Characterization of Softwood and Hardwood LignoBoost Kraft Lignins with Emphasis on their Antioxidant Activity

Jevgenija Ponomarenko,[a,b,*] Tatiana Dizhbite,[a] Maris Lauberts,[a,b] Arturs Viksna,[b] Galina Dobele,[a] Oskars Bikovens,[a] and Galina Telysheva [a]

Fractionation of softwood and hardwood LignoBoost kraft lignins, using sequential extraction with organic solvents of increasing hydrogen-bonding ability (dichloromethane, n-propanol, and methanol), was carried out. Using SEC, analytical pyrolysis, FTIR and UV/VIS spectroscopy, and chemical analytical methods, four fractions were obtained and characterized in terms of their yield, composition, functionality, lignin structural features, and antioxidant properties. In tests with free radicals ($ABTS^{\bullet+}$, $DPPH^{\bullet}$, $O_2^{\bullet-}$) and the ORAC (oxygen radical absorbance capacity) assay, the high radical scavenging capacity of the lignin's soluble fractions was demonstrated. The antioxidant activity of the fractions was tested by their influence on thermo-oxidative destruction of model polyurethane elastomers. The TGA data clearly revealed the antioxidant effect of the three fractions, with the most prominent activity for the propanol-soluble fraction. The dichloromethane fraction has potential as an antioxidant for non-polar products. Novel correlations between lignin's structural features and its radical scavenging activity were found that can be used for tuning lignin's antioxidant properties.

Keywords: LignoBoost kraft lignin; Solvent fractionation; Antioxidant properties; $ABTS^{\bullet+}$; $DPPH^{\bullet}$; $O_2^{\bullet-}$ assays; ORAC assay

Contact information: a: Latvian State Institute of Wood Chemistry, 27 Dzerbenes str., LV-1006, Riga, Latvia; b: University of Latvia, Faculty of Chemistry, Kr. Valdemara str. 48, Riga, Latvia;
** Corresponding author: jevgenijaponomarenko@inbox.lv*

INTRODUCTION

The amount of technical lignin formed as a by-product of the pulp and paper industry is about 50 million tons per year (Varanasi *et al.* 2013). Moreover, a significant increase in the separation of lignin, instead of burning of black liquor, is recognized as being a reasonable approach for maximizing kraft pulp mill output, since with the increase of black liquor stream, the existing production rates of sodium regeneration aggregates would become the production bottlenecks. However, expanding recovery boiler capacity is very expensive (Varanasi *et al.* 2013). LignoBoost technology developed by Innventia AB (Tomani 2010) is considered a prospective solution for the isolation of lignin from kraft spent liquor. Currently, the majority of lignin is used as a boiler fuel, with an estimated value of 0.18 USD•kg^{-1} (Macfarlane *et al*. 2009), which is not adequate for its high potential value as polyphenol biopolymer.

At present, the manufacture of carbon fibers from the LignoBoost kraft lignin is proposed as the most effective avenue for its utilization (Norberg *et al*. 2013). Nevertheless, other applications for this lignin have to be considered; in particular, those allowing the realization of the functional and structural features of lignin as an aromatic

polymer of natural origin are promising. One such application is the production of technical antioxidants based on lignin. As of 2011, the fields of antioxidant applications were distributed as follows: rubbers (and latex) – 53%; plastics – 36%, food and nutrition – 8%, and oil fuels – 3% (Kumar 2013). Demand for natural antioxidants is increasing, primarily due to the uncertain safety of synthetic compounds. For example, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and other synthetic antioxidants cause liver damage and have demonstrated cytotoxicity and carcinogenesis in laboratory animals (Thompson and Moldeus 1988). Therefore, consumers prefer the use of naturally originating compounds, which are considered healthier. The antioxidant markets of Japan, the US, China, and Europe have grown steadily in the past five years. Growth of sales from 2005 to 2010 was 43 percent. In July 2013, the antioxidant demand (in terms of money) was 1.5 billion USD (Butti and Vischetti 2013). The market for antioxidants is expected to grow continuously in the future due to the rise of technical standards in the plastics industry and increasing demand for processed foods. Therefore, the search for new renewable resources for the manufacture of antioxidants is an urgent task for market development. Because of their bio-degradability and much lower toxicity, naturally originating antioxidants such as polyphenols have gained major interest as suitable alternatives to synthetic ones (Malik and Krohnke 2006; Jamshidian *et al*. 2012).

The antioxidant activity of technical lignin is well documented (Lu *et al*. 1998; Pouteau *et al*. 2003; Dizhbite *et al*. 2004; Carmen *et al*. 2004; Kosikova *et al*. 2006; Pan *et al*. 2006). It is known that polymeric polyphenol lignin *in situ* serves to protect plants against chemical, biological, and mechanical stresses. As an antioxidant, lignin has two advantages: it is of natural origin and, simultaneously, it is a polymer. Antioxidant polymers are a topic of great interest for researchers in many industrials fields, such as in pharmaceuticals, the cosmetic and food industries, and the plastics industry. The interest in high-molecular weight antioxidants is connected with the possibility of forming materials with long-term stabilities (Cirillo and Iemma 2012).

The conditions of kraft delignification lead to the formation of stilbene, styrene, catechol, and biphenyl substructures in lignin macromolecules (Gellerstedt and Lindorfs 1984; Gandini 1991; Vishtal and Kraslawski 2011). The destruction of ether bonds during delignification results in increasing content of phenolic hydroxyl groups. As a result, the kraft lignin structure is enriched with moieties of potentially high antioxidant activity (Dizhbite *et al.* 2004; Boeriu *et al.* 2004). Additionally, the LignoBoost technology can produce products of high purity with very low contents of carbohydrates and extractives. Carbohydrate admixtures can decrease the phenolic hydroxyl group's content in technical lignins and modify the reaction ability of these groups due to formation of ether or phenyl-glycoside bonds with the phenylpropanoid substructures of lignin (Lawoko *et al*. 2005).

The well-known high chemical heterogeneity of technical lignin, including significant differences in lignin macromolecule chemical structure and functionality over molecular mass distribution (Bikova *et al*. 2004), dramatically decreases its applicability as an antioxidant in targeted systems. Recently, it was demonstrated that the extraction of wheat straw technical lignin with organic solvents of different polarity as well as with alkaline solutions resulted in isolation of the more homogenized products, which demonstrated good antioxidant activity (Arshanitsa *et al*. 2013; Ma *et al*. 2013). Fractionation of lignin with organic solvents of increasing hydrogen-bonding capability has been shown to be very attractive due to the opportunity to obtain lignin fractions with different polarities and decreased heterogeneity (Ropponen *et al*. 2011), as well as

solubility, which is the major drawback of most lignin applications (Pouteau *et al*. 2005; Brodin *et al*. 2009; Thring and Griffin 1995).

The aim of the present work was assessment of the fractionation of LignoBoost kraft lignins (hardwood and softwood) as a tool for a more detailed understanding of the relationship between lignin structure and antioxidant activity and for obtaining polymeric antioxidants for products/materials of different polarities, *e.g.* vegetable oil and poly-urethane (PU) elastomers.

EXPERIMENTAL

Materials

Softwood and hardwood kraft lignins were isolated from the original black liquors using the LignoBoost process by Innventia AB.

Hypoxanthine, xantine oxidase, nitroblue tetrazolium (NBT), EDTA, 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), fluorescein, and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich; dimethyl sulfoxide (DMSO), methanol, n-propanol and dichloromethane were acquired from LACHEMA; 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid diammonium salt (ABTS), *tert*-butylhydroquinone (TBHQ), and 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) came from Fluka Chemie. All chemicals used for the analyses were of analytical grade. All test solutions were freshly prepared before use.

Methods

Fractionation of lignin

Lignin was fractionated by successive extraction with organic solvents of increasing hydrogen-bonding capability, as described by Thring and Griffin (1995), using dichloromethane (d_H = 6.1 MPa^{½}), n-propanol (d_H = 17.4 MPa^{½}), and methanol (d_H = 22.3 MPa^{½}) (Hansen 2007). Lignin (10 g on a dry basis) was suspended in 40 mL of the respective solvent in a 100-mL extraction flask and agitated using an ultrasonic water bath at room temperature for 15 min. The undissolved material was filtered off and resuspended for a second identical extraction. The fractions from both steps were combined. The collected dissolved material was filtered and vacuum dried. Three parallel fractionation experiments were conducted. The yields of the fractions are shown as % of dry ash-free parent lignin. The dry weight was determined by separate oven drying of samples at 105 °C until constant weight. Ash content was determined by the combustion of samples at 700 °C for 3 h in a Carbolite ELF 11/6B furnace (UK).

Chemical analysis and FTIR

All results are expressed on a dry-weight and ash-free basis. The methoxyl group (OCH_3) content in lignin samples was determined according to the Viebock–Schwappach method. Determination of the methoxyl group content in lignins was carried out using the classical Zeisel–Viebock–Schwappach method with 57% hydroiodic acid (HI). The methyl iodide formed was determined by chromatography (Agilent 6850 GC System, column CP7506) using ethyl iodide as an internal standard (Zakis 1994). The contents of phenolic hydroxyl groups (OH_{phen}) were determined by conductometric titration,

performing the analysis with a "Radiometer analytical" titration device CDM 210 (TitraLab 90, Denmark). All procedures are described in detail by Zakis (1994).

Fourier transform infrared (FTIR) spectra of lignins were recorded in KBr pellets using a Spectrum One apparatus (Perkin Elmer); resolution: 4 cm^{-1}, number of scans: 64. The spectral condensation index (SCI) was calculated according to Faix (Faix 1991; Xiao *et al*. 2012).

Analytical pyrolysis (Py-GC/MS)

Py-GC/MS analysis was used for characterization of the parent lignins and their fractions in terms of structure of lignin macromolecule and the presence of carbohydrate and other admixtures. The analysis was performed using a Frontier Lab (Japan) Micro Double-shot Pyrolyser Py-2020iD (pyrolysis temperature 500 °C, heating rate 600 °C s^{-1} directly coupled with a Shimadzu GC/MS – QP 2010 apparatus (Japan) with capillary column RTX-1701 (Restek, USA), 60 m x 0.25 mm x 0.25 μm film (injector temperature 250 °C, ion source 250 °C with EI of 70 eV, MS scan range m/z 15 to 350, carrier gas helium at the flow rate of 1 mL min^{-1}, and split ratio 1:30). The mass of a sample was 1.00 to 2.00 mg. The oven program was 1 min at 60 °C, and then the temperature was increased at 6 °C min^{-1} to 270 °C. Finally, it was held at 270 °C for 10 min. The apparatus was modified by installation of a gas-carrier flow splitter, Vitreous Silica Outlet Splitter VSOS (SGE, Australia), to operate the FID and MS detectors simultaneously. Identification of the individual compounds was performed on the basis of GC/MS chromatograms using Library MS NIST 147.LI13, whereas the relative area of the peak of individual compounds was calculated using the Shimadzu software on the basis of GC/FID data. The summed molar areas of the relevant peaks were normalized to 100%. Relative peak areas calculated for pyrolysis products of different origin (lignin, carbohydrates, and lipophilic extractives) were used for assessment of the composition of lignin samples.

Molecular mass distribution (MMD)

The molecular mass distributions of the parent lignins and the fractions obtained were analyzed by size exclusion chromatography (SEC) in accordance with a procedure described by Ringena *et al*. (2006) using an Agilent 1100 system. Lignin samples dissolved (1 mg mL^{-1}) in the eluent (DMSO) were injected into a PolarGeL-L 300×7.5 mm column (Polymer Laboratories). The flow rate was 0.8 mL min^{-1}, and the column was maintained at 60 °C. A differential refractometer (RI) and a photometer in the ultraviolet range (λ=254 nm) were used as detectors. For calibration, polystyrene standards with varying molecular masses were used.

Differential scanning calorimetry (DSC)

The DSC method was used for determination of the glass transition temperature (T_g) of the lignins under study and their fractions. A Mettler Toledo Star^e 823 DSC apparatus (Greifensee, Switzerland) equipped with a TSO801RO sample robot was used. Samples of mass 8±1 mg were enclosed in 100-μL Al crucibles with one hole in the cover. T_g was defined using *Star^e Software Version* 9.00 in accordance with DIN standard 51007. A sample was first scanned through 140 °C (heating rate 20 °C min^{-1}), then cooled to -50 °C (quenching rate 20 °C min^{-1}) and scanned from -50 °C up to 200 °C (heating rate 10 °C per min). T_g was determined from the second scan.

Thermogravimetric analysis (TGA)

TGA was applied with the aims to characterize thermal stability of the lignins under study and their fraction. The analysis was performed in the temperature range 293 to 700 °C in a N_2 atmosphere (flow rate 50 mL min^{-1}) using the Metler Toledo Star System TGA/ADTA 851e device at a heating rate of 10 °C min^{-1}. A sample size of 8 to 10 mg was used.

Assessment of radical scavenging activity

$DPPH^{\bullet}$ radical scavenging assay was performed using the spectrophotometric method described by Dizhbite *et al*. (2004). Absorbance at 517 nm of lignins and $DPPH^{\bullet}$ solutions was measured after 15 min using a Perkin Elmer Lambda 650 UV/VIS spectrophotometer.

$ABTS^{\bullet+}$ was produced by reacting 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) with potassium persulfate ($K_2S_2O_8$) (Baltrusaityte *et al*. 2007). The $ABTS^{\bullet+}$ solution was diluted with phosphate buffered saline (PBS), pH=7.4, to obtain the absorbance of 0.800 ± 0.030 at 734 nm and mixed with the lignin solution. The absorbance at 734 nm was measured after 10 min.

$ABTS^{\bullet+}$ was produced by the reaction of ABTS with potassium persulfate ($K_2S_2O_8$) (Baltrusaityte *et al*. 2007). A stock solution of ABTS (2 mM) with a 50 mM phosphate buffered saline (PBS), constituted of 8.18 g NaCl, 0.27 g KH_2PO_4, 3.58 g $NaHPO_4$ x $11H_2O$, and 0.15g KCl in 1L of distilled water. If necessary, the pH of solutions was adjusted to 7.4 with 0.1 M NaOH. The $ABTS^{\bullet+}$ solution was produced by mixing a 50 mL stock solution with 200 µL of $K_2S_2O_8$ (70 mM) aqueous solution. The mixture was kept in the dark at room temperature during 15-16 h before use. To evaluate the antioxidant capacity, the $ABTS^{\bullet+}$ solution was diluted with PBS to reach an absorbance of 0.800 ± 0.030 at 734 nm. The sample solution (0.03 mL) was mixed with 3 mL of the $ABTS^{\bullet+}$ solution, in a 1 cm path length microcuvette. The absorbance at 734 nm was read at room temperature after 10 min, using a Perkin Elmer Lambda 25 UV/VIS spectrometer. A PBS solution was measured as a blank sample.

$O_2^{\bullet-}$ was generated in a hypoxantine/xanthine oxidase system (Moridani *et al*. 2003) using NBT (nitroblue tetrazolium) as an indicator. The reduction of the NBT concentration in the presence of lignins was measured at 560 nm for 12 min.

Free radical scavenging activity is expressed as IC_{50} (the concentration required for a 50% inhibition of the free radical). Trolox (a water-soluble derivative of vitamin E) was tested as a reference antioxidant.

The ORAC assay was performed according to the method described in Prior *et al*. (2005) with a FLUOstar Omega microplate reader (BMG LABTECH, Germany). A 96-well plate was used with blank solution, Trolox, and a lignin sample, to which the fluorescein solution was added. The microplate was covered and incubated in the microplate reader for 15 min at 37 °C. Then, fluorescence measurements (Ex. 485 nm, Em. 520 nm) were taken every 66 sec. After 3 cycles, 25 µL of AAPH solution was injected into the well using a multi-channel pipette, and the test was resumed. The measurements finished within ca. 90 min (81 cycles). The final ORAC values were calculated using a regression equation ($Y=aX+b$) correlating the Trolox concentration (50 to 200 µM in PBS) and the net area under the fluorescein decay curve. In the ORAC assay for lignin samples, the antioxidant activity is expressed in terms of Trolox equivalent (TE) antioxidant capacity, on one gram of a sample. The higher the TE value is, the higher the antioxidant capacity is.

Effect of lignin fractions on thermo-oxidative destruction of PU

Thermo-oxidative destruction of the model PU elastomer (control and containing 2.5 % lignin sample films) was studied in the temperature range 20 to 200 °C by TGA and DTA (differential thermal analysis) in an air atmosphere (flow rate 50 mL min^{-1}) using the Metler Toledo Star System TGA/ADTA 851e device at a heating rate of 10 °C min^{-1}. A sample size of 8 to 10 mg was used. The PU elastomer films were obtained as described in Arshanitsa *et al.* (2013).

Inhibition of oil oxidation

Rapeseed oil was used as a substrate. First, 0.025 g of antioxidant (lignin or standard TBHQ) was mixed in the reaction vessel of the ML OXIPRES apparatus (Mikrolab Aarhus) with 5 g of oil, and the mixture was sonicated for 30 min. Oil without additives was used as the control. Then, the reaction vessel was filled with O_2 to 0.5 MPa, placed into a furnace at 130 °C, and the pressure was recorded (Bandonienė *et al*. 2000). Lignin protection factor (PF) and antioxidant activities (AA) were calculated as,

$$PF = IP_X/IP_C \quad (1)$$

$$AA = (IP_X - IP_C)/(IP_{TBHQ} - IP_C) \quad (2)$$

where IP_X, IP_C, and IP_{TBHQ} (s) are the induction periods of oil with the lignin sample, of the control, and of the oil with TBHQ, respectively (Abdalla and Roozen 1999).

Statistical treatment of the results

All values measured are shown as an average with a confidence interval (at level of significance α=0.05). Each measurement was performed at least in triplicate. Statistical calculations were carried out using IBM SPSS Statistics 21.

RESULTS AND DISCUSSION

Fractionation and Characterization of LignoBoost Kraft Lignins

Four fractions were obtained from each softwood and hardwood lignin (Table 1): a dichloromethane-soluble fraction (F-1), a propanol-soluble fraction (F-2), a methanol-soluble fraction (F-3), and the residual fraction that was not soluble in the solvents applied (F-4). For softwood lignin, the dominating fraction was the methanol-soluble fraction (~13%). In total, the extraction procedure solubilized ~ 34% of this lignin. The solubility of hardwood lignin was much higher (the total yield of the soluble fractions was ~81%), and the propanol-soluble fraction dominated, with a yield of ~48%.

Each fraction was characterized in terms of the presence of carbohydrates and other admixtures, molecular mass distribution (MMD), thermal stability, and characteristic structural features of lignin macromolecules using analytical pyrolysis (Py-GC/MS), FTIR spectroscopy, functionality analyses, and thermogravimetrical analysis (TGA).

The fractionation of both lignins yielded fractions of increasing average molecular mass (Table 1). The residual fractions had higher M_w than the parent lignins, while the M_w of other fractions were much lower: F-1 < F-2 <F-3<< F-4.

Table 1. Yields and Molar Masses of LignoBoost Softwood and Hardwood Kraft Lignins and their Fractions*

Sample	Yield, % of parent lignin	M_n, kDa	M_w, kDa	$M_w \cdot M_n^{-1}$
Softwood Lignin				
Parent Lignin	-	0.899±0.028	12.14±0.15	13.5±0.7
F-1	9.5±0.3	0.138±0.003	0.698±0.007	5.1±0.2
F-2	11.3±0.4	0.518±0.005	2.09±0.05	4.0±0.2
F-3	12.9±0.5	1.20±0.08	4.47±0.08	3.7±0.1
F-4	66.3±0.9	4.44±0.10	18.6±0.24	4.2±0.1
Hardwood Lignin				
Parent Lignin	-	0.421±0.009	4.79±0.08	11.4±0.4
F-1	23.2±0.5	0.184±0.008	0.799±0.008	4.3±0.2
F-2	48.3±0.6	0.392±0.023	1.996±0.049	5.1±0.3
F-3	9.2±0.5	2.14±0.08	4.48±0.08	2.1±0.1
F-4	19.3±0.2	4.96±0.08	18.40±0.24	3.7±0.2

* F-1 - CH_2Cl_2 fraction; F-2 - C_3H_7OH fraction; F-3 - CH_3OH fraction; F-4 - residual fraction

Despite the large difference in the average molecular masses of the parent lignins (Table 1), the M_w values for the fractions of softwood and hardwood lignins, which were solubilized in the same solvents, had very similar values. Dichloromethane fractions from both lignins had relatively low M_n and M_w, showing that they consist mostly of oligomeric lignin-derived compounds. The molecular masses of the residual fractions were considerably higher than that of the parent LignoBoost kraft lignins, particularly for hardwood lignin. It can be assumed that this fraction contains noticeable quantities of lignin-carbohydrates complexes (Gosselink *et al*. 2004). All soluble fractions had 2 to 4 times lower polydispersity indices ($M_w \cdot M_n^{-1}$) than the parent lignins (Table 1). The methanol-soluble fractions of both lignins had the most homogeneous molecular mass distribution among the fractions studied.

The lowest glass transition temperature (T_g) value was exhibited by the lignin fraction soluble in dichloromethane (Table 2). This can be explained by the oligomeric nature of lignin in these fractions.

In terms of functionality (Table 2), dichloromethane-soluble fractions had the highest OH_{phen} content, which is the main positive factor influencing the antioxidant activity of lignin. This indicates a high potential of CH_2Cl_2-soluble fractions as antioxidants for non-polar substrates (*e.g.*, oils). However, the significantly lower thermal stability of the CH_2Cl_2-soluble fractions, which revealed itself in lowering temperatures of the thermal destruction with volatiles emission beginning (T_{start}), maximal emission rate (T_{max}) and half-volatilization temperature ($T_{50\%}$) (TGA data, Table 2), could limit their use as antioxidants for polymer materials.

Table 2. Thermal Properties and Functional Group Contents of LignoBoost Softwood and Hardwood Kraft Lignins and their Fractions*

Sample	T_g, °C	T_{start}, °C	T_{max}, °C	$T_{50\%}$, °C	Carbonized residue at 500 °C, %	-OCH_3 content, mmol g^{-1}	-$OH_{phen.}$ content, mmol g^{-1}
	Softwood lignin						
Parent lignin	157±1	168±2	378±2	501±2	55.5±3.2	4.32±0.03	2.18±0.01
F-1	55±1	218±2	370±2	385±2	36±1.8	4.04±0.04	3.39±0.02
F-2	121±1	272±2	380±2	511±2	52±3.1	5.41±0.02	2.62±0.02
F-3	156±1	280±2	394±2	580±2	49.9±2.0	4.55±0.02	2.53±0.02
F-4	162±1	286±2	384±2	600±2	56±2.3	4.50±0.01	2.26±0.02
	Hardwood lignin						
Parent lignin	113±1	217±2	365±2	418±2	43±2.1	6.09±0.03	2.43±0.02
F-1	63±1	223±2	377±2	375±2	30±1.5	5.94±0.04	2.88±0.01
F-2	123±1	234±2	365±2	472±2	49±2.5	6.47±0.03	2.38±0.01
F-3	164±1	240±2	354±2	488±2	50±2.5	6.43±0.04	2.26±0.02
F-4	168±1	239±2	402±2	550±2	54±3.0	5.25±0.03	1.76±0.02

* F-1 - CH_2Cl_2 fraction; F-2 - C_3H_7OH fraction; F-3 - CH_3OH fraction; F-4 - residual fraction

The thermal stability of the parent softwood lignin and its fractions is high enough to withstand the usual polymer thermal processing temperatures (220 to 250 °C), whereas satisfactory thermostability of hardwood lignin was observed only for the C_3H_7OH- and CH_3OH-soluble fractions.

The contents of phenolic hydroxyl groups in propanol- and methanol-soluble fractions obtained from softwood lignin were higher than that of the parent lignin and residual fraction. The methanol- and propanol-soluble fractions of hardwood lignin have OH_{phen} contents similar to that of the parent lignin. The methoxyl group contents were the highest in the propanol-soluble fractions of both lignins.

FTIR spectra of all fractions under study showed similarities with respect to the major absorption lines typical for softwood and hardwood lignins (Faix 1992), but differed in their intensity. This was exemplified by the spectra of softwood lignin samples (Fig. 1). The F-1 fraction of softwood lignin was characterized by an elevated absorption in the C-H stretching of methyl, and methylene groups ranging between 3045 and 2780 cm^{-1} were detected for the F-1 fractions (normalized peak area 61 to 63, in comparison to ~24 to 45 for other fractions and parent lignins), which could indicate that the F-1 fractions had high lipophilic contents. The spectral condensation index (SCI) increased as follows: CH_2Cl_2-soluble fraction (F-1) < C_3H_7OH-soluble fraction (F-2) < CH_3OH-soluble fraction (F-3) < residual fraction (F-4) for both lignins.

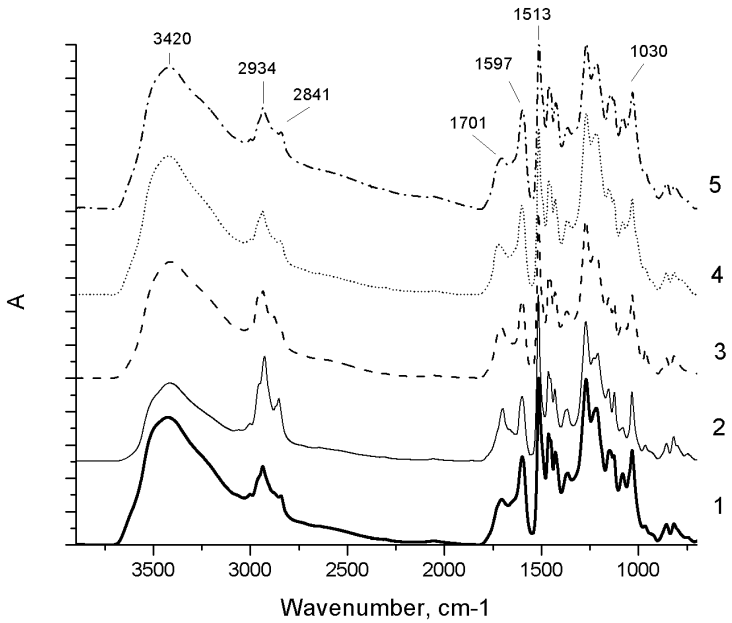

Fig. 1. FTIR spectra of LignoBoost softwood kraft lignin and its fractions: 1 – parent lignin, 2 – F-1 (CH_2Cl_2 fraction); 3 – F-2 (C_3H_7OH fraction); 4 – F-3 (CH_3OH fraction); 5 – F-4 (residual fraction)

As was expected, SCI values for softwood lignin and its fractions were higher than those for hardwood lignin samples: respectively, 0.60 and 0.44 (parent lignins), 0.50 and 0.37 (F-1), 0.57 and 0.44 (F-2), 0.63 and 0.50 (F-3), and 0.64 and 0.58 (F-4). The increasing absorption of C-O groups at 1030 cm^{-1} in the spectrum of F-4 fraction of hardwood lignin (more than twice as compared with the parent lignin) may correspond to the elevated amounts of carbohydrates.

Based on the Py-GC/MS results (Table 3), all fractions contained lignin as the predominant component, although a certain amount of carbohydrates and extractives are also present.

Table 3. Summarized Relative Abundance (%) of Lignin (L), Carbohydrates (C), and Extractives (E) Pyrolysis Products Detected for LignoBoost Kraft Lignins and their Fractions*

Sample	Proportion in the Volatiles, %		
	L-derived products	C-derived products	E-derived products
Softwood Lignin			
Parent Lignin	97.0±0.9	1.80±0.05	1.20±0.03
F-1	92.3±0.8	2.20±0.10	5.60±0.04
F-2	95.5±0.7	2.40±0.10	2.10±0.10
F-3	97.3±0.8	1.60±0.10	1.10±0.02
F-4	97.5±0.6	1.80±0.03	0.60±0.02
Hardwood Lignin			
Parent Lignin	97.4±0.5	2.50±0.04	0.20±0.01
F-1	97.9±0.8	0.70±0.02	1.50±0.01
F-2	98.6±0.5	0.70±0.03	0.70±0.03
F-3	97.4±0.8	2.20±0.03	0.50±0.05
F-4	91.5±0.2	8.50±0.10	<0.01

* F-1 - CH_2Cl_2 fraction; F-2 - C_3H_7OH fraction; F-3 - CH_3OH fraction; F-4 - residual fraction

The results of Py-GC/MS are in rather good compliance with the FTIR data concerning enrichment of dichloromethane-soluble fractions with lipophilic substances, obviously from fatty acids or other extractives contained in wood raw material. The relative content of the carbohydrate-originated volatile products varied insignificantly among the fractions, excluding the F-4 fraction of hardwood lignin, where the carbohydrate portion was the highest (Table 3); these results are also in compliance with FTIR results.

To quantitatively characterize the differences in the chemical structure of lignin in the various fractions, peak areas of individual phenols (calculated as relative percentage from normalized to 100% peak areas of all lignin-originated pyrolysis products) were summarized into groups of compounds with specific structural features (Table 4). Taking into account the effects of *para*-substitution on the antioxidant properties of monomeric phenols (Rogynskyi 1989), the structure of lignin side chains was further investigated.

The lignin-originated pyrolysis products from various fractions differ with respect to the content of methoxylated phenols and length and structure of the side chains (Table 4). The lignin components of the fractions soluble in CH_2Cl_2 contain the highest amount of methoxylated phenolic groups in comparison with other fractions and parent lignins, and, simultaneously, the highest portion of lignin moieties with oxygen-containing groups in the side chains, mostly C=O groups in the α-position.

Table 4. Summarized Distribution of Various Groups of Lignin Derived Products in Pyrolyzates of LignoBoost Kraft Lignins and their Fractions*

Sample	Groups of Phenolic Compounds				
	Guaiacol- and syringol derived compounds	With non-substituted saturated side chain	With $C_\alpha=C_\beta$ bonds in side chain	With O-containing side chains	$\frac{(PhC_1+PhC_2)}{PhC_3}$*
	Relative Content, %				
Softwood Lignin					
Parent Lignin	88.2±0.7	36.9±0.9	12.3±0.6	13.7±0.5	3.20±0.05
F-1	93.7±0.6	28.5±0.7	12.2±0.7	22.4±0.7	2.80±0.04
F-2	88.0±1.1	41.9±0.8	14.0±0.8	9.6±0.4	4.60±0.04
F-3	87.8±0.5	38.1±0.4	14.5±0.5	10.4±0.4	4.50±0.03
F-4	87.8±0.8	37.3±0.4	6.8±0.8	11.9±0.4	3.40±0.04
Hardwood Lignin					
Parent Lignin	95.9±0.4	37.3±0.7	20.8±0.4	8.3±0.3	3.10±0.02
F-1	96.9±0.4	33.6±0.5	16.7±0.5	14.2±0,6	4.60±0.04
F-2	95.7±0.3	38.2±0.8	19.5±0.4	7.3±0.2	3.60±0.03
F-3	94.9±0.6	38.0±0.4	19.7±0.3	8.0±0.3	4.30±0.03
F-4	94.8±0.8	34.0±0.8	27.5±0.9	8.8±0.2	2.30±0.02

* F-1 - CH_2Cl_2 fraction; F-2 - C_3H_7OH fraction; F-3 - CH_3OH fraction; F-4 - residual fraction
*-the ratio between the summarized relative content of phenols with shortened side chains (PhC_1+PhC_2) and the content of phenols with propanoid side chains (PhC_3)

The contents of the aromatic substructures with unsaturated side chains in softwood lignin fractions soluble in propanol and methanol are slightly higher than those for parent lignin and the dichloromethane-soluble fraction, while for the insoluble fraction (F-4), the amount of such structures is considerably lower. Conversely, in the case of hardwood lignin, the portion of compounds with double bonds in the side chains is the highest for the residual fraction. However, this index could have some uncertainty

due to the possibility of double bond formation in the process of elimination during pyrolysis.

The formation of lignin pyrolysis products with shortened side chains (C_1 and C_2) gives evidence of the destruction of β-aryl-alkyl ether bonds in lignin macromolecules, primarily during the delignification process. For all soluble fractions of hardwood lignin, especially for the CH_2Cl_2 fraction, the ratio between the summarized relative content of phenols with shortened side chains (PhC_1+PhC_2) and the content of phenols with propanoid side chains (PhC_3) was higher in comparison with parent lignins and residual fractions (Table 4).

The Py-GC/MS results characterizing lignin structural features (Table 4), together with non-lignin relative contents, SCI, M_w, and $M_w \bullet M_n^{-1}$, were used in the present study to investigate the relationship between lignin structure and antioxidant properties.

Antioxidant Properties and Relationship between Lignin Structural Features and Radical Scavenging Capacity

Antioxidant activity is a complex characteristic that depends on, in addition to the structure of the antioxidant, a set of kinetic parameters of the antioxidant and the substrate, the products of their transformations, and oxidation conditions. Correspondingly, a single universal parameter establishing the antioxidant activity of lignin in different systems at various oxidation regimes cannot exist in principle. Therefore, in the present study, the antioxidant properties of lignins and their fractions were evaluated as their capacity to scavenge the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ free radicals and the reactive oxygen species (ROS) superoxide anion radical ($O_2^{\bullet-}$). The ORAC kinetic assay was also used; this assay is partially based on the evaluation of peroxyl free radical scavenging.

Table 5. Radical Scavenging Activity of LignoBoost Kraft Lignins and Their Fractions*

Sample*	Radical Scavenging Activity				
	$DPPH^{\bullet}$		$ABTS^{\bullet+}$		$O_2^{\bullet-}$
	IC_{50}, mg L^{-1}	RSI, mol $DPPH^{\bullet}$ mol^{-1} OH_{phen}	IC_{50}, mg L^{-1}	RSI, mol $ABTS^{\bullet+}$ mol^{-1} OH_{phen}	IC_{50}, mg L^{-1}
Trolox	4.7±0.4	2.7	4.0±0.1	1.1	17.7±1.5
Softwood Lignin					
Parent Lignin	17.4±0.5	1.3	3.8±0.1	2.0	36.9±1.9
F-1	21.0±0.8	0.7	4.1±0.1	1.2	59.7±1.7
F-2	11.0±0.7	1.7	3.1±0.2	2.1	30.4±0.5
F-3	13.0±0.7	1.5	3.8±0.1	1.8	39.5±1.8
F-4	21.1±0.4	1.0	4.7±0.1	1.6	44.9±1.4
Hardwood Lignin					
Parent Lignin	15.0±0.6	1.4	4.8±0.2	1.5	32.8±1.3
F-1	17.6±1.4	1.0	4.1±0.1	1.5	51.6±2.5
F-2	11.3±0.3	1.9	3.7±0.2	1.9	27.0±1.1
F-3	12.0±0.3	1.9	4.2±0.1	1.8	23.4±1.1
F-4	17.4±0.7	1.6	6.4±0.2	1.5	31.1±1.8

*F-1 - CH_2Cl_2 fraction; F-2 - C_3H_7OH fraction; F-3 - CH_3OH fraction; F-4 - residual fraction

The results of the $ABTS^{\bullet+}$, $DPPH^{\bullet}$, and $O_2^{\bullet-}$ tests are presented (Table 5) in terms of the IC_{50} (mg L^{-1}) and Radical Scavenging Index (RSI). The lower the IC_{50} value is, the

higher is the radical scavenging activity of the compounds, as the IC_{50} displays the concentration of the tested antioxidant sample required for a 50% inhibition of the radical species.

The IC_{50} parameter is usually proposed in the related literature and has high practical importance. However, it depends on the initial concentration of the free radical. Therefore, in the present work, the RSI was used to characterize the antioxidant capacity with more reliability (Dearden *et al*. 2009). RSI corresponds to the number of radical moles scavenged by one mole of antioxidant and is independent of the radical concentration. Note that the higher the RSI is, the higher is the free radical scavenging activity. However, for lignin – a heterogenic polymer – it is not possible to calculate the radical scavenging activity on one mole of an antioxidant but, using the results of functional group analysis, this value can be presented in terms of scavenged radical moles on one mole of lignin phenolic hydroxyl groups. Results presented in this manner (Table 5, RSI) enable easier understanding of the structure-activity relationship.

Regarding $ABTS^{\bullet+}$ scavenging, practically all fractions and both parent lignins exhibit very similar free radical scavenging capacities, which were close to or even better than that for the reference antioxidant Trolox. The results obtained show that the number of $ABTS^{\bullet+}$ moles scavenged by lignins and their fractions are higher than the number of phenolic hydroxyl groups in the lignin samples. This indicates scavenging activity of the lignin phenoxyl radicals (formed as a result of scavenging) or the products of their coupling.

In the test with the free radical $DPPH^{\bullet}$, the radical scavenging activity of all lignin samples under study was significantly lower (by 2.5 to 4.5 times) in comparison with Trolox. Nevertheless, the results confirm the usefulness of using $DPPH^{\bullet}$ for differentiation of structurally similar high-molecular weight phenolic antioxidants by their radical scavenging ability, as considered recently for low-molecular weight polyphenols (Di Meo *et al.* 2013). The best $DPPH^{\bullet}$ scavenging activities in both tests were observed for the fractions soluble in methanol and propanol (Table 5). The same fractions also demonstrated the highest activity in the test with the reactive oxygen form $O_2^{\bullet-}$.

All lignin fractions showed good results in the ORAC kinetic test (Fig. 2, Table 6), at the level of Trolox or even better. Moreover, the dichloromethane-soluble fractions showed the strongest inhibition effect on fluorescein oxidation. This discrepancy with the results of other tests used can be explained using kinetic factors.

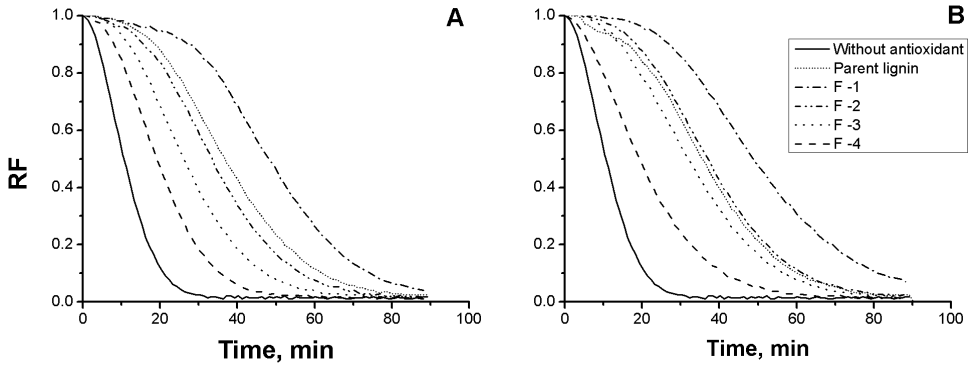

Fig. 2. ORAC test results for LignoBoost softwood (A) and hardwood (B) kraft lignins: F-1 - CH_2Cl_2 fraction; F-2 - C_3H_7OH fraction; F-3 - CH_3OH fraction; F-4 - residual fraction. RF – relative fluorescence

Decreased substrate oxidation with the lignin fractions was confirmed in tests with vegetable (rapeseed) oil and model polyurethane elastomers.

Table 6. Antioxidant Activity in the ORAC of Parent Lignins and Their Fractions*

Sample	Absorbance capacity, Trolox Equivalents** (TE), mmol per g of sample
Softwood lignin	
Parent lignin	5.8±0.3
F-1	8.6±0.6
F-2	5.1±0.2
F-3	3.5±0.4
F-4	1.7±0.2
Hardwood lignin	
Parent lignin	5.6±0.5
F-1	8.1±0.6
F-2	5.7±0.4
F-3	4.8±0.3
F-4	2.4±0.2

* F-1 - CH_2Cl_2 fraction; F-2 - C_3H_7OH fraction; F-3 - CH_3OH fraction; F-4 - residual fraction
**Antioxidant capacity calculated based on Trolox; 1 g of Trolox corresponds to 3.9 mmol TE.

The CH_2Cl_2-soluble fractions, which differ from the standard synthetic antioxidant TBHQ in terms of increased environmental safety, revealed themselves as potential protectors of rapeseed oil from oxidation. Parent lignins and other fractions were not tested due to the fact that they did not form homogenous compositions with the oil. The values of the protection factor found for softwood and hardwood CH_2Cl_2 fractions and TBHQ were 1.6, 1.7, and 2.0, respectively, and the antioxidant activity of the fractions were 80% of that for TBHQ.

The antioxidant activity of LignoBoost kraft lignins and their soluble fractions were tested with respect to thermo-oxidative destruction of model PU films. In the present study, the lignin fractions were added to PU in small amounts (2.5%), close to the level usually used in practice for technical antioxidants (0.5 to 2%). The PU films obtained were transparent, without solid inclusions, and had uniform thickness (~200 μm).

Table 7. Effect of Parent LignoBoost Kraft Lignins and their Soluble Fractions* on Thermo-Oxidative Destruction of PU Films

Sample	T_{start}, °C	T_{max}, °C	Maximal mass loss rate, mg min^{-1}
Lignin-free PU	543±2	300±2	0.99±0.05
Softwood Lignin			
PU with parent lignin	280±2	328±2	0.57±0.03
PU with F-1	290±2	333±2	0.64±0.04
PU with F-2	303±2	332±2	0.47±0.03
PU with F-3	295±2	334±2	0.50±0.02
Hardwood Lignin			
PU with parent lignin	290±2	328±2	0.56±0.05
PU with F-1	290±2	332±2	0.59±0.04
PU with F-2	299±2	330±2	0.34±0.02
PU with F-3	300±2	329±2	0.40±0.02

* F-1 - CH_2Cl_2 fraction; F-2 - C_3H_7OH fraction; F-3 - CH_3OH fraction

The TGA data (Table 7) clearly showed the antioxidant effect of parent lignins and the soluble fractions, which revealed themselves in increasing starting (T_{start}) and maximal development (T_{max}) temperatures of PU thermo-oxidative destruction, as well as the decreasing process rate on the first stage of PU thermo-oxidative degradation. The DTA data also confirmed the changes in thermo-oxidative behavior of model PU films: the exothermal maximum connected with oxidizing PU destruction caused volatile products to shift toward the high-temperature region by 25 to 30 °C.

The results of the tests on radical scavenging activity toward $DPPH^{\bullet}$ and $O_2^{\bullet-}$ (Table 5) are in conformity with the protective effect of C_3H_7OH- and CH_3OH-soluble fractions, which was somewhat stronger than for fractions soluble in CH_2Cl_2. However, this could also result from the lower thermal stability of the F-1 fractions (Table 2).

Due to the molecular complexity of lignins, it is difficult to assign their antioxidant efficiency to definite structural elements. It is known that the main factor governing lignin redox activity is the phenolic hydroxyl groups, as for other polyphenolic antioxidants (Brand-Williams *et al*. 1995; Barclay *et al*. 1997; Frankel, 1998; Salehi *et al*. 2011). For monomeric phenolic antioxidants, the presence of methoxy groups in the *ortho*-position of OH_{phen} is the important factor positively influencing antioxidant activity. The data on the effects of aliphatic hydroxyl groups and C=C bonds are contradictory (Torres *et al*. 2003; Dizhbite *et al*. 2004; Thavasi *et al*. 2006; Setzer 2011).

Consideration of the results of the radical scavenging tests together with the PY-GC/MS and chemical analysis data presents an opportunity to determine the structure-activity correlations needed for understanding and tuning of the antioxidant properties of lignin. With this aim, the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ results, together with the number of moles of scavenged radicals per mole of OH_{phen}, were used. The values of the Pearson correlation coefficient, characterizing the influence of various lignin structural features on the radical scavenging activity, are shown in Table 8.

Table 8. Pearson Correlation Coefficients between Lignin Characteristic Features and Radical Scavenging Activity in Tests with $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ Radicals

Lignin Characteristics	Pearson Correlation Coefficients	
	$DPPH^{\bullet}$	$ABTS^{\bullet+}$
Methoxyl group content	0.61*	0.09
Relative portion of lignin-derived phenols with non-substituted alkyl side chains	0.71	0.87
O-containing substitution (incl. α-C=O) in side chains	-0.87	-0.58
C_1=C_2 bond in side chains	0.65	-0.06
Ratio (PhC_1+PhC_2)/PhC_3	0.27	0.53
Carbohydrate-originated admixtures content	0.032	0.06
Extractive-originated admixtures content	0.36	0.15
Spectral condensation index (SCI)	0.22	0.02
M_w	-0.06	-0.03
$M_w \bullet M_n^{-1}$	-0.18	0.11

*Values in bold are different from 0 with a significance level α=0.05.

The OCH_3 group in the *ortho*-position to OH_{phen} positively influences the lignin radical scavenging activity in the test with $DPPH^{\bullet}$, but this is not true for $ABTS^{\bullet+}$. The significant positive effect in both tests of non-substituted lignin alkyl side chains was explained by their electron-donor properties, which promote transfer of H atoms from the

phenolic hydroxyl group. For both tests, a negative effect of O-containing side chains was observed. A positive effect of C=C bonds in the side chains was found in the test with $DPPH^{\bullet}$. No reliable correlations were found for the condensation index, molecular mass, polydispersity index, and radical scavenging activity of lignin.

The influence of carbohydrates and extractives on lignin antioxidant activity needs further investigation because the insignificantly low values of correlation coefficients found for both tests (Table 8) could be connected with small contents of these admixtures in the LignoBoost kraft lignins (Table 2).

The data obtained with the Py-GC-MS/FID method present an opportunity to identify some other relationships between lignin structural features and antioxidant properties. It was shown (Table 8) that the radical scavenging activity of lignin in the $ABTS^{\bullet+}$ test correlated positively with the ratio between relative contents of lignin-related phenols with shortened (PhC_1+PhC_2) and phenols with non-destructed (PhC_3) side chains in pyrolyzates. This could be connected with radical scavenging activity of the products of the primary lignin interaction with $ABTS^{\bullet+}$.

The difference in the factors influencing the radical scavenging activity in the tests with $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ are connected with different mechanisms of lignin interaction with these radicals. The factors governing scavenging of the radical $DPPH^{\bullet}$ by lignin are easily explained from the perspective of the PCET (proton-coupled electron transfer) mechanism, whereas a noticeable impact of the SPLET (sequential proton-loss-electron-transfer) mechanism in the interaction of lignin with radical-cation $ABTS^{\bullet+}$ could be assumed (Trouillas *et al*. 2008).

CONCLUSIONS

1. Softwood and hardwood LignoBoost kraft lignins exhibited good radical scavenging properties; however, their heterogeneity, in terms of structural and functional variations over the molecular mass distribution, decreases their antioxidant activity.
2. Solvent fractionation of the lignins under study, used for obtaining fractions with enhanced homogeneity, significantly increased the LignoBoost kraft lignin's value as a source of antioxidants for different products/materials. This was exemplified by the prominent antioxidant effects of isolated fractions in PU elastomers and rapeseed oil.
3. Some novel structure-activity correlations were found. In particular, there was a positive impact of the presence of non-substituted alkyl groups and a negative effect of O-substitution in lignin side chains on the radical scavenging activity. These results can be used for tuning lignin's antioxidant properties.

ACKNOWLEDGMENTS

The authors highly appreciate the support for this investigation given by Dr. E. Sjöholm (INNVENTIA AB) and the collegial help of MSc. A. Arshanitsa in the synthesis of model PU elastomers. Financial support from the European Social Fund within the project "Support for Doctoral Studies at University of Latvia" and Latvian budget, LZP grant 564/2012 and Government Program 2010.10-4/VPP, is gratefully acknowledged.

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Article submitted: October 28, 2013; Peer review completed: December 10, 2013; Revised version received: February 14, 2014; Accepted: February 16, 2014; Published: February 20, 2014.