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Structure and Radical Scavenging Activity Relationships of Pyrolytic Lignins

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ABSTRACT: This work deals with antioxidant properties of pyrolytic lignins against two free radicals, 1,1-diphenyl-2-picrylhydrazyl and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid). Pyrolytic lignins produced by the thermal pyrolysis of the Etek lignin were extracted from the liquid pyrolysis product and fractionated using solvents of different polarities. The main functional groups linked to the lignin skeleton have been characterized by ^{1}H NMR and ^{19}F NMR upon acetylation and trifluoromethylation, respectively. Their radical scavenging activity against targeted free radicals was evaluated in vitro, and it was correlated to the content of studied functional groups. In contrast to the extracted Etek lignin, thermal pyrolysis produces lignin adducts that have higher scavenging efficacy than the nonthermally altered lignin and even higher than that of quercetin, a well-known phenolic antioxidant. The phenyl hydroxyl and methoxyl groups appear to be the main lignin structural factors contributing to the overall scavenging properties against the DPPH and ABTS free radicals. Also, these results show that there is no correlation between the content of aliphatic hydroxyl and carbonyl groups and the antioxidant activity.

KEYWORDS: *pyrolytic lignin, acetylation, trifluoromethylation, functional groups, free radical scavenging activity*

INTRODUCTION

Lignin is a dendritic network polymer of oxygenated phenyl propene monomer units that contains a variety of linkages connecting the different aryl ethers. This structure makes lignin a potential renewable source for the production of aromatic compounds. The ratio of the different linkage types varies according to the type of biomass. For example, more β-O-4 linkages are found in hardwood lignin than in softwood lignin.[1] Additional major linkages are represented by the β-5, 5-5, 4-O-5, β-1-, α-O-4, and β-β linkages, of which the proportion varies considerably from one plant species to another and within individual parts of the plant (leaf, stem, root, seed, etc.). Etek lignin is a solid residue obtained following an acidic treatment of woods to produce fermentable sugars, as substrate for ethanol production. Its approximate composition is mainly made of 45.7% cellulose and 29.2% lignin, with extractives, hemicelluloses, and inorganics accounting for the remainder.

In the search for an alternative to petroleum feedstocks, the thermochemical processing of lignin achieves depolymerization by the cleavage of linkages to produce value-added pyrolysis oil, of which pyrolytic lignin is a major component. Pyrolysis (heating in the absence of oxygen) induces cracking, which can randomly cleave most lignin linkages, producing lignin-derived chemicals of different molecular weights, structures, and functionalities.[2]

The free radical scavenging activity of lignin as a natural antioxidant is well documented.[3−8] Several papers have mentioned lignin's protective effect against free radicals, lipid peroxidation, and DNA oxidation and its stabilizing effect on material composites and recycled materials. However, papers on the antioxidant properties of the thermally degraded pyrolytic lignin are seldom found in the literature. Therefore, the aim of the present work was to compare pyrolytic lignins to nondegraded lignin and a well-known phenolic antioxidant (quercetin) in terms of functional groups and antioxidant activity. This study is consistent with our quest for the production of renewable chemicals from the pyrolysis of biomass.

Herein, we report on the characterization and radical scavenging ability of pyrolytic lignins obtained by fast pyrolysis of commercially available Etek lignin from SEKAB (Örnsköldsvik, Sweden). Its structure is altered from natural lignin, but it still contains polyphenols and could be a potentially useful source of aromatics for the chemical industry.

We quantitatively determined the functional groups present in the isolated lignins and studied the free radical scavenging properties of each lignin fraction to determine the influence of the major structural features. To establish the structure−activity relationship, the half-inhibition values (IC_{50}) of the antioxidant activity were plotted against the content of each functional group. Resulting correlations qualitatively show that the radical scavenging properties increase for lignin having high contents of aryl hydroxyl and methoxyl groups.

METHODS

Fast Pyrolysis of Etek Lignin. The Etek lignin supplied by SEKAB E-Technology (Örnsköldsvik, Sweden) was pyrolyzed in a bubbling fluidized bed of quartz sand at 500 °C. Details about the reaction system have been described in previous papers.[9,10]

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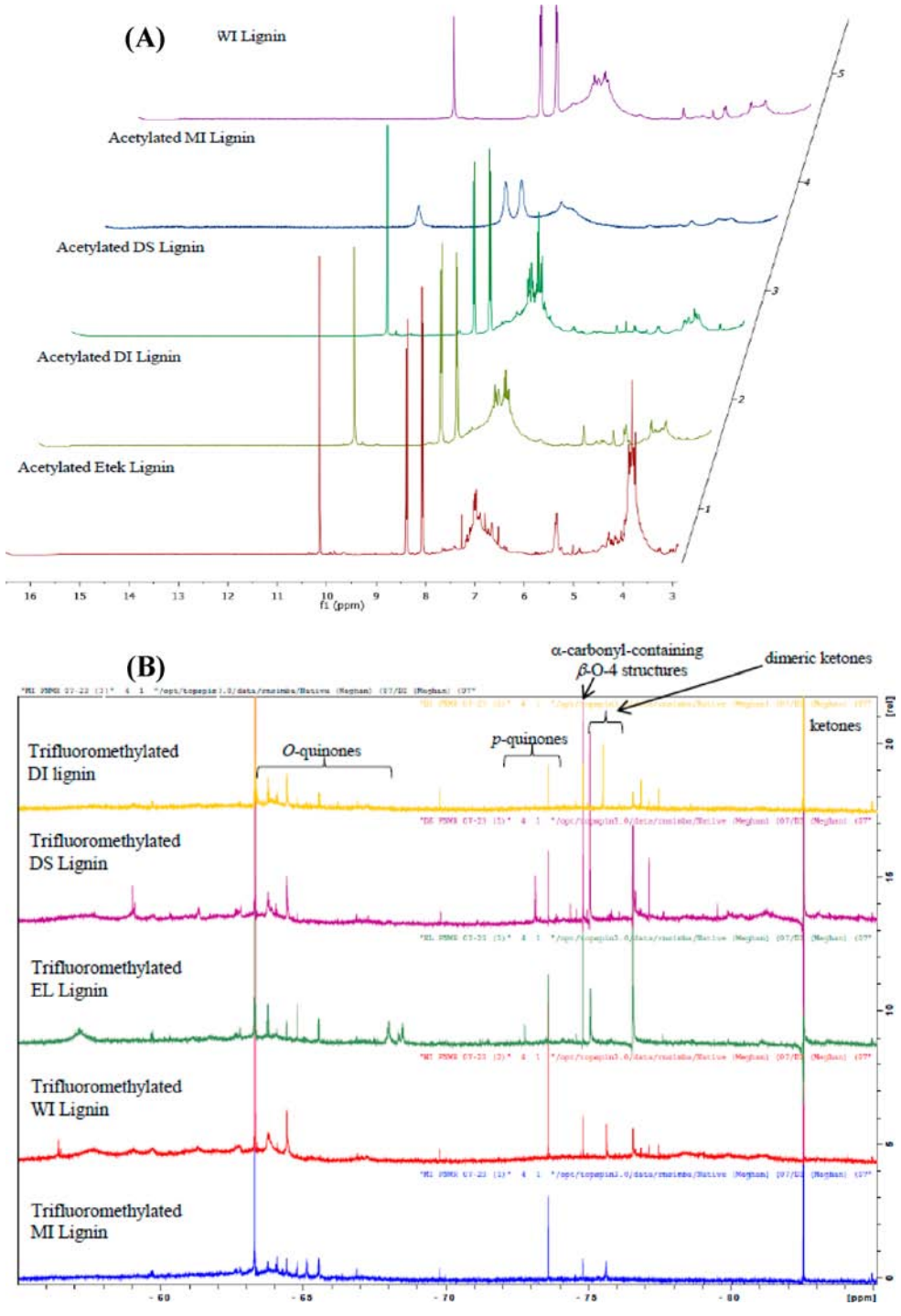

Figure 1. ^{1}H NMR 400 MHz (A) and ^{19}F NMR 376 MHz (B) spectral profile of acetylated and trifluoromethylated lignins. DI, dichloromethane insoluble; DS, dichloromethane soluble; MI, methanol insoluble; WI, water insoluble; EL, extracted Etek lignin.

Isolation and Fractionation of Pyrolytic Lignins. A solid sample made of a mixture of pyrolysis oil with sand was loaded in a porous cellulose thimble and Soxhlet extracted (70 ± 1 °C) using hexane. Nonpolar chemicals, namely, benzene, toluene, xylenes, asphaltenes, monomeric phenols, and polycyclic hydrocarbons, have been removed. To extract the lignin, the remaining cake was dissolved in methanol under shaking. The solid supernatant was filtered through Whatman filter paper no. 3 and washed with methanol to yield the methanol-insoluble (MI) lignin. Then, the filtrate was concentrated under vacuum and mixed with water to produce the water-soluble (aqueous) and water-insoluble (solid) fractions. After mixing with dichloromethane, the water-insoluble fraction was separated upon filtration to produce both dichloromethane-soluble (DS) and -insoluble (DI) fractions. The solid particles immersed in the water-soluble fraction were separated by filtration, washed by water, and dried to yield the water-immersed (WI) lignin. Extracted lignins were soaked on dry ice with methanol and freeze-dried to produce fine powders called pyrolytic lignins. The products were stored in desiccators over P_2O_5 prior to further studies.

Extraction of Etek Lignin. For comparison purposes, lignin was extracted from the same commercial Etek lignin using THF under stirring at 45 °C for 2 h (three times). The THF extract was filtered and concentrated under vacuum. The concentrate was added, dropwise, to water under stirring to yield a precipitate that was

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Table 1. Characterization and Radical Scavenging Activities of Isolated Lignins[a]

		acetylated lignin (mmol/g of lignin)			trifluoromethylated lignin (mmol/g of lignin)	IC_{50} (μg/mL)	
lignin	yield (%)	ArOH	AlkOH	MeO	C=O	DPPH	$ABTS^{\bullet+}$
EEL	12.0	7.3 ± 0.02	3.8 ± 0.06	3.8 ± 0.06	0.60 ± 1.18	188.0	310.0
MI lignin	17.2	4.6 ± 0.10	6.6 ± 0.14	nd	0.15 ± 0.41	218.0	nd
WI lignin	27.7	9.7 ± 0.82	4.6 ± 0.18	0.9 ± 0.06	0.38 ± 1.06	75.0	194.0
DI lignin	49.8	11.5 ± 0.22	3.6 ± 0.03	0.9 ± 0.03	0.16 ± 1.18	76.0	216.0
DS lignin	5.3	17.6 ± 0.32	8.2 ± 0.25	1.4 ± 0.56	0.29 ± 0.71	39.0	174.0
quercetin		nd	nd	nd	nd	51.0	98.0

[a]Characterization of acetylated lignins has been made using ^{1}H NMR. ArOH, aromatic hydroxyls; AlkOH, aliphatic hydroxyls; MeO, methoxyls; IC_{50}, half-inhibition; with EEL, the lignin extracted from the commercial Etek lignin (nonthermally altered). Pyrolitic lignins are names as follows: DI, dichloromethane insoluble; DS, dichloromethane soluble; MI, methanol insoluble; WI, water insoluble. DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid).

filtered, washed with water, and dried in a vacuum oven over P_2O_5 at 65 °C for 5 h. The obtained powder (extracted Etek lignin) was stored in a desiccator.

Quantitative ^{1}H NMR Spectral Determination of Lignin Phenolic Hydroxyl (ArOH), Aliphatic Hydroxyl (AlkOH), and Methoxyl (MeO) Groups.[11] *Acetylation of Lignin.* Purified lignin (0.5 g) was dissolved in 6 mL of pyridine/acetic anhydride (1:1, v/v) and kept in the dark for 72 h (room temperature, 25 °C). The solution was added dropwise to 120 mL of ice-cold water containing 1 mL of concentrated HCl, under constant stirring. The precipitated lignin acetate was collected on a 10 μm nylon membrane filter, washed with water, and dried over P_2O_5 under vacuum.

^{1}H NMR Analysis of Acetylated Lignins. Functional groups, phenolic hydroxyl (ArOH), aliphatic hydroxyl (AlkOH), and methoxy (MeO) groups) of lignin were estimated using ^{1}H NMR. Lignin acetate (50 mg) and *p*-nitrobenzaldehyde (NBA, 5 mg as internal standard) were dissolved in 0.5 mL of $CDCl_3$. The ^{1}H NMR spectrum was recorded on a Bruker Avance III spectrometer at 400 MHz. A total of 128 scans were collected. The content of each functional group was calculated from the ratio of the integrations for the protons of the functional group versus the protons of the internal standard according to the following established equation.[11]

$$F = \frac{\frac{I_F}{3} \times \frac{4}{I_{NBA}} \times \frac{W_{NBA}}{151} \times 1000}{W_L - \frac{I_{Ac}}{3} \times \frac{4}{I_{NBA}} \times \frac{W_{NBA}}{151} \times 42} \quad (1)$$

F is the content of each functional group (ArOH, AlkOH, and MeO), in mmol/g of lignin. I_F is the integration of protons of the functional group; the integration ranges used for the different functional groups are as follows (δ): 4.10−3.10 for MeO, 2.50−2.17 for the ArOH acetyl group, and 2.17−1.70 for the AlkOH acetyl group. Three is the number of protons of acetyl and methoxyl groups; 4 is the number of protons on the NBA benzene ring; I_{NBA} is the integration of four protons on the NBA benzene ring (8.4 and 8.2 ppm); W_{NBA} is the weight of NBA, in mg; 151 is the formula weight of NBA; W_L is the weight of the lignin acetate, in mg; I_{Ac} is the integration of protons of total acetyl groups corresponding to ArOH and AlkOH (2.50−1.70 ppm); and 42 is the formula weight of acetyl group minus one (43 − 1).

Quantitative ^{19}F NMR Spectral Determination of Lignin Carbonyl Groups.[12] *Trifluoromethylation Procedure.* To obtain trifluoromethylated lignin derivatives, 100 mg of lignin was dissolved in 10 mL of dry tetrahydrofuran (room temperature, under constant stirring). Then 600 μL of Me_3Si-CF_3 was added, and the mixture was cooled to 0 °C; 15 mg of tetramethylammonium fluoride (TMAF) was then added to initiate the reaction. The reaction mixture was stirred for 30 min at 0 °C and then at room temperature for 24 h. The resulting trifluoromethylated siloxy adducts were then hydrolyzed by adding 50 mg of TMAF at room temperature for 24 h in THF, followed by solvent evaporation under reduced pressure. The residue was water-washed and centrifuged (3 × 50 mL) prior to being dissolved in a solvent mixture (dioxane/water, 25:5, v/v) and freeze-dried under reduced pressure.

^{19}F NMR Spectroscopy. ^{19}F NMR spectra of trifluoromethylated lignin were recorded on a Bruker Avance III NMR spectrometer at 376 MHz. Derivatized lignin (15−20 mg) was dissolved in 800 μL of a solvent mixture (pyridine/deuterated chloroform, 1.6:1, v/v) and stirred with a magnetic bar until complete dissolution. Then, 100 μL of the internal standard solution (1.69 g/mL, 3,3′-bis(trifluoromethyl)-benzophenone in 1.6:1, v/v, pyridine/$CDCl_3$) was added to the mixture. Quantitative ^{19}F NMR spectra were acquired at defined conditions using a 128 scans per measurement and a 5 s delay time.

Evaluation of the Free Radical Scavenging Activities. *DPPH Free Radical Scavenging Activity.* A previously described method[3] was used to evaluate the radical scavenging capability of lignin samples with the use of 1,1-diphenyl-2-picrylhydrazyl (DPPH) as the radical generator. To 1180 μL of a 6.1 × 10^{-5} mol/L DPPH methanol solution in a test tube was added 320 μL of lignin solution (0.001−5.000 mg/mL) in 90% aqueous dioxane, and the mixture was vortexed and incubated in the dark at 25 °C for 16 min. A Varian Cary 5G UV−vis−NIR spectrophotometer (Palo Alto, CA, USA) was used to monitor the concentrations of DPPH radicals at 0 and 16 min at 515 nm. Following an established equation, the inhibition percentage (IP) of the DPPH radical was determined and plotted as a function of the lignin concentration. Obtained graphs were used to determine the lignin concentration needed to obtain 50% of the DPPH free radical inhibition (IC_{50}). Quercetin, a well-known phenolic antioxidant, was used as a positive control for a concomitant determination of antioxidant activity.

$$IP\ (\%) = \frac{OD1 - OD2}{OD1} \times 100 \quad (2)$$

OD1 is the optical density recorded at 0 min, and OD2 is the optical density measured after 16 min of incubation of the medium.

$ABTS^+$ Radical Scavenging Activity. The $ABTS^{\bullet+}$ (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging effect of isolated lignins has been determined according to a reported method.[13] Initially, the $ABTS^{\bullet+}$ radical was generated by reacting 7 mM ABTS solution in water with 2.45 mM potassium persulfate in the dark over 12−16 h. Prior to performance of the radical scavenging reaction, the absorbance of the reactant was adjusted to 0.700 ± 0.02 at 734 nm (room temperature). Then the reaction was initiated by adding 10 μL of diluted lignin solutions to 1 mL of the $ABTS^{\bullet+}$ solution. The absorbance of the mixture was recorded at 734 nm 1 and 20 min after the addition of the diluted sample. The inhibition percentage (IP) of the $ABTS^{\bullet+}$ radical was determined as the percent of the decrease of the optical density of each medium. The IC_{50} was determined as above-described. All determinations were performed in duplicate with quercetin as positive control.

RESULTS AND DISCUSSION

Lignin Structural Features. To explore how pyrolysis-induced structure modification of lignin affects its functionality (expressed as its reactivity in the presence of free radicals), a quantitative determination of functional groups (phenolic

hydroxyl, aliphatic hydroxyl, methoxyl, and carbonyl groups) has been done using the ^{1}H NMR of acetylated lignins and ^{19}F NMR of trifluoromethylated lignins (see Figure 1 for the profile). Those chemical features have been reported to positively influence the antioxidant activity of natural lignins.[3,14,15] Our results (see Table 1) show that prior to the fluidized-bed fast pyrolysis, the Etek lignin structural features were as follows: phenolic hydroxyls (ArOH, 7.3 mmol/g of lignin), aliphatic hydroxyls (AlkOH, 3.8 mmol/g of lignin), and methoxyls (MeO, 3.8 mmol/g of lignin). Following the pyrolysis, there was generally a substantial increase of ArOH and AlkOH accompanied with the loss of MeO on the pyrolytic lignins isolated. An explanation is that pyrolysis promoted the cleavage of the α- and β-ether linkages between lignin subunits, resulting in the formation of new ArOH and AlkOH. During the Etek lignin pyrolysis, the thermolytic cleavage of the methoxyl group of the guaiacyl ring led to the decrease of the methoxyl content in most PLs. The lack of methoxyls in the MI PLs suggests that this pyrolytic product possesses a chemical structure close to H-(*p*-hydroxyphenyl) type lignin. DS PL represents the lignin pyrolytic product with higher content of ArOH, AlkOH, and MeO that should increase its functionality, especially when scavenging reactive oxygen.

The ^{19}F NMR spectra of trifluoromethylated lignins displayed well-resolved signals ranging from −64 to −87 ppm. Major carbonyl-containing moieties were mainly *o*-quinones (from −62.5 to −68 ppm), *p*-quinones (between −72 and −74 ppm), α-carbonyl-containing β-O-4 structures (−74.5 ppm), dimeric ketones (from −74 to −76 ppm), and ketones (−82 ppm). Results show high carbonyl content (see Table 1) in the extracted Etek lignin. Comparison to resulting pyrolytic lignins showed carbonyl contents that were significantly decreased by the thermal treatment.

Evaluation of Lignin Free Radical's Scavenging Activities. The antioxidant activity of isolated lignins was determined as their scavenging capability against two well-known free radicals: 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$).

Influence of Lignin Functional Groups on DPPH Scavenging Activity. The spectrophotometric method based on the reduction of the stable DPPH free radicals has been used in this work as an antioxidant assay because it provides an easy, rapid, and convenient method to evaluate the antioxidants and radical scavengers.[16−18] All isolated lignins showed significant free radical scavenging activity in a dose-dependent manner (see Figure 2). Figure 2 shows similar curves for the DS lignin, quercetin (the phenolic used as the control), DI lignin, and WI lignins; the MI and Etek lignins are less reactive. The DS lignin shows the highest activity of all the samples. For screening purposes, the IC_{50} of each lignin has been calculated for better comparison to quercetin, the well-known phenolic antioxidant used. Values of DPPH IC_{50} ranged from 39 to 218 μg/mL. A higher antioxidant activity results in a lower IC_{50} value. The results (see Table 1) indicate that the DS lignin is the most effective pyrolytic lignin fraction tested for scavenging the DPPH free radicals. The IC_{50} value of the DS lignin is even lower than those of the control (quercetin) and the Etek lignin. IC_{50} values of the DI and WI lignins are not significantly different and are in an acceptable range for a potent antioxidant. This indicates that the pyrolysis of the Etek lignin yields products of higher potency as chemical antioxidants. The variance in the scavenging potency will depend on the

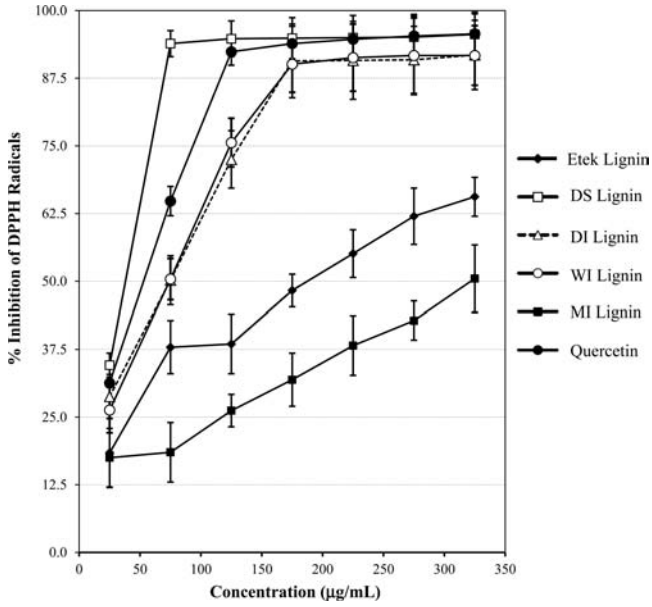

Figure 2. DPPH radical scavenging capacity of isolated lignins (Etek and pyrolytic lignins). The radical scavenging ability of various concentrations (25−325 μg/mL) of lignin was analyzed by measuring their inhibitory effects on the absorbance of the DPPH radicals. Absorbance of the reaction was measured at 517 nm. The reaction was performed in duplicates, and results were expressed as percent inhibition of the absorbance of the DPPH radicals. DS, dichloromethane soluble; DI, dichloromethane insoluble; WI, water insoluble; MI, methanol insoluble.

hydrogen-donating or electron-donating ability of structural features attached to each lignin. The DS lignin, which has shown the higher DPPH scavenging activity, bears (see Table 1 and Figure 1) the higher content of ArOH and AlkOH. It is important to note that the MI lignin that has shown the lower ArOH accompanied with a high AlkOH content had demonstrated the weaker radical scavenging activity. The fact that the MI lignin, which has a higher AlkOH content than DS, but a lower ArOH content and no MeO groups, has the lowest activity suggests that AlkOH content might not closely influence the lignin antioxidant properties. A strong relationship between MeO content and activity has been observed on the DS, DI, and WI lignins. Conversely, the higher MeO content observed from the Etek lignin did not lead to a higher DPPH radical scavenging efficiency; in addition, quercetin (the control), a flavonoid that has no MeO groups but bears five ArOH, has a scavenging activity next to the highest lignin antioxidant (DS lignin). These data indicate that the ArOH groups have the greatest effect on the antioxidant activity of the substrate. This makes sense on the basis of what is known about the stability of phenoxy radicals and the propensity for them to form in lignin-like structures. In an effort to establish a structure−activity relationship, correlations (see Figure 4) made between DPPH IC_{50} and the content of functional groups revealed significant effects of both ArOH and MeO on the lignin antioxidant activity. In contrast, because no correlation was found, it is clearly established that the AlkOH and CO contents did not positively affect the scavenging activity. This observation about AlkOH is consistent with a previous paper.[3] It implies that functional groups positively influenced the DPPH scavenging activity of isolated lignins in the following order: ArOH > MeO > AlkOH > CO.

Influence of Lignin Functional Groups on ABTS$^{\bullet+}$ Scavenging Activity. The inhibition capability of extracted lignins on the ABTS$^{\bullet+}$ radicals was studied in vitro according to the procedure described elsewhere[13] (see Figure 3 and Table 1). A different trend in the curves compared to the DPPH scavenging assay was observed. The ABTS scavenging activity was concentration dependent (see Figure 3). Quercetin, with five ArOH and a carbonyl group, demonstrated the strongest scavenging activity against the ABTS$^{\bullet+}$ free radicals with an IC_{50} value at 98 μg/mL. The DS lignin again has the highest scavenging activity among pyrolyzed lignins, with the relative order staying the same from the previous assay: DS lignin > WI lignin > DI lignin > Etek lignin > MI lignin. The IC_{50} value of the MI lignin was below the concentration range investigated in this work. No carbonyl-dependent activity was established in this study. Overall, these results strongly support the hypothesis that ArOH is the main structural feature of the lignin skeleton responsible for the observed free radical scavenging activity. However, the correlation observed between the ABTS$^{\bullet+}$ IC_{50} and the MeO content (see Figure 4) suggests that this functional group is important in the overall antioxidant activity as well. The chemistry of both (ArOH and OCH_3) functional groups provides useful information that may apply to the lignin pyrolysis products. The position of the functional groups on the aromatic ring will also have a strong influence on the radical scavenging abilities of the molecules to inductive or resonance stabilization of the resulting radicals.[19,20]

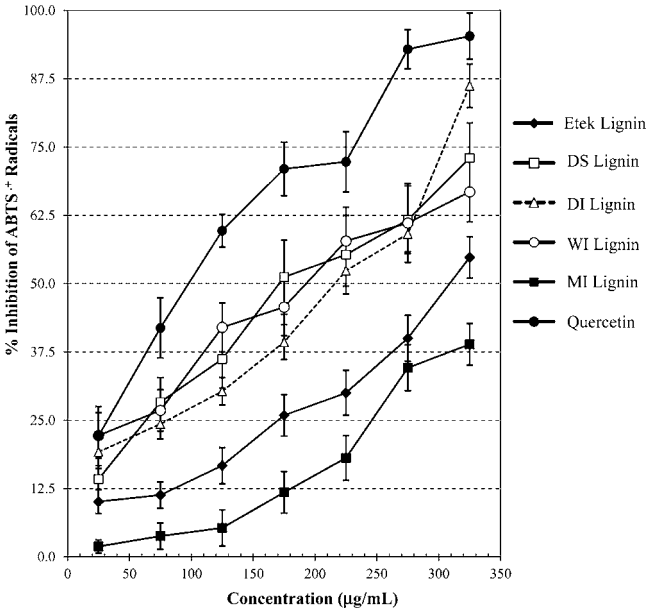

Figure 3. ABTS$^{\bullet+}$ radical scavenging capacity of isolated lignins (Etek and pyrolytic lignins). The radical scavenging ability of various concentrations (25−325 μg/mL) of lignin was analyzed by measuring their inhibitory effects on the absorbance of the ABTS$^{\bullet+}$ radicals. Absorbance of the reaction was measured at 734 nm. The analyses were performed in triplicates, and results were expressed as percent inhibition of the absorbance of ABTS radicals ± SD. DI, dichloromethane insoluble; DS, dichloromethane soluble; MI, methanol insoluble; WI, water insoluble.

This study indicates that pyrolysis of the Etek lignin produced lignin-related products with potent hydrogen-donating properties capable of acting as strong free radical scavengers. The DS lignin, the most effective antioxidant among the pyrolytic lignin fractions, demonstrated a higher DPPH scavenging efficiency than quercetin (a well-known phenolic antioxidant), suggesting that lignin pyrolysis products may serve as a green source of antioxidant chemicals for use in material composites. These results provide a good correlation between ArOH and MeO content and radical scavenging activity. With the content of other functional or unsaturated groups (which might have some effect on the lignin antioxidant

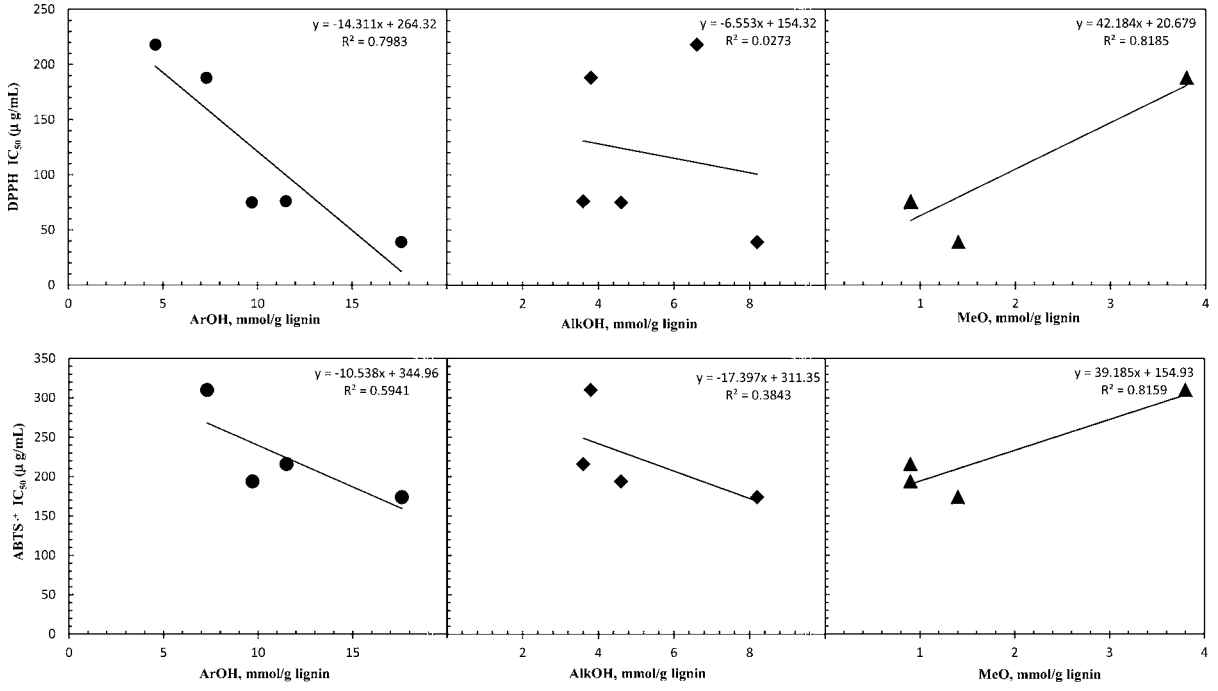

Figure 4. Correlation between functional groups and antioxidant activity of isolated lignins. Antioxidant activity (DPPH and ABTS$^{\bullet+}$) refers to the free radical scavenging activity, expressed as half-inhibition value (IC_{50}, μg/mL).

activity) unknown, some discrepancies in the scavenging activity of lignin pyrolysis products were expected. Further research is needed on lignin model compounds bearing numerous structural factors to better understand the mechanism contributing to the free radical scavenging activity of pyrolytic lignins. Therefore, further research is needed on lignin model compounds bearing the numerous structural factors involved to clearly understand the mechanism contributing to the global free radical scavenging activity of pyrolytic lignins.

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Notes

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