.3 Laccase: an overview

Laccase was first discovered by Yoshida in 1883 (83). He obtained it from the latex of the Japanese lacquer tree, Rhus vernicifera. Yoshida noted that the white sap of this tree rapidly hardened in the presence of air. He postulated that this phenomenon could be due to the presence of an enzyme in the lacquer and referred to it as “diastase.” Bertrand who further purified this substance from the latex of an Indo-Chinese lacquer tree (Rhus succedanea), coined it laccase (84). Bertrand was also the first to identify the ability of this enzyme to catalyze the oxidation of polyphenols such as p-hydroquinone and pyrogallol (85) and to postulate that laccase was a metalloenzyme (86). However, he incorrectly determined that the metal in question was manganese. Some four decades later, Keilin and Mann established that copper and not manganese was the critical metal in laccase (87).
3.4.4 Structural features of laccase

Laccase belongs to the multicopper enzyme family, which includes ascorbate oxidase and ceruloplasmin (88, 89). Laccases, both fungal and plant, are relatively large proteins with molecular masses ranging from 64-160 kDa (88, 90). The variation in molecular weight has been attributed in part to the differing glycosylation levels in these proteins. Indeed, the carbohydrate content in laccases varies and can constitute 7-45% (by wt) of the protein molecule, depending on the source of the enzyme (88, 90). In general, the carbohydrate content in plant laccases is higher than in fungal laccases (90, 91, 148). Typically in laccases the amino acid chain contains 500 amino acids (92). Table 8 summarizes some properties of some characterized laccases.

Table 8. Properties of some Fungal and Plant Laccases.

<table>
<thead>
<tr>
<th>Source</th>
<th>MW a</th>
<th>%Carb</th>
<th>Potential b</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhus vernicifera</em></td>
<td>110</td>
<td>45</td>
<td>394-434</td>
<td>93, 94, 135-137</td>
</tr>
<tr>
<td><em>Rhus succedanea</em></td>
<td>130</td>
<td>ND</td>
<td>ND</td>
<td>94, 95, 136</td>
</tr>
<tr>
<td><em>Acer pseudoplatanus</em></td>
<td>97</td>
<td>40-45</td>
<td>ND</td>
<td>96, 122, 138, 139</td>
</tr>
<tr>
<td><em>Pinus taeda</em></td>
<td>90</td>
<td>22</td>
<td>ND</td>
<td>141</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source</th>
<th>MW a</th>
<th>%Carb</th>
<th>Potential b</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Polyergus versicolor</em></td>
<td>64.65</td>
<td>10.12</td>
<td>775-785</td>
<td>97, 98, 99</td>
</tr>
<tr>
<td><em>Neurospora crassa</em></td>
<td>64-64.8</td>
<td>11-12</td>
<td>ND</td>
<td>100, 101</td>
</tr>
<tr>
<td><em>Penicillium oxysteum</em></td>
<td>59-64</td>
<td>12.5</td>
<td>ND</td>
<td>102, 103</td>
</tr>
<tr>
<td><em>Pseudoperonospora pinastri</em></td>
<td>126</td>
<td>7</td>
<td>760-790</td>
<td>104, 105</td>
</tr>
<tr>
<td><em>Myctiophthora thermophila</em></td>
<td>160</td>
<td>14</td>
<td>450-480</td>
<td>104</td>
</tr>
<tr>
<td><em>Rhizoctonia solani</em></td>
<td>132</td>
<td>10</td>
<td>680-730</td>
<td>104</td>
</tr>
</tbody>
</table>

* expressed in kDa, * expressed in mV vs. NHE.

Functionally, all the multicopper enzymes catalyze the four-electron reduction of O₂ to water (99). Optical and magnetic spectroscopy, as well as crystallography, have elucidated several features of the active site of the multicopper enzymes. It is now well accepted that laccase, the simplest of the multicopper enzymes, contains four copper (Cu (II)) (88-90, 106, 107). These cuprous ions have been classified in accordance with their
differing spectroscopic signatures. Accordingly, they have been divided into three different classes known as type 1 copper (T1), type 2 copper (T2), and type 3 copper (T3). Laccase contains one copper each of T1 and T2, and two of T3.

The so-called T1 copper, or blue copper, absorbs at 610 nm and is responsible for the blue color of the enzyme (88-90). The T1 copper can be displaced by mercury. This leads to a T1-Hg-laccase derivative where the T1 center is replaced by a redox inactive Hg$^{2+}$ ion, while maintaining the integrity of the rest of the active site (88-90, 108). The so-called T2 copper, or normal copper, can be readily examined with EPR (electron paramagnetic resonance) (88-90). Several anionic compounds such as Ni$^{2+}$, CN$^{-}$, F$^{-}$, and other halides, but not I$^{-}$, strongly interact with the T2 copper and inhibit the enzyme’s activity (88-89, 109). Furthermore, this copper can be reversibly removed leaving a laccase derivative that contains the T1 and T3 copper centers only. This laccase derivative is referred to as a T2D (type 2 depleted) laccase (88-90). The T3 copper pair has an absorption band at approximately 330 nm (88-90). The T2 and the two T3 copper centers have been shown to be in close proximity (~4 Å) and together form what is referred to as the "trinuclear copper cluster" (90, 110, 111). The binding and reduction of dioxygen occurs at this site.

It has been shown that overall, the copper ligation of the trinuclear copper cluster is very similar in the multicopper oxidases (112), the peptide ligands of the T2/T3 trinuclear cluster involve eight histidines, where six histidines coordinate the T3 copper and the other two coordinate the T2 copper (90). However, differences in the coordination of the T1 copper exist between fungal and tree laccases (90, 113). The T1 copper coordination environment in ascorbate oxidase and tree laccases involves a Cu-S from cysteine (~2.1 Å), a Cu-S from methionine (~2.8 Å), and two Cu-N from histidine (~2.0 Å). On the other hand, coordination studies on fungal laccases suggest that the T1 copper ligation involves a leucine (Leu) or phenylalanine (Phe) rather than a methionine (Met) at the axial position (113). Leu and Phe are amino acids that do not contain functional groups that can ligate to the copper. This coordination environment has been recently corroborated by the crystal structure of Coprinus cinereus laccase. The T1 copper liga-
tion for that lacase (see Figure 19) consisted of two Cu-N from histidine, one Cu-S from cysteine and, most noteworthy, no axial ligand (114).

Figure 19. A: crystal structure of lacase from *Coprinus cinereus*; B: T1 copper site of lacase from *Coprinus cinereus* (114).

In general, redox potentials of the T1 copper sites (see Table 8) in plant laccases (approx. 394-434 mV) are lower than in fungal laccases (480-790 mV) (90, 113, 148). Similarly, significant differences exist in redox potentials amongst fungal laccases. The reason(s) for these differences in redox potentials remains unclear (90). Nonetheless, it has been suggested that the variation in redox potentials may be due in part to the differing T1 copper coordination environment (113).

The T1 copper site in lacase accepts electrons from the substrate and then shuttles them to the trinuclear T2/T3, 13 Å away along a cysteine-histidine pathway, where dioxygen is reduced to water (90). The importance of the T2/T3 trinuclear copper cluster in the reduction of dioxygen to water has been established (111). It was discovered that a T2D lacase derivative did not react with oxygen, suggesting that the T2 copper center is crucial in the oxygen reduction. In addition, researchers noted that the T1Hglacase derivative, in which the T1 site is now redox inactive but the T2-T3 cluster is intact, reacts with oxygen. Thus, demonstrating that the T2/T3 trinuclear copper cluster is the minimal structural unit required for the reduction of dioxygen to water (108, 111).
Recently, Solomon (90, 115) proposed a mechanism that describes the reduction of dioxygen (see Figure 20). According to Solomon, an initial reduction by two electrons from the T3 copper center yields a peroxide intermediate. This step occurs upon the addition of oxygen. The peroxide bridges between the T2 copper and T3 copper. The water molecule present between the two type-3 coppers is believed to produce the hydroxide bridge at the oxidized T3 site. Upon further reduction of the peroxide, a water molecule is expelled. The hydroxide bridge between T3 copper and T2 copper, in the native intermediate, is then cleaved, and the resting enzyme state is regained (90, 115).

Figure 20. Proposed mechanism of the reduction of dioxygen in laccase (90, 115).
3.4.3 Substrate specificity

One of the unique features of laccases is their broad specificity for substrates. Accordingly, laccases have been shown to oxidize an array of compounds such as para-diphenols, o-diphenols, arylidamines, aminophenols (88, 148), benzenethiols (116), and hydroxyindoles (117). The reactivity of laccases isolated from different sources towards diphenols and diamines may vary. For example, while laccase isolated from *Trametes versicolor* can oxidize 1,2,4,5 tetramethoxybenzene (118), laccase from sycamore maple cannot (148).

Several synthetic substrates are commonly used to measure the activity of laccases (see Figure 21). However, it is important to note that the utility of these substrates in establishing what constitutes a laccase and what does not is ambiguous, especially when the physiological substrates of laccases are considered (148). On this basis, an enzyme that has been purified should be classified as a laccase only after establishing, for example, its structural features, substrate specificity, and reactivity to inhibitors (148).

Figure 21. Examples of synthetic substrates used to measure laccase activity. I (119), II (120), III (144), IV (121), V (138), VI (122), VII (123), VIII (124), IX (141).

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The physiological substrates (see Figure 22) of both fungi and plant laccases vary depending on the role of the enzymes. For example, the laccase present in the sap of *Rhus vernicifera* catalyzes the polymerization of urushiol into lacquer (125), while the natural substrates that serve as precursors in lignin biosynthesis are the monolignols, coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol (see Figure 1). Two compounds, 1,8-dihydroxynaphthalene (126) and parasperone A, (127) have been identified as natural substrates of fungal laccases involved in pigment deposition. In contrast, the physiological substrates of fungal laccases involved in lignin degradation and detoxification are poorly defined.

![Chemical structures](image)

Figure 22. Probable physiological substrates for fungal and plant laccases (125-127).

### 3.4.6 Plant laccase

Laccases are essentially divided into two major classes, plant and fungal, although they have been detected in several insects such as the tobacco hornworm (*Manduca sexta*) (128), the blowfly (*Calliphora vicina*) (129), and others (130). Plant laccases that have been isolated and purified to full homogeneity include peach (*Prunus persica*) (131, 132), tea (*Camellia sinensis*) (133), *Schinus molle* (134), and several species of *Rhus* (87, 135, 136, 137). More recently, laccase has been isolated from sycamore maple (*Acer pseudop-
**Fagales** (138, 139, 140) and loblolly pine (*Pinus taeda*) (141). These two latter discoveries were consequential in reexamining the function and role of laccase in lignin biosynthesis.

Historically, lignin deposition in plants was believed to be catalyzed principally, if not solely, by the action of peroxidases and not laccases. This school of thought was founded mainly on the basis of the studies reported by Nakamura (142) and Harkin and Obst (143, 144). Nakamura observed that purified laccase isolated from *Rhus vernicifera* sap could not oxidize coniferyl alcohol. Obst and Harkin reported that sections of tree stems failed to oxidize syringaldazine unless hydrogen peroxide, a necessary reagent for activating peroxidases, was added. In addition, earlier findings reported by Freudenberg (145) suggested that peroxidases always appeared to catalyze synthetic lignin or DHP (dehydrogenation polymer) formation more rapidly than laccases. These results coupled with the argument of laccases’ limited distribution in plants (146) led to the belief that these enzymes were not essential in lignin deposition.

However, the recent studies by Driouich (139) and Stempien and Obst (140, 147) on laccase isolated from sycamore maple revealed that the enzyme could polymerize monolignols into DHP. Consequently, a new theory on lignin deposition has been presented. It is now theorized that both peroxidases and laccases are involved in lignin deposition, where laccase is primarily responsible for the initial polymerization of monolignols to oligolignols (147, 148). Peroxidases, on the other hand, are believed to catalyze the reactions of oligolignols leading to the extended lignin macromolecule.

### 3.4.7 Fungal Laccase

Although laccase has been prepared from a wide variety of plants, the main source for these enzymes is fungi (137). Fungal laccases have been isolated from *Polyporus*, *Pleurotus*, *Pholiota*, *Neurospora*, and *Podospora* genera, just to name a few (149). Three major functions have been attributed to fungal laccases. They are pigment deposition, detoxification, and lignin degradation, which is of special interest in this project.
3.4.7.1 Pigment deposition

Experimental evidence has suggested that certain fungal laccases may be involved in pigment deposition during spore formation. Indeed, Clutterbuck (150) noted that mutants of *Aspergillus nidulans* lacking a laccase activity produced yellow spores rather than normal green spores. The involvement of laccase in pigment deposition has also been observed in other fungi such as *Lentinus edodes* (151), *Aspergillus parasiticus* (152), and *Cochliobolus heterostrophus* (153).

3.4.7.2 Detoxification

Evidence for the detoxification capability of laccase has been illustrated in several studies. It has been shown (158, 155) that laccase from *Botrytis cinerea* oxidizes both pterostilbene (trans 3,5-dimethoxy-4'-hydroxystilbene) and resveratrol (trans 3, 5, 4'-trihydroxystilbene). These two stilbene compounds are produced in the leaves and fruits of many species of the *Vitaceae* (climbing shrubs) only after UV exposure or after fungal infection. It is believed that these two components may be produced by plants to inhibit and resist infection caused by the fungus. It has also been suggested that during lignin degradation, phenolic type compounds toxic to fungi may be released and, as such, laccase produced by white-rot fungi may also be involved in the detoxification of these chemicals (148, 156).

3.4.7.3 Lignin degradation

The reactions of laccase with substructure lignin model compounds have been studied. It is widely believed that laccase catalyzes solely the oxidation of phenolic lignin moieties via a one-electron abstraction. The phenoxy radicals can then undergo depolymerization reactions via several degradation pathways or polymerization via radical coupling. Non-phenolic lignin compounds are not oxidized by laccase (158, 159).
Laccase has been shown to degrade phenolic β-O-4 model compounds (157). For example, Kawai et al. (158, 159) (see Figure 23) found that substrate (9) (syringylglycerol-β-guaiacyl ether) was converted mainly to the α-carbonyl dimer (10), 2,6-dimethoxyhydroquinone (11), glyceraldehyde-2-guaiacyl ether (12) via alkyl-phenyl cleavage, and to guaiacol by O-Cβ cleavage (13).

Figure 23. Possible mechanism for side chain cleavage of a phenolic β-O-4 lignin substructure model by laccase from *Coriolus versicolor* (159).

Laccase has also been shown to degrade β-1 lignin substructure model compounds (159, 160). The degradation products shown in Figure 24 were generated through three types of cleavages via phenoxy radicals. These were: Cα-Cβ cleavage, alkyl-aryl cleavage,
and Cα oxidation. Nonphenolic β-1 lignin model compounds were not oxidized by laccase.

![Chemical Structures](image)

**Figure 24.** Possible mechanisms for degradation of β-1 lignin model compounds by Cα oxidation, Cα-Cβ cleavage, and alkyl-aryl cleavage (159).

Aromatic ring cleavage of phenolic lignin model compounds has been shown to be catalyzed by laccase. Kawai et al. reacted 4,6-di(tert-butyl)guaiacol with laccase to form the quinonolactone (II) derivative shown in Figure 25 (161, 159). Kawai proposed two mechanisms that could explain the formation of this product. However, based on labeling experiments, pathway B was discarded. An isotopic experiment showed that $^{18}$O$_2$ from H$_2$O$_{18}$O was not incorporated in II, but that O$_{18}$ atom from $^{18}$O$_2$ was. Consequently, pathway A was believed to be the proper mechanism.
Figure 25. Possible mechanisms of formation of muenolactone (II) from substrate (I) (159).

3.4.8 Polymerization via laccase

Although fungal laccase has been shown to degrade and to depolymerize lignin and lignin model compounds, other reports have indicated that low molecular weight lignin compounds treated with this enzyme can be polymerized. Leonowicz et al. (162) have observed that low molecular weight lignosulphonate fractions incubated with laccase from Trametes versicolor led to polymerization. Similarly, Potthast et al. (163) have detected that creosol was polymerized upon treatment with laccase. Ikeda et al. have also shown that 2,6-dimethylphenol incubated with laccase led to polymerization (164).

Solution conditions, in particular the pH, have been shown to influence the transformation of substrates by fungal laccases (165, 166). Indeed, the products obtained upon the
incubation (see Figure 26) of vanillin and syringic acid with laccase from *Trametes versicolor* and *Rhizoconia praticola* varied depending on the pH range.

Figure 26. Products of syringic acid and vanillin upon incubation with laccase from *T. versicolor* and *R. praticola* at different pH (166).

Nonetheless, it has been noted that the polymerization activity of laccase can be circumvented by introducing other enzymes such as glucose 1-oxidase (167) and veratryl alcohol oxidase (168) into the system. These observations suggest that in nature, lignin degradation pathways may involve the synergistic action of several enzymes including
3.5 Applications of laccase

Fungal laccases are unique enzymes in that they have a broad specificity for various phenolic substrates. A plethora of studies and patents have been brought forth suggesting that these biological systems may be potential candidates for a myriad of applications. For example, using laccase from *Fuscoporia ostreata*, Bouwens et al. have presented a method for enhancing color in tea-based food products (169). Their invention is based on the ability of laccase to oxidize the colorless catechins present in fresh tea leaves and convert them to a complex mixture of yellow and orange to dark brown substances. Small and Asquith (170) have invented a process for reducing the bitterness of green or partially roasted coffee beans. Presumably, the bitterness in coffee beans is attributed to the presence of guaiacols. The bitterness can be mitigated with the use of a plant laccase by removing these compounds. Duhe and Hendrix (171) claim that laccase from *Polyporus versicolor* can be used to treat poison ivy dermatitis by its ability to degrade the principal toxin, urushiol. Pederson and Schmidt (172) have described a process that employs laccase to remove excess dye from all types of textile materials, such as natural fibers, man-made fibers, and combinations thereof. Another interesting patent brought forth by Tsuchiya (173) involves the use of laccase in combination with a mediator to reduce or remove malodorous sulfur, nitrogen, and short fatty acid-based compounds.

Arcand and Archibald (174) have presented experimental evidence demonstrating that the laccase from *T. versicolor* can partially dechlorinate a variety of mono-, di- and trichlorophenolic compounds. Similarly Bollag et al. (175) have also reported on the detoxification of certain chlorinated phenolic compounds such as 4-chloro-2-methylphenol, using laccase from *Rhizoctonia praticola*. In a similar vein, Siegmund et al. (176) demonstrated that laccase in the presence of a mediator and a surfactant could detoxify an array of toxic polycyclic aromatic hydrocarbons such as anthracene, pyrene, and others.
Hansen et al. (177) have described a process for the production of linerboard and corrugating medium leading to an increase in strength properties using laccase. Similarly, Pederson and Felby (178) have patented a laccase-based process for preparing lignocellulose-based products such as particleboard, plywood, and medium-density fiberboard. Pilot-scale studies by Lund et al. (179) have demonstrated that boards produced in the presence of laccase yielded good mechanical properties, although wax issues were encountered.

In addition to the applications mentioned above, studies by Potthast et al. (180, 181) demonstrated that laccase maybe useful in organic synthesis, such as the preparation of benzaldehydes from benzyl alcohols. Furthermore, laccase can also be used in the synthesis of vinblastine (see Figure 27), a useful drug for treating malignant diseases such as testicular cancer and leukemia (88).

![Figure 27. Synthesis of vinblastine via laccase (88).](image-url)
3.6 Laccase-mediator systems and delignification of pulps in their presence

Fungal laccase has been shown to oxidize substructure lignin model compounds; however, its overall response to pulp fibers is poor. The generally accepted explanation for this phenomenon has been presented (5, 182). The size of the enzyme is too large and, therefore, it cannot diffuse into the secondary wall of a fiber to contact the lignin. Recently, however, it was shown that it is possible to circumvent this problem by using small organic molecules, referred to as mediators, thus enhancing the delignification response in actual pulps. It is accepted that the so-called mediator is the active agent in delignification. In addition, studies have demonstrated that the specificity of laccase, which is limited to phenolic moieties, can be extended to nonphenolic substrates with the aid of mediators.

3.6.1 ABTS

The first mediator ABTS 2-2' azinobis(3-ethylbenzthiazoline-6-sulfonate), was introduced by Bourbonnais and Paice. The ability of laccase to oxidize this compound was already known, since ABTS is commonly used for activity measurements (121).

Bourbonnais and Paice (183) reported that laccase from Coriolus versicolor, in the presence of ABTS, oxidized nonphenolic lignin model compounds (see Figure 28). It has been suggested that such reactions are initiated via abstraction of a hydrogen atom from the C-α position to yield hydroxyl substituted benzyl radicals (184). These radicals are subsequently oxidized to aldehyde or ketone moieties and are further degraded subsequent to an alkaline extraction stage (185).

Veratryl alcohol was oxidized to veratraldehyde when reacted with laccase and ABTS. The incubation of an etherified β-1 dimer II with laccase and ABTS led to two products, benzaldehyde and veratraldehyde. In addition, the reaction of dimer I, a β-O-4 model compound, dimer III, with laccase and ABTS led to oxidation at the C-α position and yielded dimer IV. In the absence of ABTS, no oxidation took place with any model compounds. Similarly, in the presence of ABTS alone, no oxidation was observed; the
dark green and stable ABTS$^-$ is produced upon the oxidation of ABTS (186). This indicated that both the enzyme and the mediator must be present during the reaction.

Figure 28. Reaction between dimeric lignin model compounds and laccase/ABTS (183-185).

The laccase-ABTS concept was also carried out on actual pulps. In one study, Bourbonnais and Paice (6) reported 24% delignification when a hardwood pulp with an initial kappa number of 12.1 was reacted with laccase from *Trametes versicolor*. The study was carried out at 2% consistency in an air atmosphere at 25°C. The reduction in kappa number was gradual and reached 24% after five days of treatment. The decrease in pulp viscosity was minimal (initial= 24.2 mPa.s, final=21.9 mPa.s). The researchers also monitored the amount of methanol that was generated during this experiment. They noted that
the methanol released during the five-day period reached a plateau after the first day (21 mg/l). The detection of methanol is suggestive of demethylation reactions (6). Delignification with the laccase-ABTS system in this study was also carried out at a pH ranging from 3.5-7.0. The optimal pH for this system was found to be 4.5.

In a more recent study (187), Bourbonnais and Paice reported about a 32% delignification (after extraction stage) when a softwood kraft pulp was reacted with laccase (from *Trametes versicolor*) and ABTS under 100-400 KPa of oxygen for two hours. The experiment was carried out at a pH 5, 60°C, and 10% consistency. In the absence of the enzyme and ABTS, the extent of delignification was negligible (187, 188).

The mechanism involved in delignification using a laccase-ABTS system is as follows: laccase is activated in the presence of oxygen, and in turn the enzyme oxidizes the mediator (182, 189, 190). Subsequent diffusion of the oxidized mediator in the pulp oxidizes the lignin. It is believed that two intermediary active species are involved in delignification, where the ABTS$^+$ reacts with phenolic substrates and the ABTS$^{2-}$ oxidizes nonphenolic lignin moieties (185, 191).

The implementation of ABTS in a mill environment seems highly unlikely, since the cost is prohibitive (8, 188). Also, the extent of delignification obtained with ABTS is inferior to the newer class of mediators, which will be discussed next. Consequently, research efforts have faded away from ABTS and are shifting towards other mediators. Nonetheless, Bourbonnais and Paice demonstrated that a laccase-mediator (ABTS) system is capable of delignifying kraft pulps, both softwoods and hardwoods, and it is important to acknowledge that fact.

3.6.2 HBT

Recently, Call (192, 193) introduced a new class of mediators. His patent (7) covered aliphatic, cycloaliphatic, heterocyclic, or aromatic compounds, which contain the NO or NOH moiety. However, HBT, (1-hydroxybenzotriazole, see Figure 29), a heterocyclic
compound with the NOH moiety, was shown to be the most promising mediator for both hardwood and softwood kraft pulps, as well as for oxygen delignified softwood and hardwood kraft pulps. Cal reported reductions in kappa, as high as 60% for these types of pulps after the alkaline extraction stage, without any significant losses in viscosity. Delignification levels of this magnitude can be obtained in a single treatment (with laccase-HBT), and in one to four hours. The system can operate at a wide range of temperatures (40 to 65°C), pH (4-6.5), consistencies (1-20%), and oxygen pressures (1-10 bars). However, it seems that the optimal conditions are: 60°C, 10% consistency, pH 4.5, and 10 bars oxygen pressure. These positive results have prompted great interest in the research community for further understanding of the chemistry of a laccase-HBT system, both from a fundamental and an applied point of view.

Figure 29. 1-Hydroxybenzotriazole (HBT).

Several studies have examined the reactivity of a laccase/HBT system with nonphenolic dimeric lignin model compounds. It has been demonstrated that 3-1 model compounds are oxidized with laccase/HBT via abstraction of an H atom from the C-α position (194). On this basis, the initial oxidation pathways using either ABTS or HBT are similar. However, differences in the degradation products have been observed. While the oxidation of dimer I (see Figure 28) with ABTS led to benzaldehyde and veratraldehyde, the oxidation of the same dimer with HBT led to veratraldehyde, benzaldehyde, and dimer II (see Figure 30).
Nonphenolic β-O-4 lignin models have also been examined. Dimer III was oxidized to dimer IV in a manner similar to the laccase/ABTS system (see Figure 31). Cleavage of dimer IV was only observed after an alkaline extraction stage, however, the degradation products were not characterized (194).

Unlike Bourbonnais’ observations, Srebotnik et al. (195) reported that β-O-4 dimer V, treated with laccase from Trametes villosa and HBT (see Figure 32), was converted to compounds VI, VII, and VIII, thus suggesting that cleavage did occur. Structure VIII could have formed either by the oxidation of VI or the cleavage of VII.
Figure 32. Reaction products from the reaction of dimer V with laccase/HBT (195).

Kawai et al. (196) have also examined the reactivity of nonphenolic β-O-4 model compounds using laccase from *Trametes versicolor* and HBT (see Figure 33).

Figure 33. Reaction of an etherified model compound with laccase/HBT according to Kawai (196).
Dimers IX and X were converted to various compounds suggesting that the degradation can proceed via several pathways, such as β-ether cleavage, Cα-Cβ cleavage, and Cα oxidation.

Some of the discrepancies between the studies of Srebotnik, Bourbonnais, and Kawai may be due in part to the different reactivities of the substrates. Nonetheless, these studies once again, demonstrate that the specificity of laccase can be extended to nonphenolic substrates with the aid of mediators and that the degradation of such substrates most likely proceeds via several mechanistic pathways.

The general mechanism involved in delignification with a laccase-HBT system is similar to a laccase-ABTS system. The oxygen present in the solution oxidizes the enzyme, which in turn oxidizes HBT (193). The oxidized HBT then diffuses into the pulp and oxidizes the lignin. The next cycle begins when oxygen is reduced to water. This generally accepted mechanism is shown in Figure 34 (193). The active delignification mechanism for a laccase-HBT system has been shown to be radical based. Using EPR, Potthast proposed that the active functionality in delignification is the NO\(^\cdot\) (radical), generated in situ from the action of laccase and HBT (197).

![Figure 34. Proposed mechanism, laccase-HBT in biobleaching.](image)

Sealey, et al. (197) also demonstrated that the mechanism of delignification is radical based. Superoxide dismutase (SOD), a radical scavenger, was added to an oxygen-laccase-HBT-pulp system and was shown to interfere with delignification. Their results indicated that as the dosage of SOD was increased from 29,000 to 116,000 U, the ability of the laccase-HBT system to reduce the kappa number decreased. Interestingly, at a SOD
dose of 116,000U, no delignification took place. A control experiment was also carried out where a dose of 116,000U of a thermally denatured SOD was added to the system. A denatured SOD is ineffective as a radical scavenger. Delignification was not inhibited, which again supports that the mechanism of delignification is radical based.

Sealey and Ragauskas (198, 199) have characterized the nature of the residual lignin before and after a laccase-HBT treatment. A softwood kraft pulp with an initial kappa number of 23.5 was reacted with laccase (from *Polyporus versicolor*) in the presence of HBT for 24 hr at 45°C, and at 10 bars of oxygen pressure. Subsequent alkaline extraction yielded a pulp with a final kappa of 17.3. The residual lignin from the starting pulp and the enzyme-treated pulp were extracted via acid hydrolysis and the 13C NMR spectra were recorded. The spectra revealed that the laccase treated-pulp had fewer methoxyl groups when compared to the starting pulp. In addition, an enrichment in β-O-4 lignin structures was observed, suggesting that these lignin moieties are unreactive towards LMS. This observation contradicts the results noted on model compound studies. On this basis, it is possible that the affinity of LMS in a polymeric system to β-O-4 structures is redirected towards other lignin functional groups.

In that same study, the two residual lignins were also subjected to a phosphorylating agent, 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane. By phosphorylating the residual lignin, the 31P NMR spectra can be recorded. This technique is useful for canvassing various hydroxyl groups in residual lignins (200). The results obtained from the 31P NMR spectra revealed that the residual lignin from the enzyme treated pulp contained substantially fewer phenolic hydroxyl groups than the residual lignin isolated from the starting pulp. These results suggested that the mediator preferentially attacks phenolic substrates. Further analysis of C-5 noncondensed and condensed lignin groups showed that a laccase-HBT system oxidizes both types of phenolic groups (198). The reactivity of condensed structures in a laccase-mediator system demonstrates a unique feature of this system since such structures are typically unreactive towards conventional oxidative bleaching technologies such as oxygen delignification (47).
The reactivity of laccase and laccase/HBT towards phenolic condensed lignin structures has been demonstrated by Tamminen et al. (201). A pine kraft pulp (kappa no. 24.7) and a two-stage oxygen delignification pine kraft pulp (kappa no. 8.6) were treated with laccase and laccase/HBT. The residual lignins isolated after the treatments were subjected to oxidative degradation. Their results indicated decreases in phenolic 5-5 condensed lignin structures and hence are consistent with Sealey and Ragauskas' NMR data. Another essential observation was the enrichment in p-hydroxyphenyl-type compounds, suggesting that such structures are not reactive towards laccase/HBT treatments.

The substantial reduction in phenolic lignin moieties has also been reported by Poppius-Levin et al. (202, 203). A pine kraft pulp (kappa no. 24.7) was reacted with laccase (from Trametes hirsuta) in the presence of HBT at 45°C for 2 hours under five bars of oxygen pressure at a pH of 4.5. Subsequent alkaline extraction yielded a pulp with a final kappa of 15.4. The residual lignins were extracted and analyzed by FTIR. These results supported the findings of Sealey and Ragauskas that HBT preferentially attacks free phenolic sites. Accordingly, Poppius et al. reported a 42% drop in free phenolic groups for the residual lignin isolated from the laccase-HBT treated pulp (202).

Sealey and Ragauskas (204) have investigated the fate of the mediator. In one experiment, laccase and HBT were reacted in the absence of pulp. A new compound was detected from a proton NMR spectrum. The new compound was later confirmed to be benzotriazole (BT). In order to further understand the formation of BT, Sealey and Ragauskas carried out an experiment where they reacted laccase (from Polyporous versicolor) and HBT in a NMR tube, while maintaining the temperature constant at 45°C. This technique enabled them to monitor the generation of benzotriazole (BT) over time. Their results indicated that the conversion of HBT to BT peaked at 11% after 11 hours. Another experiment was performed between laccase and HBT, this time under 145 psi of oxygen pressure. The reaction was carried out for 24 hr and again, Sealey and Ragauskas detected an 11% conversion of HBT to BT.
The next experiment they carried out was designed to determine whether BT was a delignifying agent. Laccase and BT were reacted in the presence of a softwood pulp for 24 hr at 45°C under 145 psi of oxygen pressure. They noted that BT was inactive as a delignifying agent, since a decrease in the kappa number was not detected. Finally, an experiment was carried out to determine the fate of the mediator in the presence of pulp-laccase-HBT and oxygen. A softwood kraft pulp was reacted with laccase and HBT at pH 4.5 for 24 hr at 45°C under 145 psi of oxygen pressure. Subsequent to this treatment, the bleach effluents were concentrated and acetone extracted in order to recover the mediator structures (i.e., both benzotriazole and hydroxybenzotriazole). A similar extraction procedure was performed on the pulp. Based on the results, 99% (65% by mass from effluent and 34% by mass from the pulp) of the mediator-type structures (i.e., hydroxybenzotriazole and benzotriazole) were recovered. The most interesting observation that they report is that 84% of the mediator (HBT) was converted to benzotriazole during the 24-hour experiment. It is suggested that the conversion of HBT to BT is detrimental to delignification (204). Others have also detected the formation of BT (199, 205).

Potthast has proposed that BT generated from HBT during biobleaching can be formed by the coupling of transient HBT radicals with lignin-type structures (phenoxy radicals). The mechanism by which HBT can be reduced to BT is shown in Figure 35 (205).

![Figure 35. Proposed mechanism for generating BT (205).](image-url)
Research efforts are currently fading away from HBT since its implementation on a mill scale seems far from promising. Cyclic-voltametry studies conducted by Paice (189), have shown that HBT does not form a reversible redox. An irreversible redox suggests that HBT is not catalytic. Amann has also demonstrated that HBT can inhibit laccase (8). This claim has been corroborated by the disappearance of aromatic amino acids such as tyrosin and tryptophan in laccase from *Trametes versicolor* treated with HBT. This may be attributed to the high reactivity of the HBT (NO radical), which renders it less specific in its chemistry. Based on these trends, HBT may be considered far from the ideal mediator, even though its delignification potential in biobleaching is impressive.

### 3.6.3 NHA and VA

Amann (8) recently introduced two new mediator, *N*-acetyl-*N*-phenylhydroxylamine (NHA) and violuric acid (VA) (see Figure 36). Preliminary results published by Amann suggest that NHA and VA may be potential candidates as mediators. A softwood kraft pulp (initial kappa=15.7) was treated with laccase/NHA, laccase/HBT, and laccase/VA in a TCF sequence (LEQP, i.e., L = LMS, E = extraction Q = chelation, and P = peroxide). In addition, a control sequence was performed by exchanging the LE with an oxygen delignification stage (O). The results shown in Table 9 illustrate that the presence of NHA and VA were effective mediators, and that the losses in viscosity were not very significant.

![Chemical structures of VA and NHA](image)

**Figure 36. Chemical structures of VA and NHA (8).**
Table 9. Bleaching response of VA, HBT, and NHA in a LEQP sequence (8).

<table>
<thead>
<tr>
<th></th>
<th>Kappa</th>
<th>Viscosity (dm³/Kg)</th>
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<tr>
<td>Control (OLOP)</td>
<td>5.1</td>
<td>845</td>
</tr>
<tr>
<td>VA</td>
<td>5.5</td>
<td>890</td>
</tr>
<tr>
<td>NHA</td>
<td>5.7</td>
<td>920</td>
</tr>
<tr>
<td>HBT</td>
<td>5.8</td>
<td>880</td>
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</table>

Other studies have reported similar delignification benefits using NHA and VA. Fu et al. (206) treated a hardwood kraft pulp with an initial kappa of 17.5 with laccase/NHA and recorded 43% delignification. Similarly, Sealey (207) reported 45% delignification after treating a SW kraft pulp (kappa no. 14) with NHA and 54% delignification with VA, along with insignificant losses in viscosity.

In an effort to explain the various reactivities of mediators, Amann measured the redox potential of ABTS, HBT, NHA, and VA (see Table 10). All values fell within a narrow range. This suggests that no conclusive relationship can be established between redox potential and mediator reactivity, although the redox potential must be an important variable.

Table 10. Redox potentials of mediators (193).

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Redox potential (V): vs. NHE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTS</td>
<td>0.74/1.15</td>
</tr>
<tr>
<td>HBT</td>
<td>1.13</td>
</tr>
<tr>
<td>VA</td>
<td>0.97</td>
</tr>
<tr>
<td>NHA</td>
<td>1.00/1.21</td>
</tr>
</tbody>
</table>

Based on lignin model compounds, Freudenrich et al. (208) reported that dimer A (see Figure 37), upon treatment with laccase from *Trametes versicolor* and either NHA or VA and subsequent to an alkaline extraction, was converted to products B and C. These results demonstrate that VA and NHA mediate the fragmentation of etherified lignin model compounds.
Figure 37. Reaction of an etherified lignin model dimer with VA and NHA (208).

Although these results suggest that NHA and VA are potential candidates for laccase-mediated delignification systems, they are still not considered ideal. Freudenreich et al. have demonstrated that VA and NHA are reduced to alloxan and NA, respectively (see Figure 38). Alloxan and NA do not lead to delignification, suggesting that these mediators are not catalytic (208).

Figure 38. Degradation products of VA and NHA (208).

Pfüller et al. (209) have reported that VA, like HBT, may also inhibit the laccase. Laccase activity using HBT, VA, and NHA were measured at various concentrations of med-
The results shown in Figure 39 indicate that both HBT and VA acid were potent inactivators of laccase, whereas the inactivation due to NHA was insignificant.

Furthermore, ESR studies have shown that NHA is very quickly oxidized by laccase and that the radical decays rapidly (208). In turn, this may suggest that when using NHA, higher mediator doses may be required.

Figure 39. Inactivation of laccase by HBT, VA, and NHA (209).

Despite the studies reported so far, the fundamental delignification chemistry of a laccase/ NHA and laccase/ VA remain overall poorly defined and require further study. There is an evident need for understanding the fundamental delignification chemistry of such biobleaching systems using the latest generation of mediators. Although NHA and VA may not be suitable for commercial applications, the understanding of their delignification chemistry may lead in the future to noninhibiting and catalytic mediators with great delignification potential.
4. DISSERTATION OBJECTIVES

The goal of this research proposal was to investigate the fundamental delignification chemistry of laccase-NHA and laccase-VA systems on high lignin content conventional softwood kraft pulps. This research was novel in two aspects. First, at the inception of this research program, there were no reported investigations targeted towards understanding the delignification chemistry of LMS employing high-kappa kraft pulps. Second, at the time this project was initiated, the delignification chemistry of NHA and VA had not been investigated. On this basis, the findings reported in this thesis were consequential contributions to the LMS delignification chemistry body of knowledge.

The main objectives of this research proposal were to:

1. Determine the effectiveness of LMS on high-kappa conventional softwood kraft pulps.
2. Investigate the nature of the residual lignin before and after enzymatic treatments.
3. Improve the LMS conditions to enhance the delignification response.
4. Investigate the role of quinones during LMS.
5. Compare LMS delignification vs. O₂ delignification.
6. Examine structural changes in residual lignin treated with LMS.
5. THESIS FORMAT

The author has elected to present the research results in the format of publications and conference proceedings. Because of this format, there will be some duplication of the introduction and experimental sections. In addition, it should be noted that these papers have been changed usually only slightly, from the published or submitted version to incorporate improvements as a result of the thesis examining committee.

The results and discussion section of this thesis is divided into eight chapters. All eight chapters have been published and presented in various journals and at different conferences. Supporting data and documentation, as well as additional results stemming from other research activities, can be found in the Appendices of this thesis. The eight publications comprising the core of this thesis are titled:


Publication 1 describes a preliminary study on LMS biobleaching of high-kappa kraft pulps. This study conclusively demonstrated that LMS is viable even under most draconian conditions with respect to initial pulp lignin content. The effects of time, dose of mediator, dose of laccase and the presence of extractives on delignification are discussed.

Publication 2 describes and compares the structural changes in residual lignins isolated from an LMSNHA and an LMSHBT treated softwood kraft pulp (kappa no. = 33.8). The benefits of oxidatively reinforcing the extraction stage with oxygen (O), peroxide (P), and oxygen and peroxide (E+P+O) were explored vis-à-vis brightness and delignification. In addition, the nature of residual lignins, isolated after LMSNHA(E+P+O) and LMSHBT(E+P+O) treatments, was investigated. This study established that the two N-hydroxy based mediators exhibited different selectivity towards lignin functional groups.

Furthermore, the brightening benefits of the LMS treated pulps stemming from the oxidative reinforced alkaline extractions strengthened the hypothesis that quinonoid structures must play an important role in LMS.

Publication 3 was a duplicate of publication 2, with respect to the experimental protocol and conditions, except for the starting brownstock. This investigation delved into the structural changes of residual lignins isolated from LMSNHA and LMSHBT delignified
high-kappa softwood kraft pulps. The different selectivity of NHA and HBT for lignin functional groups was also observed in this study. More importantly, however, the NMR spectral data demonstrated that the nature of the residual lignin, which is a function of the extent of delignification, may influence the response of LMS. Publications 2 and 3 were the first papers to ever describe the nature of residual lignins isolated from LMS_{NHA} delignified, both high- and low-, kraft pulps.

Publication 4 describes the two-step central composite experimental design that was carried out to improve the LMS_{NHA} delignification response on high-kappa kraft pulps. Based on the experimental conditions used in this study, it was demonstrated that time was not a statistically significant variable (at the 95% confidence level). In addition, the dose of enzyme was reduced by more than 80%. Despite the improvements in delignification that were achieved, this study demonstrated that VA can outperform NHA with respect to delignification. Pulp yield benefits are also discussed in this chapter.

Publication 5 compares the delignification response between O_{2} and LMS_{VA} using the high-kappa kraft pulp. The results demonstrated that the delignification response of the biobleaching system was comparable to an O_{2} stage, and highlighted the efficiency of an LMS with respect to operating conditions. Equally impressive, was the retention in the end viscosity after the LMS treatment—a unique feature of LMS.

Publication 6 discusses and compares the structural changes in residual lignins isolated after subjecting the high-kappa kraft pulp to LMS_{NHA}, LMS_{HBT}, and LMS_{VA} (using the improved conditions from publication 4) treatments. This study supported the trends noted in publication 3 with respect to NHA and HBT. In addition, this study described for the first time the nature of the residual lignin after an LMS_{VA} treatment. The data revealed that VA led to more exhaustive depletion of lignin functional groups and exhibited a different selectivity towards lignin moieties than both NHA and HBT.

Publication 7 describes the semi-quantification of quinones generated during LMS. The presence of quinones had been speculated and theorized by several groups, but never act-
ually quantified nor validated. This study provided the first spectroscopic data on quinones and on their formation during LMS.

The last study, publication 8 and the addendum describe the treatment of isolated residual lignin with laccase and LMSVANB1417. Spectral data revealed that the lignin treated with laccase only was highly transformed. Further structural changes were not detected in the presence of any of the three mediators, suggesting that in solution, the mediator may not necessary, and that the chemistry is dominated by the laccase alone.
6. EXPERIMENTAL

6.1 Wood source and pulps

The wood source that was employed to prepare the pulps for this project originated from two logs of loblolly pine (*Pinus taeda*). The logs, which were selected from a tree farm (Union Camp, Savannah, GA, now International Paper), were approximately 25 years old and void of any visual diseases. The logs (approx. 1.5-m long sections) were debarked with a two-handed knife, cut into approximately 0.5-m bolts, and split into quarter sections with an axe. The quarter sections were fed into a 4 ft Carriage chipper. The chips were then screened with a shaker box screen; accept chips fit through a 25-mm-diameter hole but not through a 13-mm-diameter hole. A sample of chips was used to measure the thickness of the accept chips; over 90% of the screened chips were between 2 and 8 mm thick. The chips were air dried for approximately one week, frozen, bagged, and finally shipped to the pulping facilities at Potlatch Corp., Cloquet, MN. A total of 10 conventional cooks, modified with a 3-hour impregnation stage at 130°C, were performed. The reader should note that despite the impregnation stage, the pulps will be referred to throughout the manuscript as “conventional softwood kraft pulps” for simplicity. Two kinds of pulps were prepared: low- and high-lignin-content kraft pulps. The conditions and results of the low- (target kappa no. 30) and high-kappa (target kappa no. 70) cooks are described in Tables 11 and 12, respectively.

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<td>19.5</td>
<td>19.5</td>
<td>19.5</td>
<td>19.5</td>
</tr>
<tr>
<td>Liquor wood</td>
<td>4.1</td>
<td>4.1</td>
<td>4.1</td>
<td>4.1</td>
<td>4.1</td>
</tr>
<tr>
<td>Cook temperature (°C)</td>
<td>172</td>
<td>172</td>
<td>172</td>
<td>172</td>
<td>172</td>
</tr>
<tr>
<td>H-factor</td>
<td>1100</td>
<td>1317</td>
<td>1451</td>
<td>1529</td>
<td>1552</td>
</tr>
</tbody>
</table>

Average kappa number of 5 cooks* 33.8

* The pulps from the five cooks were combined, washed and screened (using a laboratory 12x43 Sprout Waldren flat screen), dewatered, fluffed, bagged, and stored at 4°C. The kappa number represents the average kappa number of the combined pulps.
Table 12. Cooking conditions and cook results for the high-kappa pulp.

<table>
<thead>
<tr>
<th>Cook number</th>
<th>100268</th>
<th>100269</th>
<th>100270</th>
<th>100271</th>
<th>100272</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD wood wt (Kg)</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Active Alkali (%)</td>
<td>18.5</td>
<td>18.5</td>
<td>18.5</td>
<td>18.0</td>
<td>18.0</td>
</tr>
<tr>
<td>Liquor: wood</td>
<td>4.1</td>
<td>4.1</td>
<td>4.1</td>
<td>4.1</td>
<td>4.1</td>
</tr>
<tr>
<td>Cook temperature (°C)</td>
<td>172</td>
<td>172</td>
<td>172</td>
<td>172</td>
<td>172</td>
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<tr>
<td>H-factor</td>
<td>574</td>
<td>573</td>
<td>553</td>
<td>554</td>
<td>548</td>
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<tr>
<td>Average kappa number of 5 cooks*</td>
<td>75.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The pulps from the five cooks were combined, washed and screened (using a laboratory 12x43 Sprout Waldron flat screen), dewatered, fluffed, bagged, and stored at 4°C. The kappa number represents the average kappa number of the combined pulps.

6.2 Chemicals

All chemicals were purchased from Aldrich, VWR, and Acros and used as received except for p-dioxane, NHA, and laccase. p-Dioxane was freshly distilled over NaBH₄ prior to lignin isolations and LMS reactions on residual lignins. NHA was synthesized in accordance with Oxley et al. method (210). The synthesis is described in section 6.4. Laccase (NS 51002) from Trametes villosa was supplied by Novo Nordisk Biochem and used as received. The laccase was kept frozen at -20°C before use. All NMR solvents and solutions were dried over 4 Å molecular sieves for 24 hr before acquiring NMR spectra.

6.3 Equipment

Biobleaching experiments were performed in a 300-, 1000- (see Figure 41), or 2000-mL-capacity Parr reactor depending on the amount of pulp used. The 300-mL vessel was typically loaded with 10-15 g of pulp (o.d. basis), whereas the 1000- and 2000-mL reactors were usually charged with 15-30 g and 40-60 g (o.d. basis), respectively. The Parr reactors were constructed from 316 stainless steel and were equipped with a stirrer and a pressure gauge. The reactors were connected to a 4842 temperature controller. Laccase activity was measured using a Shimadzu UV-160A. NMR experiments were performed on a Bruker DMX 400 with a 5-mm QNP probe. NMR spectral acquisition and analysis...
were controlled by Bruker’s XWINNMR 2.1 software running on a SGI (Silicon Graphics Indigo) server using the Irix 7.0 operating system.

Figure 40. Photograph of a 1000-mL-capacity Parr reactor with various components. Safety rupture disc, gas release valve, bomb cylinder, and other internal bomb fittings are not visible in this photograph.

6.4 NHA Synthesis

The mediator (see Figure 42) was prepared via the reduction of nitrobenzene to yield N-phenylhydroxylamine followed by an acylation step to afford N-acetyl-N-phenylhydroxylamine (NHA).
Figure 41. Two step synthesis of NHA (210).

6.4.1 Preparation of N-phenylhydroxylamine

A 500-mL, three-necked, round-bottom flask, equipped with a thermometer, a condenser, and a magnetic stir bar, was charged with wet 5% rhodium on carbon (1.1 g, Aldrich catalogue no. 33,017-5), tetrahydrofuran (THF) (200.0 mL, Aldrich catalogue no. 44,340-9), and nitrobenzene (34.3 mL, Aldrich catalogue no. 25,237-9). The mixture was cooled to 15°C, with the aid of an ice-water bath, while stirring. Hydrazine hydrate (16.5 mL, Aldrich catalogue no. 22,581-9) was then introduced over time (30 min) into the mixture from a pressure-equalized addition funnel, while the temperature was maintained below 30°C. The reaction mixture was allowed to react for an additional two hours before it was filtered. The filtered solution was then immediately transferred to a cooled 1000-mL, three-necked round-bottom flask equipped with a thermometer and a magnetic stirrer bar, to carry out the acylation step.

6.4.2 N-acetyl-N-phenylhydroxylamine

To the N-phenylhydroxylamine solution was added a slurry of sodium bicarbonate (42g/40 mL of water). The solution was allowed to cool down (< 4°C using a water-salt-ice bath) while it was mixed vigorously. Acetyl chloride (23.6 mL, Aldrich catalogue no. 11,418-9) was slowly introduced (over 1 hr) from a pressure-equalized addition funnel while maintaining the temperature below 0°C. The reaction mixture was further stirred
for an additional 30-35 minutes before sodium hydroxide (20 g in 200 mL of water) was added, keeping the temperature below 20°C. The aqueous phase was separated, the THF phase was diluted with an equal volume of petroleum ether, and the aqueous phase was separated again. The organic phase was extracted with a 10% solution of sodium hydroxide (2 x 50 mL). The aqueous phases were combined, washed with methylene chloride (200 mL), and then neutralized with concentrated hydrochloric acid. The mixture was extracted with methylene chloride (3 x 100 mL). The extracts were combined, dried over magnesium sulfate, filtered, and concentrated under reduced pressure (approx. 30-40 mL). Crystallization was induced by first adding 100 mL petroleum ether to the solution. The cloudy solution was then stored overnight in the freezer. The crystals were filtered, washed with petroleum ether, and air-dried in the fume hood. A 13C NMR spectrum is shown in Figure 42. Peak assignments were in accord with the literature (207). In addition, the measured melting point (67-68°C) was also in agreement with the available literature (207, 210).

6.5 Laccase activity

The activity of the laccase, from Trametes villosa (Novo, NS 519/2) was measured by monitoring the rate of oxidation of syringaldazine. One unit of activity (U) was defined as the change in absorbance at 530 nm of 0.001 per minute, per mL of enzyme solution, in a 100-mM phosphate buffer (2.2 mL) and 0.216 mM syringaldazine in methanol (0.3 mL). The test was performed at room temperature (207).
6.6 LMS delignification of kraft pulps procedure

The following section describes the LMS delignification procedure employing the 1000-mL-capacity Parr reactor. The same protocol was used when the LMS experiments were carried out in the 2000- and 300-mL capacity reactor. Time, dose of mediator, and dose of laccase are specified in each publication since they varied from study to study.

A 1000-mL capacity Parr reactor, equipped with a pressure gauge, a stirrer, and a heating mantle and connected to a temperature controller, was charged with 15-30 g of pulp (o.d. basis). The pulp consistency was adjusted to 5-10% by adding deionized water. The slurry was then heated to a temperature of 45°C while stirring and was maintained at that temperature throughout the incubation time. The mediator dose was added and the reac-
tion mixture was allowed to mix (approx. 3-5 min). The pH of the system was then adjusted to 4.5 with either glacial acetic acid or a saturated solution of sodium bicarbonate. The proper dose of laccase was introduced into the well-stirred mixture before sealing the reactor and pressurizing the system with $O_2$ (145 psig). Subsequent to the enzymatic treatment, the pulp was removed from the reactor, filtered, and washed thoroughly with distilled water (12 L). The pulp was either subjected to an alkaline extraction, to another bleaching treatment (e.g., $O_2$-delignification), or simply used as is.

**Note:** In the first three publications, the LMS treatments were performed at a consistency of 9-10%. However, difficulties were always encountered when adjusting the pH of the slurry and when stirring. Hence, having additional water in the reaction mixture should allow for easier control of the treatment. To address this issue, a comparative study was initiated to determine if the delignification response of an LMS is influenced by the consistency of the pulp slurry (5% vs. 10% consistency). A total of 16 LMS$_{NL}(E)$ treatments (of which eight were at 5% consistency and eight at 10% consistency) were performed in accordance with the general procedure discussed in this section. The results (see Appendix I) revealed that under the experimental conditions chosen in this study, there was no statistical difference between the 5% and 10% consistency experiments with respect to delignification. Consequently, all LMS treatments in the subsequent publications were carried out at 5% consistency (except when stated otherwise).

6.7 **Alkaline extraction stage**

Alkaline extraction stages (E) were performed in 4-mm-thick heat-resistant Kapak pouches at 10% consistency, 80°C, for 1 hour, and employing 2.5% charge (o.d. basis) of NaOH. The procedure was as follows:

**Alkaline extraction (E) procedure**

1. The pulp was placed in the Kapak pouch.
2. The deionized water (some was saved for rinsing) and the 1.0N NaOH were mixed in a beaker.
3. The water/NaOH solution was introduced into the bag.
4. The beaker was rinsed and that solution was added to the slurry
5. The pouch was sealed, kneaded, and placed in a water bath.
6. The bag was kneaded every 10-15 minutes throughout the duration of the treatment.
7. After the treatment, the pulp was removed from the pouch, filtered, and thoroughly washed with the deionized water.

Peroxide reinforced alkaline extraction stages were performed in accordance with the procedure above, except hydrogen peroxide (0.5%, o.d. basis) was introduced in Step 2.

Oxygen reinforced extraction stages (E+O) were performed in the 300-mL-capacity Parr reactor at 10% consistency, 80°C, for 1 hour, and employing 2.5% charge (o.d. basis) of NaOH. The reactor was pressurized with oxygen to 60 psig. The procedure was as follows:

E+O procedure
1. The pulp was placed in the 300-mL-capacity vessel.
2. Deionized water (1/2 of required amount) was added to the pulp.
3. The slurry was stirred and heated to 80°C.
4. The 1.0N NaOH and the remaining water (10 mL were saved as rinse water) were mixed in a beaker.
5. The solution in the beaker was added to the slurry.
6. The beaker was quickly rinsed and that solution poured into the reaction mixture.
7. The reactor was immediately and quickly sealed and pressurized.
8. After the treatment, the treated pulp was filtered and thoroughly washed with deionized water.

Oxygen and peroxide reinforced alkaline extractions (E+P+O) were performed identically with the procedure described above, except hydrogen peroxide (0.5%, o.d. basis) was introduced in Step 4.

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Note: The hydrogen peroxide (30% solution) should be handled with an ice/water bath in order to reduce the degradation of the chemical. The bottle should be placed back in the refrigerator as soon as possible.

The terminal (or exit) pH after each extraction and reinforced extraction was determined. Measured pH values were always greater than or equal to 10.5.

6.8 Pulp properties

LMS-treated pulps were characterized for kappa number, brightness, and viscosity in accordance with TAPPI Standard Methods T236 or UM246 (211, 212), T452 (213), and T230 (214), respectively. Kappa and viscosity measurements were performed in duplicates. Brightness measurements consisted of five readings per handset.

6.9 Isolation of Residual lignin

Residual lignin from brownstock samples and LMS-treated pulps was isolated with the acid hydrolysis method (27, 44, 215, 216). This technique is frequently employed since it is relatively rapid and yields a lignin of relatively high purity, free of carbohydrate contamination. The isolation procedure was as follows:

Isolation procedure (see Figure 43)

A 5-L, three-necked round-bottom flask containing boiling chips and fitted with a Friedelstein condenser was charged with 100 g of air-dried pulp (o.d. basis). A 0.1 N HCl solution mixture of p-dioxane/water (9:1) was added to the pulp so that the final consistency of the slurry was 4%. The reaction mixture was refluxed for two hours under an argon atmosphere before it was cooled and filtered through a 4-L-capacity coarse fritted-glass Buchner funnel covered with filter paper (to avoid plugging). The resulting filtrate was passed through a fine-fritted-glass Buchner funnel packed with celite to remove any pulp fines. The lignin/water/dioxane mixture was then neutralized with an aqueous saturated solution of sodium bicarbonate to an apparent pH value of 5.0-5.5 and concentrated under reduced pressure to approximately 10% of the initial volume. Deionized water (approx. 400 mL) was added and the mixture was concentrated again under reduced pressure to
remove the last traces of \( p \)-dioxane. The resulting lignin-water mixture was transferred to a 1-L beaker, diluted with deionized water to a volume of approximately 800 mL, acidified to a pH of 2.0-2.5, and transferred to 250-mL-capacity centrifuge bottles. The bottles were frozen, thawed, and centrifuged. The supernatant was siphoned, and fresh acidified water was added. This step was repeated three times, each time combining the lignin into fewer bottles so that in the third wash one bottle contained all the lignin. The lignin was then freeze-dried for 1-2 days, after which it was characterized with NMR. Lignin yields were calculated as follows:

\[
\% \text{lignin yield} = \left( \frac{\text{mass of lignin isolated}}{\text{initial kappa of brownstock x 0.15}} \right) \times 100.
\]

Lignin yields ranged from 45.4-49.0%.

Figure 43. Acid hydrolysis apparatus (217).

6.10 LMS reactions on isolated residual lignins

Residual lignin (500 mg) was dissolved in a 1:1 solution of freshly distilled \( p \)-dioxane: distilled water. The solution was transferred to a Teflon beaker that was then placed in the 300-mL capacity Parr reactor. The reaction solution was heated to 45°C while it was...
stirred and was maintained at that temperature throughout the reaction period. The appropriate dose of NHA, HBT, or VA was added and the apparent pH was adjusted to 4.5 with glacial acetic acid. After mixing the solution for approximately five minutes, the required dose of laccase was added. The reactor was sealed and pressurized with oxygen to 145 psig. Subsequent to the two-hour treatment, the Teflon jacket was removed, the reacted solution was transferred into a 500-mL round-bottom flask, and it was concentrated under reduced pressure. The remaining mixture was placed in a 250-mL centrifuge bottle and diluted to approximately 180 mL with fresh distilled water. The pH was adjusted to 2.0-2.2 with 1.0 N HCl. The bottle was frozen, thawed, and centrifuged. The supernatant was decanted and fresh acidified water (pH 2.0-2.2) was added. This washing cycle was repeated three times. The washed lignin still contained in the centrifuge bottle was subjected to an alkaline extraction (E). The E stage was performed by introducing approximately 180 mL of fresh distilled water into the bottle. The pH was adjusted to 11 with 1.0 N NaOH. The bottle was then capped and placed in a water bath heated to 80°C. Subsequent to the treatment (1.5 hr), the solution bottle was allowed to cool (approx. 15 min.), and the pH was adjusted (from ≥10.5 to 2.0-2.2) using 1.0 N HCl. The precipitated lignin was then washed three times via the freeze-thaw-centrifuge method described above, freeze-dried for 1-2 days, and finally characterized with 31P- and 13C-NMR spectroscopy.

6.11 Characterization of residual lignins

6.11.1 31P NMR

31P NMR technique is an efficient and facile technique for quantifying lignin hydroxyl groups such as aliphatic, condensed and noncondensed phenolic, and carboxylic acid hydroxyl groups (200, 218). 31P NMR experiments were carried out in accordance with established literature method (58, 59, 219). The procedure was as follows:

1. A solvent solution (50 mL) of 1.6:1 of pyridine to deuterated chloroform was prepared.

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2. The solvent solution (25 mL) was used to prepare a mixture solution containing 100 mg of cyclohexanol (internal standard) and 90 mg of chromium acetylacetonate (relaxation agent).

3. Previously dried lignin (20-25 mg) was accurately weighed into a 4-mL vial that contained a magnetic spin bar. All lignins were dried under vacuum (30-mm Hg) at 40°C for 24 hours.

4. An aliquot of the solvent solution (400 μl) and an aliquot of the mixture solution (150 μl) were introduced into the vial, (sealed with a Teflon cap) containing the lignin and the spin bar, from two different Hamilton syringes.

5. The mixture in the vial was mixed for few minutes (approx. 5 min).

6. 2-Chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (70 μl; phosphorylating agent) was introduced into the vial. The vial was shaken for approximately 20 seconds before it was transferred to a 5-mm NMR tube to record the NMR spectrum.

Quantitative $^{31}$P NMR spectral acquisition was performed using an inverse gated decoupling (IGD) pulse sequence to suppress the nuclear Overhauser effect. All spectra were recorded using a 90° pulse angle, a 25-second delay between pulses, a sweep width of 61.9 ppm, a time domain size of 32,678, and a line broadening of 4 Hz, at room temperature. A minimum of 150 scans was acquired. Chemical shifts were calibrated relative to the cyclohexanol peak signal centered at 145.1 ppm. Integration regions that were used to assign the signals are tabulated in Table 13.

<table>
<thead>
<tr>
<th>Functional Group</th>
<th>Integration Region (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclohexanol (internal standard)</td>
<td>145.4-144.0</td>
</tr>
<tr>
<td>Aliphatic OH</td>
<td>150.0-145.3</td>
</tr>
<tr>
<td>Condensed phenolic OH</td>
<td>144.0-141.0</td>
</tr>
<tr>
<td>Guaiacyl phenolic OH</td>
<td>141.0-137.0</td>
</tr>
<tr>
<td>COOH</td>
<td>136.2-134.0</td>
</tr>
</tbody>
</table>
6.11.2 $^{13}$C NMR

$^{13}$C NMR samples were prepared by placing the dry lignin (100-170 mg) in a 4-mL vial containing a magnetic spin bar. DMSO-d$_6$ (450 μL) was introduced in the vial and the mixture was allowed to stir for approximately 20 min. The sample was then transferred to a 5-mm NMR tube. Spectral acquisition was performed using the following conditions: 100 pulse sequence, 90° pulse angle, 14-s delay between pulses, 220-ppm sweep width, 32,678 time domain, 10-Hz line broadening, 50°C, and 8-10,000 scans. Integration regions used to analyze the NMR spectra are illustrated in Table 14 (220, 221). All chemical shifts were calibrated relative to the DMSO peak at 39.5 ppm.

<table>
<thead>
<tr>
<th>Functional Group</th>
<th>Integral Region (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-COOH</td>
<td>178.0-162.5</td>
</tr>
<tr>
<td>C3, C4 (C-Ar-O)</td>
<td>154.0-140.0</td>
</tr>
<tr>
<td>C1, (C-Ar-C)</td>
<td>140.0-127.0</td>
</tr>
<tr>
<td>C5, (C-Ar-C)</td>
<td>127.0-123.0</td>
</tr>
<tr>
<td>C6, (C-Ar-H)</td>
<td>123.0-117.0</td>
</tr>
<tr>
<td>C5, (C-Ar-H)</td>
<td>117.0-114.0</td>
</tr>
<tr>
<td>C2, (C-Ar-H)</td>
<td>114.0-106.0</td>
</tr>
<tr>
<td>Aliphatic C-O (Cβ in β-O-4)</td>
<td>90.0-78.0</td>
</tr>
<tr>
<td>Aliphatic C-O,Cα in β-O-4</td>
<td>78.0-67.0</td>
</tr>
<tr>
<td>Aliphatic C-OR</td>
<td>67.0-61.0</td>
</tr>
<tr>
<td>Aliphatic C=O, Cα (β-O-4)</td>
<td>61.0-57.0</td>
</tr>
<tr>
<td>OCH$_3$</td>
<td>57.0-54.0</td>
</tr>
<tr>
<td>Cβ in β-β and Cβ in β-5</td>
<td>54.0-52.0</td>
</tr>
<tr>
<td>CH$_2$ in Diaryl methane</td>
<td>29.5-27.0</td>
</tr>
</tbody>
</table>

6.11.3 $^{31}$P NMR for o,p-quinones

6.11.3.1 Background

The combined content of o-, p-quinones in residual lignins isolated from the brownstock and LMS-treated kraft pulps was determined in accordance with a procedure recently developed independently by Zawadzki (72), Zawadzki et al. (73, 223), Zhang and Gellersted (224), and Argyropoulos and Zhang (225). The procedure involves the derivatiza-
tion of both ortho- and para-quinones with trimethylphosphite followed by $^{31}$P NMR analysis. The proposed tagging mechanism of $\alpha$-quinonoid structures is believed to proceed by an initial attack of the trimethylphosphite phosphorus at the carbonyl oxygen to yield intermediate II (see Figure 44). Further cyclization of II to III, followed by hydrolysis, leads to the stable open-chain phosphate ester IV that can be detected by $^{31}$P NMR.

An analogous mechanism (see Figure 45) has been proposed for tagging $\rho$-quinonoid structures.
An initial attack of the trimethylphosphite (intermediate VI), followed by a methyl shift, leads to the open-chain phosphate ester VII. Two isomeric forms can result from the reaction of trimethylphosphite with both ortho- and para-quinones.

6.11.3.2 Derivatization of residual lignins with trimethylphosphite

The derivatization procedure was performed in accordance with Zawadzki’s method (72). Residual lignins (30 mg) previously dried under vacuum (30 mm Hg) at 40°C were placed in a tared 4-mL vials (vial + Teflon cap). The sealed vials containing the lignins were dried once more as described above and weighed again. The vials were placed in an open-top dessicator and subjected to a continuous flow of argon. As a precaution, the stream of argon was passed through drierite in order to trap any moisture incoming with the gas. Then 500 μL of 50% TMP/DMF (v/v) was introduced into the vials under the inert atmosphere with a dried 1000-μL syringe. The dessicator was covered with aluminum foil and the reaction was allowed to proceed for seven days. Excess trimethylphosphite was removed by first introducing into the vials 250 μL of DMSO with a 500-μL syringe and placing them under vacuum (30 mm Hg) at 45°C until the samples were nearly dry (approx. 6 hr). The treated lignin samples were then dissolved in 500 μL of 60% DMSO-d6/pyridine (v/v) containing tri-meta-tolylphosphate (0.7 mg/mL) and chromium-acetylacetonate (0.9 mg/mL). Deionized water (5 μL) was then introduced and the lignin solutions were mixed for 12 hours before recording the 31P NMR spectra.

6.11.3.3 31P NMR acquisition conditions

31P NMR spectra of derivatized lignins were acquired using a 90° pulse angle, a 5-second pulse delay, an inverse gated broad-band proton decoupling sequence, and a 12-Hz line broadening. A minimum of 1000 scans was recorded per spectrum at 305K. The internal standard, tri-meta-tolyl-phosphate, had a sharp signal at -16.3 ppm. The combined ortho- and para-quinone signal was centered at -2.5 ppm.
6.12 NMR error analysis

NMR error analysis was established by isolating residual lignins either from brownstock samples or LMS-treated samples and analyzing the spectroscopic data three separate times. Least significant difference (LSD = \(2t^*s\)), where \(t\) is the critical student t value at the 95% confidence level, and \(s\) is the standard deviation, were calculated. LSD values were obtained for each of the functional groups analyzed by \(^{31}\)P NMR and \(^{13}\)C NMR. These values were then used to determine if the differences in the measured lignin functional groups were statistically significant. These values are tabulated in publications 2, 3, 6, and 8.
7. RESULTS AND DISCUSSION

7.1 Publication 1. Biobleaching of High-Lignin-Content Kraft Pulps via Laccase-Mediator Systems


Abstract
The response of a commercial softwood kraft pulp with an initial kappa no. of 97.5 to laccase/N-hydroxybenzotriazole (HBT) treatments was investigated. A series of 84 enzymatic treatments were performed to determine how the bio-delignification of high kappa pulps is influenced by reaction time, dose of laccase, dose of HBT, and pulp extractives. According to the analysis of variance, time, dose of mediator, dose of laccase, and extractives, all had a statistical effect on delignification at a 95% confidence level. Also, the interaction between the variable dose of laccase and extractives, as well as that between the variable dose of mediator and dose of laccase had a statistical effect on delignification. The results from this preliminary study revealed that a laccase-HBT system can effectively delignify high kappa pulps. A drop in kappa number greater than 16 points was observed in some treatments.
Introduction
The production of chemical pulps has been dramatically altered over the past decade in response to new environmental regulations and consumer activism. Although current pulp manufacturing technologies address required environmental performance regulations, new challenges and opportunities are developing. The need for improved manufacturing efficiencies, enhanced wood utilization practices, and continuing environmental concerns has become one of the central research themes of the late 1990's. Recently, significant interest has developed in the production of bleached kraft pulp originating from high-lignin-content pulps. The primary factor contributing to this research is the well-known loss of pulping selectivity when attempting to remove the last vestiges of lignin in pulps by kraft delignification. Several recent publications have examined the improved yield benefits of utilizing a single or double oxygen stage to delignify high lignin content pulps (3, 227). Bokstrom and Norden (228) have shown that overall yield improvements of 2% can be achieved by stopping a kraft cook at kappa number of 57 instead of the typical value of 27. The high-kappa pulp was then further delignified using a two-stage oxygen system followed by a typical D(EOP)D_3D sequence.

Unfortunately, chemical consumption and environmental considerations severely limit the types of delignification technologies that can be employed with high-kappa pulps. To date, the two most promising delignification technologies for high-lignin-content pulps consist of using oxygen delignification or modifying the pulping process (i.e., AQ, polysulfide, AQ/polysulfide). This paper examines a third alternative, the development of a delignification technology for high-lignin-content pulps.

The use of laccase and a chemical mediator has been shown to be very effective at delignifying typical kraft pulps. By employing a mixture of laccase and N-hydroxybenzotriazole, a 40-60% delignification of hardwood and softwood kraft pulps has been achieved (193, 198). Furthermore, these results are achieved under mild temperatures (40-55°C) and oxygen pressures (50-140 psig). The mechanism of laccase/mediator delignification is summarized in Figure 46. Studies by Sealey et al. (197), Paice et al. (190), and Poppius-Levlin et al. (229) support this general mechanism.
Figure 46. Proposed mechanism for laccase mediator delignification of kraft pulps.

In principal, the laccase mediator system is catalytic but recent studies by Bourbonnais et al. (192), Sealey and Ragauskas (204), and Potthast (205) have shown that the N-hydroxybenzotriazole is eventually converted to benzotriazole, an inactive agent for the biobleaching stage (see Figure 47).

Figure 47. Conversion of HBT to BT.

Despite these limitations, studies by Call and Muche (230), Sealey et al. (198), and Poppius-Levin et al. (231) have shown that the laccase/mediator biobleaching system is very selective for lignin removal and appears not to degrade cellulose. Based on these considerations, the use of a laccase/mediator system appeared to be a very attractive technology for the delignification of high kappa kraft pulps.
Experimental

Materials. N-hydroxybenzotriazole (HBT), glacial acetic acid, acetone, and 1.0 N NaOH were commercially purchased and used as received. Laccase was isolated from a *Porphyridium* fungus and was provided by Novo Nordisk. The enzyme was frozen to -20°C until use. Once thawed, the activity of the enzyme was measured, and the proper dose was added to the pulp.

Furnish. All laccase-mediated treatments were performed on a commercial softwood Kraft pulp. Prior to the enzymatic treatments, the pulp was washed and screened. A portion of the brownstock (K = 97.5) was acetone extracted for 24 hours and thoroughly washed to remove the residual acetone. The acetone-extracted pulp (K = 95.9) was then used for additional laccase treatments.

Laccase Activity. The activity of the laccase was measured by monitoring the rate of oxidation of syringaldazine. One unit of activity was defined as the change in absorbance at 530 nm of 0.001 per minute, per mL of enzyme solution, in a 100 mM phosphate buffer (2.2 mL) and 0.216 mM syringaldazine in methanol (0.3 mL). The test was performed at 23°C. The activity of the enzyme used in this study was 1.87E+06 (U/mL of enzyme solution).

Laccase-HBT Treatment-General Description. A 1000-mL capacity Parr Bomb, equipped with a pressure gauge and a stirrer, was charged with 10.0 g of never-dried pulp (solids basis). The pulp consistency was adjusted to 9% by adding distilled water. The slurry was then heated to a temperature of 45°C and was maintained at that temperature for the duration of the enzymatic treatment. The mediator dose was then added to the heated slurry and was allowed to mix for 5 minutes before the pH of the system was adjusted to 4.5 with glacial acetic acid. The proper dose of enzyme was then added and allowed to mix for a minute before the bomb was closed and pressurized with oxygen (145 psig). Subsequent to the laccase treatment, the pulp mixture was filtered, thoroughly
washed with distilled water (12 L/10g O.D.) and subjected to an alkali extraction. The alkali extraction was performed at 2% NaOH on O.D. pulp for 1 hour at 70°C.

Pulp Characterization. The lignin content of the brownstock, as well as that of the laccase-treated pulps, was expressed as a kappa number and was determined in accordance with Tappi Method T-236 (211). Each reported kappa number in this report represents the average of two individual measurements.

Experimental Design. A total of 84 laccase-mediator treatments were performed. The brownstock was subjected to enzymatic treatments at various times, doses of mediator, and enzyme. The experimental protocol for the brownstock is shown in Table 15. The acetone extracted pulp was also subjected to the same experimental schedule as in Table 15. However, the experiments were performed only at 1, 2, and 4 hr intervals (see Table 16).

Table 15. Experimental protocol for brownstock.

<table>
<thead>
<tr>
<th>% HBT on o.d. pulp</th>
<th>Laccase dose (U/10g of o.d. pulp)</th>
<th>2.7x10^6</th>
<th>5.4x10^5</th>
<th>1.0x10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1,2,4,6 hours</td>
<td>1,2,4,6 hours</td>
<td>1,2,4,6 hours</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1,2,4,6 hours</td>
<td>1,2,4,6 hours</td>
<td>1,2,4,6 hours</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1,2,4,6 hours</td>
<td>1,2,4,6 hours</td>
<td>1,2,4,6 hours</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1,2,4,6 hours</td>
<td>1,2,4,6 hours</td>
<td>1,2,4,6 hours</td>
<td></td>
</tr>
</tbody>
</table>
Table 16. Experimental protocol for acetone extracted brownstock.

<table>
<thead>
<tr>
<th>Laccase dose (U/ 10 g of o.d. pulp)</th>
<th>2.7 x 10^8</th>
<th>5.4 x 10^8</th>
<th>10.8 x 10^8</th>
</tr>
</thead>
<tbody>
<tr>
<td>% HBT on o.d. pulp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1,2,4 hours</td>
<td>1,2,4 hours</td>
<td>1,2,4 hours</td>
</tr>
<tr>
<td>2</td>
<td>1,2,4 hours</td>
<td>1,2,4 hours</td>
<td>1,2,4 hours</td>
</tr>
<tr>
<td>4</td>
<td>1,2,4 hours</td>
<td>1,2,4 hours</td>
<td>1,2,4 hours</td>
</tr>
<tr>
<td>6</td>
<td>1,2,4 hours</td>
<td>1,2,4 hours</td>
<td>1,2,4 hours</td>
</tr>
</tbody>
</table>

Results and Discussion

The biobleaching of kraft pulps with laccase/mediator systems continues to receive strong interest, in part due to the discovery of new mediators for laccase. Since Call’s discovery of N-hydroxybenzotriazole as a mediator for laccase delignification, several alternative and significantly improved mediators have been found for laccase (8).

The intent of this study was to investigate the response of a high-kappa kraft pulp to a laccase- mediator treatment. The mediator employed for these studies was N-hydroxybenzotriazole (HBT). Although other mediators have been developed for laccase that exhibit improved delignification properties, we selected HBT as the mediator for this study since its fundamental biobleaching chemistry is perhaps the best understood of all the mediators currently available for kraft pulps. This paper describes some of the first successful attempts at delignifying high kappa kraft pulps with a laccase- mediator system.

The laccase/ HBT biobleaching studies were performed on a commercial softwood kraft pulp with an initial kappa of 97.5. A portion of the brownstock was saved and subjected to an acetone extraction prior to enzymatic treatments to free the brownstock from extractives and to determine whether or not extractives had an effect on delignification. The starting kappa no. of the acetone-extracted pulp was 95.9. These two pulps were
then used to perform a total of 84 enzymatic treatments at various doses of laccase and HBT, and at different times (see Tables 15 and 16).

The preliminary data generated from these experiments were subjected to an analysis of variance to determine if the four variables (time, dose of mediator, dose of laccase, and extractives) had a significant effect on delignification. The analysis was also extended to investigate several interaction effects between these variables on delignification. The interaction of time with the other variables (i.e., extractives, HBT, laccase) had to be excluded from the analysis due to the unbalanced experimental design. These excluded interactions were added to the residual error. The analysis of variance is shown in Table 17.

Table 17. ANOVA for delta kappa -type III sums of squares*.

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>DF</th>
<th>Mean Square</th>
<th>F-ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main effects</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A: Extractives</td>
<td>19.4</td>
<td>1</td>
<td>19.4</td>
<td>20.0</td>
<td>0.0000</td>
</tr>
<tr>
<td>B: HBT</td>
<td>519.2</td>
<td>3</td>
<td>173.1</td>
<td>178.2</td>
<td>0.0000</td>
</tr>
<tr>
<td>C: Laccase</td>
<td>15.5</td>
<td>2</td>
<td>7.7</td>
<td>8.0</td>
<td>0.0008</td>
</tr>
<tr>
<td>D: Time</td>
<td>16.5</td>
<td>3</td>
<td>5.5</td>
<td>5.6</td>
<td>0.0017</td>
</tr>
<tr>
<td><strong>INTERACTIONS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB</td>
<td>5.3</td>
<td>3</td>
<td>1.8</td>
<td>1.8</td>
<td>0.1555</td>
</tr>
<tr>
<td>AC</td>
<td>28.7</td>
<td>2</td>
<td>14.4</td>
<td>14.8</td>
<td>0.0000</td>
</tr>
<tr>
<td>BC</td>
<td>26.6</td>
<td>6</td>
<td>4.4</td>
<td>4.6</td>
<td>0.0007</td>
</tr>
<tr>
<td><strong>RESIDUAL</strong></td>
<td>61.2</td>
<td>63</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TOTAL (corrected)</strong></td>
<td>737.3</td>
<td>83</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Statgraphics Plus (version 1.4) was used to generate the analysis of variance.

Based on the probability values (P-values) shown in Table 17, the four variables, namely, time, the dose of HBT, the dose of laccase, and the extractives content, each had a significant effect on delignification at a 95% confidence level. Interestingly, the interaction between the variable extractives and HBT did not have a significant effect on delignification (at 95% confidence level). On the other hand, the interaction between the variable extractives and laccase (AC, see Table 17), as well as the interaction between HBT and
lacase (BC, see Table 17), did have a statistically significant effect on delignification. Each of these effects will be discussed next.

**Effect of the presence of extractives on delignification.** The analysis of variance revealed that the presence of extractives had a statistical effect on delignification. Indeed, when considering all the experiments performed in this study, the kappa number fell from 97.5 to 84.5 (Δ kappa = 13) for the brownstock, and from 95.9 to 84.0 (Δ = 11.9) for the acetone-extracted pulp. Although, there is a statistical significance, change in delignification due to the presence of extractives is minor.

**Effect of HBT Dose on Delignification.** The variable HBT was also shown to have a statistical effect on delignification. Figure 48 depicts the delignification response at the various doses of HBT in the presence of lacase. As the dose of HBT increases, so does delignification. The drop in kappa number substantially increased as the mediator dose was increased from 1 to 6%. Previous studies have shown that HBT can be converted to benzotriazole and that it is not a delignifying agent (204, 205). Hence, the increase in mediator dose should improve delignification, excluding the enzyme interaction, the data in this study supported this conclusion.

**The Effect of Laccase Dose on Delignification.** The laccase dose was also shown to have a statistical effect on delignification. This observation is graphically depicted in Figure 49. Delignification increased as the enzyme dose was increased from 2.7 x 10⁶ U to 5.4 x 10⁶ U. However, when the laccase dose was further doubled from 5.4 x 10⁶ U to 10.8 x 10⁶ U, delignification seems to level off. This observation suggests that at a laccase dose higher than 5.4 x 10⁶ U, the system is saturated, making any further addition of enzyme ineffective at improving delignification.

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Figure 48. Delta kappa vs. % HBT (on o.d. pulp)

The delta kappa values are based on the average of all experiments, which include all
doses of laccase, times, and extractives content.

Figure 49. Delta kappa vs. dose of laccase.

The delta kappa values are based on the average of all experiments, which include all
doses of HBT, times, and extractives content.
**The Effect of Reaction Time on Delignification.** The time variable also had a statistically significant effect on delignification. The data shown in Figure 50 reveals an increase in delignification as the reaction time is increased from 1 to 6 hr. Although the effect of time on delignification is statistically significant, most of the lignin is removed after a 1 hr treatment, thus highlighting the efficiency of the system. A further increase in reaction time does not yield much higher levels of delignification. In essence, the data seems to suggest that delignification approaches a plateau at reaction times greater than one hour.

![Figure 50: Delta kappa vs. time](image)

The delta kappa values are based on the average of all experiments, which include all doses of HBT, laccase, and extractives content.

**Interaction effect of the extractives content and dose of laccase on delignification.**

The effect of both variables extractives and laccase yielded some very interesting results. An examination of Figure 51 reveals that, in the absence of extractives, lower delignification levels were observed when a dose of $5.4 \times 10^6$ U and $10.8 \times 10^6$ U of laccase were employed. At the lower dose of laccase ($2.7 \times 10^6$ U), this effect was not observed. The reasons for this phenomenon remain unclear.
Figure 51. Effect of extractives and dose of laccase on delignification *.

* The delta kappa values are based on the average of all experiments, which include all doses of HBT and all times.

Interaction effect of HBT dose and laccase dose on delignification. The interaction between the variables HBT and laccase on delignification is shown in Figure 52. Delignification increased as the dose of HBT was increased regardless of the dose of laccase. Although the kappa drop was higher at the 2% dose of HBT than at the 1% dose, delignification was to a large extent constant at all three levels of laccase. At the 4% and 6% doses of HBT, delignification was more pronounced than at the lower levels of HBT. Interestingly, at these doses of HBT, more laccase was required to achieve a better delignification response. This observation supports some of the earlier findings on the capability of HBT to inhibit the enzyme (8). Hence, as more HBT is added to the system, more laccase is needed to achieve substantial delignification.
Figure 52. Effect of laccase and HBT on delignification.

The delta kappa values are based on the average of all experiments, which include all reaction times and extractives.

Conclusions

Although preliminary in nature, this study is the first of its kind to examine the effects of a laccase-mediator system on high kappa pulps. The data presented in this report demonstrated that a laccase/N-hydroxybenzotriazole treatment could efficiently remove lignin from high kappa pulps. The main variables, time, dose of laccase, dose of HBT, and the extractives content, all had a statistical effect on delignification. An increase in the dose of HBT from 1% to 6% (on O.D. pulp) yielded a substantial increase in delignification (∆kappa = 8.9 vs. 15.4). Most of the lignin was removed within the first hour. However, small increases in delignification were seen for each increment in reaction time. A statistical effect on delignification was observed as the dose of laccase was increased from $2.7 \times 10^6$ to $10.8 \times 10^6$ U. However, the delignification response seemed to level off at a laccase dose of $5.4 \times 10^6$ U. The key interactions between the major variables were bet-
ween the dose of laccase and extractives and between the dose of mediator and the dose of laccase. Further investigations will be necessary to understand the trends seen between laccase and extractives. Furthermore, additional mechanistic studies will be performed to investigate the reactions between laccase/mediator and extractives on high lignin content pulps. Future studies will address these issues and also examine alternative mediators that will exhibit improved stability and delignification properties for high lignin content pulps.

Acknowledgments

The gift of the laccase from Novo Nordisk Biochem as well as the donation of the pulp from Mr. Eugene Johnson at Riverwood International is gratefully appreciated. The authors would also like to thank the Gunnar and Lillian Nicholson Faculty Exchange Fund, the Institute of Paper Science and Technology, and its member companies for their financial support. Portions of this work were used by F.S.C. as partial fulfillment of the requirements for the Ph.D. degree at the Institute of Paper Science and Technology.
7.2 Publication 2. The Effects of Oxidative Alkaline Extraction Stages after LaccaseHBT and LaccaseNHA Treatments—An NMR Study of Residual Lignins


Abstract

Two laccase-mediator systems (LMS) were performed with 1-hydroxybenzotriazole (HBT) and N-acetyl-N-phenylhydroxylamine (NHA), as the mediators, on a southern softwood conventional kraft pulp with an initial kappa of 33.8. The LMSHBT and LMSNHA treated pulps were then subjected to various reinforced alkaline extraction stages with oxygen (E+O), peroxide (E+P), and peroxide/oxygen (E+P+O). The kappa data suggested that both LMSHBT and LMSNHA are effective at delignifying kraft pulps. However, under the conditions employed in this study, a greater level of delignification was obtained with an LMSHBT stage than with an LMSNHA stage. The viscosity measurements confirmed the selectivity of LMS. Oxidative reinforcement of the alkaline treatments was beneficial for regaining the loss in brightness from LMS when the mediator was either NHA or HBT. 31P NMR spectral analysis of phosphorylated residual lignins revealed a greater enrichment of carboxylic acid groups after an LMSHBT than after an LMSNHA stage. Depletion of free phenolic groups was evident after either an LMSHBT or an LMSNHA treatment. However, it was greater after an LMSNHA stage.
Introduction

In response to environmental regulations, research efforts in pulp and paper have been directed toward finding novel delignification and bleaching technologies with minimal impact on the environment. Currently, alternative bleaching technologies to chlorine include hydrogen peroxide, oxygen, chlorine dioxide, and ozone (232). Although these chemical bleaching agents have been successfully implemented in mills around the world, the constant search for other bleaching methods is still very active. The potential for using enzymatic treatments was realized in the mid 80s, when it was discovered that xylanase pretreatments of pulps prior to their subsequent bleaching with chlorine, chlorine dioxide, and hydrogen peroxide could yield substantial savings in bleaching chemicals (233). More recently, the use of lignin-degrading enzymes, such as laccase, has been shown to be more effective than xylanases.

Historically, the use of laccase for bleaching kraft pulps had limited acceptance due to the minimal delignification that could be achieved. This inefficiency was attributed to the size of the enzyme and, therefore, to its inability to diffuse into the pulp fibers to catalyze the oxidation of lignin (5). Fortunately, this problem was circumvented when Bourbonnais and Paice discovered that laccase in the presence of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) could delignify kraft pulps (183, 187). The introduction of HBT by Call, (193, 7) which followed that of ABTS, demonstrated that much higher levels of delignification could be achieved with HBT than with ABTS. Since then, various research groups have been focusing their attention on laccase-mediator systems (163, 182, 204, 234, 235). More recently, mediators such as violuric acid, NHA, and others have been discovered (8, 236).

Despite these advancements in biobleaching systems, much remains to be discovered and learned. Further understanding of the fundamental chemistry of LMS delignification is, therefore, of paramount importance. This paper examines the effects of LMSNHA, LMSHBT, and of subsequent oxidatively reinforced alkali extraction stages on the structure of residual lignin.

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Experimental

Materials. All materials were purchased from Aldrich Chemical Co., Milwaukee, WI, and used as received, except for 2-propanol, NHA, and laccase. 2-Propanol was freshly distilled over NaBH₄ prior to using it for the lignin isolation experiments. NHA was synthesized in accordance with Oxley's method (210). Laccase, from Trametes villosa, was donated by Novo Nordisk Biochem. The conventional southern softwood kraft pulp was prepared at Potlatch Corp. facilities in Cloquet, MN. The wood source originated from Pinus taeda and was acquired from Union Camp. The wood was approximately 25 years of age, void of visual disease and of compression wood. The chips were cooked to an H-factor of 1390 using 19.5% active alkali and a 4.1 liquor:wood ratio. The pulp was thoroughly washed, screened, centrifuged, fluffed, and stored at 4°C prior to LMS bleaching treatments.

Enzyme Assay. Laccase activity was measured by monitoring the rate of oxidation of syringaldazine. One unit of activity (U) was defined as the change in absorbance at 530 nm of 0.001 per min per mL of enzyme solution, in a 100 mM phosphate buffer (2.2 mL) and 0.216 mM syringaldazine in methanol (0.3 mL). The procedure was carried out at 23°C. The activity of the laccase used in this study was 1.87E+06 U/mL of enzyme solution.

Laccase-Mediator Delignification Procedure. A 1000-ml capacity Parr reactor equipped with a stirrer, a pressure gauge, a heating mantle, and connected to a temperature controller was charged with 15 g of never-dried fibers (solid basis). The pulp consistency was adjusted to 9% by adding distilled water. The slurry was then heated to a temperature of 45°C and was maintained at this temperature throughout the incubation period. HBT (2 x 10⁻³ moles) was then added (or 2.2 x 10⁻² moles of NHA when NHA was used) to the heated slurry. Subsequent to mixing the slurry (ca. 5 min), the pH was adjusted to 4.5 with glacial acetic acid. Laccase was then added (372,000 U per gram of o.d. pulp) and the reactor was sealed and pressurized with oxygen to 145 psig. Subsequent to the four-hour treatment, the pulp was thoroughly washed and subjected to vari-
ous reinforced alkaline stages (E*). All E* stages were performed for one hour at 80°C. These stages are summarized in Table 1. Kappa, brightness, and viscosity measurements were performed on the extracted pulps in accordance with TAPPI methods T236, T452, and T230, respectively (211, 213, 214).

Control experiments were also conducted on the brownstock in the absence of laccase and mediator to evaluate the effect of E* treatments. The conditions for the E, E+O, E+P, and E+P+O stages are summarized in Table 18.

<table>
<thead>
<tr>
<th>Table 18. Summary of Extraction Stage Conditions* #.&lt;br&gt; Extraction Stage (E*)</th>
<th>%NaOH (o.d. basis)</th>
<th>%H₂O₂ (e. d. basis)</th>
<th>O₂ (psig)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>2.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E+O</td>
<td>2.5</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td>E+P</td>
<td>2.5</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>E+P+O</td>
<td>2.5</td>
<td>0.5</td>
<td>60</td>
</tr>
</tbody>
</table>

* All E* were applied to LMSHBT and LMSNFA treated pulps.<br> # Similarly, All E* conditions were applied on the kraft softwood brownstock (initial kappa # = 33.8). These experiments served as controls.

**Laccase-Mediator Procedure for Lignin Isolation Purposes.** In order to isolate the residual lignin, larger batches of LMS treated pulps were needed. In order to accomplish this task, a 2000-mL instead of the 1000-mL capacity Parr reactor was employed. In this case, the reactor was charged with 60 g of never-dried fibers (solid basis). The experimental protocol for the larger batches was identical to the one described above, except 8.9 x 10³ moles of HBT and NHA were added instead of 2.2 x 10⁴ moles.

**Isolation of Residual Lignins.** The isolation of residual lignins was carried out in accordance with standard literature methods (64). A 5000-mL three-necked round bottom flask equipped with a Friedel's condenser was charged with 50 g of o.d. pulp (air-dried). The consistency of the pulp was adjusted to 4% by adding a 0.10N (HCl) 9.1 p-dioxa-
ane-water solution. The slurry was then refluxed for 2 hr under an argon atmosphere. Subsequent to the treatment, the pulp was filtered and the filtrate was passed through celite to remove any fines. The filtrate was then neutralized and concentrated under reduced pressure to approximately 10% of the original volume. Water (ca. 400 mL) was added and the mixture was concentrated again under reduced pressure to remove the last traces of p-dioxane. The solution’s pH was then adjusted to 2.5 with 1.00 N HCl. The precipitate (i.e., the lignin) was collected, washed several times, and freeze-dried. Lignin yields ranged from 45.4 to 48.3%.

**Characterization of Residual Lignins.** The residual lignins isolated from the brownstock (kappa # 33.8) and from LMS<sub>HD</sub> (E), LMS<sub>BD</sub> (E+P+O), LMS<sub>NH</sub> (E), and LMS<sub>NH</sub> (E+P+O) treated pulps were phosphorylated and characterized by 31P NMR in accordance with established literature methods (44, 59). NMR data was acquired with a DMX400 MHz Bruker spectrometer.

**NMR Error Analysis.** The NMR error analysis was performed by repeating the isolation of the brownstock residual lignin three times under identical conditions and comparing the results. The isolated lignin samples were then phosphorylated and analyzed by 31P NMR, as described above. A least significant difference (LSD) value at a 95% confidence interval was calculated by using the standard deviations along with the Student-t value. The LSD values for the functional groups acquired by 31P NMR are illustrated in Table 19.

### Table 19. 31P NMR least significant difference values.

<table>
<thead>
<tr>
<th>Functional group</th>
<th>Average (mmol/g lignin)</th>
<th>St. dev.</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxyl OH</td>
<td>0.23</td>
<td>0.003</td>
<td>0.013</td>
</tr>
<tr>
<td>Guaiacyl OH</td>
<td>1.02</td>
<td>0.025</td>
<td>0.095</td>
</tr>
<tr>
<td>Condensed OH</td>
<td>0.82</td>
<td>0.016</td>
<td>0.060</td>
</tr>
<tr>
<td>Aliphatic OH</td>
<td>1.40</td>
<td>0.019</td>
<td>0.069</td>
</tr>
</tbody>
</table>
Results and Discussion

The purpose of this study was to compare the delignification efficiency of an LMS\textsubscript{HBT} and LMS\textsubscript{NHA} treatment. This was accomplished by using the same molar equivalence of mediators for all LMS\textsubscript{HBT} and LMS\textsubscript{NHA} treatments (note: all other experimental conditions such as dose of enzyme, temperature, time, \textit{O}\textsubscript{2} pressure, and pH were held constant). These experimental conditions were selected so that the difference in biobleaching could be attributed to the mediators. In addition, the effects of reinforcing the subsequent alkaline extraction stages to the jaccote treatments with oxygen and peroxide were examined. These changes in biobleaching were studied by measuring the physical and optical properties of the pulps, and the structural changes of the residual lignins. HBT was chosen as the reference mediator since it is probably one of the most studied mediators currently available for kraft pulps.

All LMS treatments were performed on a laboratory-prepared southern softwood conventional kraft pulp with an initial kappa of 33.8. The LMS\textsubscript{HBT} and LMS\textsubscript{NHA} pulps were then subjected to various reinforced alkaline extraction stages (see Table 18), and the extent of delignification, brightness, and viscosity were measured after LMS (E*) treatments. The residual lignins were then isolated from the brownstock and from LMS\textsubscript{HBT} (E), LMS\textsubscript{HBT} (E+P+O), LMS\textsubscript{NHA} (E) and LMS\textsubscript{NHA} (E+P+O) treated pulps and characterized via \textsuperscript{31}P NMR.

Physical and Optical Properties of LMS Treated Pulps. The changes in TAPPI brightness and viscosity results are depicted in Figures 53 and 54, respectively. Oxidative reinforcement of the alkaline extraction stage was beneficial after either an LMS\textsubscript{HBT} or an LMS\textsubscript{NHA} treatment. These results are in agreement with the trends seen by Paice \textit{et al.} when they incubated a softwood kraft pulp with LMS\textsubscript{HBT} and subsequently treated it with a QP stage (237). The largest increases in brightness were observed after an (E+P) and an (E+P+O) stage. Interestingly, relative to the brownstock (BS), an LMS\textsubscript{NHA} (E) treatment suffered a greater loss in brightness than an LMS\textsubscript{HBT} (E) treatment. This greater loss in brightness may be attributed to a higher content of quinone-type structures.
Figure 53. TAPPI brightness after LMS_{HBT}(E^*) and LMS_{NHA}(E^*) treatments on a softwood kraft pulp with a starting kappa number of 33.8. BS = brownsstock residual lignin.

Figure 54. Viscosity after LMS_{HBT}(E^*) and LMS_{NHA}(E^*) treatments on a softwood kraft pulp with a starting kappa number of 33.8. BS = brownstock residual lignin.

Viscosity measurements are in agreement with previously reported data (197, 229) and once again confirm the selectivity of a laccase-mediator system. Although both treatments were selective, the LMS_{NHA} system exhibited a higher degree of selectivity than the LMS_{HBT} system. This trend was evident despite the type of reinforcement used in the extraction stage.

The delignification results shown in Figure 55 suggest that both an LMS_{HBT} and LMS_{NHA} treatment yielded substantial levels of delignification. However, under the conditions
used for this study. HBT was more effective than NHA. The reinforcement of the alkaline stage further extended the level of delignification. Interestingly, reinforcement of the alkaline extraction stage with both peroxide and oxygen seemed to have narrowed the lignin content difference between the two LMS systems.

![Graph](image)

Figure 55. % Delignification after LMS_{HBT}(E^*) and LMS_{NHA}(E^*) treatments on a softwood kraft pulp with a starting kappa number of 33.8.

**Control Experiments.** Past literature results indicate that in order for an LMS system to yield significant levels of delignification, both the mediator and the laccase must be present (202, 237). In this study, we carried out a series of control experiments, which enabled us to discriminate between the effects of LMS and the oxidatively reinforced alkaline extraction stages on the brownstock. The alkaline extraction conditions for these control experiments are summarized in Table 18. The results, shown in Table 20, demonstrate that in all cases, an LMS stage before an E* improves delignification. The improved delignification effects were unfortunately accompanied by a decrease in pulp brightness and a slight decrease in viscosity.
Table 20. Kappa, Tappi Brightness and Viscosity of Control Experiments, LMS\textsubscript{NH\textsubscript{4}}(E\textsuperscript{*}) and LMS\textsubscript{SHT}(E\textsuperscript{*}) Treatments.

<table>
<thead>
<tr>
<th>Pulp</th>
<th>Kappa\textsuperscript{1}</th>
<th>Brightness\textsuperscript{2} (%)</th>
<th>Viscosity\textsuperscript{3}(cp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brownstock (BS)</td>
<td>33.8</td>
<td>24.5</td>
<td>33.7</td>
</tr>
<tr>
<td>BS(E)</td>
<td>31.7</td>
<td>25.6</td>
<td>27.5</td>
</tr>
<tr>
<td>BS(E+O)</td>
<td>28.9</td>
<td>26.4</td>
<td>28.8</td>
</tr>
<tr>
<td>BS(E+P)</td>
<td>27.4</td>
<td>30.2</td>
<td>24.6</td>
</tr>
<tr>
<td>BS(E+P+O)</td>
<td>25.1</td>
<td>31.9</td>
<td>22.5</td>
</tr>
<tr>
<td>LMS\textsubscript{SHT}(E)</td>
<td>25.9</td>
<td>20.8</td>
<td>25.2</td>
</tr>
<tr>
<td>LMS\textsubscript{SHT}(E+O)</td>
<td>23.1</td>
<td>23.6</td>
<td>25.6</td>
</tr>
<tr>
<td>LMS\textsubscript{SHT}(E+P)</td>
<td>21.2</td>
<td>30.8</td>
<td>24.8</td>
</tr>
<tr>
<td>LMS\textsubscript{SHT}(E+P+O)</td>
<td>19.9</td>
<td>31.7</td>
<td>22.3</td>
</tr>
<tr>
<td>LMS\textsubscript{NH\textsubscript{4}}(E)</td>
<td>28.6</td>
<td>15.9</td>
<td>30.4</td>
</tr>
<tr>
<td>LMS\textsubscript{NH\textsubscript{4}}(E+O)</td>
<td>26.3</td>
<td>18.9</td>
<td>32.2</td>
</tr>
<tr>
<td>LMS\textsubscript{NH\textsubscript{4}}(E+P)</td>
<td>24.1</td>
<td>23.6</td>
<td>27.3</td>
</tr>
<tr>
<td>LMS\textsubscript{NH\textsubscript{4}}(E+P+O)</td>
<td>22.0</td>
<td>25.6</td>
<td>27.9</td>
</tr>
</tbody>
</table>

\textsuperscript{1} The pooled standard deviation of all kappa measurements was 0.1.
\textsuperscript{2} The pooled standard deviation of all brightness measurements was 0.41.
\textsuperscript{3} The pooled standard deviation of all viscosity measurements was 0.58.

\textsuperscript{31}P NMR. Based on the physical and optical properties, an LMS\textsubscript{NH\textsubscript{4}} and an LMS\textsubscript{SHT} treatment followed by an (E+P+O) extraction stage yielded the highest brightness and level of delignification, whereas enzymatic treatments followed by a simple E stage yielded the opposite. The next step was for us to further our understanding of LMS\textsubscript{NH\textsubscript{4}} and LMS\textsubscript{SHT} by studying structural changes of the residual lignins.

\textsuperscript{31}P NMR was used to evaluate the structural changes in phosphitylated residual lignins isolated from the brownstock and after an LMS\textsubscript{SHT} (E), LMS\textsubscript{SHT} (E+P+O), LMS\textsubscript{NH\textsubscript{4}} (E) and LMS\textsubscript{NH\textsubscript{4}} (E+P+O) treatment. \textsuperscript{31}P NMR is an facile and effective method for evaluating various types of hydroxyl groups such as those present in carboxyl, free phenolic, condensed phenolic and aliphatic lignin moieties.

It is clearly evident from the data shown in Figure 56 that relative to the brownstock lignin, the LMS\textsubscript{SHT} (E) and LMS\textsubscript{NH\textsubscript{4}} (E) lignins were both enriched in carboxyl groups.

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However, this enrichment was more pronounced with HBT than with NHA. As expected, reinforcement with peroxide and oxigen further increased the content of carboxylic acid of both LMS\_NHA and LMS\_HBT residual lignins. However, this increase was more substantial when NHA was used.

![Graph showing residual lignin content after treatments](image)

**Figure 56. Carboxyl OH groups in residual lignins isolated after LMS\_HBT(E), (E+P+O), and LMS\_NHA(E), (E+O+P) treatments.**

We had earlier suggested that the greater loss in brightness after an LMS\_NHA treatment than after an LMS\_HBT could be attributed to a greater content in quinone-type structures. If our speculation in regard to this matter is correct, then the greater increase in carboxylic acid content after the LMS\_NHA (E+P+O) treatment stage could be attributed to the well-known ring opening reactions of quinones to generate muconic acid-type structures.

Inspection of Figures 57 and 58 suggests a depletion of guaiacyl and condensed phenolic hydroxyl groups, and is consistent with trends seen by other researchers when HBT was employed (199, 202). This decrease was greater with NHA than with HBT. In turn, this may suggest that the oxidative selectivity of LMS\_NHA toward phenolic lignin structures may be different than that of LMS\_HBT. Reinforcement of the alkaline extraction stages seems to narrow the gap between the two systems.
Figure 57. Guaiacyl OH groups in residual lignins isolated after LMS_{HBT}(E), (E+P+O), and LMS_{NH}(E), (E+O+P) treatments.

Figure 58. Condensed OH groups in residual lignins isolated after LMS_{HBT}(E), (E+P+O), and LMS_{NH}(E), (E+O+P) treatments.

The aliphatic lignin hydroxyl groups content, shown in Figure 59, also decreased relative to the brownstock. This decrease is consistent with recent observations of side chain oxidation and fragmentation of model compounds during LMS (E) treatments reported by Freudenchick et al. (208) and Li et al. (238). Reinforcement of the alkaline extraction
stages with peroxide and oxygen did not further deplete these types of groups, as expected.

Figure 59. Aliphatic OH groups in residual lignins isolated after LMS_{HBT}(E), (E+P+O), and LMS_{NH}_{A}(E), (E+O+P) treatments.

Overall, the structural analysis of the residual lignins suggests that the oxidative chemistry of an LMS_{NH}_{A} and LMS_{HBT} system is different. This is supported by the observed differences in the structure of the isolated residual lignins after the alkaline extractions. If the LMS bio-delignification chemistry was proceeding via the same pathway, when either mediator was used, then the residual lignins after an LMS_{NH}_{A}(E) and an LMS_{HBT}(E) would have had identical $^{13}$P{NM}{R} spectra, and this was not the case. Obviously, the same rationale applies to the residual lignins isolated after an LMS_{NH}_{A}(E+P+O) and LMS_{HBT}(E+P+O) treatments.

Conclusions

In summary, these results confirmed the reported effectiveness of HBT and NH_{A} as mediators in LMS systems. Based on the conditions used in this study, we observed that HBT yielded higher levels of delignification than NH_{A}. The oxidatively reinforced alkaline extraction stages were shown to be very beneficial and seem to narrow the gaps between the two LMS systems.
Overall, the structural analysis of the residual lignins was consistent with the delignification properties of the pulp. The improvements in the LMSHBT vs. LMSNHA systems were reflected in the NMR analysis of the lignin samples. For example, the residual lignin isolated after an LMSHBT(E) treatment was enriched in lignin carboxylic acid moieties more so than after an LMSNHA(E) treatment. The increased delignification observed when using an E+P+O stage after the LMS treatment was accompanied with increased amounts of carboxylic acid groups in the residual lignin.

The spectral analysis of the residual lignin samples after LMSNHA(E*) and LMSHBT(E*) treatments indicated that NHA, as a mediator, has different selectivity than HBT. Studies into the effect of quinone-type structures on LMS systems are given in chapter 7.

Acknowledgements

The authors would like to thank the Institute of Paper Science and Technology and its member companies for their support for these ongoing studies. The authors would also like to express their gratitude to Union Camp for supplying the Pinus taeda tree, Potlatch Corp. for pulping the chips, and Novo Nordisk Biochem for furnishing the laccase needed for these studies. Portions of this work were used by FSC as partial fulfillment of the requirements for the Ph.D. degree at the Institute of Paper Science and Technology.
7.3 Publication 3. The Kismet of Residual Lignins During LMS Delignification of High-Kappa Kraft Pulps


Summary

A series of laccase-mediator treatments (LMS) with 1-hydroxybenzotriazole (HBT) and N-acetyl-N-phenylhydroxylamine (NHA) as the mediators were performed on a laboratory prepared southern softwood conventional kraft pulp (kappa = 75.4). Subsequent to the LMS treatments, the treated pulps were subjected to various oxidatively reinforced alkaline extraction stages (E*). The kappa results suggested that both LMSHBT and LMSNHA treatments delignified this high-kappa pulp. The E* stages were beneficial in countering the darkening effect observed after the LMS treatments. Structural changes in residual lignins isolated before and after laccase-mediator (LMSNHA, E*) and LMSHBT (E*) treatments were explored. The spectral analysis of phosphitylated residual lignins revealed an increase in carboxylic acid content and a depletion of phenolic hydroxyl groups in non-condensed at C-5 lignin moieties. Aliphatic hydroxyl groups were substantially decreased when NHA was used. Overall, it appears that LMSHBT and LMSNHA treatments on high-kappa kraft pulps primarily attack phenolic hydroxyl groups in non-condensed at C-5 lignin structures.
Introduction

As pulp producers continue to address environmental concerns, other areas of interest are re-emerging. In particular, wood utilization practices are becoming consequential as the availability of and accessibility to inexpensive fibers will diminish in the long run. These issues will be vital if pulp producers are to remain competitive in this global market.

Research efforts have begun to focus on developing novel manufacturing technologies that address these issues. One of the most promising approaches to improving the economics of kraft pulp production consists of increasing overall pulp yields. This can be achieved by halting the kraft cook at a relatively high kappa (> 45) prior to reaching the terminal phase. The pulp is then subjected to a single or double oxygen stage before it is bleached. Jang et al. (3) and others have shown that this approach can improve the overall yield of bleached kraft pulps by 2-4 % (227, 228).

As an alternative to oxygen delignification, we have recently begun exploring the feasibility of delignifying high-kappa pulps via lignin degrading enzymes, more specifically with laccase (239, 240). Historically, the use of laccase for delignifying kraft pulps was limited due to the size of the enzyme and its inability to diffuse into pulp fibers to oxidize the residual lignin (5).

Fortunately, this barrier was overcome when Bourbonnais and Paice (6) realized that laccase in the presence of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), a mediator, could delignify both hardwood and softwood kraft pulps with high selectivity. The introduction of 1-hydroxybenzotriazole (HBT) by Call (7) further demonstrated the high delignification capability and high selectivity of a laccase-mediator system (LMS) on conventional pre- and post O2 kraft pulps. Research into the delignification chemistry of kraft pulps via an LMS,ABTS and an LMS,HBT system has been and continues to be extensively examined (163, 184, 194, 202, 204, 205, 229, 241, 242).

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More recently, several other compounds, such as \( N \)-acetyl-\( N \) phenylhydroxylamine (NHA), violuric acid (VA), and others have been reported to act as mediators (see Fig. 60) (8, 236). Despite these advancements, much remains to be learned about the chemistry of laccase mediated delignification systems.

![Figure 60. Structures of mediators employed in this study.](image)

The purpose of this study was to examine the delignification chemistry of LMS systems on a high-lignin content kraft pulp using NHA and HBT as mediators. The LMS\(_{\text{HBT}}\) system served as a reference to the understudied LMS\(_{\text{NHA}}\) system. The conditions of the alkaline extraction stage were varied so that the effects of peroxide (E+P), oxygen (E+O), and peroxide and oxygen (E+P+O) on the LMS treated pulp could be established. The outcome of the LMS(E+) treatments was determined by measuring the changes in lignin content and brightness of the treated pulps. In addition, the structural changes in residual lignins isolated after LMS\(_{\text{NHA}}\)(E), LMS\(_{\text{HBT}}\)(E) and LMS\(_{\text{NHA}}\)(E+P+O), LMS\(_{\text{HBT}}\)(E+P+O) treatments were ascertained by \(^{31}\)P NMR.

### Experimental

**Materials and Methods.** All materials were purchased from Aldrich Chemical Co., Milwaukee, WI, and used as received, except for \( p \)-dioxane, NHA and laccase. \( p \)-Dioxane was freshly distilled over NaBH\(_4\) prior to using it for the lignin isolation experiments. NHA was synthesized in accordance with Oxley’s method (210). A conventional southern USA softwood kraft pulp was prepared from *Pinus taeda* chips at Potlatch Corp facilities in Cloquet, MN. The chips were cooked to an H-factor of 573 using 18.5 % active
alkali. The pulp was thoroughly washed, screened, centrifuged, fluffed, and stored at 4°C prior to LMS bleaching treatments. Laccase, from Trametes villosa, was donated by Novo Nordisk Biochem, Franklinton, NC.

**Enzyme assay.** Laccase activity was measured by monitoring the rate of oxidation of syringaldazine. One unit of activity (U) was defined as the change in absorbance at 530 nm of 0.001 per minute per mL of enzyme solution, in a 100 mM potassium phosphate buffer (2.2 mL) and 0.216 mM syringaldazine in methanol (0.3 mL, pH 6.7). The procedure was carried out at 23°C. The activity of the laccase was 1.87E+06 U/mL of enzyme solution.

**Laccase-mediator delignification procedure.** A 1000-mL capacity Parr reactor equipped with a stirrer, a pressure gauge, a heating mantle, and connected to a Parr 4842 temperature controller was charged with 15 g of o.d. fibers. The pulp consistency was adjusted to 9% by adding distilled water. The slurry was then heated to a temperature of 45°C and was maintained at this temperature throughout the incubation period. HBT (2.2 x 10⁻³ moles) was then added (or 2.2 x 10⁻³ moles of NHA) to the heated slurry. Subsequent to mixing the slurry (ca. 5 minutes), the pH was adjusted to 4.5 with glacial acetic acid. Laccase was then added (372,000 U per gram of o.d. pulp) and the reactor was sealed and pressurized with oxygen to 145 psi. After the four-hour treatment, the pulp was thoroughly washed and subjected to various oxidatively reinforced alkali extraction stages (E⁺). All E⁺ stages were performed for one hour at 80°C in 4mm thick heat-sealable Kapak pouches. The E⁺ conditions are summarized in Table 21. Kappa and brightness measurements were performed on the extracted pulps in accordance with TAPPI methods T236 and T452, respectively (TAPPI Test Methods 1999).

**Table 21. Summary of extraction stage conditions.**

<table>
<thead>
<tr>
<th>Extraction Stage</th>
<th>%NaOH (o.d basis)</th>
<th>%H₂O₂ (o.d basis)</th>
<th>O₂ (psi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>2.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E⁺O</td>
<td>2.5</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td>E⁺P</td>
<td>2.5</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>E⁺P⁺O</td>
<td>2.5</td>
<td>0.5</td>
<td>60</td>
</tr>
</tbody>
</table>

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Hexenuronic acid content in brownstock. The content of hexenuronic acids in the brownstock was indirectly measured in accordance with a modified procedure reported by Vuorinen et al. (243). In brief, a 1000-mL round bottom flask was charged with 25 g of pulp (o.d. basis). The pulp consistency was adjusted to 3% by adding distilled water. The pH was then lowered to 3 using a 4.0 N solution of sulfuric acid. The slurry was refluxed for three hours at 100°C. The change in kappa number before and after the treatment was then determined and served as an indirect measurement of hexenuronic acids (see Table 22).

<table>
<thead>
<tr>
<th>Initial kappa number of</th>
<th>Kappa number after</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>75.4</td>
<td>73.6</td>
<td>2.4</td>
</tr>
<tr>
<td>75.4</td>
<td>74.0</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Control experiments. Control experiments (see Table 23) were performed on the brownstock in accordance with the LMS experimental protocol, except no laccase was employed. The treated pulps were then subjected to the E* stages under the conditions outlined in Table 21.

Table 23. Summary of control experiments.

<table>
<thead>
<tr>
<th>Experiment a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brownstock followed by E stage</td>
</tr>
<tr>
<td>Brownstock treated with HBT and followed by E stage</td>
</tr>
<tr>
<td>Brownstock treated with NHA only and followed by E stage</td>
</tr>
<tr>
<td>Brownstock treated with HBT only and followed by E+O</td>
</tr>
<tr>
<td>Brownstock treated with NHA only and followed by E+O</td>
</tr>
<tr>
<td>Brownstock treated with HBT only and followed by E+P</td>
</tr>
<tr>
<td>Brownstock treated with NHA only and followed by E+P</td>
</tr>
<tr>
<td>Brownstock treated with HBT only and followed by E+P+O</td>
</tr>
<tr>
<td>Brownstock treated with NHA only and followed by E+P+O</td>
</tr>
</tbody>
</table>

a. Mediator treatments were performed without the laccase (see LMS and extraction procedures for experimental details).
Laccase-mediated procedure for lignin isolation purposes. In order to isolate the residual lignin from the LMS treated pulps, larger batches were needed. A 2000-ml capacity Parr reactor was employed and was charged with 60 g of never-dried fibers (solid basis). The experimental protocol for the larger batches was identical to the one described above, except \(8.9 \times 10^{-3}\) moles of HBT and NHA were added instead of \(2.2 \times 10^{-3}\) moles.

Isolation of residual lignins. The isolation of residual lignins was carried out following standard literature methods (27, 64, 215). In brief, a 5000-ml three-necked round bottom flask was charged with 50 g of o.d. pulp and the consistency was adjusted to 4% by adding a 0.10 N HCl 9:1 p-dioxane water solution. The slurry was then refluxed for two hours under an argon atmosphere. The pulp was filtered and the filtrate was filtered through celite, neutralized, and concentrated under reduced pressure to approximately 10% of the original volume. Water (ca. 400 ml.) was added and the mixture was concentrated again under reduced pressure to remove the last traces of p-dioxane. The solution's pH was then adjusted to 2.5 with 1.00 N HCl. The precipitated lignin was collected, washed several times, and freeze-dried. Lignin yields ranged from 45.4 to 48.3%. Lignin yields were calculated as follows:

\[
\% \text{ lignin yield} = \left( \frac{\text{mass c't lignin isolated}}{\text{(initial kappa of brownstock)} \times 0.15} \right) \times 100
\]

Characterization of residual lignins. The residual lignins isolated from the brownstock (kappa # 75.4) and from LMSHIT (E), LMSHIT (E+P-O), LMSHIT (E) and LMSHIT (E+P+O) treated pulps were phosphorylated and characterized by \(^{31}\)P NMR in accordance with established literature methods (59, 244). NMR data were acquired with a DMX400 MHz Bruker spectrometer.

NMR error analysis. The NMR error analysis was conducted by isolating the residual lignin from the brownstock three separate times under identical conditions and comparing the results. The isolated lignin samples were then phosphorylated and analyzed by \(^{31}\)P NMR. A least significant difference (LSD) value at a 95% confidence interval was obt-
ained by using the standard deviation along with the Student-t value. The calculated LSD values for the functional groups acquired by $^{31}$P NMR are illustrated in Table 24.

<table>
<thead>
<tr>
<th>Functional group</th>
<th>Average (mmol/g)</th>
<th>SD</th>
<th>LSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxyl OH</td>
<td>0.19</td>
<td>0.006</td>
<td>0.037</td>
</tr>
<tr>
<td>Non-condensed at C-5 pheno-</td>
<td>0.91</td>
<td>0.013</td>
<td>0.078</td>
</tr>
<tr>
<td>Condensed at C-5 phenolic</td>
<td>0.69</td>
<td>0.022</td>
<td>0.133</td>
</tr>
<tr>
<td>Aliphatic OH</td>
<td>1.73</td>
<td>0.007</td>
<td>0.042</td>
</tr>
</tbody>
</table>

Table 24. $^{31}$P NMR Least significant difference values.

Results and Discussion

To date, research efforts into laccase-mediator systems have focused on the bio-delignification of low-lignin content kraft pulps with kappa numbers ranging from 10-33. These studies have been primarily directed at developing novel environmentally compatible bleaching technologies. Our research interests lie in applying LMS towards kraft process improvements. We have previously shown that an LMS treatment could efficiently delignify high-lignin content kraft pulps (239, 240). The utilization of such technology may have positive ramifications on wood utilization practices, since LMS treatments have been repeatedly shown to exhibit a high selectivity towards lignin. The purpose of this study was to further understand the fundamental LMS delignification chemistry of high-kappa kraft pulps with HBT and NHA. All LMS$_{NHA}$ (E*) and LMS$_{HBT}$ (E*) treatments were performed under identical conditions. This enabled us to relate the changes in biobleaching to the mediator. These changes were assessed by determining structural differences in the residual lignins as well as by measuring the kappa and brightness of the treated pulps.

Hexenuronic acids in the brownstock. It is well known that the presence of hexenuronic acids (HexA) in kraft pulps, especially in hardwood kraft fibers, has an impact on the kappa number. Indeed, Vuorinen et al. (233) reported that HexA contribute as much as 50% to the kappa number of Scandinavian hardwood kraft pulps. Clearly, in such cases, a kappa number would not be a good reflection of the lignin content. In this study, we proceeded to indirectly measure the HexA content in the brownstock in accor-
dance with the literature (243). The change in kappa number before and after the acid hydrolysis treatments averaged 2.15% (see Table 22). Consequently, this indicates that the kappa number is a good reflection of the lignin content in the pulp used in this study.

**Extent of delignification and brightness results**

*Delignification results from control experiments.* It has been shown that in order to achieve substantial delignification with an LMS treatment, both the mediator and the laccase must be present in the system, and that a treatment in the presence of only laccase has a minimal effect on delignification (188). In this study, we performed a series of control experiments in the absence of laccase. The pulps were first treated in the presence of HBT and/or NHA (MS_{HBT} and MS_{NHA}) and then subjected to the E+ stages. In addition, an alkaline extraction (E) was carried out on the untreated brownstock (BS(E)). The kappa numbers measured subsequent to the MS_{HBT}(E), MS_{NHA}(E), and BS(E) treatments were the same (see Fig. 61). Clearly, this indicates that the presence of the mediator alone does not delignify the pulp. Furthermore, we can conclude that the decrease in kappa number of pulps treated with either mediator and followed by an E+O, E+P, and E+P+O stage is attributed to the oxidative reinforcement and not to the mediator.

*Delignification results from LMS treated pulps.* The LMS (E+) delignification kappa data depicted in Figure 61, clearly indicate that both an MS_{HBT} and MS_{NHA} delignified the high-kappa pulp. However, based on the experimental conditions employed in this study, the use of HBT yields a higher degree of delignification than NHA. The use of oxidatively reinforced alkali extractions after both the MS_{HBT} and MS_{NHA} treatments further enhanced this effect. The use of an (E+P+O) alkaline extraction stage seems to narrow the difference of the kappa number after an MS_{NHA} and an MS_{HBT} treatment.

The addition of peroxide in the alkaline extraction stage leads to both brightening and delignification. This differs from the typical response of D_i pulps to E+P treatments, where the peroxide essentially brightens the pulp and does not significantly delignify it (75). In our case, the alkaline peroxide response could be due to several factors, including the presence of transitional metals in the pulp.
Figure 61. Kappa results of control treatments in the absence of laccase (MS-NHA and MS-HBT) and LMS treated pulps using NHA and HBT (LMS-NHA and LMS-HBT) followed by the alkaline extraction stages E, E+O, E+P, and E+P+O.

N.B.: Initial kappa # of brownstock was 75.4. The kappa of the brownstock subsequent to an alkaline extraction stage (E) was 72.3.

A general comparison of the LMS delignification results and the control studies that are summarized in Figure 62 indicate that both the mediator and laccase must be present in the system in order to achieve substantial delignification.

Brightness results from LMS treated pulps. Figure 63 illustrates the brightness values for the LMS (E) and LMS (E*) treated pulps. Pulp darkening was observed subsequent to the LMS_HBT (E) and LMS_NHA (E) treatments. However, the loss in brightness was more pronounced when NHA was used. Pulp darkening after LMS_HBT and LMS_HBT stages on low-lignin content pulps has been reported by several researchers (202, 237).
We speculate that the pulp darkening may be attributed to a greater content of quinone type structures generated during an LMS-NHA than during an LMS-HBT treatment. The oxidatively reinforced alkali extraction stages were effective at countering this loss in brightness, especially when peroxide was used (i.e., E+P and E+P+O). Evidently, the oxidative reinforcement of an alkaline extraction stage leads to the destruction of chromophores.

**Analysis of phosphorylated residual lignins.** Having characterized the LMS (E^*) treated pulps for lignin content and brightness, we proceeded further with our study by examining the structural changes in the residual lignins. The residual lignins from the brownstock and from LMS-HBT (E), LMS-HBT (E+P+O), LMS-NHA (E), and LMS-NHA (E+P+O) treated pulps were isolated, phosphorylated, and characterized via ³¹P NMR. This facile and effective technique enabled us to canvass several important lignin functional groups, including carboxylic acid groups, aliphatic hydroxyl groups, and phenolic hydroxyl groups in non-condensed and condensed at C-5 lignin moieties. Figure 63 illustrates phenolic lignin moieties that were quantified using this procedure.
Figure 63. Phenolic hydroxyl groups in C-5 condensed and non-condensed at C-5 lignin structures.

Carboxylic acid groups. The results shown in Figure 64 clearly indicate that relative to the brownstock residual lignin, the carboxylic acid groups content increased after an LMS\textsubscript{HBT} (E) and LMS\textsubscript{NHA} (E) treatment on the high-kappa pulp.

![Graph showing carboxylic acid groups content]

Figure 64. Carboxylic acid hydroxyl groups in residual lignins isolated from the brownstock as well as from LMS treated pulps using NHA and HBT (LMS-NHA and LMS-HBT) followed by the alkaline extraction stages E and E+P+O.

An analogous increase in carboxylic acid groups has been reported in previous LMS\textsubscript{HBT} work using low-lignin content kraft pulps (199). Our results also showed that the reinforcement of the alkaline extraction stage with peroxide and oxygen further increased the content of carboxylic acid groups. The content of carboxyl groups after an LMS\textsubscript{NHA}
(E+P+O) was greater than after an LMS\textsubscript{HBT} (E+P+O). This difference must be due to the different delignification chemistry of the two mediators.

**Phenolic hydroxyl groups in lignin structures non-condensed at C-5.** The data shown in Figure 65 indicate that the residual lignins isolated after an LMS\textsubscript{NHA} (E) and LMS\textsubscript{HBT} (E) treatment were depleted of noncondensed at C-5 lignin structures with respect to the brownstock lignin. Nonetheless, the decrease in this moiety was more pronounced with NHA than with HBT with a simple E stage.

![Figure 65](image)

**Figure 65. Phenolic hydroxyl groups in non-condensed lignin structures at C-5 in residual lignins isolated from the brownstock as well as from LMS treated pulps using NHA and HBT (LMS-NHA and LMS-HBT) followed by the alkaline extraction stages E and E+P+O.**

The oxidative reinforcement of the alkaline stage after an LMS\textsubscript{HBT} further decreased the non-condensed groups by an additional 31.5%. The depletion of these lignin structures has also been noted on low-lignin content pulps (199, 202). An (E+P+O) stage after the LMS\textsubscript{NHA} treatment did not yield any further decrease of phenolic hydroxyl groups in non-condensed at C-5 lignin structures, as one would anticipate when additional oxidants are introduced in an alkaline extraction stage. These results suggest that there are differences

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in the fundamental chemistry between an LMS$_{\text{NHA}}$ and an LMS$_{\text{HBT}}$ treatment. Further studies will be required to elucidate these differences.

**Phenolic hydroxyl groups in C-5 condensed lignin structures.** Inspection of Figure 66 reveals that relative to the brownstock residual lignin, the concentration of phenolic hydroxyl groups in C-5 condensed lignin structures after an LMS$_{\text{HBT}}$ (E) and an LMS$_{\text{NHA}}$ (E) treatment was comparable. The reinforcement of the alkaline extraction stage after an LMS$_{\text{HBT}}$ treatment substantially decreased the content of phenolic hydroxyl groups in these condensed lignin structures. A decrease of this magnitude was not observed after an LMS$_{\text{NHA}}$ treatment.

![Figure 66. Phenolic hydroxyl groups in C-5 condensed lignin structures in residual lignins isolated from the brownstock as well as from LMS treated pulps using NHA and HBT (LMS-NHA and LMS-HBT) followed by the alkaline extraction stages E and E+P+O.](image)

Overall, the phenolic hydroxyl data in non-condensed and condensed lignin structures suggest that the LMS delignification chemistry on high-kappa pulps exhibits a higher selectivity towards non-condensed at C-5 lignin structures than towards condensed C-5 lignin moieties. These results are in general agreement with recent studies on LMS$_{\text{HBT}}$
Aliphatic hydroxyl groups. The results shown in Figure 67 illustrate a decrease in the content of aliphatic hydroxyl groups after an LMS$_{\text{NHA}}$ (E) and an LMS$_{\text{HBT}}$ (E) treatment relative to the brownstock residual lignin. However, the decrease was greater with NHA than with HBT.

![Graph showing aliphatic hydroxyl groups content]  

Figure 67. Aliphatic hydroxyl groups in residual lignins isolated from the brownstock as well as from LMS treated pulps using NHA and HBT (LMS-NHA and LMS-HBT) followed by the alkaline extraction stages E and E+P+O.

This observation is indicative of side chain oxidation, and is consistent with Freudenreich et al. (208) and Li et al. (238) recent observation of side chain oxidation and fragmentation of model compounds during LMS treatments. The benefits associated with oxidatively reinforcing the alkali extraction stages were not evident.

Conclusions

In summary, the delignification response of the pulps clearly indicates that an LMS treatment can be effectively employed on high-lignin content kraft pulps. Oxidative rein-
forçement of the alkali extraction stages is beneficial in delignifying and countering the
darkening phenomenon observed after LMS treatments. Based on the spectral analysis of
residual lignins, an LMS\textsubscript{NHA} (E) treatment led to a greater decrease in the content of phe-
nolic hydroxyl groups in non-condensed at C-5 lignin structures than an LMS\textsubscript{HBT} (E)
stage. Nonetheless, the results seem to indicate that both an LMS\textsubscript{NHA} and an LMS\textsubscript{HBT}
principally favors the oxidation of free phenolic moieties. Oxidation of side chain ali-
phatic hydroxyl groups was more pronounced after an LMS\textsubscript{NHA} than after an LMS\textsubscript{HBT}
treatment.

Our LMS studies on high-kappa kraft pulps suggest that differences exist in the delignifi-
cation chemistry of NHA and HBT, despite the fact that both mediators operate via nit-
roxy radicals (205). As previously discussed, the formation of quinones could be occur-
dring during the LMS treatment. This hypothesis is supported by the brightness response
of the pulps to E vs. (E+P+O). In addition, the structural changes in the phenolic hy-
droxyl content of the LMS (E*) residual lignin are consistent with our hypothesis. Further
studies are ongoing to confirm this important issue.

Acknowledgements
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Paper Science and Technology.

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7.4 Publication 4. The path forward to practical nascent laccase biobleaching technologies


Abstract
The advantages of chemical bleaching agents are well established and include many factors such as ease of use, reliability, acceptable selectivity, and bleaching costs. Given these considerations, the successful implementation of a biobleaching stage in a modern kraft mill must meet several criteria including cost, dependability, and pulp performance properties. Research studies into the biobleaching of kraft pulps promise new bleaching technologies that could reduce capital expenditures and yield bleached products with superior physical pulp properties and negligible impact on the environment.

Over the past several years, the utilization of laccase-mediated systems (LMS) for delignifying kraft pulps has steadily improved in terms of delignification capabilities, enzyme and mediator dosages, and bleached pulp properties. Our studies have been primarily directed at understanding the fundamental chemical processes involved in LMS biodelignification. A better understanding of the enzymatic and chemical mechanisms involved in the LMS system will improve the overall biobleaching effect and hasten its application in commercial bleaching operations. This paper reports on improving the LMS system using violuric acid (VA) or N-acetyl-N-phenyl-hydroxylamine (NHA) as mediators. The bleaching properties of LMS were investigated in terms of brightness, lignin content, and pulp viscosity.
Introduction

Bleached kraft pulp production is a dominant global industry that contributes 50% of the total world wood pulp production. Over the past decade, this part of the fiber line has been dramatically changed to improve the overall environmental performance of bleached kraft pulp operations. As a result, the demand for elemental chlorine has substantially decreased and the consumption of chlorine dioxide, hydrogen peroxide, oxygen, and ozone has increased. With the introduction of these new bleaching technologies, the research focus for future bleaching opportunities has begun to change. A research issue that is taking on added importance revolves around the need to reduce capital expenditures associated with bleaching kraft pulps. As discussed by several industry leaders in the recent TAPPI Journal Industry Leaders Interview Series (245, 246, 247), the production of wood pulp is not providing sufficient return on equity for the amounts of capital required to produce bleached kraft pulp. Furthermore, there is a growing belief that this issue will not be addressed by the incremental research studies of the past. New pulping, bleaching, and papermaking technologies need to be developed that can substantially reduce the capital requirements of the pulp mill of the future. Certainly, under this vision, alternative technologies such as biopulping and biobleaching of kraft pulps appear attractive. The temperature, pH, and pressure requirements of many oxidoreductases, coupled with their catalytic properties, suggest that biopulping/bleaching systems with low-capital requirements and low operating costs could be developed for the pulp mill of the future.

Several research groups are actively investigating biobleaching systems that could be employed to remove lignin from kraft pulps. Hemicellulase-aided bleaching is the most well-established economically feasible biotechnical application in the pulp and paper industry. The enzymatic treatments, based on xylanases (4) and mannanases (248), introduce modifications in the carbohydrate structures, leading to enhanced delignification in subsequent chemical bleaching operations. The mechanism is based on the partial depolymerization of hemicelluloses, which impede the chemical removal of residual lignin from pulp fibers. The benefits obtained by the enzymatic treatment depend on the type of
raw material, pulping process, and bleaching sequence. The enzymatic treatment leads to reductions in chemical consumption, maintains product quality, and has been commercially implemented.

Several other promising enzymatic delignification technologies are under active investigation, including the use of laccase-mediator systems (183), manganese peroxidase (249), and EPOC (i.e., mixture of lipase/ketone/fatty acid/H2O2) (250). Mn-peroxidase is secreted by white-rot fungi and participates in the degradation of lignin (251). Ebara et al. have shown that this treatment reduced the total effective chlorine charge required to obtain a pulp of 85% brightness in a (CD)ED sequence by 51, 66, and 69% for normal- and low-lignin-content softwood pulps and hardwood pulps (252). Call has presented data indicating that the EPOC system can delignify commercial kraft pulps by 40% under mild conditions and short retention times. Laccase-mediator bleaching technology has advanced significantly over the past decade. Since the initial reports highlighting the need of employing a mediator with laccase to achieve efficient delignification, significant improvements in this technology have come forth. Advances in mediator design (8, 253), fundamental LMS chemistry (190, 199, 254, 255, 256), and pulp bleachability have demonstrated that (LMS)E treatment of conventional HW and SW kraft pulps can achieve 20-55% delignification (194, 229, 257).

The application of LMS technology toward high kappa kraft pulps is another promising opportunity. Studies by Parthasarathy (258), Bokstrom et al. (228), and others have shown that by halting the kraft cook prior to reaching the residual phase, pulp yield can be improved. For example, Magnotta, et al. (259) reported that halting a SW kraft cook at kappa 44 and then performing an O0-stage, followed by D(U+O+P)D, provides a 3.8% yield increase over O0(U+O+P)D for a 30-kappa SW kraft pulp. By extension, an LMS-stage could potentially replace an O or OO-stage for high kappa kraft pulps and provide improved delignification properties over a typical O-stage. Our initial studies in this field have demonstrated that LMS treatments can remove 16-38% of the lignin from SW kraft pulps ranging in kappa numbers from 70 to 90 (239, 260). Interestingly, we have also demonstrated that a double LMS treatment can extend the biodeignification effect, re-
moving 53% of the lignin from a 73-kappa SW kraft pulp via (LMS)(LMS)(E). In addition, the overall performance of an LMS(E) can be significantly improved by oxidatively reinforcing the E-stage with hydrogen peroxide and/or oxygen (240, 261). This effect is due, in part, to the in-situ formation of quinones (262, 263) during the LMS stage, which can then be oxidatively removed with alkaline peroxide. This manuscript further explores the biodelignification effects using a new generation (8) of N-hydroxy-based laccase mediators with high-lignin content SW and HW kraft pulps. The effects of biodelignification are explored in terms of % delignification, yield, and fundamental LMS bleaching chemistry.

**Experimental**

**Materials.** All materials employed in this study were purchased from Aldrich Chemicals, Milwaukee, WI, and used as received except for N-acetyl-N-phenylhydroxylamine (NHA) and laccase. NHA was synthesized in accordance with Oxley’s method (210) and laccase from *Trametes villosa* was donated by Novo Nordisk Biochem.

**Furnish.** Table 25 provides a brief description of the pulps employed in this study. In brief, the LMS_{NHA} and LMS_{VA} treatments were performed on a series of high-lignin-content laboratory-prepared conventional batch southern softwood and hardwood kraft pulps. In addition, two commercial hardwood and softwood kraft pulps were employed with the LMS_{VA} system.

**Table 25. Description of Kraft Pulps Employed**

<table>
<thead>
<tr>
<th>Pulp</th>
<th>Kappa #</th>
<th>Viscosity/cP</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW-lab prepared</td>
<td>73.4</td>
<td></td>
</tr>
<tr>
<td>SW-lab prepared</td>
<td>50.0</td>
<td>45.0</td>
</tr>
<tr>
<td>SW-commercial</td>
<td>27.5</td>
<td>24.2</td>
</tr>
<tr>
<td>HW-lab prepared</td>
<td>26.9</td>
<td>67.6</td>
</tr>
<tr>
<td>HW-commercial</td>
<td>10.8</td>
<td>21.6</td>
</tr>
</tbody>
</table>

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Laccase Assay. Laccase activity was measured by monitoring the rate of oxidation of syringaldazine. One unit of activity (U) was defined as the change in absorbance at 530 nm of 0.001 per minute, per mL of enzyme solution, in a 100 mM phosphate buffer (2.2mL) and 0.216 mM syringaldazine in methanol (0.3mL). The procedure was carried out at 23°C. The activity of the enzyme was 1.87 E+05 (U/mL of enzyme solution).

Laccase-mediator treatment procedure. All LMS experiments were carried out using a 1000-mL capacity Parr reactor equipped with a pressure gauge, a stirrer, and a temperature controller. The pulp (20g o.d. basis) was placed in the reactor and the consistency was adjusted to 5% using distilled water. The slurry was then heated to 45°C while mixing and was maintained at that temperature throughout the incubation time. The appropriate dose of mediator was added and further mixing (approx. 5 min) was allowed. The pH was then adjusted to 4.5 and the appropriate dose of laccase was added. The reactor was sealed and pressurized with oxygen (10 bar). Subsequent to the treatment, the pulp was removed from the reactor and thoroughly washed with distilled water. The treated pulp was subjected to an alkaline extraction stage and then characterized.

Pulp Characterization. The delignification response of the LMS\textsubscript{NLAE} (E) treatments was expressed as kappa number. Kappa numbers were determined in accordance with TAPPI Standard Method T-236 (211) Each reported kappa number represents the average of two individual measurements. Typically, the kappa number of duplicates varied by +/- 0.3 kappa units.

Experimental Design. A two-phase central composite design (CCD) was carried out in this study. A total of 33 LMS\textsubscript{NLAE} (E) treatments were performed at various times, doses of NHA, and laccase. The data obtained from Phases I and II were then subjected to conventional multiple linear regression analysis. Regression models from each phase were constructed from a set of variables consisting of the original variables, together with their squares and pairwise cross products. The regression models contained only those terms that were justified by a significance test at a 95% confidence level (t-tests) and that yielded the highest value of the multiple correlation coefficient (R²). In addition, the lack
of fit for the two regression models was calculated and was determined to be insignificant at the 95% confidence level.

**Results and Discussion**

The delignification response of LMS treatments is critically dependent on the properties of the mediator. Although a host of mediators have been proposed for LMS delignification, some of the most effective mediators are N-hydroxy-based structures (see Figure 68).

![Figure 68. N-Hydroxy-based laccase mediators.](image)

The first part of this investigation dealt with improving the LMS conditions using NHA as the mediator. This task was accomplished by carrying out a two-phase central composite design. In Phase I, 20 enzymatic treatments were performed at various times, doses of NHA, and laccase. In Phase II, 13 treatments were carried out at various doses of NHA and laccase and at a constant time of 2 hours. Phases I and II, along with the kappa results, are summarized in Tables 26 and 28 respectively. In order to simplify the regression analysis, the original variables (i.e. time, % NHA, and dose of laccase) were nondimensionalized and coded. Tables 27 and 29 define the relationships for the coded variables for Phases I and II respectively.
### Table 26. LMS(Y)* experimental conditions and data for Phase I.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time (^b)</th>
<th>% NHA (^c)</th>
<th>Latexase (^d) soluble</th>
<th>Kappa</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.0</td>
<td>1.0</td>
<td>1.0</td>
<td>73.4</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>1.0</td>
<td>9.0</td>
<td>68.1</td>
</tr>
<tr>
<td>3</td>
<td>2.0</td>
<td>4.0</td>
<td>9.0</td>
<td>67.2</td>
</tr>
<tr>
<td>4</td>
<td>2.0</td>
<td>4.0</td>
<td>1.0</td>
<td>63.7</td>
</tr>
<tr>
<td>5</td>
<td>6.0</td>
<td>4.0</td>
<td>9.0</td>
<td>67.5</td>
</tr>
<tr>
<td>6</td>
<td>6.0</td>
<td>1.0</td>
<td>1.0</td>
<td>67.1</td>
</tr>
<tr>
<td>7</td>
<td>6.0</td>
<td>4.0</td>
<td>1.0</td>
<td>63.0</td>
</tr>
<tr>
<td>8</td>
<td>6.0</td>
<td>1.0</td>
<td>9.0</td>
<td>68.4</td>
</tr>
<tr>
<td>9</td>
<td>4.0</td>
<td>2.0</td>
<td>3.0</td>
<td>66.7</td>
</tr>
<tr>
<td>10</td>
<td>4.0</td>
<td>2.0</td>
<td>3.0</td>
<td>66.0</td>
</tr>
<tr>
<td>11</td>
<td>4.0</td>
<td>2.0</td>
<td>3.0</td>
<td>66.4</td>
</tr>
<tr>
<td>12</td>
<td>4.0</td>
<td>2.0</td>
<td>3.0</td>
<td>66.9</td>
</tr>
<tr>
<td>13</td>
<td>4.0</td>
<td>2.0</td>
<td>3.0</td>
<td>66.6</td>
</tr>
<tr>
<td>14</td>
<td>4.0</td>
<td>2.0</td>
<td>3.0</td>
<td>66.0</td>
</tr>
<tr>
<td>15</td>
<td>7.4</td>
<td>2.0</td>
<td>3.0</td>
<td>66.0</td>
</tr>
<tr>
<td>16</td>
<td>4.0</td>
<td>6.4</td>
<td>3.0</td>
<td>63.8</td>
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<tr>
<td>17</td>
<td>4.0</td>
<td>2.0</td>
<td>19.0</td>
<td>69.2</td>
</tr>
<tr>
<td>18</td>
<td>0.6</td>
<td>2.0</td>
<td>3.0</td>
<td>67.1</td>
</tr>
<tr>
<td>19</td>
<td>4.0</td>
<td>0.6</td>
<td>3.0</td>
<td>68.5</td>
</tr>
<tr>
<td>20</td>
<td>4.0</td>
<td>2.0</td>
<td>0.5</td>
<td>65.3</td>
</tr>
</tbody>
</table>

\(^a\) Stages were performed with 2.5% NaOH, for 1 h, at 80°C and 10% consistency.

\(^b\) Time in hours

\(^c\) % mediator on o.d. basis.

\(^d\) mL of enzyme solution per 10 g of o.d. fiber.
Table 27. Definition of the relationships for the coded variables for Phase I.

<table>
<thead>
<tr>
<th>Original Variable</th>
<th>Coded Variable</th>
<th>Coding Relationship</th>
<th>Sample Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time, h</td>
<td>T</td>
<td>( T = (\text{time-4})/2 )</td>
<td>-1 when Time = 2.00; 0 when Time= 4.00; +1 when Time = 6.00; -1.682 when Time = 0.64; +1.682 when Time = 7.40</td>
</tr>
<tr>
<td>NHA, %</td>
<td>M</td>
<td>( \log M = 0.301 \text{NHA} + 0.301 )</td>
<td>-1 when NHA = 1.00; 0 when NHA= 2.00; +1 when NHA = 4.00; -1.682 when NHA = 0.62; +1.682 when NHA = 6.40</td>
</tr>
<tr>
<td>Laccase, mL</td>
<td>L</td>
<td>( \log L = 0.477 \text{Laccase} + 0.477 )</td>
<td>-1 when Laccase = 1.00; 0 when Laccase = 3.00 +1 when Laccase = 9.00; -1.682 when Laccase = 0.47; +1.682 when Laccase =19.60</td>
</tr>
</tbody>
</table>

The final regression model obtained for Phase I is as follows:

Kappa = 66.33 -1.26 M +1.23 L + 0.74 M*L + 0.28 L²

The R² value obtained is 0.960 and the standard deviation is 0.37. The variable time was not included in the regression model since this term was not statistically significant (at the 95% confidence level). In turn, this highlights the efficiency of an LMS, in that most of the delignification takes place in a relatively short time. Figure 69 demonstrates that there is good agreement between the observed and predicted values.

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Figure 69. Predicted kappa by the regression mode vs. the observed kappa for Phase I.

The best delignification response was observed at a 4% dose of NHA and at 1 mL of laccase/10 g of o.d pulp, resulting in 9.8 kappa unit drop with respect to the brownstock kappa. Although the results were promising, we believed that delignification could be further improved and, thus, we proceeded to carry out Phase II of the experimental design. Table 28 summarizes our results from Phase II.

The final regression model for Phase II is as follows:

\[
\text{Kappa} = 63.55 - 1.86 M + 0.91 L + 0.37 M^2 + 0.69 M^2
\]

The R² value obtained was 0.984 and the standard deviation was 0.28. As in Phase I, there was a good agreement between the predicted and observed kappa numbers (see Figure 70).
Table 28. Experimental conditions and data for Phase II*.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Timeb</th>
<th>% NHAc</th>
<th>Enzymed</th>
<th>Kappa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting pulp</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>73.4</td>
</tr>
<tr>
<td>1</td>
<td>2.0</td>
<td>2.00</td>
<td>0.50</td>
<td>65.3</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>2.00</td>
<td>1.50</td>
<td>66.5</td>
</tr>
<tr>
<td>3</td>
<td>2.0</td>
<td>6.82</td>
<td>1.00</td>
<td>62.2</td>
</tr>
<tr>
<td>4</td>
<td>2.0</td>
<td>4.00</td>
<td>1.00</td>
<td>63.8</td>
</tr>
<tr>
<td>5</td>
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<td>4.00</td>
<td>1.0</td>
<td>63.7</td>
</tr>
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<td>1.00</td>
<td>67.8</td>
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<td>7</td>
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<td>1.70</td>
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</tr>
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<td>8</td>
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<td>4.00</td>
<td>0.29</td>
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<td>0.50</td>
<td>61.1</td>
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<td>1.00</td>
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<td>6.00</td>
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<td>63.8</td>
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<td>4.00</td>
<td>1.00</td>
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</tr>
<tr>
<td>13</td>
<td>2.0</td>
<td>4.00</td>
<td>1.00</td>
<td>63.0</td>
</tr>
</tbody>
</table>

*E stages were performed with 2.5% NaOH, for 1 h, at 80°C and 10% consistency.

bTime in hours

c% mediator on o.d. basis.

dmL of enzyme solution per 10 g of o.d. fiber.

Table 29. Definition of the relationships for the coded variables for Phase II.

<table>
<thead>
<tr>
<th>Original Variable</th>
<th>Coded Variable</th>
<th>Coding Relationship</th>
<th>Sample Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHA, %</td>
<td>M</td>
<td>M = (NHA-4)/2</td>
<td>-1 when NHA = 2.00, 0 when NHA = 4.00; +1 when NHA = 6.00, -1.414 when NHA = 1.17, +1.414 when NHA = 6.83</td>
</tr>
<tr>
<td>Laccase, ml.</td>
<td>L</td>
<td>L = (Laccase-0.5)</td>
<td>-1 when Laccase = 0.50, 0 when Laccase = 1.00; +1 when Laccase = 1.50, -1.414 when Laccase = 0.29, +1.414 when Laccase = 1.71</td>
</tr>
</tbody>
</table>
Figure 70. Predicted kappa by the model vs. observed kappa for Phase II.

Based on our experimental conditions in Phase II, the best delignification response was observed at a dose of 6% of NHA and 0.50 mL of enzyme solution. These conditions enabled us to reduce the kappa number by additional 2.50 units using 50% less of enzyme solution. The dose of mediator had to be increased from 4% to 6%. In turn, this suggests that an LMS\textsubscript{NHA} treatment is not truly catalytic. Freudenreich et al. (208) have recently shown that NHA can be reduced to a nondelignifying agent, and that may explain in part, the need for higher doses of NHA.

Although we anticipated that the delignification properties of LMS\textsubscript{NHA} could be further improved, preliminary concurrent investigative studies employing violuric acid (VA) as a LMS mediator indicated that this agent exhibited superior biodelignification properties.
By employing identical LMS conditions and the same pulp as in Phase II (i.e. 0.5mL/10-g o.d. pulp; 6% molar equivalence of NHA), an LMS

\[ \text{VA}(E) \]

was found to substantially outperform the LMS\text{NHA} system. Indeed, we were able to reduce the pulp kappa number by 34.0 units. In addition, we found the superiority of VA holds true regardless of the LMS conditions and the starting kappa number. For example, an LMS\text{VA}(E) treatment (4% molar equivalence of NHA on o.d. pulp; 5% consistency; 3 mL of laccase solution/10 g of o.d. pulp; 45°C; 2 hr; extraction: 2.5% NaOH on o.d. pulp; 80°C; 2 hr) on a 50-kappa SW kraft pulp led to a drop of 17 kappa units. When the identical treatment was carried out using NHA, we observed a total drop of 7.2 kappa units.

A notable observation of these studies is the dramatic differences in delignification efficiency despite the fact that both VA and NHA are N-hydroxy-based mediators. Following these investigations, we further explored the response of the LMS\text{VA}(E) system to laccase charge. The results are shown in Figure 71. In general, the behavior of an LMS\text{VA} treatment was consistent with the trends observed with the LMS\text{NHA} system.

\[ \text{Figure 71. Delignification of 27.5 kappa SW kraft pulp with LMS}\text{VA}(E^*)^6. }\]

\[
\begin{align*}
\text{Kappa} & \quad \text{Beemstock} & \quad \text{E} & \quad \text{LMS-VA} & \quad \text{LMS-VA} & \quad \text{LMS-VA} & \quad \text{LMS-VA} \\
& & \quad \text{(E-P)} & \quad \text{(E-P)} & \quad \text{(E-P)} & \quad \text{(E-P)} & \quad \text{(E-P)} \\
10 & & & & & & \\
5 & & & & & & \\
0 & & & & & & \\
\end{align*}
\]

\( ^6 \text{VA1 used 1.87 E+04 U of laccase, VA2 used 5.61 E+06 U laccase, VA3 used 11.22 E+06 U of laccase, VA4 used 22.4 E+06 U of laccase. The charge of VA was 4%: The E-stage was at 80°C, 10% csc, 2.5% NaOH, for 1.5 h. The (E+P) stages were performed in a similar manner except 0.5% H_2O_2 was added. } \)
Our interest in LMS treatments is based not solely on its delignification response but also on its low reactivity with cellulose. Indeed, treatment of an LMS system with a fully bleached pulp was shown by Haynes (264) to have no impact on pulp viscosity, suggesting that the LMS system is highly specific for lignin. Nonetheless, the reactivity of LMS toward cellulose may differ with an unbleached kraft pulp where lignin-derived radicals are formed. This high bleaching specificity should provide very high pulp bleaching yields. To test this hypothesis, a series of HW and SW kraft pulps were treated to an LMS_{10}(E+P) sequence and gravimetric yields were determined. The results shown in Table 30 demonstrate an improved selectivity and yield benefits with an LMS_{10}(E+P) stage for both high- and low-kappa kraft pulps.

Hexenuronic acid analysis of the two HW kraft pulps was performed in accordance with a modified procedure established by Vuorinen et al. (243). The analysis indicated that the contribution of these groups to the initial kappa number was 18.2% (initial kappa no. 26.9) and 24.0% (initial kappa no. 10.8), respectively. It is known that an LMS treatment does not oxidatively remove hexenuronic acid groups (265). Hence, further substantial delignification could be accomplished after an LMS_{10}(E+P) treatment if the two HW kraft pulps had been treated with a mild acid stage.

<table>
<thead>
<tr>
<th>Pulp</th>
<th>SW 50.0 kappa</th>
<th>SW 27.5 kappa</th>
<th>HW 26.9 kappa</th>
<th>HW 10.8 kappa</th>
</tr>
</thead>
<tbody>
<tr>
<td>45.0 cP</td>
<td>61.1</td>
<td>65.4</td>
<td>70.4</td>
<td></td>
</tr>
<tr>
<td>24.2 cP</td>
<td>61.1</td>
<td>65.4</td>
<td>70.4</td>
<td></td>
</tr>
<tr>
<td>% Delign.</td>
<td>42.6</td>
<td>25.8</td>
<td>99.9</td>
<td></td>
</tr>
<tr>
<td>% Viscosity Loss</td>
<td>20.4</td>
<td>45.9</td>
<td>99.4</td>
<td></td>
</tr>
<tr>
<td>Yield</td>
<td>99.9</td>
<td>100.0</td>
<td>99.1</td>
<td></td>
</tr>
</tbody>
</table>

*(E+P) stage was for 1.5 h, 80°C, 10% csc, 0.5% H_2O_2, 2.5% NaOH, gravimetric yield corrected for loss of lignin (i.e., mass lignin lost = 0.15% (initial – final kappa #).
The pulp bleachable ability of the LMS$_{VA}$-treated pulps was examined using a DED sequence. The results, summarized in Figure 72 and Table 31, indicated that the LMS-treated pulps can achieve +80 TAPPI brightness values employing ECF bleaching technologies.

Figure 72. Changes in TAPPI brightness bleaching SW kraft pulp (kappa 30) with LMS$_{VA}$ (E+P+O)DED and LMS$_{VA}$ LMS$_{VA}$ (E+P+O)DED.

Table 31. Bleaching conditions employed for LMS$_{VA}$ (E+P+O)DED and LMS$_{VA}$ LMS$_{VA}$ (E+P+O)DED.

<table>
<thead>
<tr>
<th>LMS$_{VA}$</th>
<th>(E+P+O)</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% charge VA. See experimental section for remaining details</td>
<td>70°C, 10% csC, 1 hr, 0.05% MgSO$_4$, 2.5% NaOH, 15 min 60 psi, reduce pressure by 12 psi every 5 min</td>
<td>D$_0$: 70% csC, 30°C, 45 min, D$_1$: 70°C, 3 hr, 10% csC, see Fig. 73 for ClO$_2$ charges in brackets</td>
</tr>
</tbody>
</table>

Conclusions

The results of this study yield a series of conclusions. First, high-kappa pulps respond favorably to both LMS$_{NHA}$ and LMS$_{VA}$, although the latter mediator exhibits a superior biodelignification response. For the LMS$_{NHA}$ system studied, the reaction is complete within 36 minutes and high doses of lactase do not provide improved delignification. In
Contrast, higher doses of mediator provide improved lignin removal. These experimental trends appear to also hold true with the LMS$_{VA}$ system. The high selectivity of the LMS$_{VA}$ system provides exceptionally high pulp yields after alkaline extraction. Finally, the LMS-treated pulps are amendable to ECF bleaching conditions providing +80 brightness pulps. Despite the significant delignification that is achievable with NHA and VA on high- and low-kappa pulps, these mediators cannot be considered ideal. Hence, this creates once again an opportunity for finding other mediators compatible with laccase.

Acknowledgments
The authors would like to thank U.S. Department of Energy (DOE) and the member companies of the Institute of Paper Science and Technology for their support of these ongoing studies. This manuscript was prepared, in part, with the support of DOE Cooperative Agreement No. DE-FC36-99GO10374. However, any opinions, findings, conclusions, or recommendations expressed herein are those of the author(s) and do not necessarily reflect the views of DOE. Also, we would like to thank Drs. Dimmel and Lucia for their guidance, Novo Nordisk Biochem for supplying the laccase and Pulpitch Corp. for providing one of the laboratory-prepared kraft pulps employed in this study. Portions of this work were used by F.S.C. as partial fulfillment of the requirements for the Ph.D. degree at the Institute of Paper Science and Technology.
7.5 Publication 5. Laccase-Mediator Systems and Oxygen Delignification-A Comparative Study


Abstract

A conventional SW kraft pulp with an initial kappa number of 73.4 was subjected to a series of treatments using laccase-violuric acid (LMSVA = L), oxygen, and a combination of both systems. The treated pulps were characterized for kappa, brightness, and viscosity. Based on the experimental conditions employed in this study, an L, LE, and LLE exhibited superior retention in pulp viscosity than an O or a double O. The delignification response obtained with an LLE and an OLE treatment was comparable to a OO stage. Relative to the brownstock, the laccase treated pulps were significantly lower in brightness. However, an E stage after an L treatment alleviated this effect. Overall, the application of laccase-mediator systems on high-kappa pulps may be a viable technology that can yield substantial delignification without detrimental ramifications on viscosity.
Introduction

The bleaching of kraft pulps is a pivotal operation in the production of high-value paper products. Over the last two decades, research activities in pulp and paper have largely focused on environmental issues. As these issues continue to be addressed, new opportunities are developing. There has been a renewed interest in pulping and bleaching yields research (3, 228, 259, 266, 267, 268). Improvements in yields will have positive ramifications on wood utilization practices, as well as on operating and capital cost associated with the production of kraft pulps. One promising method for improving pulp yield consists of halting the kraft cook at a relatively high kappa (> 40). The pulp is then treated with a single or double oxygen stage before it is bleached.

Recently, we have begun exploring competing enzymatic systems, more specifically laccase-mediator systems (LMS), as potential cost-effective alternatives to oxygen delignification (239, 240). Several research groups (188, 8, 192, 197, 229, 241) have established the high selectivity of LMS and the ability to achieve substantial levels of delignification (> 45% delignification) on low-lignin content SW and HW kraft pulps (kappa # < 30). Based on these findings, we hypothesized that an LMS could delignify high-lignin content kraft pulps. Our initial results (239) shown in Figure 73 supported this claim.

![Figure 73. Effect of 1-hydroxybenzotriazole (HBT) charge on delignification of a 97.5 kappa commercial SW kraft pulp with constant laccase charge.](image)

Further LMS studies on a conventional SW kraft pulp (kappa # 73.4) demonstrated that violuric acid (VA) was a superior mediator with respect to both 1-hydroxybenzotriazole
(HBT) and N-acetyl-N-phenylhydroxylamine (NHA) (262). These results are summarized in Figure 74.

![Graph showing Kappa number of pulps subjected to LMS followed by an alkaline extraction, using equal molar doses of NHA, HBT and VA.]

Figure 74. Kappa number of pulps subjected to LMS followed by an alkaline extraction, using equal molar doses of NHA, HBT and VA.

This report summarizes our continued efforts in examining LMS systems on high-kappa kraft pulps. The objective of this study was to compare the delignification response of an LMS_{VA}, and a two stage LMS_{VA} to an oxygen and a double oxygen treatment. In addition, the delignification response of sequence treatments with both systems was also investigated.

Materials and Methods

Materials. All materials were purchased from Aldrich Chemical Co., Milwaukee, WI, and used as received, except for NHA. NHA was synthesized in accordance with Oxley’s (210) method. Laccase from Trametes villosa, was donated by Novo Nordisk Biochem, Franklinton, NC.

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Furnish. A conventional southern USA softwood kraft pulp (kappa # 73.8) was prepared from *Pinus taeda* chips at Potlatch Corp. facilities in Cloquet, MN. The chips were cooked to an H-factor of 573 using 18.5% active alkali. The pulp was thoroughly washed, screened, centrifuged, fluffed, and stored at 4°C prior to carrying out experiments.

**Methods**

**Enzyme assay.** Laccase activity was measured by monitoring the rate of oxidation of syringaldazine. One unit of activity (U) was defined as the change in absorbance at 530 nm of 0.001 per minute per mL of enzyme solution, in a 100 mM potassium phosphate buffer (2.2 mL) and 0.216 mM syringaldazine in methanol (0.3 mL, pH 6.7). The procedure was carried out at 23°C. The activity of the laccase was 1.87 E + 06 U/mL of enzyme solution.

**Laccase-mediator delignification procedure.** A 300-mL capacity Parr reactor equipped with a stirrer, a pressure gauge, a heating mantle, and connected to a Parr 4842 temperature controller was charged with 10 g of o.d. fibers. The pulp consistency was adjusted to 10% with distilled water. The slurry was then heated to 45°C and was maintained at this temperature throughout the incubation period. VA (4.4 mmol/10 g of o.d. pulp) was then added to the heated slurry. Subsequent to mixing the slurry (approx. 5 minutes), the pH was adjusted to 4.5 with glacial acetic acid or saturated sodium bicarbonate solution. Laccase (93,500 U, or 0.05 mL of enzyme solution/g of o.d. fiber) was added, and the reactor was sealed and pressurized with oxygen to 145 psig. After a mixing period of 1 hour, the pulp was removed from the reactor and thoroughly washed with distilled water (12 L per 10 g of o.d. pulp). The treated and washed pulp was either followed by subsequent treatments (oxygen delignification or LMS) or simply subjected to an alkaline extraction stage (E).

**Alkaline extraction stage.** Alkaline extractions (E) were carried out for 1 hour at 80°C, 10% consistency in 4-mm thick heat-sealable Kapak pouches. All E treatments employed 2.5% charge (o.d. basis) of NaOH.

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Oxygen delignification. Oxygen delignification was carried out in a 300-mL capacity Parr reactor. All treatments were conducted at 95 °C, 10% consistency, for 1 hour. A charge of 2.5% NaOH (o.d. basis) was employed. Subsequent to a treatment, the pulp was thoroughly washed with distilled water.

Hexenuronic acid content in brownstock. The content of hexenuronic acids in the brownstock was indirectly measured in accordance with a modified procedure reported by Vuorinen et al. (269). In brief, a 1000-mL round bottom flask was charged with 25 g of pulp (o.d. basis). The pulp consistency was adjusted to 3% by adding distilled water. The pH was then lowered to 3 using concentrated sulfuric acid. The slurry was refluxed for three hours at 100°C. The change in kappa number before and after the treatment was then determined and served as indirect measurement of hexenuronic acids (see Table 32). Clearly, the hexenuronic acid content of this pulp was negligible.

Table 32. Changes in Kappa # after acid treatment of brownstock.

<table>
<thead>
<tr>
<th>Replicate #</th>
<th>Initial</th>
<th>Final kappa</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>73.4</td>
<td>71.5</td>
<td>2.6</td>
</tr>
<tr>
<td>2</td>
<td>73.4</td>
<td>71.9</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Pulp characterization. The kappa, brightness, and viscosity before and after treatments were measured in accordance with TAPPI Test Methods T236 and UMM246, T452, and T230, respectively (211-214). Only the terminal viscosity of the treated pulps was measured. The initial viscosity of the starting material could not be determined using TAPPI Test Method T230 due to the high-lignin content in the pulp. Kappa and viscosity measurements were duplicated. Each brightness value was based on the average of five readings.

Experimental design. A conventional SW kraft pulp with an initial kappa of 73.8 was subjected to a series of L (L = LMS1A) and oxygen delignification treatments. The list of experiments is summarized in Table 33.

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Table 33. Summary of treatments.

<table>
<thead>
<tr>
<th>Treatment #</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L</td>
</tr>
<tr>
<td>2</td>
<td>LE</td>
</tr>
<tr>
<td>3</td>
<td>LLE</td>
</tr>
<tr>
<td>4</td>
<td>O</td>
</tr>
<tr>
<td>5</td>
<td>OO</td>
</tr>
<tr>
<td>6</td>
<td>OLE</td>
</tr>
<tr>
<td>7</td>
<td>LO</td>
</tr>
<tr>
<td>8</td>
<td>OLO</td>
</tr>
</tbody>
</table>

Results

Based on our previous studies (240, 262), we have demonstrated that an LMS_{HAA}, an LMS_{HBT}, and an LMS_{VA} can yield substantial delignification when applied on high-kappa pulps. However, under the experimental conditions used in our studies, we noted that VA outperformed both NHA and HBT (262). This study summarizes our continued research efforts in this field. A conventional SW kraft pulp (kappa # 73.4) was subjected to LMS_{VA}, oxygen, double oxygen, and a combination of LMS and oxygen treatments. The pulps were then characterized for viscosity, kappa, and brightness.

Kappa. The delignification results shown in Figure 75 clearly demonstrate than an LMS system can yield substantial delignification on a high-kappa pulp. As expected, the delignification effect was further enhanced after an E stage, as the NaOH solubilizes the oxidized lignin. The data also suggest that under the experimental conditions employed in this study, an oxygen stage subsequent to an LMS treatment did not yield substantial delignification. This inefficiency could be linked to the oxidized nature of the residual lignin after an LMS. We have previously shown that the nature of the residual lignin of an LMS treated pulp is enriched with carboxylic acid groups and with quinone type structures, and is depleted of phenolic hydroxyl groups (240, 262). Hence, if an LMS treated pulp is further subjected to an oxygen delignification stage, then we might expect that some of the oxygen chemistry will be impeded. Presumably, the alkalinity of the oxygen system is directed towards the ionization of acid groups, the solubilization of

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already oxidized lignin material, and the destruction of quinone type structures. Hence, less oxygen delignification chemistry occurs. The same rationale could also be applied to the observed delignification response of the O stage after an OL treatment (i.e., OLO).

![Kappa numbers of brownstock (BS) and pulps treated with O, L, and O/L.](image)

Based on the experimental protocol used in this study, the delignification response of the LLE and OLE sequence was comparable to that of a OO stage and better than that of a single O stage. In addition, the LE treatment responded more favorably than a single O.

Overall, the LMS delignification system exhibits an additive effect that is reflected in the percent delignification of the LE and LLE treatments. Clearly, these results highlight the efficiency of the laccase-mediator system.

**Viscosity.** Accompanying the substantial delignification response of a laccase-mediator system was a high retention in pulp viscosity. The viscosity results shown in Figure 76 indicate that L, LE, and LLE treatments are much more selective than an O or a OO stage. An L treatment applied after an oxygen delignification stage was more beneficial.
to the viscosity than when applied before, which re-emphasizes the uniqueness of the enzymatic system.

![Graph showing viscosity of pulps treated with O, L, and O/L.]

Figure 76. Terminal viscosity of pulps treated with O, L, and O/L.

The incorporation of an L treatment between a double oxygen stage was also beneficial, since the terminal viscosity was higher than that of a double oxygen treatment. Furthermore, the viscosity of an OLO treatment was comparable to a single O stage. In turn, this may prove to be an attractive approach in the future for obtaining the typical enhanced delignification benefits from a double oxygen stage without further loss in pulp viscosity. Nonetheless, further experimental work will be needed to support this claim.

**Brightness.** The brightness data shown in Figure 77 demonstrate that an LMS treatment leads to pulp darkening. This effect is attributed to the formation of quinone structures during an L treatment (262). The alkaline extraction stage after an L treatment was beneficial in regaining some of the brightness lost, obviously because of the well-known ability of NaOH to destroy quinone type structures. The combination of LMS with oxy-
gen had less of a detrimental effect on brightness than LMS alone. Overall, the bright-
ness results suggest that if an LMS system is to be a viable technology then the darkening
effect will need to be addressed. We believe that further bleaching with peroxide could
be a viable approach in dealing with this problem. However, further studies will be
needed to validate this claim.

![Brightness of brownstock (BS) and pulps treated with O, L, and O/L.](image)

Figure 77. Brightness of brownstock (BS) and pulps treated with O, L, and O/L.

Conclusions

In summary, our results suggest that substantial delignification with high retention in
pulp viscosity can be achieved via a laccase-violuric acid system. This study examined
the delignification capabilities of an LMS system to deal with high-kappa kraft pulps.
The observed selectivity of the LMS system should provide a technology in the future to
delignify kraft pulps with high yields.

It is now well established that the yield benefits results when stopping a kraft cook prior
to reaching the residual phase can be achieved with SW kraft pulps of kappa # 40-50.
Based on our studies using a kappa 75 SW kraft pulp, we believe that a 50% delignifica-

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tion level using the lower kappa pulps (i.e. kappa # 40-50) is readily achievable with retention of pulp viscosity. Nonetheless, several research issues remain to be addressed. The loss of brightness during an LMS treatment of high-kappa pulps is a high-priority issue that will need to be examined.

Acknowledgements

The authors would like to thank Drs. Dimmel and Lucia for their guidance as well as the Institute of Paper Science and Technology and its member companies for their support for these ongoing studies. The authors would also like to express their gratitude to Potlatch Corp. Cloquet, MN for pulping the chips, and Novo Nordisk Biochem Franklin, NC for furnishing the laccase needed for these studies. Portions of this work were used by FSC as partial fulfillment of the requirements for the Ph.D. degree at the Institute of Paper Science and Technology.
7.6 Publica8on 6. Insight into the deligniﬁcation chemistry of laccase-mediator systems on high-lignin-content kraft pulps


Abstract

A high lignin content softwood kraft pulp was reacted with laccase in the presence of 1-hydroxybenzotriazole, N-acetyl-N-phenylhydroxylamine, and violuric acid. The deligniﬁcation response with violuric acid was superior to both 1-hydroxybenzotriazole and N-acetyl-N-phenylhydroxylamine. Spectral analysis of residual lignins isolated before and after the biobleaching treatments revealed that the reacted lignins were highly oxidized and that the magnitudes of structural changes were most pronounced with the laccase violuric acid biobleaching system. An increase in the content of carboxylic acid groups and a decrease in methoxyl groups were noted with all three laccase mediator systems. The oxidation pathway is directed primarily towards phenolic noncondensed lignin units for all three laccase-mediated systems. Nonetheless, the biobleaching system with violuric acid was also reactive towards phenolic condensed lignin structures.
Introduction

The bleaching of kraft pulps is an integral component of manufacturing high-value products in the pulp and paper industry. Historically, bleaching of kraft pulps was accomplished primarily using chlorine or hypochlorous acid. However, due to environmental concerns, these bleaching agents are being displaced by chemicals that are more environmentally benign. Currently, pulp bleaching operations employ ozone, chlorine dioxide, hydrogen peroxide, and oxygen to remove residual lignin and brighten kraft pulp fibers (232). Despite the relatively rapid conversion of pulp bleaching operations to elemental chlorine free technologies, many research opportunities exist to further improve this important industrial process. One promising strategy in the future for pulp bleaching may involve biological systems. The catalytic nature of enzymes and their relatively low temperature and operating pH profiles suggest that enzymatic bleaching technologies could result in the development of low-cost pulp bleaching processes.

Currently, several enzymatic bleaching technologies including manganese peroxidase, lignin peroxidase, and laccase are being actively pursued by researchers (188, 270). Although all of these biobleaching technologies have advanced over the past decade, laccase biobleaching treatments have achieved some of the most significant results, removing in some cases more than 50% of the lignin in low-lignin content kraft pulps (199, 202, 270). We have recently reported that laccase-catalyzed delignification of high-lignin content kraft pulps is also possible and that this technology can provide distinct pulp-yield benefits (239, 240, 260).

It is now well established that laccase biobleaching of kraft pulps can only occur in the presence of a low-molecular weight mediator. This is commonly attributed to pulp fiber dimensions, which prevent the diffusion of laccase into the fiber to allow for lignin oxidation (5). Hence, laccase biobleaching of pulp fibers involves the oxidation of a mediator by laccase and the oxidized mediator then diffuses into the fiber and oxidatively de-
grades lignin. The oxidized lignin is removed from the pulp after a subsequent alkaline extraction treatment.

The efficacy of a laccase mediator system (LMS) for delignification is critically dependent on the type of mediator employed. The first reported mediator for LMS delignification was 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), its bleaching chemistry has been extensively explored (163, 182-184, 6, 187, 194, 256). Additional mediators have since been discovered that significantly improve the overall delignification effect of an LMS-stage (8, 7, 236). Many of these mediators contain an N-hydroxy functionality, and the active delignification species is believed to be the nitroxyl radical (205, 208). The first of these N-hydroxy mediators was 1-hydroxybenzotriazole (HBT). It was shown that with a laccase-HBT treatment delignification could be extended by 20-30% over ABTS (7, 270). The LMS delignification chemistry using HBT and low-lignin content kraft pulps and lignin model compounds has been extensively studied (163, 195, 196, 199, 203, 242, 254, 271, 272). Essential observations with respect to lignin structural changes are that HBT preferentially oxidizes the phenolic component of lignin, introduces carboxylic acid groups, and can oxidize aliphatic lignin moieties. Recently, N-acetyl-N-phenylhydroxylamine (NHA) and violuric acid (VA) (8, 208) were introduced as potential N-hydroxy mediators. Nonetheless, the delignification chemistry of LMS employing VA and NHA vis à vis lignin structural changes remains unknown. To provide a fundamental basis from which new LMS bleaching technologies can be further advanced, we have examined the changes in lignin structure before and after an LMS treatment using NHA and VA (see Figure 78), employing a high-lignin-content softwood kraft pulp. In addition, these results were compared to LMS treatments employing HBT as mediator.
Figure 78. Chemical structure of NHA, HBT, and VA.

**Experimental**

**Materials and methods.** 1-Hydroxybenzotriazole, violuric acid, and all other chemicals and solvents were purchased from Aldrich, Milwaukee, WI, and used as received except for p-dioxane and NHA. p-Dioxane was freshly distilled over NaBH₄ before use. NHA was synthesized in accordance with Oxley et al. (210). Laccase from *Trametes villosa* was donated by Novo Nordisk Biochem. A conventional southern USA softwood kraft pulp was prepared from *Pinus taeda* chips at Potlatch Corp. facilities in Cloquet, MN. The chips were cooked to an H-factor of 573 using 18.5% active alkali. The pulp was thoroughly washed, screened, centrifuged, flushed, and stored at 4°C prior to LMS bleaching treatments.

**Enzyme assay.** Laccase activity was measured by monitoring the rate of oxidation of syringaldazine. One unit of activity (U) was defined as the change in absorbance at 530 nm of 0.001 per minute per ml. of enzyme solution in a 100 mM potassium phosphate buffer (2.2 mL) and 0.216 mM syringaldazine in methanol (0.3 mL, pH 6.7). The procedure was carried out at 23°C. The activity of the laccase was 1.87E+06 U/mL of enzyme solution.

**Laccase-mediator general procedure (LMS).** A 2000-mL-capacity 316 Parr reactor equipped with a stirrer, a pressure gauge, and a heating mantle and connected to a Parr...
4842 temperature controller was charged with 60 g of pulp (oven dry weight). Distilled water (1,137 mL) was added. The pulp slurry was heated to 45°C and was maintained at this temperature throughout the duration of the treatment. VA, NHA, or HBT (0.0206 mol) was then added to the heated slurry. Subsequent to mixing the pulp slurry (approx. 5 minutes), the pH was adjusted to 4.5 with glacial acetic acid or saturated sodium bicarbonate solution. Laccase (93,500 U, or 3 mL of enzyme solution) was added, and the reactor was sealed and pressurized with oxygen to 145 psi. After a reaction time of 2 hours, stirring was halted, the reactor was depressurized, and the reacted pulp was removed from the reactor and thoroughly washed with distilled water (4,800 mL). The treated pulp was either followed by either extracted with acidic dioxane, or an alkaline extraction was followed by an acidic dioxane extraction.

Alkaline extraction. Pulp fibers (60 g, oven dry weight) were extracted with an aqueous 0.5 N NaOH (75 mL) solution for 1 hour at 80°C with mixing.

Control experiments: mediator systems (MS). Control experiments (MS) were carried out in the absence of laccase and in the presence of mediators. These treatments were carried out in accordance with the laccase-mediator delignification procedure described above, except no laccase was employed.

Pulp characterization. The lignin content of the kraft pulps was determined by a KMnO₄ titration of the pulp in accordance with TAPPI Standard Methods T236-cm85 (211). This value, known as the kappa number, is an indirect measurement of the lignin content where % mass lignin = 0.15 x kappa number.

Isolation of residual lignins. The isolation of residual lignins was carried out following standard literature methods (64, 215-216). In summary, a 5-L, three-necked round bottom flask containing boiling chips and fitted with a Friedelchs condenser was charged with 50 g of air-dried pulp (oven dry weight). A solution 0.1 N HCl of 9.1 p-dioxane water (1,440 mL) was added to the pulp. The reaction mixture was refluxed for two hours under an argon atmosphere before it was cooled and filtered through a 4-L-capacity
coarse fritted-glass Buchner funnel covered with filter paper. The resulting filtrate was passed through a fine-fritted-glass Buchner funnel packed with celite to remove any pulp fines that may be present. The lignin/water/dioxane mixture was then neutralized with an aqueous saturated solution of sodium bicarbonate to an apparent pH value of 5.0-5.5, and concentrated under reduced pressure to approximately 10% of the initial volume. Deionized water (approx. 400 mL) was added and the mixture was concentrated again under reduced pressure to remove the last traces of p-dioxane. The resulting lignin-water mixture was transferred to a 1-L beaker, diluted with deionized water to a volume of approximately 800 mL, acidified to a pH of 2.0-2.5 with an aqueous 1N HCl solution, and transferred to 250-mL capacity centrifuge bottles. The bottles were frozen, thawed, and centrifuged. The supernatant was siphoned, and fresh acidified water (approx. 200 mL, pH 2.0-2.5 using 1 N HCl) was added. This step was repeated three times, each time reducing the volume of wash water by 30%. The lignin was then freeze-dried and characterized by NMR. Lignin yields were calculated as follows:

\[ \% \text{ lignin yield} = \frac{\text{mass of lignin isolated}}{\text{initial kappa of brownstock}} \times 0.15 \times 100 \]

Lignin yields ranged from 45.4-49.0%.

**Characterization of residual lignins.** The residual lignins isolated from the brownstock, MS, LMS, and LMS(E) were analyzed using a 400 MHz Bruker DMX spectrometer. Quantitative \(^{13}\text{C}\) NMR spectra were acquired and analyzed in accordance with established literature methods (221). In brief, lignin (160-168 mg) was dissolved in 450 μL of DMSO-d6 before being transferred into a 5-mm NMR tube. \(^{13}\text{C}\) NMR spectra were recorded with an inverse gated decoupling sequence, 90° pulse angle, 14-s pulse delay, 23,000-Hz sweep, 10-12,000 transients, at 50°C. The fourier transformed spectra were integrated in accordance with reported chemical shifts for lignin functional groups. The integrals were normalized to the aromatic signals, which were assumed to have 6 carbons (221).

The lignin samples were also characterized by quantitative \(^{31}\text{P}\) NMR. Sample phosphitylation and spectral acquisition and analysis were carried out following established literature methods (59, 244). \(^{31}\text{P}\) NMR spectra were recorded using an inverse
gated decoupling sequence, 90° pulse angle, 25-s pulse delay, 13,000-Hz sweep, and 150 transients, at room temperature. NMR spectral acquisition and analysis were controlled by Bruker’s XWINNMR 2.1 software running on a SGI (Silicon Graphics Indigo) server using the Irix 7.0 operating system.

Typical accuracy values reported in the literature (22L, 244) for integrating the various lignin functional groups by $^{13}$C and $^{31}$P NMR range from ±3-5% and ±1.3-1.9%, respectively. In this study, the error analysis was based on reproducing the LMS$_{NDE}(E)$ treatment three separate times and the lignin isolation and spectral acquisition and analysis three separate times. The $^{13}$C and $^{31}$P NMR standard errors were 2.0% and 1.2%, respectively.

Results and discussion

Laccase-mediator systems continue to receive increasing attention as this biobleaching process is environmentally compatible, highly selective, and effective with respect to delignification. Research activities have primarily focused on understanding the delignification chemistry on low-lignin-content kraft pulps since it is envisioned that, if implemented, LMS would displace a conventional bleaching stage. For the last few years, we have been exploring LMS on high-lignin content kraft pulps as a potential technology for improving pulp yields in the future. The objective of this study was to examine the delignification chemistry of the newest generation of N-hydroxy mediators, more specifically, VA and NHA, and compare these results against HBT. The delignification response of LMS was assessed by measuring the lignin content of the pulp, via kappa measurements, before and after LMS treatments. Residual lignins isolated after LMS$_{VA}$, LMS$_{NHA}$, and LMS$_{HBT}$ reactions were characterized via $^{31}$P and $^{13}$C NMR.

Delignification response. The delignification response of LMS treatments employing NHA, HBT, and VA as mediators was established using an acetone-extracted softwood kraft pulp with an initial kappa number of 71.4.
Furthermore, control experiments (MS) were carried out in accordance with the LMS procedure using all three mediators, except laccase was not used. The results shown in Figure 79 clearly indicate that a treatment in the presence of a mediator led to no delignification. Similarly, this lack of activity has also been reported on pulps treated solely with laccase (188, 242). On this basis, enzymatic delignification of kraft pulps necessitates the presence of both laccase and mediator.

![Graph showing kappa numbers of MS, LMS, and LMS(E) treated kraft pulps using VA, NHA, and HBT as mediators. The initial kappa number of the brownstock was 71.4.](image)

Based on the results summarized in Figure 79, the delignification response of the LMS_{NHA} and LMS_{HBT} system was similar. The effectiveness of LMS_{VA} vis-à-vis delignification is evident as the LMS_{VA} system outperformed both the LMS_{HBT} and LMS_{NHA} treatments. Additional drops in lignin content of the pulp subsequent to the alkaline extractions were expected since the alkaline conditions enhance the dissolution of oxidized lignin fragments. The factors contributing to the enhanced LMS_{VA} delignification response remain unknown. Several studies have attempted to correlate the delignification
efficiency of mediators with isolated key parameters such as dissociation energies (201, 242), radical half-lives (205, 208), steric effects (208), and redox potentials (8, 273). However, to date such correlations have been difficult to establish, although undoubtedly these parameters must be important factors. On this basis, it may be appropriate to presume that the effectiveness of a mediator cannot be elucidated solely by examining one parameter at a time. Rather, it may be hypothesized that several parameters must be working in concert and that what differentiates one mediator from another may be attributed to various synergistic effects. To further define the fundamentals of LMS delignification on the kraft pulp used in this study, we elected to characterize the structure of residual lignins isolated before and after bioleaching treatments.

**NMR analysis.**

Residual lignins were isolated from the starting pulp as well as the MS, LMS, and LMS(E) treated pulps and analyzed employing NMR techniques that characterized the predominant structures in lignin. Figure 80 summarizes the lignin functional groups examined in this study.

![Diagram showing lignin functional groups](image)

**Figure 80.** Aliphatic hydroxyl groups, β-O-4 aryl ether linkages, and phenolic hydroxyl groups in condensed and noncondensed lignin structures.

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Lignin samples were phosphitylated and quantitatively analyzed by $^{31}$P NMR. $^{31}$P NMR is a facile, highly reproducible, and rapid technique that canvasses various lignin functional groups such as carboxylic acid, aliphatic, and condensed and noncondensed phenolic.

Residual lignins isolated from the various treatments were also characterized by $^{13}$C NMR. Figures 81 and 82 are typical $^{13}$C and $^{31}$P NMR spectra of brownstock lignin.

Figure 81. Typical $^{13}$C NMR of brownstock residual lignin
Figure 82. Typical $^{31}$P NMR of brownstock residual lignin

Control experiments. Control experiments that consisted of reacting the starting pulp solely with mediators employing the LMS conditions led to no delignification. These observations were also supported by the $^{31}$P NMR data, since structural changes in phenolic and aliphatic lignin moieties were not evident. Similarly, an increase in the content of carboxylic acid groups, a mark of oxidative bleaching systems, did not take place. The $^{31}$P NMR results are illustrated in Figure 83.

The $^{13}$C NMR spectral analyses of these lignins further corroborated the fact that the system was inactive in the presence of a mediator and in the absence of laccase. Indeed, the content of β-O-4 aryl ether, methoxyl, and carboxylic groups in the MS and brownstock residual lignins was unchanged. Overall, the NMR results are consistent with the delignification trends illustrated in Figure 79. On this basis, the presence of laccase and a mediator is the minimal requirement for transforming the lignin polymer.
Figure 83. $^{31}$P NMR results of residual lignin isolated from softwood kraft pulp and from kraft pulp treated with NHA, HBT, and VA in the absence of fericase.

**LMS and LMS(£) experiments**

**Carboxylic acid groups.** The $^{31}$P NMR results shown in Figure 84 indicate that relative to the brownstock residual lignin, the content of carboxylic acids increased after LMS treatments with all three mediators. An increase in such moieties is indicative of oxidation reactions and is a mark of oxidative bleaching systems. A closer examination of the data demonstrates that the highest concentration of carboxylic acids was observed after an LMSVA treatment. This trend was further supported from the $^{13}$C NMR spectral data shown in Figure 85.
Figure 84. Carboxylic acid groups in residual lignins isolated from brownstock and LMS, and LMS(E) treated pulps employing NHA, HBT, and VA as mediators.

Figure 85. Carboxylic acid groups determined by $^{13}$C NMR in residual lignins isolated from brownstock and LMS, and LMS(E) treated pulps employing NHA, HBT and VA as mediators.
On this basis, the data highlight the oxidative effectiveness of violuric acid and further corroborates the enhanced delignification properties of this system, which was previously illustrated in Figure 79.

**Aliphatic hydroxyl groups from \(^{31}\)P NMR.** Inspection of Figure 86 reveals that the content of aliphatic hydroxyl groups in residual lignins isolated after treatment with LMS\(_{\text{NHA}}\), LMS\(_{\text{HBT}}\), and LMS\(_{\text{VA}}\) substantially decreased with respect to the brownstock lignin. However, the greatest change was observed when VA and NHA were employed as mediators. A decrease in aliphatic hydroxyl groups is indicative of side chain oxidation and validates the recent observation of side chain oxidation and fragmentation of lignin model compounds treated with laccase-mediator systems (208, 238).

![Aliphatic hydroxyl groups in residual lignin](image)

**Figure 86.** Aliphatic hydroxyl groups in residual lignin isolated from brownstock and LMS, and LMS(E) treated pulps employing NHA, HBT, and VA as mediators.

**Phenolic hydroxyl groups in noncondensed at C5 lignin units from \(^{31}\)P NMR.** Figure 87 clearly demonstrates that all three residual lignins isolated after LMS treatments were depleted of phenolic hydroxyls in C5 noncondensed lignin structures. This decrease was most pronounced when VA was employed as the mediator.
Analogous diminutive trends of phenolic noncondensed lignin structures have been reported on low-lignin content kraft pulps studies using HBT (199, 201, 254) and NHA (254) as the mediator.

![Diagram]

**Figure 87.** Phenolic hydroxyls in noncondensed at C5 lignin groups in residual lignin isolated from brownstock and LMS, and LMS(E) treated pulps employing NHA, HBT, and VA as mediators.

**Phenolic hydroxyl groups in C5 condensed lignin structures from \(^{31}\)P NMR.** Figure 88 indicates that the content of phenolic hydroxyl groups in C5 condensed lignin structures decreased after LMSVA. In contrast, these lignin moieties were resistant towards LMSNHA and LMSHBT treatments. On this basis, the \(^{31}\)P NMR results suggest that the selectivity of the LMSVA system is different than that of LMSHBT and LMSNHA. The overall trend of these LMS systems indicates that the preferential types of phenolic substrates are of a noncondensed nature. Phenolic lignin moieties condensed at C5 have been shown to be reactive toward LMS treatments on low-lignin content kraft pulps employing HBT (199, 201, 254) and NHA (254) as mediators. Therefore, it may be appropriate to presume that the selectivity of an LMS towards various lignin functional groups is influenced by the content and type of lignin present in the pulp.
The lack of reactivity of phenolic C5 condensed structures suggests that the residual lignin after LMS treatments on high-lignin content kraft pulps is of a condensed character. This claim is further supported by the enrichment in the ratio of C5 condensed to non-condensed lignin moieties relative to the brownstock residual lignin, shown in Table 34.

![Bar chart](image)

Figure 88. Phenolic hydroxyls in condensed at C5 lignin groups in residual lignin isolated from brownstock and LMS, and LMS(E) treated pulps employing NHA, HBT, and VA as mediators.

<table>
<thead>
<tr>
<th>Residual lignin isolated from:</th>
<th>Ratio of condensed to noncondensed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brownstock</td>
<td>0.85:1.00</td>
</tr>
<tr>
<td>LMS_NHA</td>
<td>1.12:1.00</td>
</tr>
<tr>
<td>LMS_HBT</td>
<td>0.99:1.00</td>
</tr>
<tr>
<td>LMS_VA</td>
<td>1.22:1.00</td>
</tr>
<tr>
<td>LMS_NHA(E)</td>
<td>1.06:1.00</td>
</tr>
<tr>
<td>LMS_HBT(E)</td>
<td>0.94:1.00</td>
</tr>
<tr>
<td>LMS_VA(E)</td>
<td>1.17:1.00</td>
</tr>
</tbody>
</table>

Table 34. Ratio of C5 condensed to C5 non-condensed lignin units.

β-O-4 aryl ether lignin structures and methoxyl groups from 13C NMR. Figure 89 illustrates the content of β-O-4 aryl ether lignin structures determined by 13C NMR. The
results indicate that such structures are resistant to LMS treatments. Similar trends have been reported in previous LMS studies using HBT as the mediator on low-lignin content kraft pulps (198, 199). In contrast, reports on lignin model compound studies have indicated that such structures are reactive towards LMS (194-196). The factors contributing to this inactivity in kraft pulps remain unknown. However, it may be possible that this behavior is related to a selectivity phenomenon. Although a dimeric model compound may respond to an LMS treatment, a different scenario may be envisioned when dealing with the actual lignin macromolecule that contains an array of functional groups. On this basis, an LMS may have a higher affinity towards other functional groups than $\beta$-O-aryl ether and, in turn, this may explain the resistance observed in this study and in the literature.

Figure 89. Content of $\beta$-O-aryl ether groups in residual lignins isolated from brownstock and LMS, and LMS(E) treated pulps employing NHA, HBT, and VA as mediators.

The trends shown in Figure 90 reveal a decrease in the content of methoxy groups. These results are consistent with previous LMS studies on low-lignin content kraft pulps employing ABTS and HBT as mediators (6, 188, 199).
Figure 90. Content of methoxy groups in residual lignins isolated from brownstock and LMS, and LMS(E) treated pulps employing NHA, HBT, and VA as mediators.

Conclusions

In summary, these results established that LMSVA outperforms LMSHBT and LMSNHA with respect to delignification of high-lignin-content-kraft pulps. The effectiveness of VA was further supported by the magnitude of lignin structural changes with respect to NHA and HBT. Spectral analyses of residual lignins isolated before and after LMS treatments, employing VA, NHA, and HBT as mediators, revealed that these mediators preferentially react with phenolic noncondensed lignin structures, whereas condensed phenolic lignin structures were to a large extent inactive. Nonetheless, in the presence of laccase and VA, our results indicate a decrease in the latter type of lignin functional groups, suggesting that the selectivity between these mediators may be different.

Acknowledgments

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Corp., Cloquet, MN, for pulping the chips; and Novo Nordisk Biochem, Franklinton, NC, for furnishing the laccase needed for these studies. Portions of this work were used by F.S.C. as partial fulfillment of the requirements for the Ph.D. degree at the Institute of Paper Science and Technology. A.J.R. also wishes to thank J.B. Stothers for his guidance and help through his research career.
7.7 Publication 7. Formation of Quinonoid Structures in Laccase-Mediator Reactions


Abstract

A softwood kraft pulp (kappa # 71.4) was subjected to a series of laccase-mediator treatments using 1-hydroxybenzotriazole (HBT), N-acetyl-N-phenylhydroxylamine (NHA), and violuric acid (VA). Based on the experimental conditions used in this study, the highest delignification response was observed with VA. Losses in brightness were observed after all three LMS systems, and were attributed to the formation of quinonoid structures. The residual lignins were isolated and derivatized with trimethylphosphite. The $^{31}$P NMR spectral analyses confirmed the formation of quinones from LMS$_{VA}$NHAHBT. The decrease in quinones after an LMS(E) could be attributed, in part, to a Michael addition of hydroxide ion to quinones.
Introduction

Over the last two decades, research efforts in pulping and bleaching have largely focused on environmental issues. As these concerns continue to be addressed, new research opportunities are developing. One area of active research focuses on improving pulp yields from pulping and bleaching operations (3, 228, 259, 266-268). The benefits in enhancing pulp yields are fourfold, including improved wood utilization practices, reduced pulp manufacturing capital costs, reduced operating costs, and improved profitability. An attractive approach for improving pulp yields consists of halting the kraft cook prior to reaching the terminal phase. In the terminal phase of pulping, the selectivity between lignin removal and carbohydrate degradation is significantly reduced resulting in loss of pulp carbohydrates. Stopping a kraft cook prior to the terminal phase reduces carbohydrate losses but yields a pulp with high lignin content (pulp kappa number of 40-50). A promising strategy for removing the lignin from such pulps prior to bleaching consists of employing a single- or a double-oxygen stage. Several research groups have reported that pulp yields can be increased in the range of 2-6% by employing this approach (3, 227, 267).

Recently, we have begun investigating the potential of employing lignin degrading enzymes, more specifically, laccase-mediator systems (LMS), to delignify high kappa kraft pulps (239, 240). Laccase has been shown to effectively oxidize phenolic compounds and phenolic lignin model compounds (88, 89, 116, 159-161). However, laccase alone is ineffective in delignifying pulp fibers (188). This inefficacy is attributed to the size of the enzyme and, hence, to its inability to diffuse in a pulp fiber and oxidize the lignin (5). The limitations of diffusion were shown to be circumvented by the addition of low molecular weight compounds, also known as mediators. Bourbonnais et al. first demonstrated this approach when they reported that laccase in the presence of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) could delignify kraft pulps (274). In addition, based on model compound studies, it was shown that the specificity of the laccase-mediator system could be expanded to non-phenolic substrates (180, 183-184). The proposed mechanism for this mediator assisted biodelignification process involves laccase oxidizing ABTS and, in turn, the oxidized ABTS diffuses into the pulp fiber and reacts with the
lignin. The reduced ABTS is then reoxidized by laccase. A host of alternative mediators have now been reported in the literature (8, 7, 236). Some of the more effective mediators are N-hydroxybenzotriazole (HBT), violuric acid (VA), and N-acetyl-N-phenylhydroxylamine (NHA). Typically, these mediators have been employed with laccase on low-lignin content kraft and sulfite pulps, as well as re lignin model compounds (163, 187, 199, 203, 205, 208, 228, 242, 254, 270-271).

The ability of these LMS treatments to remove lignin from high-lignin content pulps has remained largely unexplored. We have recently demonstrated that an LMS treatment, using HBT or NHA as mediators, can remove significant amounts of lignin from high-kappa kraft pulps (239-240). NMR analysis of the residual lignin in the pulp after an LMS treatment indicated that the bio-delignification treatment extensively oxidizes C-5 noncondensed phenolic lignin structures, whereas C-5 condensed phenolic lignin structures were overall resistant to oxidation. In addition, side chain oxidation did occur on the propane-linking unit of lignin. The primary product detected from these oxidative treatments has been the formation of carboxylic acid groups (239, 240).

The presence of quinone groups in an LMS treated pulp has been frequently proposed (203, 205, 239-240, 237, 254). Lignin model compounds studies with laccase indicate that this can occur (275). Recently, Poppius-Levin et al. (203, 276) presented FT-IR data suggesting that the residual lignin from LMS treated pulp has an enriched level of quinonoid structures.

The formation of quinones in LMS treated pulps could readily explain the substantial increases in brightness when a kraft pulp is first subjected to LMS and then treated with alkaline hydrogen peroxide (237, 240, 254). It is well established that alkaline hydrogen peroxide readily reacts with para- and ortho-quinones (277, 278). The removal of these intensively colored bodies from kraft pulp with alkaline peroxide would significantly improve the brightness of the pulp. The purpose of this study was to determine the relative amounts of quinones in residual lignins isolated from a softwood high-kappa kraft pulp before and after LMS treatments, using HBT, NHA, and VA as mediators.
Experimental

Methods and Materials

Chemicals. All chemicals were purchased from Aldrich Co., Milwaukee, WI, and used as received, except for p-dioxane, NHA, and laccase. P-Dioxane was freshly distilled over NaBH₄ prior to using it for lignin isolation experiments. NHA was synthesized in accordance with Oxley's method (210). Laccase from Trametes villosa was donated by Novo Nordisk Biochem, Franklinton, NC.

Furnish. The softwood kraft pulp employed in this study originated from a 25-year-old Pinus taeda tree that was donated by Union Camp (now International Paper), Savannah, GA. The chips were cooked at Potlatch Corp. facilities in Cloquet, MN, to an H-factor of 573 using 18.5% active alkali. The pulp was thoroughly washed, screened, centrifuged, flushed, and stored at 4°C. Prior to executing the experimental design called for in this study, the pulp was Soxhlet extracted with acetone for 24 hours and then thoroughly washed with distilled water to remove the residual acetone.

Hexenuronic acid in pre-acetone extracted brownstock. The content of hexenuronic acids in the brownstock was indirectly measured in accordance with a modified procedure reported by Vuorinen et al. (269). In brief, a 1000-mL round bottom flask was charged with 25 g of pulp (o.d. basis). The pulp consistency was adjusted to 3% by adding distilled water. The pH was then lowered to 3 using 4.0 N sulfurous acid. The slurry was refluxed for three hours at 100°C. The change in kappa number before and after the treatment was then determined and served as an indirect measurement of hexenuronic acids (see Table 35).
Table 35. Changes in Kappa # After Acid Treatment of Brownstock.

<table>
<thead>
<tr>
<th>Replicate #</th>
<th>Initial Kappa</th>
<th>Final Kappa</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>73.4</td>
<td>71.5</td>
<td>2.6</td>
</tr>
<tr>
<td>2</td>
<td>73.4</td>
<td>71.9</td>
<td>2.0</td>
</tr>
</tbody>
</table>

**Laccase assay.** Laccase activity was measured by monitoring the rate of oxidation of syringaldazine. One unit of activity (U) was defined as the change in absorbance at 530 nm of 0.001 per minute per milliliter of enzyme solution, in a 100 mM potassium phosphate buffer (2.2 mL) and 0.216 mM syringaldazine in methanol (0.3 mL, pH 6.7). The procedure was carried out at 23°C. The activity of the laccase was 1.87E + 06 U/mL of enzyme solution.

**Laccase-mediator delignification procedure (LMS).** A 2000-mL capacity Parr reactor equipped with a stirrer, a pressure gauge, a heating mantle, and connected to a Parr 4842 temperature controller was charged with 60 g of o.d. fibers. The pulp consistency was adjusted to 5% with distilled water. The slurry was then heated to 45°C and was maintained at this temperature throughout the incubation period. VA, NHA, or HBT (4.4 mmol/10g of o.d. pulp) was then added to the heated slurry. Subsequent to mixing the slurry (approx. 5 minutes), the pH was adjusted to 4.5 with glacial acetic acid or saturated sodium bicarbonate solution. Laccase (93,500 U, or 0.05 mL of enzyme solution/g of o.d. fiber) was added, and the reactor was sealed and pressurized with oxygen to 145 psig. After a mixing period of 1 hour, the pulp was removed from the reactor and thoroughly washed with distilled water (12 L per 10 g of o.d. pulp). The treated and washed pulp was either followed by a subsequent alkaline extraction stage or simply used as is.

**Alkaline extraction stage (E).** Alkaline extractions were performed in 4 mm-thick Kapak heat sealable pouches for 1 hour, at 80°C, and 10% consistency. All E treatments employed 2.5% charge of NaOH.

**Control experiments in the absence of laccase (MS).** Control experiments were performed in the absence of laccase and in the presence of VA, HBT, and NHA. These
treatments were carried out in accordance with the laccase-mediator delignification procedure described above, except no laccase was employed.

**Pulp characterization.** The brownstock, MS, LMS, and LMS(E) pulps were characterized for kappa number and brightness in accordance with standard TAPPI Standard Methods T236-cm85 and T452-om98, respectively (211, 213).

**Isolation of residual lignins.** The isolation of residual lignins was carried out following standard literature methods (27, 64, 213). In summary, a 5000-mL three-necked round bottom flask was charged with 50 g of o.d. pulp and the consistency was adjusted to 4% by adding a 0.10 N HCl 9:1 p-dioxane:water solution. The slurry was then refluxed for two hours under an argon atmosphere. The pulp was filtered and the filtrate was filtered through celite, neutralized, and concentrated under reduced pressure to approximately 10% of the original volume. Water (approx. 400 mL) was added and the mixture was concentrated again under reduced pressure to remove the last traces of p-dioxane. The solution’s pH was then adjusted to 2.5 with 1.00 N HCl. The precipitated lignin was collected, washed several times, and freeze-dried. Lignin yields ranged from 46.3 to 49.0%. Lignin yields were calculated as follows: % lignin yield = (mass of lignin isolated/initial kappa of brownstock x 0.15)) x 100. The calculated lignin yields were corrected for initial hexemuronic acid groups content.

**Derivatization of residual lignins with trimethylphosphite (TMP).** Derivatization of residual lignins with trimethylphosphite was performed in accordance with Zawadzki’s method (72, 223). In brief, a 30 mg sample of lignin previously dried at 40°C under vacuum for 24 hours was treated with 500 µL of 50% TMP/DMF (v/v) under an argon atmosphere for 7 days. Subsequent to the incubation period, excess trimethylphosphite was removed by first adding 250 µL of DMSO and then placing the lignin solution under vacuum at 45°C until the sample was nearly dry (approx. 6 hours). The treated lignin samples were then dissolved in 500 µL 60% of DMSO-d5/pyridine (v/v) containing trimeta-tolylphosphate (0.7 mg/mL) and chromium–acetylacetone (0.9 mg/mL). Deion-
ized water (5 μL) was then added and the lignin solution was allowed to mix for 12 hours prior to acquiring the 31P NMR spectrum.

31P NMR of derivatized residual lignins. 31P NMR spectra of derivatized lignins were acquired using a 90° pulse, a 5-second pulse delay, inverse-gated broad-band proton decoupling and 1000 scans per spectrum (approx. 1 hr 36 min total acquisition time) (72-223). All 31P NMR spectra were recorded on a DMX 400 MHz Bruker spectrometer.

Results and Discussion

Extent of Delignification and Brightness. The delignification and brightness responses of laccase-mediator systems employing HBT, NHA, and VA on a softwood kraft pulp (kappa # 71.4) were evaluated before and after an alkaline extraction stage (E). In addition, a series of control experiments in the absence of the laccase were carried out. The kappa and brightness measurements relative to the initial brownstock are summarized in Table 36.

Table 36. Kappa and TAPPI Brightness for a softwood kraft pulp treated with MS⁎, LMS⁎ and LMS(E)† using HBT, NHA, and VA as mediators*.  

<table>
<thead>
<tr>
<th>Pulp/Treatment</th>
<th>Kappa #</th>
<th>St.dev</th>
<th>TAPPI Brightness</th>
<th>St.dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brownstock</td>
<td>71.4</td>
<td>0.19</td>
<td>18.4</td>
<td>0.11</td>
</tr>
<tr>
<td>MSNHA</td>
<td>71.3</td>
<td>0.11</td>
<td>18.5</td>
<td>0.20</td>
</tr>
<tr>
<td>MSHBT</td>
<td>71.0</td>
<td>0.18</td>
<td>18.7</td>
<td>0.33</td>
</tr>
<tr>
<td>MSVA</td>
<td>71.2</td>
<td>0.08</td>
<td>18.5</td>
<td>0.15</td>
</tr>
<tr>
<td>LMSNHA</td>
<td>67.0</td>
<td>0.29</td>
<td>7.8</td>
<td>0.43</td>
</tr>
<tr>
<td>LMSHBT</td>
<td>65.3</td>
<td>0.11</td>
<td>11.6</td>
<td>0.45</td>
</tr>
<tr>
<td>LMSVA</td>
<td>53.6</td>
<td>0.09</td>
<td>9.8</td>
<td>0.39</td>
</tr>
<tr>
<td>LMSNHA(E)</td>
<td>58.4</td>
<td>0.37</td>
<td>10.7</td>
<td>0.21</td>
</tr>
<tr>
<td>LMSHBT(E)</td>
<td>57.4</td>
<td>0.01</td>
<td>15.5</td>
<td>0.35</td>
</tr>
<tr>
<td>LMSVA(E)</td>
<td>45.1</td>
<td>-</td>
<td>13.7</td>
<td>0.37</td>
</tr>
</tbody>
</table>

⁎MS treatment in the absence of laccase but in the presence of mediator.
†LMS treatment in the presence of both laccase and mediator
‡LMS(E) treatment in the presence of both laccase and mediator and followed by an alkaline extraction stage (E).
*see experimental section for details.
The results clearly indicate that in the absence of laccase and in the presence of the mediator only, delignification did not occur. In addition, previous LMS studies have demonstrated that the delignification response of a laccase treatment in the absence of a mediator is insignificant (188, 242). Hence, both the mediator and the laccase must be present in order to achieve delignification. Based on the experimental conditions employed in this study, VA was a superior mediator with respect to HBT and NHA on this high-kappa kraft pulp. The extent of delignification of both NHA and HBT was comparable.

It is well known that a high content of hexenuronic acids (HexA) has an adverse impact on the kappa number since HexA consume potassium permanganate (269). As summarized in Table I, the change in kappa number after the acid stage was approx. 2%, implying that the HexA content is insignificant and that the kappa numbers in this study were a good reflection of the lignin content.

Accompanying the LMS delignification, the treated pulps suffered a loss in brightness. The brightness data shown in Table 36 indicate that the LMS treatment always darkens the pulp with respect to the brownstock. This effect was most significant with NHA and VA. The extraction stage with sodium hydroxide improved the final brightness of the LMS treated pulps relative to the brownstock; however, it never exceeded the initial brightness. Based on our previous studies (240, 254), we have shown that this darkening effect can be further alleviated with the reinforcement of the extraction stage with peroxide, and with peroxide and oxygen. This effect is consistent with quinones being produced in LMS stage.

Quinone Content. The role of quinones in the observed LMS delignification chemistry was explored by isolating the residual lignin from the SW kraft brownstock, and after the MS, LMS, and LMS(E) treatments, as described in the experimental section. The combined content of ortho- and para-quinones was examined using a trimethylphosphite derivatization procedure and $^{31}$P NMR. Studies by Zawadzki and Ragauskas (72-73, 226, 279), Argyropoulos and Zhang (225), and Zhang and Gellerstedt (224) have shown that trimethylphosphite can readily be used to tag ortho- and para-quinones and after hydroly-
sis yield stable phosphate ester adducts. The adducts are detected via $^{31}$P NMR experiments; adduct levels provide a semi-quantitative determination of the quinone content. The combined ortho- and para-quinone data are presented in Figure 91.

Figure 91. Semiquantitative quinone content of residual lignins isolated from the brownstock (BS) and after MS, LMS, and LMS(E) treatments using NHA, HBT, and VA as mediators.

The experimental results indicate that the content of quinone structures in the brownstock is quite low. Our value is comparable to that reported by Zawadzki (73). Treatment of the pulps in the presence of mediators and oxygen failed to introduce any further quinones into the residual lignins.

Repeating these experiments in the presence of both laccase and mediator led to an approximate 2.7-fold increase in detectable quinone structures when NHA and VA were used. The relative trend also suggests that the content of quinones was lower when HBT was employed.

The subsequent alkaline extraction stage reduced the quinone content, on average, by approximately 21%. The loss of quinones during the alkaline extraction stage can be attributed to the reactivity of NaOH with such structures. The nucleophilic addition of
hydroxide ion to quinonoid structures can result in increased solubility. This type of chemistry can lead to the formation of hydroxy substituted catechols via a Michael addition of hydroxide anions and also to alpha-hydroxy-carboxylic acid cyclopentadiene structures (see Figure 92) (37, 280). The latter structures are postulated to stem as a result of a nucleophilic addition of HO⁻ followed by a benzylic acid rearrangement.

Figure 92. Proposed sites of addition of hydroxide anions to quinone structures (37, 280).

Despite the loss in quinone structures subsequent to the extraction stage, the data suggest that the residual lignin still contained approximately 50% more quinone structures than the brownstock.

One possible explanation for this observation could be attributed to the proposed propensity of o- and p-benzoquinones to undergo condensation reactions leading to the formation of bi-phenyl linkages (37). As a result, the solubility of such structures may be adversely affected. Another possible explanation may be linked to the ability of catechols to readily oxidize back to quinone structures.
Conclusions

In summary, this study provides some of the first spectroscopic data that establishes conclusively the formation of quinones in LMS and LMS(E) treated softwood kraft pulps. The data provide an explanation, in part, for the darkening of kraft pulps after an LMS stage and its subsequent partial brightening after an LMS(E) stage. The observed formation of quinones after an LMS stage is also consistent with the reported brightness benefits of alkaline peroxide bleaching of LMS treated pulps. The formation of quinones and the darkening effect of pulps are important aspects of the chemistry of LMS delignification. This issue will need to be addressed and further understood if LMS technology is to be implemented commercially.

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7.8 Publication 8. Laccase-lignin reactions


Abstract

Residual lignin samples isolated from a SW kraft pulp (kappa no. 71.4) were subjected to laccase and laccase-mediator treatments using HBT, NHA, and VA as mediators. Structural changes relative to the brownstock lignin were measured via $^{13}$C NMR and $^{31}$P NMR. $^{31}$P NMR spectral analysis of residual lignins treated with laccase and with laccase in the presence of mediators revealed a depletion in phenolic lignin groups, non-condensed and condensed at C-5, as well as in aliphatic hydroxyl groups. $^{13}$C NMR spectral data revealed a decrease in methoxy groups and a substantial increase in carboxylic acid groups.
Introduction

The pulp and paper industry continues steadily and successfully to displace traditional chlorine and hypochlorous acid bleaching methods with more environmentally compatible technologies such as ECF and TCF. Despite these remarkable advances, new challenges and opportunities are emerging. There is a renewed interest in improving pulping and bleaching yields of kraft pulps, since the accessibility to inexpensive fibers is expected to decrease in the long run. One promising method for improving overall pulp yields consists of halting a kraft cook at a relatively high kappa and then treating the pulp with a single or a double oxygen stage before it is bleached. This type of approach has been reported to improve the overall yield of bleached kraft pulps by 2.4% (3, 228).

For the last few years, our research activities have been directed at examining laccase-mediator systems as alternatives to oxygen delignification for improving pulp yields (239-240). In the past, the use of laccase for delignifying kraft pulps was restricted due to the size of the enzyme and thus to its inefficacy in diffusing into pulp fibers to react with the lignin (5). This limitation was first overcome with the aid of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), a mediator (6). Since this initial discovery, research efforts into laccase-mediator systems (LMS) have intensified and remain very active on lignin model compounds and low-lignin-content kraft pulps (163, 208, 234, 242, 254, 265, 281). The application of LMS for delignifying high-kappa kraft pulps remains to a large extent understudied. Our initial studies on high-kappa kraft pulps demonstrated that LMS treatments using 1-hydroxybenzotriazole (HBT) as the mediator could efficiently delignify such furnish (239). Further LMS studies using N-acetyl-N-phenylhydroxylamine (LMSNHAc), violuric acid (LMSVa), and IBT (LMSIBT) as mediators revealed that VA outperforms both HBT and NHA vis-à-vis delignification (262). Nonetheless, all three LMS systems lead to pulp darkening. Our studies have established that this effect is attributed, in part, to the formation of quinonoid-type structures during LMS (262). Structural analysis of phosphitylated residual lignins isolated subsequent to LMS treatments (with VA, NHA, or HBT) using a high-kappa SW kraft pulp revealed that all three mediators preferentially attack noncondensed at C-5 phenolic lignin struc-
tures. C-5 condensed phenolic lignin structures exhibited resistance towards an LMS<sub>SHBT</sub> and an LMS<sub>NHA</sub> treatment, but less towards LMS<sub>VA</sub>. A decrease in aliphatic hydroxyl groups and an increase in carboxylic acid groups relative to the brownstock lignin were also evident with all three mediators. Nonetheless, VA was the most potent mediator when considering the magnitude of change in lignin functional groups.

This report summarizes our continued LMS research efforts. The purpose of the present study was to examine the structural changes of residual lignins isolated from a SW kraft pulp (kappa no. 71.4) and then treated with LMS<sub>VA</sub>, LMS<sub>SHBT</sub>, and LMS<sub>NHA</sub>.

**Experimental**

**Materials** All materials used in this study were purchased from Aldrich Chemical Co., Milwaukee, WI, and used as received except for <em>p</em>-dioxane, <em>N</em>-acetyl-<em>N</em>-phenylhydroxylamine (NHA), and laccase. <em>p</em>-Dioxane was distilled over NaBH<sub>4</sub>, and NHA was synthesized in accordance with Oxley’s method (210). Laccase from <em>Trametes villosa</em> was donated by Novo Nordisk Biochem, Franklinton, NC.

**Enzyme Assay.** Laccase activity was measured by monitoring the rate of oxidation of syringaldazine. One unit of activity (U) was defined as the change in absorbance at 530 nm of 0.001 per minute per mL of enzyme solution, in a 100 mM potassium phosphate buffer (2.20 mL) and 0.216 mM syringaldazine in methanol (0.30 mL, pH 6.7). The procedure was carried out at 23°C. The activity of the laccase was 3.8E+07 U/mL of enzyme solution. The activity of the laccase was also measured in several solutions of water:dioxane as shown in Figure 93.
Isolation of residual lignins. Residual lignins were isolated from an acetone-extracted, never-dried, laboratory-prepared SW kraft pulp with a starting kappa number of 71.4 in accordance with established literature method (27, 64). In brief, a 5000-mL three-necked-round bottom flask was charged with 120 g of pulp (o.d. basis). The consistency was adjusted to 4% by adding a 0.10 N HCl 9:1 p-dioxane-water solution. The slurry was then refluxed for two hours under an argon atmosphere. The pulp was filtered and the filtrate was filtered through celite, neutralized, and concentrated under reduced pressure to approximately 10% of the original volume. Water (ca. 400 mL) was added and the mixture was concentrated again under reduced pressure to remove the last traces of p-dioxane. The solution’s pH was then adjusted to 2.5 with 1.00 N HCl. The precipitated lignin was collected, washed three times, and freeze-dried. A total of four isolations were carried out. The residual lignins were combined and then used to carry out the enzymatic treatments.

LMS procedure on residual lignins. Residual lignin (500 mg) was dissolved in 150 mL of a 1:1 solution of p-dioxane-distilled water. The solution was first transferred to a Teflon beaker, which was then placed into a 300-mL-capacity Parr reactor. The lignin solution was heated to 45°C and was maintained at this temperature throughout the incubation period (2 hours). NHA (1.85 x 10⁻⁵ moles) was then added (or 1.85 x 10⁻⁶ moles of...
HBT or VA) and the pH was adjusted to 4.5 using glacial acetic acid. After mixing the solution (approx. 5 minutes), laccase (2 mL of enzyme solution) was added and the reactor was sealed and pressurized with oxygen to 145 psi. Subsequent to the treatment, the dioxane was removed under reduced pressure. The lignin-water solution was then diluted to approximately 250 mL using fresh distilled water, and the pH was adjusted to 2.2 with 1.00 N HCl. The precipitated lignin was washed three times using acidified water. The treated lignin was then subjected to an alkaline extraction stage.

**Extraction stage of LMS treated residual lignins.** Subsequent to the LMS treatments, the residual lignins were subjected to an alkaline extraction stage. The lignins were diluted to approximately 180 mL using distilled water. The initial pH was adjusted to 11 using a 1.00 N NaOH, and the lignin solutions were treated for 1.5 hours at 80°C. Following the treatment, the lignin solution was acidified to pH 2.2 using 1.00 N HCl, centrifuged, and decanted. The precipitated lignin was washed three times using fresh acidified water (pH 2.2), freeze-dried, and then characterized.

**Characterization of residual lignins.** The treated residual lignins as well as the starting residual lignin were characterized by $^{31}$P NMR and $^{13}$C NMR in accordance with established literature methods (59, 221, 282). NMR data were acquired with a DMX 400 MHz Bruker spectrometer.

**Results and Discussion**

The bleaching of kraft pulps with LMS continues to be extensively studied. Knowledge on residual lignins isolated and characterized subsequent to LMS treatments of kraft pulps has substantially expanded over the past few years. Furthermore, our studies have demonstrated that LMS treatments deplete high-kappa pulps primarily of noncondensed C-5 phenolic lignin groups, as well as of side chain aliphatic hydroxyl groups, leading to the formation of carboxylic acids and $\alpha$, $\beta$-quinones. This study further explores the reactions of laccase and LMS with residual lignins in the absence of kraft pulp fibers.

Residual lignin samples isolated from a SW kraft pulp (kappa no. 71.4) were treated with laccase or LMS using VA, NHA, and HBT as the mediators. The treatments involved
placing the lignin, laccase, and/or the mediator in a 1:1 water:dioxane solution for 2 hours at 45°C in a pressurized Parr reactor (145 psi of oxygen). The treated lignins were recovered and treated to an alkaline extraction stage at 80°C for 1.5 hours. Lignin structural changes were evaluated via $^{13}$C and $^{31}$P NMR. Figures 94-97 summarize the most important changes observed in lignin structures. As illustrated in Figure 94, the carboxylic acid content of the lignin treated with laccase and LMS substantially increased under the conditions employed.

![Graph showing carboxylic acid content per aromatic ring](image)

**Figure 94.** Carboxylic acid content per aromatic ring; 2000 µL of laccase per 500 mg of lignin.

This increase in acid groups was accompanied by depletion in phenoxy groups, as shown in Figure 95. Both condensed and noncondensed C-5 phenolic groups were reactive towards laccase. Although we have previously observed this reactivity towards lignin from low-kappa pulps, the same did not hold true on high-lignin-content kraft pulps. Indeed, LMS treatments of high-kappa pulps yielded residual lignins predominantly depleted of noncondensed C-5 phenolic groups. Several factors may be contributing to this difference in selectivity, including lignin content, mass-transfer effects, and reaction parameters.
Accompanying the loss in phenolic hydroxyl groups was a substantial decrease in aliphatic hydroxyl group content, as seen in Figure 96. This trend can be attributed to side chain oxidation of the lignin and corroborates LMS lignin model compound studies. The LMS treatments in this study were found to slightly decrease the methoxy group content of the recovered lignins, suggesting that demethoxylation took place (see Figure 97).
Figure 96. Aliphatic hydroxyl group content (mmol/g lignin); 2000 µL of laccase per 500 mg of lignin.

Figure 97. Methoxyl content per aromatic ring; 2000 µL of laccase per 500 mg of lignin.
Conclusions

This study demonstrates that, overall, LMS treatments on residual lignins follow the same general oxidative trends as have been observed with kraft pulp fibers. In addition, these results suggest that laccase or an LMS treatment could be employed to modify the structure of lignin. This technology may have future applications to tailor the properties of lignin for commercial applications.

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7.9 Addendum to publication 8

The following results were presented at the 6th European workshop on lignocelluliosics and pulp (EWLP) but were not incorporated in the manuscript due to size limitations.

$^{31}$P NMR error analysis. The LSD values were determined by repeating the LMS VS(E) treatment three separate times. The results are summarized in Table 37.

<table>
<thead>
<tr>
<th>Table 37. $^{31}$P NMR LSD values from LMS VS(E) treatment in triplicate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Functional group</td>
</tr>
<tr>
<td>Carboxyl OH</td>
</tr>
<tr>
<td>Non-condensed at C-5 OH</td>
</tr>
<tr>
<td>Condensed at C-5 OH</td>
</tr>
<tr>
<td>$p$-Hydroxyphenyl OH</td>
</tr>
<tr>
<td>Aliphatic OH</td>
</tr>
</tbody>
</table>

185
Control experiments. Control experiments were performed using the procedures and conditions described in publication 8, except no laccase was employed. The $^{31}$P NMR data of various functional groups are shown in Figure 98. These results clearly indicate that the nature of the residual lignin was unaltered when treated solely with mediator.

![Figure 98. $^{31}$P NMR data of control experiments. Residual lignin treated with mediator in the absence of laccase. SM= starting material.](image)

Treatments of residual lignin at various doses of laccase. Lignin samples were subjected to laccase, and LMS treatments employing the exact procedures and conditions described in publication 8, except for the dose of laccase. Enzyme solution doses of 20, 500, and 2000 μl. were employed.

Laccase and LMS treatment: 20 μl. of enzyme solution. Lignin functional groups were characterized before and after a laccase treatment employing 20 μl. of enzyme solution per 500 mg of residual lignin. Analysis of the $^{31}$P NMR results (see Figure 99) revealed that changes in lignin functional groups relative to the starting residual lignin, were not evident. Similarly, residual lignins subjected to LMS treatments employing VA, NHA and HBT and 20μl. of laccase (see Figure 100) were also unaltered.

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Figure 99. % Change in lignin functional groups determined by $^{31}$P NMR before and after a laccase treatment using 20 μL of laccase solution per 500 mg of lignin.

Figure 100. Lignin functional groups (mmol/g lignin) content determined by $^{31}$P NMR before and after laccase (20 μL per 500 mg of lignin), and LMS treatments. SM = starting material.
These unanticipated observations were hypothesized to be linked in part, to the activity of laccase in dioxane:water at 45°C. Consequently, laccase activities were measured under an environment similar to a laccase-residual lignin treatment. Laccase (20μl) was added to an Erlenmeyer flask containing 150 mL of a 1:1 dioxane:water solution. The flask containing the dioxane:water mixture was first heated to 45°C. Laccase was then introduced, the solution was swirled twice, a sample was withdrawn, and the activity was measured. This measurement represented the activity at time zero (t = 0 min). A second measurement was taken at t = 30 min. The results shown in Figure 101 clearly highlight the effect of temperature and time on the activity of the laccase in a dioxane:water solution. A 50% decrease in activity was noted by simply heating the laccase in dioxane water at 45°C (t = 0). Laccase activity could not be detected after 30 minutes of incubation. On this basis, there must be a minimal dose requirement of laccase to initiate modification to the lignin polymer.

![Graph showing activity of laccase in dioxane:water at different conditions](image)

**Figure 101.** Activity measurements of laccase in water: dioxane, at room temperature, and at 45°C (at time =0, and time =30 minutes). ND= non-detectable; activity at room temperature was 22.3E+06 U/mL of enzyme solution.

**Laccase and LMS treatments: 500 vs. 2000 μL of enzyme solution.** The experimental protocol described in publication 8 was repeated with 500 μL and 2000 μL of laccase. The 31P NMR results shown in Figure 103 demonstrated that relative to the starting material, the treated residual lignin samples were significantly modified. However, the magnitude in change was more pronounced when the higher dose of laccase was employed.

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Figure 102. % Change in lignin functional groups determined by $^{31}$P NMR before and after laccase(E) (500 and 2000 µL per 500 mg of lignin) treatments.

Note. These percent changes are normalized to the starting values.

Further analysis of $^{31}$P NMR spectra of the enzymatically treated residual lignins revealed that $p$-hydroxyphenyl lignin groups were resistant to laccase and LMS (see Figure 103). Similar data on LMS$_{HBT}$ treated kraft pulps as well as laccase and LMS$_{HBT}$ treated residual lignins, have been reported by Tamminen et al. (201)
The $^{13}$C NMR spectral data indicate that the residual lignins were enriched in β-O-4 type structures, suggesting that, under the experimental conditions employed in this study, such moieties are unreactive towards laccase and LMS. Analogous findings have been reported by Sealey and Ragauskas (199) on LMS$_{100}$ treated kraft pulps.

The increasing trend in the ratio of substituted to unsubstituted aromatic lignin groups suggests that the laccase and LMS treated residual lignins maybe more condensed than the untreated lignin. These results are summarized in Figure 105.
Figure 104. β-O-4 content in residual lignins before and after laccase(E) and LMS(E) treatments using 2000 μL of laccase per 500 mg of lignin. Content determined by $^{13}$C NMR.

Figure 105. Ratio of substituted to unsubstituted aromatic groups before and after laccase(E) and LMS(E) treatments using 2000 μL of laccase per 500 mg of lignin. Ratio determined by $^{13}$C NMR.
The most notable observation that can be drawn based on the data presented in publication 8 and the addendum is the ineffectiveness of the mediators in LMS treatments of residual lignins. Indeed, the presence of VA, HBT, or NHA did not lead to any further structural changes in residual lignins. It may then be inferred, that in solution, diffusion issues are secondary and hence, the role of the mediator is insignificant. The laccase becomes the principal player involved in the degradation of lignin. Tamminen et al. (201) characterized the nature of residual lignins first isolated from oxygen delignified kraft pulp and then treated with laccase and \( \text{LMS}_{\text{HBT}} \). Their results also indicated that the presence of HBT did not yield further lignin degradation.
LMS treatments on high-kappa kraft pulps led to substantial delignification without detrimental impact on viscosity. The efficiency of the LMS was evident since most of the lignin was removed under one hour at 45°C. Of the mediators investigated, violuric acid was the most effective promoter delignification. A comparative study between oxygen delignification and violuric acid revealed that under relatively mild conditions, a single or a double LMSVA treatment is comparable to a single or a double O stage. Of great notability was the much higher end viscosity of LMSVA treated pulps in comparison to the end viscosity of oxygen-treated pulps. This research program also demonstrated the pulp yield benefits associated with the use of LMSVA. Accompanying these distinguished attributes, however, was a decrease in brightness levels. This trend was noted regardless of the mediator employed and the starting kappa number of the brownstock. The loss in brightness was mitigated with the reinforcement of the alkaline extractions with oxygen, peroxide, and oxygen peroxide, suggesting that this deleterious effect can be overcome. ECF bleaching sequences in conjunction with LMSVA led to final TAPPI brightness readings of +80.

The loss in brightness was most likely related to an increase in colored quinone structures as a result of the LMS. This research conclusively established and quantified for the first time the formation of quinonoid structures during LMS and their decrease subsequent to an extraction stage. On this basis, the brightness benefits associated with the oxidative reinforcement of the alkaline extractions were attributed to quinones.

The spectral analyses of residual lignins isolated after LMS treated high-kappa kraft pulps revealed that HBT, VA, and NHA preferentially attack phenolic lignin moieties. In addition, a substantial decrease in aliphatic hydroxyl groups was also noted, suggesting side chain oxidation. In all cases, an increase in carboxylic acid was observed. Of notable importance was the different selectivity of NHA, VA, and HBT towards lignin functional groups, despite the common N-OH moiety. C-5 condensed phenolic lignin groups were overall resistant to an LMSNHA, HBT treatments but, to a lesser extent to an LMSVA. The inactiveness of these condensed lignin moieties was not observed when low-kappa
kraft pulps were biobleached, suggesting that the LMS chemistry is influenced by the extent of delignification.

Treatment of isolated residual lignins with laccase led to a modified lignin. All functional groups, excluding carboxylic acids, detectable by $^{31}$P NMR substantially decreased. $^{13}$C NMR spectral analysis revealed a decrease in methoxyl groups, and a substantial increase in carboxylic acid groups. However, β-O-4 lignin structures were resistant to a laccase treatment. The substituted to unsubstituted aromatic group ratio indicated that the modified residual lignin maybe of a condensed nature relative to the starting material. The presence of VA, NHA, or HBT with laccase did not lead to any further lignin structural changes. This suggests that in solution, under the experimental conditions employed, the alteration of the lignin polymer proceeded mainly by the action of laccase and that the mediator’s role was secondary.

In summary, the results reported in this research project demonstrated the potential benefits of biotechnology for future applications in wood utilization practices.

Finally, this research program has presented important features on the delignification chemistry of LMS$_{NHA}$ and LMS$_{VA}$ that are momentous contributions to the overall LMS chemistry knowledge base.
9. RECOMMENDATION FOR FUTURE WORK

Fundamental research, in collaboration with academia, industry, and government personnel, into laccase biobleaching must continue if a viable biological-based technology is to be commercially implemented.

The results presented in this research program were positive; however, the implementation of LMS on a commercial scale is far from practiced realization. This is due, in part, to the non-catalytic nature of the biobleaching system employing N-hydroxy based mediators. On this basis, research targeted to address this issue is warranted. In addition, further understanding of the differing selectivity of these N-hydroxy mediators may lead to new and efficient ones that may or may not contain the N-OH moiety. The biobleaching of the future must be catalytic, cost-effective, and virtuous with respect to selectivity and delignification.

In addition, the toxicology of mediators will need to be addressed in the near future to ensure the environmental compatibility of laccase-mediator systems.
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Auburn University: Prof. Dr. Thomas Elder
Hercules: Mr. Bill Hancock

I would also like to sincerely thank Mrs. Catherine Ragauskas, for her genuine friendship, kindess, support, and great hospitality. To Emma, Alyse, and Luke Ragauskas, you always put a smile on my face. To my childhood friend Mr. Youssef Bahnam and my dearest friends Ms. Ruya Ataç and Mr. Jim Hayden for always being supportive.

I am endlessly grateful for the support and love of my entire family especially, my mother Mrs. Gilberte M. Chakar, my father, Dr. Selim R. Chakar, and my two sisters Ms. Nadine S. Chakar and Ms. Maya S. Chakar. I would also like to extend the same appreciation to my newest family members, Mrs. Lois Koenig and Ms. Kristine Koenig. I am also indebted to my uncle Dr. Jean Zincovitch and Aunt Mrs. Beatrice Zincovitch for taking good care of me.

Most of all, to perhaps the least recognized person throughout my tenure at IPST but who played a crucial part in the success of this project, my dear and loving wife Mrs. Melissa C. Chakar. Her love, support, patience and sacrifices made all this possible.
11. REFERENCES


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250. Call, H.P. New Enzymatically Mediated Delignification and Bleaching Systems.

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Appendix 1. LMS at 5% and 10% consistency - A comparative study

Objective. To establish if the delignification response of LMS treatments carried out at 5% and 10% consistency is the same or different.

Conditions. Mediator used: NHA; Mediator dose: 2% (o.d. basis); Temperature = 45°C; Time = 2 hr, O₂ pressure = 145 psig; pH = 4.5

Results:

Table A.1. Kappa results of LMS₅₀₇₅ treatments

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<th>Average</th>
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Table B.1. Data sorted by % consistency

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Average 1: 66.4 Average 2: 66.5

Standard deviation 1: 0.35573 Standard deviation 2: 0.35565

Variance 1: 0.126543 Variance 2: 0.126486

222
Statistics

Variance test
F-ratio 1.000226
critical F @0.025 0.2
critical F @0.975 4.99

Accept that variance 1 = variance 2.

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</table>

\(^a\) Variable 1: kappa at 5% consistency
\(^b\) Variable 2: kappa at 10% consistency

Since the t statistic (-0.36549, see Table C.1) lies within the acceptance region +/- 2.144789 (see Table C.1) the null hypothesis (i.e., Ho: average 1 = average 2, see Table B.1.) is accepted. The two means are equal at the 95% confidence level.
### Table A.2: Kappa results after LMS treatments using non-acetone-extracted brownstock

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<th>time (hr)</th>
<th>Kappa 1</th>
<th>Kappa 2</th>
<th>average Kappa</th>
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Appendix 3.

$^{31}$P NMR spectra from publication 2

Brownstock lignin- trial 1. NMR File saved as u/chakar/ P31-5-FSC1E

Brownstock lignin- trial 2. NMR File saved as u/chakar/ P31-5-FSC2E
Brownstock lignin-trial 3. NMR File saved as u/chakar/P31-5-FSCJE

LMS$_{\text{HBT}}$ (E) NMR File saved as u/chakar/P31-5-fsocbht

229
LMS_HET (E+P+O). NMR File saved as u/chakar/ P31-5-ficeophb

LMS_HIA (E). NMR File saved as u/chakar/ P31-5-ficEnhhaa
LMS_{NH}_{2} (E+P+O). NMR File saved as u/chakar/ P31-5-fscEopnha
Appendix 4

$^{31}$P NMR spectra from publication 3

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Brownstock residual lignin (trial 2)  NMR file saved as u/chakar/p31-5-BSHK2

232
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LMS_{nrr(E)} NMR file saved as u/chakar/p31-5-HKEHBT
$\text{LMS}_{\text{HBOF}(E+P+O)}$. NMR file saved as u/chakar/p31-5-HKEPOHBT

$\text{LMS}_{\text{NE1}(E)}$. NMR file saved as u/chakar/p31-5-HKENHAA

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## Multiple Regression Report - Central Composite Design Phase 1

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## Regression Coefficient Section

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Appendix 6

Supporting data for publication 6. $^{31}$P and $^{13}$C NMR spectral and numerical data

Appendix 6 contains spectral and numerical data for publication 6. $^{13}$C NMR values were obtained by integrating the aromatic area and calibrating it to 6. $^{31}$P NMR values were calculated as follow.

The signal area from the internal standard cyclohexanol (145.1 ppm) which was due to 6.01E-04 g of cyclohexanol was calibrated to 1.0. Cyclohexanol (FW = 100.16 g/mol) contains one OH group, hence, 6.01E-04 g of cyclohexanol divided by 100.16 g/mol was the number of moles (5.9994E-06) of hydroxyl groups present in the internal standard in the sample. Since the integration area of cyclohexanol was set to 1.0, each unit area in the spectrum was equal to 5.9994E-06 moles of hydroxyl groups. Lignin functional groups in each spectrum were quantified by first multiplying the integration area by 5.9994E-06 and then dividing by the weight of the lignin. This value was then multiplied by a 1000 to convert the units from mol per gram to mmol per gram lignin.
$^{31}$P NMR of brownstock residual lignin. File saved as disk5/chakar/p31-5-acs1-97

$^{31}$P NMR of MS-HIA. File saved as disk5/chakar/p31-5-2r-97
$^{31}$P NMR of MS$_{oht}$. File saved as disk5/chakar/p31-5-3r-97

$^{31}$P NMR of MS$_{va}$. File saved as disk5/chakar/p31-5-4r-97

244
$^{31}$P NMR of LMSH1A: File saved as disk5/chakar/p31-5-5r-97

$^{31}$P NMR of LMSH1T: File saved as disk5/chakar/p31-5-6r-97
$^{31}$P NMR of LMS_VA. File saved as disk5/chakar/p31-5-7r-97

$^{31}$P NMR of LMS_{Nat(E)} trial no 1. File saved as disk5/chakar/p31-5-8r-97
$^3\text{P NMR of LMS}_{\text{imr}(E)}$. File saved as disk5/chakar/p31-5-9r-97

$^3\text{P NMR of LMS}_{\text{v}}(E)$. File saved as disk5/chakar/p31-5-10r-97
$^3$P NMR of LMS$_{10a}(E)$ trial no. 2. File saved as disk5/chakar/p31-5-11r-97

$^3$P NMR of LMS$_{10a}(E)$ trial no. 3. File saved as disk5/chakar/p31-5-12r-97
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<th>MSVA</th>
<th>LMS_{diff}</th>
<th>LMS_{opt}</th>
<th>LMS_{opt}(E)</th>
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$^{13}$C NMR of brownstock residual lignin. File saved as: disk5/chakar/c13-5-SFbs

$^{13}$C NMR MSHBT. File saved as: disk5/chakar/c13-mshtbt
$^{13}$C NMR $MS_{NEA}$. File saved as: disk5/chakar/c13-msnea

$^{13}$C NMR $MS_{VA}$. File saved as: disk5/chakar/c13-msva
$^{13}$C NMR of brownstock residual lignin. File saved as: disk5/chakar/c13-5-SFhs

$^{13}$C NMR MS$_{HHT}$. File saved as: disk5/chakar/c13-msHbt

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$^{13}$C NMR LMS$_{NH\alpha}(E)$ - trial no. 3. File saved as: disk5/chakar/e13-5-Inhae3

$^{13}$C NMR LMS$_{VA}(E)$. File saved as: disk5/chakar/e13-5-lvioe
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<th>MI 50</th>
<th>MI 80</th>
<th>MI 85</th>
<th>MI 89</th>
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<th>MI 105</th>
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<td>2.03</td>
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$^{31}$P brownstock residual lignin. File name saved as chakar/disk5/p31-tmp1-acd

$^{31}$P MS data. File name saved as chakar/disk5/p31-tmp2-acd
$^{31}$P MSNMR. File name saved as chakar/disk5/p31-tmp3-acs

$^{31}$P MSVA. File name saved as chakar/disk5/p31-tmp4-acs
$^{31}$p LMS$_{NBA}$  File name saved as chakar/disk5/p31-tmp5-acs

$^{31}$p LMS$_{PHT}$  File name saved as chakar/disk5/p31-tmp6-acs
$^{3}{\text{P}}$ LMS$_{\text{VA}}$. File name saved as chakar/disk5/p31-tmp7-acx

$^{11}{\text{P}}$ LMS$_{\text{NitE}}$. File name saved as chakar/disk5/p31-tmp8-acx
$^{31}$P LMS_{\text{ref}}(E). File name saved as chakar/disk5/p31-mp9-acs

$^{31}$P LMS_{\text{val}}(E). File name saved as chakar/disk5/p31-mp10-acs
Appendix 8. $^{31}$P and $^{13}$C NMR supporting spectral data for publication and addendum 8

$^{31}$P NMR laccase only(E) (2000 uL/500mg lignin). File saved as disk5/chakar/p31-ewlp-lacE

$^{31}$P NMR LMSHVC(E) (2000 uL/500mg lignin). File saved as disk5/chakar/p-ewlp-l-hbt-E

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$^{31}$P NMR LMS$_{V,L}(E)$ (2000 μL/500 mg lignin) trial no. 1. File saved as disk5/chakar/p-ewlp-l-val-E

$^{31}$P NMR LMS$_{STL}(E)$ (2000 μL/500 mg lignin) File saved as disk5/chakar/p-ewlp-lnhaE-2
$^{13}$C NMR brownstock lignin (E). File saved as disk5/chakan/e-elp-bs-E

$^{13}$C NMR laccase (E) (2000 uL/500mg lignin). File saved as disk5/chakan/e-elplp-lacE
$^{13}$C NMR LMS_{ntr}(E) (2000 ul/500mg lignin). File saved as disk5/chakar/e-cwlp-hbtE-2

$^{13}$C NMR LMS_{val}(E) (2000 ul/500mg lignin). File saved as disk5/chakar/e-elp-lmsvaE2

$^1$H NMR data. 20 µL laccase solution per 500 mg of lignin$^a$.

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<th>Lignin wt (g)</th>
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<th>[C5 non-condensed]$^b$</th>
<th>[Carboxyl]$^b$</th>
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<td>0.71</td>
<td>0.87</td>
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<tr>
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<td>1.61</td>
<td>0.74</td>
<td>0.89</td>
<td>0.25</td>
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<tr>
<td>LNS$_{b}$</td>
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<td>LNS$_{e}$</td>
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$^a$ No alkaline extraction followed the enzymatic treatments.

$^b$ brackets stand for concentration of OH in lignin functional groups in mmol/g lignin.
### 31P NMR data. 2000 µL and 500 µL laccase solution per 500 mg of lignin.

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<th>Description</th>
<th>Lignin wt (µg)</th>
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<th>[C5 noncond]</th>
<th>[Carboxyl]</th>
<th>[α-hydroxyphenyl]</th>
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<tr>
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<td>0.82</td>
<td>0.98</td>
<td>0.31</td>
<td>0.12</td>
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<tr>
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<tr>
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<td>0.0254</td>
<td>1.45</td>
<td>0.79</td>
<td>0.95</td>
<td>0.33</td>
<td>-</td>
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<td>1.47</td>
<td>0.79</td>
<td>0.95</td>
<td>0.31</td>
<td>-</td>
</tr>
<tr>
<td>Laccase(E)</td>
<td>0.0239</td>
<td>0.92</td>
<td>0.49</td>
<td>0.44</td>
<td>0.33</td>
<td>0.10</td>
</tr>
<tr>
<td>LMS1,4,6(E)</td>
<td>0.0241</td>
<td>0.94</td>
<td>0.60</td>
<td>0.38</td>
<td>0.45</td>
<td>0.09</td>
</tr>
<tr>
<td>LMS1,2,6(E)</td>
<td>0.0242</td>
<td>0.86</td>
<td>0.56</td>
<td>0.40</td>
<td>0.38</td>
<td>0.10</td>
</tr>
<tr>
<td>LMS1,3,6(E)</td>
<td>0.0240</td>
<td>0.93</td>
<td>0.53</td>
<td>0.33</td>
<td>0.52</td>
<td>0.10</td>
</tr>
<tr>
<td>LMS1,3,2,E2</td>
<td>0.0238</td>
<td>0.90</td>
<td>0.52</td>
<td>0.32</td>
<td>0.49</td>
<td>0.09</td>
</tr>
<tr>
<td>LMS1,2,6(E)</td>
<td>0.0236</td>
<td>0.97</td>
<td>0.57</td>
<td>0.35</td>
<td>0.53</td>
<td>0.11</td>
</tr>
</tbody>
</table>

500 µL laccase solution per 500 mg of lignin

| Laccase(E) | 0.0239 | 1.70 | 0.86 | 0.86 | 0.33 |

- Weights were corrected for Na, S, and N content; see table below
- C5 cond = C5 condensed
- C5 noncond = C5 noncondensed
<table>
<thead>
<tr>
<th>Description</th>
<th>Lignin wt (g)</th>
<th>%N</th>
<th>%S</th>
<th>%Na</th>
<th>% Total</th>
<th>Corrected wt(g)</th>
</tr>
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<tbody>
<tr>
<td>HS</td>
<td>0.0261</td>
<td>0.08</td>
<td>0.84</td>
<td>0</td>
<td>0.92</td>
<td>0.0259</td>
</tr>
<tr>
<td>LMP(1)</td>
<td>0.0255</td>
<td>0</td>
<td>0.74</td>
<td>0.68</td>
<td>1.42</td>
<td>0.0251</td>
</tr>
<tr>
<td>MS100(E1)</td>
<td>0.0254</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MS50(E1)</td>
<td>0.0251</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MS50(E2)</td>
<td>0.0255</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Laccase1E1</td>
<td>0.025</td>
<td>3.11</td>
<td>0.82</td>
<td>0.65</td>
<td>4.58</td>
<td>0.0239</td>
</tr>
<tr>
<td>LMP100(E1)</td>
<td>0.0253</td>
<td>4.03</td>
<td>0.74</td>
<td>0</td>
<td>4.77</td>
<td>0.0241</td>
</tr>
<tr>
<td>LMP90(E1)</td>
<td>0.0254</td>
<td>3.94</td>
<td>0.82</td>
<td>0</td>
<td>4.76</td>
<td>0.0242</td>
</tr>
<tr>
<td>LMP30(E1)</td>
<td>0.0255</td>
<td>5.19</td>
<td>0.74</td>
<td>0</td>
<td>5.93</td>
<td>0.0234</td>
</tr>
<tr>
<td>LMP30(E2)</td>
<td>0.0253</td>
<td>5.29</td>
<td>0.76</td>
<td>0</td>
<td>6.05</td>
<td>0.0238</td>
</tr>
<tr>
<td>LMP30(E3)</td>
<td>0.025</td>
<td>4.9</td>
<td>0.74</td>
<td>0</td>
<td>5.44</td>
<td>0.0236</td>
</tr>
</tbody>
</table>

500 μL laccase solution per 500 mg of lignin

Laccase(E1) 0.0245 1.11 0.8 0.59 2.50 0.0239
\(^{13}\)C NMR data. 2000 µL lacase solution per 500 mg of lignin.

<table>
<thead>
<tr>
<th>Region</th>
<th>Functional group</th>
<th>Integral region (ppm)</th>
<th>BS(ES)</th>
<th>Lactase(ES)</th>
<th>LMSup(E)</th>
<th>LMSne(E)</th>
<th>LMSav(E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>COOH</td>
<td>178.162.5</td>
<td>0.21</td>
<td>1.00</td>
<td>1.04</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>C3,4 -(C-Ar-C)</td>
<td>154.0-140.0</td>
<td>1.83</td>
<td>1.77</td>
<td>1.71</td>
<td>1.73</td>
<td>1.64</td>
</tr>
<tr>
<td>3</td>
<td>C1 -(C-Ar-C)</td>
<td>140.0-127.0</td>
<td>1.42</td>
<td>1.59</td>
<td>1.69</td>
<td>1.78</td>
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<tr>
<td>4</td>
<td>C5, -(C-Ar-C)</td>
<td>127.0-123.0</td>
<td>0.50</td>
<td>0.54</td>
<td>0.55</td>
<td>0.57</td>
<td>0.55</td>
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<tr>
<td>5</td>
<td>C6, -(C-Ar-H)</td>
<td>123.0-127.0</td>
<td>0.81</td>
<td>0.75</td>
<td>0.73</td>
<td>0.81</td>
<td>0.79</td>
</tr>
<tr>
<td>6</td>
<td>C5, -(C-Ar-H)</td>
<td>117.0-114.0</td>
<td>0.53</td>
<td>0.47</td>
<td>0.45</td>
<td>0.38</td>
<td>0.43</td>
</tr>
<tr>
<td>7</td>
<td>C2, -(C-Ar-H)</td>
<td>114.0-106.0</td>
<td>0.91</td>
<td>0.88</td>
<td>0.86</td>
<td>0.73</td>
<td>0.91</td>
</tr>
<tr>
<td>2-4</td>
<td>Substituted aromatic C</td>
<td>154.0-123.0</td>
<td>3.75</td>
<td>3.90</td>
<td>3.95</td>
<td>4.09</td>
<td>3.86</td>
</tr>
<tr>
<td>5-7</td>
<td>Unsaturated aromatic C</td>
<td>123.0-106.0</td>
<td>2.25</td>
<td>2.10</td>
<td>2.05</td>
<td>1.91</td>
<td>2.14</td>
</tr>
<tr>
<td>8</td>
<td>Aliphatic C-O (Cβ in β-1,3)</td>
<td>90.0-78.0</td>
<td>0.48</td>
<td>0.45</td>
<td>0.46</td>
<td>0.48</td>
<td>0.67</td>
</tr>
<tr>
<td>9</td>
<td>Aliphatic C-O (Cα in β-1,4)</td>
<td>78.0-67.0</td>
<td>0.48</td>
<td>0.71</td>
<td>0.91</td>
<td>0.80</td>
<td>0.96</td>
</tr>
<tr>
<td>10</td>
<td>Aliphatic C-OR</td>
<td>67.0-61.0</td>
<td>0.21</td>
<td>0.29</td>
<td>0.45</td>
<td>0.33</td>
<td>0.43</td>
</tr>
<tr>
<td>11</td>
<td>C7 (β-1,4)</td>
<td>61.0-57.0</td>
<td>0.27</td>
<td>0.34</td>
<td>0.46</td>
<td>0.41</td>
<td>0.49</td>
</tr>
<tr>
<td>12</td>
<td>OCH3</td>
<td>57.0-54.0</td>
<td>0.82</td>
<td>0.75</td>
<td>0.76</td>
<td>0.74</td>
<td>0.78</td>
</tr>
<tr>
<td>13</td>
<td>Cβ in β-1,3 and Cβ in β5</td>
<td>54.0-32.0</td>
<td>0.11</td>
<td>0.18</td>
<td>0.22</td>
<td>0.18</td>
<td>0.23</td>
</tr>
<tr>
<td>14</td>
<td>CH3 in Diaryl methane</td>
<td>29.5-27.0</td>
<td>0.08</td>
<td>0.21</td>
<td>0.18</td>
<td>0.20</td>
<td>0.22</td>
</tr>
</tbody>
</table>
Appendix 9. $^{31}\text{P}$ NMR chemical shifts of various oximes

Objective: To measure the $^{31}\text{P}$ NMR shifts of compounds I-XXI shown below:

![Chemical structures of compounds I-XXI](image_url)
<table>
<thead>
<tr>
<th>Oxime</th>
<th>Name</th>
<th>Source*</th>
<th>Catalog</th>
<th>FW</th>
<th>CAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>2,3-butanedione oxime, 97%</td>
<td>Ald</td>
<td>112135</td>
<td>101.11</td>
<td>57-71-6</td>
</tr>
<tr>
<td>II</td>
<td>Acetone oxime</td>
<td>Ald</td>
<td>A10507</td>
<td>73.10</td>
<td>126-06-0</td>
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<tr>
<td>III</td>
<td>4-methyl-2-pentanone oxime, 99%**</td>
<td>Ald</td>
<td>408093</td>
<td>115.18</td>
<td>105-44-2</td>
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<tr>
<td>IV</td>
<td>Pinacolone oxime, 98%</td>
<td>Ald</td>
<td>442887</td>
<td>115.18</td>
<td>2475-93-6</td>
</tr>
<tr>
<td>V</td>
<td>12-Tricosanone</td>
<td>Aldrc</td>
<td>S553700</td>
<td>353.63</td>
<td>-</td>
</tr>
<tr>
<td>VI</td>
<td>1,3-dibenzyl ketoxime, 98%</td>
<td>Lan</td>
<td>0914</td>
<td>225.29</td>
<td>1788-31-4</td>
</tr>
<tr>
<td>VII</td>
<td>2-hydroxyiminopropanic acid</td>
<td>Aldrc</td>
<td>S492523</td>
<td>103.08</td>
<td>-</td>
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<tr>
<td>VIII</td>
<td>5-(Hydroxymethylene) azelaic acid</td>
<td>Aldrc</td>
<td>S906654</td>
<td>217.22</td>
<td>-</td>
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<tr>
<td>IX</td>
<td>2,5-hexadiene dioxime</td>
<td>Aldrc</td>
<td>S378496</td>
<td>144.17</td>
<td>2157-57-5</td>
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<tr>
<td>X</td>
<td>Diethyl oximinoalane**</td>
<td>Aldrc</td>
<td>S651575</td>
<td>189.17</td>
<td>6829-41-0</td>
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<tr>
<td>XI</td>
<td>2,4-dimethyl-pentan-3-one-oxime**</td>
<td>Aldrc</td>
<td>S37762</td>
<td>129.2</td>
<td>-</td>
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<tr>
<td>XII</td>
<td>2,2,4,4-tetramethyl-3-pentanone oxime**</td>
<td>Ald</td>
<td>415510</td>
<td>157.26</td>
<td>7754-22-5</td>
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<tr>
<td>XIII</td>
<td>3-methyl-1-butanone oxime**</td>
<td>Aldrc</td>
<td>S383327</td>
<td>143.23</td>
<td>22457-23-4</td>
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<tr>
<td>XIV</td>
<td>Acetophenone oxime, 98%</td>
<td>Lan</td>
<td>4529</td>
<td>135.16</td>
<td>613-91-2</td>
</tr>
<tr>
<td>XV</td>
<td>Benzophenone oxime, 98+</td>
<td>Lan</td>
<td>0817</td>
<td>197.24</td>
<td>574-66-3</td>
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<tr>
<td>XVI</td>
<td>p-Benzoguone dioxime, 97%**</td>
<td>Lan</td>
<td>11881</td>
<td>138.13</td>
<td>105-11-3</td>
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<tr>
<td>XVII</td>
<td>Benzaldehyde oxime, 98%</td>
<td>Lan</td>
<td>4563</td>
<td>121.14</td>
<td>932-90-1</td>
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<tr>
<td>XVIII</td>
<td>Cinnamaldehyde oxime, 98%</td>
<td>Lan</td>
<td>5026</td>
<td>147.18</td>
<td>13372-81-1</td>
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<tr>
<td>XIX</td>
<td>Benzoil dioxime, 98%</td>
<td>Lan</td>
<td>2003</td>
<td>240.26</td>
<td>23873-81-6</td>
</tr>
<tr>
<td>XX</td>
<td>Cyclohexanone oxime, 97%**</td>
<td>Lan</td>
<td>11221</td>
<td>113.16</td>
<td>100-64-1</td>
</tr>
<tr>
<td>XXI</td>
<td>1-phenyl-1,2-propanedioxime, 99%</td>
<td>Ald</td>
<td>220094</td>
<td>163.18</td>
<td>119-51-7</td>
</tr>
</tbody>
</table>

* Ald = Aldrich, Aldrc = Aldrich rare chemical, Lan = Lancaster

** added 70uL of derivatizing agent, otherwise (i.e., no **) 140uL was added

NMR sample preparation and acquisition conditions: Sample preparation was carried out in accordance with the method described in section 6.11.1 of the experimental section, except for the amount of derivatizing reagent used. Either 70 uL or 140 uL of the agent was used. The amount of derivatizing agent is specified in Table A. All chemical shifts were referenced to the signal at 132.2 ppm.
Compound I. NMR file saved as: Chakar/disk.5/p2,3butaoxime

Compound II. NMR file saved as: chakar/disk 5/ p-acetoneox2
Compound III  NMR file saved as: chakar/disk5/ p-4-met-2pento

Compound IV  NMR file saved as: chakar/disk5/ p31-pinac oxim
Compound IV; sample chakar/disk 5/ p31-pinac oxim recorded 24 hr later, and saved as chakar/disk 5/ p-pinacolone 2

Compound V. NMR file saved as chakar/disk 5/ p-trico-oxime
Compound VI. NMR file saved as chakar/disk5/ p-dibenzylktox

Compound VII. NMR file saved as chakar/disk5/ p-2hydroxioxi
Compound VIII. NMR file saved as chakar/disk5/ p-hydroxyazeal

Compound IX. NMR file saved as chakar/disk5/ p-2,5hexdioxi2
Compound X. NMR file saved as chakar/disk5/ p-diethylxoximi

Compound XI. NMR file saved as chakar/disk5/ 2,4-dimepentre3ox
Compound XII. NMR file saved as chakan/disk5/p-tetpentaoxim

Compound XIII. NMR file saved as chakan/disk5/p-heptaoxime
Compound XIV. NMR file saved as chakar/disk5/ p31-acetophoxi
Compound XV. NMR file saved as chakar/disk5/ p31-benzophoxi

Compound XVI. NMR file saved as chakar/disk5/ p31-benzoquoxi

Compound XVII. NMR file saved as chakar/disk5/ p31-bnzaldheox
Compound XVIII. NMR file saved as chakar/disk5/p31-cinnamoxim

Compound XIX. NMR file saved as chakar/disk5/p31-benzildiox
Compound XX. NMR file saved as chakar/disk5/ p31-cyclooxxime

Compound XXI. NMR file saved as chakar/disk5/ p-phenylooxeox
Appendix 10. Other publications


FUNDAMENTAL INVESTIGATION OF LACCASE MEDIATOR DELIGNIFICATION ON HIGH LIGNIN CONTENT KRAFT PULPS

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Institute of Paper Science and Technology
500 10th Street NW
Atlanta, GA 30318
USA
arthur.ragauskas@ipsst.edu

ABSTRACT
The fundamental bleaching chemistry of high and low-kappa softwood (SW) kraft pulps via laccase-mediator systems (LMS) was explored. The high-kappa pulps responded more favorably to LMS than the low-kappa pulps. The residual lignin from the low and high kappa pulps was enriched in acid groups after LMS(E*) treatments. Accompanying this effect was a decrease in the phenoxy content. This decrease was more pronounced in the case of the low kappa pulps.

INTRODUCTION
The bleaching of kraft pulps is an integral component in the production of high-value paper products. Over the last decade, research efforts have been largely focused on environmental issues, but as these challenges have been addressed, new research opportunities are being developed. Kraft pulping and bleaching yields have historically been important research topics that have now taken on renewed interest. Improved pulp yield offers the opportunity to enhance kraft wood utilization practices and reduce operating and capital costs associated with the production of kraft pulps. Pulp yields can be improved by a variety of techniques including modified chipping techniques, pulping additives, and halting the kraft pulping process at high kappa numbers. The latter process attempts to stabilize the high recoverability of the initial and bulk phase of a kraft cook. Typically, the cook is halted at or before reaching the residual phase. This yields 6% kraft pulp with kappa numbers in the range of 40-50. The remaining lignin is then removed by oxygen delignification. Jamel et al. (1) and others (2, 3) have shown that this approach can effectively improve the overall yield of bleached kraft pulp by 2-3%.

Recently, our research group has begun to explore several competing technologies that could increase pulp yields. One attractive approach is to use biobleaching technologies as a potential cost-effective alternative to oxygen delignification. The high selectivity of LMS delignification of low kappa kraft pulps (kappa > 30 for SW and >15 for HW) has been established by several researchers (4, 5, 6, 7). In addition, the LMS stage has been shown to remove more than 45% of the residual lignin present in low-lignin kraft pulps. By extension, we have hypothesized that an LMS stage could remove significant amounts of lignin from high-kappa kraft pulps with high selectivity. The results of our initial investigations supported our hypothesis that an LMS stage could be employed to remove lignin from high-kappa kraft pulps (8). Figure 1 summarizes some of the results of applying an LMS stage to a SW kraft pulp with an initial kappa of 97.5. This report further examined the chemistry involved in delignification of high-kappa kraft pulps. A series of LMS-stages were performed on a 73.4-kappa conventional southern SW kraft pulp and a 13.8-kappa conventional southern SW kraft pulp. Both pulps originated from the same tree. The pulps were then extracted using an E, EO, EP, and EPO stage. The nature of the residual lignin was also examined.

![Figure 1. Effect of HBT charge on delignification of a 97.5 kappa SW kraft pulp with constant laccase charge.](image)

EXPERIMENTAL
Materials
All materials were purchased from Aldrich and used as received except for p-dioxane, NH3A, and laccase. p-Dioxane was freshly distilled over NaOH prior to using it. NH3A was synthesized in accordance with Oxley's method (9). Laccase, from Trametes versicolor was donated by Novo Nordisk Biochem.

Enzyme Assay
Laccase activity was measured by monitoring the rate of oxidation of syringaldazine.
One unit of activity (U) was defined at the change in absorbance at 530 nm of 0.001 per minute, per ml of enzyme solution, in a 100 mM phosphate buffer (2.2mM) and 0.216 mM metyrapone in methanol (0.3mM). The procedure was carried out at 23°C.

Laccase-Mediator Delignification Procedure

A 2000 ml capacity Parr reactor, equipped with a pressure gauge, a stirrer, a heating jacket, and a digital thermometer, was charged with 60 g of never-dried pulp (solid basis). The pulp consistency was adjusted to 9% by adding distilled water. The slurry was then heated to a temperature of 45°C and was maintained at this temperature for the duration of the enzymatic treatment. A 2% dose of HBT (on o.d. weight) was then added (when NHAA was used, a 2% dose was added on the same molar equivalence as HBT) to the heated slurry. Subsequent to mixing the slurry (about 5 minutes), the pH was adjusted to 4.5 with glacial acetic acid. Laccase was then added (9.2 mg of laccase per 10 g of o.d. pulp). The reactor was then sealed and pressurized with oxygen to 145 psig. Subsequent to the four hour treatment, the pulp was thoroughly washed with water and subjected to various reinforced alkali stages (E*). All extraction stages were conducted at 10% consistency for one hour and at 80°C. The E* stages are summarized in Table 1. Kappa and brightness measurements were performed on the extracted pulps in accordance with T236 and T212 (10), respectively.

Table 1 Summary of E* conditions

<table>
<thead>
<tr>
<th>Extraction stage</th>
<th>% NaOH (o.d. basis)</th>
<th>% H₂O₂ (o.d. basis)</th>
<th>O₂ (psig)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>2.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EO</td>
<td>2.5</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td>EP</td>
<td>2.5</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>EPO</td>
<td>2.5</td>
<td>0.5</td>
<td>60</td>
</tr>
</tbody>
</table>

Isolation of Residual Lignins

The isolation of residual lignin from kraft pulps was carried out in accordance with standard literature methods (11). A 5L three-necked round bottom flask, equipped with a Friedrich condenser and charged with 30 g of o.d. pulp (air-dried). The consistency of the pulp was adjusted to 4% by adding 0.10N NaOH (7.9 g), 1-propanol-water solution. The slurry was then refluxed for 2 hours under an argon atmosphere. Subsequent to the treatment, the pulp was filtered and the filtrate was filtered through celite. The filtrate was then neutralized and concentrated under reduced pressure to approximately 10% of the original volume. Water (400 ml) was added and the mixture was concentrated again under reduced pressure. The solution's pH was then adjusted to 2.5 with 1.00N HCl. The precipitate (i.e. the lignin) was collected, washed several times, and freeze-dried.

Characterization of Residual Lignins

The residual lignins isolated from the untreated brownstock (33.8 kappa and 75.4 kappa) and from LMS₉₀(B), LMS₈₀(E), LMS₂₀(E), and LMS₀₀(E) treated pulps were characterized by ¹³C NMR. The NMR data was acquired with a DMX400 MHz Bruker spectrometer. The ¹³C NMR experiments were conducted in accordance with standard literature methods (12).

RESULTS AND DISCUSSION

Based on our previous studies, we were confident that an LMS stage could be used to dekappa high-kappa kraft pulps. As the next step in investigating LMS delignification of high-lignin content kraft pulps, we proceeded to examine the chemistry of LMS delignification of low kappa (33.8) and high kappa (75.4) lignin content kraft pulps. Although a variety of compounds can be employed in an LMS stage, two of the most effective mediators we have are 1-hydroxynaphthalene (HBT) and N-acetyl-N-phenylhydroxylamine (NHAA). The low-kappa and high-kappa kraft pulps were dekappaed under exactly the same LMS conditions using NHAA and HBT. Subsequent to the LMS stage, the pulps were extracted with an E, EO, EP, and EPO stage. The changes in delignification and brightness gains are summarized in Figures 2-5.

![Figure 2: Delignification of the kappa 75.4 SW kraft pulp with LMS followed by E* stage.](image-url)

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The results from LMS delignification of the high- and low-kappa pulps bear a strong resemblance. In each case, the use of HBT as a mediator provided superior delignification under our experimental conditions. The use of an oxidatively reinforced alkaline extraction stage improved the observed delignification effects. Interestingly, the use of hydrogen peroxide in an EP-stage was found to substantially improve the delignification effect. This differs from the response frequently observed with D₂O pulps in which the EP-stage principally brightens the pulps. It is also interesting to note that the use of an EPO stage narrows the difference of the kappa number for the HBT and NHA A LMS-treated pulps. Nonetheless, the high-kappa pulp always exhibited an improved bleachability after LMS(EPO) in comparison to the low-kappa pulp.

Treatment of the high and low kappa pulps with either HBT or NHA A in an LMS stage retains in a darkening of the kappa pulps. Pulp brightness values did not respond favorably to either an E or EO stage but the use of peroxide in an EP or EPO stage yielded a favorable response in brightness. Clearly, the use of peroxide in an EP or EPO stage results in lignin and chromophore removal.

Residual Lignin Analysis
To further explore the fundamental principles involved in controlling the observed delignification effects for the LMS(EPO) stages reported in Figures 2–5, we elected to isolate lignin from the starting low- and high-kappa brownstocks. In addition, the residual lignin from the LMS(E) and LMS(EPO) stages was isolated. In all cases, the lignin samples were isolated from the pulp using an acidic p-dioxane/water extraction procedure (11). Typically, this procedure yielded 45–48% of the lignin from the kraft pulps. The resulting samples were then derivatized and analyzed by NMR. Among the many functional groups that can be analyzed following this protocol, we were able to rapidly determine the amounts of carboxyl and phenolic groups present in the residual lignin. Figure 6 summarizes the changes in carboxyl acid groups from the residual lignin samples isolated from the high-kappa pulps. This analysis indicates that the residual lignin after LMS(E) and LMS(EPO) stages is enriched in acid groups. Differences in carboxyl content for the LMS(E) (EPO) and LMS(NHA A(EPO) suggest that the different mediators may be altering the types of chemistry involved in lignin removal.
Figure 6. Carboxyl content of the kappa 75.4 SW kraft pulp for brownstock and LMS(E) and LMS(EPO) treated pulps.

Analysis of the residual lignin from the low kappa brownstock, LMS(EPO) and LMS_{MAXX}(EPO) pulps (see Fig. 7) bears a comparable trend to the high-kappa pulp data. However, the relative differences are amplified for the low-kappa pulps. This result implies that the differences in lignin from the high- and low-kappa pulps affect the delignification chemistry during the LMS(E) stage.

Figure 7. Carboxyl content of the kappa 33.8 SW kraft pulp for brownstock and LMS(E) and LMS(EPO) treated pulps.

The NMR analysis also permitted us to monitor changes in phenoxyl content for the various kraft pulps. The results of this analysis are presented in Figures 8 and 9.

Figure 8. Phenoxyl content of the kappa 75.4 SW kraft pulp for brownstock and LMS(E) and LMS(EPO) treated pulps.

The phenoxy content analysis of the residual lignin suggests that the LMS(E) stage consumed from phenoxyl groups in lignin. The LMS_{MAXX}(EPO) treatment of the high kappa SW kraft pulp removed additional phenoxyl groups, but this depletion was not observed with the LMS_{MAXX}(EPO) sequence.

Interestingly, the loss of phenoxyl groups for the various LMS(E) stages for the low-kappa SW kraft pulp was more pronounced than the high-kappa pulp. This is an unexpected result since the low kappa pulp was not as bleachable as the high-kappa kraft pulp under the experimental conditions employed in this study.

Figure 9. Phenoxyl content of the kappa 33.8 SW kraft pulp for brownstock and LMS(E) and LMS(EPO) treated pulps.

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CONCLUSIONS
Our results suggest that the LMS(E*) delignification of Kraft pulps can be effectively implemented for high- and low-kappa Kraft pulps. The use of additional oxidant in the extraction stage after an LMS-stage is clearly beneficial for both systems. Therefore, this technology could be potentially developed into a viable alternative to oxygen delignification of high-kappa Kraft pulps.

The relative changes in carbonyl and phenolic content for the high- and low-kappa Kraft pulps suggest that the nature of residual lignin influences the effectiveness of an LMS(E*) treatment. Furthermore, differences were noted in the structure of residual lignin from LMSlow(E*) and LMShigh(E*). This implies that the different N-hydroxy tannin mediators may be exhibiting different delignification chemistries. Further studies will be needed to elucidate the complex chemistry of LMS with high-kappa Kraft pulps.

ACKNOWLEDGMENTS
The authors would like to thank the Institute of Paper Science and Technology and its member companies for their support of these ongoing studies. Also, we would like to thank Novo Nordisk Biochem for supplying the laccase and Polatich Corp for pulping the chips used in this study. Portions of this work were used by F.S.C. in partial fulfillment of the requirements for the Ph.D. degree at the Institute of Paper Science and Technology.

REFERENCES
EVALUATION OF HEXENURONIC ACIDS IN U.S. KRAFT PULPS

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ABSTRACT
The studies by Baehret et al. (1) have established the importance of hexenuronic acids to kraft bleeding operations. Although the contribution of hexenuronic acids to Northern European pulps has been extensively reported, the content of hexenuronic acids in U.S. kraft pulps has not been as well documented. This study examined the contribution of hexenuronic acids to commercial U.S. SW and HW kraft pulping and bleeding operations. Based on a mild acid hydrolysis procedure, the content of hexenuronic acids contributed 33-67% to the kappa number of commercial HW kraft pulps, whereas for SW kraft mill pulps, the hexenuronic acids contributed only 5-12% of the pulp kappa number.

I. INTRODUCTION
The formation of hexenuronic acids during kraft pulping conditions was initially postulated by Clayton (2). These classical studies were directed at investigating the degradation of hardwood 4-O-methyl-D-gluco- pyranosyluronic acids (3) under aqueous alkali conditions at 170°C. Johnson and Samuelson (3) employed 2-0-(4-O-methyl-D-glucopyranosyluronic acid)-D-xylitol as a model compound to provide evidence for the formation of hexenuronic acids upon treatment with alkali at elevated temperatures. Subsequent studies by Simkovski et al. (4) provided spectroscopic data that alkaline degradation of L-arabinobio-4-O-methyl-D-glucurono-D-xylitol involved the elimination of 4-O-methyl-D-glucuronic acid residues, yielding, in part, hexenuronic acid. Despite these early studies, the importance of hexenuronic acids to pulp bleaching studies was not fully appreciated until the research results reported by Jiehet al. (5). Subsequent to these reports, it became readily apparent that hexenuronic acids contributed to pulp bleachability (6, 7), influenced the retention of reprocess elements in kraft pulps (8, 9), contributed to the formation of oxalic acids during ozone bleaching (10), and impacted pulp brightness values (11). Accompanying these investigations, were significant improvements in the methodology for detecting and removing hexenuronic acids from kraft pulps (12).

Despite these advances, the contribution of hexenuronic acids to SW and HW kraft pulping operations in the U.S. has been a largely unreported issue. Past studies by Hanna et al. (13) and Steiner et al. (14) have indicated that laboratory cooks of North American wood sources can yield pulps that have hexenuronic acids contributing 10-40% to the pulp kappa number. The present study surveyed the contribution of hexenuronic acids to the pulp kappa number of several U.S. kraft pulp operations.

II. EXPERIMENTAL

Materials - All pulps were acquired from commercial U.S. HW and SW kraft pulping operations. The pulps were extensively washed with deionized water until the filtrate was pH neutral and colorless. Laboratory cooks were prepared from wood chips that originated from a single sweetgum tree.

Kraft pulping. Laboratory kraft pulps were prepared according to standard laboratory conventional batch and R901 cooking procedures. Conventional kraft pulps were prepared employing an H-factor of 1,800-800. The H-factor for the R901 pulps varied from 1,000-200.

Hexenuronic acid analysis. The presence of hexenuronic acids in kraft pulps was evaluated indirectly using a modified literature method. In brief, the pulps were placed at a pH 3 formic acid/sodium formate buffer solution yielding a final consistency of 3%. The mixtures were refluxed for 2 and 5 hours. The treated pulps were filtered, washed, and analyzed for kappa number in accordance with TAPPI Standard Method T236-cm85. Kappa number measurements were performed in duplicate and typically these values differed by less than 2%. In selected cases, the initial acid hydrolysate effluents were analyzed for 2-furoic acid using standard 1H/13C methods (6).

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III. RESULTS AND DISCUSSION

The contribution of hexosameric acids to the measured kappa number of several commercial pulps has been reported in the literature. Typically, the contribution of hexosameric acids to HW kraft pulps has been found to be approximately 30-35% of the pulp kappa number for northern Scandinavian pulp mills (3). For SW kraft pulps, the northern Scandinavian pulp mills yield fibers that contain 10% hexosameric acid based on kappa number analysis (15). Allison et al. (15) have reported comparable values for commercial SW kraft pulp mills employing Radiata pine as a fiber source. This paper examined the level of hexosameric acids from U.S. kraft pulp mill operations. A total of 14 pulps were examined for hexosameric acid content. In each case, the pulps were refluxed in pH 3 buffered solution for 2 and 5 hours and pulp kappa number was determined. The results of this analysis are summarized in Table 1.

<table>
<thead>
<tr>
<th>Pulp</th>
<th>Kappa #</th>
<th>HW</th>
<th>Pulp</th>
<th>Kappa #</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hawthorne</td>
<td>2 h, A</td>
<td>5 h, A</td>
</tr>
<tr>
<td>Kraft</td>
<td>10.9</td>
<td>6.3</td>
<td>4.9</td>
<td>14.1</td>
</tr>
<tr>
<td></td>
<td>11.6</td>
<td>11.0</td>
<td>8.2</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>13.6</td>
<td>9.5</td>
<td>9.1</td>
<td>14.2</td>
</tr>
<tr>
<td></td>
<td>13.4</td>
<td>9.6</td>
<td>8.3</td>
<td>13.2</td>
</tr>
<tr>
<td>Pulp 02</td>
<td>10.6</td>
<td>7.3</td>
<td>5.9</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>8.5</td>
<td>2.8</td>
<td>-</td>
<td>21.5</td>
</tr>
<tr>
<td>Kraft</td>
<td>30.0</td>
<td>27.4</td>
<td>20.4</td>
<td>23.7</td>
</tr>
</tbody>
</table>

1 Refluxed pulp in pH 3 buffered solution for 2 or 5 hours.

The industrial HW kraft pulps exhibited varying responses to an A-stage treatment with reductions in kappa number varying from 67-33%. Undoubtedly, this is due in part to the different HW sources and different cooking technologies employed at the various mills. Commercial SW kraft pulps were found to have substantially reduced amounts of hexosameric acids contributing to the pulp kappa number. These results are consistent with literature results, although in general many of the HW pulps exhibited exceptionally high levels of hexosameric acids contributing to the pulp kappa number.

To further explore the formation of hexosameric acids as a function of kraft cooking technologies, a series of laboratory cooks was performed from a common HW source employing conventional and simulated RH1 cooking conditions. The pulps from these cooks were then analyzed for their response to an A-stage. Figure 1 summarizes the results of these studies. For both the RH1 and conventional cooks, the contribution of hexosameric acids to the kappa increases as delignification is extended to approximately kappa 13. Extending delignification reduces the level of hexosameric acids presumably due to a slow degradation occurring during the cook. Analysis of the effluents for 2-furaldehyde, by UV/VIS, confirmed the kappa number trends observed after mild acid hydrolysis (see Figure 2).

![Figure 1: Contribution of hexosameric acids to pulp kappa number for laboratory kraft cooks.](image-url)
The data in Figures 1 and 2 suggest that the extent of delignification is one of the most significant factors contributing towards the hexameric acid content for birch-cooked pulps.

Figure 2. Concentration of hexameric acids in µmol/g pulp

IV. CONCLUSIONS

The results presented in this report demonstrate the importance of hexameric acids to U.S. HW pulp bleaching operations. For some HW pulping operations, hexameric acids appear to be the dominant component contributing to the kappa number. In these cases, a substantial component of pulping operations is being directed at removing these unsaturated sugars and not lignin. The well-documented ability of an A-stage to selectively remove hexameric acids in kraft pulps provides a significant opportunity to reduce the operating and capital costs associated with bleaching such pulps.

V. ACKNOWLEDGMENTS

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VI. REFERENCES


Low kappa factors (0.05 KF) of chlorine and chlorine dioxide were employed to chemically pretreat softwood kraft pulp and associated residual lignin before an oxygen delignification stage. Quasi-pretreatments using nitrogen dioxide were performed in parallel and all results were compared to high kappa factor treatments (KF = 0.20) of pulp and lignin to exaggerate and examine the lignin structural changes contributing to the pulp delignification response during an oxygen stage. The principal spectroscopic method chosen to investigate the chemical changes in lignin was $^{31}$P NMR. One of the most significant results arising from these studies was the relatively constant content (< 30% change) of the condensed phenolics despite the efficacy of the chemical pretreatment stage for the lignins. Interestingly, the free phenolics were not appreciably consumed, strongly suggesting that these moieties should be the focus of any future attempts to maximize the performance of an oxygen stage.
The issue of overcoming the limits to oxygen delignification has received increased research attention recently. Since oxygen provides significant environmental and economic benefits, reportedly being able to increase yield, successfully reduce lignin levels and thus provide bleaching cost savings while maintaining compatibility with recovery operations, increased delignification without compromising yield or pulp properties is a very desirable goal [1-10]. One promising avenue for enhanced delignification that has witnessed considerable research attention is the use of pretreatments to improve the bleaching response of pulp in an oxygen stage [11-13]. Pretreatments may be defined as low kappa factor (KF, low molecular chlorine multiples/kappa of pulp) pulp bleaching stages that follow pulping to increase pulp bleachability without adversely affecting pulp properties.

The current research was conducted to elucidate the chemical basis for the limits in oxygen delignification through an analysis of various significant functional groups in lignin. These groups were analyzed after chemical treatments that employed low and high kappa factors of chlorine and chlorine dioxide in addition to an intermediate kappa factor of nitrogen dioxide. Gierer and others have provided the fundamental chemical underpinnings for the structural changes imparted to lignin during oxygen delignification [14-16]. Highly reactive hydroxyl radicals, for example, can react with aromatic and aliphatic lignin structures to generate organic radicals which are purportedly susceptible to attack by superoxide. Superoxide has been suggested to be involved in the scission of aromatic, conjugated, and aliphatic (side chains) lignin structures. Ring opening and side chain elimination reactions can induce carboxylic acid formation and enhance lignin solubility in alkaline conditions. The resistance of lignin removal after 30% delignification during an oxygen stage has been extensively studied and has in part been attributed to carbon-carbon bond structures that are recalcitrant to degradation. For example, dimeric arylpropane units containing saturated side chains such as bicerosol display a reduced reactivity in oxygen systems [17]. In addition, while Lai reports that diphenylmethane lignin units are notoriously stable, he contends that condensed phenolic lignin structures are unstable, while Argyropoulos has maintained that condensed phenolic structures are the major factor limiting oxygen delignification [18-24]. Much of the work that remains to be done in this area requires an increased understanding of the role and fate of the lignin structures that are activated or that remain inert during an oxygen stage.
Herein, we report the structural changes that occur to the residual kraft lignin of two mill pulps, manufactured by EMCC® (extended modified kraft cooking) and CC (conventional cooking) technologies, after both chemical pretreatments (using varying kappa factor charges of chlorine, chlorine dioxide, and nitrogen dioxide) and followed by oxygen delignification. Specifically we have correlated the chemical structural changes of the lignin to the delignification efficiency observed for the two industrial pulps. We have obtained quantitative $^{31}$P NMR spectra and elemental analyses of all lignins in an effort to identify the structural factors responsible for the inactivity and reactivity of lignin during oxygen delignification.

**Methods**

**Pulping and Oxygen Delignification.** Kraft pulps were manufactured by industrial sponsors using typical EMCC® (MK) and Conventional Cooking (CK) technologies. The kappa numbers measured for the MK and CK pulps were 23.2 and 22.4, respectively.

Oxygen delignification runs were conducted on a 300-ml PARR Instruments Pressure Reactor employing the following conditions: 60 minutes, 100°C, 100 ps, 2.33% NaOH charge (relative to mass of pulp), 10% consistency (when applicable), and subject to mild (5 Hz) impeller-blade stirring. The headspace in the PARR reactor was thoroughly flushed with oxygen before application of pressure. Lignin oxygen runs were done by dissolving 75 mg of each lignin into 60 mL of alkaline charged water and stirring. The starting and ending pHs for the runs ranged from 11.3 to approximately 10. All pulps and lignins were removed after the runs and allowed to cool before either a thorough distilled water wash or acid precipitation, respectively.

**Lignin Isolation.** The residual lignins of the MK and CK pulps were isolated by a slightly modified acid hydrolysis procedure that involved a 1 hour reflux of the pulps in an 0.1 N HCl solution containing 91% dioxane water for $P$-dioxane was distilled over sodium borohydride powder for one hour immediately before use. After reflux, the supernatant was collected and the dioxane was removed under reduced pressure. The pH of the remaining water suspension was adjusted to 2.0, the
resultant lignin suspension was frozen to increase lignin coalescence, and allowed to thaw after 24 hours. The precipitate was collected by centrifugation. The preceding process was repeated in triplicate using fresh water rinses of pH ∼ 2.0. The resultant lignin was lyophilized and collected. Product recovery yields were based on the pulp starting kappas (total lignin content) and typically ranged from approximately 40 to 60%.

**High, Intermediate, and Low Kappa Factor Pretreatments.** The pulp pretreatments were conducted at 0.05 kappa factor using freshly prepared chlorine or chlorine dioxide. In order to amplify and more fully explain the chemical effects induced by these pretreatments, higher kappa factors of 0.20 were used, in addition to the use of an intermediate kappa factor of 0.10 for a nitrogen dioxide pretreatment. Nitrogen dioxide pretreatments were accomplished by introducing the appropriate amount of a sodium nitrite solution followed by nitric acid. All pulp pretreatments were conducted in sealed bags and were run at 70°C for 30 minutes while maintaining a final pH of approximately 2.0. All pretreatment conditions used for the pulps were applied to the residual lignins, except that the lignin was dissolved in 9:1 dioxane-water and stirred at ambient temperature in round bottom flasks. All kappa values have an experimental error of approximately 3%. Bleachability in this work will refer to a given level of delignification at constant conditions (caustic, temperature, and time of reaction).

**Recovery of Lignin from Pretreatment and Oxygen Bleaching.** Lignin pretreatment and oxygen bleaching runs were performed after the pulp runs to provide a fundamental basis for the delignification observed in the pulps through analysis of discrete lignin functional groups. Thus, the residual lignins from the MK and CK pulps were systematically isolated and investigated through 31P NMR. Since our objective was to provide an accurate summary of the fate of the lignin structural subunits after pretreatment and a subsequent oxygen stage, it was necessary to recover as much of lignin as possible after pretreatment and oxygen bleaching. All lignins were therefore recovered as quantitatively as possible by exhaustive ethyl acetate extraction as described by Assgari and Argyropoulos [22]. Any remaining aqueous phase that remained after chemical reactions in either dioxane/water or alkaline water was removed under reduced pressure and DMF was added to the remaining precipitate to specifically dissolve low molecular weight lignin fragments [22]. The DMF layer was filtered to remove insoluble salts and the remaining
solution was added drop-wise to diethyl ether to precipitate the lignin. Lignins were lyophilized and vacuum-oven dried before NMR analyses. Recovery yields for the lignins after both pretreatment and oxygen bleaching runs typically ranged from 55-75%.

Quantitative Lignin $^{31}$P NMR Analyses. Spectral characterization of the residual lignins was accomplished on a 400 MHz Bruker DX Spectrometer employing published procedures [25]. All residual lignins were dissolved in a solution of 650 µL of pyridine/CDCl$_3$ (v/v 1.6/1) that contained either cyclohexanol or endo-N-hydroxy-5-norbornene-2,3-dicarboximide as an internal standard and chromium acetylacetonate as an internal relaxation agent. The samples were phosphorylated with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane. Regions for integration have been reported elsewhere [26]. The integration values have a reproducibility of approximately 95%.

Results and Discussion

Pulp Studies

The initial studies focused on determining the effect of pulp pretreatments on the delignification responses of MK and CK pulps. Shown in Figure 1 are the delignification responses of the pulps after oxygen delignification.

![Delignification Graph]

*Figure 1. The levels of oxygen delignification obtained for the MK and CK pulps as a function of pretreatment.*
The oxygen control level of delignification achieved for both pulps is approximately 45%. The low KF chlorine and chlorine dioxide pretreatments increase the delignification by an additional 30% over the threshold levels, and additionally the MK pulp has a better delignification response than the CK pulp by over 2 kappa units. One of the more remarkable findings in the above figure is the high delignification achievable by a relatively modest NO₂ pretreatment. Obviously, modest NO₂ treatments (KF = 0.10) tremendously boost delignification in a subsequent oxygen stage in a manner comparable to high kappa factor pretreatments using chlorine and chlorine dioxide (KF = 0.20). The enhancement of the performance of an oxygen stage by varying concentrations of NO₂ has been well described in the literature [27-29]. These results validate the ability of this particular treatment to predispose lignin to enhanced oxygen delignification. It is noteworthy that this data provide evidence that the MK pulps display slightly better bleachability than the CK pulps (on the order of at least 10%).

Carboxylic Acid Content

The carboxylic acid group is typically associated with a significant increase in the oxidation state of lignin. It is an important structural change that occurs in any bleaching process since it the primary way of imparting an enhanced degree of solubility to lignin and typically follows ring opening, aliphatic cleavage, or other oxidative fragmentation of lignin. Shown in Table 1 is a list of the carboxylic acid group changes for all the lignins analyzed in this study. A chlorine dioxide (D) pretreatment caused approximately 30% increase in the overall acid content of both the CK and MK lignins, not unlike what has been previously observed in D bleaching [30]. However, chlorine (C) pretreatments did not induce the generation of similar acid levels.
Table 1. The carboxylic acid content for pretreated and post-oxygen (expressed as pretreatment/KF/O) treatment residual MK and CK lignins expressed in mmoles/gram of lignin. The recovery yields for these lignins ranged between 55 and 75% of the original lignin mass.

<table>
<thead>
<tr>
<th>CARBOXYLIC ACID CONTENT (mmoles/g lignin)</th>
<th>MK</th>
<th>CK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown Stock</td>
<td>0.30</td>
<td>0.31</td>
</tr>
<tr>
<td>Oxygen Control</td>
<td>0.76</td>
<td>0.90</td>
</tr>
<tr>
<td>Cl₂0.05</td>
<td>0.24</td>
<td>0.26</td>
</tr>
<tr>
<td>Cl₂0.05/O</td>
<td>0.70</td>
<td>0.80</td>
</tr>
<tr>
<td>ClO₂0.05</td>
<td>0.41</td>
<td>0.43</td>
</tr>
<tr>
<td>ClO₂0.05/O</td>
<td>1.04</td>
<td>1.01</td>
</tr>
<tr>
<td>NO₂0.10</td>
<td>0.24</td>
<td>0.22</td>
</tr>
<tr>
<td>NO₂0.10/O</td>
<td>0.76</td>
<td>0.58</td>
</tr>
<tr>
<td>Cl₂/20</td>
<td>0.21</td>
<td>0.25</td>
</tr>
<tr>
<td>Cl₂0.20/O</td>
<td>0.51</td>
<td>0.54</td>
</tr>
<tr>
<td>ClO₂0.20</td>
<td>0.72</td>
<td>0.65</td>
</tr>
<tr>
<td>ClO₂0.20/O</td>
<td>1.39</td>
<td>1.09</td>
</tr>
</tbody>
</table>

We found from elemental analysis, however, that there was a heavy incorporation of chlorine, up to 15% in the lignins, which partially offset the introduction of acid groups. Also, it was found by Lachenal et al. that at low KFs of chlorine, acid level increases are not as appreciable as for a D pretreatment [31]. In fact, the generation of carbon dioxide and carbonate have been found to be significant pathways during chlorinations of pulp [31]. Interestingly, carboxylic acid generation was not found to be as significant in NO₂ pretreatments. Again, this was not surprising since significant incorporation of nitrogen (approximately 5-10%) was found and a previously described mechanism of NO₂ reactions with lignin as shown in Figure 2 provides a partial explanation [29]. NO₂ has been postulated by Walding et al. to induce significant depolymerization through nitrification reactions. In fact, Samuelson has found that the tendency to delignify from such treatments owes not so much to

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increased hydrophilicity by acid incorporation, as to extensive lignin degradation into fragments smaller than found in ordinary bleaching sequences.

![Lignin degradation diagram](image)

**Figure 2.** The hydrolysis of nitrated lignin during an oxygen stage that follows a NO₃ pretreatment as described in Walding's work is shown above. It is expected to be facile since the highly electronegative nitrated lignin substituent can be displaced by the α-hydroxyl group under alkaline conditions.

The discrepancy between the levels of acid between CK and MK (higher for MK, opposed to trends shown in Table 1) after an oxygen stage can be explained by the greater abundance of aliphatic hydroxyls in MK over CK by more than 10%. The α-hydroxyls can participate in a base-induced intramolecular expulsion of an adjoining β-nitrated ring leaving a vicinal diol that can further oxidize to a terminal acid during oxygen delignification.

The control oxygen lignins demonstrated a 2-3-fold increase in acid levels and the CK lignin had approximately 15% higher levels. The D pretreated MK lignin shows an enhanced carboxylic acid content beyond what is observed for the oxygen control that is consistent with the slightly better bleachable of its associated pulp (see Figure 1). Employing a high KF pretreatment before oxygen exaggerates the acid differences between the starting and oxygen treated lignins consistent with the pulp data. An important difference in lignin structure to account for the slightly better bleachability of the MK over CK are the higher levels of condensed phenolics and aliphatic hydroxyls of the MK lignin. The phenolics are primary sites
of reactivity for chlorine dioxide and the increased levels in the MK may explain the increased delignification response of the MK pulp.

**Phenolic Content**

Lignin contains both condensed and non-condensed (free) phenolic lignin structures which have tremendous importance in the overall response of lignin to oxidants. Several of the structures of these important functionalities which comprise the focus of our NMR investigations are shown in Figure 3. The salient difference between condensed and non-condensed structures is the substitution pattern at the 5-position of two aryloxypropanoid units.

![Diagram of phenolic structures](image)

**Figure 3.** The structures of some typical condensed and non-condensed (free) phenolic units in residual kraft lignins are displayed. Notice that the salient differences occur in the substitution pattern at the 5-carbon of the lignin aromatic subunits, where condensed structures have C-C bonds, whereas non-condensed have C-H bonds.

Remarkably, although C pretreatments (both high and low KFs) did not increase carboxylic acid levels, they nonetheless tended to afford higher levels of condensed phenolics. Shown in Figure 4 are the actual levels of the condensed phenolics for the pretreated lignins. Table 2
provides a comparative analysis of the phenolic levels of all of the lignins. The levels of condensed phenolics in the C pretreatment are more significant than observed in the D pretreatment. This suggests that one of the potential side reactions of a C pretreatment is the formation of coupling products by radical reactions [31, 32]. Nonetheless, this pretreatment does not prevent the delignification efficiency associated with a subsequent oxygen stage.

Table 2. The non-condensed and 5-condensed phenolic content for the pretreated and post-oxygen (italics) residual MK and CK lignins expressed in mmol/gram of lignin.

<table>
<thead>
<tr>
<th>Phenolic Content</th>
<th>MK</th>
<th>CK</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-Condensed</td>
<td>Condensed</td>
</tr>
<tr>
<td>Brown Stock</td>
<td>0.93</td>
<td>0.89</td>
</tr>
<tr>
<td>Oxygen Control</td>
<td>0.60</td>
<td>0.64</td>
</tr>
<tr>
<td>ClO2/0.05</td>
<td>0.67</td>
<td>0.60</td>
</tr>
<tr>
<td>ClO2/0.05/Oxygen</td>
<td>0.44</td>
<td>0.63</td>
</tr>
<tr>
<td>ClO2/0.05</td>
<td>0.68</td>
<td>0.69</td>
</tr>
<tr>
<td>ClO2/0.05/Oxygen</td>
<td>0.53</td>
<td>0.58</td>
</tr>
<tr>
<td>NO2/0.10</td>
<td>0.20</td>
<td>0.43</td>
</tr>
<tr>
<td>NO2/0.10/Oxygen</td>
<td>0.30</td>
<td>0.52</td>
</tr>
<tr>
<td>ClO2/0.20</td>
<td>0.29</td>
<td>0.69</td>
</tr>
<tr>
<td>ClO2/0.20/Oxygen</td>
<td>0.27</td>
<td>0.58</td>
</tr>
<tr>
<td>ClO2/0.20</td>
<td>0.36</td>
<td>0.54</td>
</tr>
<tr>
<td>ClO2/0.20/Oxygen</td>
<td>0.34</td>
<td>0.46</td>
</tr>
</tbody>
</table>
Figure 4. The content of condensed phenolics in the pretreated residual MK and CK lignins.

An experimental result that provides insight for the level of bleachability is the level of carboxylic acid for the lignins after a control oxygen stage. The CK lignin has a larger carboxylic acid content than the MK lignin, and is in fact more bleachable as a pulp. The MK lignin, however, has more condensed phenolic structures than the CK that may partially explain its diminished bleachability, a cogent argument that has been the subject of numerous investigations [20, 22, 23, 36-38]. Yet, a C/O stage appears to refute this latter argument since the MK pulp is slightly more bleachable, although it has a higher level of condensed structures. This slight increase in reactivity may well be due to other structural features such as the aliphatic hydroxyl groups (vide infra).

Condensed phenolic structures have received considerable attention as a major source that contribute to the inactivity of lignin during oxygen delignification. The current work has examined their role in the context of a preactivation step (pretreatments) of the lignin before oxygen and found that they remain relatively intransigent throughout the chemical bleaching/oxygen steps. Shown in Figure 5 are the levels of condensed phenolics for the lignins after an oxygen stage.

The condensed levels do not vary more than 30% throughout. Moreover, the delignification efficiency observed in the pulps is not compromised as a result of these structural components.
The data strongly indicate that these structures are robust and therefore stable, and are not intrinsically the primary constituents that limit the reactivity of lignin.

**Figure 5. The content of condensed phenolics in the post-oxygen stage residual MK and CK lignins.**

In fact, even high kappa factor pretreatments do not significantly affect their relative distribution with respect to total phenolics as evidenced in Figure 6. In light of the previous data, Figure 6 supports the observation that condensed phenolics are not depleted as much as free phenolics, yet, free phenolics do not change dramatically enough to account for the delignification observed in the pulps. In fact, the pretreatment phenolic ratios are quite constant, testifying to the relative robustness of both the condensed and non-condensed phenolic contents.

**Figure 6. The ratio of condensed phenolics content to total phenolic content in the post-oxygen stage residual MK and CK lignins.**
Again, the consumption of the non-condensed phenolics is surprisingly inefficient. Most work strongly indicates that these are the primary reactive sites for D and O stages [32, 33]. As shown in Table 2 (vide supra), the relative change in the non-condensed phenolics as part of a pretreatment is not appreciable with regard to the bleachability results for the pulps. This result suggests that the phenolic sites are not being consumed as would be expected to account for the decrease in lignin content and that alternative explanations may account for these drops. Perhaps lignin exhibits phenolic sites of differing reactivity based on their electrochemical potentials and environmental constraints and sites that contribute to increased oxidation/solubilization are activated by a pretreatment.

Aliphatics

As shown in Figure 7, the aliphatic levels for the pretreated lignins diminish slightly, but this is not unusual considering that most of the pretreatments do not attack the aliphatic side chains appreciably. Chlorine, however, is known to attack side chains and efficiently deplete this functionality which is demonstrated in the pretreatment. Interestingly, although MK has a greater proportion of condensed structures, suggesting that its bleachability is hindered, it is nevertheless more bleachable than CK, perhaps as a result of the disparity in aliphatic levels between the two lignins. An exaggerated high kappa factor C pretreatment, moreover, extensively consumes the aliphatics, further supporting the latter argument for the heightened bleachability of MK versus CK pulps.

![Figure 7. The content of aliphatic hydroxyl functionalities in the pretreated and post-oxygen stage residual MK and CK lignins.](image)

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Conclusions

The free phenolics of residual lignin are surprisingly not appreciably consumed in an oxygen bleaching stage following a pretreatment stage of chlorine, chlorine dioxide, or nitrogen dioxide despite the enhanced bleachability of pretreated pulps. The NMR data strongly imply that more than 50% of these units are resistant to oxidation, while the concentration of condensed phenolics remain relatively constant. The major difference between the MK and CK lignins are the higher levels of condensed phenolics in the MK lignin which may partially assist during lignin oxidation for the C and D pretreatments. The MK pulps are slightly easier to bleach and the NO₂ pretreatment was extremely effective in promoting the bleachability of the pulps, which may be a consequence of its ability to fragment lignin efficiently via nitration. Condensed phenolics are nonetheless quite resistant to degradation and appear to remain in the lignin samples despite the pretreatments. Their relative robustness does not, however, appear to be the main rationale for the inactivity of lignin toward oxygen delignification, but serves to suggest that the nature and reactivity of the free phenolics deserve increasing scrutiny.

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References


Influence of hexenuronic acids on US bleaching operations

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ABSTRACT: Acid hydrolysis of commercial US HW kraft pulps demonstrated that between 20-55% of the kappa number of these pulps could be attributed to hexenuronic acids. For SW kraft pulps, the contribution of hexenuronic acids to the pulp kappa number was not as substantial. Examination of the xylan and hexenuronic acid content of the kraft pulps indicated that there was a correlation between these two components. Finally, analysis of HW kraft pulps for hexenuronic acid groups indicated that formation of this unsaturated hemicellulose could be influenced by the extent of delignification.

EXECUTIVE STATEMENT: This study examines the role of hexenuronic acids in US HW and SW kraft pulps. The presence of hexenuronic acids was found to be a dominant component of the pulp kappa number for HW kraft pulps, whereas, for SW kraft pulps, it was found to be a minor component. Acid hydrolysis studies on HW kraft pulps suggest that it may be possible to reduce the apparent pulp kappa number by 20 - 55% with a mild acid treatment prior to bleaching. The level of hexenuronic acids in a HW kraft pulp can be influenced by the extent of delignification.

INTRODUCTION: Although fundamental bleaching research has largely focused on lignin chemistry for the past decade, recent studies have begun to readdress this issue and direct more attention toward carbohydrate chemistry. These studies have shown that hemicelluloses influence a variety of important properties throughout pulping, bleaching, and papermaking. The formation of hexenuronic acids during pulping (see Figure 1) is currently a very active research area that has been shown to have practical mill considerations. Early studies by Clayton examined the behavior of 4-O-methyl-α-D-glucuronoxylans from poplar, white birch, and white elm with alkali at 175°C and found indirect evidence for the formation of hexenuronic acids (I).

1. Alkali-catalyzed formation of hexenuronic acids.
Subsequent studies by Johansson and Samuelson (2) and Simkovic et al. (3) provided additional experimental evidence supporting the formation of hexenuronic acids from 4-O-methyl-β-D-glucuronoxylans. Finally, Telemar et al. (4) isolated and characterized hexenuronic acids from an enzymatically hydrolyzed unbleached kraft pulp.

Studies by Vuorinen et al. (5) and Buchert et al. (6) further explored the chemistry of hexenuronic acids in kraft pulps. They demonstrated that hexenuronic acids contribute approximately 50% to the kappa number of several northern Scandinavian kraft pulps. Furthermore, these unsaturated sugars were shown to readily consume electrophilic bleaching chemicals such as chlorine dioxide and ozone. A series of well-designed acid hydrolysis studies identified reaction conditions under which hexenuronic acids could be removed from kraft pulps (see Figure 2) without significantly impacting their physical properties.

2. Proposed (5) acid hydrolysis pathway for hexenuronic acid.

Following these reports, several investigators began to explore the impact of hexenuronic acids on pulp bleaching operations. Li and Gellerstedt (7) have examined the contribution of hexenuronic acids to a pulp’s kappa number. Roberts (8) and da Silva Filho et al. (9) have reported that the presence of hexenuronic acids is a principal factor controlling the affinity of pulp fibers for nonprocess elements. Senior et al. (10) reported that the use of AQ in a kraft cook could influence the amount of hexenuronic acids present in hardwood kraft pulps. Nilvebrant and Reimann (11) have shown that hexenuronic acids can contribute to oxalic acid formation from a Z-stage. Lachenal and Chirat (12) have demonstrated that the differing rates of reaction for chlorine dioxide with lignin and
hexemuronic acid can be employed to improve HW kraft bleaching operations. Vuorinen et al. have studied the impact of hexemuronic acids in ECF and TCF bleaching operations (13). Despite this significant body of work, few reported studies have examined the presence of hexemuronic acids from commercial North American kraft pulping operations. This paper examines the presence of hexemuronic acids in commercial SW and HW kraft pulps from US operations and evaluates how pulping parameters may influence their formation.

EXPERIMENTAL

Materials
All chemical reagents were commercially purchased and used as received. Commercial hardwood and softwood kraft pulps were acquired from several mills, principally in the US southeast. In addition, a series of softwood and hardwood batch and extended modified kraft cooks were performed in the laboratory. The laboratory cooks were all performed from sweetgum or loblolly pine wood sources. All pulps were extensively washed prior to a hot-acid stage.

Laboratory Kraft Cooks
Conventional HW kraft cooking trials were performed at a 4:1 liquor/wood ratio using 30% white liquor sulfurity (active alkali basis). Sweetgum chips and cooking liquor were heated to 165°C for 90 minutes and kept at that temperature until the desired H-factor was reached.

RDH kraft cooking trials were performed using a fully automated control system employing white liquor with 30% sulfurity (active alkali basis) and a cooking temperature of 165°C. White liquor addition was split into warm and hot black liquor pretreatments. The wood chips were pretreated with warm and hot black liquors before cooking with white liquor. At the end of the cook, hot cooking liquor inside the digester was displaced back to the hot and the warm black liquor accumulators using washer filtrate from a brownstock washing system. Table 1 summarizes some of the important HW kraft cooking parameters and pulp properties.

A series of extended modified continuous laboratory cooks were performed using loblolly pine wood chips. The cooking conditions used for these pulps has been previously described by Froass et al. (14).

Acid Hydrolysis
The content of hexemuronic acids in kraft pulps was measured indirectly by refluxing the pulps in a formic acid/sodium formate buffer solution (pH: 3.0) for 2 and 5 hours. The acid treated pulps were all analyzed for kappa number. Selected pulps were analyzed for viscosity values after a 5 hour acid hydrolysis stage. Selected acid hydrolysis effluents were analyzed by UV/Vis for the presence of 2-furoic acid (5). In addition, selected kraft pulps were analyzed for their xylan content following standard literature methods (15).
Table I: HW kraft pulping conditions and pulp properties.

<table>
<thead>
<tr>
<th>Cook Type</th>
<th>%EA charged on wood as Na₂O</th>
<th>H-factor</th>
<th>Kappa Number</th>
<th>Total Yield (%)</th>
<th>Brightness (ISO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional Kraft</td>
<td>15.7</td>
<td>1,800</td>
<td>11.4</td>
<td>48.8</td>
<td>33.1</td>
</tr>
<tr>
<td></td>
<td>14.9</td>
<td>1,400</td>
<td>11.8</td>
<td>49.5</td>
<td>34.9</td>
</tr>
<tr>
<td></td>
<td>13.6</td>
<td>1,100</td>
<td>14.4</td>
<td>50.3</td>
<td>32.4</td>
</tr>
<tr>
<td></td>
<td>12.8</td>
<td>800</td>
<td>15.2</td>
<td>51.0</td>
<td>32.8</td>
</tr>
<tr>
<td>RDH Kraft</td>
<td>NA</td>
<td>1,006</td>
<td>7.8</td>
<td>46.5</td>
<td>38.8</td>
</tr>
<tr>
<td></td>
<td>14.5</td>
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<td>12.3</td>
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</tr>
<tr>
<td></td>
<td>13.9</td>
<td>300</td>
<td>13.4</td>
<td>50.0</td>
<td>37.3</td>
</tr>
<tr>
<td></td>
<td>13.8</td>
<td>400</td>
<td>13.8</td>
<td>NA</td>
<td>36.8</td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>250</td>
<td>16.6</td>
<td>50.7</td>
<td>34.3</td>
</tr>
<tr>
<td></td>
<td>11.2</td>
<td>200</td>
<td>22.9</td>
<td>52.0</td>
<td>32.3</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

Although the presence of hexuronic acids has been well documented for Northern European kraft pulps, the presence of these unsaturated sugars in North American fiber sources has not been as extensively studied. To examine this issue, we acquired pulp samples from several mills in the US. Each pulp sample was exhaustively washed and analyzed for kappa number and viscosity. The pulps were then reduced at 3% consistency in a pH 3-buffered solution for 2 and 5 hours. Under these conditions, hexuronic acids are released from the hemicellulose chains and are converted to 2-carboxy-2-furaldehyde and 2-furoic acid. As shown in Figure 3, all the commercial hardwood kraft pulps exhibited a significant reduction in kappa number after acid hydrolysis ranging in value from 22% to 53%. Interestingly, the ease of acid-catalyzed removal of hexuronic acids from kraft pulp was found to vary substantially for some pulps. For most of the hardwood kraft pulps examined, a two-hour acidic treatment significantly reduced the kappa number of the pulp. For the pulp from Mill B, a five-hour acid stage was required to maximize the drop in kappa number. The factors contributing to this diversity in response to a hot acid-stage remains yet to be determined.

Acid hydrolysis of commercial softwood kraft pulps was not as effective as reducing the kappa number of the pulps as shown in Figure 4. Repeating the acid stage with a series of laboratory-prepared loblolly pine, continuous extended modified kraft pulps yielded comparable results as summarized in Figure 5. The results from acid hydrolysis of SW mill and lab kraft cooks suggest that only a small proportion of the pulp kappa number can be attributed to acid-sensitive hexuronic acid groups. These results suggest that the minor presence of hexuronic acids is further reduced as delignification of SW kraft pulps is extended from kappa number 30 to a kappa number of 20.
3. Kappa number of commercial HW kraft pulps before and after refluxing in pH 3 aqueous buffer solution for 2 and 5 h.

4. Kappa number of commercial softwood kraft pulps before and after refluxing in an pH 3 aqueous buffer solution for 2 and 5 h.
Kappa number of laboratory-prepared, extended modified softwood kraft pulps before and after refluxing in aqueous formic acid-sodium formate solution for 2 and 5 h.

The acid hydrolysis stage not only lowers the kappa of the kraft pulps but also reduces the pulp viscosity values. The HW kraft pulps typically incurred viscosity losses of 10 to 35% after refluxing in the hot acid stage for 5 h. For the SW kraft pulps, the acid hydrolysis resulted in 20–25% decreases in viscosity values. The acid hydrolysis conditions employed were directed at maximizing the removal of acid-sensitive functional groups from the pulps. Based on literature considerations, the observed losses in viscosity could be minimized with additional optimization studies (16).

Along with the kappa number analysis of each kraft pulp, the xylan content of each starting pulp was determined. Figure 6 presents the results of relating the xylan content of the kraft brownstocks to the drop in kappa number after a 5-hour acid hydrolysis treatment. These results suggest that there is a correlation between the xylan content of the starting pulp and the contribution of hexuronic acids to the kappa number of the pulp. This result is consistent with the proposed mechanism of hexuronic acid formation and its contribution to pulp kappa number.
6. Relationship between xylan content of softwood and hardwood kraft pulps and the decrease of kappa number upon 5-hour acid hydrolysis stage.

To examine how kraft-cooking technologies can influence the formation of hexenuronic acids for hardwood kraft pulps, we preformed a series of conventional and extended modified batch cooks. These cooks were all accomplished from a common sweetgum wood source to minimize experimental variability due to the fiber source. Table 1 summarizes some of the important cooking and pulp parameters for these hardwood kraft pulps.

The conventional and RDH HW kraft pulps were refluxed in a pH 3 buffered solution for 5 hours, and the kappa number of the pulps was analyzed before and after the hot acid stage. The results of the kappa number analysis are summarized in Figure 7. This data suggests that differences in batch cooking do not significantly alter the formation of hexenuronic acids in kraft pulps, although there does appear to be a small reduction in the formation of hexenuronic acid groups for the RDH pulps. Of greater significance is the observed increase in kappa number that can be attributed to hexenuronic acid groups as the final pulp kappa number is reduced. The acid hydrolysis data presented in Figure 7 suggests that the HW kraft pulps with a kappa number of approximately 14 have the largest amount of hexenuronic acids contributing to the observed kappa number. As the delignification is extended, the pulps respond less favorably to an acid hydrolysis stage. This is presumably due to a loss of acid-sensitive hexenuronic acid groups to the kraft cooking conditions.
% Delignification After Acid Hydrolysis

Kraft Pulp (Brownstock Kappa #)

7. Change in kappa number for HW kraft pulps upon acid hydrolysis (5 hour at 100°C, 3% icec, and buffer pH 3 solution) versus starting kappa number of conventional and RDH kraft pulps.

Along with the kappa number analysis of the acid hydrolyzed pulps, the hydrolysis effluents were analyzed by UV/Vis for the presence of 2-furoic acid. As summarized in Figure 2, 2-furoic acid can be used to measure the amounts of hexemuronic acids released from the pulp during a hot acid stage. The results of this analysis are summarized in Figure 8, and this data agrees with the drop in kappa number observed for the acid hydrolyzed pulps. It furthermore supports the hypothesis that for the sweetgum pulps studied, the content of hexemuronic acid groups in pulp increases as delignification is extended from kappa number 30 to approximately 14. Further depletion of hexemuronic acids was observed as delignification was extended. This is consistent with studies by (66) that reported that the hexemuronic acids undergo a slow degradation during the latter part of a kraft cook.
8. Hexenuronic acid content of unbleached RDH and conventional kraft pulps from sweetgum as determined by measuring the concentration of 2-furoic acid in the effluents from acid hydrolysis stages of the HW kraft pulps.

CONCLUSIONS

The response of commercial US HW kraft pulps to a hot acid stage suggests that hexenuronic acids are an important component to the kappa number, contributing approximately 20 – 55% of the measured pulp kappa number. These values are comparable to those reported for commercial HW pulp mills in northern Scandinavia. The hexenuronic acid content of southern US SW kraft pulps mills is significantly less than what has been reported in other parts of the world. In part, this is undoubtedly due to differences in pulping technology and cooking conditions. Certainly, future studies into pulp bleachability of North American hardwood kraft pulps must take into account hexenuronic acids and not focus solely on lignin.
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REFERENCES


EXTENDING THE LIMITS OF OXYGEN DELIGNIFICATION

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ABSTRACT

Oxygen bleachable of a low (kappa 26.6) and high (kappa 56.2) lignin content SW Kraft pulp was evaluated under conventional oxygen, extended O2, and (E=O=O=O-)-O) conditions. The bleachable of the high kappa pulp was found to be superior to that of the low kappa pulp. At a constant charge of caustic, the relative percent delignification was found to be O=O-<E=O2< E=O2< E<O) for the low and high kappa pulp. The mild conditions of the latter sequence yielded pulps with higher viscosity values. Hexenuronic acids contributed 22% to the kappa number of the low lignin content brownstock and negligibly to the high lignin content brownstock. Changes in pulp bleachable of the high and low kappa SW Kraft pulps were further investigated by isolating and characterizing the residual lignin of the pulps and the bleaching effluents. 13C NMR analysis of the kraft brownstock residual lignins indicated that the high kappa pulp had a greater amount of 11-0-aryl ethers and methoxyl groups in comparison to the low kappa pulp. The high kappa pulp had less condensed aromatic units and diphenyl methane functional groups in the lignin. These results support the hypothesis that condensed phenolics influence pulp bleachableability for O delignification. Analysis of the effluent lignins provided evidence for the differences in pulp bleachableability under varying reaction conditions. Differences in pulp bleachableability were attributed to differences in the structure of lignin and hexenuronic acid present in the high and low kappa brownstocks.

INTRODUCTION

The benefits of oxygen delignification for the production of bleached Kraft pulps have been recognized for well over four decades. Research studies over this period have examined many applied and fundamental components of oxygen delignification.[1-3,4,5] Initial studies examined the basic chemistry of oxygen delignification and process engineering parameters. As the incorporation of an O stage into pulp bleaching operations became common practice, research studies were generally directed at improving the selectivity of oxygen delignification and/or improving the overall environmental performance of Kraft bleaching operations via oxygen delignification. As environmental issues have now been addressed, research has once again returned to extending the performance of this proven technology.

Among the future opportunities for oxygen delignification is the possibility of using an O stage to improve overall pulp yields. Several researchers have noted that it is possible to improve pulp yields by halting the Kraft cook prior to the residual phase and to remove the additional lignin via oxygen delignification. In general, for SW Kraft pulps, this requires employing an O stage on a brownstock pulp having a kappa number > 40. The overall wood savings following this approach has been reported to be in the range of 2 – 6% for a modern pulp mill.[6] Coupling this approach with modified Kraft cooking conditions such as the use of polysulfide, AO, or surfactant-based chip pretreatment has been reported to increase pulp yields by 4-5%. Magratta et al. have reported improved selectivity with an extended oxygen OO system on high kappa Kraft pulps.[7] Early-pulping Kraft pulps with a kappa number of 80-90, greater than 100, has been demonstrated that an OO system can increase pulp yields > 4% prior to ECF bleaching. Bobkostrom et al. have reported that commercial OO systems can achieve greater than 70% delignification with a kappa 30 SW Kraft pulp.[8] Interestingly, lab and mill studies suggest that an OO stage can attain greater delignification and selectivity in comparison to a one-stage oxygen system.

At the other end of the oxygen delignification technology spectrum lies the use of a "mini-O". This system typically removes lesser amounts of lignin but requires less capital investment. Hirst has reported that the use of a 0.55-kappa-factor D pretreatment stage followed by a higher than normal temperature in the (E=O) stage can achieve approximately 50% delignification of an SW Kraft pulp.[9] McKenzie reported that the commercial use of a mini-oxygen delignification system prior to a conventional O stage provided an additional 25% delignification of an SW Kraft pulp.[10]
Despite the diversity of oxygen delignification technologies, it has been generally assumed that the delignification chemistry involved in this process is primarily due to the oxidative destruction of phenolics. The precocious model compound studies of Gierer, Gratel, Ljunggren, Gellerstedt, and others into the chemistry of oxygen delignification have certainly detailed how phenolic lignin-like structures can be oxidized under an O-stage [15, 12, 13]. Typically, oxidative reactions involve the loss of an electron from the phenolate anion to oxygen and subsequent attack of the phenolate radical by oxygen (see Figure 1).

![Fig. 1. Fundamental O-delignification chemistry](image)

The initial electron transfer process is known to generate superoxide and this leads to the generation of several other oxidative species, including hydrogen peroxide anion and hydroxyl radicals [14]. The presence of several types of oxidative chemical agents in an O-stage dramatically increases the complexity of the delignification chemistry occurring in an O-stage.

Structural analysis of residual lignin after an O-stage has further defined the delignification chemistry involved in this process. Studies by Gellerstedt et al. [15], Moe and Ragauskas [16], and Hegart and Argyropoulos [17] have all noted that the structure of lignin after a conventional O-stage is not dramatically altered. In general, non-condensed phenolics are diminished after an O-stage and the residual lignin is enriched in condensed structures and acid groups. Increases in carbonyl groups for oxygen delignified pulps have been reported by Zawadzki and Ragauskas [18], Lachenal et al. [19], and Gellerstedt et al. [15]. Although these results are consistent with model compound studies, it appears that, overall, residual lignin is less reactive to an oxygen stage than model compounds would suggest. These differences between model compounds and residual lignin studies have been attributed to possible mass transfer effects.

This paper examines the reactivity of a high and low kappa SW kraft pulp to conventional, extended, and mini-O oxygen delignification technologies. Pulp bleachability was assessed by characterizing the pulps in regard to physical pulp properties and fundamental lignin structures. The results of varying these operational parameters serve to highlight the structures in lignin that are involved in oxygen delignification chemistry and to provide a basis from which improved extended oxygen delignification systems can be designed.

**METHODS AND MATERIALS**

An industrial SW kraft pulp with a kappa # of 26.6 and a laboratory prepared SW kraft with a kappa # of 56.2 were employed for all studies in this paper. The mill pulp had a viscosity value of 29.6 cP and the lab pulp had a viscosity value of 42.6 cP. Prior to the oxygen delignification studies, the pulps were extensively washed until the wash water was pH neutral and colorless.

**Metals Analysis of Kraft SW Brownstocks**

Nonprocess elements of the two kraft brownstocks were determined using standard ICP methods [20]. Table 1 summarizes the results of ICP analysis.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Low Kappa SW Kraft Brownstock</th>
<th>High Kappa SW Kraft Brownstock</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni</td>
<td>720</td>
<td>884</td>
</tr>
<tr>
<td>K</td>
<td>359</td>
<td>88</td>
</tr>
<tr>
<td>Mg</td>
<td>315</td>
<td>727</td>
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<td>1</td>
</tr>
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**Hexenuronic Acid Analysis of Kraft SW Brownstocks**

The high and low kappa SW kraft pulps were analyzed for hexenuronic acid content by refluxing in pH 3 solution, following literature methods [21]. Hexenuronic acids contributed approximately 22.5 and 1.0% to the kappa number of the low and high SW kraft pulps, respectively.

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Oxygen Delignification

The Kraft pulps were oxygen delignified in a stirred Parr Reactor. Table II summarizes the delignification conditions.

<table>
<thead>
<tr>
<th>TABLE II. BLEACHING CONDITIONS*</th>
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<tr>
<td>Stage</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>OO</td>
</tr>
<tr>
<td>(ii) 60</td>
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<td></td>
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<tr>
<td>(E+O)D</td>
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MgSO₄ charge: 0.3%, Consistency: 12%, I.L. represents the NaOH charges for the 26.6 kappa SW pulp and H designates the NaOH charges employed for the 56.2 kappa SW pulp. Caustic charge was split between two (E+O) stages, the (E+O) pulp was washed and then treated to a D stage employing a 0.05 N.HCl charge of CrO₃, at 10% ccg and 70°C, for 30 min, with a terminal pH 2.2. The (E+O)D pulp was washed prior to the second (E+O) stage.

Delignified pulps were analyzed for kappa number following TAPPI method om-246 [22]. Typical experimental standard deviations for this procedure were determined to be ±0.0% for the low kappa pulps (+30) and ±0.6% for the high kappa pulps (+30). Pulps viscosity values were determined in accordance with TAPPI Standard T-230 om-89 [19] and standard deviations were ±0.4 for the low kappa pulps and ±1.5 for the high kappa pulps.

Lignin Isolation

Oxidized lignin from the oxygen delignification effluents was isolated by concentrating the bleach effluents to a fourth of their initial volume and acidiifying to a pH of 1.5 using 1.00 N. H₂SO₄. The precipitated lignin was removed by ultra-centrifuging, washed twice with pH 2.00 sulfuric acid solution, and then freeze dried. The isolated material was then further dried under high vacuum at 45°C prior to NMR analysis.

Isolation of residual lignin from the houwstock. O, OO, and (E+O)D(E+O) pulps were accomplished employing standard literature methods [23]. In brief, air-dried pulp (30 – 50 g oven dry weight) was added to an aqueous 1.00 N HCl (100 ml), p-dioxane (900 ml) solution. The pulp slurry was refluxed for 2 hr under an argon atmosphere and then cooled, filtered, and concentrated. The precipitated lignin was isolated by ultracentrifuging, washed with acidic water (pH 2), and then dried. This procedure afforded, on average, 40-55% yield of residual lignin, based on mass recovery of lignin and starting pulp kappa number.

¹³C NMR Analysis of Residual Lignin

Quantitative ¹³C NMR spectra were recorded with an inverse gated 90° pulse sequence, a 144 delay, a TD of 32k, and a sweep width of 330 ppm [24]. The NMR experiments were performed at 90°C on samples containing 150-300 mg lignin/mL of DMCO-d₆. The Fourier transformed spectra were integrated in accordance with reported chemical shifts for lignin functional groups. The integrals were normalized to the aromatic signals, which were assumed to have 6 carbons.

³¹P NMR Analysis of Residual Lignin

Lignin samples were phosphorylated with 2-chloro-4,4,5,5-tetramethyl-1,3,2- dioxaphospholane following the literature method [17] and analyzed by ³¹P NMR.

RESULTS

Oxygen Bleachability of Kraft Pulps

Previous studies reported by Möe and Ragauskas [16] have shown that the bleachability of high kappa Kraft pulp (i.e., ITC, conventional, and PS/AQ) can be readily correlated to the initial kappa number of the pulp. This study further explores the bleachability of SW Kraft pulps under oxygen delignification conditions employing conventional O, extended oxygen O, and the mini-O sequence (E+O)D(E+O) with a 0.05 kappa factor in the D-stage. The experimental conditions were selected from previous literature reports [7,8,9]. To minimize the number of experimental variables, the consistency of all O stages was maintained at 12% and a constant charge of MgSO₄ was employed. The oxygen pressure was maintained at 80 psi for 60 min. for the OO experiments. This later sequence required an initial O₃ pressure of 130 psi for 20 minutes at 80°C and was then lowered to 60 psi for 60 minutes at 105°C. All oxygen delignification studies were stirred at a constant
rate throughout the experiment. The low kappa pulp was O-degalinified using 1.5%, 2.5%, and 3.5% charge of caustic. Oxygen delignification of the high kappa pulp was accomplished with caustic charges of 2.5%, 4.1%, and 5.8%. In the OO experiments, the reactor was charged with all the caustic at the beginning of the first stage, whereas the (E+O)(E+O)+O sequence had the charge split between the two (E+O) stages. Hexenuronic acid analysis of the high kappa birchwood suggested a negligible presence of this sugar in the pulp. In contrast, the kappa number analysis of the low lignin content brownstock suggested that hexenuronic acids contributed 23% to the starting kappa number.

The delignification response for the low kappa SW Kraft pulp under varying oxygen conditions is summarized in Figure 3. It is interesting to observe that the different operating conditions for the O and OO stages allowed the latter system to slightly improve the overall extent of delignification. The mild conditions of an (E+O) stage yielded 13% delignification using a 1.5% charge of caustic and 25% delignification with a 3.5% charge of NaOH. The use of a low charge of ClO₂ (45/55) and splitting the caustic charge between the two (E+O) stages significantly improved the response of the pulp towards delignification in the second (E+O) stage. This effect is presumably due to the ability of chlorine dioxide to degrade hexenuronic acids and lignin fragments prior to the second (E+O) stage.

Fig. 3. Changes in kappa number for low lignin content SW Kraft pulp before and after O, OO, (E+O), and (E+O)+(E+O).

An examination of the extent of delignification for the high kappa pulp (Fig. 4) under alkaline oxygen conditions indicates that the OO stage outperformed the O stage at all three caustic application levels (3.5%). The temperature/oxygen profiling in the OO stage improves delignification for the high kappa pulp. The delignification efficiency of the mini-O sequence was comparable effective for the high kappa and low kappa Kraft pulps.

Fig. 4. Changes in kappa number for high lignin content SW Kraft pulp before and after O, OO, and (E+O)(E+O).

Changes in delignification in terms of pulp bleachability (i.e., A kappa/charge of caustic) for the low and high kappa SW Kraft pulps are summarized in Figure 5.

Fig. 5. Pulp bleachability of 26.6 (LK) and 56.2 (HK) kappa SW Kraft pulp for O, (E+O), and OO.

These results clearly demonstrate that the high kappa pulp responds more favorably to the alkaline oxygen conditions than the low kappa pulp. The low bleachability response of the (E+O) stage emphasizes the need for a low charge of ClO₂.

The changes in pulp viscosity for the high and low kappa pulps for O, OO, and (E+O)(E+O) are summarized in Figure 6.
Although the O and OO sequences yielded relatively comparable amounts of delignification for the low kappa pulp, the OO exhibited improved viscosities. Interestingly, the sequence (E\textsuperscript{-}\textsuperscript{O})\textsubscript{2}A\textsubscript{E}E(E\textsuperscript{-}\textsuperscript{O}) using 3.5% NaOH provided the same amount of delignification that the O stage yielded with 1.5% caustic but had an improved viscosities value. For the high kappa pulp the OO stage is clearly superior with respect to delignification and viscosity retention. Comparison of the viscosity changes for the low and high kappa pulps cannot be solely attributed to lignin and pulp carbohydrates given the differences in meta content for the two pulps (see Table 1).

**Lignin Analysis**

In general, the high kappa brownstock exhibited improved pulp bleachability over the low kappa brownstock. The role of residual lignin structure in determining pulp bleachability was assessed by isolating lignin from each brownstock employing an acidic dimethyl extraction procedure. The residual lignin samples were then analyzed by quantitative \(^{13}\text{C}\) NMR spectroscopy. Figure 7 presents the spectral data obtained from the residual lignin isolated from the high kappa kraft pulp.

The results of this analysis provide a facile means of characterizing a variety of functional groups present in the residual lignin samples and these results are summarized in Table III.

**Table III: Residual lignin functional group analysis via \(^{13}\text{C}\) NMR**

<table>
<thead>
<tr>
<th>Brownstock</th>
<th>High Kappa</th>
<th>Low Kappa</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18%</td>
<td>0.03</td>
<td>0.09</td>
</tr>
<tr>
<td>Mass%</td>
<td>0.89</td>
<td>0.81</td>
</tr>
<tr>
<td>C1% in 70% and C1% in 5%</td>
<td>0.14</td>
<td>0.13</td>
</tr>
<tr>
<td>C2 in C=O</td>
<td>0.32</td>
<td>0.27</td>
</tr>
<tr>
<td>Aromatic C=H 1.96</td>
<td>2.05</td>
<td>1.06</td>
</tr>
<tr>
<td>Axid</td>
<td>0.17</td>
<td>0.31</td>
</tr>
</tbody>
</table>

The signal intensity of functional groups was made relative to the signal intensity of the aromatic carbon, which was given a value of 6.

The differences in residual lignin structure for the two pulps are consistent with past investigations into the structure of residual lignin from kraft pulps with varying kappa numbers [25]. The increased content of diphenyl methane units and the changes in Aromatic C=H-Aromatic C=H both suggest an increase in amounts of condensed phenolics for the low kappa kraft brownstock. \(^{13}\text{C}\) NMR analysis of the phosphitylated lignins supported the \(^{13}\text{C}\) NMR analysis as the ratio of C5-condensed phenolics C5-condensed phenolics was 1.00:0.87 for the high kappa kraft lignin and 1.00:0.93 for the low kappa pulp. These results support the hypothesis that the low-kappa pulp has greater amounts of condensed phenolic structures.

With regards to oxygen bleachability, the increased presence of condensed lignin structures in the low kappa pulp appears to be a key contributor to reducing pulp bleachability. This result is consistent with past studies from our group and others [15-18].

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Another approach to evaluating the fundamental oxidative chemistry involved in O, OO, and (E)O(=O)(E)=O sequences studied in this report is to examine the nature of the oxidized lignin fragments in the effluents. This was accomplished by acid precipitating the oxygen effluents and \( ^{31}P \) NMR analyzing the phosphitylated residues. Table IV summarizes the bleach effluents that were selected for analysis.

**TABLE IV. BLEACH EFFLUENTS ISOLATED, PHOSPHITYLATED, AND ANALYZED BY \(^{31}P\) NMR.**

<table>
<thead>
<tr>
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<th>Low Kappa Pulp</th>
<th>High Kappa Pulp</th>
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<tr>
<td></td>
<td>Conventional O, 1.5 and 3.5% NaOH</td>
<td>Conventional O, 2.5 and 5.8% NaOH</td>
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<tr>
<td></td>
<td>(E)O(=O)(=O)O, 3.5% NaOH</td>
<td>(E)O(=O)O(=O)O, 5.8% NaOH</td>
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<tr>
<td></td>
<td>OO, 3.5% NaOH</td>
<td>OO, 5.8% NaOH</td>
</tr>
</tbody>
</table>

For the samples analyzed, the acid group content of the effluents was increased by 200-340% with respect to the acid group content of the residual lignin at the brownstock. This result is a simple reflection of the oxidative chemistry involved in the oxygen stages studied. In addition, the aromatic hydroxyl group content of the effluent lignins increased between 10 and 50%. An increase in anhydrohydroxyl groups (i.e., hydroxyl groups attached to the linking propene-chain of lignin) suggests that the oxygen bleaching chemistry involved in an O, OO, or (E)O(OH)O(=O) stage is primarily directed at the aromatic hydroxyl groups and not oxidative chemistry on the side-chain. Of greater interest are the oxidative chemistries involving phenolic groups. The \(^{31}P\) NMR method employed allows a facile measurement of C5 condensed and noncondensed phenolics. Changes in phenoxyl content of the C5-delignified effluents with respect to the phenoxyl content of the brownstock residual lignins are summarized in Figures 7 and 8.

The \(^{31}P\) NMR effluent data indicate that for the O and OO-treatments of the low- and high kappa pulp, the C5 noncondensed phenolics are more readily depleted than the C5 condensed phenolics with respect to the brownstock residual lignins. This result implies that condensed phenolics are resistant to oxygen delignification conditions.

Fig. 7. Changes in phenoxyl content of recovered effluent lignins after treatment of low kappa SW Kraft pulp with O, OO, and (E)O(OH)O, Brownstock pulp had a C5 noncondensed, C5 condensed phenoxyl content of 0.88 and 0.82 mmol/kg of lignin, respectively.

Increased oxidative removal of condensed phenolics requires increasing amounts of caustic. The loss of phenolics in the (E)O(OH)O stage was less severe and is probably due to the milder conditions of this procedure.

Fig. 8. Changes in phenoxyl content of recovered effluent lignins after treatment of high kappa SW Kraft pulp with O, OO, and (E)O(OH)O. Brownstock pulp had a C5 noncondensed, C5 condensed phenoxyl content of 0.84 and 0.73 mmol/kg of lignin, respectively.

The \(^{31}P\) NMR spectra of the phosphitylated bleach effluents and Kraft brownstock residual lignins also suggested that hydroxyl-phenolic structures were resistant to oxygen delignification conditions. Although only low amounts of these structures are present in the
brownstock lignin (± 0.07 mmol/g) they were enriched in the bleach effluents by approximately 10%. The stability of p- hydroxyphenyl units to oxygen conditions was further explored by adding phenol to a conventional oxygen stage and recovering this chemical after the cook. GC analysis of the recovered phenol indicated recovery yields of >98%. This result further supports the above NMR evidence that p-hydroxyphenyl lignin units are resistant to oxygen delignification conditions.

SUMMARY

The oxygen delignification studies confirmed the proposed benefits for an OO stage delignification system for both low kappa and high kappa pulps. The use of a mini-O system to remove lignin from Kraft pulps having a kappa # < 30 continues to be a promising technology. Analysis of the residual lignin structure of Kraft brownstocks and bleach effluents emphasize the important role condensed phenolics play in controlling oxygen delignification technology. In addition, p-hydroxyphenyl units may also hinder O-delignification. Although the use of vigorous oxygen delignification conditions can extend lignin removal, future advancements in O-delignification will need to develop alternative technologies that selectively remove lignin resistant functional groups such as condensed phenolics.

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REFERENCES


