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Structural Characterization and Antioxidant Activity Evaluation of Lignins from Rice Husk

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In recent years, lignin and extractives from herbaceous plants and crops are receiving increasing attention for their renewability and large annual biomass stock. It is worth noting that only a few studies deal with the chemical characterization of rice husk, a side product of one of the most important crops with regard to human nutrition. Thus, in this study lignin from rice husk was isolated and characterized. Two different extraction procedures were optimized and tested: acidolysis and alkaline enzymatic (AE). The different lignins isolated were fully characterized by means of gravimetric, chromatographic (GPC), and spectroscopic (^{31}P NMR, 2D-HSQC-NMR) analyses with the aim to compare yields, sample purity, and chemical properties, recognized as key parameters for future development. Notwithstanding the extraction procedure, the results highlighted that rice husk lignin is mainly formed by guaiacyl and *p*-hydroxyphenyl units. The acidolytic approach showed an appreciable lignin recovery and high purity, whereas the AE lignin sample was found to be rich in residual polysaccharides and oxidized functionalities. Moreover, different rice husk extracts, along with acidolysis lignin and AE lignin specimens, were assayed for their antioxidant activity by means of a DPPH radical scavenging test.

KEYWORDS: Rice husk; lignin; antioxidants; nuclear magnetic resonance; gel permeation chromatography

INTRODUCTION

A suitable renewable feedstock for the chemical and energy industry is extremely important for the sustainable development of society. Nowadays, the current prosperity of chemicals is based on cheap and steady feedstock supply, especially nonrenewable fossil resources such as crude oil, coal, and natural gas. At present, renewable matters such as lignocellulosic materials, the most abundant biomass resource in the world, are foreseen as principal alternatives to fossil resources. The lignocellulosic biomass, which represents about 50% of the global biomass, has an annual production estimated at 10−50 billion of tons (*1*) and is predominantly originated by low-cost agricultural and forestal wastes. The main chemical components of lignocellulosic materials are cellulose, hemicellulose, lignin, and phenolic extractives, with a minor amount of other compounds such as ashes, proteins, starch, terpenes, waxes, resins, fatty acids, and other extractives (*2*, *3*).

In recent years, herbaceous plants have received increasing attention for two primary reasons: annual renewability and large annual biomass stock (1550 million tons/year worldwide) (*4*). Among others, rice is one of the most cultivated crops in the world with a global production of about 680 million tons/year (www.fao.org FAOSTAT Database, 2008). Italy produces approximately 1.4 million tons/year of rice, with 90% of this production concentrated in northern Italy, mostly in Lombardy and Piedmont (www.politicheagricole.gov.it). Rice husk, the outer cover of rice grain, is among the principal processing side products of the rice milling industry and accounts for about 20% by weight of rice. On average, rice husk is composed by 22% lignin, 38% cellulose, 18% hemicelluloses, 2% extractives, and 20% ashes (*5*), but its chemical composition may differ because disparate variables (geographic area, climatic conditions, type of paddy, soil chemistry, fertilizers) are involved in the crop growth. Rice husk does not possess a remarkable commercial interest, and its price is very low (30−40 €/ton in Italy, www.enterisi.it). Because of the elevated ash and lignin contents, rice husk is not appropriate as animal feed raw material. Therefore, it is for the most part it serves as animal litter, soil fertilizer, or combustible for the production of electricity and heat, creating environmental pollution. Recently, many efforts have been made to try to valorize rice husk by exploiting its characteristics as an abrasive surface or as a high ash containing material. Recent studies have demonstrated that rice husk can be burned under controlled conditions to obtain a large amount of silica (about 95% of the total ash content), which may find application in a variety of end products such as building materials, adsorbent phase for the treatment of wastewaters, solid phase for supported enzymes, and filler (*6*, *7*). Moreover, the polysaccharide fraction has been suggested for various applications, such as adhesives, films, and biofuel production (*8*, *9*).

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On the contrary, despite its widespread availability, industrial applications of lignin and phenolic extractives are rather limited, and it has been reckoned that only 1−2% of it is addressed to the development of innovative biobased products (*10*). The aim of our research is to increase this percentage, exploiting agro-industrial lignin wastes as a renewable feedstock to substitute for synthetic additives and fillers in adhesives, resins, thermoplastics, and composites (*11*, *12*).

Thus, in this study lignin and extractives from rice husk were isolated and characterized. Rice husk extractives have been already proved to possess antioxidant activity (*13*, *14*). Three solvents (water, ethanol, and acetone) were tested in different extractions with the aim to isolate extractives in a different solubility range, which would presumably possess different antioxidant properties. The extractives thus recovered were assayed for their antioxidant activity by means of a DPPH radical scavenging test. With regard to the lignin isolation, two different extraction procedures were tested: acidolytic and alkaline enzymatic. The acidolytic isolation method has been taken into account as a simple and well-defined procedure for the isolation of a representative and pure lignin sample. The alkaline enzymatic method has been envisaged as an economic and industrial applicable extraction procedure and therefore investigated to recognize the best experimental conditions for the maximization of yield and purity.

Afterward, the different lignins were fully characterized by means of gravimetric, chromatographic (GPC), and nuclear magnetic resonance analyses (^{31}P NMR and 2D-HSQC-NMR). Alkaline enzymatic lignin (AEL) and acidolysis lignin (AL) samples were also subjected to DPPH colorimetric assay to assess their radical scavenging activity.

MATERIALS AND METHODS

Reagents and Materials. Rice husk was kindly provided by a local factory, Gariboldi S.p.A.. All of the reagents and the solvents (ACS grade) were purchased from Sigma-Aldrich and used as received without further purification.

Rice Husk Preparation. Rice husk (100 g) was ground in a laboratory blender and passed through a 1 mm screen. The ground husk was then stored at −20 °C until needed, as the NREL/TP-510-42620 method suggests (Laboratory Analytical Procedures of the National Renewable Energy Laboratory, U.S. Department of Energy, available online at http://www.nrel.gov/biomass/pdfs/42620.pdf).

Assessment of Rice Husk Composition. The amount of extractives was evaluated according to the NREL/TP-510-42619 procedure (http://www.nrel.gov/biomass/pdfs/42619.pdf), based on a two-step Soxhlet extraction which allows for the removal of, first, water-soluble material and then the ethanol-soluble portion. Acid-insoluble and -soluble lignins were determined gravimetrically and spectrophotometrically, as suggested by the NREL/TP-510-42618 method (http://www.nrel.gov/biomass/pdfs/42618.pdf), performing the hydrolysis of the material for 1 h at 121 °C in an autoclave, whereas ash content was defined according to the ISO 1762:2001 procedure. The protein content was measured according to the NREL/TP-510-42625 method (http://www.nrel.gov/biomass/pdfs/42625.pdf), based on the determination of organic nitrogen by the Kjeldahl methodology. Finally, total carbohydrate content was calculated from the mass balance.

Extractive Isolation for DPPH Colorimetric Assay. Extractive isolation was performed by means of solvents possessing different polarities to cover a solubility range. Each extraction was performed in duplicate. Dried ground rice husk (2.5 g) was subjected to a Soxhlet extraction for 6 h with 170 mL of either water, ethanol, or acetone. After the extraction, the solutions were oven-dried at 105 °C and weighed. Thereafter, each residue was resuspended in 5 mL of methanol, filtered on a tared 0.45 μm GHP filter, and centrifuged in a tared centrifuge tube (11000 rpm, 2 min) to eliminate methanol-insoluble materials. The new concentration of the extractives was calculated, and all of the methanolic solutions were kept at 4 °C until needed for the DPPH colorimetric assay.

Acidolysis Lignin. Dry, extractive-free (NREL/TP-510-42619) blended rice husk (5 g) was milled in a planetary ball mill for different periods of time (5, 10, 15, 20, 30 h) at 300 rpm, using a 100 mL zirconium-grinding bowl (zirconium dioxide 95%) in the presence of 8 zirconium balls (10 mm in diameter each). The lignin extraction was performed according to a modification of the milled wood method developed by Holmbom and Stenius (*15*). In a two-neck round-bottom flask, 1 g aliquots of milled rice husk samples from the milling time experiments (5, 10, 15, 20, 30 h) were refluxed under nitrogen for 2 h in 30 mL of 0.1 M HCl dioxane/water solution (9:1) and then allowed to cool to room temperature. The insoluble material remaining after lignin solubilization was collected by centrifugation (3000 rpm, 15 min). The supernatant was added dropwise into 250 mL of 0.01 M HCl aqueous solution, which was then kept at 4 °C overnight to allow for a complete lignin precipitation. The precipitate was collected by centrifugation (3000 rpm, 15 min), washed with acidified distilled water (pH 2), and freeze-dried.

Alkaline Enzymatic Lignin Extraction. Different extracting temperatures (70, 80, 90 °C), NaOH concentrations (0.1, 0.2, 0.3 M), and reaction periods (1, 2, 4 h) were tested to assess the best extraction conditions with regard to lignin yield and purity. With regard to the optimization, if not otherwise specified, extractive-free (NREL/TP-510-42619) ground rice husk (1 g) was submitted to an alkaline cooking in 20 mL of aqueous 0.2 M NaOH under continuous stirring at 90 °C. After 4 h, the insoluble material left was filtered off through a Gooch crucible (porosity 1) and washed with a NaOH aqueous solution of the same concentration as the processing liquor. The filtrate was acidified with 5 M HCl until pH 3 was reached to allow the lignin to precipitate. The insoluble matter was collected by centrifugation (12000 rpm, 5 min), washed with deionized water, and freeze-dried. The recovered lignin was then subjected to two 3-h cellulolytic cycles with crude cellulase from *T. reesei* ATCC 26921 (50 U/g) at 40 °C, each time collected by centrifugation (12000 rpm, 5 min), and finally freeze-dried.

Evaluation of the Radical Scavenging Activity of Extractives and Lignin. The radical scavenging activity of rice husk extractives, AL and AEL specimens, was determined by means of a spectroscopic assay involving the consumption of the stable free radical originated by DPPH in a methanolic solution. The colorimetric assay was performed according to a modification of the method developed by von Gadow et al. (*16*). Two milliliters of a DPPH methanolic solution (6.1×10^{-5} M, daily prepared) was transferred in a cuvette, and the absorbance (A_0) was registered at 515 nm using a Shimadzu UV-2101PC spectrophotometer. Thereafter, different dosages of a 0.5 mg/mL methanolic solution of either water, ethanol, or acetone rice husk extractives (25, 50, 100, 200 μL each) were added, and then the cuvette was kept in the dark after mixing. When the antioxidant activity of lignins was measured, different dosages of lignin samples (0.5 mg/mL of either AEL or AL, 100, 300, 400, 500 μL each) were dissolved in a dioxane/water solution (9:1) instead of methanol. After 15 min, the absorbance (A) of the solutions was measured at 515 nm. The inhibition percentage of the free radical DPPH$^{\bullet}$ (% I) was calculated according to the following formula:

$$\% I = [(A_0 - A)/A_0] \times 100 \quad (1)$$

Methanolic solutions (dioxane/water 9:1 solutions when the lignins were concerned) of BHA, BHT, quercetin, and rutin were tested as reference antioxidants (0.5 mg/mL). The different extractive concentrations tested, expressed as micrograms per milliiter, were plotted on a log dose−inhibition curve. Thus, the resulting linear calibration curves (water extractives, R^2 = 0.9926; ethanol extractives, R^2 = 0.9898; acetone extractives, R^2 = 0.9974; AL, R^2 = 0.9980; AEL, R^2 = 0.9968) were used to derive the half-maximal inhibitory concentration (IC_{50}).

Lignin Acetylation. Approximately 60 mg of each extracted lignin was acetylated in 2 mL of an acetic anhydride: pyridine solution (1:1, v/v) kept overnight at 40 °C. After stripping with ethanol, toluene, and chloroform (15 mL × 3 times, each solvent), the samples were dried in a vacuum and solubilized in THF or DMSO-d_6 for GPC and 2D-HSQC-NMR analyses, respectively.

^{31}P NMR Analysis. Accurately weighed samples (~20 mg) were dissolved in a pyridine/deuterated chloroform solution (1.6:1 v/v, 800 μL) containing 1 mg/mL of chromium(III) acetylacetonate [Cr(acac)$_3$], along with 100 μL of an e-HNDI solution (121.5 mM, $CDCl_3$/pyridine 4.5:0.5) as

the internal standard. Approximately 100 μL of anhydrous DMF was added to the alkaline lignin specimens to improve their scarce solubility. Then, 100 μL of the derivatizing agent 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane was added (*17*). ^{31}P NMR spectra were recorded on 800 μL samples at 298 K on a Bruker Avance 500 MHz instrument. The ^{31}P NMR data reported in this paper are the averages of three experiments. The maximum standard deviation of the reported data was 2×10^{-2} mmol/g, whereas the maximum standard error was 1×10^{-2} mmol/g.

GPC Analysis. The evaluation of the peak molecular weight (M_p), number-average molecular weight (M_n), and weight-average molecular weight (M_w) was performed according to the methodology developed by Himmel (*18*). The average molar mass of a polydispersed polymer, M, results from several possible methods averaging the different species present, according to the formula

$$M = \Sigma N_i M_i^{n+1} / \Sigma N_i M_i^{n} \quad (2)$$

where N_i is the number of molecules of molecular weight M_i. The averages can be expressed as M_n (number-average molecular weight, $n = 0$), and as M_w (weight-average molecular weight, $n = 1$). The peak molecular weight, M_p, is defined as the molecular weight of the species with maximum N_i. Moreover, the ratio $I = M_w/M_n$, defined as the polydispersity index, was also calculated. The analyses were performed on a Waters 600 E liquid chromatograph connected to a HP1040 ultraviolet diode array (UV) detector set at 280 nm. The injection port was a Rheodyne loop valve equipped with a 20 μL loop. The GP column system was composed by a sequence of an Agilent PL gel 5 μm, 500 Å, and an Agilent PL gel 5 μm, 10^4 Å. The solvent used was tetrahydrofuran (Fluka, 99.8%). PL Polymer Standards of polystyrene from Polymer Laboratories were used for calibration. The PS-calibration curve was tested by means of acetylated dimeric (β-5 and 5,5′ lignin substructure) (*19*, *20*), tetrameric (*21*), and hexameric (kindly given by Prof. Jussi Sipila, University of Helsinki, Finland) lignin model compounds. The acetylated lignin samples were dissolved in THF (1 mg/mL) and analyzed at a flow rate of 1 mL/min. The M_p, M_n, and M_w values reported are the average of three analyses (M_w, 1000 g/mol; M_n, M_p, 70 g/mol, $P = 0.05$, $n = 3$).

2D-HSQC-NMR Analysis. 2D-HSQC spectra were run in DMSO-d_6 on acetylated samples, to avoid the material fractionation before the spectroscopic analysis and to increase both the solubility and the chemical shift dispersion of the side-chain units (*22*). The inverse detected ^{1}H−^{13}C correlation spectra (HSQC) were measured on a Bruker Avance 500 MHz instrument at 313 K. The spectral width was set at 6 kHz in F2 and 27 kHz in F1. Altogether, 128 transients in 256 time increments were collected. The polarization transfer delay was set at the assumed coupling of 140 Hz, and a relaxation delay of 2 s was used. The spectra were processed using Π/2 shifted squared sinebell functions in both dimensions before Fourier transformation. The assignment of predominant signals in 2D-HSQC-NMR spectra was based on the chemical shift data of lignin model compounds and milled wood lignin (MWL), as reported in the literature (*23*, *24*).

RESULTS AND DISCUSSION

The purpose of this work was to investigate different extractives and lignin isolation procedures as a first step to build an industry-accessible, low environmental impact process to exploit rice husk derivatives in a variety of profitable fields, such as antioxidants, ecocomposites, chemicals, and biofuels. Three solvents (water, ethanol, and acetone) were tested in different extractions with the aim to isolate extractives in different solubility ranges and assess their antioxidant properties. Two methods were applied for lignin isolation: acidolytic and alkaline enzymatic. The acidolytic method has been used to isolate a pure lignin fraction to be used as a reference specimen for the characterization of rice husk. On the contrary, the alkaline enzymatic lignin extraction has been optimized in view of a possible industry-accessible process, and the characterization results have been compared with the acidolytic lignin used as reference.

A preliminary characterization of native rice husk highlighted the following composition: 4.7% of extractives (sum of water, 3.5%, and ethanol, 1.2%, extractives), 26% of lignin (sum of acid insoluble, 23.3%, and acid soluble, 2.7%, lignins), 16.8% of ashes, and 52.6% of carbohydrates. In the sample no proteins were detected. The considerable amount of lignin and the large amount of ash content (wood ash content generally comprises between 3 and 5%), constituted by around 85−90% of amorphous silica (*5*), are noteworthy. These percentages reflect the biological function of rice husk, which is a physical protection of rice grains from external damage caused by environmental conditions, parasites, and herbivores. The output of this compositional estimation is close to the one reported by Chandrasekhar et al. (*5*), taking into account that slight differences may be related to different environmental and cultivating conditions as well as to the rice plant species.

Table 1. Radical Scavenging Activity of Water, Ethanol, and Acetone Extractives from Rice Husk, Expressed as IC_{50} Concentration as a Function of Weight (Second Column) and Phenolic Content (Fourth Column)[a]

	IC_{50} (μg/mL)	PhOH (mmol/g)	IC_{50} (nmol of PhOH/mL)
rice husk extractives			
water	82.9	0.59	48.9
ethanol	112.4	1.02	114.6
acetone	195.2	1.22	238.1
reference			
quercetin	1.9	16.54	31.4
rutin	4.1	6.55	26.9
BHA	6.8	5.55	37.7
BHT	8.6	4.54	39.0

[a] Values referred to methanolic solutions.

Radical Scavenging Activity of Water, Ethanol, and Acetone Extractives. Plenty of studies have demonstrated that several plant extracts, mainly composed of phenolic structures, possess interesting antioxidant (*25*, *26*) and antimicrobial properties (*27*). On the basis of these studies, three different solvents (water, ethanol, and acetone) were tested in the isolation of extractives from rice husk to cover a solubility range and appreciate if the antioxidant ability changed depending on the extraction solvent. To test the radical scavenging ability of the extracts a DPPH radical scavenging test was chosen, because the oxidation is often initiated by a free radical attack. Moreover, the DPPH method is rapid, simple, sensitive, and reproducible and does not require special instrumentation. The antioxidant activity of rice husk extractives was compared to that of commonly used antioxidants of both natural (quercetin, rutin) and synthetic origin (BHT, BHA).

Table 1 summarizes the calculated IC_{50} values expressed as micrograms (extract or reference) per milliliter. Despite the lower phenolic content (estimated by quantitative ^{31}P NMR analysis for the three different extractives types), among the other water-soluble extractives were found to be the most powerful radical scavenger. If the same data are regarded as a function of the total phenolic moiety (nmol (phenols)/mL), the IC_{50} values for water extractives and reference antioxidants were found to be quite similar, proving a close relationship between the number of phenolic functionalities and scavenged DPPH radicals. Although the solubility properties may play a crucial role with regard to the chemical characteristics of the isolated products, it is not clear why ethanol and acetone extractives showed much higher IC_{50} values. According to other studies (*28*, *29*), it seems that a direct correlation between the content of the main antioxidant compounds (total polyphenols) and the total antioxidant potential should not be taken for granted. Moreover, common phenols released as a consequence of lignin degradation such as coumaric acid, vanillin, and vanillic acid are proved to react very poorly with the DPPH free radical with a slow kinetic reaction (*30*).

Table 2. Yield, Purity, Ash Content, Average Molecular Weight Index, and Labile Hydroxyl Composition of Acidolysis Lignin Extracted from Differently Milled Rice Husk Samples

	milling time					
	0 h	5 h	10 h	15 h	20 h	30 h
yield (%)	16.0	26.8	34.0	31.2	46.3	41.9
purity (Klason %)	>85	>85	>85	>85	>85	>85
ash (%)	<2	<2	<2	<2	<2	<2
GPC						
M_n (g/mol)	9000	7900	8300	9900	10200	9300
M_w (g/mol)	31500	30300	29500	37200	41000	36300
M_p (g/mol)	4800	4700	5100	5400	5100	4900
I	3.5	3.8	3.5	3.8	4.0	3.9
^{31}P NMR						
aliphatic −OH (mmol/g)	3.08	2.89	3.40	2.98	3.03	2.88
S−OH + cond PhOH (mmol/g)	0.23	0.21	0.34	0.27	0.23	0.31
G−OH (mmol/g)	0.47	0.61	0.70	0.61	0.65	0.60
P−OH (mmol/g)	0.66	0.66	0.74	0.65	0.65	0.63
COOH (mmol/g)	0.23	0.22	0.23	0.22	0.27	0.23

Table 3. Optimization of Alkaline Lignin Extraction: Effect of Different Reaction Temperatures and NaOH Concentrations on Yields, Purity, and Morphological and Chemical Features[a]

	reaction temperature			NaOH concentration		
	70 °C	80 °C	90 °C	0.1 M	0.2 M	0.3 M
yield (%)	11.2	15.3	22.3	11.2	22.3	29.1
purity (Klason %)	65.2	65.2	74.3	49.7	74.3	77.9
ash (%)	<2	<2	<2	<2	<2	<2
GPC						
M_n (g/mol)	11300	12300	12000	7400	12000	13600
M_w (g/mol)	106000	113000	96300	39000	96300	115000
M_p (g/mol)	4200	4600	4600	3800	4600	4500
I	9.4	9.2	8.0	5.4	8.0	8.4
^{31}P NMR						
aliphatic −OH (mmol/g)	1.23	0.86	2.58	0.77	2.58	3.71
S−OH + cond PhOH (mmol/g)	0.06	0.06	0.18	0.05	0.18	0.13
G−OH (mmol/g)	0.14	0.10	0.34	0.09	0.34	0.38
P−OH (mmol/g)	0.15	0.07	0.23	0.08	0.23	0.14
COOH (mmol/g)	0.27	0.20	0.62	0.16	0.62	0.59

[a] If not otherwise indicated, reaction period, concentration of NaOH, and reaction temperature set at 4 h, 0.2 M, and 90°C, respectively.

Lignin Isolation: Acidolysis Lignin. Besides the extractives isolation, some biomass treatments allow the recovery of a quite pure lignin fraction, which may also find huge industrial feedback (*31*), being the major lignocellulosic biomass component after carbohydrates.

A first approach investigated for lignin isolation was an acidolytic extraction performed on rice husk samples subjected to different milling periods. **Table 2** reports yields and composition of the various acidolysis lignin samples (AL). All of them were also characterized by GPC analyses to identify any significant variation in the molecular weight distributions and by means of quantitative ^{31}P NMR spectroscopy to determine the amount of aliphatic hydroxyls, condensed and syringyl phenolic moieties (S−OH + cond PhOH), guaiacyl units (G−OH), *p*-coumaryl alcohols (P−OH), and carboxylic acid functionalities (COOH) as well.

The overlap of the GPC profiles (not shown) displayed an appreciable overlay of the different chromatograms, with the more extensively milled rice husk lignin sample (15, 20, 30 h) being the richer in high molecular weights. This qualitative observation is confirmed by the average molecular weight calculation (M_n, M_w, M_p), reported in **Table 2**. Although extractions carried out on larger particles may be supposed to result in the isolation of a lower molecular weight lignin fraction, as observed in wood lignin (*32*), the GPC profiles and the average molecular weight indices demonstrated an overall uniformity in the morphological properties of the examined samples. This observation could account for a lignification process that is not discriminating with respect to different regions of the husk. The homogeneity of the acidolysis lignins is furthermore supported by the ^{31}P NMR analyses (**Table 2**), which showed similar chemical features in terms of aliphatic hydroxyls, phenols, and acidic functionalities content value for all of the examined specimens. These results demonstrate that the milling time, and thus the lignocellulosic powder granule size, had no influence on the morphological and chemical characteristics of lignin, whereas the extraction yield was greatly affected.

Lignin Isolation: Optimization of Alkaline Lignin Extraction. In the past few years, experimental endeavors have been directed toward the exploitation of lignocellulosic materials by nonconventional methods, which are more concerned with the environment and the industrial applicability than those used on a laboratory scale. The use of NaOH solutions is a common method to process wood and lignocellulosic nonwoody materials to remove lignin (*33*). Moreover, it is worth noting that, besides lignin, the alkaline treatment is able to solubilize other lignocellulosic components such as hemicelluloses, residual extractives, and ashes. Often, the acidic precipitation is not sufficient to remove all contaminants, mainly polysaccharides such as hemicelluloses, which coprecipitate along lignin. Some of these carbohydrates could be hydrolyzed by means of an enzymatic treatment, improving lignin purity.

Therefore, a second part of the study was dedicated to the optimization of the alkaline extraction conditions for rice husk lignin. To reduce the residual polysaccharide content, all of the alkaline lignin specimens collected were subjected to a cellulolytic treatment as a further purification step. A mild alkaline treatment was chosen to avoid potential modifications in the lignin structure due to drastic conditions. The experimental conditions under investigation were the extraction time, the reaction temperature, and the concentration of NaOH. If not otherwise specified, extraction time, temperature, and NaOH concentration were fixed at 4 h, 90 °C, and 0.2 M, respectively. The effects of the various processing conditions on yield, purity, morphological features (GPC), and labile hydroxyls composition (^{31}P NMR) on rice husk alkaline lignin were investigated. Yields and purities of the examined samples were almost all between 15 and 25% and between 65 and 75%, respectively. Only a slight increment in yield and purity was detected with increasing extraction time (data not reported), whereas a rise in both the reaction temperature and the NaOH molarity resulted in the extraction of a larger and purer lignin fraction (**Table 3**). For all conditions tested were noted particularly high M_w values with respect to those found with the acidolytic extraction. Otherwise, M_n and M_p values were found to be almost equal for both procedures, suggesting the presence of residual polysaccharide chains linked to the lignin macromer, which might have lowered the polymer retention time. Results from quantitative ^{31}P NMR analyses led to more interesting conclusions. The variable "extraction time" did not seem to be an important affecting factor because the only consequence of its increment was an otherwise expected heightened oxidation degree (data not shown). When the variables "reaction temperature" and "NaOH concentration" (**Table 3**) were involved, pretty low values of aliphatic hydroxyls, phenolic moieties, and acidic functionalities were detected at 70 °C, 80 °C, and 0.1 M, probably because of an especially limited solubility and purity of these samples related to excessively mild extraction conditions. If the temperature was raised to 90 °C or, otherwise, the concentration

of NaOH was brought to 0.2 or 0.3 M, aliphatic alcohol and phenolic moiety content values moved upward, as well as the carboxylic acid content, as a consequence of a major oxidation. In the effort to find the best compromise between yield and purity, the optimum extraction conditions were recognized as the following: 4 h, 90 °C, and 0.3 M NaOH.

Comparison between AL and AEL Samples. Table 4 displays an overview of the results obtained for both the optimized acidolysis lignin (20 h of ball milling) and the alkaline enzymatic lignin (4 h, 90 °C, and 0.3 M NaOH + cellulase). The best results with regard to gravimetric analyses (yield, purity, ash) were identified in the AL sample (46.3, 86, <2%), which showed an appreciable lignin recovery, high purity, reduced carbohydrate fraction, and low ash content. However, considering the absence of milling, the result from the AEL protocol could be considered competitive (29.1, 77.9, <2%). The average molecular weight indices, along with the overlapped GPC profiles of AL and AEL specimens (not shown), provide evidence of an AEL sample characterized by a molecular weight distribution notably shifted toward higher molecular weight if compared to the acidolytic one, maybe due to the presence of residual carbohydrate. The samples were also characterized by means of quantitative ^{31}P NMR spectroscopy. **Figure 1**, along with **Table 4**, shows that rice husk lignin is mainly formed by guaiacyl and *p*-hydroxyphenyl units, not depending by the applied extraction procedure. A higher content of aliphatic hydroxyls and acidic functionality, along with a modest amount of phenolic moieties, was observed for the alkaline extraction. The higher content of aliphatic alcohol, in addition to the presence of several peaks between 145 and 150 ppm in the ^{31}P NMR spectrum of AEL, was related to the presence of carbohydrate impurities maybe connected to the lignin fraction. This observation is in agreement with the GPC result, and it could also explain the lower value of free phenols: part of them could be involved in the lignin−carbohydrate bond that the alkaline treatment is not able to cleave. Moreover, the lower value of P−OH in the AEL sample could be related to the alkaline cleavage of *p*-coumaryl ester bonds, also observed for wheat

Table 4. Comparison among Yields, Compositional Evaluation, and Morphological and Chemical Features of Rice Husk Lignin Specimens AL and AEL by Gravimetric, GPC, and ^{31}P NMR Analyses

	AL	AEL
milling time (h)	20	blended
yield (%)	46.3	29.1
purity (Klason %)	86.0	77.9
ash (%)	<2	<2
carbohydrate (%)	12.0	20.0
GPC		
M_n (g/mol)	10200	13600
M_w (g/mol)	41000	115000
M_p (g/mol)	5100	4500
I	4.0	8.4
^{31}P NMR		
aliphatic −OH (mmol/g)	3.03	3.71
cond PhOH + S−OH (mmol/g)	0.23	0.13
G−OH (mmol/g)	0.65	0.38
P−OH (mmol/g)	0.65	0.14
COOH (mmol/g)	0.27	0.59

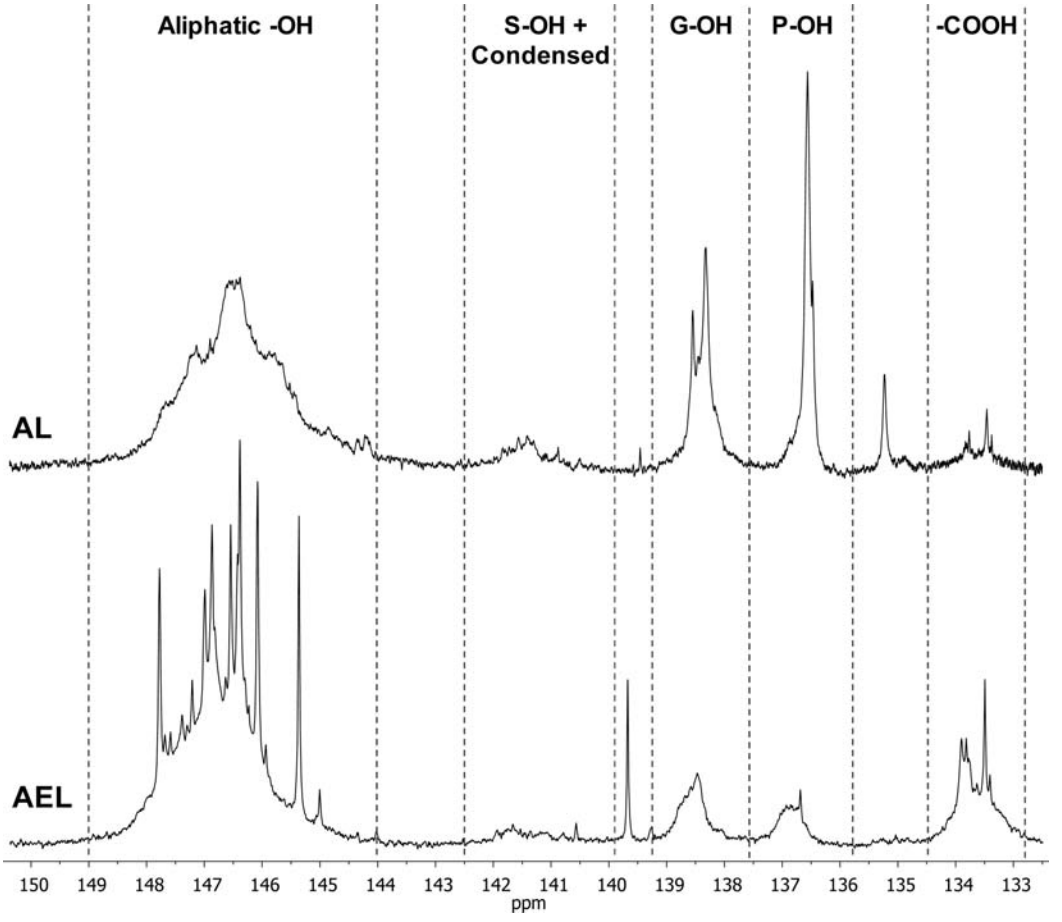

Figure 1. Comparison among ^{31}P NMR spectra of AL and AEL samples. Approximate integration ranges are included for aliphatic hydroxyls (Aliphatic −OH), syringyl and condensed phenolic units (S−OH + cond), guaiacyl phenols moieties (G−OH), *p*-coumaryl units (P−OH), and carboxylic acid functionalities (−COOH).

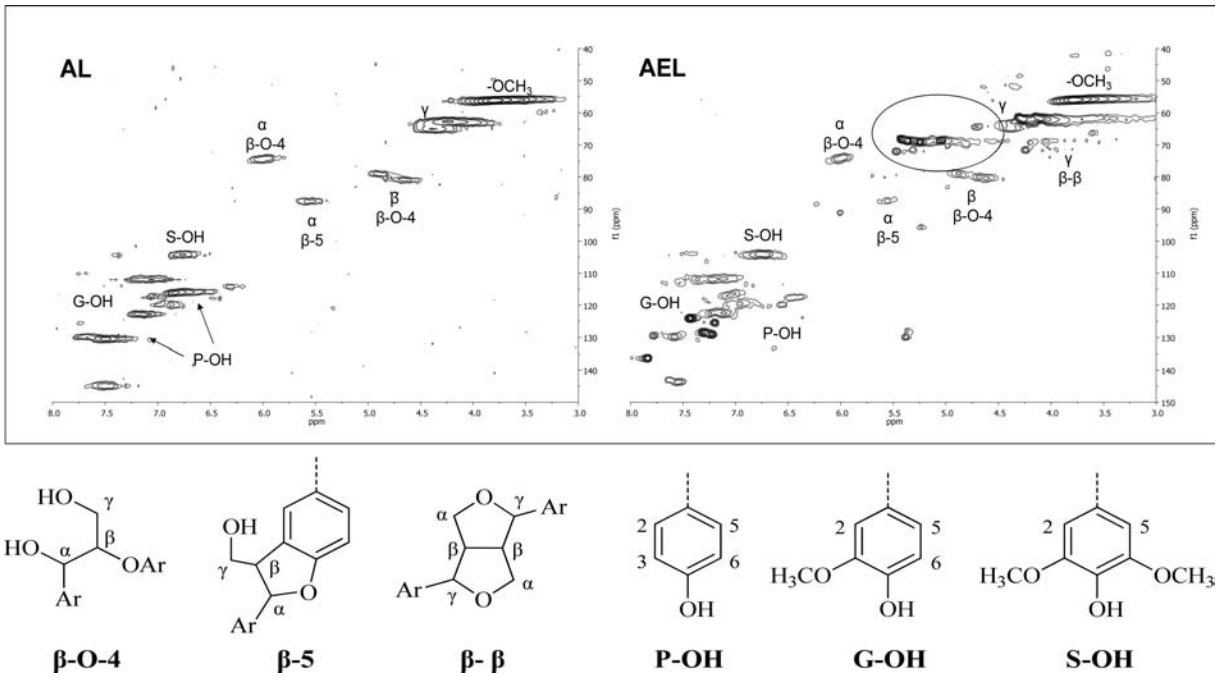

Figure 2. 2D-HSQC-NMR spectra of acetylated AL and AEL samples from rice husk, showing the aliphatic side chain (α, γ, β) for the main lignin interunits (β-O-4, β-5, β-β), the aromatic region (S syringyl, G guaiacyl, and P *p*-coumaryl), and the signals assigned to partially oxidized residual carbohydrate (circled).

straw (*34*). AL and AEL specimens were further analyzed by 2D-HSQC-NMR spectroscopy to identify the principal intermonomeric bonds and to evaluate any significant differences in the two polyphenols' connectivity. The main intermonomeric units in lignin include arylglycerol-β-arylether (β-O-4), phenylcoumaran (β-5), pinoresinol (β-β), and dibenzodioxocine (5-5′-O-4). The spectra, reported in **Figure 2**, highlighted that both the lignins contain arylglycerol-β-arylether units (β-O-4). Cross-peaks relating to other principal intermonomeric bonds (β-5, β-β) were also identified as well as, in the aromatic area, cross-peaks relating to the syringyl, guaiacyl, and *p*-coumaryl units. Even if HSQC could not be used for quantification, it is possible to observe that the P−OH aromatic cross-peaks are well visible in the AL spectrum, while in the AEL spectrum they appear not definied, confirming ^{31}P NMR data. With regard to the AEL specimen, an abundant presence of intense correlation signal at $\delta_C/\delta_H \sim 70-75, 5.0-5.5$ (oval shape), related to partially oxidized residual carbohydrates is noted. Comprehensively, gravimetric and spectroscopic analyses were consistent with an AEL sample still rich in carbohydrates, even after the cellulolytic treatment, and also containing a large amount of oxidized functionalities, originated either by cellulose degradation or lignin side-chain oxidation (or both).

In recent years, agricultural byproducts have been tested as fillers for the production of polymer matrix composites (*35*, *36*). These fillers are not only inexpensive but also able to minimize environmental pollution. Moreover, biodegradable lignocellulosic fillers possess several advantages compared to inorganic additives, such as greater deformability, lower density, and reduced cost. It has been demonstrated that lignin can improve both biodegradability and physical and mechanical properties when added to these materials; moreover, due to its antioxidant activity, it can also act as a stabilizer, preventing polymer aging (*37*). On the basis of these studies, a DPPH radical scavenging test was performed on the AL and AEL samples, and their IC_{50} values were compared to those of reference antioxidants (quercetin, rutin, BHA, BHT) (**Table 5**). When the radical scavenging activity was expressed as a function of the total phenolic content, lignins, and references, IC_{50} values were found to be overall similar, demonstrating a close relationship between number of phenolic functionalities and scavenged DPPH radicals.

Table 5. Radical Scavenging Activity of AL and AEL Specimens, Expressed as IC_{50} Concentration as a Function of both Weight (Second Column) and Phenolic Content (Fourth Column)[a]

	IC_{50} (μg/mL)	PhOH (mmol/g)	IC_{50} (nmol of PhOH/mL)
rice husk lignin			
AL	37.2	1.53	56.8
AEL	52.6	0.65	32.6
reference			
quercetin	1.8	16.54	30.4
rutin	4.3	6.55	28.3
BHA	4.5	5.55	24.7
BHT	10.4	4.54	47.4

[a] Values referred to dioxane/water solutions (9:1).

Future Developments. Chemical modifications aimed at the introduction of lipophilic chains represent potential approaches that may enhance the affinity of lignocellulosic materials toward polymeric systems. As a future development, the alkaline enzymatic rice husk lignin specimen, chemically modified through the introduction of lipophilic side chains, will be investigated as a potential strengthening additive for biodegradable polymers.

ABBREVIATIONS USED

AL, acidolysis lignin; AEL, alkaline enzymatic lignin; GPC, gel permeation chromatography; M_n, number-average molecular weight; M_w, weight-average molecular weight; M_p, peak molecular weight; *I*, polydispersity index; cond PhOH, condensed phenols; S−OH, syringyl phenols; G−OH, guaiacyl phenols; P−OH, *p*-coumaryl phenols; HSQC, heteronuclear single-quantum coherence.

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