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V. Aguié-Béghin **(INRA)**
L. Foulon **(INRA)**
P. Soto **(AgroParisTech)**
D. Crónier **(INRA)**
E. Corti **(AgroParisTech)**
F. Legée **(IJPB)**
Laurent Cézard **(IJPB)**
Brigitte Chabbert **(INRA)**
Marie-Noëlle Maillard **(AgroParisTech)**
Wouter J. J. Huijgen **(ECN)**
Stéphanie Baumberger **(IJPB)**

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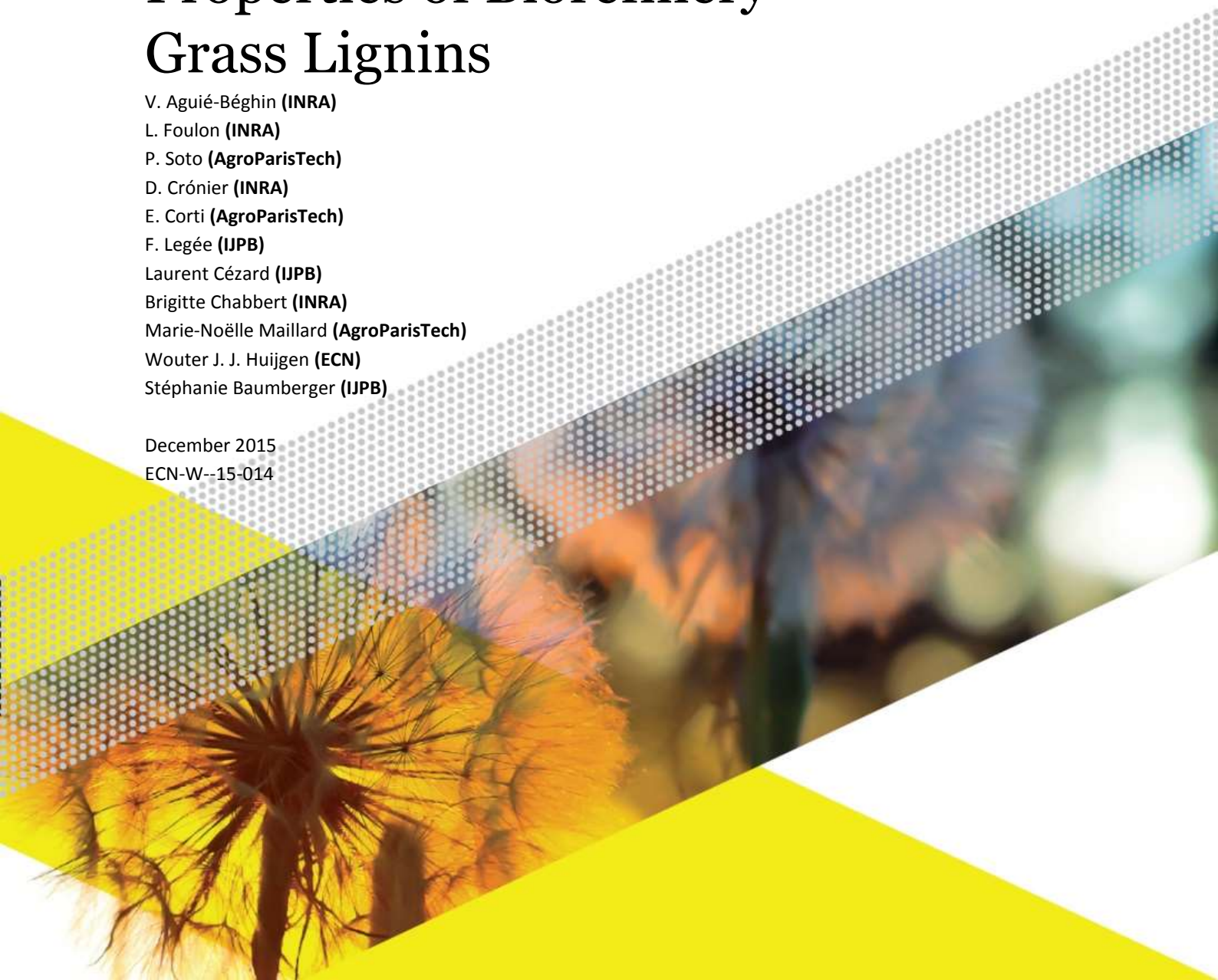

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Use of Food and Packaging Model Matrices to Investigate the Antioxidant Properties of Biorefinery Grass Lignins

Véronique Aguié-Béghin,[†,‡] Laurence Foulon,[†,‡] Paola Soto,[§,∥] David Crônier,[†,‡] Elena Corti,[§,∥] Frédéric Legée,[#] Laurent Cézard,[#] Brigitte Chabbert,[†,‡] Marie-Noëlle Maillard,[§,∥] Wouter J. J. Huijgen,[⊥] and Stéphanie Baumberger*[,#]

[†]INRA, UMR614 Fractionnement des AgroRessources et Environnement, F-51100 Reims, France

[‡]Université de Reims Champagne-Ardenne, UMR614, Fractionnement des AgroRessources et Environnement, F-51100 Reims, France

[§]AgroParisTech, UMR1145 GENIAL, F-91300 Massy, France

[∥]INRA, UMR1145 GENIAL, F-91300 Massy, France

[⊥]Energy Research Centre of The Netherlands (ECN), Westerduinweg 3, 1755 LE Petten, The Netherlands

[#]Institut Jean-Pierre Bourgin (IJPB), INRA, AgroParisTech, CNRS, Université Paris-Saclay, RD10, 78026 Versailles Cedex, France

ABSTRACT: The antioxidant properties of grass lignins recovered from an alkaline industrial process and from different ethanol organosolv pretreatment processes were compared using two types of tests: (i) classical radical 2,2′-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) scavenging tests in dioxane/water or ethanol and (ii) tests involving multiphasic systems (lipid dispersion in water or cellulose film suspended in ethanol). These multiphasic systems were representative of food and packaging matrices in view of high-value applications. All lignins, in solution or in the film, effectively scavenged radicals. Moreover, they were competitive with a food commercial rosemary extract to protect linoleic acid against oxidation. Whereas the DPPH$^{\bullet}$ test in dioxane was not discriminant, differences appeared between lignins when the test was performed in ethanol or with the multiphasic systems. Moreover, radical scavenging activity was preserved in the film even after its immersion in ethanol. Structural analysis of lignins revealed that low-molar-mass phenolics, namely *p*-hydroxycinnamic acids and lignin depolymerization products, governed lignin antioxidant properties in the multiphasic systems.

KEYWORDS: *biorefinery, cellulose nanocomposites, ethanol, food antioxidant, grass lignins, hydrogel films, organosolv, radical scavenging, soda, wheat straw*

INTRODUCTION

Biorefinery lignins are multifunctional phenolic compounds produced during the conversion of lignocelluloses into cellulosic fibers, biofuels such as bioethanol, biopolymers, or platform sugars and derivatives.[1] Development of value-added applications of these byproducts is a major challenge in improving the economic viability of the lignocellulose conversion process, thus bringing commercial lignocellulosic biorefineries a step closer to reality. Among the different functional properties of lignins, increasing interest has been given to their radical scavenging properties, with respect to formulation of plastics[2−4] and cosmetics.[5] Indeed, their phenolic structure enables them to trap radicals in either solid or liquid media, thus preventing polymers and biomolecules from degradation through reactive oxygen species. Lignins represent a renewable low-cost alternative to non-renewable toxic antioxidants such as bisphenol A or expensive natural antioxidants. Though the toxic potential of industrial lignins is recognized as low, based on various bioassays,[6,7] little attention has been paid to their possible use in food preservation. Nevertheless, they are very promising feedstock to develop potential value-added products, in particular as components of polymer composites[8] or of polysaccharide (e.g., xanthan and chitosan) hydrogel films, used as antioxidant carriers or active packaging.[9,10] A challenge is to find new antioxidant polymers to replace the synthetic antioxidants (butylated hydroxyanisole, butylated hydroxytoluene) used today under strict regulation due to their potential health risk.

Previous studies focusing on organosolv lignins[11−13] and technical lignins from pulp and paper industry (lignosulfonates, Kraft, and alkali lignins)[5,14] have highlighted variations in radical scavenging properties as a function of lignin botanical origin and isolation process. However, deciphering the relationships between lignin structure and lignin radical scavenging properties is often hindered by the interdependence of lignin structural parameters, such as molar mass distribution and phenolic group content.[11] Wheat straw, and more generally grass stems, are major agro-resources for the development of a European lignocellulose biorefinery.[15] Depending on the biorefinery system, these feedstocks can undergo various chemical or mechanical pretreatments in order to facilitate further conversion of the biomass into valuable products.

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Alkaline and organosolv pretreatments have the advantage of increasing enzyme accessibility to polysaccharides while providing a lignin stream available for chemical production or material application. Moreover, these types of pretreatments limit the formation of carbohydrate-derived bioconversion inhibitors compared to acidic or steam explosion pretreatments.[16] Although the antioxidant properties of lignins have been previously demonstrated, there is a need to compare lignin from different lignocellulose fractionation processes but similar botanical origin while exploring potential value-added applications.

The objective of this study was to investigate the radical scavenging properties of biorefinery lignins obtained from a single type of biomass, grasses, by alkaline and ethanol organosolv delignification processes already implemented at pilot or industrial scales. Antioxidant capacity was checked by the standard radical scavenging test using solution of 2,2′-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$). In addition, complex matrices were used to investigate the antioxidant properties of lignins in conditions representative of high-value targeted applications: (i) a lipid-based dispersion considered as model of food matrices and (ii) a cellulose-based solid matrix as model of biodegradable polymer carrier for release of antioxidant. Lignins were compared in terms of composition and structure, radical scavenging activity with respect to DPPH$^{\bullet}$, and protection of lipids against oxidation. Cellulose−lignin nanocomposite films were characterized by their water sorption capacity and antioxidant activity using the DPPH$^{\bullet}$ test. This work shows the relevance of the complex model matrices to understand lignin radical scavenging process and opens new prospects for lignin valorization.

MATERIALS AND METHODS

Materials. All solvents were purchased from CARLO ERBA and were of analytical grade (absolute ethanol, dioxane, 96% sulfuric acid) or specific grade (pestipur methylene chloride (CH_2Cl_2) and ethyl acetate (EtOAc) for pesticide analysis; stabilized tetrahydrofuran (THF) for HPLC).

The alkali (AL) lignin was a commercial lignin (Protobind 1000, Green-Value SA, Switzerland) recovered from a mix of wheat (*Triticum sp.*) straw and sarkanda (*Saccharum officiarum*) grass according to a patented process.[17] Part of the lignin sample (100 g) was submitted to a fractionation process by partial dissolution in a CH_2Cl_2/EtOAc 50/50 v/v mixture (500 mL, 1 h) in order to remove low-molar-mass phenolics. After filtration on a glass fiber filter (Whatman, France), the solid residue was washed twice with 50 mL of the fractionation solvent before freeze-drying and recovery of the AL extracted sample.

The organosolv (OS) lignins OS-A, OS-B, and OS-C, recovered from different ethanolic delignification processes of wheat straw, were provided by the Energy Research Centre of The Netherlands (ECN). All lignins were produced in batch autoclaves (20 L for OS-A and 2 L for both OS-B and OS-C). Specified reaction times excluded the heating and cooling times involved. Heating times for the 2 and 20 L autoclaves were ∼0.75 h (OS-B) and ∼4 h (OS-A), respectively. The time for cooling to below 50 °C was less than 1.5 h and more than 7 h for the 2 and 20 L autoclaves, respectively. A typical temperature profile for the production of OS-B in the 2 L autoclave has been published.[18] OS-A was obtained by direct ethanol treatment (210 °C, 90 min, 50% w/w aqueous ethanol, 10 L kg^{-1} dw, no catalyst added) following a published procedure.[19] OS-B was produced using a two-step fractionation approach consisting of (1) pre-hydrolysis of wheat straw for hemicellulose valorization (175 °C, 30 min, 10 L kg^{-1} dw, 20 mM H_2SO_4) and (2) autocatalytic organosolv delignification (200 °C, 60 min, 60% w/w aqueous ethanol, 10 L kg^{-1} dw).[18] OS-C was obtained by an alternative direct organosolv process using a lower temperature than that used for OS-A (140 °C, 120 min, 60% w/w aqueous ethanol, 10 L kg^{-1} dw, 60 mM H_2SO_4).[20]

The rosemary extract was a commercial extract rich in carnosic acid.

The cellulose nanocrystals were obtained from ramie fibers (*Boehmeria nivea*) as previously described.[21]

Lignin Chemical Analysis. All analyses were performed in duplicate.

Carbohydrate Analysis. Carbohydrate analysis was carried out by high-performance anion-exchange chromatography (HPAEC) with 2-D-deoxyribose as internal standard. The lignin samples (10 mg) were treated with sulfuric acid (H_2SO_4, 12 M) for 2 h at 20 °C and then diluted to 1 M for 2 h at 100 °C. All the samples were filtered (polytetrafluoroethylene (PTFE), 0.45 μm) and then injected onto a Dionex CarboPac PA-1 anion exchange column (4 × 250 mm, 10 μm size particles) and detected by pulsed amperometry (PAD 2, Dionex). Detector sensitivity was increased by postcolumn addition of 300 mM NaOH. Standard solutions of L-arabinose, D-galactose, D-glucose, and D-xylose were used for calibration.[22]

Molar Mass (MM) Distribution. The mass-average MM (M_w), number-average MM (M_n), and polydispersity (PD = M_w/M_n) of each lignin sample were estimated after acetylation via high-performance size-exclusion chromatography (HPSEC) with photodiode array detector (Waters 2996) coupled to a differential refractometer (Waters 410) and THF (1 mL min^{-1}) as eluent.[23] Acetylated samples were solubilized in THF, filtered on a PTFE membrane (0.45 μm), and injected on three successive PL gel-mixed B columns with an exclusion mass range from 200 to 10 × 10^6 g mol^{-1} (Agilent Technologies, Les Ulis, France), using the relative calibration method based on the elution of styrene and eight polystyrene standards in MM range from 104 to 900 000 g mol^{-1}. Estimated MM averages of the acetylated lignins were calculated on the basis of the differential refractometer detection.

Thioacidolysis. Thioacidolysis of lignins (15 mg) was carried out according to the literature,[24] using heneicosane ($C_{21}H_{44}$, Fluka) as internal standard (IS). Lignin-derived *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) thioacidolysis monomers were analyzed as their trimethylsilyl derivatives by a gas chromatography−mass spectrometry (GC-MS) instrument (Saturn 2100, Varian) equipped with a poly(dimethylsiloxane) column (30 m × 0.25 mm; SPB-1, Supelco) and using the following heating program: 40 to 180 °C at 30 °C min^{-1}, then 180 to 260 °C at 2 °C min^{-1}. The mass spectrometer was an ion trap with a ionization energy of 70 eV and positive mode detection. Quantitative determination of H, G, and S monomers was performed from ion chromatograms reconstructed at m/z 239, 269, and 299, respectively, as compared to the IS signal measured from the ion chromatogram reconstructed at m/z (57 + 71 + 85). The molar yield of the detected thioethylated monomers was calculated on the basis of the Klason lignin content of the sample, determined according to a published procedure.[25]

Phenolic Acid Determination (Free, Esterified, and Etherified). Total etherified, esterified, and free *p*-hydroxycinnamic acids (ferulic acid (FA) and *p*-coumaric acid (CA)) were determined by submitting a lignin sample (50 mg) to severe alkaline treatment (5 mL of 4 M NaOH, 170 °C for 2 h in an autoclave). Both free and esterified phenolic acids were determined by subjecting the same amount of lignin to a mild alkaline treatment (5 mL of 2 M NaOH, room temperature, 20 h with mechanical stirring). In both cases, the released phenolic acids were purified from the reaction medium by precipitation with the Carrez reagent, followed by analysis by liquid chromatography coupled to UV detection (LC-UV) using ethyl vanillin as IS.[26] The phenolic compounds were separated using a 150 mm × 4.6 mm HyPURITY C18 (5 μm) column (Thermo Scientific, France) with a water−acetonitrile gradient (0.1% formic acid) as mobile phase and at 0.8 mL min^{-1} flow rate. Quantification was performed using a photodiode array/UV−visible detector (Spectro System UV 6000 LP from Thermo Finnigan). Peak assignment was performed using an LCQ deca (Thermo Quest) mass spectrometer detector equipped with an electrospray ionization (ESI) interface. Free phenolic acids and other phenolic monomers were determined by organic solvent extraction followed by GC-MS analysis using a method

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adapted from Lepifre et al. (2004).[27] The lignin sample (30 mg) was suspended in 1 mL of Milli-Q water for 2 h at ambient temperature before pH setting at 3−4 by 6 M HCl and extraction by 3 × 3 mL of a CH_2Cl_2/EtOAC 50/50 v/v mixture. The extract was evaporated to dryness under reduced pressure and then dissolved in 2 mL of the extraction solvent. The solutions were analyzed by GC-MS after silylation (addition of 50 μL of BSTFA and 10 μL of pyridine to 10 μL of solution) and using nonadecane ($C_{19}H_{40}$, Fluka) as IS. The same equipment and column as described above for thioacidolysis were implemented. Content in ethyl esters of CA and FA was assessed using for ethyl esters the same response coefficient as their non-esterified counterparts.

Quantitative ^{31}P NMR. Derivatization of the lignin samples with 2-chloro-4,4′,5,5′-tetramethyl-1,3,2-dioxaphospholane (Sigma-Aldrich, France) was performed according to Granata et al. (1995).[28] NMR spectra were acquired on a Bruker Biospin Avance III 500 MHz spectrometer. To improve resolution, a total of 256 scans were acquired with a delay time of 6 s between successive pulses. The spectra were processed using Topspin 3.1 from Bruker Biospin. All spectra were manually phase-corrected and calibrated with observed signal from the reaction product between water and 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane in pyridine/$CDCl_3$ at 132.2 ppm. Signals were assigned by comparison with ^{31}P NMR chemical shifts ranges reported in the literature.[29]

Lignin Solubility Tests. Lignin solubility was determined gravimetrically by dispersing 100 mg of lignin in 10 mL of pure ethanol or 90% v/v aqueous dioxane (20 °C, 30 min on a carousel) and then centrifuging the suspension (20 °C, 20 min, 4000*g*) and drying the solid residue at 40 °C for 48 h. Solubility was determined in duplicate, based on the amount of solid residue.

After evaporation of ethanol under nitrogen flux and then freeze-drying, each soluble fraction was acetylated and solubilized in THF solvent for the determination of MM distribution as described above for initial lignins.

Preparation of Cellulose−Lignin Films. Cellulose−lignin films were prepared from mixtures of cellulose nanocrystals (CN) and lignin in dioxane/water (0.45/0.55 v/v) in order to obtain a final cellulose/lignin mass ratio of 5 with the addition of Fenton's reagent (1 mM $FeSO_4$, 0.1 mM H_2O_2).[21] The pH of the solution was adjusted to 3 with 96% sulfuric acid. The mixture was stirred with a magnetized bar during 4 h at ambient temperature before film fabrication. CN−lignin films were prepared by casting onto a horizontal PTFE plate and then drying under a nitrogen flow overnight at room temperature. Average film thickness, measured with a dual-thickness gauge (precision of ±0.001 mm) (Käfer GmbH, Villingen, Germany), was 30 ± 5 μm.

Assessment of Lignin and Film Antioxidant Activity. *Preparation of Lignin Solutions for the Antioxidant Tests.* A lignin sample was weighed in a 2 mL microfuge tube and the solvent (90/10 v/v dioxane/water mixture or absolute ethanol) was added in order to obtain concentrations between 0.25 and 5 mg mL^{-1}. The dispersion was homogenized using a vortex (Heidolph TOP-MIX 94323, Fisher Scientific Bioblock, Vaulx Milieu, France) for 30 s at 20,000 Hz and then centrifuged (Biofuge Fresco Heraeus, Kendro) at 10,000*g* for 10 min at 20 °C. The supernatant ("lignin dioxane/water extract" or "lignin ethanolic extract", respectively) was recovered, and its radical scavenging activity was measured.

Measurement of the Free Radical Scavenging Activity of Lignins by DPPH• Test. The free radical scavenging activity of lignins was evaluated by measuring their reactivity toward the stable free radical DPPH• according to a published method.[30] In a quartz cuvette, 77 μL of lignin solution (lignin dioxane/water extract or lignin ethanolic extract) was added to 3 mL of a 6 × 10^{-5} mol L^{-1} DPPH• solution, prepared daily in dioxane/water (90/10, v/v) or ethanol. The absorbance at 515 nm of each sample was monitored using an UV−visible single-beam spectrophotometer (Secomam S250, Ales, France), until reaching a plateau. A blank was prepared under the same conditions, using 77 μL of solvent instead of lignin extracts. All kinetics were obtained from at least six solutions, prepared from three different lignin preparations. The kinetics of disappearance of DPPH• was obtained by calculating at each time the difference between the absorbance of the blank solution and the absorbance of the sample. When the absorbance reached a plateau, the percentage of residual DPPH• was calculated and plotted vs the concentration of soluble lignin in the sample tested. The concentration of antioxidant extract needed to reduce 50% of the initial DPPH• (noted EC_{50}, with EC standing for efficient concentration) was determined from this curve.

Measurement of the Antioxidant Activity of Lignin Ethanolic Extracts in Lipid-Based Systems. The capacity of lignins to protect free linoleic acid in a dispersed system was determined spectrophotometrically by following the production of conjugated diene hydroperoxides generated by the action of a hydrophilic azo-radical initiator, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), according to a method adapted from Peyrat-Maillard et al. (2003).[31] An aliquot (30 μL) of substrate solution (16 mM linoleic acid in a 0.05 M borate buffer pH 9) and 10 μL of freshly prepared lignin ethanolic extract were added to 2.81 mL of 0.05 M phosphate buffer, (pH 7.4), previously thermostated at 37 °C. An aliquot (150 μL) of AAPH solution (40 mM) was then added, and the progress of oxidation was monitored by recording the absorbance increase at 234 nm vs a blank cuvette containing the same mixture except substrate solution. All kinetics were obtained from at least nine solutions (from 0.5 to 3.5 mg mL^{-1}), prepared from three different lignin preparations. The antioxidant power was defined as the slope of the curve representing the inhibition time of oxidation vs the concentration of antioxidant and was expressed in min mL mg^{-1} lignin.

Measurement of the Free Radical Scavenging Activity of Lignins in Cellulose-Based Films. The free radical scavenging activity of CN−lignin films was evaluated by measuring their reactivity toward the stable free radical DPPH• according to a method adapted from Domenek et al. (2013).[4] The test involved immersing a 0.5 cm^2 film (corresponding to 1.45 mg ± 0.03 mg of film containing 0.24 mg of lignin) in a cuvette, then adding 30 μL of absolute ethanol and 3 mL of a 6 × 10^{-5} mol L^{-1} DPPH• ethanolic solution, and following the absorbance (515 nm for 210 min) at room temperature vs a blank sample without film. The number of moles of residual DPPH• was determined after 210 min. In the above measurement, the antioxidant activity resulted both from compounds that had diffused into the ethanol solution and from compounds which were still present in the film. In order to distinguish between the two families, a piece of film (0.5 cm^2 with a mass of 1.45 mg ± 0.03 mg) was placed in 3 mL of absolute ethanol. After 210 min of infusion, the supernatant, corresponding to migrating substances, was mixed with 30 μL of a 6 × 10^{-3} mol L^{-1} DPPH• solution, and the absorbance at 515 nm of the mixture was measured during 210 min. The film residue after infusion, containing compounds having not migrated, was carefully mixed with 30 μL of absolute ethanol and 3 mL of a 6 × 10^{-5} mol L^{-1} DPPH• solution before absorbance measurement to determine the residual activity of the film. The number of reduced DPPHH moles was determined at the steady-state achievement of each kinetic (210 min) to calculate the antioxidant activity of lignin fractions fixed in cellulose matrix and released in ethanol medium. All the experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Origin and Principal Characteristics of the Lignin Samples. *Difference in Delignification Processes.* The set of lignins was selected according to the different types of delignification process used for their recovery (alkaline vs ethanol organosolv) and for the difference in process severity regarding the OS lignins. The AL lignin was a commercial sample (Protobind 1000) selected for its interest regarding applications in materials, in particular as an antioxidant.[4,32] The OS lignins were produced at pilot scale in order to compare different process conditions. OS-C was recovered from a process operating at lower temperature (140 °C) than OS-B and OS-A (175 and 210 °C, respectively). In contrast, OS-A was recovered from an autocatalytic organosolv process typified by both high temperature (210 °C) and long process time (90

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Table 1. Neutral Sugar (NS) Contents (%) and Structural Characteristics (S/G Ratio and S+G Yield in μmol g^{-1} KL) of the Grass Organosolv (OS) and Alkali (AL) Lignin Samples

	OS-A	OS-B	OS-C	AL	AL extracted[a]
total NS[b]	0.38 ± 0.10	0.61 ± 0.10	2.30 ± 0.01	4.02 ± 0.20	4.43 ± 0.10
arabinose	0.03 ± 0.01	0.07 ± 0.01	0.84 ± 0.01	0.67 ± 0.02	0.73 ± 0.02
galactose	0.04 ± 0.02	0.03 ± 0.01	0.08 ± 0.00	0.17 ± 0.02	0.18 ± 0.02
glucose	0.18 ± 0.02	0.33 ± 0.02	0.28 ± 0.01	0.65 ± 0.02	0.67 ± 0.02
xylose	0.13 ± 0.02	0.18 ± 0.01	1.10 ± 0.01	2.53 ± 0.08	2.85 ± 0.05
S/G[c]	1.55 ± 0.19	1.34 ± 0.07	0.36 ± 0.01	0.94 ± 0.02	0.96 ± 0.01
S+G yield[c]	32.5 ± 0.3	113 ± 6	150 ± 12	194 ± 1	202 ± 17

[a]Extraction process by CH_2Cl_2/AcOEt 50/50 v/v mixture. [b]Monomer mass content expressed with respect to the total dry sample mass. [c]According to thioacidolysis and GC-MS analysis of the thioethylated guaiacyl (G) and syringyl (S) monomer derivatives (H units molar proportion <0.1%).

min). Not only the reaction time was longer than that of the two other OS lignins, but especially the time required to heat-up and cool-down the larger batch autoclave reactor that was used (20 L). In order to compensate the low temperature of the process, an acidic catalyst was used specifically in the OS-C process. Comparison of the lignin samples was performed not to investigate precisely the influence of the process parameters but to identify lignin characteristics associated with different fractionation processes.

Common Structural Features and Composition of the Samples. Due to their botanical origin, all samples exhibited common features characteristics of lignins coming from grasses (Table 1 and Figure 1): the presence of syringyl (S) and guaiacyl (G) units (S/G ratio on average of 1.05 ± 0.52; Table 1), the presence of *p*-hydroxycinnamic acids (CA and FA total amount of 1.2−2.8%; Figure 1), and the contamination by arabinoxylans (0.2−3.6%; Table 1). The low carbohydrate content of the OS samples (Table 1) was consistent with values previously obtained for organosolv lignins, in particular from wheat straw.[33] It decreased from OS-C to OS-A, suggesting increasing cleavage of lignin−hemicellulose bonds as well as possibly increased degradation of hemicellulose carbohydrates as a consequence of increasing temperature of the process, as previously observed.[11,12,34] In contrast, the alkaline and milder conditions implemented to recover AL resulted in less cleavage of lignin−hemicellulose bonds as well as possibly co-precipitation of carbohydrates with lignin and explained the high residual carbohydrate content.

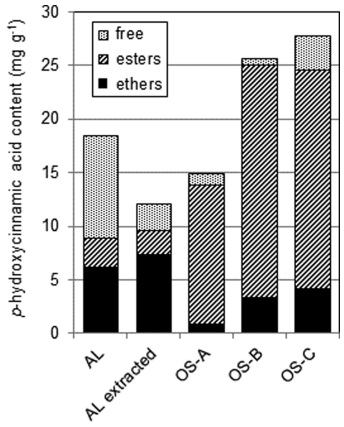

Figure 1. Composition (mg g^{-1}) in free, esterified, and etherified total *p*-hydroxycinnamic acids of the alkali (AL) and organosolv (OS) lignin samples.

Lignin Depolymerization Degree. The main difference between the samples lay in their estimated average MMs. Though MM estimation by HPSEC using polystyrene calibration does not provide accurate absolute mass values, it can be used for comparative purpose.[35] The highest estimated M_w and PD were observed for the AL lignin (M_w = 5090 g mol^{-1}, in agreement with Strassberger et al., 2015[36]), with values about twice as high as those of OS lignins (Table 2). The OS lignins showed decreasing estimated M_w in the order OS-C > OS-B > OS-A (44% decrease of M_w and 24% decrease of PD from OS-A to OS-C; Table 2), suggesting an increasing degree of depolymerization. This was confirmed by both the increase in phenolic OH proportion (Table 3) and the decrease in thioacidolysis yields (4−6 times lower for OS-A than for OS-B and OS-C; Table 1). Indeed, thioacidolysis yield reflects the proportion of ether labile bonds of the β-O-4 type, the first target of the lignin depolymerization processes releasing free phenolic hydroxyls from the bond cleavage. During OS treatments, S-type ether structures are more rapidly cleaved than G ones,[37] in agreement with the increased proportion of S free phenolic groups in eucalyptus lignins recovered from organosolv pretreatments of increasing severity.[34] This explains

Table 2. Solubility, Estimated Mass- and Number-Average Molar Mass (M_w and M_n; g mol^{-1}), and Polydispersity (M_w/M_n) of the Organosolv (OS) and Alkali (AL) Lignin Samples

		molar mass distribution[a]					
		total fraction			ethanol-soluble fraction		
sample	solubility in ethanol (% wt)	M_w	M_n	M_w/M_n	M_w	M_n	M_w/M_n
OS-A	67.3 ± 2.6	1590	1215	1.3	1100	590	1.9
OS-B	64.7 ± 2.6	2140	1440	1.5	1340	753	1.8
OS-C	48.0 ± 0.2	2810	1710	1.7	1710	821	2.1
AL	49.5 ± 0.2	5090	1670	3.0	1480	625	2.4
AL extracted[b]	40.0 ± 0.2	3160	1720	1.8	1340	647	2.1

[a]Determined by size-exclusion chromatography. [b]Extraction process by CH_2Cl_2/AcOEt 50/50 v/v mixture.

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Table 3. ^{31}P NMR Analysis[a] of the Organosolv (OS) and Alkali (AL) Lignin Samples and Their Respective Soluble Ethanolic Fractions

lignin	aliphatic OH	phenolic OH	phenolic/aliphatic ratio
Total Fraction			
Os-A	1.2 (27.1)	2.5 (59.9)	2.2
Os-B	1.6 (37.5)	2.2 (52.6)	1.4
Os-C	1.8 (47.2)	1.8 (47.5)	1.0
AL	1.6 (36.9)	2.0 (45.5)	1.1
AL extracted	1.8 (39.3)	2.1 (43.7)	1.3
Ethanol-Soluble Fraction			
Os-A	1.1 (29.6)	2.1 (60.1)	2.0
Os-B	1.1 (34.5)	1.9 (58.2)	1.7
Os-C	1.6 (47.3)	1.7 (49.7)	1.1
AL	1.3 (27.0)	2.6 (53.3)	2.0
AL extracted	1.3 (27.5)	2.5 (53.6)	2.0

[a]Amount (mmol g^{-1}) of aliphatic and phenolic (syringyl, guaiacyl, and phenol) OH groups in lignin samples and their respective soluble ethanolic fractions evaluated by quantitative ^{31}P NMR analysis. Results in percentage are given in parentheses.

the twice lower S/G ratio obtained here compared to native grass lignins.[38] However, recondensation reactions and α-ethoxylation involving preferentially G units are likely to occur during the process, as evidenced by 2D NMR in Miscanthus organosolv lignins.[39] The lowest S/G ratio was observed for OS-C (0.36). It was found to be even lower than the ratios reported for ethanol OS lignins from Miscanthus (0.63−0.66)[39] and wheat straw (0.41−0.55),[33] suggesting less recondensation in this sample.

Thus, according to these results on lignin structure, the severity of the OS treatment was found to be lower for the low-temperature operating process, even if an acidic catalyst was implemented in that case.

Radical Scavenging Properties of Lignins in Solution. *Similarity of EC_{50} Determined in Aqueous Dioxane for All Lignin Samples.* The EC_{50} parameter is defined as the antioxidant concentration necessary to reduce 50% of the initial DPPH$^{\bullet}$ radicals.[30] In a preliminary screening approach, this parameter was chosen as the most frequently used and convenient parameter to assess the radical scavenging activity of lignins. All the lignins showed similar scavenging activity toward DPPH$^{\bullet}$ in aqueous dioxane, with an average EC_{50} value of 0.33 ± 0.04 g of dispersed lignin per L (Table 4). This result was unexpected in view of the variations in the phenolic/aliphatic hydroxyl ratio, a parameter previously shown to be correlated to the radical scavenging properties of ethanol OS poplar lignins[11] and *Miscanthus* lignins[12] in solution. Nevertheless, it suggested that other structural parameters counterbalanced the effect of the higher ratio observed for OS-A and AL or that the structural variations were not sufficient to impact radical scavenging in the conditions employed for the test.

Table 4. Free Radical Scavenging Activity of Organosolv (OS) and Alkali (AL) Lignin Samples toward DPPH$^{\bullet}$ in 90/10 v/v Dioxane/Water

sample	EC_{50} (g total fraction L^{-1})
OS-A	0.31 ± 0.07
OS-B	0.29 ± 0.03
OS-C	0.32 ± 0.02
AL	0.36 ± 0.01
AL extracted[a]	0.37 ± 0.02

[a]Extraction process by CH_2Cl_2/AcOEt 50/50 v/v mixture.

For comparison, some pure phenolic monomers were characterized in parallel to the lignin samples using the same test conditions (data not shown). The lignin EC_{50} was found to be similar to that of *p*-coumaryl alcohol (EC_{50} = 0.25 g L^{-1}) and only about 5−7 times less efficient than that of pure ferulic and sinapic acids (EC_{50} = 0.05 g^{-1}) and sinapic acid (EC_{50} = 0.05 g L^{-1}), which are well-known phenolic antioxidants.[30,31] The efficiency of the lignins was also in the same order of magnitude as those described in the literature for other co-products from agro-industry, such as skins of tomatoes, carrots, apples, or oranges.[40]

EC_{50} values were reported previously for different wood[41−43] and bamboo lignins.[44] Though direct comparison is often hampered by differences in the DPPH$^{\bullet}$ test conditions (solvent and concentration) and in the way EC_{50} is expressed, the values obtained by Dizhbite et al. (2012)[42] could be compared to ours, expressed in concentration per mol DDPH$^{\bullet}$ present in the system. This comparison suggested that the hardwood AL lignins tested by thpse authors displayed a higher antioxidant efficacy than the grass lignins of our study (6−11 g L^{-1} mol^{-1} DPPH$^{\bullet}$ vs 124−154 g L^{-1} mol^{-1} DPPH$^{\bullet}$). However, Pan et al. (2006)[11] observed for OS poplar lignins values of 13−280 g L^{-1} mol^{-1}, similar to that obtained here. Preliminary tests that we performed on spruce and maize milled-wood lignin, both isolated under the same conditions, confirmed the higher activity of hardwood lignins compared to grass lignins (data not shown). Further study would now be required to investigate more precisely the influence of botanical origin on the antioxidant properties of industrial lignins.

Antioxidant Activity of Lignin Ethanolic Extracts Competitive with a Commercial Rosemary Extract. The advantage of aqueous dioxane for the test was that it totally dissolved all the lignin samples, which was a prerequisite to assess the possible influence of lignin structure and sample composition. However, from a more applied point of view, performing the test in ethanol had the advantages that ethanol is a solvent compatible with the production of antioxidant extracts for food application and that it facilitates the dispersion of antioxidants in a liquid hydrophobic environment.

According to the DPPH$^{\bullet}$ scavenging kinetics (Figure 2), all the lignin ethanolic extracts recovered from a 5 mg mL^{-1} lignin dispersion were found effective to scavenge DPPH$^{\bullet}$ in ethanol, competitively with a commercially available antioxidant rosemary extract currently used as a food additive (EU regulation 1333/2008). A similar kinetic behavior was observed for the different lignin extracts (Figure 2), but comparison of the percentage of residual DPPH$^{\bullet}$ reached at the steady state of the reaction indicated a slightly higher activity of AL compared to the three OS ethanolic extracts. When expressed relative to the total initial lignin amount, the free radical scavenging activity of lignins depended both on their solubility in ethanol and on the radical scavenging activity of the ethanolic extract. Therefore, it is necessary to consider lignin fractionation due to extraction by ethanol.

Enrichment of the Ethanolic Extracts in Low MM Compounds. Lignin solubility in absolute ethanol ranged between 49.5 ± 0.2% for AL lignin and 67.3 ± 2.6% for OS-A (Table 2). The value obtained for AL lignin was consistent with the yield of methanol-soluble fraction reported for the same lignin (Protobind 1000).[45] Whatever the lignin, the estimated

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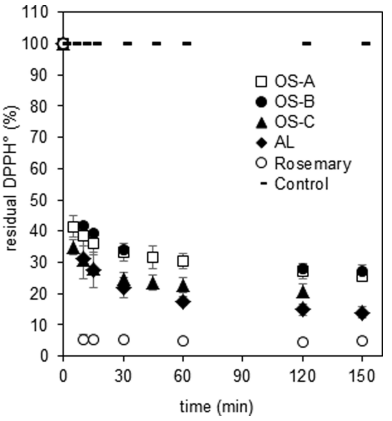

Figure 2. Kinetics of free radical scavenging toward DPPH• in ethanol solution of the lignin ethanol extracts recovered from 5 mg mL^{-1} of alkali (AL) and organosolv (OS) lignin dispersions.

MM averages of the ethanol-soluble fractions were systematically lower than those of the initial lignins, in agreement with a selective extraction of lower MM compounds. The estimated M_w values of the ethanol-soluble fractions (1090−1700 g mol^{-1}; Table 2) corresponded to the equivalent MM of monolignol pentamers to nonamers, and the M_w decrease due to extraction was more pronounced for AL (factor 3.5) than for OS lignins (factor 1.5). In the case of OS lignins, the M_n decrease was more pronounced than that of M_w (factor 2 vs factor 1.5), suggesting that a higher proportion of low-MM compounds was extracted by ethanol in OS lignins than in AL. Interestingly, ethanol extraction reduced the differences in MM between the AL and OS samples, which shows that such a treatment could be used to standardize MM distribution in addition to providing fractions enriched in oligomers. In view of these results regarding lignin ethanol-soluble fractions, it was concluded that the highest activity of the AL sample in ethanolic solution resulted from the chemical composition of the soluble fraction and not from its yield or MM distribution. Indeed, AL exhibited the lowest solubility and similar estimated average MM compared to the OS samples. Furthermore, comparison of the phenolic/aliphatic OH ratio in the initial lignins and in their ethanol-soluble fractions revealed an enrichment in phenolic OH for AL lignin (ratio twice as high in the ethanol-soluble fraction as in the initial sample), whereas it was unchanged for OS lignins (Table 3). These results showed that ethanol fractionation of AL lignin selectively extracted compounds with higher OH phenol content and highest antioxidant activity.

Thus, the radical scavenging tests performed in solution confirmed the performance of all the lignin samples and indicated that the low-MM compounds concentrated in the AL ethanol extract were more active than those of the OS lignin extracts. In order to check these properties in conditions closer to those of the targeted applications, the lignins were implemented in multiphasic systems.

Use of Lignins as Antioxidant in Multiphasic Systems. *Prevention of Lipid Oxidation in Oil/Water Dispersions.* Due to their radical scavenging capacity, lignins could prevent lipid oxidation. Indeed, several industrial lignins of distinct origins (bagasse alkali Granit lignin, softwood kraft Curan 100 lignin, steam explosion lignin, and lignosulfonates) were shown to reduce lipid oxidation induced by hydrogen peroxide in normal human red blood cells.[14] In this previous study, the AL lignin led to the best performance. More recently, lignins isolated from oil palm fronds were found to limit the oxidation of methyl linoleate induced by 2,2′-azobis(isobutyronitrile).[46] In the current study, the capacity of grass lignins to prevent lipid oxidation was assessed through the capacity of their ethanol-soluble fractions to prevent oxidation of linoleic acid dispersed in an aqueous phase, using 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) as oxidation initiator. This system was selected as representative of an emulsified matrix in cosmetic or food products.[31] All the lignin ethanolic extracts delayed the production of conjugated dienes from the oxidation of linoleic acid compared to a control system without lignin (Figure 3).

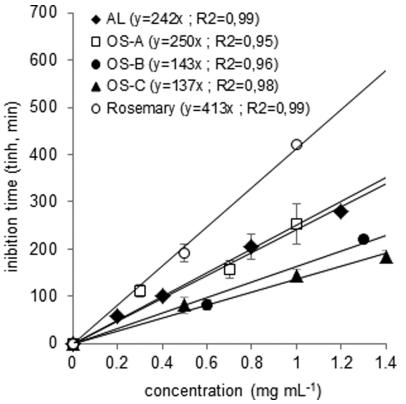

Figure 3. Effect of ethanolic extracts from alkali (AL) and organosolv (OS) lignins and reference rosemary extract on 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced linoleic acid oxidation: inhibition time oxidation vs antioxidant concentration in the ethanolic extract added to the dispersed system.

This protective effect resulted in a lengthening of the duration of the induction phase of oxidation relative to the control. The induction phase increased linearly with the concentration of lignin added to the reactional medium. Its duration was found similar to that of the commercial rosemary extract (Figure 3). Both AL and OS-A extracts were found to be the most active compared to OS-B and OS-C extracts. By contrast, the DPPH• test in ethanol could not discriminate the OS extracts (Table 4). This result suggests that radical scavenging mechanisms were different in the emulsified system and stresses the importance of performing the antioxidant tests in systems representative of the targeted application. The lower MM averages of the OS-A ethanol-soluble fraction compared to its OS-B and OS-C counterparts could be responsible for a facilitated diffusion of antioxidant molecules in the system and their location at the oil−water interface. The subsequent higher concentration of antioxidant molecules in contact with lipids could be responsible for the enhanced lipid protecting effect.

Functionalization of Antioxidant Carrier Cellulose-Based Films. In order to assess the antioxidant properties of the grass lignins dispersed in a polymer matrix, DPPH• scavenging tests were performed in ethanol on cellulosic films containing lignins. These films were selected for their interest as multifunctional materials for active antioxidant packaging. The reference cellulose film deprived of lignin was transparent (transmittance in visible wavelength range >95%). This transparency decreased slightly when lignins were incorporated in cellulose films due to an intense yellow color related to the lignin content (16.7% w/w). The homogeneous yellow coloration was associated with a brightness aspect (Figure

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4b), which suggested a good dispersion of lignins in the cellulose matrix and a smooth surface. The good dispersion was

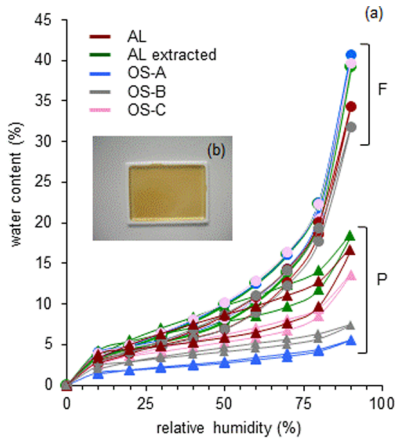

Figure 4. (a) Water sorption isotherms of cellulose−alkali (AL) and cellulose−organosolv (OS) lignin films (F) obtained by treatment with Fenton's reagent and (P) corresponding initial powder mix. (b) Picture of the cellulose−AL lignin film.

favored by the film preparation process, which included an oxidative treatment by addition of Fenton's reagent in the polymeric medium before film casting. Indeed, this oxidative treatment was shown to induce some grafting of lignin onto the surface of CNs.[21] Moreover, it advantageously increased the water retention capacity of the films (F) compared to the initial nontreated powder mix (P) (water sorption isotherms, Figure 4a) conferring properties of hydrogels.

The capacity of the films to scavenge radicals in liquid medium in contact with the film was assessed by immersing the films into an ethanolic DPPH• solution and following the DPPH• reduction for 210 min, until a steady state was reached.[4] All the initial lignins incorporated in the cellulose matrix caused radical scavenging activity to the film, and the total antiradical activity of the film ranged between 18.9 and 40.7 μmol of reduced DPPHH per mg film, in the following order: OS-A > OS-B > AL > OS-C (black circles, Figure 5). Interestingly, this order corresponded to the decreasing order

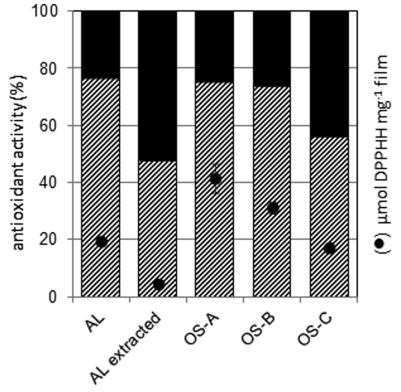

Figure 5. DPPH• scavenging activity of cellulose films incorporated with alkali (AL) and organosolv (OS) lignins. Values are given as cumulative antioxidant activity fixed in cellulose matrix (black bars) and released in ethanol medium (hatched bars). (●) Total reduced DPPHH (μmoles mg^{-1} of film; same axis values as on the left axis) generated by the film.

of ethanol solubility and increasing order of MM averages of the ethanol-soluble fractions (Table 2). In contact with ethanolic solutions during its immersion, the composite films are prone to swell, which facilitates the migration of low-MM phenolic compounds with radical scavenging properties. This phenomenon is consistent with the observation that the radical scavenging capacity of the films increased with decreasing MM averages and increasing ethanol solubility of lignins.

Calculation was performed to predict radical scavenging activity of the films, taking into account both the film lignin content (16.7% wt) and the radical scavenging capacity of the initial lignin sample in ethanol (% residual DPPH• reached at the plateau, Figure 2). Assuming that the radical scavenging activity of the film was only due to that of the ethanol-soluble lignin fraction, the predicted values for AL and OS-C accounted for 77% and 71% of the film activity, respectively. This underestimation suggested that some ethanol-soluble lignin fraction was trapped in the film matrix and thus inaccessible for DPPH• scavenging in the liquid phase or at the solid−liquid interface. Such trapping is consistent with the evidence of lignin grafting onto cellulose.[21] By contrast, the predicted values for OS-A and OS-B exceeded the observed film activity, with 166% and 131% of the activity recovered, respectively. This result suggested either that the lignin nonsoluble fraction contributed to the film activity or that the film preparation increased the proportion of ethanol-soluble compounds and/or their DPPH• scavenging activity. Under the oxidative conditions of the film preparation (presence of Fenton's reagent), lignin structural modifications are likely to occur, which could account for these changes. Among them, the cleavage of lignin β-aryl ether bonds by Fenton's reagent at ambient temperature, previously observed on model compounds,[47] could increase the free phenolic group content in lignins and subsequently antioxidant properties. In addition, the oxidative cleavage of ether bonds could release low-MM lignin compounds likely to diffuse more easily within the swollen polymer matrix. The low MM averages and polydispersity of OS-A and OS-B might increase their susceptibility toward the Fenton's reagent, in agreement with the increased antioxidant capacity observed specifically with these two samples.

In order to assess the real contribution of the soluble lignin fractions, the antiradical activity of the ethanolic extracts recovered after the immersion of the film for 210 min in ethanol deprived of DPPH• was measured. The activity of the film residue was deduced from the total activity of the film and the activity of the film ethanolic extract. The majority of the activity turned out to be due to the lignin ethanol-extractable fraction (hatched bars, Figure 5), the activity of the film residue accounting for 23−52% of the total activity of the film (black bars, Figure 5).

Contribution of *p*-Hydroxycinnamic Acids to Lignin Antioxidant Properties. *Origin and Potential Contribution of p-Hydroxycinnamic Acids in the Samples.* In grass cell-walls, *p*-hydroxycinnamic acids are covalently linked to the polymer network, *p*-coumaric acid as ester of lignin S units and ferulic acid as both ester of arabinose in hemicelluloses and ether of lignin G units.[48] During the biorefinery process, these *p*-hydroxycinnamic acids can either remain bonded to lignin and arabinoxylans or be released. Subsequently, the released acids might co-precipitate with lignin in their free form, possibly with some modifications (e.g., ethylation in case of ethanol organosolv process[33]). Though CA and FA are known for their antioxidant properties,[30] their contribution to the

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antioxidant properties of industrial grass lignins have not been investigated so far. More generally, their effect on lignin properties has been neglected to date and taken into account only in a few studies.[27] As ethanol-soluble compounds, free *p*-hydroxycinnamic acids were likely to contribute to the antiradical activity of lignin ethanolic extracts and of the films. In addition, phenolic acids esterified to lignin could possibly increase lignin antiradical activity, as long as their phenolic group is free.

Variations in p-Hydroxycinnamic Acid Contents. A difference linked to the alkaline conditions was the lower content in esterified *p*-hydroxycinnamic acids and higher content in free ones, as a consequence of ester bond hydrolysis in alkaline conditions (Figure 1). Free CA and FA accounted for 52% of the total CA and FA in AL and only for 1−4% in the OS samples (Table 5). In contrast to AL samples, OS samples contained *p*-hydroxycinnamic acids mainly present under their bound forms, as esters and ethers. Among the esters, *p*-hydroxycinnamic ethyl esters (both CA and FA esters) previously mentioned by Bauer et al. (2012)[39] were found in all CH_2Cl_2/AcOEt OS lignin extracts. Such structures are likely to be formed by esterification of the free *p*-hydroxycinnamic acids by ethanol under acidic conditions. The twice lower content in free and esterified *p*-hydroxycinnamic acids in OS-A compared to OS-B and OS-C (Figure 1) could account for the severity of the conditions. In addition, 91% of the extractible CA or FA was under ethylated form in this sample, versus 31% and 67% in OS-B and OS-C, respectively (Table 5).

Table 5. Composition in Free, Esterified, and Etherified *p*-Coumaric Acid (CA) and Ferulic Acid (FA) of the Organosolv (OS) and Alkali (AL) Lignin Samples (mg g^{-1}, Standard Deviation < 5%)

	OS-A	OS-B	OS-C	AL	AL extracted[a]
free[b]					
CA	0	0.21	0.43	6.14	1.81
FA	0.10	0.16	0.60	3.47	0.69
ethyl CA	0.30	0.10	1.03		
ethyl FA	0.69	0.07	1.10		
esterified[c]					
CA	8.92	17.61	14.81	1.70	1.13
FA	4.06	4.15	5.66	0.95	1.11
etherified[d]					
FA	0.92	3.38	4.21	6.26	7.36

[a]Extraction process by CH_2Cl_2/AcOEt 50/50 v/v mixture. [b]Determination by direct extraction with CH_2Cl_2/AcOEt 50/50 v/v mixture and LC-MS using the response coefficients of the non-ethylated forms for ethyl CA and ethyl FA. [c]Determination by mild alkaline hydrolysis and LC-MS with correction for the contribution of free CA and FA. [d]Determination by severe alkaline hydrolysis and LC-MS with correction for the contribution of esters and free CA and FA.

Besides *p*-hydroxycinnamic acids and their ethylated forms, vanillin and syringaldehyde (total content 0.58−2.98 mg g^{-1}) were found in both AL and OS lignin extracts. Other free phenolic monomers were detected specifically in the AL sample (benzaldehyde, vanillic and syringic acids, <0.60 mg g^{-1}, and acetosyringone, 3.77 mg g^{-1}), as described by Lepifre et al. (2004).[26] Whereas phenolic aldehydes are likely to be produced by oxidation of *p*-hydroxycinnamic acids and alcohols, benzylic acids and ketones might be present initially in the samples and released in the alkali medium.

In view of the presence of free phenolic monomers in the AL lignin, this sample was specifically submitted to an extraction aiming at recovering only lignin oligomers and polymers.

Properties of the Extracted AL Lignin. Extraction by a CH_2Cl_2/EtOAc 50/50 v/v mixture reduced by 4 times the free *p*-hydroxycinnamic acid content of AL from 9.6 mg g^{-1} down to 2.5 mg g^{-1} (Figure 1) but did not affect lignin content and structure, in either the total or ethanol-soluble fractions (Tables 1 and 3). However, PD of the initial sample was reduced about 2-fold after extraction, as a consequence of the elimination of low-MM compounds (Table 2). In spite of the high EC_{50} of pure FA (0.05 g L^{-1}), removing most FA units from AL had little impact on EC_{50} determined in aqueous dioxane (Table 4), due to the low total mass content of free and esterified FA in the initial sample (0.44%). In contrast, the pre-extraction step totally suppressed the activity of the lignin when incorporated in the cellulose-based films (Figure 5). This confirmed the crucial role of the low-MM extractible compounds in this polymer system, independently of the total phenolic hydroxyl content (similar between AL and AL extracted; Table 3). This result is consistent with our previous study on PLA films containing the same AL lignin (Protobind 1000) where the radical scavenging properties of the film in ethanol was found correlated to the amount of free phenolic monomers released by the extrusion thermal treatment.[4] Interestingly, both in this previous study and in the current one, the films kept some residual radical scavenging activity after ethanol extraction. This suggested that some of the AL lignin compounds extractable by the CH_2Cl_2/EtOAc mixture were still present in the film after immersion of the film in ethanol.

In conclusion, whatever system tested (solution, lipid dispersion, or film suspension), all selected lignins were found able to scavenge free radicals in the liquid phase. The structural differences observed between the lignins had no effect on the radical scavenging activity determined with DPPH$^{\bullet}$ radical solutions in aqueous dioxane, which was found similar to that of pure *p*-coumaryl alcohol. By contrast, differences appeared when the tests were performed by dispersing lignins in ethanol and became even more prominent when lignins were incorporated in a cellulose matrix acting as solid carrier.

The antioxidant protective effect of various lignins incorporated in polymer matrices had been previously reported.[3] However, the investigated antioxidant effect was targeted toward the polymer matrices and not toward a liquid medium in contact with the film. In this previous study, low MM averages and total OH contents were found to be beneficial to antioxidant properties, thanks to a better miscibility of lignins in the matrices. In the current study, the mobility of lignins within cellulose matrices and their solubility in ethanol appeared as primary criteria for a protective effect toward the liquid medium in contact with the film. This was found to favor two types of lignins: highly depolymerized organosolv ethanol lignins with low MM averages and alkali lignins containing free *p*-hydroxycinnamic acids.

From an applied industrial point of view, the results show that ethanol extracts obtained from biorefinery grass lignins could compete with commercial rosemary antioxidant extracts for high-value applications. Moreover, self-assembling polysaccharide films could be engineered as active hydrogels for controlled release of antioxidant molecules through simple chemical functionalization. Further investigation is now

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required to ensure the control of the release and assess possible toxicity of the released compounds.

AUTHOR INFORMATION

Corresponding Author

*Phone: +33 (0) 1 30 83 37 78. E-mail: stephanie.baumberger@versailles.inra.fr.

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Notes

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ECN
Westerduinweg 3
1755 LE Petten
The Netherlands

P.O. Box 1
1755 LG Petten
The Netherlands

T +31 88 515 4949
F +31 88 515 8338
info@ ecn.nl
www.ecn.nl