JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Characterization of *Miscanthus giganteus* Lignin Isolated by Ethanol Organosolv Process under Reflux Condition

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Supporting Information

ABSTRACT: *Miscanthus giganteus* lignin was extracted by an organosolv process under reflux conditions (4 h) with varying concentrations of ethanol (65%, 75%, 85%, 95%) and 0.2 M hydrochloric acid as catalyst. The resulting lignin was extensively characterized by size exclusion chromatography (SEC), Fourier-transform infrared spectroscopy (FTIR), gas chromatography−mass spectrometry (GC/MS), two-dimensional nuclear magnetic resonance spectroscopy (2D-NMR), and chemical analysis (residual sugars, Klason lignin, ash). The predominant linkage units present were *β*-*O*-4′ (82−84%), resinol (6−7%), and phenylcoumaran (10−11%). The 65% ethanol solvent system gave the lowest lignin yield (14% of starting biomass) compared to 29−32% of the other systems. Increasing ethanol concentration resulted in decreasing carbohydrate content of the lignins (3.6−1.1%), a higher solubility in tetrahydrofuran (THF), a slight reduction of the molecular weight (M_w 2.72−2.25 KDa), an increasing *α*-ethoxylation, and an increase in ethoxylated phenylpropenoic compounds (*p*-coumaric and ferulic acid), but the S/G ratio of the monolignols (0.63, GC/MS) and Klason lignin content (86−88%) were unaffected. An extraction method for these ethyl-esterified phenylpropenoids and smaller molecular weight lignin compounds was developed. The effect of reaction time (2, 4, and 8 h) was investigated for the 95% ethanol solvent system. Besides increased lignin yield (13−43%), a slight increase in M_w (2.21−2.38 kDa) and S/G ratio (0.53−0.68, GC-MS) was observed. Consecutive extractions suggested that these changes were not from lignin modifications (e.g., condensations) but rather from extraction of lignin of different composition. The results were compared to similar solvent systems with 95% acetone and 95% dioxane.

KEYWORDS: *Miscanthus giganteus, lignin, organosolv, ethanol, acetone, dioxane, NMR spectroscopy, FTIR spectroscopy, SEC, thioacidolysis*

INTRODUCTION

Lignocellulosic biomass essentially consists of a closely associated network of large molecular weight polysaccharides (cellulose and hemicellulose) and lignin. Lignin is a complex and irregular macromolecule mainly built from three monolignols (*p*-coumaryl, coniferyl, and sinapyl alcohol). These units are randomly connected by various bonds, such as several types of ether (e.g., *β*-*O*-4′, *α*-*O*-4′) and carbon−carbon linkages (e.g., *β*−*β*′, *β*-5′, and 5−5′), whose formation is mediated by laccases and peroxidases.[1,2] Conversion of lignocellulosic biomass into fuels or other useful chemical products is a rapidly expanding research area. The polysaccharides are hydrolyzed chemically and/or enzymatically into hexoses and pentoses which are then fermented into biofuels. Lignin interferes in this process by reducing accessibility of substrate and irreversibly binding hydrolytic enzymes.[3] Lignin is also the precursor for toxic phenolic fermentation inhibitors which are formed during conventional dilute acid pretreatment steps.[4] Furthermore, to date, the utility of lignin is limited: the left-over lignin portion of the biomass is mainly combusted for steam generation, and only a small fraction is used to produce chemicals such as vanillin or lubricants.[5,6] Therefore, separation of lignin from polysaccharides before pretreatment and carbohydrate hydrolysis as well as conversion of lignin into higher value products would greatly improve the economics of the overall biomass conversion process. Lignin removal can be achieved in part, for example, by means of organosolv processes or ionic liquid pretreatments at the cost of some carbohydrate losses. As a first step in the lignin conversion, the polymer has to be broken down into smaller molecules. Chemical catalysts are most promising for selectively breaking lignin linkages.[7−9] These catalysts could also help in lignin removal from biomass by reducing the size of the lignin polymer, resulting in better solubility in the solvents used for extraction. A drawback in the development of such catalysts is the unavailability of commercial standards larger than dimeric units. Hence, lignin has to be extracted from plants, and various methods have been developed for this purpose.

Milled wood lignin (MWL), also known as Björkman lignin, is extracted from finely ball-milled biomass with aqueous dioxane.[10,11] It is considered to be representative of the original lignin, although contrary views have been expressed.[12] MWL usually gives low yields [13] and contains a significant amount of carbohydrate contamination.[14] A modification of MWL is cellulolytic enzyme lignin (CEL), where the carbohydrate content of ball-milled biomass is lowered by cellulolytic enzyme treatment before solvent extraction.[15]

The CEL procedure was further improved by adding a mild acid hydrolysis step in an organic solvent after enzymatic digestion.[16] This enzymatic mild acidolysis lignin (EMAL) usually gives higher yields and less carbohydrate contamination

Received: June 4, 2012
Revised: July 20, 2012
Accepted: July 23, 2012
Published: July 23, 2012

compared to MWL and CEL. All these methods usually require intensive ball milling of the biomass for a period of hours to weeks.[17,18]

Brauns' lignin, an organosolv lignin, is based on extraction of only ground wood with 95% ethanol but results in low yield and low molecular weight lignin.[19] Use of ethanol with addition of hydrochloric acid as catalyst [20] and dioxane organosolv lignin preparations,[21−23] generated by refluxing biomass at atmospheric pressure with an acid catalyst, have also been described. In most cases, further tedious purification steps, e.g., reprecipitation in diethyl ether and petroleum ether washing, were applied to obtain the final lignin preparation.[24] Other lignin extraction methods use high concentrations of organic acids (formic acid, acetic acid) with hydrochloric acid as catalyst [25] or complete dissolution of biomass and precipitation of dissolved lignin (DWL, dissolved wood lignin).[26] Recently, organosolv lignin extractions using ethanol/water mixtures in the range of 40−95% (w/w) at high temperatures (160−190 °C) with either acid[27−31] or base catalyst [32] have widely been applied. Organosolv delignification without addition of catalysts usually requires higher temperature.[33] Most studies have mainly aimed at developing pretreatment methods for maximum lignin removal from biomass rather than at optimizing properties of the resulting lignin.

Miscanthus giganteus is a C4 perennial grass with high yields and has great potential as a feedstock for biofuel production.[34] It has advantages, such as little nitrogen or herbicide requirement, up to 15 years of lifetime, low moisture content at harvest, and low susceptibility to pests and diseases.[35,36] In our studies, we extracted lignin from *Miscanthus* by an ethanol organosolv process at ambient pressure and moderate heat and compared it to acetone and dioxane extraction under similar conditions. Our goal was to investigate this process that does not require pressurized vessels, large amounts of toxic, or highly flammable chemicals and has only minimal demands for equipment and time. The procedure should be easy and safe to reproduce and should result in high lignin quantities that can be used as a standard for chemical and biochemical catalysis work.

METHODS

Materials. *M. giganteus* was provided by the Energy Biosciences Institute, University of Illinois at Urbana−Champaign, USA. It was ground using an SM2000 cutting mill (Retsch, Haan, Germany) to pass a 2 mm sieve size screen. All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA).

Lignin Extraction. *M. giganteus* (2 mm) was extracted three times each with water and ethanol at 100 °C using an ASE 350 automated solvent extractor (Dionex, Sunnyvale, CA, USA). Settings: 100 mL cell size, 20 g of biomass, 120% rinse, 3 cycles per solvent, 60 s nitrogen purge after extraction. Extracted biomass was ground using a ZM200 rotor mill (Retsch, Germany) passing a 0.5 mm sieve size. The composition of the extracted and dried *Miscanthus* was 44.1% glucan, 22.2% xylan, 1.91% arabinan, 21.4% Klason lignin, 2.75% acetyl. Ground and dried biomass (25 g) was refluxed in a 0.5 L round-bottom flask with a reflux condenser with 175 mL of either 65%, 75%, 85%, or 95% ethanol/5% 0.2 M HCl (v/v) or 95% acetone/5% 0.2 M HCl (v/v) or 95% dioxane/5% 0.2 M HCl (v/v) (using a nitrogen atmosphere for dioxane extraction) for 2−8 h, depending on the experiment. The mixture was filtered through a glass microfiber filter (Whatman International Ltd., Maidstone, U.K.), and solids were washed three times with 50 mL of ethanol, acetone, or dioxane, depending on the experiment. The combined filtrate and washes were concentrated to about 100 mL by evaporation at reduced pressure. This solution was introduced into 1 L of vigorously stirred HCl−acidified water (5 mL of 4 M HCl in 1 L of water). The precipitate formed overnight was collected by centrifugation (3200*g*). It was resuspended five times with 25 mL of DI water and successively centrifuged and then freeze dried.

Molecular Weight Analysis by Size Exclusion Chromatography (SEC). Lignin samples were dissolved in tetrahydrofuran (THF, 1 mg/mL) and analyzed on a GPC 50 Plus system (Agilent Technologies, Santa Clara, CA, USA) with a UV detector and autosampler. All lignins prepared in this study were soluble in THF at 1 mg/mL. The columns used were a series of two 300 mm × 7.5 mm i.d., 3 μm, MesoPore, with a 50 mm × 7.5 mm i.d. guard column of the same material (Agilent Technologies). Samples were eluted at 30 °C with 1 mL/min THF and detected at 280 nm. The system was calibrated using polystyrene samples (EasiVial PS-L) in the range of M_p 38 640−162 Da (Agilent Technologies). Molecular weight at peak top (M_p), number-averaged molecular weight (M_n), weight-averaged molecular weight (M_w), and polydispersity (M_w/M_n) were determined by the Cirrus software (Agilent Technologies).

Monolignol Analysis by Thioacidolysis and Gas Chromatography/Mass Spectrometry (GC/MS). Lignin (2 mg) and 200 μL of thioacidolysis reagent (2.5% boron trifluoride diethyl etherate/10% ethanethiol in 1,4-dioxane) were incubated under a nitrogen headspace at 100 °C for 4 h.[37] After cooling on ice, 150 μL of 0.4 M sodium bicarbonate was added, the solution was vortex mixed, and 1 mL of water was added. The mixture was extracted three times with each 0.5 mL of ethyl acetate, the combined extracts were dried over sodium sulfate, and 200 μL was incubated with 20 μL of pyridine and 100 μL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) including 1% trimethylchlorosilane (TMCS) at 70 °C for 20 min.

Samples were analyzed by GC/MS (Agilent Technologies). The column used was a 30 m × 250 μm i.d., 0.25 μm, VF-5 ms (Agilent Technologies). Configuration: 320 °C injector temperature, oven-programmed isothermal 260 °C for 3 min, then with 30 °C/min to 320 °C, 1 min isothermal, 1 mL/min helium flow rate. MS detection settings: single-ion monitoring (SIM) *m*/*z* 239, 269, and 299.

Nuclear Magnetic Resonance (NMR) Spectroscopy Analysis. NMR spectra were acquired on an Avance 800 MHz spectrometer (Bruker BioSpin, Billerica, MA, USA). Lignin (∼60 mg) was dissolved in 0.6 mL of deuterated dimethyl sulfoxide (DMSO-d_6); solvent peak was used as internal reference (δ_C 39.5, δ_H 2.49 ppm). Standard Bruker implementations of 2D NMR (correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC), and heteronuclear single quantum coherence-total correlation spectroscopy (HSQC-TOCSY)) experiments were used for structural elucidation and assignment verification. HSQC experiments had the following parameters: acquired from 10 to 0 ppm in F2 (^{1}H) using 2048 data points for an acquisition time (AQ) of 128 ms, 220−5 ppm in F1 (^{13}C) using 512 increments (F1 acquisition time 5.8 ms) of 128 scans with a 1 s interscan delay (D1). A squared cosine-bell apodization function was applied in both dimensions. HSQC cross-peaks were assigned by identifying correlated resonances and comparing their chemical shifts with literature values. A semiquantitative analysis of the HSQC cross-peak intensities was performed separately for the different regions of the spectra. In the aliphatic oxygenated region, interunit linkages were estimated from C_α−H_α correlations to avoid possible interference from homonuclear ^{1}H−^{1}H couplings and the relative abundance of side chains involved in interunit linkages and terminal structures was calculated (with respect to total side chains). In the aromatic region, ^{1}H−^{13}C correlations of $S_{2,6}$ and G_2 were used to calculate the S/G ratio (the G integrals were doubled). Volume integration of peaks in HSQC plots was accomplished using Bruker's TopSpin 3.0 software.

Compositional Analysis. Lignin (50 mg) or ball-milled *Miscanthus* after extractions (50 mg) was incubated at room temperature with 0.5 mL of 72% (w/w) sulfuric acid; the suspension was vortex mixed every 15 min. After 1 h, 14 mL of deionized water was added and the mixture was autoclaved for 60 min. A sugar recovery standard containing the same sulfuric acid concentration was prepared in a similar way and coautoclaved with the samples.[38] The mixture was kept in the refrigerator overnight, and 2 mL of the clear

supernatant was filtered (0.45 μm, polyethersulfone) and used for high-performance liquid chromatography (HPLC) analysis.

The precipitated solids were resuspended by vortex mixing, and the suspension was filtered, extensively rinsed with deionized water, and dried at 105 °C overnight; the weight (m_1) was determined. The filter and solids were then incubated at 575 °C for 3 h, and the weight (m_2) was determined. The difference $m_1 - m_2$ resulted in Klason lignin (ash corrected).

For ash determination, 50 mg of lignin was incubated on a preweighed aluminum pan at 575 °C for 3 h and the weight was determined.[39]

For HPLC, samples were analyzed at 50 °C and eluted with 0.005 M sulfuric acid at a flow rate of 0.6 mL/min on a 1200 series liquid chromatography instrument equipped with a refractive index detector (Agilent Technologies). The column used was a 300 mm × 7.8 mm i.d., 9 μm, HPX-87H, with a 30 mm × 4.6 mm i.d. guard column of the same material (Bio Rad, Richmond, CA, USA).

Fourier Transform Infrared Spectroscopy (FTIR). Lignin samples were spotted on a diamond crystal and analyzed on a Nicolet 6700 FTIR spectrophotometer (Thermo Scientific, San Jose, CA, USA) in attenuated total reflection (ATR) mode. Spectra were recorded from 750 to 2000 cm^{-1} with a resolution of 4 cm^{-1}, and 32 spectra were averaged per sample and analyzed using the OMNIC software (Thermo Scientific).

Lignin Fractionation. Lignin (100 mg) was vigorously vortex mixed with 5 mL of ethyl acetate (range 0−50% (v/v)) in methyl *tert*-butyl ether (MTBE) in an ultrasonic bath for 3 min. The suspension was centrifuged (3200*g*), the supernatant was removed, and the residue was freeze dried and weighed.

RESULTS AND DISCUSSION

Lignin Extraction and Recovery. Isolation of *Miscanthus* lignin by refluxing at ambient pressure in the presence of 0.2 M hydrochloric acid was performed using 65%, 75%, 85%, and 95% ethanol as well as 95% acetone and 95% dioxane. Ethanol organosolv lignin (EOL) recoveries (% of original Klason lignin) for the 95%, 85%, and 75% ethanol preparations with 4 h reflux time were very similar (32%, 31%, and 29%, respectively), but in the case of the 65% ethanol solvent it was about one-half (14%) (Figure 1). The different ethanol concentrations resulted in a fairly similar extraction of lignin from *Miscanthus* (range 38−44%) (Figure 2). In particular, the

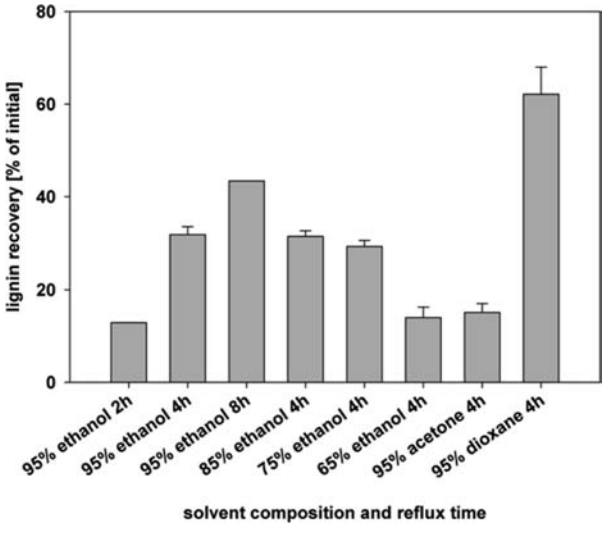

Figure 1. Recovered precipitated and washed lignin (% of initial) after organosolv extraction in the presence of 0.2 M HCl as a function of solvent composition and reflux time.

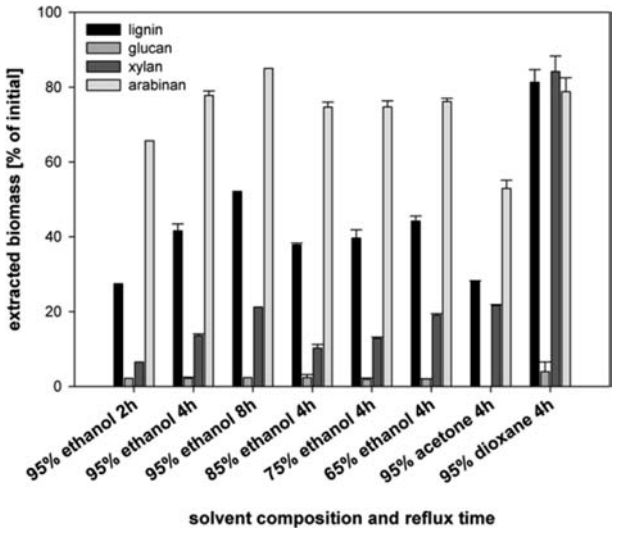

Figure 2. Extraction of lignin, glucan, xylan, and arabinan from biomass (% of initial) after organosolv extraction in the presence of 0.2 M HCl as a function of solvent composition and reflux time.

similar lignin reduction with 95% ethanol (42%) and 65% ethanol (44%) is contrary to the observed lignin recoveries (32% and 14%, respectively). An obvious reason for this low lignin yield after the 65% ethanol extraction was attributed to formation of colloidal suspensions of lignin in the water washes that would not clear even after prolonged centrifugation. An increased formation of this colloidal lignin has been observed in our studies with decreasing ethanol concentration, and this effect has been reported previously.[40] All four ethanol concentrations resulted in about the same reduction of glucan (range 1.9−2.4%) and arabinan (range 75−78%) but varied in the decrease of xylan (range 10−19%) with the lowest xylan reduction with 85% ethanol (10%). Extraction time also played a role in EOL recovery. The 2 h extraction gave less than one-half (13%) compared to the 4 h extraction (32%), and 8 h extraction resulted in 43% EOL (Figure 1).

The 95% acetone extraction gave lower acetone organosolv lignin (AOL) yields (15%) than most of the ethanol solvent systems, but 95% dioxane resulted in 62% dioxane organolsov lignin (DOL) (Figure 1). For the acetone solvent system, the low yield is probably due to the significantly reduced extraction temperature (~60 °C) compared to the ethanol systems (range 82−85 °C) that led to less lignin fragmentation and less lignin solvolysis and therefore less overall delignification (28%) (Figure 2). The higher temperature (~95 °C) and better solubilization ability of dioxane resulted in a very remarkable reduction of lignin (81%) and hemicelluloses (84% xylan and 79% arabinan) (Figure 2), and a high percentage of the extracted lignin was precipitated in acidified water.

Chemical Analysis. All EOL with 4 h reflux time had similar Klason lignin content (range 86−89%) and low ash (range 0.04−0.05%) (Table 1). The residual carbohydrate content (sum of glucan, xylan, and arabinan) decreased from 3.6% to 1.1% with increasing ethanol concentration. The same trend was observed for acetyl groups (decrease from 0.35% to 0.13%). Interestingly, xylan exhibited a strong decline (from 2.4% to 0.3%), but arabinan was only reduced moderately (0.8% to 0.7%). Arabinose/arabinan is a side chain of arabinoxylan and is involved in cross-linking hemicelluloses and lignin.[41] We suspect that some of the residual arabinose is

Table 1. Lignin Composition and Characteristics as a Function of Solvent Composition and Extraction Time

	solvent composition and extraction time							
	ethanol						acetone	dioxane
parameter	65% (4 h)	75% (4 h)	85% (4 h)	95% (2 h)	95% (4 h)	95% (8 h)	95% (4 h)	95% (4 h)
linkages (NMR)								
β-*O*-4′ (A) [%][a]	39	31	24	14	6	4	81	68
β-*O*-4′ (A′) [%][a]	45	51	59	68	77	80		
resinol (B) [%][a]	6	7	6	7	7	6	8	17
phenylcoumaran (C) [%][a]	10	11	10	11	10	10	11	15
S/G ratio (NMR)[a]	0.54	0.54	0.56	0.54	0.52	0.61	0.61	1.08
S/G ratio (GC/MS)[b]	0.63	0.66	0.63	0.53	0.63	0.68	0.79	2.18
SEC								
M_p [kDa]	1.86	1.88	1.87	1.84	1.84	1.84	1.80	2.05
M_w [kDa]	2.72	2.54	2.46	2.21	2.25	2.38	2.17	2.31
M_n [kDa]	1.52	1.45	1.40	1.35	1.24	1.17	1.42	1.24
polydispersity (M_w/M_n)	1.79	1.75	1.76	1.64	1.81	2.03	1.53	1.86
chemical analysis								
Klason lignin [%]	87.1	88.7	86.2	89.4	86.2	88.1	89.7	91.9
glucan [%]	0.37	0.27	0.25	0.22	0.13	0.11	0.27	0.36
xylan [%]	2.38	1.38	0.67	0.40	0.30	0.26	0.81	0.58
arabinan [%]	0.84	0.82	0.78	0.81	0.66	0.57	1.67	0.19
acetyl [%]	0.35	0.23	0.20	0.27	0.13	0.12	0.35	0.16
ash [%]	0.04	0.05	0.05	0.06	0.05	0.06	0.11	0.06

[a]Values are calculated from HSQC 2D NMR. [b]Values are calculated from thioacidolysis and GC/MS analysis.

still covalently bound to lignin, and the reduction in xylan content is based on the easier cleavage of xylose−xylose and xylose−arabinose linkages compared to the cross-linking arabinose−ferulic/*p*-coumaric acid ester bonds. AOL and DOL had slightly higher Klason lignin values (90% and 92%, respectively), but residual carbohydrate levels (2.8% and 1.1%) and ash content (0.11% and 0.06%) were comparable to EOL.

Miscanthus lignin has a very low amount (4%) of *p*-hydroxyphenol (H) units and mainly consists of guaiacyl (G) and syringyl (S) units.[42] Monolignol analysis of the *Miscanthus* used for extraction of lignin and of all the lignins in this study by thioacidolysis resulted in about 1−2% H units (% of sum (H + G + S); data not shown). The S/G ratio of the ethanol organosolv lignins measured by thioacidolysis did not change significantly with ethanol concentration (range 0.63−0.66) (Table 1). These values are comparable with reported S/G values for *Miscanthus* MWL (0.7).[14] In AOL and DOL, measured S/G ratios were higher (0.79 and 2.18, respectively). A higher S/G ratio could be caused by a decrease in G units that are normally more susceptible to condensation reactions than S units, where both ring positions 3 and 5 are occupied by methoxy groups. The higher reflux temperature of the dioxane preparation (∼95 °C) could be a reason for increased condensation in the DOL compared to EOL. Since in the AOL preparations a much lower extraction temperature (∼60 °C) was applied, less condensation should occur and a lower or similar S/G ratio than for the EOL could be expected. Therefore, it remained unclear whether the higher S/G ratios in AOL and DOL compared to EOL were due to lignin modifications or different lignin extraction properties of the solvents used.

Molecular Size Analysis. Despite different ethanol concentrations, all EOL with a 4 h reflux time showed similar molecular weight at maximum peak (M_p, range 1.84−1.88 kDa) (Table 1, Figure 3A) as well as a similar polydispersity index (M_w/M_n, range 1.75−1.81) (Table 1), suggesting a similar and narrow molecular weight distribution. With increasing ethanol concentration a gradual decrease in calculated weight-average molecular weight (M_w, 2.72−2.25 kDa) and number-average molecular weight (M_n, 1.52−1.24 kDa) was observed. Thus, the lignin obtained had a lower molecular weight than previously reported *Miscanthus* EOL (M_w 3.2−6.5 kDa) obtained under much higher temperatures (170−190 °C)[13] or *Miscanthus* acetosolv (M_w 6.02 kDa) or formosolv lignins (M_w 6.66 kDa)[14] but higher than *Miscanthus* cellulolytic enzyme lignin (M_w 1.37 kDa) and alkaline ethanol lignin (M_w 1.20 kDa).[43] A peak at longer retention time (around 1060 s) was present in all EOL but was remarkably high in the 95% EOL (Figure 3A). This peak had a calculated molecular weight ≈ 0.2 kDa, was absent in AOL and DOL (Figure 3B), and was identified by GC/MS (data not shown) as a mixture of mainly ethyl esters of *p*-coumaric (MW 192) and ferulic acid (MW 222). Obviously, a higher ethanol concentration favored formation of ethyl-esterified phenylpropenoic compounds derived from either lignin or hemicellulose. Due to their low water solubility, these derivatives were coprecipitated together with the larger lignin compounds. Longer reflux time favored formation of these molecules: The size of the peak behind these structures in the SEC chromatogram almost doubled when extraction time was doubled (Figure 3C). Interestingly, the increase of the extraction time also led to lignin with a slightly increased molecular weight. Although the 2−8 h extractions had identical M_p (1.84 kDa) (Table 1), the gradual growth of M_w from 2.21 and 2.25 to 2.38 kDa (Table 1) and the gradual expansion of the SEC trace to lower retention times (higher M_w) (Figure 3C) from 2 to 8 h indicated that the size of the lignin was affected by the extraction time. Acidic organosolv processes preferably cleave *α*-aryl ether bonds over *β*-aryl ether bonds, resulting in a transient *α*-carbocation that can react in different ways.[42,44,45] Condensation reactions can occur, and this could result in, e.g., phenylcoumaran structures via intrachain bond (Route 1) (Figure 4) or polymerization via interchain bond

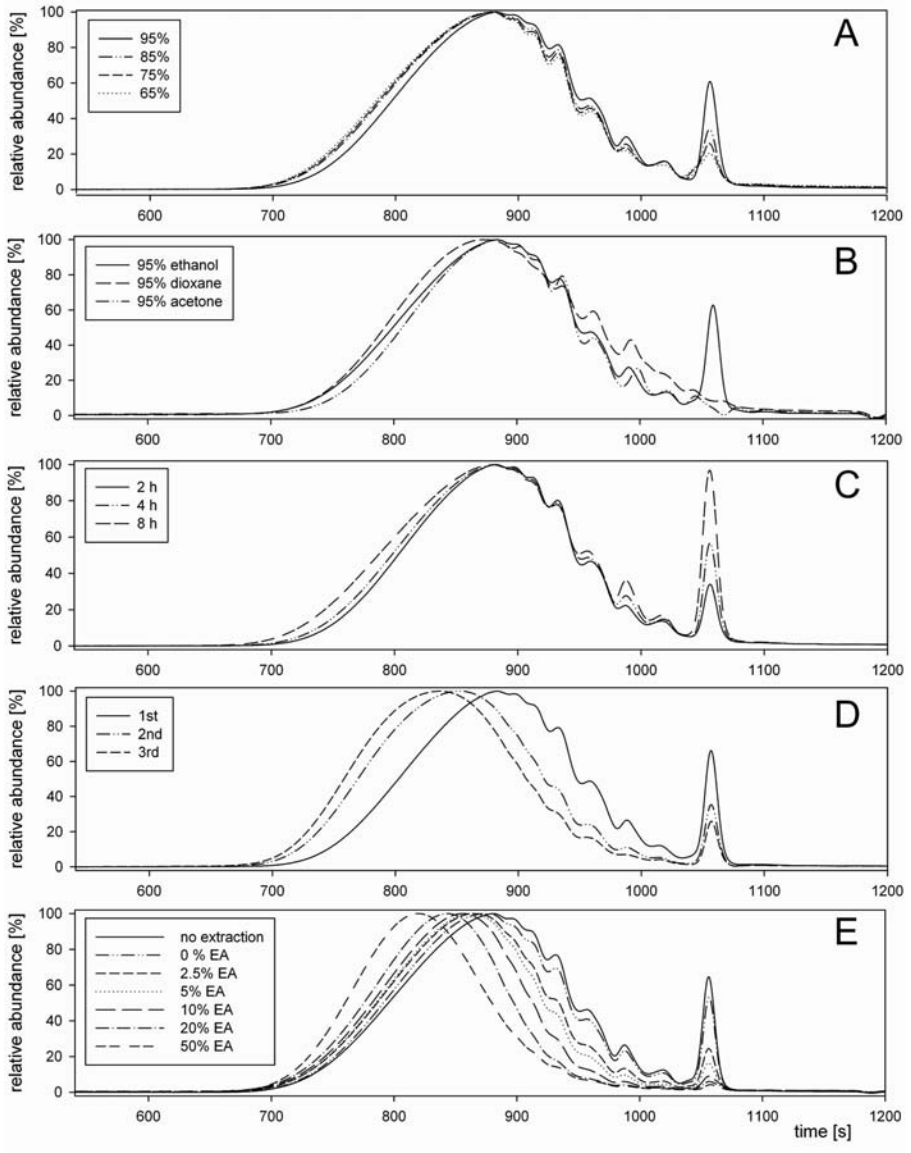

Figure 3. Size exclusion chromatograms of lignin preparations (dissolved in THF, 1 mg/mL) obtained after (A) extraction of *Miscanthus* with 95%, 85%, 75%, and 65% ethanol/0.2 M HCl (4 h reflux), (B) extraction of *Miscanthus* with ethanol, dioxane, and acetone (all 95%/0.2 M HCl, 4 h reflux), (C) extraction of *Miscanthus* with 95% ethanol/0.2 M HCl for 2, 4, and 8 h, (D) 3 successive 4 h extractions of *Miscanthus* with 95% ethanol/0.2 M HCl (fresh solvent for each extraction), and (E) extraction of *Miscanthus* with 95% ethanol/0.2 M HCl for 4 h (no extraction) and extraction of this resulting 95% EOL with 0%, 2.5%, 5%, 10%, 20%, and 50% ethyl acetate (EA) in methyl *tert*-butyl ether (MTBE).

formation, e.g., with an electron-rich carbon of another aromatic ring (Route 2) (Figure 4). Although intramolecular bond formation is highly favored over intermolecular, the latter could result in an increase of the molecular weight. Without added nucleophile formation of structures of the Hibbert's ketone type can be observed (Route 3) (Figure 4). The extraction solvent ethanol could serve as a nucleophile and, since present in excess, should preferably lead to substitution at the α-carbon (Route 4) (Figure 4), which would suppress condensation of lignin. NMR analysis confirmed a high content of α-ethoxylation, so intra- and intermolecular condensations of lignin are not very likely. Furthermore, it can be expected that condensation of lignin only takes place when lignin is solubilized and not in solid state. In order to test this, biomass was consecutively extracted with 0.2 M HCl in 95% ethanol 3 times for 4 h each. Solvent was removed after every extraction period; the biomass was thoroughly washed and then extracted with fresh solvent. In this way, dissolved lignin was not exposed to heat and acid for more than 4 h. A gradual decrease of the lignin obtained from the first, second, to the third extraction (60%, 25%, and 15%, respectively, % of sum of lignin of all three extractions; data not shown) was observed. A shift from longer to shorter retention times and thus increased molecular weight lignin was observed from the first to the third extraction (Figure 3D). The S/G ratio of the lignin obtained after the second and third extraction (0.69 and 0.89, HSQC 2D NMR, data not shown) was also higher than in the first 4 h extraction (0.51). The higher S/G ratio (and therefore a lower G content) could either be a sign of more condensed structures that were less readily hydrolyzed during extraction or simply indicate lignin with a different S and G composition, e.g., derived from extraction of lignin from different layers of the cell wall. Hence, it can be assumed that the increase of molecular weight after prolonged extraction time is probably due to extraction of

dx.doi.org/10.1021/jf302409d | J. Agric. Food Chem. 2012, 60, 8203−8212

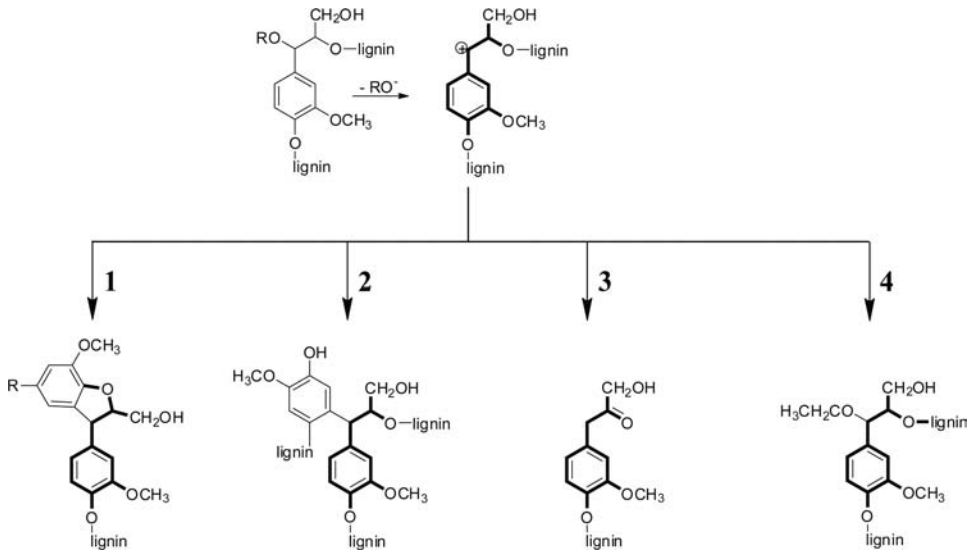

Figure 4. Solvolytic α-ether/α-hydroxy (OR) cleavage and possible fate of the transient α-carbocation. Route 1: intrachain condensation with C5 of a guaiacyl unit to form phenylcoumaran-type structures. Route 2: interchain condensation with an electron-rich carbon of another lignin molecule. Route 3: formation of Hibbert's ketone-type structures. Route 4: nucleophilic addition of the solvent (ethanol).

lignin of different molecular size and composition. As displayed in Table 1, comparison of 95% EOL and DOL revealed the same M_n (1.24 kDa) and similar M_w (2.25 and 2.31 kDa, respectively) and polydispersity index (1.81 and 1.86, respectively), but the higher M_p of DOL (2.05 kDa) indicates a lignin of slightly higher molecular weight. The AOL had a slightly lower M_p (1.80 kDa) and M_w (2.17 kDa) but higher M_n (1.42 kDa), resulting in a lower polydispersity (1.53), translating in an even narrower distribution of the molecular weight. Overall, EOL, AOL, and DOL can be considered to be very similar lignins with respect to molecular weight distribution.

NMR Analysis. The *Miscanthus* lignin samples, from different isolation procedures, were analyzed by 2D NMR. HSQC spectra (oxygenated side chain region) of the different lignins are shown in Figure 5, and the main lignin correlation assignments are listed in Table 2.[46,47] The side chain region of all spectra showed the presence of the major interunit linkages: β-aryl ether (structure A) (Figure 5), resinol (structure B), and phenylcoumaran (structure C) as previously reported,[14] but spirodiene structures (structure D) have not been detected in any lignins examined in this study. However, this structure has also been detected only in low amounts (0.5%) in acetosolv and formosolv lignins from *Miscanthus*.[14] It is known that *Miscanthus* lignin has a very high content of β-*O*-4′ aryl ether linkages (up to 93% reported for MWL[14]). This could also be seen in the HSQC spectra; all showed prominent signals corresponding to β-*O*-4′ ether units (Figure 5). Interestingly, all EOL preparations show additional peaks in comparison to AOL and DOL, which were determined by further NMR experiments to be from a α-ethoxylated β-*O*-4′ substructure (A′). Apparently, the A′ substructure was present in the ethanosolv lignin preparations due to treatment under acidic conditions with ethanol (Route 4) (Figure 4). The substructure α-ethoxylated β-*O*-4′ (A′) was determined by analysis of both HMBC and HSQC-TOCSY experiments. The C_α−H_α correlations were observed at δ_C/δ_H 79.8/4.45 and 80.9/4.54 ppm, while the C_β−H_β correlations were observed at δ_C/δ_H 82.1/4.39 and 83.1/4.30 ppm when linked to G and at 84.4/4.20 and 84.6/4.11 ppm while linked to S units. H_α in A′ gave HMBC correlations to the C_γ at δ_C 59.3 ppm and also to a methylene at δ_C/δ_H 63.6/3.32, which further gave a TOCSY correlation with a methyl group at δ_C/δ_H 15.0/1.08 ppm (not shown in Figure 5). The relative abundances of the main interunit linkages (as a percentage of the total side chains) were calculated from the HSQC spectra (Table 1). Volume integration allows for estimation of the differences in interunit linkage composition and showed a decrease in signals of substructure A in the HSQC spectrum (Figure 5), and the gradual change of unsubstituted β-*O*-4′ structures (A) to substituted (A′) ratio from 39:45 to 6:77 (Table 1) with increasing ethanol concentration underlines this finding. A similar trend was observed when extraction time was increased from 2 over 4 to 8 h. Longer extractions yielded lower detected A:A′ ratios. As mentioned above, an increase in phenylcoumaran structures could be a sign of condensation reactions. Resinol substructures are mainly formed during lignin synthesis from dimerization reactions coupling monolignols and are usually not formed during lignin modification via condensation. Since the ratio of resinol/phenylcoumaran was fairly constant in all EOL preparations (range 0.6−0.7) we conclude that, in addition to the expected reduction of lignin molecular size, α-ethoxylation is the only major modification in the ethanosolv lignin process for the conditions studied. AOL also had a high β-*O*-4′ aryl ether (A) content (81%), similar to the sum of A and A′ for the EOL (range 82−84%). The lower β-*O*-4′ aryl ether content of DOL (68%) could be explained by a higher cleavage of these bonds as a result of the higher reflux temperature condition applied. In addition, the S/G ratio was calculated and confirmed the similar S/G ratio of the EOL preparations (range 0.52−0.56) (Table 1) and the higher ratio of AOL (0.61) and DOL (1.08).

HSQC spectra also reveal differences in the carbohydrates present when using different solvents for isolation. The major carbohydrates in the 95% EOL lignin are α- and β-arabinofuranose. Those carbohydrates are linked to the *p*-coumarate unit; the hydroxycinnamic acid acylates the *O*-5-position of the arabinosyl units (established by HMBC). Another observation was that the anomeric position was ethoxylated. In the AOL, the acetone reacts selectively with *cis*-

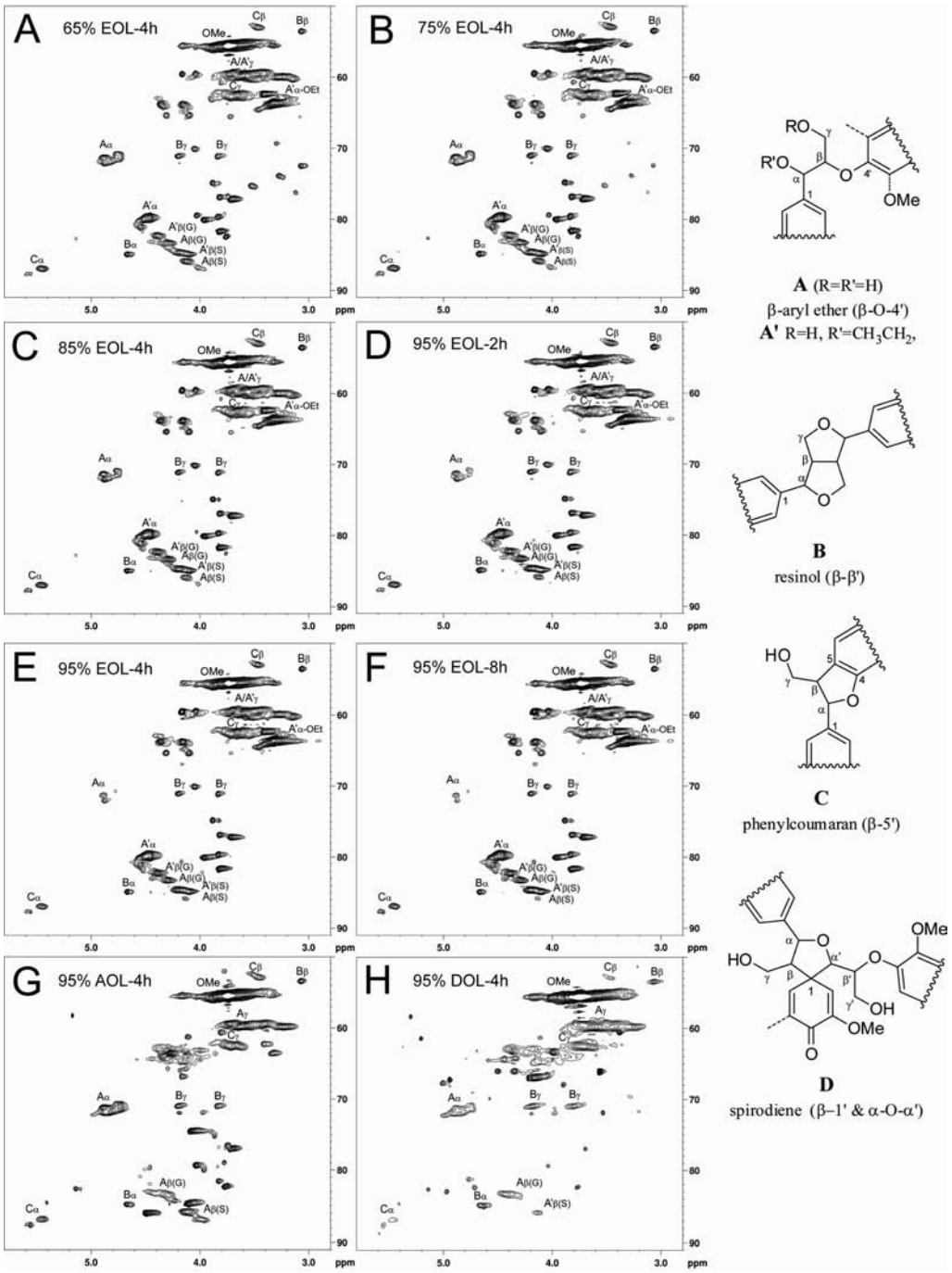

Figure 5. HSQC NMR spectra (δ_C/δ_H 50−91/2.8−5.8 ppm) of the organosolv lignins isolated from *M. giganteus*, and chemical structures of the lignin substructures A (*β*-aryl ether), A′ (*α*-ethoxylated *β*-aryl ether), B (resinol), C (phenylcoumaran), and D (spirodiene): (A) 65% EOL-4 h, (B) 75% EOL-4 h, (C) 85% EOL-4 h, (D) 95% EOL-2 h, (E) 95% EOL-4 h, (F) 95% EOL-8 h, (G) 95% AOL-4 h, and (H) 95% DOL-4 h (see Table 2 for lignin signal assignment).

vicinal hydroxyl groups in the carbohydrates to form acetonide and the DOL contain less carbohydrates.

FTIR Spectroscopy. The FTIR spectrum of the EOL lignins (Figure 6A) showed typical lignin bands at 1592/1602, 1507/1510, and 1421 cm^{-1} derived from aromatic ring vibrations in addition to C−H deformation vibrations (1456 cm^{-1}). Other bands were aromatic ring breathing of condensed S and G rings (1326 cm^{-1}) and G rings (1264 cm^{-1}), aromatic in-plane bending of S (1123 cm^{-1}) and G units (1030 cm^{-1}), C−C plus C−O plus C═O stretch (1224 cm^{-1}), and C−H out of plane deformation of G and S units (832 cm^{-1}). The band at 1705 cm^{-1} could be derived from C═O stretching vibrations of unconjugated ketones, carbonyls, or ester groups or conjugated aldehydes and carboxylic acids.[48] This band has previously been assigned to formation of Hibbert's ketones (Route 3) (Figure 4) as a result of high-severity ethanolsov conditions.[13] It is noteworthy to mention that most lignin IR bands below 1430 cm^{-1} are complex. They are not derived from one single structural feature, and the intensity is rather from various vibration modes, which complicates interpretation.[48]

dx.doi.org/10.1021/jf302409d | J. Agric. Food Chem. 2012, 60, 8203−8212

Table 2. Assignments of the Main $^{1}H-^{13}C$ Correlation Signals in the HSQC Spectra of Organosolv Lignin from *M. giganteus* (Oxygenated Aliphatic Area)

labels	δ_C/δ_H (ppm)	assignment[a]
C_β	52.8/3.47	C_β-H_β in phenylcoumaran substructures (C)
B_β	53.5/3.07	C_β-H_β in resinol substructures (B)
OMe	55.4/3.74	C−H in methoxyls
A/A'_γ	59.3/3.46 and 3.70	C_γ-H_γ in β-*O*-4′ substructures (A/A′)
C_γ	62.3/3.65	C_γ-H_γ in phenylcoumaran substructures (C)
A′-OEt	63.6/3.32	C−H in α-OEt β-*O*-4′ substructures (A′)
B_γ	70.9/3.83 and 4.20	C_γ-H_γ in resinol substructures (B)
A_α	71.6/4.87	C_α-H_α in β-*O*-4′ substructures (A)
A'_α	79.7/4.46 and 80.9/4.54	C_α-H_α in β-*O*-4′ substructures (A′)
$A'_{\beta(G)}$	82.1/4.39 and 83.1/4.30	C_β-H_β in β-*O*-4′ linked to a G unit (A′)
$A_{\beta(G)}$	83.5/4.28	C_β-H_β in β-*O*-4′ linked to a S unit (A)
$A'_{\beta(S)}$	84.4/4.20 and 84.6/4.11	C_β-H_β in β-*O*-4′ substructures (A′)
B_α	84.8/4.67	C_α-H_α in resinol substructures (B)
$A_{\beta(S)}$	85.7/4.14	C_β-H_β in β-*O*-4′ linked to a S unit (A)
C_α	86.8/5.46	C_α-H_α in phenylcoumaran substructures (C)

[a]Signals were assigned by comparing with the literature.[46,47]

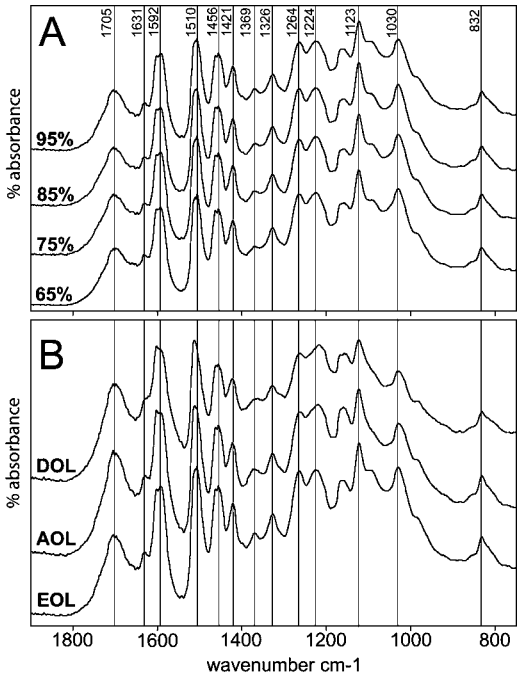

Figure 6. FTIR spectra of (A) 65%, 75%, 85%, and 95% ethanol organosolv lignin (EOL) with 4 h reflux time and (B) EOL, acetone organosolv lignin (AOL), and dioxane organosolv lignin (DOL) with 4 h reflux time.

FTIR spectra of the EOL with a 4 h extraction time were very similar. No major differences in bands attributed to either G or S structures were observed, reflecting the similar G/S ratio determined by GC/MS and NMR (Table 1). An increase of the band at 1369 cm^{-1} (possibly aliphatic C−H stretch in CH_3) and the shoulder at 1095 cm^{-1} (possibly C−O deformation in aliphatic ethers) from 65% to 95% EOL could be an indication of increased ethoxylation of the α-carbon.

In the spectra of AOL and DOL the intensity of 1264 and 1030 cm^{-1} (G units) was lower compared to 1224 cm^{-1} and might describe the lower G content and thus a higher S/G ratio of these lignins compared to EOL where these three bands displayed about the same intensity (Figure 6B). Increasing extraction time was reflected in only minor changes of the FTIR spectra.

Solubility. Organosolv lignins are known to have better solubility in many organic solvents than intact lignins due to their reduced molecular size.[45] A common solvent used for SEC analysis of lignins is THF. Therefore, a high solubility in THF means that the sample can directly be analyzed without further acetyl derivatization steps. The 95% EOL had the highest solubility in THF of all EOL prepared. A concentration of 50 mg/mL showed only minor sediment, whereas 65−85% EOL had clearly visible insoluble material. The higher solubility of the EOL with increasing ethanol concentrations could be due to the decreasing carbohydrate content and increasing ethoxylation of the α-hydroxy group of the β-*O*-4′ linkages, resulting in a less polar lignin. AOL and DOL also showed no precipitate at a concentration of 50 mg/mL, although neither exhibited α-ethoxylation. The carbohydrate content of the DOL was the same as 95% EOL (1.1%), but in AOL (2.8%) it was similar to the levels of the 75% EOL (2.5%). Since the carbohydrates in AOL are present as acetonides, as shown by NMR, their polarity is greatly reduced. Therefore, it could be concluded that the lower carbohydrate content (lower polarity) is correlated with higher THF solubility of these lignins.

Lignin Fractionation. When used as a substrate for catalysis studies, the peak at retention time of 1060 s (Figure 3a) of the 95% EOL could interfere with detection of small molecular weight compounds. Therefore, a method that selectively removes lignin compounds of low molecular weight was developed. We found that the 95% EOL obtained had a very low solubility in methyl *tert*-butyl ether (MTBE) but a higher solubility in ethyl acetate (EA). Figure 3E shows the SEC trace of the starting material and the respective residual lignin residues after extraction with mixtures of EA in MTBE. The higher the concentration of the EA in MTBE in the extraction mixture the higher the amount of lower molecular weight compounds extracted and the higher the molecular weight of the residual lignin (shift to lower retention times). Extraction with MTBE alone (0% EA) lowered the peak at high retention times (*p*-coumaric/ferulic acid ethyl ester) only to a minor extent. A concentration of 5% EA in MTBE reduced this peak by about 80% and resulted in a lignin with a significantly higher molecular weight (M_p 2.21 kDa, M_n 1.80 kDa, M_w 2.83 kDa) (Table 3) compared to the starting material (M_p 1.84 kDa, M_n 1.24 kDa, M_w 2.25 kDa). With 50% EA in MTBE, lignin with an even higher molecular weight (M_p 3.44 kDa, M_n 2.64 kDa, M_w 3.72 kDa) (Table 3) was obtained, but the yields were low (around 10%). The 5% EA in MTBE mixture extracted only about 13% of the initial lignin and was deemed a good compromise of reducing the ethyl ester monomers and keeping the yields of the 95% EOL. NMR analysis of this extracted 95% EOL revealed that the overall properties were preserved.

In conclusion, extraction of *Miscanthus* lignin by ethanol−water mixtures at reflux conditions is a relatively safe and fast process that results in lignin of high purity, high solubility, and good yield. Except for size reduction and partial α-ethoxylation of the β-*O*-4′ linkages the structure of the lignin is mostly unaltered. Therefore, extraction can be applied, e.g., by

Table 3. SEC Characteristics (M_p, M_w, M_n, polydispersity M_w/M_n) of Residual Lignins Obtained from Extraction of 100 mg of 95% EOL with 5 mL of 0%, 2.5%, 5%, 10%, 20%, and 50% (v/v) Ethyl Acetate (EA) in Methyl *tert*-Butyl Ether (MTBE)

	M_p	M_w	M_n	M_w/M_n
no extraction	1.84	2.25	1.24	1.81
0% EA	1.96	2.48	1.37	1.81
2.5% EA	2.17	2.74	1.67	1.64
5% EA	2.21	2.83	1.80	1.57
10% EA	2.35	3.01	2.04	1.48
20% EA	2.66	3.29	2.33	1.41
50% EA	3.44	3.72	2.64	1.41

researchers in need for lignin as a reference substrate in chemical and biochemical catalytic studies.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Information showing the solubility of EOL preparations, additional 2D NMR spectra (aromatic region) of organosolv lignins and from 2D NMR experiments establishing the linkage between Ara*f* and *p*-CA/FA and modification of hydroxyl groups of arabinose by acetone, assignments of signals in the aromatic area, and FTIR spectra of 95% EOL from 2, 4, and 8 h extraction. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was funded by the Energy Biosciences Institute.

Notes

The authors declare no competing financial interest.

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