

Article

Microwave-Assisted Lignin Isolation Using the Enzymatic Mild Acidolysis (EMAL) Protocol

Luca Zoia, Marco Orlandi, and Dimitris S. Argyropoulos

J. Agric. Food Chem., **2008**, 56 (21), 10115-10122 • DOI: 10.1021/jf801955b • Publication Date (Web): 15 October 2008

Downloaded from <http://pubs.acs.org> on November 26, 2008

More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

[View the Full Text HTML](#)



ACS Publications
High quality. High impact.

Microwave-Assisted Lignin Isolation Using the Enzymatic Mild Acidolysis (EMAL) Protocol

LUCA ZOIA,^{†,§} MARCO ORLANDI,[†] AND DIMITRIS S. ARGYROPOULOS^{*,§}

Dipartimento di Scienze dell'Ambiente e del Territorio, Università degli Studi di Milano-Bicocca, Piazza della Scienza 1, 20126 Milano, Italy, and Organic Chemistry of Wood Components Laboratory, College of Natural Resources, North Carolina State University, Raleigh, North Carolina 27695

The use of microwaves is explored in an effort to further improve the recently developed lignin isolation protocol termed EMAL (enzymatic mild acidolysis lignin). Because the presence of the lignin–carbohydrate linkages seems to be rather pronounced within wood, a microwave reactor was used to replace traditional refluxing during the mild acidolysis step. This was done in an attempt to augment the selectivity of this step toward cleaving lignin–carbohydrate bonds as well as reducing the overall intensity of this step toward inducing changes in the lignin structure, thus affording lignin in greater yields and purities. Consequently, in this study the yields, purities, and structures of lignins isolated from spruce (softwood) by the EMAL protocol under various microwave conditions were examined. The variables studied included microwave power, microwave heating time, hydrochloric acid concentration and water content of the reaction medium. Microwave heating afforded EMAL samples of high purity (90%, comparable to the conventional protocol) but in significantly greater gravimetric yields. Quantitative ³¹P NMR and SEC data confirmed that the structure of lignin was similar to that obtained by traditional EMALs, with comparable contents of β-aryl ether bonds, phenolic hydroxyls (condensed and uncondensed), and carboxylic acids.

KEYWORDS: EMAL; ³¹P NMR; SEC; lignin; Norway spruce; microwave

INTRODUCTION

Lignin is a complex natural polymer resulting from the oxidative coupling of primarily (4-hydroxyphenyl)propanoids (1). The current theory is that the lignin polymer is formed by combinatorial-like phenolic coupling reactions, via a radical generated by peroxidase–H₂O₂, where monolignols react endwise with the growing polymer (2). Such “random” dehydrogenative reactions produce a heterogeneous and highly cross-linked macromolecule, built up of different interunit linkages such as β-O-4, β-β, β-5, β-1, 5-5, and 4-O-5 (1). Furthermore, lignin is covalently linked to carbohydrates (3, 4), forming a lignin–carbohydrate network made up of benzyl ether (3, 5), benzyl ester (3, 6, 7), and phenyl glycoside (8–10) bonds. Although lignin has been studied for more than 100 years, its structural details continue to emerge (11). One of the most important problems in the elucidation of the lignin structure has been the isolation of the total lignin from wood in a chemically unaltered form (11–14). Early lignin preparation techniques used strong mineral acids to attain high lignin yields (15). Such drastic conditions, however, were found to cause irreversible reactions that severely altered the structure of the

isolated material. Currently, the most widely used techniques aimed at isolating lignin from wood, inasmuch a chemically unaltered form as possible, are based on the extraction of ball-milled wood by neutral solvents (12, 16). Whereas milled wood lignin (MWL) is extracted from finely milled wood without any previous treatment (16, 17), cellulolytic enzyme lignin (CEL) utilizes cellulolytic enzymes to remove most of the carbohydrate fractions prior to aqueous dioxane extraction of ball-milled wood meal (12, 14).

Recent progress toward isolating lignin from wood has shown that a novel procedure using the combination of enzymatic and mild acidolysis, enzymatic mild acidolysis lignin (EMAL), isolates lignin that is more representative of the total lignin present in milled wood (11, 18, 19). In an effort to further improve the yields and purities of the EMAL protocol, a microwave reactor was used with the aim of replacing the traditional refluxing usually applied during the mild acidolysis step. This was done in anticipation that the lignin–carbohydrate bonds may be more severely cleaved, thus affording lignin in greater yields and purities (19). Microwave irradiation has been successfully applied in organic chemistry (20) with reported more rapid reaction speeds, higher yields under milder reaction conditions, and improved product purities (20–22). Traditionally, organic synthesis at elevated temperatures is carried out by conductive heating with an external heat source (such as an oil bath, sand bath, or heating jacket). This is a comparatively

* Author to whom correspondence should be addressed [telephone (919) 515-7708; fax (919) 515-6302; e-mail dsargyro@ncsu.edu].

[†] Università degli Studi di Milano-Bicocca.

[§] North Carolina State University.

slow and inefficient method for transferring energy into a system because it depends on the thermal conductivity of the various materials that must be penetrated and results in the temperature of the reaction vessel being higher than that of the reaction mixture. In contrast, microwave irradiation produces efficient internal heating (in core volumetric heating) by direct coupling of microwave energy with the molecules (e.g., solvents, reagents, catalysts) that are present in a reaction mixture. Because the reaction vessels employed are typically made of (nearly) microwave transparent materials such as borosilicate glass, quartz, or Teflon, an inverted temperature gradient as compared to conventional thermal heating results. The very efficient internal heat transfer results in minimized wall effects, which may lead to, for example, diminished catalyst deactivation and reagent decomposition. The dielectric heating is generated by two major mechanisms: a dipolar polarization mechanism and a conduction mechanism. The possibility of performing reactions within a very short time period by the direct interaction of microwave energy with the reaction mixture as opposed to indirect energy transfer (using an oil bath or similar device) offers potentially reduced energy consumption and time savings, but, most significantly, increased efficiency and specificity of reactions.

In this study we have explored the influence of microwave heating during the mild acidolysis step in the EMAL procedure. To do this, EMALs with microwave heating systems were isolated from softwood and characterized by wet chemical procedures, quantitative ^{31}P NMR, and size exclusion chromatography. Comparison of the chemical structure of EMAL with traditional heating and EMAL with microwave heating revealed only subtle differences, whereas the yields of lignin were increased by up to 40% compared to the traditional EMAL procedure.

MATERIALS AND METHODS

Isolation of EMALs. EMALs were isolated from ball-milled wood as previously reported (18).

Microwave EMALs Isolation. Microwave EMALs were isolated from spruce (softwood). For the microwave-assisted EMAL procedure a microwave reactor Microdigest A300 V 2.X (Prolabo) was used (microwave power from 0 to 200 W; microwave emission frequency, 2.45 GHz \pm 50 MHz). Four hundred milligrams of material, after enzymatic hydrolysis, was mixed together with 200 mg of glass beads in a borosilicate glass flask designed for the above reactor. Then 25 mL of aqueous solutions (containing 80, 85, or 90% of Dioxane) with different HCl concentrations (0.01 or 0.025 mol/L) was added. Argon was bubbled in the solution for 5 min before each treatment. Then the solution was subjected to different microwave treatments in which the power (1, 5, 10, or 15%) and time of the experiment (60, 120, or 240 min) was varied. The suspension was cooled to room temperature and centrifuged for 2 h (2054 g). The supernatant was carefully withdrawn, neutralized with sodium bicarbonate to a pH of around 7, and finally added dropwise into a 1 L of acidified deionized water (pH 2) under vigorous stirring. The precipitated lignin was allowed to equilibrate with the aqueous phase overnight; the precipitated lignin was recovered by centrifugation (2054 g; 1 h), washed (two times) with deionized water, and freeze-dried. After freeze-drying, the lignin was kept in a vacuum oven at 40 °C, prior to further analyses. The parameters explored were the time of microwave treatment, the power of the microwave source, the water content within the dioxane solution during the mild acidolysis step, and the role of the HCl concentration.

Determination of Lignin Purity. The purities of the samples were calculated by summing the acid-insoluble (Klason lignin) and acid-soluble lignin contents, measured according to the method reported by Yeh et al. (23). The values reported are the average of three analyses \pm 1.0% ($P = 0.05$, $n = 3$).

Acetobromination Derivatization Procedure. Acetobromination was used as the derivatization method of choice for all samples prior to size exclusion measurements (19). Approximately 2.5 mL of a mixture composed of 8 parts of acetyl bromide and 92 parts (v/v) of glacial acetic acid was added to about 10 mg of a lignin sample in a 15 mL round-bottom flask. The flask was sealed and placed in a water bath set at 50 °C for 2 h with continuous magnetic stirring. The solvent was rapidly evaporated at 25–28 °C in a rotary evaporator connected to a high-vacuum pump and a cold trap. The residue was immediately dissolved in tetrahydrofuran (5 mL) and subjected to size exclusion analyses.

Size Exclusion Chromatography (SEC). SEC of samples was performed on size exclusion chromatographic system (Waters system) equipped with a UV detector set at 280 nm. The analyses were carried out at 40 °C using THF as the eluent at a flow rate of 0.75 mL/min. A 150 μL volume of the sample dissolved in THF (1 mg/mL) was injected into HR5E and HR 1 columns (Waters) connected in series. The HR5E column specifications allow for molecular weights of up to 4×10^6 g/mol to be reliably detected. The SEC system was calibrated with polystyrene standards in the molecular weight range of $(890-1.86) \times 10^6$ g/mol, and Millenium 32 GPC software (Waters) was used for data processing. The values reported are the average of three analyses \pm 5000 g/mol ($P = 0.05$, $n = 3$).

Quantitative ^{31}P Nuclear Magnetic Resonance. Quantitative ^{31}P NMR spectra of all lignin preparations were obtained using published procedures (19, 24, 25). To improve resolution, a delay time of 5 s was used, and a total of 256 scans were acquired. The values reported are the average of three analyses \pm 0.05 mmol/g ($P = 0.05$, $n = 3$).

Statistical Analyses of Data. The use of factorial analysis of variance (ANOVA) was made to determine the effects of the various experimental variables (time of treatment, microwave power, HCl concentration, water content in reaction medium) examined in this work on the yield and purity of the lignin obtained (XLSTAT 2008 software).

RESULTS AND DISCUSSION

In our efforts to better understand the lignin isolation process from wood using the microwave-assisted EMAL protocol, we aimed at increasing the yield and purity of the EMAL samples with minimum possible irreversible changes on the chemical structure of the lignin. During the present study we evaluated the yields, purities, molecular weights, and various structural characteristics of the lignin samples isolated via microwave heating during the EMAL mild acidolysis step.

To be able to compare the efficiency of different solvents to generate heat from microwave irradiation, their capabilities to absorb microwave energy and to convert the absorbed energy into heat must to be taken into account. These factors may be considered using the loss angle, δ , which is usually expressed in the form of its tangent, $\tan \delta = \epsilon''/\epsilon'$, where ϵ'' is the dielectric loss, indicative of the efficiency with which electromagnetic radiation is converted into heat, and ϵ' is the dielectric constant, describing the ability of molecules to be polarized by the electric field and store electrical energy (20). The loss factor ϵ'' provides a convenient parameter for comparing the ability of different materials to convert microwave into thermal energy. Solvents such as dioxane, without a permanent dipole moment, are more or less microwave transparent (21). Water is a moderate microwave absorbing solvent. As such, altering the dioxane concentration of the reaction mixture could affect not only the solubility of the lignin but also the energy transfer process. Furthermore, the effect of introducing ions into the system (H^+ and Cl^-) is known to lead to a marked increase in the dielectric heating rate due to the ionic conduction mechanism (20). Consequently, in an effort to arrive at recommended set of conditions, we explored the influence of different parameters, applied during the microwave step, on the data.

Lignin Yield and Purity. The yields of microwave heating EMAL from spruce at different times of treatment, microwave

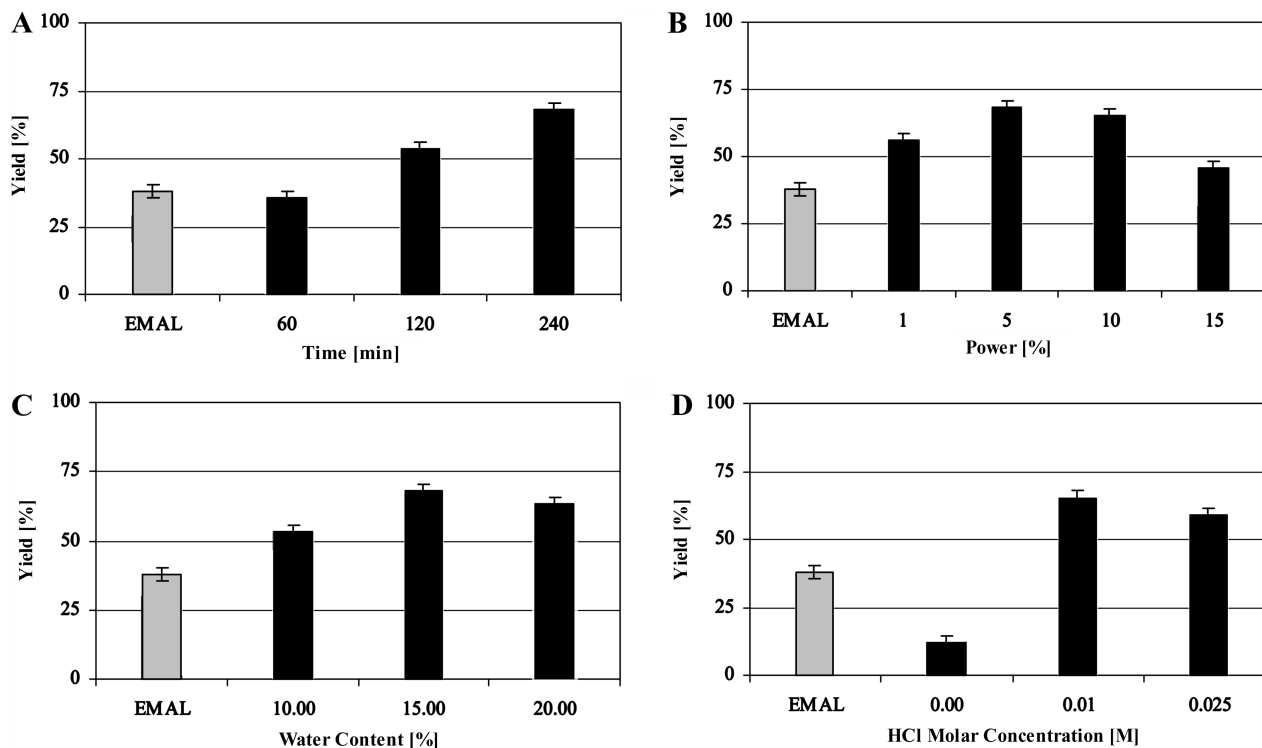


Figure 1. Gravimetric yields of EMALs produced by microwave-assisted heating (black bars): for different lengths of time of microwave treatment (A); for different microwave power settings (B); for different water contents present within the reaction media (C); for different HCl concentrations present within the reaction solution (D). For comparative purposes the yields obtained when using the traditional refluxing step during the EMAL protocol are also shown (gray bars). Fixed levels for the unchanged variables: time of treatment, 240 min; power, 5%; water content, 15%; HCl concentration, 0.01 M.

Table 1. Factorial Analysis of Variance (ANOVA) Examining the Effect of Time of Treatment (60, 120, and 240 min), Microwave Power (1, 5, 10, and 15%), HCl Concentration (0.00, 0.01, and 0.025 M), Water Content (10, 15, and 20%), and Their Interaction on Yield and Purity of Isolated EMAL^a

| variable | <i>P</i> value (yield) | <i>P</i> value (purity) |
|-------------------------------------|------------------------|-------------------------|
| time of treatment | 0.033 | 0.050 |
| microwave power | 0.055 | 0.136 |
| water content | 0.073 | 0.046 |
| HCl concentration | 0.019 | 0.066 |
| time of treatment × microwave power | 0.116 | 0.038 |

^aThe *P* value is the standard one-tailed test probability of the null hypothesis that there is no difference among the levels of a variable.

power source, water content, and HCl concentration within the reaction medium are shown in **Figure 1**.

In accordance with our earlier observations (18), the yields of using traditional reflux heating during the EMAL protocol applied on spruce (38% w/w, based on the amount of Klason plus UV lignin contents of the starting wood and the isolated lignin) were found to be greater than those of the corresponding MWL (12%) and CEL (15%). The combination of enzymatic and mild acidolysis permits isolation of lignin that may be more representative of the total lignin present in milled wood (11, 18, 19).

The statistical analyses performed using the factorial ANOVA that describes the influence of the explored variables on the yield and purity are shown in **Table 1**.

The data of **Figure 1** show that the use of microwaves can liberate in a more efficient way lignin from lignin-carbohydrate complexes that are known to preclude lignin isolation in higher yields (19). The data of **Figure 1A** indicate that increasing the time of microwave treatment increases the yield of EMAL (*P* value from ANOVA 0.033 < 0.05). Because the traditional EMAL procedure uses a refluxing heating step of 120 min, we

explored shorter and longer reaction periods under microwave conditions. At the same time (120 min) the yield of microwave EMAL was found to be 50%, whereas longer microwave treatments (240 min) further increased the yield to about 68%. In terms of lignin yield, the microwave procedure offered yields similar to those of traditional heating but within 50% less time (60 min). **Figure 1B** shows that the power of the microwave source plays a role in the isolation procedure. The *P* value from ANOVA is 0.055 (>0.05), indicating that the effect of microwave power on yield seems not to be important; however, for all power levels investigated (240 min of time treatment), the yields were higher than those obtained for the traditional EMAL protocol with the optimum being at 5% power. Further increases in power (over 5%) caused a decrease in lignin yield. ANOVA indicates that there was no apparent interaction with the variables time and power (*P* value = 0.116).

The water content within the reaction medium was found to be considerably less important in affecting the yield (**Figure 1C**) with a *P* value of 0.073. Finally, **Figure 1D** shows the effect of HCl concentration on the yield of EMAL (*P* value = 0.019). The EMAL protocol, using traditional heating, requires an HCl concentration of 0.01 mol/L. By adding HCl in the reaction medium at the same level the yield was found to increase from 12% (HCl concentration = 0.00 mol/L) to 68%. However, further increases from 0.01 to 0.025 surprisingly decreased the yield from 68 to 55%. This could be rationalized on the basis that, at higher HCl concentrations, acid degradation of lignin might occur, resulting in actual delignification and the generation of low molecular weight lignin fragments that are not recovered during the ensuing lignin isolation procedure (11).

Lignin samples isolated from wood still contain associated carbohydrates and other nonlignin contaminants, regardless of the isolation and purification procedures applied (11, 12, 14). Lignin-carbohydrate linkages exist in wood and are known to

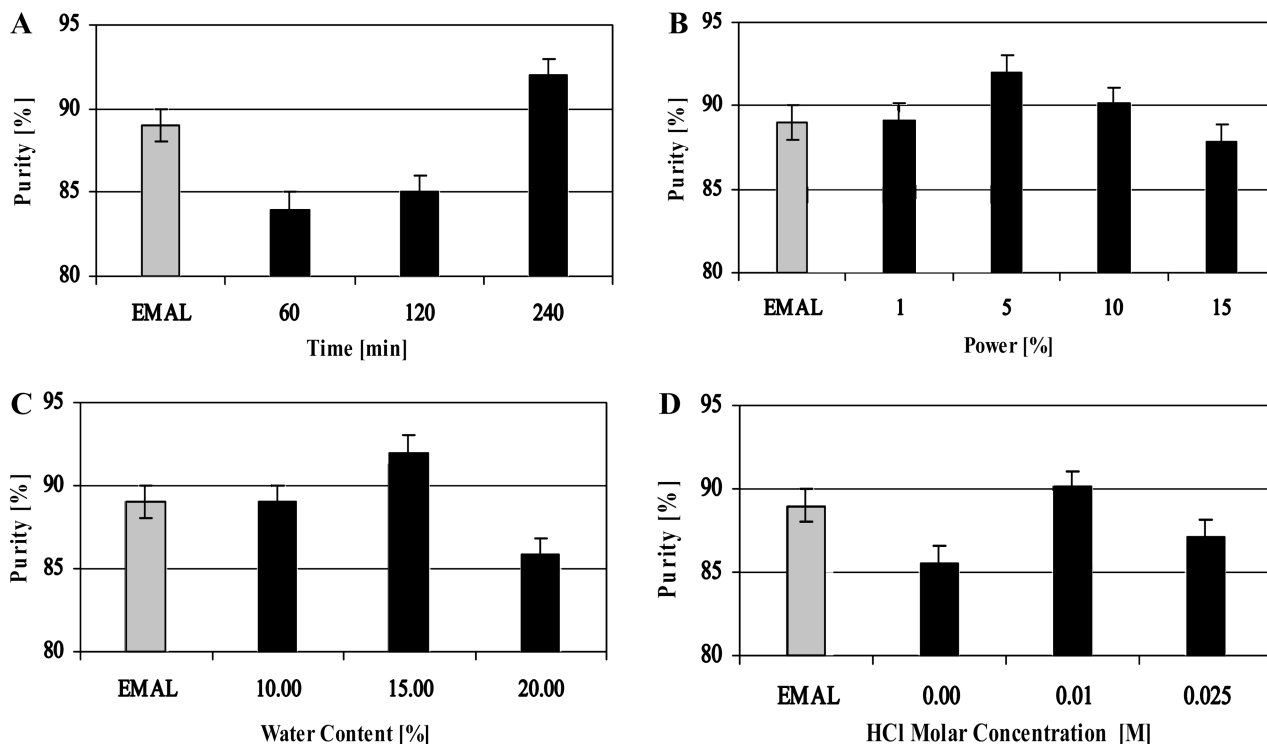


Figure 2. Purities of EMALs produced by microwave-assisted heating based on Klason and UV lignin contents: for different periods of microwave treatment (**A**); for different microwave power settings (**B**); for different water contents within the reaction medium (**C**); for different HCl concentrations within the reaction medium (**D**). For comparative purposes the purity obtained when using the traditional refluxing step during the EMAL protocol is also shown. Fixed levels for the unchanged variables: time of treatment, 240 min; power, 5%; water content, 15%; HCl concentration, 0.01 M.

be of benzyl ester, benzyl ether, and phenyl glucoside types (*I*). Such interactions between lignin and carbohydrates preclude the isolation of lignin in high yields and purities. All of these lignin-carbohydrate bonds are, however, susceptible to acid hydrolysis. Under the conditions used for EMAL isolation, the complete cleavage of the phenyl glucoside bonds has been shown to occur on model compounds, whereas nonphenolic benzyl ether structures were found to be more stable under similar conditions (26, 27). The rates of these reactions may actually be affected to different degrees under microwave heating, conditions contributing to the observed benefits. Model compound work to verify the stated hypotheses is underway in our laboratory.

The additional virtues of microwave heating are apparent when one compares the purities of the EMAL samples obtained under different conditions. The results from ANOVA indicate that the effect of the experimental variables on the purity is less important than the effect on the yield (**Table 1**).

Figure 2 shows the purity data as a function of different treatment times, microwave powers, water contents, and HCl concentrations.

Applying microwaves at 5% power level for 60 and 120 min showed that the purities of samples were lower than those of samples obtained by traditional EMAL. However, after 240 min of microwave heating, the purity of the recovered EMAL was 92%. This value is slightly higher than that obtained for traditional reflux heating (**Figure 2A**). Under variable microwave power conditions the purities of the isolated EMALs were found to be approximately 90% (**Figure 2B**). Microwave power seems not to influence the purity, with a *P* value of 0.136 (**Table 1**). However, the ANOVA indicates a strong interaction between time and power (*P* value = 0.038): the positive effect of prolonged time of treatment on purity is not observed at high microwave powers. Because the dielectric properties of the

medium are known to change when different amounts of water are present (21), it is not surprising to note that water affected the purity of the isolated EMAL significantly (*P* value = 0.046). For different water contents the best purity obtained was when 15% of water was present in the solution: low water contents (10%) and higher water contents (20%) gave lignin purities of 88 and 86%, respectively (**Figure 2C**). These data may be explained on the basis that at lower water contents (10%) the ability to hydrolyze lignin-carbohydrate bonds was decreased, whereas at higher water contents (20%) the solubility of the lignin-carbohydrate complexes was increased: both phenomena led to more impure lignin. The effect of HCl concentration (*P* value = 0.066) on the purities of the isolated EMALs (**Figure 2D**) showed that in the absence of HCl (0.00 mol/L) the hydrolyses of lignin-carbohydrate bonds did not occur (the value of yield in **Figure 1D** and purity in **Figure 2D** are low), whereas the data for 0.025 mol/L HCl showed that an increase in the acid concentration did not simply result in an increase of the EMAL purity (11). When one considers the yield and purity data, one arrives at the conclusion that longer treatment time improves the isolation of lignin, whereas high power values and acid concentration could reduce the yield via a lignin degradation mechanism.

Molecular Weight Distribution and Functional Group Distribution Analyses. The weight-average molecular weights (M_w) accumulated from SEC analyses of the EMALs obtained under different conditions of microwave heating are shown in **Figure 3**.

Acetobromination was used as the derivatization method of choice for all samples prior to size exclusion measurements to ensure complete solubility of the lignin in the THF used as the mobile phase for the size exclusion measurements (19). In our earlier work, we have reported that the EMAL procedure affords lignins that are enriched in high molecular weight components

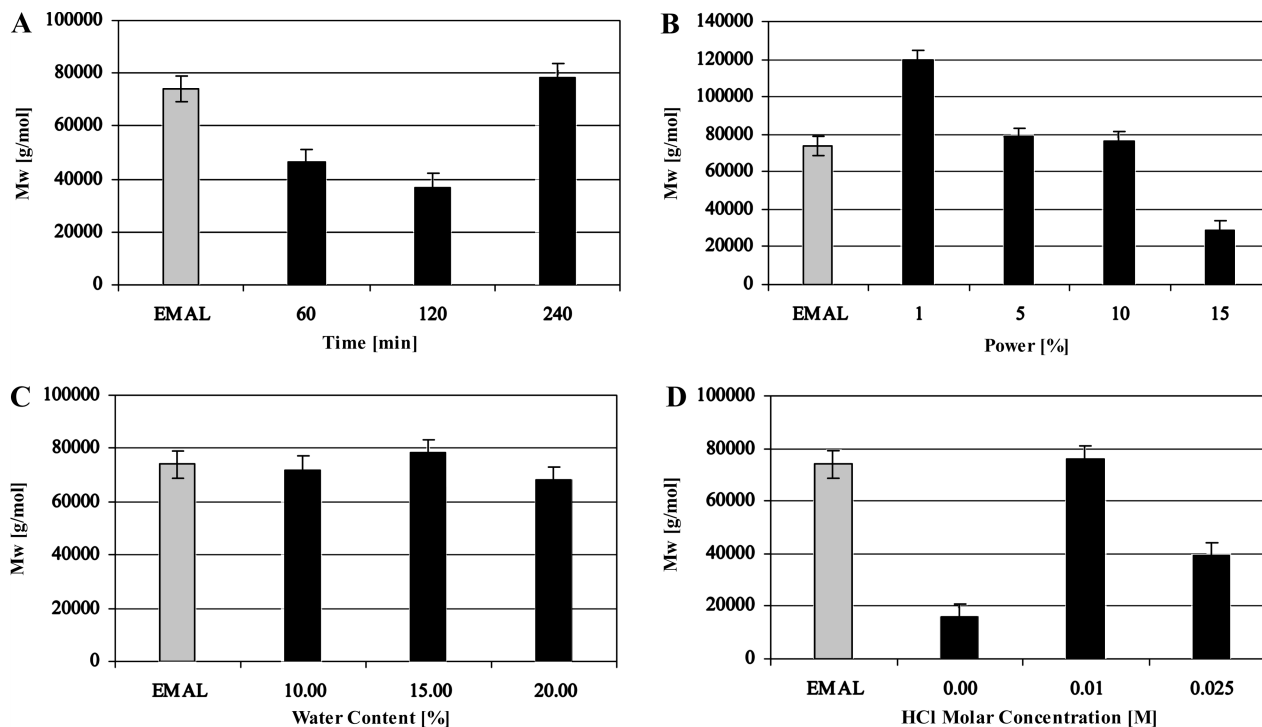


Figure 3. Weight-average molecular weight values (M_w) of EMALs produced by microwave-assisted heating as obtained via size exclusion analyses: for different periods of microwave treatment (A); for different levels of microwave power (B); for different water contents present in the reaction medium (C); for different HCl concentrations present in the reaction medium (D). For comparative purposes the M_w obtained when using the traditional refluxing step during the EMAL protocol is also shown. Fixed levels for the unchanged variables: time of treatment, 240 min; power, 5%; water content, 15%; HCl concentration, 0.01 M.

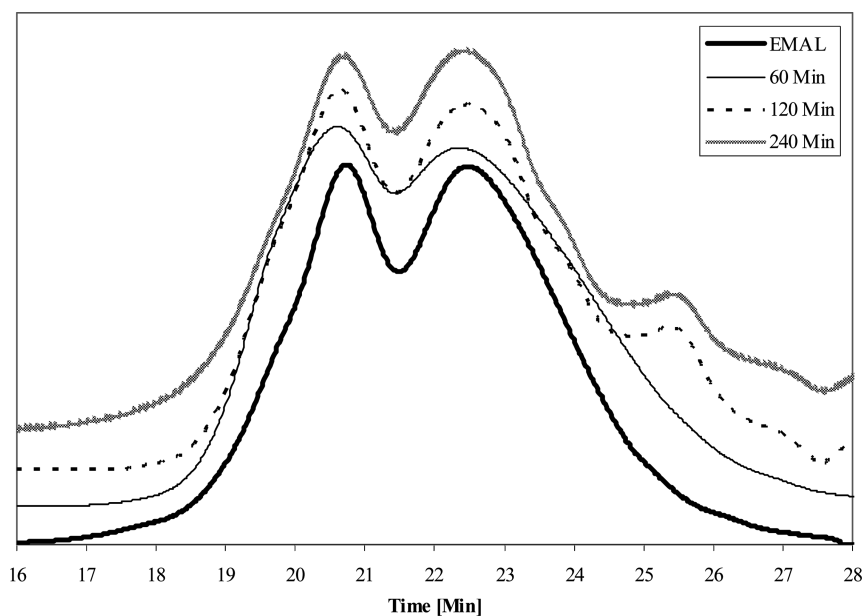


Figure 4. Elution profiles in size exclusion analyses of EMAL samples produced by microwave-assisted heating for different periods of treatment.

compared with those obtained for milled wood (MWL) and cellulase enzyme (CEL) lignins (18). These data supported the hypothesis that the concerted effect of cellulolytic action and mild acidolysis allowed for the isolation of lignin fragments that are not accessible by either the MWL or CEL procedure. The shapes of the molecular weight distribution curves of the microwave-assisted EMAL samples were found to be nearly identical to those obtained via the conventional reflux heating, that is, bimodal distributions. However, the molecular weight values of EMALs obtained after heating with microwaves for 60 and 120 min were significantly lower than those obtained

for EMAL and after 240 min of microwave irradiation (Figure 3A). Figure 4 shows a series of size exclusion chromatograms for the EMALs isolated as a function of time of microwave treatment. Although these profiles seem to be similar for all samples, some variations in the high and low molecular weight regions are obvious upon close examination. Apparently, the average molecular weight for EMAL isolated after a prolonged microwave irradiation (240 min) compares well to that of EMAL isolated with the traditional reflux protocol. This was in agreement with the data reported in Figures 1A and 2A, where the yields and purities at different times of treatment are shown:

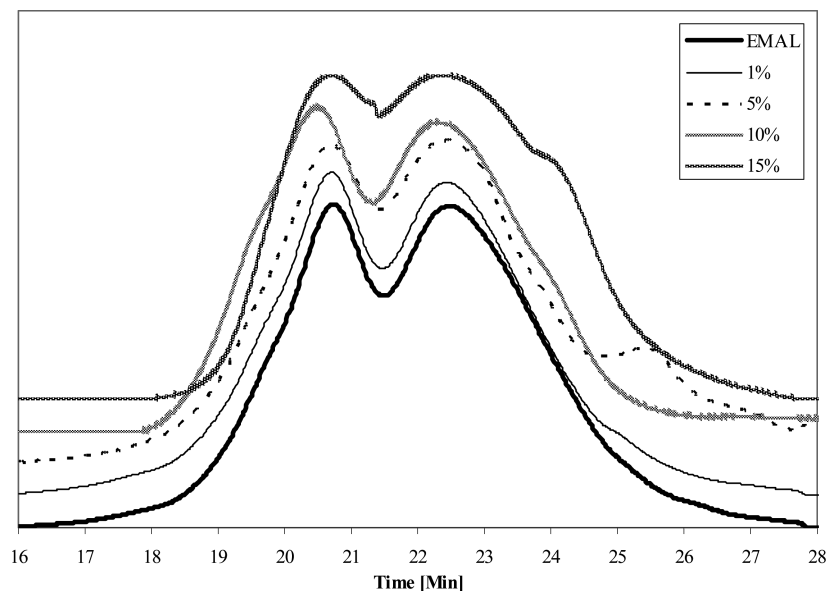


Figure 5. Elution profiles in size exclusion analyses of EMAL samples produced by microwave-assisted heating for different levels of microwave power.

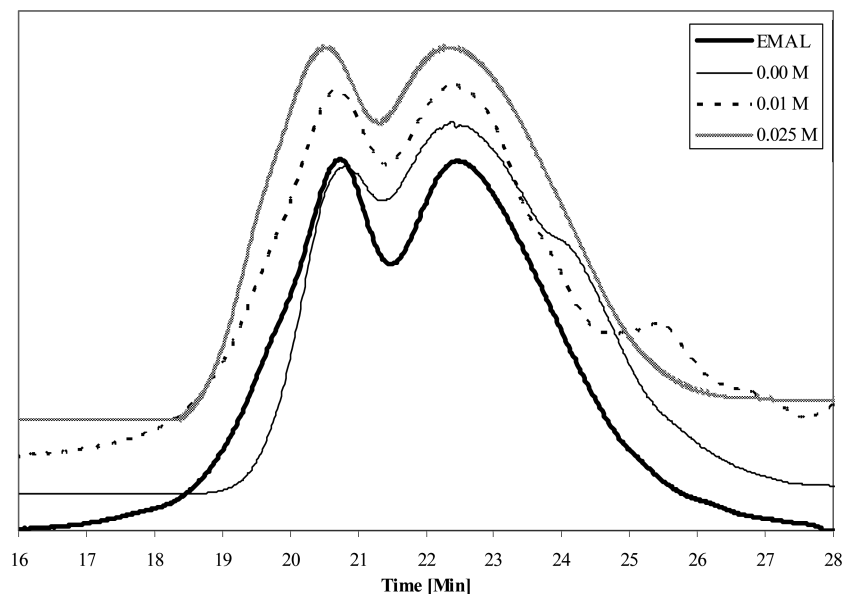


Figure 6. Elution profiles in size exclusion analyses of EMAL samples produced by microwave-assisted heating for different HCl concentrations in the reaction medium.

short times of treatment (60 and 120 min) led to lower yields and purities than longer times (240 min).

The M_w value of EMAL, obtained by microwaving at 1% of power level, is high (120,000 g/mol) compared to the traditional EMAL procedure. These data could be explained by the presence of a high molecular weight fraction (**Figure 5**) related to carbohydrate impurities. What appears to be clear is that increasing the microwave power beyond 5% causes the molecular weight of the isolated EMAL to significantly decrease (**Figure 3B**). Lignin degradation at elevated microwave power levels is a distinct possibility. Furthermore, the data of **Figure 5** display an obvious difference in the elution profiles when different levels of microwave power were applied. A significant loss of the high molecular weight fraction is apparent with a concomitant increase in the low molecular weight area. This behavior was clear for samples isolated at 10 and 15% power levels. Therefore, the data in **Figure 2A**, which relates the yields

at different powers, are rationalized as follows: high power leads to lignin degradation, decreasing the yield of the isolation procedure.

By varying the amount of water present in the reaction medium, no significant effects in the actual molecular weights of the obtained EMALs were observed (**Figure 3C**). Finally, **Figure 3D** shows that in the absence of HCl the isolated EMAL is of an extremely low molecular weight, most likely pointing to the significance and necessity of cleaving the lignin–carbohydrate bonds prior to lignin isolation. At HCl concentrations higher than 0.01 mol/L, that is, 0.025 mol/L, the M_w was found to significantly decrease, most likely due to acidic lignin degradation as evidenced and correlated by the yield data of **Figure 1A**. **Figure 6** shows the different profiles in SEC by varying HCl concentration on reaction medium. In the absence of HCl the isolated lignin was of a very low molecular weight without any fraction of higher molecular weight. This is in accordance with the fact that, without the hydrolysis of the lignin–carbohydrates

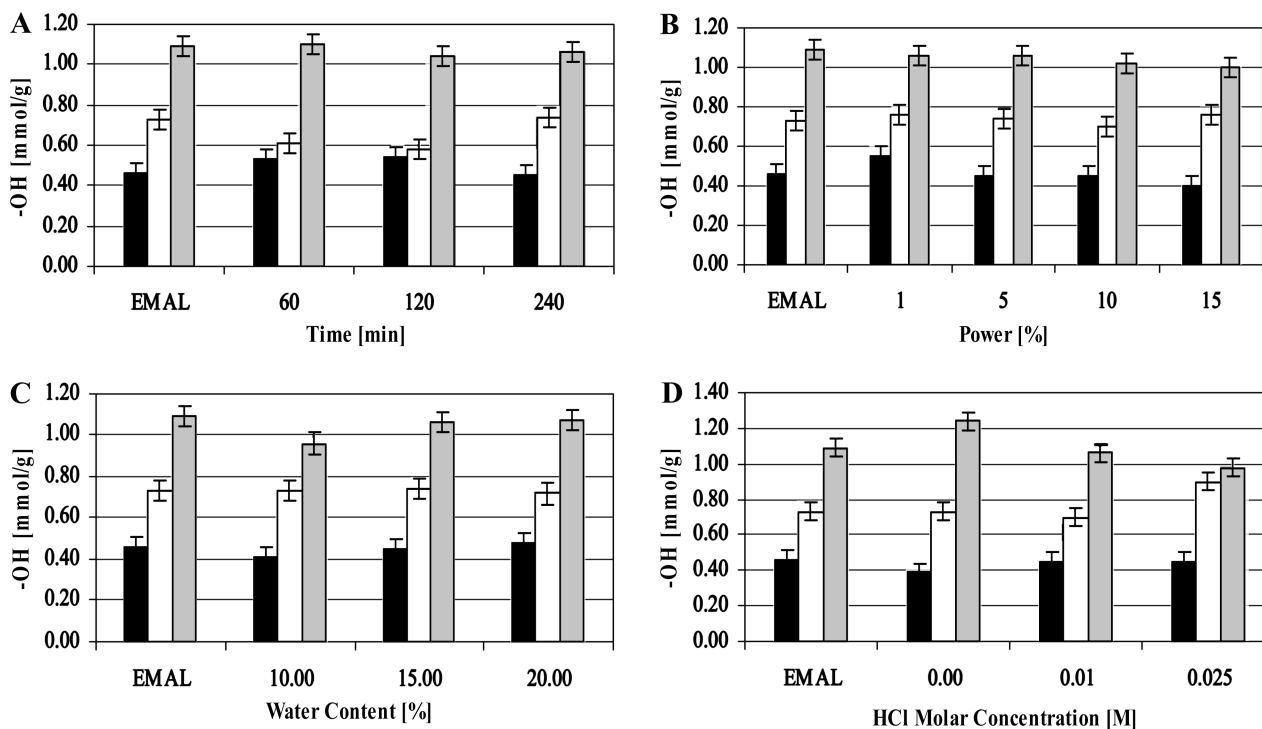


Figure 7. Condensed phenol (black bars), noncondensed guaiacyl phenol (white bars), and total β -aryl ether (gray bars) contents for EMALs produced via microwave-assisted heating (data obtained with ^{31}P NMR and expressed in mmol/g): for different periods of microwave treatment (**A**); for different microwave power levels (**B**); for different water contents present in the reaction medium (**C**); for different HCl concentrations present in the reaction medium (**D**). For comparative purposes the same functional groups contents present on EMAL when using the traditional refluxing step during the EMAL protocol are also shown. Fixed levels for the unchanged variables: time of treatment, 240 min; power, 5%; water content, 15%; HCl concentration, 0.01 M.

complexes, the isolation of the lignin is precluded. When the HCl concentration is increased to 0.01 mol/L, the elution profile becomes similar to that of traditional EMAL. Further increase of the HCl concentration to 0.025 mmol/L decreased the amount of high molecular weight fraction present in lignin.

Quantitative ^{31}P NMR spectroscopy is a reliable means to determine the amounts of various hydroxyl groups within a lignin macromolecule. The quantitative distribution of such functional groups is obtained after phosphorylation of lignin with 2-chloro-1,3,2-dioxaphospholane (reagent I) or 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (reagent II) (24, 25). These methods allowed the quantification of β -aryl ethers, the condensed phenolic hydroxyls, and the uncondensed *p*-hydroxyl, guaiacyl, and syringyl phenols as well as carboxylic acids present in the various EMAL samples isolated. These data are a valuable and facile way of understanding the degree and type of modification on the lignin structure afforded during the different microwave treatments. For all samples in this work no significant variations in carboxylic acids and uncondensed *p*-hydroxyl phenols were apparent.

Overall, the data of **Figure 7** show that the β -aryl ether and condensed and uncondensed phenolic group contents present on the EMAL isolated after 240 min of microwave treatment are similar to those of traditional EMAL samples (**Figure 7A**). After 60 and 120 min of microwave irradiation, the amounts of uncondensed phenols were found to be slightly lower (**Figure 7A**). These data could be compared with the purity data of **Figure 2A**, and as such it could be correlated to the partial cleavage of the lignin–carbohydrate linkages, most likely phenyl glucosides. With regard to the values of β -aryl ether bonds a slight decrease was apparent with increasing microwave treatment time.

Figure 7B shows that the amount of condensed phenols was somewhat reduced with increasing microwave power.

With regard to the uncondensed phenols and the β -aryl ether groups, there was a significant trend: high levels of microwave power (over 5%) resulted in higher amounts of phenols and lower amounts of β -aryl ethers, indicating lignin degradation; in agreement with the SEC data (**Figure 3B**). Most significantly, however, the effect of HCl concentration is seen to significantly participate in defining the lignin structure (**Figure 7D**). In the absence of HCl, the amount of β -aryl ethers was found to be 1.22 mmol/g. Using an HCl concentration of 0.025 mol/L and under microwave reflux conditions, the amount of β -aryl ether bonds was found to be 25% lower than in its absence, and the amount of uncondensed phenols was found to be as high as 0.9 mmol/g. This indicates severe degradation of the lignin structure, and it is also correlated with the low M_w value obtained during the size exclusion chromatographic analyses of **Figure 3D**.

Conclusion. The EMAL protocol has been shown to offer much higher gravimetric lignin yields and purities than those of the corresponding MWL and CEL. This work demonstrates that it is possible to further improve the yields and purities of the EMAL protocol by using microwave heating during the refluxing step of the methodology. Using Norway spruce as the wood species of choice, we arrived at a set of recommended conditions for such a treatment. It is thus recommended that the concentration of HCl and the amount of water present in the reflux stage be kept identical to the EMAL procedure, whereas the 5% microwave power over an extended period of time (4 h) needs to be applied for the mild acid hydrolysis step. These conditions may extend the isolation yield of softwood EMAL up to about 70%, without introducing any additional chemical modifications on the lignin (as evidenced by SEC and quantitative ^{31}P NMR data). Overall, it appears that low microwave power affects the efficiency of heating, minimizing

wall effects and energy consumption. It is likely that this form of direct energy transfer in the reflux medium increases the rate of hydrolysis of the lignin–carbohydrate linkages via an ion conduction mechanism exerted on the HCl by the magnetic field.

LITERATURE CITED

- (1) Fengel, D.; Wegener, G., Eds. *Ultrastructure and reactions. In Wood Chemistry*; de Gruyter: Berlin, Germany, 1989; 613 pp.
- (2) Ralph, J.; Lundquist, K.; Brunow, G.; Lu, F.; Kim, H.; Schatz, P.; Marita, J.; Hatfield, R.; Ralph, S.; Christensen, J.; Boerjan, W. Lignins: natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids. *Phytochemistry* **2004**, *3*, 29–60.
- (3) Yaku, F.; Yamada, Y.; Koshijima, T. Lignin–carbohydrate complex. Part IV. Lignin as a side chain of the carbohydrate in Bjorkman LCC. *Holzforschung* **1981**, *35*, 177–181.
- (4) Lawoko, M.; Henriksson, G.; Gellerstedt, G. New method for the quantitative preparation of lignin–carbohydrate complex from unbleached softwood kraft pulp: lignin–polysaccharide networks I. *Holzforschung* **2003**, *57*, 69–74.
- (5) Eriksson, O.; Goring, D.; Lindgren, B. O. Structural studies on the chemical bonds between lignin and carbohydrates in spruce. *Wood Sci. Technol.* **1980**, *14*, 267–279.
- (6) Watanabe, T.; Koshijima, T. Evidence for an ester linkage between lignin and glucuronic acid in lignin–carbohydrate complexes by DDQ-oxidation. *Agric. Biol. Chem.* **1988**, *52*, 2953–2955.
- (7) Lundquist, K.; Simonson, R.; Tingsvik, K. Lignin–carbohydrate linkages in milled-wood lignin preparations from spruce wood. *Sven. Papperstidn.* **1983**, *86*, 44–47.
- (8) Yaku, F.; Yamada, Y.; Koshijima, T. Lignin–carbohydrate complex, part II. Enzymatic degradation of acid polysaccharides in Bjorkman LCC. *Holzforschung* **1976**, *30*, 148–156.
- (9) Kondo, R.; Sako, T.; Limori, T.; Imamura, H. Formation of glycosidic lignin–carbohydrate complex in the dehydrogenative polymerization of coniferyl alcohol. *Mokuzai Gakkaishi* **1990**, *36*, 332–338.
- (10) Xie, Y.; Yasuda, S.; Wu, H.; Liu, H. Analysis of the structure of lignin–carbohydrate complexes by specific ¹³C tracer method. *J. Wood Sci.* **2000**, *46*, 130–136.
- (11) Wu, S.; Argyropoulos, D. S. An improved method for isolating lignin in high yield and purity. *J. Pulp Pap. Sci.* **2003**, *29*, 235–240.
- (12) Chang, H.; Cowling, E.; Brown, W. Comparative studies on cellulolytic enzyme lignin and milled lignin of sweet gum and spruce. *Holzforschung* **1975**, *29*, 153–159.
- (13) Lee, Z.; Meshitsuka, G.; Cho, N.; Nakano, J. Characteristics of milled wood lignins isolated with different milling times. *Mokuzai Gakkaishi* **1981**, *27*, 671–677.
- (14) Ikeda, T.; Holtman, K.; Kadla, J.; Chang, H.; Jameel, H. Studies on the effect of ball milling on lignin structure using a modified DFRC method. *J. Agric. Food Chem.* **2002**, *50*, 129–135.
- (15) Adler, E. Lignin chemistry—past, present and future. *Wood Sci. Technol.* **1977**, *11*, 169–218.
- (16) Bjorkman, A. Studies on finely divided wood I. Extraction of lignin with neutral solvents. *Sven. Papperstidn.* **1956**, *59*, 477–485.
- (17) Bjorkman, A. Lignin and lignin–carbohydrate complexes extraction from wood meal with neutral solvents. *Ind. Eng. Chem.* **1957**, *49*, 1395–1398.
- (18) Guerra, A.; Filippinen, I.; Lucia, L. A.; Argyropoulos, D. S. Comparative evaluation of three lignin isolation protocols for various wood species. *J. Agric. Food Chem.* **2006**, *54*, 9696–9705.
- (19) Guerra, A.; Filippinen, I.; Lucia, L. A.; Saquing, C.; Baumberger, S.; Argyropoulos, D. S. Toward a better understanding of the lignin isolation process from wood. *J. Agric. Food Chem.* **2006**, *54*, 5939–5947.
- (20) Lindstrom, P.; Tierney, J.; Wathey, B.; Westman, J. Microwave assisted organic synthesis—a review. *Tetrahedron* **2001**, *57*, 9225–9283.
- (21) Dallinger, D.; Kappe, C. O. Microwave-assisted synthesis in water as solvent. *Chem. Rev.* **2007**, *107*, 2563–2591.
- (22) De la Hoz, A.; Diaz-Ortiz, A.; Moreno, A. Microwaves in organic synthesis. Thermal and non-thermal microwave effects. *Chem. Soc. Rev.* **2005**, *34*, 164–178.
- (23) Yeh, T.; Tatsuhiko, Y.; Capanema, E.; Chang, H.; Chiang, V.; Kadla, J. Rapid screening of wood chemical component variations using transmittance near-infrared spectroscopy. *J. Agric. Food Chem.* **2005**, *53*, 3328–3332.
- (24) Granata, A.; Argyropoulos, D. S. 2-Chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane, a reagent for the accurate determination of the uncondensed and condensed phenolic moieties in lignins. *J. Agric. Food Chem.* **1995**, *43*, 1538–1544.
- (25) Argyropoulos, D. S. Quantitative phosphorus-31 NMR analysis of lignin: a new tool for the lignin chemist. *J. Wood Chem. Technol.* **1994**, *14*, 45–63.
- (26) Lawoko, M.; Henriksson, G.; Gellerstedt, G. Characterization of lignin–carbohydrate complexes from spruce sulfite pulps. *Holz-forschung* **2006**, *60*, 162–165.
- (27) Kosikova, B.; Joniak, D.; Kosakova, L. The properties of benzyl ether bonds in the lignin–saccharidic complex isolated from spruce. *Holzforschung* **1979**, *33*, 11–14.

Received for review June 27, 2008. Revised manuscript received September 10, 2008. Accepted September 12, 2008.

JF801955B