# Isolation and Chemical Structure Characterization of Enzymatic Lignin from *Populus deltoides* Wood

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**ABSTRACT:** Cellulytic enzymes were used for the isolation and structural characterization of *Populus deltoides* wood lignin as a fast growing and important species in wood processing technology. The isolation was based on the hydrolysis and partial solubilization of wood xylan and cellulose using combination of *Thricoderma lanuginosus* xylanase, *Aspergillus* sp. plus, *A. niger* cellulase, and almond glycosidase, followed by lignin purification using *Bacillus licheniformis* alkaline protease (for hydrolysis of cellulase contamination). The structure of enzymatic lignin (EL) was elucidated using chemical analysis, Py-GC/MS, FTIR, and quantitative $^{13}$C-NMR techniques. Different lignin structures of acetylated and nonacetylated lignin preparation were calculated. *P. deltoides* EL has been determined to have an h : g : s ratio of 5 : 60 : 35. Also, *P. deltoides* EL contained 0.59/Ar of β-O-4 moieties with small amounts of other structural units such as pino/syringyresinol (0.05/Ar), phenylcoumaran (0.05/Ar), and spirodienone (0.01/Ar). The degree of condensation was estimated at 20%. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 118: 469–479, 2010

**Key words:** enzymatic lignin; enzymatic sequence; quantitative $^{13}$C-NMR; Py-GC/MS; hardwood

## INTRODUCTION

Eastern cottonwood (*Populus deltoides),* one of the largest eastern hardwoods, is short-lived but the fastest growing commercial forest species in North America and Asia. Among all poplar species, *P. deltoides* has gained most important all over the world, due to its rapid growth rate, relative ease of vegetative propagation and wood quality. Eastern cottonwood is one of the few hardwood species that is planted and grown specifically for pulp and paper production.[1] There is not much information on the structure of fast growing hardwood lignins, especially in case of *P. deltoides.*[2,3]

Most softwood lignins consist predominantly of guaiacyl (g) units, whereas the structure of hardwood lignins is more complicated due to the presence of both guaiacyl (g) and syringyl (s) units (Fig. 1). Nimz[4] proposed the structure of beech lignin on the basis of nonquantitative $^{13}$C-NMR studies, whereas Adler[5] has drawn a scheme of birch lignin based predominantly on the results of permanganate oxidation (Table I).

Capanema et al.[8] reported a detailed investigation regarding chemical structure of *Eucalyptus grandis* milled wood lignin through quantitative $^{13}$C-NMR. However, despite extensive investigations during the past five decades, the structure of fast growing hardwood species lignin, is not studied.

MWL preparation has long been used as a standard method for lignin structure studies. The MWL preparation from protolignin offers almost a moderate yield.[9] Yield is a key factor in lignin structural study, since the extracted lignin should represents total lignin of the plant. Detailed studies on the process of MWL as the predominant lignin model in lignin investigations have shown that milling time has a significant effect on the yield.[2,10] Actually, the yield of MWL preparation could be increased by increasing the milling time.[11] It is reported that yield of extraction could be as high as 34% after 28 days of ball milling.[9] However, ball milling reduces the degree of polymerization, creating new free-phenolic hydroxyl groups through cleavage of β-aryl ether linkages as well as increasing α-carbonyl groups via side-chain oxidation.[11]

Efforts to improve the process of isolating lignin, in a relatively unaltered state, aimed at selectively

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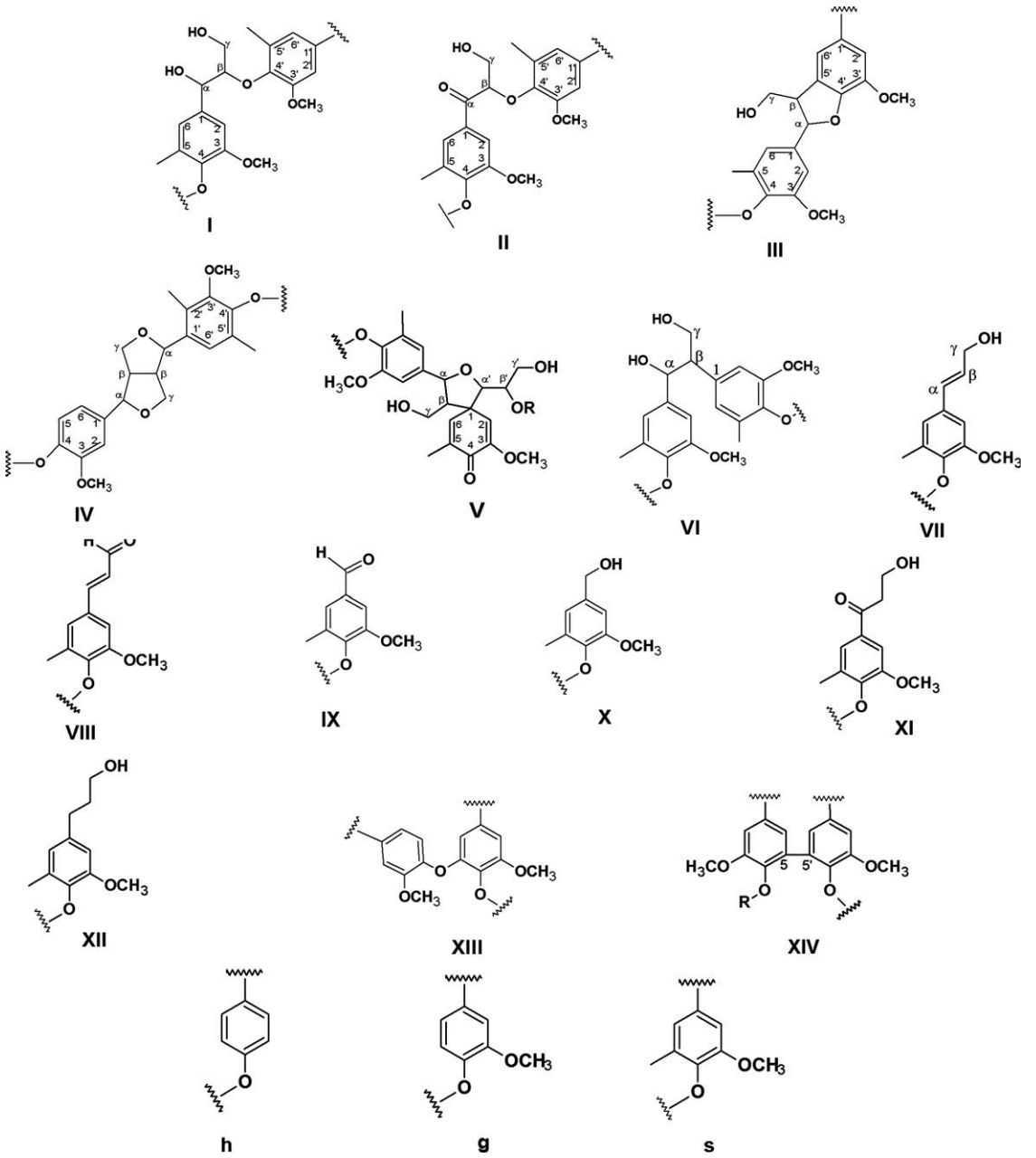


**Figure 1** Lignin substructures.

degrading the wood polysaccharides were started with cellulytic enzyme lignin (CEL) isolation.[12] Cellulolytic enzyme lignin (CEL) preparation, especially for hardwoods, has not been used as widely as MWL in structural studies of lignin. Study of hardwood lignins was restricted to some eucalypt species.[9,13] CEL has a higher molecular weight, lower condensation and higher β-O-4′ interlinkage compared to MWL.[14] The higher yield of CEL extracted from wood helps us understand the lignin structure as a whole. The major drawback of cellulytic enzyme lignins isolated with endocellulase is the presence of carbohydrate and protein impurities.[15] However, it seems this type of isolation is the most suitable way for releasing a lignin structurally representative of the *in situ* lignin in wood.

In this study, a cocktail of enzymes was used for isolation and purification of lignin from *P. deltoides*. The effect of multistage enzyme treatment and chemical purification in extraction of residual lignin from pulp was investigated.[16] The aim of the present study is to use a multistage enzymatic procedure to isolate lignin in high yield from relatively short time ball milled poplar wood that could be used for chemical structure characterization.

**TABLE I**
**Brief Review of Various Moieties Amount in Hardwood Lignins (per 100 Aromatic Unit)**

| Unit | Beech[4] | Birch[5] | *E. globulus*[6] | *E. grandis*[7] | *E. grandis*[8] | *P. deltoides* (present work) |
|---|---|---|---|---|---|---|
| β-*O*-4/α-*OH* | | | | | 55 | 43 |
| β-*O*-4/α-*CO* | | | 4 | | 2 | 3 |
| phenylcoumaran | 6 | 6 | 3 | 10 | 3 | 5 |
| pino/syringyresinol | 5 | 3 | 5 | 4 | 3 | 5 |
| β-1/α-*OH* | 15 | 7 | | | 2 | 3 |
| spirodienone: guaiacyl | | | | | 1 | – |
| syringyl | | | | | 4 | 2 |
| β-*O*-4 *total* | 65 | 60 | 56 | 47 | 61 | 59 |
| γ-*O*-alkyl *total* | | | | | 23 | 27 |
| 5-5′ | 2 | 4.5 | 3 | 6 | 3 | 4 |
| OMe | 136 | 164 | 164 | 145 | 160 | 130 |
| Total OH | | 186 | 117–121 | 138 | 144 | 153 |
| Primary | | 86 | 68 | 75 | 70 | 69 |
| Secondary | | 80 | 20 | 35 | 55 | 73 |
| Phenolic | | 20 | 29–30 | 28 | 19 | 11 |
| Total carbonyl | | | 24 | 19 | 17 | 6 |
| Nonconjugated | | | 10 | 8 | 3 | <1 |
| COOH | | | 4 | | 5 | 16 |
| Degree of condensation | | | 18 | 50 | 21 | 20 |
| ***s : g : h*** | | 50 : 50 | 84 : 14 : 02 | 50 : 50 | 62 : 36 : 2 | 35 : 60 : 5 |

## MATERIALS AND METHODS

### Materials

*P. deltoides* plantation wood (12 years old) was used as a raw material for lignin isolation. Sapwood was grounded to pass a 20-mesh screen in a Wiley mill, Soxhlet-extracted with acetone for 48 h and then sonicated for 10 min in cold water followed by centrifugation and drying in ambient conditions (Wiley wood). Wiley wood was then grounded for 168 h in a 1 gal porcelain jar (rotary ball mill) using alumina balls under a nitrogen atmosphere. The following commercial enzymes were used in lignin isolation and purification procedures: *Thricoderma lanuginosus* xylanase as a purified endo 1,4-β-xylanase (EC: 3.2.1.8, 2500 u/g), *Aspergillus* sp. cellulase as endocellulase (EC: 3.2.1.4, 1000 u/g), *Aspergillus niger* Novozyme 188 from Novozymes as exoglucanase (EC: 3.2.1.91, 250 u/g), almonds β-glucosidase G4511 as glucosidase (EC: 3.2.1.21, 40 u/mg) and *Bacillus licheniformis* alkaline protease (Subtilisin Type VIII, 15 u/g). All the enzymes were purchased from Sigma chemical company (St. Louis, MO). 1,4-dioxane was distilled over sodium before utilization. All chemicals were purchased from Merck Company (Darmstadt, Germany) as laboratory grade and were used as received.

### Enzymatic treatments

To prepare an enzymatic lignin (EL) extract, ball milled wood was treated by the following sequence of enzyme treatments:

#### Xylanase treatment

Ball milled wood (40 g) was suspended in phosphate buffer (800 mL, pH 7, 50 m*M*) and 4 g (2500 U/g) of xylanase was added and incubated (300 rpm) under continuous stirring for 24 h at 50°C. After centrifugation (10,000 rpm, 15 min) the residue was washed (3×) carefully with deionized water.

#### Endocellulase and exoglucanase treatment

Residue from the xylanase treatment was suspended in phthalate buffer (pH 5.5, 50 m*M*) and 4 mL (1000 u/g) of endocellulase (EC: 3.2.1.4) and 4 mL (250 u/g) of cellulase enzyme (EC: 3.2.1.91) was added and incubated under continuous stirring (300 rpm) for 48 h at 45°C. After centrifugation (10,000 rpm, 15 min) the residue was washed carefully with deionized water.

#### Glucosidase treatment

Residue from above stage was suspended in acetate buffer (pH 5, 50 m*M*) and 100 units of glucosidase (EC: 3.2.1.21, 40 unit/mg) was added and incubated under continuous stirring (300 rpm) for 48 h at 37°C. After centrifugation (10,000 rpm, 15 min) the residue was washed carefully with deionized water.

### Isolation and purification of lignin

Purification of the nonhydrolysable residue which contained contaminating protein was done according to Ibarra et al.[16] by suspending the residue in alkaline protease. The reaction was conducted at pH 8.5 (tris buffer) and 37°C, with gentle stirring (150 rpm) for 24 h. The solution was centrifuged (10,000 rpm, 15 min) and the insoluble part was recovered. The residue was suspended in 96% aqueous 1,4-dioxane for 3 × 24 h with continuous stirring. The combined

**TABLE II**
**Yield, Purity and Elemental Composition of EL**

| Characteristics | EL |
|---|---|
| Yield (%)[a] | 38 |
| Purity (%)[b] | 94.4 |
| Carbohydrates (%) | 6.9 |
| C (%) | 57.6 |
| H (%) | 6.2 |
| O (%) | 33 |
| N (%) | <0.3 |
| OH (phenolic)[c] | 0.11 |
| OH(Aliphatic)[c] | 1.42 |
| C=O (c) | 0.05 |
| $C_9$ formula | $C_9H_{8.87}O_{3.78}(OCH_3)_{1.27}$ |
| Molecular weight of c9 unit | 234.4 |

[a] Based on acetyl bromide lignin content of extracted ground wood meal (19.2%).
[b] Purity based on acetyl bromide lignin content of the samples.
[c] Per aromatic unit (C9).

aliquots were concentrated under reduced pressure and freeze dried to obtain crude EL. Crude EL was dissolved in least amount of 90% glacial acetic acid (10 mL) followed by precipitation into stirring ethyl ether to obtain a purified EL (400 mL).

### Lignin acetylation

The acetylation of EL was done by dissolving the sample into pyridine/$Ac_2O$ (1 : 1, v : v) overnight in room temperature. Pyridine was removed by azeotropic distillation with ethanol under reduced pressure.[10] The evaporation was repeated 7 times to ensure complete removing of pyridine from the sample.

### Analysis

#### Lignin content and elemental analysis

Lignin content of wood and lignin were determined by acetyl bromide method according to the method described by Hatfield et al.[17] Briefly, samples were dissolved in 25% solution of acetyl bromide in acetic acid and after 4 h treatment in 50°C the dissolved lignin was determined by UV–vis spectroscopy in 280 nm. Elemental analysis (C, H, and N) was performed by a CHN elemental analyzer. Oxygen content was determined as

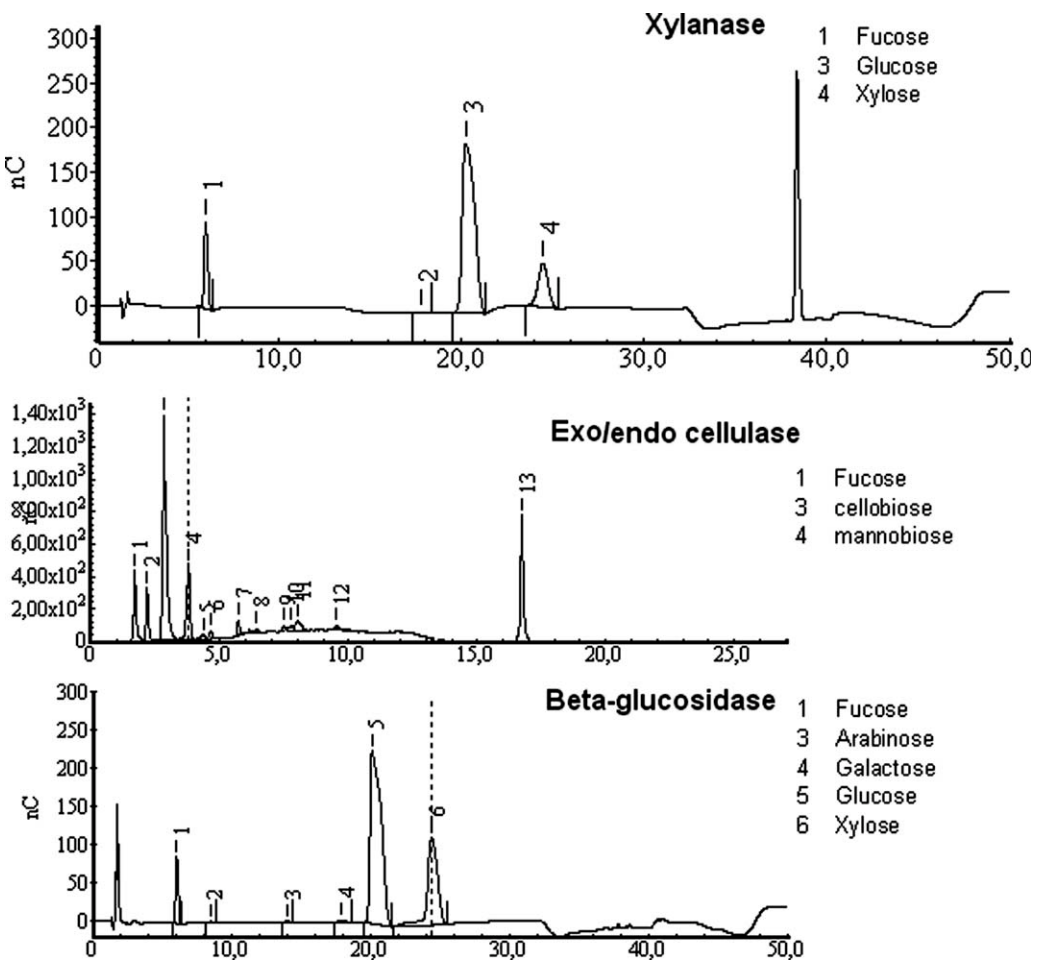


**Figure 2** HPAEC chromatogram of dissolved sugars during enzyme treatments.

**TABLE III**
**Main Sugar Monomer Composition of Extracted EL (%)**

| | Rhamanose | Arabinose | Xylose | Mannose | Galactose | Glucose |
|---|---|---|---|---|---|---|
| EL | 0.14 | 0.23 | 4.25 | 0.19 | 0.27 | 0.82 |

100–(C + H + N), assuming no other elements were present. Methoxyl, carbonyl, and hydroxyl functional groups were determined quantitatively by $^{13}$C-NMR.

### Sugar content determination with HPAEC and GC

Sugar dissolution profile during enzyme treatment was monitored by analyzing the enzyme hydrolysates with high-performance anion-exchange chromatography (HPAEC). Filtrates was dissolved in water and analyzed by HPAEC-PAD using a Dionex DX-500 system (Dionex, Sunnyvale, CA) equipped with a pulsed amperometric detector and CarboPac PA-1 column. Dissolved sugars were determined relative to individual standard samples and fucose as an internal standard. The carbohydrate content of the extracted lignin was determined using a modified alditol-acetate gas chromatographic method.[18] Lignin sample was acidolyzed with 72% sulfuric acid in 100°C and the released sugars were reduced and acetylated by $NaBH_4$ and Acetic anhydride, respectively. Gas chromatography was performed using a Shimadzu GC 14-A (Shimadzu, Kyoto, Japan) equipped with a column DB-5 of 15 m × 0.32 mm. The carrier gas was helium at a flow rate of 2.0 mL min$^{-1.}$ The injection temperature was 220°C with a split ratio of 30 : 1, and the FID detector temperature was 240°C. The oven temperature was raised from an initial 210°C to 230°C at 2°C min$^{-1.}$ The concentration of sugar monomers was determined relative to inositol as an internal standard using response factors derived from pure compounds.

### Gel permeation chromatography

Gel permeation chromatography (GPC) analysis of acetylated sample was performed on an Agilent 1100 HPLC system (Agilent Technologies, Foster City, CA) at ambient temperature using three PLGEL columns connected in series. Tetrahydrofuran (THF) was the eluent (0.5 mL/min), and fractions were monitored using a refractive index detector. The acetylated lignins were dissolved in THF at a concentration of 1 mg/mL, and 250 μL of this solution was injected into the HPLC. Molecular weight determinations were made using polystyrene as a calibration standard.

### FTIR spectra

FTIR of lignin KBr pellet (2 mg of lignin sample in 200 mg of KBr) was recorded on a Bruker Equinox-55 spectrometer (Bruker Optik, Ettlingen, Germany). Absorbance was recorded at a resolution of 8 cm$^{-1}$ for a total of 64 scans per measurement.

### Py-GC/MS

Pyrolysis of EL (~100 mg) was performed with a Curie-point flash pyrolyzer (Frontier Laboratories) equipped with an Agilent 6890 GC/MS system (Agilent Technologies, Foster City, CA) using a 30 m × 0.25 m DB-5 column (film thickness 0.25 mm). The pyrolysis was done at 500°C. The oven temperature was programmed from 40°C (1 min) to 300°C at 30°C min$^{-1}$. Helium was the carrier gas (1 mL min$^{-1}$). The injector temperature was kept at 280°C while the GC/MS interface was kept at 300°C. The compounds were identified by comparing their mass spectra with those of the Wiley and NIST libraries and reported in the literature.[19,20]

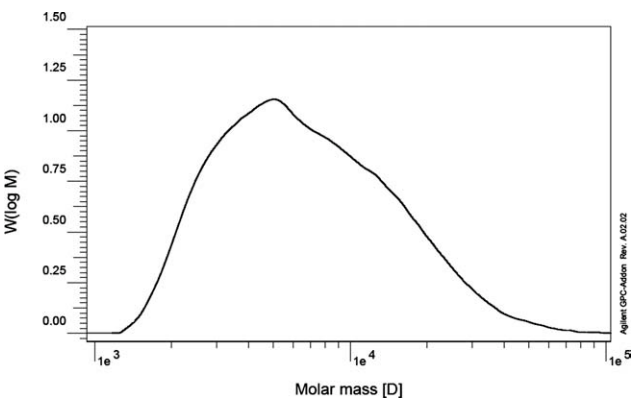


**Figure 3** GPC chromatogram of representative EL.

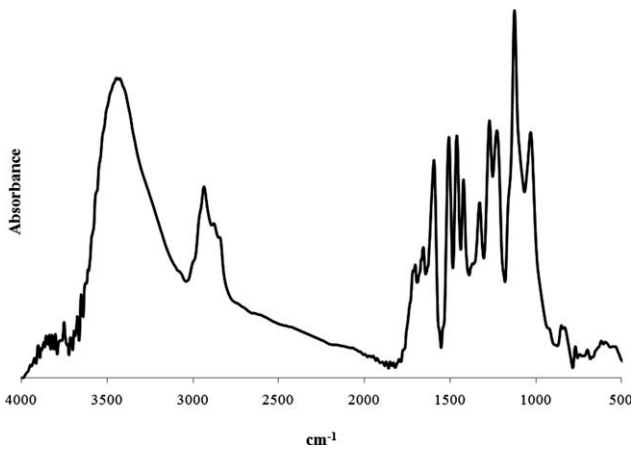


**Figure 4** FTIR spectra of EL from *P. deltoides*.

**TABLE IV**
**Bond Assignments in the FTIR of EL Lignin**

| Absorption | | |
|---|---|---|
| Wave number ($cm^{-1}$) | EL | Bond origin |
| 1727 | 0.045 | C=O stretch in unconjugated C=O |
| 1656 | 0.052 | Stretching of C=O conjugated to aromatic rings, C=O stretching in amides |
| 1594 | 0.088 | Aromatic skeletal vibration |
| 1507 | 0.097 | Aromatic skeletal vibration |
| 1461 | 0.122 | C—H in $-CH_3$ and $-CH_2-$ |
| 1422 | 0.080 | Aromatic skeletal vibration |
| 1371 | 0.040 | Symmetric bending of aliphatic C—H |
| 1328 | 0.071 | s ring plus g ring condensed |
| 1270 | 0.104 | g ring |
| 1226 | 0.1 | C—C plus C—O stretch |
| 1030 | 0.1 | Aromatic C—H, C—O in primary alcohols |
| 890 | 0.007 | β-glycosidic linkages in pyranose units |
| 851 | 0.021 | Aromatic C—H |

### Quantitative $^{13}$C-NMR

The NMR spectra of non-acetylated and acetylated lignin were recorded on a Bruker Avance 400 MHz spectrometer at 300 K using DMSO-$d_6$ as the lignin solvent. Twenty percent concentration solution of lignin was placed in a 10-mm diameter NMR tube, a 75° pulse width, a 1.4 s acquisition time, and 12 s relaxation delay were used. A total of 10,000 scans were collected. Decoupling only was made only during the acquisition.

## RESULTS AND DISCUSSION

### Elemental analysis and sugar content

Table II contains information regarding the elemental and chemical compositions of the enzymatic lignin preparation. A good yield extraction (38%, 168 h ball milling) of enzymatic lignin compared to conventional milled wood lignin indicates the effectiveness of cellulytic enzymes sequence in improving the lignin accessibility of wood cell wall by the extracting solvent (i.e., 1,4-dioxane). Guerra et al.[9] reported a maximum of 34% MWL extraction yield after extensive ball milling (28 days) of *E. globules* wood. Therefore, the reduced ball milling time for enzymatic lignin preparation does not affect the overall yield of lignin isolation. These conditions can ensure that the prepared lignin is relatively suitable for chemical structure elucidation of wood. In general, the extraction yield is dependent on raw material, milling time, and accessibility of the media (i.e., lignin) by the solvent. Cellulytic enzymes selectively dissolve the sugars of the wood cell wall and increase its porosity for lignin access by the solvent. The yield of cellulytic extraction is ranging from 20% to 80%.[14] Albeit a higher yield of extraction could be achieved by increasing the milling time, such protocol always contains oxidation and condensation reactions which lead to degradation of lignin macromolecule structure.[9] Figure 2 shows the dissolved sugars from wood meal during different enzyme treatments, analyzed with HPAEC. Sugar dissolution profile in enzymatic treatment shows the effectiveness of enzymatic stages in carbohydrate hydrolysis process. The sugar content of prepared enzymatic lignin is a little high amount (6.9%), since already it has been reported that cellulytic enzyme extracted lignin contains carbohydrate contamination (Table II).[15] The composition and amount of individual main sugar monomers in prepared EL analyzed by GC is shown in Table III. The elemental analyses were formulated to represent the contribution of lignin and resulted in the $C_9H_{8.87}O_{3.78}(OCH_3)_{1.27}$ formulae for EL. High oxygen content of EL may be due to

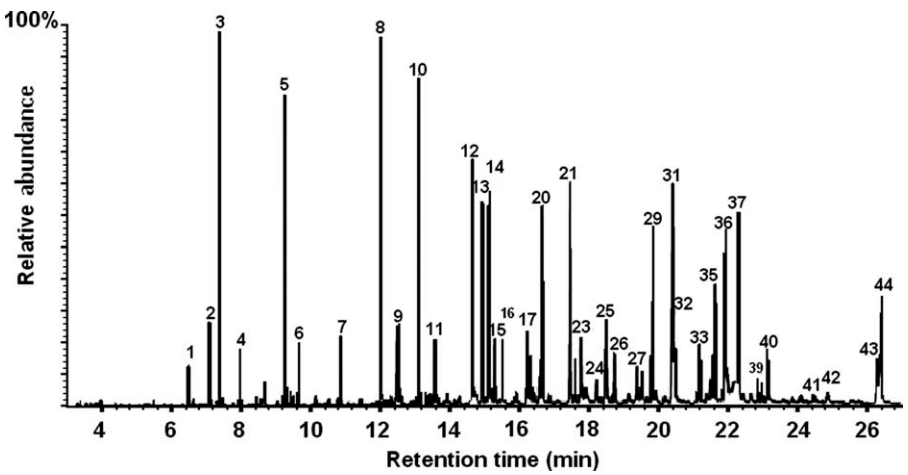


**Figure 5** Py-GC/MS chromatogram of a representative EL isolated from *P. deltoides* wood. The numbers refer to the compounds listed in Table IV.

contaminants (e.g., carbohydrates) or unwanted oxidation reaction during long term enzymatic treatment. It is noteworthy that the nitrogen content of the EL is below detection limit, showing that contamination by enzyme should not be a problem in this analysis and protease treatment was completely effective in lignin purification from protein contaminants. The phenolic and aliphatic hydroxyl and carbonyl contents were estimated using quantitative $^{13}$C-NMR. The phenolic and aliphatic hydroxyl groups content of EL were 0.11 and 1.42, per aromatic ring, respectively.

### Molecular weight distribution

Molecular weight distribution of acetylated EL is shown in Figure 3. The acetylation was done by conventional anhydride acetic/pyridine method. Chromatogram pattern indicates that prepared enzymatic lignin with current procedure has low and high molecular weight moieties and high molecular weight fragments in EL are more abundant (Fig. 3). Molecular weight at modal point of chromatogram of EL is 9100 g/mol. These observations confirm the effectiveness of purification method in EL preparation, since the mild treatment does not remove small fractions of isolated lignin. The less regular pattern in EL may indicate some degree of aggregation and partial solubilization of some lignin-carbohydrate complex (LCC) fragments in THF even after acetylation of the sample. This may explain the less quality of observed chromatogram for EL.

### FTIR and Py-GC/MS

The FTIR spectrum of the prepared *P. deltoids* lignin is shown in Figure 4. On the basis of extensive studies, the most important spectral absorptions of chemical bonds in lignin have been determined.[21] Table IV shows the origin and the amount of each absorption band for EL preparation.

These spectra elucidate that the extracted and purified lignin have the least extra absorptions caused by nonlignin impurities (1656 $cm^{-1}$ and 890 $cm^{-1}$; Table IV).

Py-GC/MS is a sensitive technique that enables a reproducible characterization of the intractable and involatile macromolecular complexes like lignin. Pyrogram of extracted enzymatic lignin is shown in Figure 5. The identities and relative molar abundances of the released lignin compounds are listed in Table V. Guaiacol, syringol and *p*-hydroxycinnamyl type phenols, derived from the g, s, and h lignin units (Fig. 1), respectively, were identified. The g phenols were released in higher abundances than the respective s phenols. The uncorrected molar s/g ratio obtained from the molar areas of all the lignin derived compounds is as 0.67, although the values were on average 1.1-fold higher than $^{13}$C-NMR data. Concerning the s/g ratios, Sarkanen and Hergert[22] pointed out those degradation methods in general overestimate the amounts of s units. This is due to the low condensation degree of s units within the macromolecule. Accordingly, all degradation techniques lead to an elevated amount of s units and, as a consequence, the uncorrected s/g ratios overestimate the true s/g ratios in lignin.

**TABLE V**
**Identification and Relative Molar Abundance of the Compounds Identified in the Py-GC/MS of EL from *P. deltoides* Wood**

| No. | Compound | Relative abundance (%) | Origin |
|---|---|---|---|
| 1 | 4-Hydroxybenzaldehyde | 0.8 | h |
| 2 | Phenol | 1.5 | h |
| 4 | Methylphenol | 1.2 | h |
| 6 | Dimethylphenol | 1.4 | h |
| 3 | Guaiacol | 9 | g |
| 5 | 4-Methylguaiacol | 7 | g |
| 7 | 4-Ethylguaiacol | 1.5 | g |
| 8 | 4-Vinylguaiacol | 8.3 | g |
| 9 | Eugenol | 1.5 | g |
| 11 | *cis*-Isoeugenol | 1.3 | g |
| 12 | trans-Isoeugenol | 4.9 | g |
| 14 | Vanillin | 4.7 | g |
| 15 | Propine-guaiacol | 1.5 | g |
| 16 | Propine-guaiacol | 1.4 | g |
| 17 | Homovanillin | 1.5 | g |
| 19 | Vanillic acid methyl ester | 0.5 | g |
| 20 | Acetoguaiacone | 2.6 | g |
| 22 | Guaiacyl-acetone | 1 | g |
| 24 | Propiovanillone | 0.4 | g |
| 25 | Guaiacyl vinyl ketone | 2.2 | g |
| 30 | Dihydroconiferyl alcohol | 0.2 | g |
| 32 | *cis*-Coniferyl alcohol | 0.8 | g |
| 36 | *trans*-Coniferyl alcohol | 4.3 | g |
| 37 | *trans*-Coniferaldehyde | 4.7 | g |
| 10 | Syringol | 8.1 | s |
| 13 | 4-Methylsyringol | 4.2 | s |
| 18 | 4-Ethylsyringol | 1 | s |
| 21 | 4-Vinylsyringol | 4.2 | s |
| 23 | 4-Allylsyringol | 1.1 | s |
| 26 | *cis*-Propenylsyringol | 0.9 | s |
| 27 | Propine-syringol | 0.6 | s |
| 28 | Propine-syringol | 0.7 | s |
| 29 | *trans*-Propenylsyringol | 3.2 | s |
| 31 | Syringaldehyde | 4.2 | s |
| 33 | Homosyringaldehyde | 1 | s |
| 34 | Syringic acid methyl ester | 0.5 | s |
| 35 | Acetosiryngone | 2.2 | s |
| 38 | Syringylacetone | 0.5 | s |
| 39 | Propiosyringone | 0.4 | s |
| 40 | Syringyl vinyl ketone | 1 | s |
| 41 | Dihydrosinapyl alcohol | 0.3 | s |
| 42 | *cis*-Sinapyl alcohol | 0.3 | s |
| 43 | *trans*-Sinapyl alcohol | 1.5 | s |
| 44 | *trans*-Sinapaldehyde | 4 | s |

### Structural analysis of lignins with quantitative $^{13}$C-NMR

Quantitative $^{13}$C-NMR was used to estimate the abundance of each inter-unit linkage and functional

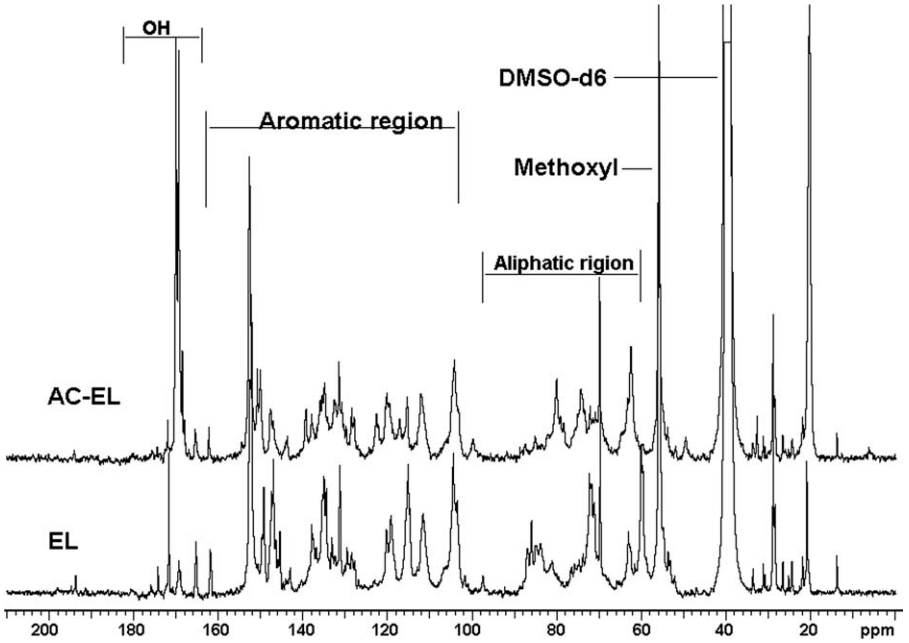


**Figure 6** $^{13}$C-NMR spectra of EL and AC-EL.

groups in prepared lignins. $^{13}$C-NMR spectra of the nonacetylated and acetylated EL are shown in Figure 6. Corresponding peak assignments identified for the provided lignin is shown in Tables VI and VII. Signals were assigned by comparing with the literature.[3,8] The $^{13}$C-NMR spectra can be divided into structural unit regions and integrated to obtain structural information. Table VIII lists the calculated various spectral regions identified in the quantitative $^{13}$C-NMR spectra with results reported as number of units per aromatic ring. Calculations were adapted from the published literature.[8] Integration was performed by setting a value of 6.12 for the aromatic region (159.6–101 ppm) and the side-chain carbons.

The amount of β-O-4 moieties (Structures I and II in Fig. 1) in EL was estimated as 0.59/Ar (per aromatic unit; Table VIII). The amount of side chain carbons of phenyl propane units was also estimated for EL as 2.72/Ar. The theoretical amount of these carbons is 3.

Methoxyl group (OMe) content was estimated from the integral at 56–54 ppm as 1.27/Ar (Tables V). The aromatic region can be divided into oxygenated aromatic carbons ($C_{Ar—O}$), aromatic carbon–carbon ($C_{Ar—c}$), and protonated aromatic carbons (125–102 ppm).[8]

$^{13}$C-NMR data of EL shows that the contribution of *p*-hydroxy phenolic (h), guaiacyl (g) and syringyl (s) units in *P. deltoides* is in order of 5 : 60 : 35, respectively. This order is in accordance with Py-GC/MS analysis in which the amount of s unit structures was lower than g structures (Table V). Surprisingly, high amount of guaiacyl units (g) regarding to syringyl units (s) is a interesting results since analysis of other hardwood lignins such as Eucalypt species indicate a higher amount of syringyl (s) than guaiacyl (g).[8,13] Recent study made by Rencoret et al.[23] show a similar order for Paulownia wood lignin which is also a fast growing species like *P. deltoides*. It seems that fast growing hardwood species unlike the other hardwoods lignin contains a low amount of syringyl units. The total condensed units in lignins can be estimated from the amount of aromatic carbon–carbon ($C_{Ar—c}$) and protonated aromatic carbons. Carbon–Carbon aromatic region consists of the $C_1$ of side chain and $C_5$ of condensed aromatic moieties such as phenylcoumaran (structure III) or biphenyl (structure XIV). Since benzyaldehyde structures overlapped by $C_\beta$ in cinnamylaldehyde moieties (VIII), estimating the degree of condensation by direct integration of this region always contains some error. Estimating the degree of condensation using protonated aromatic region (125–102 ppm) is more precise than aromatic carbon–carbon ($C_{Ar—c}$).[8]

The amount of experimental protonated aromatic carbons ($C_{Ar—H}$) in the lignin preparation was estimated to be 2.40/Ar (Table VI). The theoretical amount of $C_{Ar—H}$ atoms can be calculated from the h : g : s ratio considering the contribution of two carbons for s and h units and three carbons for g units in this region (Table VIII). The difference between the theoretical and experimental values gives the amount of condensed moieties as 20% (Table VIII). Extended milling causes more condensation reactions in lignin. Therefore, by reducing the milling

**TABLE VI**
**Signal Assignment in the Spectrum of Nonacetylated EL**

| No. | Chemical shift | EL | Assignment |
|---|---|---|---|
| 1 | 200-196 | 0.02 | α–CO except II |
| 2 | 196-194 | 0.03 | C=O in II and VIII |
| 3 | 194-191 | 0.02 | IXα |
| 4 | 178-175 | 0.02 | Vs |
| 5 | 173-171 | 0.09 | Aliphatic COOR |
| 6 | 170-168 | 0.07 | Conjugated COOR |
| | 166-164 | 0.05 | α CO in ArCOOH |
| | | | $C_{3,5}$ in etherified s units |
| | | | $C_{3,5}$ in etherified XIII |
| 7 | 155-150 | 0.95 | $C_3$ in etherified XIV |
| | | | $C_\alpha$ in VIII, $C_4$ in conjugated CO/COOR g units |
| | | | $C_{3,6}$ in V of g units, $C_{3,5}$ in V of S units |
| 8 | 150.5-148 | 0.25 | $C_3$ in noncondensed g units |
| | 148-144 | 0.57 | $C_3$,$C_4$,$C_5$ in g units |
| | 144-141 | 0.09 | $C_4/C_{4'}$ in 5-5 units (XIV) |
| | 136-134 | 0.6 | $C_1$ in g β-O-4 |
| 9 | 113-109 | 0.47 | $C_2$ in g units |
| 10 | 109-100 | 0.93 | $C_{2,6}$ in s units |
| | | | $C_{2,6}$ in XIII |
| 11 | 88-82 | 0.69 | $C_\beta$ in I and II (Cα′ in V with β-O-4) |
| | | | Cα in III |
| | | | Cα in IV |
| 12 | 78-70 | 1.02 | Cα in I |
| | | | Cα in VI |
| | | | Cγ in IV, C in carbohydrates |
| 13 | 65.5-61 | 0.35 | Cγ in V |
| | | | Cγ in VI |
| | | | Cγ in III |
| | | | Cα in X |
| 14 | 56-54 | 1.27 | —$OCH_3$ |
| 15 | 54-52 | 0.16 | $C_\beta$ in III |
| | | | $C_\beta$ in IV |
| 16 | 34-32 | 0.03 | $C_\beta$ in XII |
| **Clusters** | | | |
| 1 | 220-210 | <0.01 | Nonconjugated C=O |
| 2 | 200-190 | 0.05 | Conjugated C=O |
| 3 | 162-142 | 1.89 | $C_4$ in h units |
| | | | $C_{3,5}$ in s units |
| | | | $C_{3,4}$ in g units (except XIII) |
| | | | $C_{3,5}$ in XIII, |
| | | | $C_{3,6}$ in V of g units, |
| | | | $C_\alpha$ in VIII |
| 4 | 125-101 | 2.40 | $C_{Ar—H}$ |
| 5 | 90-61 | 2.56 | Alk—O—, $C_\beta$ in V |
| 6 | 90-78 | 0.9 | Alk—O—Ar, α—O—Alk |
| 7 | 77-65 | 1 | γ—O—Alk, $OH_{sec}$ |
| 8 | 65-58 | 0.95 | $OH_{prim}$ + $C_\beta$ in V |

See Figure 1 for the presented structures.

time and application of a selective enzymatic sequence the structure of lignin was less degraded during the extraction process. Moreover, condensation in lignin is a very important aspect in reactivity of lignin during pulping, since by increasing the condensation degree the reactivity of lignin structure in alkaline conditions is reduced considerably.

From the $^{13}$C-NMR spectra, the signal for phenylcoumaran sub-structures (III) (87–85.5 ppm) estimated to be 0.05/Ar. Signal of 50–48 ppm of acetylated lignin belongs to phenylcoumaran (III) and β-1 (VI) sub-structures. Therefore, the amount of β-1 moieties can be easily estimated as 0.03/Ar (Table VIII). Integration of 200–190 ppm region belongs to carbonyl (—C=O) groups and it is estimated as 0.05 per aromatic ring for EL (Table VII). In particular, integrations of the peak at 194 ppm representing cinnamyl aldehyde (VIII) and α-carbonyl type β-O-4 sub-structures (II) is 0.03 per aromatic ring. The presence of α-carbonyl type structures indicates that oxidation probably occurs during the milling or enzymatic extraction process. The presence of benzaldehyde structures such as vanillin (IX) by Py-GC/MS (Table V) further suggests that the milling process leads not only to side-chain oxidation but also to small structural degradation.[24]

**TABLE VII**
**Signal Assignment in the Spectrum of Acetylated EL**

| No. | Chemical shift | EL | Assignment |
|---|---|---|---|
| 1 | 200-196 | – | α—C=O exept II |
| 2 | 194-193 | 0.01 | C=O in II and VIII |
| 3 | 193-190 | – | $C_\alpha$ in XI |
| 4 | 182.5-180 | – | $C_4$ in V of g units |
| 5 | 178-175 | 0.02 | $C_4$ in V of s units |
| 6 | 170.4-169.3 | 0.69 | Primary aliphatic OH |
| 7 | 169.3-168.3 | 0.73 | Secondary aliphatic OH |
| 8 | 168.3-166 | 0.18 | Phenolic OH, conjugated COOR |
| 9 | 158-156 | – | $C_4$ in etherified h units |
| 10 | 148-144.5 | 0.28 | $C_4$ in etherified h units except XIII conjugated g units |
| 11 | 144.5-142 | 0.07 | $C_3$ in III, $C_4$ in conjugated etherified s units |
| | | | $C_4$ in conjugated XIII |
| 12 | 113-109 | 0.43 | $C_2$ in g units |
| 13 | 105-101 | 0.66 | $C_{2,6}$ in s units, $C_{2,6}$ in XIII |
| 14 | 87-85.5 | 0.05 | $C_\alpha$ in III |
| 15 | 60-59 | 0.01 | Cγ in XI |
| 16 | 58-54 | 1.30 | —$OCH_3$ |
| 17 | 54-53 | 0.06 | $C_\beta$ in IV |
| 18 | 50.5-47 | 0.08 | $C_\beta$ in VI, $C_\beta$ in III |
| **Clusters** | | | |
| 1 | 210-200 | <0.01 | Nonconjugated C=O |
| 2 | 200-190 | 0.05 | Conjugated C=O |
| 3 | 162-142 | 1.76 | $C_4$ in h units |
| | | | $C_{3,5}$ in s units |
| | | | $C_{3,4}$ in g units (except XIII) |
| | | | $C_{3,5}$ in XIII, |
| | | | $C_{3,6}$ in V of g units, |
| | | | $C_\alpha$ in VI |
| 4 | 125-101 | 2.40 | $C_{Ar—H}$ |
| 5 | 90-61 | 2.37 | Alk—O— |
| 6 | 90-78 | 0.9 | Alk—O—Ar, α—O—Alk |
| 7 | 77-65 | 1 | γ—O—Alk, $OH_{sec}$ |
| 8 | 65-61 | 0.89 | $OH_{prim}$ |

See Figure 1 for the presented structures.

**TABLE VIII**
**Calculation of Various EL Moieties by Quantitative $^{13}$C-NMR**

| Structure | Calculation | No. of moieties per aromatic ring ($C_9$) |
|---|---|---|
| α-CO/β-O-4 (II) | $(I196\text{-}193)_{na}$ | 0.03 |
| Spirodienone structures (V) | $(I\ 182.5\text{-}180) = V_g$ | <0.01 |
| | $(I\ 177.5\text{-}175) = V_s$ | 0.02 |
| Pino/syringyresinol (IV) | $(I\ 54\text{-}52)_{ac}/2$ | 0.05 |
| | $(I\ 54\text{-}52)_{na}$-III/2 | |
| Phenylcoumaran (III) | $(I\ 87\text{-}85.5)_{ac}$ | 0.05 |
| β-1 (VI) | $(I\ 50\text{-}48)_{ac}$-III | 0.03 |
| 5-5′ (XIV) | (I 144-141)na-III | 0.04 |
| β-O-4/α-OH (I) | $(I\ 78\text{-}70)_{na}$-VI-IV − 3× carbohydrates | 0.43 |
| Ar-$CH_2$OH structures (X) | $(I\ 65.5\text{-}61)_{na}$-III-VI-V | 0.2 |
| β-O-4 total | $(I\ 88\text{-}82)_{na}$ − III-IV | 0.59 |
| γ-O-Alk total | (I 77-65)-$OH_{sec}$ | 0.27 |
| Alk-O-Ar | (I 90-77)-IV-V(×2) | 0.81 |
| Phenolic OH | $(I\ 168.3\text{-}166.1)_{ac}$-COOR conj | 0.11 |
| Total OH | (I 171.6-166.1) | 1.53 |
| Side chain | (I 100-45)-OMe-5.5(carb%)+ | 2.72 |
| | $(I\ 174\text{-}165)_{na}$+(I 210-190) | |
| s | $(I\ 106\text{-}100)_{ac}$: 2-$V_s$ | 0.35 |
| | OMe-!+h | |
| g | $(I\ 113\text{-}111)_{ac}+V_s$ | 0.60 |
| h : g : s (100% base) | g = 1-s-h | 5 : 60 : 35 |
| Degree of condensation | [3g+2(s+h)]-(I126-102) | 0.2 |
| Etherified total | 1-OHph-V | 0.87 |
| s-etherified | Total s –PhOHs-$V_s$ | 0.23 |
| g-etherified | Total g –$PhOH_g$-$V_g$ | 0.54 |
| g-etherified noncondensed | $(I\ 151\text{-}149)_{na}$ | 0.41 |
| 4-O-5′$_{et}$ ($XIII_{et}$) | $g_{et}$-$(I\ 149\text{-}143)_{ac}$ | 0.19 |

ac, acetylated; nc, nonacetylated.

The oxygenated aliphatic region can also provide more detailed structural information about the quantities of inter-unit linkages in the lignin preparations. The spectral region of 145.5–141 ppm represents etherified and non-etherified $C_4$ carbons of biphenyl (5–5′) groups (XIV) and $C_3$ carbons of phenylcoumaran (β-5) substructures (III). Integration of this region results in value of 0.09/Ar. The degree of condensation as determined by integration of the aromatic methine region (127–103 ppm) was 0.20 (Table VII). It means that 20% of the extracted lignin structure is condensed. On the basis of these values, either the total amounts of 5–5′ (XIV) and β-5 subunits (IIV) are slightly underestimated or other structural moieties such as 4-O-5′ (XIII) are contributing to condensation in the lignin polymer. From the above integrations, EL contains 0.04, 5–5′ substructure per aromatic ring. These values agree with those reported in the literature.[16,25]

Widespread condensation probably does not occur due to the rigidity of the solid lignin matrix; however, condensation may still occur in structures of close proximity. It has been reported that the initial release of lignin during milling occurs primarily from regions of high lignin content, presumably the cell corners and middle lamella.[26] These regions are known to contain a higher degree of condensation than the lignin contained in the secondary wall. Because EL is produced by degradation of carbohydrates, it should be derived primarily from the secondary wall.

Spirodienone is a newly discovered structural unit in lignin and has a characteristic chemical shift of 181 ppm in the $^{13}$C-NMR spectrum representing the

**TABLE IX**
**Structural Characteristics of *P. deltoides* EL and Lignin Isolated from *E. globulus* (6)**

| | Relative abundance (% Side-chains involved) | |
|---|---|---|
| Linkage | *E. globulus* (6) | *P. deltoides* |
| β-*O*-4 structures (I) | 69 | 59 |
| Resinols (IV) | 3 | 5 |
| Phenylcoumarans (III) | 2.9 | 5 |
| Spirodienones (V) | 2.8 | 2 |
| β-1 structures (VI) | 2 | 3 |
| Dibenzodioxocins (XIV) | 0 | 4 |
| Percentage of lignin units | | |
| h (%) | 2 | 5 |
| g (%) | 36 | 60 |
| s (%) | 62 | 35 |
| s/g ratio | 1.72 | 0.58 |
| Phenolic content (%) | 19 | 11 |
| Phenolic/aliphatic hydroxyls ratio | 0.14 | 0.07 |

See Figure 1 for the presented structures.

β-carbon.[6] Integration indicates amount of 0.01 per aromatic unit for this structure in enzymatic lignin (Table VII). Compared to other hardwoods commonly used for papermaking, such as eucalypt wood,[3] the lignin from Poplar presents almost a same proportion of β-O-4 ether linkages, and a lower proportion of carbon–carbon linked (condensed) structures (such as β-β, β-5, 5–5, and β-1) and a lower s/g ratio. Since the β-O-4 ether linkages are cleaved to a high extent during alkaline cooking, while condensed linkages resist alkaline cooking conditions,[7] the relatively low content of condensed structures in the lignin from poplar wood will fascinate this wood more to pulp and paper industry. Table IX shows the comparison of different linkages between *E. globulus* lignin and *P. deltoides* analyzed in present study.

## CONCLUSIONS

*P. deltoides* lignin prepared by sequential enzymatic treatment has regular structure, fair molecular weight but possibly less dissolved in GPC solvent after derivitization (i.e., acetylation) and much less protein impurity and little amount of carbohydrates. The C, H, N, O analysis of lignin reveals an average per $C_9$ unit formulae of $C_9H_{8.87}O_{3.78}(OCH_3)_{1.27}$ for *P. deltoides*. Enzymatic lignin of *P. deltoides* wood has been characterized by Py-GC/MS and $^{13}$C-NMR techniques. The G units amount was considerably higher than S units with a h : g : s molar composition of 5 : 60 : 35. The main structure present in *P. deltoides* lignin is β-O-4 which accounts for 59% of side chains, followed by pino/syringyresinol (IV) and phenylcoumaran (III) that involved each 5%. Other structures like spirodienone (IV), β −1 (VI) and 5–5′ (XIV) were present 2, 3 and 4%, respectively. Comparing to common used species for pulping softwoods and hardwood such as eucalypt, *P. deltoides* contains almost a good proportion of β-O-4 ether linkages and low content of condensed (C—C linkaged) structures.

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## References

1. Little, E. L. Checklist of United States Trees (Native and Naturalized), Agriculture Handbook 541; U.S. Department of Agriculture: Washington DC, 1979.
2. Ikeda, T.; Holtman, K.; Kadla, J. F.; Chang, H. M.; Jameel, H. J Agric Food Chem 2002, 50, 129.
3. Rencoret, J.; Marques, G.; Gutierrez, A.; Ibarra, D.; Li, J.; Gellerstedt, G.; Santos, J. I.; Jimenez Barbero, J.; Martinez, A. T.; Del Rio, J. C. Holzforschung 2008, 62, 514.
4. Nimz, H. H. Angew Chem Int Ed Engl 1974, 13, 313.
5. Adler, E. Wood Sci Technol 1977, 11, 169.
6. Gellerstedt, G.; Zhang, L. J. Wood Chem Technol 1992, 12, 387.
7. Gierer, J. Wood Sci Technol 1985, 19, 289.
8. Capanema, E. A.; Balakshin, M. Y.; Kadla, J. F. J Agric Food Chem 2005, 53, 9639.
9. Guerra, A.; Filpponen, I.; Lucia, L. A.; Argyropoulos, D. S. J Agric Food Chem 2006, 54, 9696.
10. Fujimoto, A.; Matsumoto, Y.; Chang, H. M.; Meshitsuka, G. J Wood Sci 2005, 51, 89.
11. Wu, S.; Argyropoulos, D. S. J Pulp Pap Sci 2003, 29, 235.
12. Chang, H.; Cowling, E.; Brown, W. Holzforschung 1975, 29, 153.
13. Guerra, A.; Lucia, L. A.; Argyropoulos, D. S. Holzforschung 2008, 62, 24.
14. Hu, Z.; Yeh, T. F.; Chang, H.-M; Matsumoto, Y.; Kadla, J. F. Holzforschung 2006, 60, 389.
15. Holtman, K. M.; Chang, H. M.; Kadla, J. F. J Agric Food Chem 2004, 52, 720.
16. Ibarra, D.; Del Río, J. C.; Gutiérrez, A.; Rodríguez, I. M.; Romero, J.; Martínez, M. J.; Martínez, Á. T. Enzyme Microb Technol 2004, 35, 173.
17. Hatfield, R. D.; Grabber, J.; Ralph, J.; Brei, K. J Agric Food Chem 1999, 47, 628.
18. Blakeney, A. B.; Harris, P. J.; Henry, R. J.; Stone, B. A. Carbohydr Res 1983, 113, 291.
19. Faix, O.; Meier, D.; Fortmann, I. Holz Roh-Werkstoff 1990, 48, 351.
20. Ralph, J.; Hatfield, R. D. J Agric Food Chem 1991, 39, 1426.
21. Faix, O. In Methods in Lignin Chemistry; Lin, S. Y., Dence, C. W., Eds.; Springer-Verlag: Berlin, 1992; pp 104–132.
22. Sarkanen, K. V.; Hergert, H. L. Lignins, Occurrence, Formation, Structure and Reactions; Sarkanen, K. V., Ludwig, C. H., Eds.; Wiley: New York, 1971; pp 43–94.
23. Rencoret, J.; Marques, G.; Gutiérrez, A.; Nieto, L.; Jiménez-Barbero, J.; Martínez, Á. T.; Del Río, J. C. Indus Crops Prod 2009, 30, 137.
24. Adler, E.; Brunow, G.; Lundquist, K. Holzforschung 1987, 41, 199.
25. Evtuguin, D. V.; Pascoal Neto, C.; Silva, A. M. S.; Dominges, P. M.; Amado, F. M. L.; Robert, D.; Faix, O. J Agric Food Chem 2001, 49, 4252.
26. Balakshin, M.; Yu., E. A.; Goldfarb, B.; Frampton, J.; Kadla, J. F. Holzforschung 2005, 59, 488.