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Evaluating Laccase-Facilitated Coupling of Phenolic Acids  
to High-Yield Kraft Pulps

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# Evaluating Laccase-Facilitated Coupling of Phenolic Acids to High-Yield Kraft Pulps

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In an effort to alter the physical properties of high-yield kraft, fibers were treated at high consistency (20%) with laccase and syringic, vanillic, or 4-hydroxybenzoic acid. Treatment with laccase and 4-hydroxybenzoic acid resulted in a 20-point increase in kappa number and a 100% increase in bulk acid groups. ESCA analysis of the treated and untreated pulp revealed that the laccase-grafted fibers had a two-fold enrichment in acid groups strongly suggesting a laccase-facilitated coupling of 4-hydroxybenzoic acid to the fiber surface. A model system consisting of lignin-coated cellulosic fibers was developed to determine changes to the lignin structure during laccase grafting. <sup>31</sup>P NMR analysis of lignin from the model system revealed an increase in acid groups with concomitant decrease in phenolic hydroxyl groups.

## **Introduction**

Currently, the pulp and paper industry relies on two principal methods to achieve the properties desired in final products and to create new products. The first approach involves altering fiber morphology by varying fiber type and applying mechanical treatments to fibers. The second approach involves changing fiber chemistry by varying pulping parameters and applying chemical pre-treatments and papermaking additives. Since these methods can sometimes be impractical and require large capital investments, the pulp and paper industry continues to explore new techniques to improve cost effectiveness and to create new and improved products. Enzyme treatments to custom modify pulp fibers to alter both fiber chemistry and fiber morphology represent an attractive alternative technology to traditional approaches to achieve desired paper products.

Laccase (benzenediol:oxygen oxidoreductase) is an oxidative enzyme that participates in the oxidation of phenolic compounds, aminophenols, polyphenols, polyamines, certain inorganic ions, and aryl diamine<sup>1</sup> compounds with the concomitant reduction of oxygen to water. Laccase has been investigated thoroughly for their potential to biobleach kraft pulps.<sup>2,3,4</sup> Laccase is also capable of polymerizing lignin preparations.<sup>5,6</sup> The proposed mechanism of lignin polymerization by laccase is the formation of phenoxy radicals by abstraction of a hydrogen atom followed by a series of radical polymerization reactions. Laccase's non-specific substrate requirements and its polymerization capabilities make it a prime candidate for the polymerization of reactive compounds with lignin. One of the major obstacles for

laccase catalyzed grafting of low molecular weight compounds onto lignin has been the inability to dissolve lignin in water.

Lund et al.<sup>7</sup> reacted vanillylamine with softwood kraft lignin and laccase in dioxane:water solutions and concluded from size-exclusion chromatography studies that laccase polymerized the vanillylamine to the kraft lignin. In a later study, Lund and Ragauskas<sup>8</sup> used <sup>31</sup>P NMR to analyze the reaction of lignin model compounds with laccase and dissolved kraft lignin in dioxane:water mixtures. Remarkably, these authors found that the reaction of lignin with water-soluble guaiacol sulfonate resulted in the lignin macromolecule becoming water-soluble. This work presented strong evidence in favor of the ability of laccase to polymerize compounds with lignin in solution. In these studies, lignin was coupled with model compounds in a homogenous system where both components are in solution. Unfortunately, the need for organic solvents in a homogenous system is unattractive for potential commercial applications.

Yamaguchi et al.<sup>9</sup> utilized laccase to polymerize various phenolic compounds to form dehydrogenative polymers (DHP). The DHPs were subsequently coupled to thermomechanical pulp (TMP) with peroxidase for the formation of paperboard. After hot-pressing for board formation, the ply-bond strength of the paperboard produced from DHP-coupled TMP increased twofold. Yamaguchi et al.<sup>10</sup> also found an increase in tensile strength of paper after laccase pre-treatment of thermomechanical pulps (TMP) with subsequent combination with a vanillic acid DHP. The reaction was also performed with

milled wood lignin (MWL); the molecular weight of the MWL increased after combination with the DHP. These authors rationalized their results as a chemical bonding between the lignin and the DHP that is facilitated by laccase during the laccase pre-treatment of the TMP. Unfortunately, the nature of the bonding between the DHP and the MWL and the chemical changes on the surfaces of the treated fibers were not investigated.

High lignin content kraft pulp fibers are frequently employed for the production of linerboard. Laine<sup>11</sup> has shown that lignin on high-yield kraft fibers is localized primarily at the surface of the fibers. Laine attributed the surface lignin content to a re-deposition of lignin onto fiber surfaces during the kraft pulping process. In the work presented here, it was hypothesized that the high lignin content on the surface of high-yield kraft pulps may present potential sites for grafting when fibers are reacted with laccases. Laccase was reacted with phenolic compounds in the presence of high-yield kraft pulp to modify the surface properties of the pulp fibers. The treated fibers were then analyzed by conductometric titration, kappa number determination, and electron spectroscopy for chemical analysis (ESCA). In addition, the changes to lignin chemical structure occurring during the reaction were explored by <sup>31</sup>P NMR analysis of a lignin-fiber preparation.

## Materials and Methods

**Pulps.** A linerboard softwood kraft pulp was obtained from a commercial facility located in the southeastern U.S.A. The commercial pulp was exhaustively washed until the filtrate was pH neutral and colorless. Pulp was air-dried and Soxhlet-extracted for 24 hrs with acetone with subsequent washing with water prior to all treatments and lignin isolation procedures.

**Laccase.** Laccase was donated by Novo Nordisk Biotech, Raleigh, N.C. The activity of laccase was measured by monitoring the rate of oxidation of syringaldazine. The change in  $A_{530\text{nm}}$  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 (0.300 mL) was set to one unit (U) of activity. This test was done at 23°C.

**Pulp Treatments.** Pulp (25.0g) was suspended at 20.0% consistency (mass pulp/mass pulp+mass water) in a Kapak bag and combined with 6.00% (by mass) addition of phenolic acid (vanillic acid, syringic acid, or 4-hydroxybenzoic acid). The mixture was stirred and the pH was adjusted to 4.5. The pulp slurry was then immersed in a water bath set to 45°C, after which 5.00 mL of laccase ( $4.25 \times 10^7$  U/mL) was added and the bag was sealed and the pulp mixture was allowed to react for 2.00 hours. The experiments performed are outlined in Table 1. After treatment, the pulp samples were filtered and washed until the filtrate was colorless and pH neutral. Pulps were then stored at -4°C for subsequent kappa number determination, conductometric acid titration, and electron spectroscopy for chemical analysis. As a control, samples of filter paper macerated in a Waring blender were treated

with laccase and phenolic acids using the same method as the commercial kraft pulp. The lignin content of the kraft pulps was determined by  $\text{KMnO}_4$  titration of the pulp following TAPPI method T-236 and expressed as a “kappa number”. This value is an indirect measurement of lignin content: % lignin content =  $0.15 \times \text{kappa number}$ . Bulk acid group content was measured in milli-equivalents per gram (meq/g) by literature titration methods.<sup>12</sup>

**Time Trial Pulp Treatments.** Ten grams of each sample were placed in an extraction thimble and extracted with acetone. A small sample of approximately 1.50 g was removed at 1.00, 2.00, 4.00, 12.0 and 24.0 hours. These samples were washed and weighed and then measured for acid group content literature titration methods.<sup>12</sup>

**ESCA Analysis.** Electron Spectroscopy for Chemical Analysis (ESCA) measurements were performed at the Helsinki University of Technology, Finland, following literature methods.<sup>13</sup> The instrument used was an AXIS 165 (by KRATOS Analytical). Prior to ESCA analysis, handsheet samples were acetone-extracted in Soxhlets for 12 hours to remove extractives. Each measurement included two spectra: wide and high-resolution calcium and oxygen spectra. Two small pieces were cut from the middle of a  $200\text{-cm}^2$  sheet and attached to the sample holder via a metal clip. The samples were evacuated in the prechamber overnight for removal (degassing) of water and to ensure that all samples were measured under standard conditions. Three to five measurements were made at different points on the sample. The analysis volume is less than 1 square millimeter in diameter and between 2 and 10.0 nm in depth (the latter depends on the kinetic energy of the electron signal studied). The high-



resolution spectra were charge-corrected using the tabulated value of 285.0 eV for the C 1s (1s orbital) components (C-C bond). Component fitting was done with four Gaussian peaks, all having the same peak width at half maximum; distance between peaks (relating to chemical shifts of known species) was also held constant during the iterations. ESCA standard deviation is  $\pm 0.080\%$ .

**Lignin Isolation Procedure.** Residual lignin was isolated from acetone-extracted, never-dried, softwood kraft pulp with a starting kappa of 90.9 following established literature methods.<sup>14,15</sup> In brief, a 3000-mL three-necked round-bottom flask was charged with 50.0 g of pulp (o.d. basis). The consistency was adjusted to 4.00% by adding a 0.100 N HCl 9:1 p-dioxane:water solution. Next the slurry was refluxed for two hours under an argon atmosphere. The pulp was then filtered and the filtrate was filtered through celite, neutralized, and concentrated under reduced pressure to approximately 10.0% of the original volume. Water (approx. 200 mL) was added and the mixture was concentrated again under reduced pressure to remove the last vestiges of p-dioxane. The pH of the lignin solution was then adjusted to pH 2.5 with 1.00 N HCl. The precipitated lignin was collected, washed three times with dilute HCl at pH 2.5, and freeze-dried.

**Lignin Impregnated Cellulosic Fibers.** Five grams (5.00g) of filter paper (no. 4 Whatman) was fiberized in a Waring blender for 1.00 min. These fibers were combined with 200 mg of lignin and suspended at 15.0% csc in dioxane in a round-bottom flask. The dioxane was

removed gradually under reduced pressure at 30°C. This yielded cellulose fibers impregnated with lignin.

#### **Reaction of Lignin-Impregnated Cellulosic Fibers with Laccase and Phenolic Acids.**

The lignin-impregnated fibers were reacted in the same manner as high-yield kraft pulp fibers (Table 1). Each phenolic acid (88.0 mg) was added to a Kapak bag containing the pulp slurry at 5.00% csc. The samples were pH adjusted to 4.50 and then brought to 45.0°C in a water bath. Laccase (2.00 mL) was added to the pulp mixture and the reaction was allowed to continue for 2.00 hours. Upon completion, each sample was washed with water until the filtrate was clear. The lignin was reisolated from these fibers by soaking in 1:1 mixture of distilled dioxane and water for 12.0 hours and then filtering to remove the fibers. The filtrate was concentrated under reduced pressure and then combined with water for a final volume of 200 mL. The pH of the mixture was adjusted to 2.5 for acid precipitation of the lignin. These samples were then centrifuged for 25.0 min and decanted. Lignin samples were then Soxhlet-extracted in an extraction thimble with diethyl ether for 2.00 hours and allowed to air-dry.

**<sup>31</sup>P Nuclear Magnetic Resonance (NMR).** NMR data was acquired with a DMX 400 MHz Bruker spectrometer. Lignin was phosphitylated with 2-chloro-4,4,5,5-tetramethyl-1,2,3-dioxaphospholane and analyzed by quantitative <sup>31</sup>P NMR. The quantitative <sup>31</sup>P NMR measurements were carried out following literature methods.<sup>16,17</sup> All spectra were recorded at 25°C. Typical recording conditions employed a 30° pulse with an inverse gated decoupling

pulse sequence, 14 s pulse delay, and 200 transients. All data were collected from single samples and assignments were based on literature values for lignin functional groups.<sup>16,17</sup>

## **Results and Discussion**

The effects of modifying the fiber chemistry of lignocellulosic pulps have been well established over the past three decades. Several literature reports<sup>18,19,20,21,22</sup> and patents<sup>23,24,25</sup> have demonstrated distinct changes in fiber properties by grafting acid groups onto pulp fibers. Barzyk et al.<sup>26</sup> have shown that the incorporation of acid groups onto the surface of fully bleached kraft fibers improves specific bond strength of resulting paper sheets. Gatenholm<sup>27</sup> affected the swelling properties of cellulosic fibers by the introduction of various amounts of carboxylic acid groups by succinylation of fibers. Finally, Sato<sup>28</sup> was able to graft amino acids to cellulose to increase the ability of cellulose to adsorb heavy metals. In summary, recent research studies have clearly shown that fiber modification is an avenue of research that has numerous theoretical and practical implications.

The ability to alter the chemical functionality of lignocellulosic fibers enzymatically has been explored by several researchers. Oksanen<sup>29</sup> applied both hemicellulases and cellulases to kraft pulp fibers and found that cellulases increased density of paper sheets made from these fibers. Mansfield<sup>30</sup> has shown that cellulase can be used to increase the tensile strength of paper sheets made from Douglas-fir (*Pseudotsuga menziesii*) kraft pulps. The use of oxidoreductase enzymes to modify the physical properties of pulp fibers has been less

extensively explored. The research results of Felby et al.<sup>31</sup> are perhaps the most notable in this field as they demonstrated that laccase pre-treatment of linerboard grade pulp fibers could improve the modulus of elasticity of resulting boards by 15-25%. These laccase-induced strength benefits were attributed to free-radical initiated bonds formed by laccase reacting with lignin at the fiber surface.

To further explore the ability of laccase to modify the chemical functionality of high-lignin-content kraft pulps, this paper examines the possibility of grafting phenolic derivatives onto kraft pulps. Utilizing the single electron oxidative capabilities of laccase it was envisaged that treatment of phenoxy compounds with laccase would generate phenoxy radicals that could react with lignin on the surface of kraft pulps. The benzoic acids studied in this report are summarized in Table 1. In each case, a phenolic acid was treated with laccase and a high-lignin-content kraft pulp for 2.00 h at 45°C. Upon the completion of each reaction, the treated fibers were washed extensively and analyzed for kappa number, bulk acid-group content and surface carboxylic acid-group content. As shown in Figure 1, the kappa number increased for the pulps that were treated with laccase and a phenolic acid. Pulp fiber treated with laccase and 4-hydroxybenzoic acid exhibited the largest increase in kappa number (i.e., 21 pt). The kappa number determination is a measure of the oxidative properties of the pulp when treated with  $\text{KMnO}_4$ . An increase in kappa number after a laccase/benzoic acid treatment can be attributed to the coupling of phenolic benzoic groups onto the pulp fibers. Yamaguchi et al.<sup>10</sup> observed a similar result when measuring sulfuric acid lignin content after

combining laccase activated TMP fibers with DHP of vanillic acid. They also concluded that the increase in sulphuric acid lignin indicated an attachment of DHP to TMP fibers.

The phenolic compounds used in this study all possessed carboxylic acid functionality, and conductometric titration of the treated pulp samples was utilized to quantify the amount of acid groups that were attached to the pulp fibers. The method applied here was shown by Katz et al.<sup>12</sup> to be effective in measuring carboxylic acid groups in pulp fibers. Figure 2 clearly shows that laccase treatment of kraft pulps in the presence of phenolic acid compounds was effective in increasing the bulk acid group content of pulp fibers. Pulp treatment with 4-hydroxybenzoic + laccase almost doubled the acid group content of the pulp fibers. These results correlated with the kappa number measurements of post-laccase-treated fibers.

It was apparent that 4-hydroxybenzoic acid was the most effective compound in increasing both the kappa number and bulk carboxylic acid content; therefore, further investigations were focused on this compound. The ineffectiveness of vanillic acid and syringic acid was tentatively attributed to steric hindrance of the methoxy groups or the reactivity of the phenoxy radical. Ishihara<sup>32</sup> and Leonowicz et al.<sup>33</sup> showed that vanillic and syringic acid undergo fewer polymerization reactions with laccase at a pH below 6.9. At this point it was unclear whether the incorporation of 4-hydroxybenzoic acid was occurring at the fiber surface; therefore, electron spectroscopy for chemical analysis (ESCA) was performed to reveal changes to fiber surface chemistry.

The results of ESCA measurements are shown in Figure 3. It is clear that laccase treatment with 4-hydroxybenzoic acid increased the acid content at the fiber surface. There was a 75-80% increase over the control sample. This was significant since the laccase-facilitated attachment of phenolic compounds was envisaged to occur on the lignin at the surface of the fiber. The acid group content at the fiber surface doubled compared to the control without laccase. These results are similar to those obtained in the conductometric titration. It should also be noted that the increase in acid content after treatment with laccase and 4-hydroxybenzoic acid was present even after 12 hours of Soxhlet extraction of the treated fibers. These results present direct evidence suggesting the formation of chemical bonding between the phenolic acid and the pulp fibers. To test this further, a time study was performed where pulps were Soxhlet-extracted with acetone for 48 hours to determine whether the acid groups on the pulp were retained.

As shown in Figure 4, there was an initial loss of acid groups in the sample followed by stabilization at a level that remained significantly higher than the control sample. These results supported those of the ESCA analysis; however, the results to this point do not reveal whether or not lignin was the main target for coupling of the phenolic acids to the fiber. To determine whether the bulk of the attachment of phenolic acids to pulp fibers occurred on cellulose or lignin, a control experiment was performed utilizing fully bleached kraft pulp. This pulp was reacted with 4-hydroxybenzoic acid at the same conditions employed with high-kappa pulps. The results on Figure 5 show that acid group content increased with those samples treated with laccase and 4-hydroxybenzoic acid; however, the

increase was only approximately 10-20% compared to the 50-80% increase in acid groups observed during treatment of the highly lignified fibers.

To elucidate the mechanism behind the increase in acid groups when reacting laccase and 4-hydroxybenzoic acid with high-yield kraft pulp, filter paper fibers were impregnated with lignin with the intention of using these lignin-impregnated fibers for reaction with laccase and 4-hydroxybenzoic acid. It was hypothesized that the lignin-impregnated fibers could be used to replace the high-yield kraft pulp fibers used in the previous reactions. The lignin from the lignin-impregnated fibers was subsequently removed and analyzed using  $^{31}\text{P}$  NMR. As shown in Figure 5, after a 2-hour extraction with ether, the carboxylic acid groups of the lignin increase after treatment with laccase and 4-hydroxybenzoic acid. These results correlate with the previous results of the conductometric acid titration (Figure 2) and the ESCA measurements on the high-yield kraft pulp (Figure 3).

The results in Figure 7 show a decrease in the condensed and non-condensed phenolic moieties at C5 in the presence of laccase. Chakar and Ragauskas<sup>34</sup> have shown a similar decrease in the non-condensed and condensed phenolics at C5 when reacting laccase with lignin in dioxane:water (1:1) solutions. The decrease in phenolics was attributed to the oxidation of these lignin structures to quinones. It is interesting to note that although the laccase reaction in this work was performed in two phases at atmospheric pressure, the results still correlate with those obtained when reacting laccase and lignin in solution with oxygen pressure.

Without performing the laccase reaction in an oxygen pressure vessel, Lund and Ragauskas<sup>8</sup> observed a decrease in condensed and non-condensed phenolic moieties with the addition of laccase in solution. Despite the addition of phenolic additives to the solution, laccase decreased the non-condensed and condensed phenolics at C5 both in the work here and that of Lund and Ragauskas.<sup>8</sup> This result is surprising since the condensed phenols would be expected to increase with the coupling of the phenolic acids. This strongly suggests that the increase in acidic groups on the lignin is less pronounced than the overall oxidation of phenolic groups in lignin by laccase.

## **Conclusions**

The results show that laccase treatment of high-yield kraft pulps in combination with phenolic acids results in increases in carboxylic acid groups, especially at the fiber surface. The data presented soundly supports laccase-facilitated grafting of phenolic acids to lignin on fiber surfaces in a two-phase solid/liquid system.

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**Table 1: Treatment Regime Applied to High-Kappa Kraft Pulp Fibers and Lignin Impregnated Cellulosic Fibers**

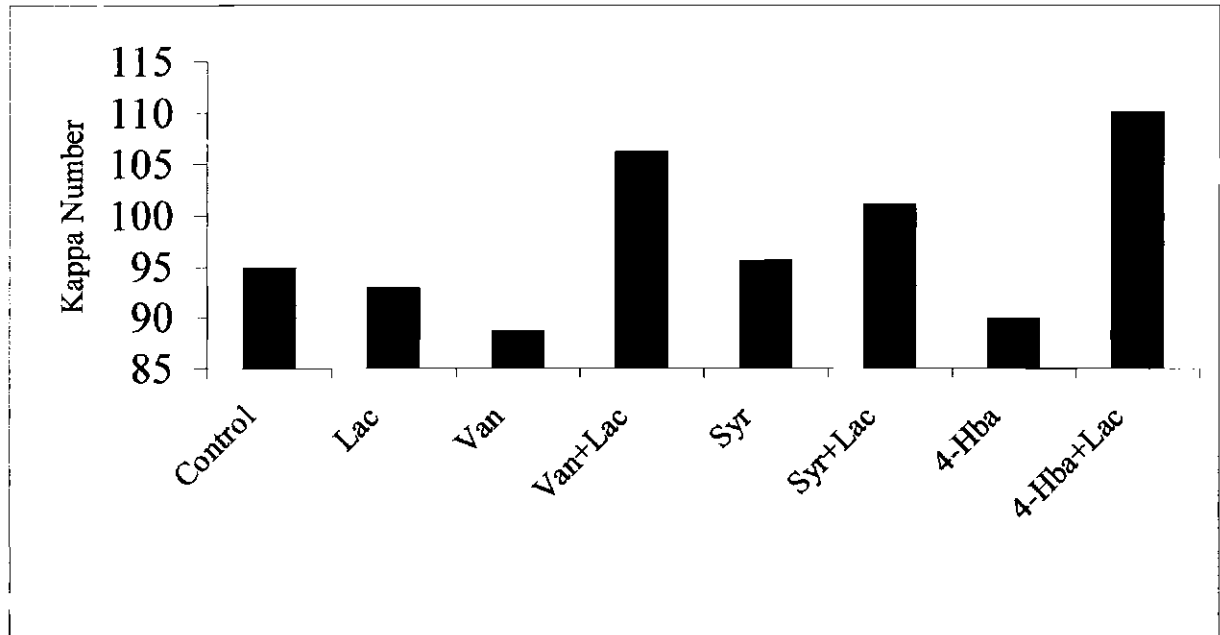
Treatment	Symbol
Control	Control
Laccase+Pulp	Lac
Vanillic Acid + Pulp	Van
Vanillic Acid + Laccase + Pulp	Van + Lac
Syringic Acid	Syr
Syringic Acid + Laccase + Pulp	Syr + Lac
4-Hydroxybenzoic Acid + Pulp	4-HBA
4-Hydroxybenzoic Acid + Laccase + Pulp	4-HBA + Lac

**Table 2:** <sup>31</sup>P NMR Error Analysis<sup>a</sup>

Functional Group	Average (mmole/g lignin)	Least Significant Difference @95% Confidence Interval
Carboxylic Acid	0.51	0.06
Non-condensed at C5	0.61	0.08
Condensed at C5	0.79	0.03

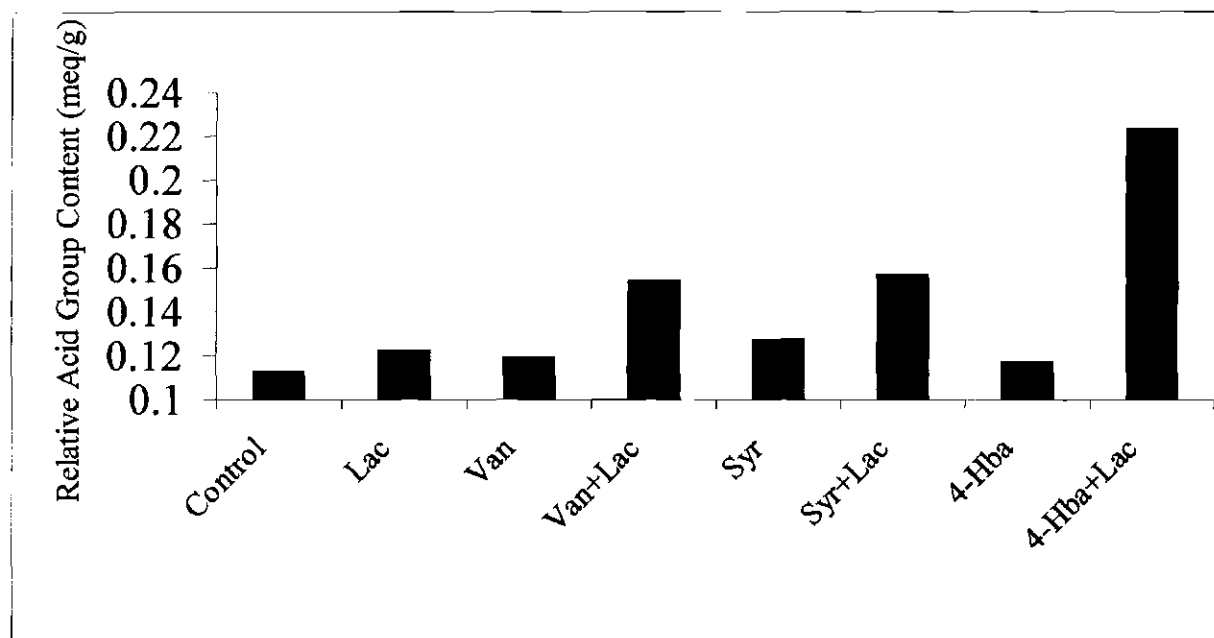
<sup>a</sup> <sup>31</sup>P NMR error analysis obtained by repeating the 4-hydroxybenzoic acid + laccase treatment three times and performing <sup>31</sup>P NMR on each treatment. The least significant difference (LSD) at a 95% confidence interval was obtained from the standard deviation along with the Student's t-value.

**Figure 1:** Results of Kappa Number Measurements of High-Kappa Kraft Pulp Treated with Laccase and Phenolic Acid Compounds

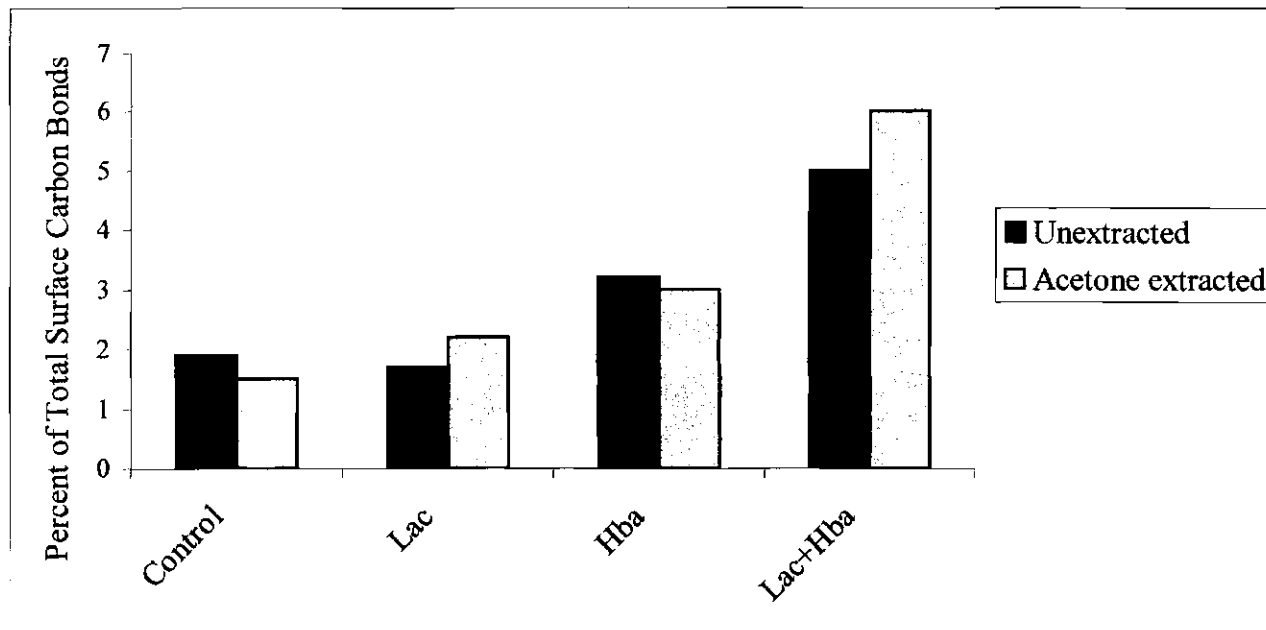




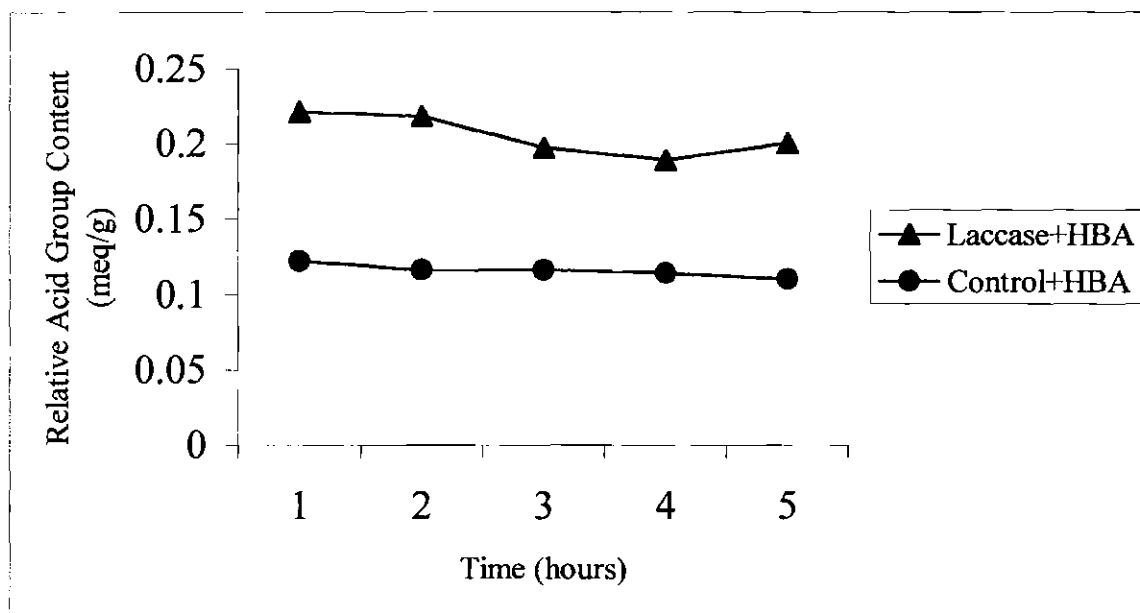
**Figure 2: Results of Conductometric Titration of High-Yield Kraft Pulp Fibers Treated with Laccase and Phenolic Acid Compounds to Determine the Bulk Acid Groups on Pulp Fibers<sup>a</sup>**



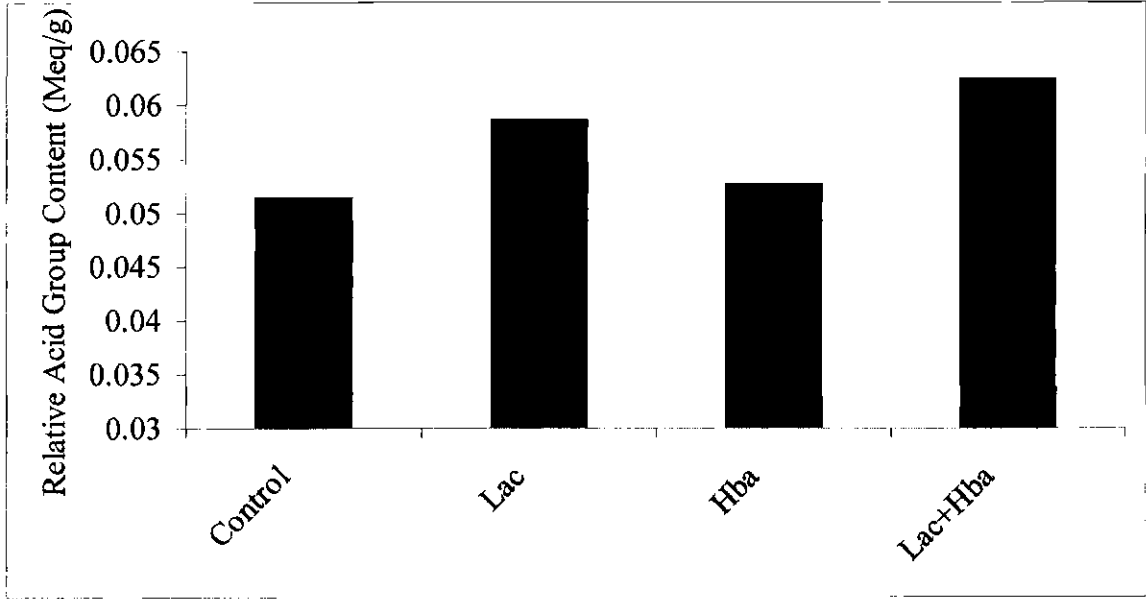
**Figure 3:** ESCA Analysis of Handsheets from High-Yield Kraft Pulp Fibers Treated with Laccase and 4-Hydroxybenzoic Acid to Determine Surface Acid Group Content



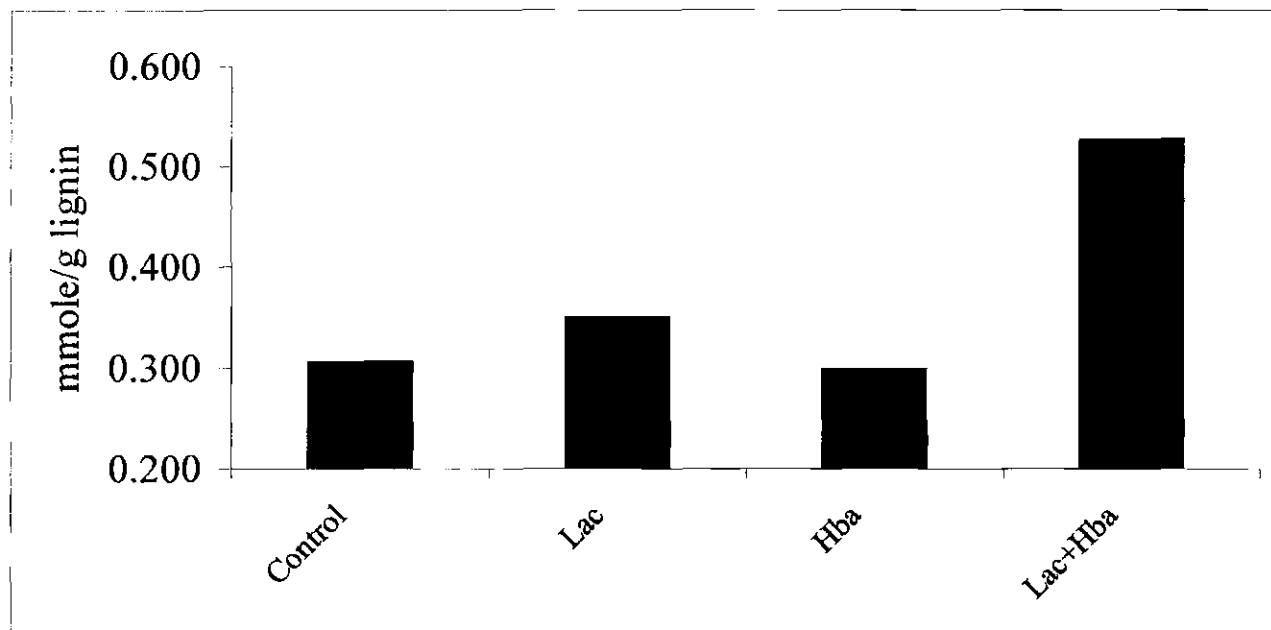
**Figure 4:** Twenty-Four-Hour Acetone Extraction of Pulp Sample Treated with Laccase and 4-Hydroxybenzoic Acid Measured by Conductometric Acid Titration to Determine the Stability of Acid Groups Over Time



**Figure 5:** Treatment of a Bleached Kraft Pulp Containing only 0.30% Lignin with Laccase and 4-Hydroxybenzoic Acid



**Figure 6:**  $^{31}\text{P}$  NMR Analysis to Determine the Amount of Carboxylic Acid Moieties in Lignin Samples Reisolated from the Lignin/Filter Paper Fiber Preparation



**Figure 7:**  $^{31}\text{P}$  NMR Analysis to Determine the Amount of Non-condensed and Condensed Moieties at C5 in Lignin Samples Reisolated from the Lignin/Filter Paper Fiber Preparation

