JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Accurate and Reproducible Determination of Lignin Molar Mass by Acetobromination

Janne Asikkala,[†] Tarja Tamminen,[‡] and Dimitris S. Argyropoulos*[,†,§]

[†]Department of Chemistry, P.O. Box 55, 00014, University of Helsinki, Helsinki, Finland

[‡]VTT, P.O. Box 1000, 02044 VTT, Espoo, Finland

[§]Organic Chemistry of Wood Components Laboratory, Departments of Chemistry and Forest Biomaterials, North Carolina State University, Raleigh, North Carolina 27695, United States

ABSTRACT: The accurate and reproducible determination of lignin molar mass by using size exclusion chromatography (SEC) is challenging. The lignin association effects, known to dominate underivatized lignins, have been thoroughly addressed by reaction with acetyl bromide in an excess of glacial acetic acid. The combination of a concerted acetylation with the introduction of bromine within the lignin alkyl side chains is thought to be responsible for the observed excellent solubilization characteristics acetobromination imparts to a variety of lignin samples. The proposed methodology was compared and contrasted to traditional lignin derivatization methods. In addition, side reactions that could possibly be induced under the acetobromination conditions were explored with native softwood (milled wood lignin, MWL) and technical (kraft) lignin. These efforts lend support toward the use of room temperature acetobromination being a facile, effective, and universal lignin derivatization medium proposed to be employed prior to SEC measurements.

KEYWORDS: *lignin, molar mass determination, size exclusion chromatography, acetyl bromide, ^{31}P NMR, acetobromination, derivatization, acetylation, kraft lignin, milled wood lignin*

INTRODUCTION

The molar mass determination of lignin has been a challenge because of its complex nature, variable polarity, and partial solubility in a variety of common solvent systems. Size exclusion chromatography (SEC) is a documented, versatile tool used for the determination of the molar mass of lignins and other biopolymers offering an estimation of the weight average molar mass (M_w), the number average molar mass (M_n), and the polydispersity index of lignins.[1−8] Over the years, several SEC procedural details have been examined with the aim to provide guidance as to an optimal and universal system selection that could be applied to most lignins irrespective of type and botanical origin. For example, in a recent round robin effort[9] aimed at delineating such variables, by using cross-linked polyvinyl styrene columns, THF as the mobile phase, and acetylation as the derivatization method of choice, it was recommended that the acetylation should be conducted over a period of 6 days (at room temperature) so as to ensure the complete dissolution. Such precautions outline the inherent experimental limitations imposed when solubility considerations for lignins in THF are considered. One main issue of concern related to the accurate and reliable determination of the molar mass of lignins are the documented polar and nonpolar associative interactions occurring in lignin in a variety of solvent systems such as water−NaOH, DMF, DMSO, and THF.[1−8] In cases where associative interactions do not induce distortion, direct analysis (without derivatization) can still however be considered as a satisfactory solution, provided that the optimal column material and analysis parameters are applied.[10]

One common method to prevent and control the formation of polar associative interactions within lignin in organic media is offered by acetylation using acetic anhydride in pyridine.[2] The remaining challenge however that has not been thoroughly addressed in the literature is the complete and universal solubility of the derivatized lignins within the SEC solvent system. Obviously, this is a very important consideration, since incomplete solubility will result in erroneous and irreproducible data. This is the case because the acetic anhydride pyridine, despite being widely used, is known to offer questionable solubility if not executed properly, that is, long reaction times.[9] The use of acetyl bromide in wood and lignin chemistry is well-known, since this is used as a reactive solvent in methods offered to determine lignin content in biomass[11] and in determining lignin structural features via the derivatization followed by a reductive cleavage (DFRC) methodology.[12−14]

In this study, we critically examined and developed a universal derivatization system for most lignins (irrespective of their nature and botanical origin) that may be used in obtaining reproducible molar mass and distribution information. Our approach involves the use of acetyl bromide in glacial acetic acid creating within a very short reaction period and at room temperature lignin derivatives that are highly soluble in THF, thus addressing the solubility and reactivity concerns enumerated by the use of acetic anhydride in pyridine acetylation procedures.[9,15−17]

Received: April 10, 2012
Revised: August 6, 2012
Accepted: August 8, 2012
Published: August 8, 2012

MATERIALS AND METHODS

Chemicals. Softwood and hardwood kraft lignins were from MeadWestvaco (Richmond, VA, USA), Indulin AT, and PC-1369. Norway spruce milled wood lignin was softwood wood isolate by the traditional Bjorkman process.[18] All the other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received.

Lignin Acetobromination Procedure. A lignin sample (10 mg) was weighed into a predried reaction vessel, where 2.3 mL of glacial (anhydrous) acetic acid was added. The reaction mixture was stirred for variable periods of time ranging from 15 min to 20 h, and then, 0.25 mL (3.38 mmol) of acetyl bromide was added. The reaction mixture was finally stirred at room temperature for 20 h (or any desired reaction time). Acetic acid and excess of acetyl bromide were evaporated with an efficient rotary evaporator followed by high vacuum drying at 25−30 °C for 30−45 min.

Lignin Acetylation with Acetyl Chloride Procedure. This procedure was similar to the acetobromination, but acetyl chloride (3.52 mmol) was used instead of acetyl bromide.

Partial Lignin Acetylation with Acetic Acid Catalyzed by HBr. A lignin sample (10 mg) was weighed into a predried reaction vessel, where 2.5 mL of 25% (v/v) HBr in acetic acid was added. The reaction mixture was stirred at room temperature for 20 h. Acetic acid and HBr were evaporated with an efficient rotary evaporator followed by high vacuum drying at 25−30 °C for about 30−45 min.

Lignin Acetylation Using Acetic Anhydride/Pyridine. A lignin sample (10 mg) was weighed into a predried reaction vessel, where 2−3 mL of anhydrous pyridine was added. The reaction mixture was stirred for about 15−20 min, and 0.25 mL (2.65 mmol) of acetic anhydride was added. The reaction mixture was stirred at room temperature for 20 h. Acetic acid and excess pyridine were evaporated with an efficient rotary evaporator followed by high vacuum drying at about 25−30 °C for about 30−45 min.

Solubility of the Acetylated Lignin in THF. The solubility of the samples in THF was assessed by dissolution of 10 mg of an acetylated sample to 10 mL of pure THF followed by filtration via a 0.45 μm syringe filter. The filter was then rinsed with excess THF to ensure that all of the soluble lignin was flushed out from the filter. Then, the THF was evaporated carefully, and the solid was weighed.

Molar Mass Determination. Molar mass was determined by using organic size exclusion chromatography (SEC). Samples were dissolved in THF (HPLC grade, without stabilizer). Sample concentration was 1 mg/mL. After dissolution, samples were filtered using a syringe filter (PTFE, 0.45 μm pore size). After filtration, samples were injected into the SEC system. The SEC system (Agilent, Santa Clara, CA, USA) used includes a degasser, pump, auto sampler, column oven (Agilent 1100 series), diode array UV detector (Agilent 1050 series), and refractive index detector (Agilent 1200 series). The mobile phase was THF (HPLC grade, without stabilizer) with a flow rate of 0.5 mL/min. The columns used were 300 mm × 7.8 mm i.d., Styragel HR-5E and Styragel HR-1, connected in series with a 30 mm × 4.6 mm i.d. guard column of the same material (Waters, Milford, MA, USA). The system was calibrated with polystyrene standards (500, 890, 1000, 4000, 9000, 42 300, 177 000, 434 000, 1 270 000 Da) using UV detection at 280 nm. Molar masses of the samples were calculated using wavelength at 280 nm. The Agilent Chemstation (rev. A. 10.02) with Agilent SEC add on (rev. A 02.02.) were used to calculate the molar mass distributions.

NMR Analyses. A typical quantitative ^{31}P NMR analysis procedure was obtained as previously reported.[19,20,22] The ^{31}P NMR spectra were recorded using inverse gated proton decoupling sequences on a Varian Unity Inova 600 spectrometer (600 MHz proton frequency). The probe used was a 5 mm direct detection broadband probe head. ^{31}P spectra were collected with 256 transients using 90° pulse flip angle, 85000 Hz spectral width, 1 s acquisition time, 6 s relaxation delay, and at 27 °C.

The reported ^{13}C NMR spectra were recorded with a Varian Mercury 300 MHz spectrometer (300 MHz proton frequency). The probe was a 5 mm ASW PFG probe head. ^{13}C spectra were collected using 10 000 transients at 90° pulse flip angle, 20 000 Hz spectral width, 1 s acquisition time, 3 s relaxation delay, and at 27 °C

RESULTS AND DISCUSSION

The facility of dissolution of solid biomass as well as a variety of lignins in acetyl bromide is well-known. The extreme reactivity however of acetyl bromide raises questions of unwanted lignin reactions and molar mass integrity. Possible reactions, such as condensation, acidolysis, and/or halogenations, need therefore to be investigated for their possible occurrence within lignin under the proposed reaction conditions.[10,11,15] In our efforts to elucidate and verify that such chemistries do not detrimentally affect the molar mass measurements, we critically examined and compared the proposed derivatization methods with conventional acetylation using a variety of technical and native lignin samples. More specifically, we used one softwood milled wood lignin and two technical lignins from the kraft process from both softwoods and hardwoods. All lignins were subjected to conventional acetylation and the proposed acetobromination as well as other treatments aimed to answer the posed questions. Our selection of milled wood lignin was critical in the sense that this is the most structurally intact, sensitive, and representative lignin to that present in wood. Alternatively, the two examined kraft lignins are anticipated to be less reactive, since they have already been subjected to the rather severe alkaline pulping conditions.

For the purposes of defining the reactivity profiles of the enumerated lignins, we used four different treatments. Acetylation with acetic anhydride in pyridine and room temperature dissolution of the lignin in acetyl bromide/glacial acetic acid represented the current standard procedure for acetylation and the proposed protocol, respectively. In addition, we examined the use of acetyl chloride in acetic acid and treatment in 30% HBr in acetic acid as two conditions capable of promoting accentuated side reaction on the lignin. More specifically, our contention was that acetyl chloride, as an alternative to acetyl bromide,, needs to be examined since it produces the HCl byproduct that is of lower reactivity than HBr. Furthermore, HBr in acetic acid was also used in an effort to exaggerate the effects of acidic hydrolysis and condensation reactions on lignin.[10,11,15]

During this effort, all reactions were carried out at room temperature for 20 h. After each reaction, the solvent system and the unreacted reagents were rapidly and completely removed by using an efficient rotary evaporator followed by high vacuum drying at 40 °C for about 20 min. For the NMR experiments, the pyridinium acetate (from acetic anhydride/pyridine acetylations) had to be removed by aid of toluene and hexane azeotropes. The treated lignins were dissolved in tetrahydrofuran, and the resulting solutions were filtered. An aliquot of 30 μL was then injected into the SEC column (highly cross-linked styrene−divinylbenzene copolymer particles) that employed a photo diode array (PDA) UV detector that stored the complete absorption data from 190 to 800 nm, and 280 nm wavelength was used to calculate the molar mass distributions. Narrow distribution polystyrene standards, covering the weight average molar mass range from 500 to 1 270 000 Da were used to calibrate the system and calculate the actual molar mass averages reported.

In our hands, the Norway spruce milled wood lignin was only partially soluble (54%) after 20 h, and even when acetylated over a period of 6 days,[9] only about 60% was soluble in THF, while its complete dissolution was evident when acetyl bromide

in glacial acetic acid was used to acetobrominate the sample. Figure 1A shows the actual SEC chromatograms of the milled wood lignin derivatized with acetic anhydride and acetyl bromide, respectively. As anticipated, due to the enumerated solubility considerations, the observed molar mass distribution curves show different intensities. Overall, the acetylated MWL shows a lower response to the acetobrominated lignin at the same elution volume due to the lower amount of dissolved (and consequently eluted) material. Notably, the degree of derivatization on the OH groups in both lignins was monitored with quantitative ^{31}P NMR[19,20] and was found to be complete (Figure 1B,C). Irrespective of the mode of derivatization, the molar mass distribution curves show two resolved peaks at the higher and lower molar mass ranges. Interestingly, for a reason still unknown, a small but discernible high molar mass tail was found to be present in the partially soluble acetylated milled lignin sample. Cathala et al.[21] suggests that there are distinct structural differences between high and low molar mass regions in lignin and these structural differences are shown in differences in solubility and inherently they will show in the observed chromatograms. Overall, it is not surprising that the two derivatization methods offer different molar mass averages, since their solubility in THF are so vastly different (Table 1).

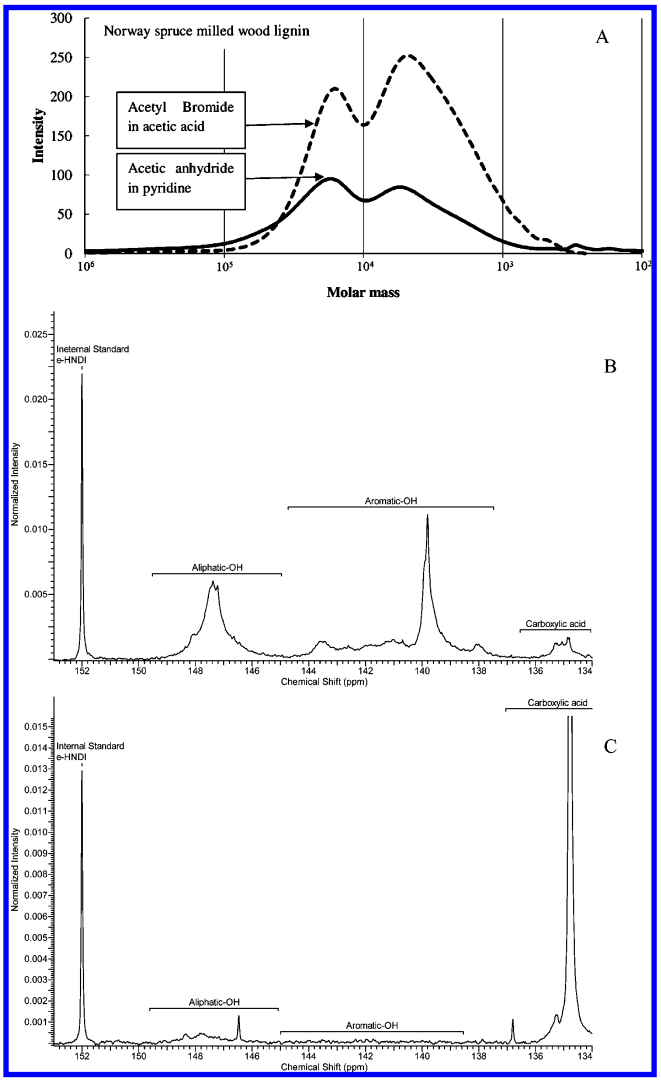

Figure 1. Size-exclusion chromatograms of Norway spruce milled wood lignin acetylated over with acetic anhydride/pyridine and acetobrominated with acetyl bromide/glacial acetic acid, for 20 h at room temperature (A). ^{31}P NMR after phosphitylation (B and C). Initial Indulin AT softwood kraft lignin (B) and its acetylated counterpart using with the proposed acetobromination method over a period of 30 min (C).

In contrast to milled wood lignin, softwood and hardwood kraft lignins showed similar solubility in THF, irrespective of the mode of derivatization (Table 1 and Figure 2). Also the size-exclusion chromatograms for them were nearly identical resulting in nearly similar molar mass averages. This is most likely due to the fact that technical lignins compared to milled wood lignin are structurally degraded during the kraft process and the molar mass is already so small that the solubility is easily achieved.[22]

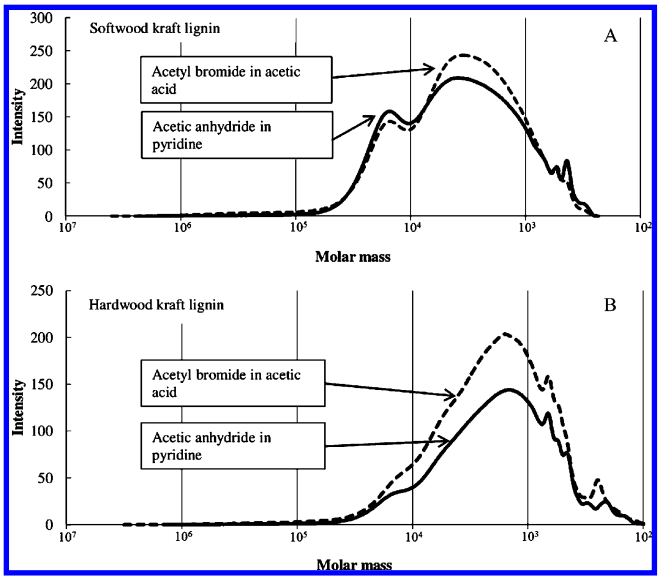

Figure 2. Size-exclusion chromatograms of softwood (A) and hardwood (B) kraft lignin acetylated over with acetic anhydride/pyridine and acetobrominated with acetyl bromide/glacial acetic acid, for 20 h at room temperature.

Beyond the above comparisons that showed the obvious solubility differences operating in milled wood lignin for

Table 1. Molar Mass Values of Norway Spruce Milled Wood Lignin and Softwood and Hardwood Kraft Lignins Using Different Acetylation Methods: Acetic Anhydride in Pyridine and Acetyl Bromide in Acetic Acid

	milled wood lignin		softwood kraft lignin		hardwood kraft lignin	
	acetic anhydride in pyridine	acetyl bromide in acetic acid	acetic anhydride in pyridine	acetyl bromide in acetic acid	acetic anhydride in pyridine	acetyl bromide in acetic acid
M_n (g/mol)	1500	3000	1600	1700	1000	1000
M_w (g/mol)	20000	10000	6500	8000	3300	3900
M_p (g/mol)	17000	5000	3900	3500	1500	1600

dx.doi.org/10.1021/jf303003d | J. Agric. Food Chem. 2012, 60, 8968−8973

acetylated and acetobrominated samples, two more conditions were examined in order to further understand any possible side reactions under acetobromination conditions, affecting its molar mass. One of them was acetyl chloride in glacial acetic acid, since this is a reagent similar to acetyl bromide but of considerably reduced reactivity.[23] In addition, pure HBr was used (catalyzed by the presence of glacial acetic acid) in order to ensure that any HBr catalyzed and induced changes in lignin were visualized and documented. As such, they become a point of reference when looking for such changes that may be occurring under the recommended acetobromination conditions. Figure 3A shows the size-exclusion chromatograms obtained when milled wood lignin was subjected to these reagents with apparent remarkable differences among them.

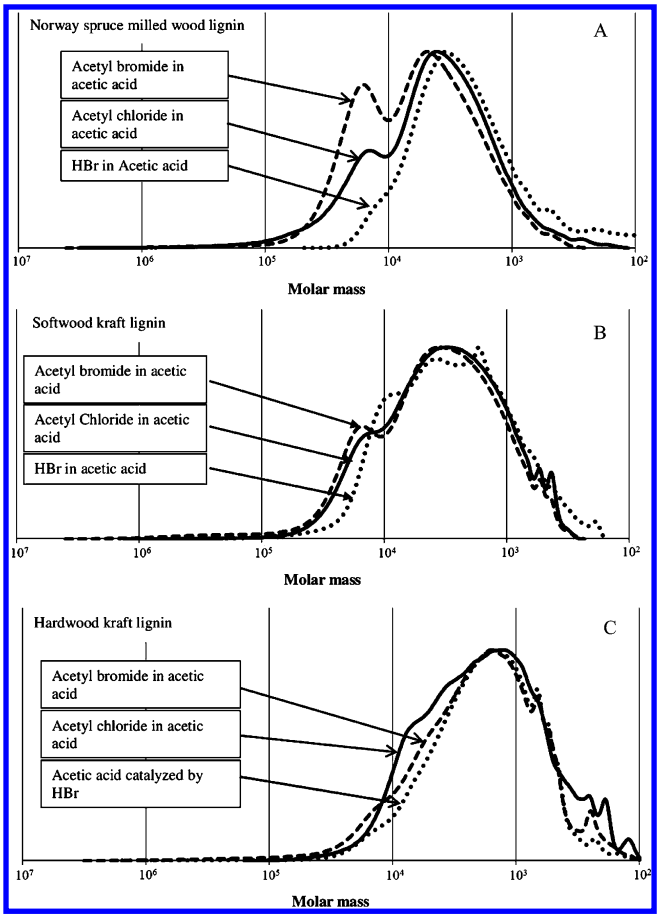

Figure 3. Size-exclusion chromatograms of Norway spruce milled wood lignin (A), softwood kraft lignin (B), and hardwood kraft lignin (C) treated with acetyl bromide, acetyl chloride and HBr in glacial acetic acid for 20 h at room temperature.

The difference observed in the chromatograms between the acetobrominated milled wood lignin to that treated with acetyl chloride can be explained by their different solubilities. This is because treating milled wood lignin with acetyl chloride produced a derivative that was only poorly soluble in THF compared to its acetobrominated analogue. One way to rationalize for these solubility differences is the documented chemistry that actually operates on milled wood lignin when subjected to acetyl bromide.[12] More specifically, Lu et al.[12−14] have shown in detail, with the aid of model compounds, that such conditions acetylate the lignin phenolic and aliphatic OH groups but also selectively brominate the α-positions of the β-ether bonded lignin substructures, substituting hydroxyl groups. In our view, the polar bromine group induced the augmented solubility characteristics obtained after acetobromination.

To further assess any possible side reactions caused by the acetyl bromide acetylation compared to acetic anhydride/pyridine acetylations detailed ^{13}C NMR were acquired from MWL lignins acetylated with both methods (Figure 4). The

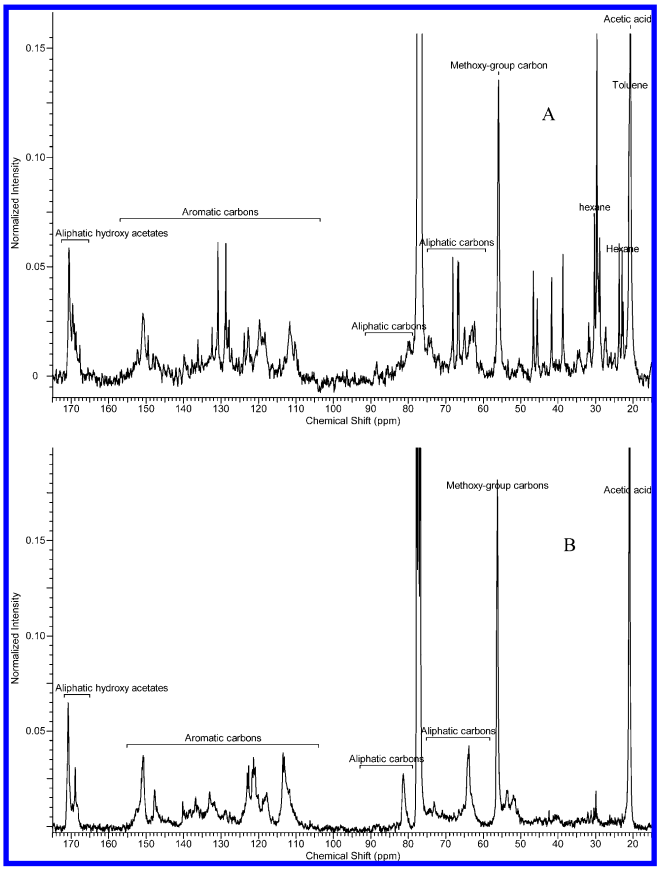

Figure 4. ^{13}C NMR spectra and signal assignments of Norway spruce milled wood lignin acetylated with acetic anhydride in pyridine (A) and acetyl bromide (B).

same solubility problem persisted in $CDCl_3$ as in THF. The MWL acetylated with acetic anhydride pyridine was not completely soluble in $CDCl_3$, which makes the direct comparison of lignins challenging. According to literature accounts,[28−30] the area from 166 to 171 ppm is responsible for the acetylated aliphatic hydroxyl groups. In this region, the 169.5−170.5 ppm range shows some changes; more specifically, the AcBr acetylated MWL is missing a signal that could be due to the α-hydroxy, which has been exchanged with bromine.[12−14] The region from 141 to 160 ppm is assigned[28−30] to oxygenated aromatic carbons. As anticipated, no differences between the two acetylation methods are evident. This was also true for the aromatic carbon−carbon region (125−141 ppm). The aliphatic carbons of the lignin side chains, whose signals appear in the region from 60 to 80 ppm, showed some changes between the two acetylation methods, but overall, these differences were of the same nature as those observed as described for the acetylated OH groups observed between 169.5 and 170.5 ppm. In all, the ^{13}C NMR spectra acquired for MWL samples derivatized with the two methods that we examined here showed structural changes consistent

dx.doi.org/10.1021/jf303003d | J. Agric. Food Chem. 2012, 60, 8968−8973

with the anticipated derivatization chemistries. No other apparent chemical effects that could have a serious impact on the molar mass of the lignin derivatives were apparent.

The chemistry of acetyl chloride on the lignin should be distinct from its acetyl bromide analogue, and as such, no solubility advantages are apparent. Furthermore, Figure 3A demonstrates that HBr as such causes significant depolymerization of softwood milled wood lignin.

Similar experiments to those carried out with milled wood lignin above were also carried out with softwood and hardwood kraft lignins in order to elucidate any side reactions operating on such lignins when acetyl bromide is used. Similar to milled wood lignin, HBr as such induced some differences to the SEC curves also in the case of kraft lignins, probably due to structural changes under these severe conditions[24] (Figure 3 B, C). Acetyl chloride yielded chromatograms similar to those from acetyl bromide. This indicates that the OH groups in kraft lignin can be readily acetylated in acetyl bromide aiding solubility under mild and rapid conditions.

As already discussed, a round robin effort aimed at defining the optimum acquisition conditions for the complete lignin derivatization and its accurate and reproducible molar mass determination arrived at the conclusion that the acetylation step using pyridine/acetic anhydride needs to be carried out over a period of six days in order to ensure complete acetylation.[9] In our hands, however, even after six days of acetylation with acetic anhydride/pyridine, the solubility of softwood milled wood lignin in tetrahydrofuran was still rather limited (60%). Interestingly, this partial solubility was found to be in effect even after 20 h of reaction time despite the fact that most of the hydroxyl groups in lignin had already been acetylated as observed by ^{31}P NMR. This could be due to the possibility that the milled wood lignin may contain carbohydrate linkages via an α-ether type that need to be cleaved in order for complete solubility to be attained in organic media.

Since the reactivity of acetyl bromide in glacial acetic acid is significantly higher than that of acetic anhydride/pyridine, we examined the possibility of carrying out the acetobromination reaction at room temperature in a few hours instead of days. Such a reaction protocol would have the added advantage of reducing the probability of unwanted side reactions. Therefore, we carried out a series of acetobromination reactions at several time intervals. Short acetobromination reaction times (0.5, 1, 2, 3, 4, 6 h) were selected to be examined in order to determine the optimum reaction time. Milled wood lignin was selected for these experiments, since it has already been demonstrated as representing the most delicate of the examined samples (Figure 5) and as such it is well-positioned to reveal any possible structural alterations within it. In addition, milled wood lignin was found to be the least soluble after acetylation, thus ensuring that it would show any possible incomplete acetylation effects and also outline the favorable effects of acetobromination.

The SEC curves in Figure 5 suggest that there are no significant differences between short and longer reaction times. As such, shorter acetobromination reaction times were selected. Our work showed that, even after 30 min of acetobromination at room temperature, the sample became completely soluble in THF and all of its hydroxyl groups were completely acetylated, according to ^{31}P NMR data after phosphitylation. Short reaction times give similar molar mass distribution than acetic anhydride pyridine acetylation, and in addition, possible acid catalyzed side reactions are diminished (Figure 5).

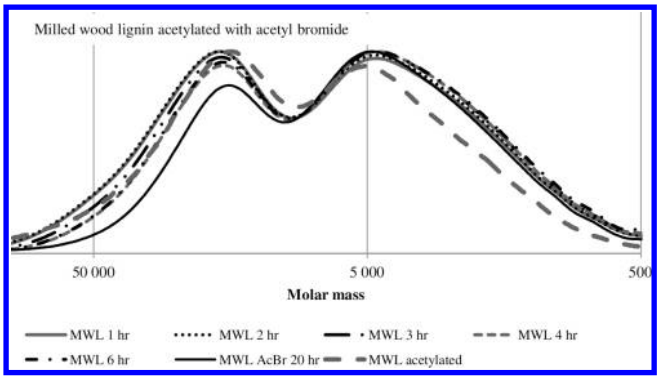

Figure 5. Series of size-exclusion chromatograms aimed to demonstrate the effect of acetobromination time on softwood milled wood lignin with acetyl bromide/glacial acetic acid compared to the acetylation of the same sample with acetic anhydride/pyridine. Since the chromatographic differences for the acetobrominated samples were rather small between the range of times from 30 min to 6 h, the data supports short reaction times.

During this work, it was noted (using ^{31}P NMR) that the acetylation of milled wood lignin was completed in acetic anhydride/pyridine after about 20 h of reaction. Despite the complete and documented derivatization chemistry observed, the acetylated sample was still only partly soluble in THF. Therefore, some other factor that is affecting its solubility should be operational. Perhaps the degree of oxidation of the lignin and the actual abundance of COOH groups within the lignin may also be contributing to its solubility as already discovered with such samples when treated under oxidative conditions.[25−27] The documented extreme solubility of acetobrominated lignins could be due to the presence of the bromine group within the lignin as previously discussed.[12] Finally, since the acetobromination protocol proposed involves no specific purification procedure other than the use of high vacuum to remove the rather volatile low molar mass compounds, the possible effect of acetic acid and/or acetyl bromide impurities on the actual molar mass was examined. More specifically, a series of experiments was carried out where molar mass distribution was calculated in the presence of variable small amounts of acetic acid in the samples. As anticipated, the minor amounts of acetic acid residues would have negligible effects on the determined molar mass (Table 2).

Table 2. Comparison of Molar Mass of Softwood Kraft Lignin with Added Acetic Acid

	pure sample	acetic acid added to the sample (1%)
M_n (g/mol)	1700	1500
M_w (g/mol)	8000	7000
M_p (g/mol)	3500	3300

Finally, it should be noted that the stability of acetobrominated samples was also evaluated by allowing samples to stand at room temperature for about one week. Repeated chromatograms gave results similar to those of fresh samples. However, after one month of aging at room temperature, some notable effects of sample degradation became apparent.

Norway spruce milled wood lignin and softwood and hardwood kraft lignins were reacted and derivatized with various reagents in order to arrive at an improved protocol that may serve as a universal derivatization step for lignin prior to its molar mass determination using THF as the eluent. The

association effects that in some cases are known to hamper analysis of underivatized lignins could be effectively eliminated using acetylation with acetic anhydride/pyridine, but the solubility of the acetylated lignins remained limited in THF. The use of acetyl bromide in glacial acetic acid gave completely THF soluble samples with demonstrated minimal structural alterations when a short reaction time (0.5 h) and room temperature were used. Overall, acetobromination does not seem to affect the hydrodynamic coils of the lignin polymers. Consequently, the molar masses observed when the acetobromination derivatization was used were practically identical to those obtained when acetic anhydride/pyridine were used with the advantage that the acetobromination reaction time could be as low as 30 min compared to 6 days when acetic anhydride/pyridine was used.

AUTHOR INFORMATION

Corresponding Author

*Phone: 1 919 515 7708; fax: 1 919 515 6302; e-mail: dsargyro@ncsu.edu.

Funding

This study is part of LigniVal projects in the Tekes BioRefine program. Tekes, Metso Power Oy, Oy Metsä-Botnia Ab, Stora Enso Oyj, Roal Oy is acknowledged for financial support.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Dr. Alistair King, Dr. Sami Heikkinen, and Dr. Jussi Sipilä are acknowledged for their expertise with the NMR measurements.

REFERENCES

(1) Norgren, M.; Edlund, H.; Wågberg, L. Aggregation of lignin derivatives under alkaline conditions. Kinetics and aggregate structure. *Langmuir* **2002**, *18*, 2859−2865.

(2) Cathala, A.; Saake, B.; Faix, O.; Monties, B. Association behaviour of lignins and lignin model compounds studied by multidetector size-exclusion chromatography. *J. Chromatogr., A* **2003**, *1020*, 229−239.

(3) Guerra, A.; Gaspar, A.; Contreras, I. S.; Lucia, L.; Crestini, C.; Argyropoulos, D. S. On the propensity of lignin to associate, Part I: a size exclusion chromatographic study with lignins derivatives isolated from different plants species. *Phytochemistry* **2007**, *68*, 2570−2583.

(4) Sarkanen, S.; Teller, D. C.; Hall, J.; McCarthy, J. L. Lignin 18. Associative effects among organosolv lignin components. *Macromolecules* **1981**, *14*, 426−434.

(5) Sarkanen, S.; Teller, D. C.; Abramowski, E.; McCarthy, J. L. Lignin 19. Kraft lignin component conformation and associated complex configuration in aqueous alkaline solution. *Macromolecules* **1982**, *15*, 1098−1104.

(6) Sarkanen, S.; Teller, D. C.; Stevens, C. R.; McCarthy, J. L. Lignin 20. Associative interactions between kraft lignin components. *Macromolecules* **1984**, *17*, 2588−2597.

(7) Connors, W. J.; Sarkanen, S.; McCarthy, J. L. Gel chromatography and association complexes of lignin. *Holzforschung* **1980**, *34*, 80−85.

(8) Contreras, S.; Argyropoulos, D. S.; Lucia, L. On the propensity of lignin to associate static light scattering measurements on native lignin. *Biomacromolecules* **2008**, *9*, 3362−3369.

(9) Baumberger, S.; Abaecherli, A.; Fasching, M.; Gellerstedt, G.; Gosselink, R.; Hortling, B.; Li, J.; Saake, B.; De Jong, E. Molar mass determination of lignins by size-exclusion chromatography: towards standardization of the method. *Holzforschung* **2007**, *61*, 459−468.

(10) Mattinen, M.-L.; Suortti, T.; Gosselink, R.; Argyropoulos, D. S.; Evtuguin, D.; Suurnäkki, A.; de Jong, E.; Tamminen, T. Polymerization of different lignins by laccase. *BioResources* **2008**, *3*, 549−565.

(11) Iiyama, K.; Wallis, A. F. A. An improved acetyl bromide procedure for determining lignin in woods and wood pulps. *Wood Sci. Technol.* **1988**, *22*, 271−280.

(12) Lu, F.; Ralph, J. Derivatization followed by reductive cleavage (DFRC Method), a new method for lignin analysis: protocol for analysis of DFRC monomers. *J. Agric. Food Chem.* **1997**, *45*, 4655−4660.

(13) Lu, F.; Ralph, J. DFRC method for lignin analysis. 1. New method for β-aryl ether cleavage: lignin model studies. *J. Agric. Food Chem.* **1997**, *45*, 4655−4660.

(14) Lu, F.; Ralph, J. The DFRC method for lignin analysis. 2. Monomers from isolated lignins. *J. Agric. Food Chem.* **1998**, *46*, 547−552.

(15) Brauns, F. E. *Chemistry of Lignin*; Academic Press: New York, 1952.

(16) Björkman, A. Lignin and lignin-carbohydrate complexes extraction from wood meal with neutral solvents. *Ind. Eng. Chem.* **1957**, *49*, 1395−1398.

(17) Hatfield, R. D.; Grabber, J.; Ralph, J.; Brei, K. Using the acetyl bromide assay to determine lignin concentrations in herbaceous plants: some cautionary notes. *J. Agric. Food Chem.* **1999**, *47*, 628−632.

(18) Björkman, A. Studies on finely divided wood. Part 1. Extraction of lignin with neutral solvents. *Sven. Papperstidn.* **1956**, *59*, 477−485.

(19) Argyropoulos, D. S. Quantitative phosphorus-31 NMR analysis of lignin: a new tool for the lignin chemist. *J. Wood Chem. Technol.* **1994**, *14*, 45−63.

(20) King, A. W. T.; Jalomäki, J.; Granström, M.; Argyropoulos, D. S.; Heikkinen, S.; Kilpeläinen, I. A new method for rapid degree of substitution and purity determination of chloroform-soluble cellulose esters, using ^{31}P-NMR. *Anal. Meth.* **2010**, *2*, 1499−505.

(21) Cathala, B.; Saake, B.; Faix, O.; Monties, B. Association behaviour of lignins and lignin model compound studied by multidetector size-exclusion chromatography. *J. Chromatogr., A.* **2003**, *1020*, 229−239.

(22) Granata, A.; Argyropoulos, D. S. 2-Chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane a reagent for the accurate determination of the uncondensed and condensed phenolic moieties in lignins. *J. Agric. Food Chem.* **1995**, *43*, 1538−1544.

(23) Greenwood, N. N.; Earnshaw, A. *Chemistry of the Elements*, 2nd ed.; Butterworth/Heineman: Oxford, U.K., 1997; pp 789−809.

(24) Sjöström, E. *Wood Chemistry: Fundamentals and Applications*; Academic Press Limited: London, 1993.

(25) Sun, Y.; Argyropoulos, D. S. A. Comparison of the reactivity and efficiency of ozone, chlorine dioxide, dimethyldioxirane and hydrogen peroxide with residual kraft lignin. *Holzforschung* **1996**, *50*, 175−182.

(26) Asgari, F.; Argyropoulos, D. S. Fundamentals of oxygen delignification, part II, kinetics of functional group formation/elimination in residual kraft lignin. *Can. J. Chem.* **1998**, *76*, 1606−1615.

(27) Sun, Y.; Argyropoulos, D. S. A. Fundamentals of high pressure oxygen and low pressure oxygen-peroxide (EOP) delignification of softwood and hardwood kraft pulps; a comparison. *J. Pulp Pap. Sci.* **1995**, *21*, 185−190.

(28) Holtman, K. M.; Chang, H.-M.; Jameel, H. Quantitative ^{13}C NMR characterization of milled wood lignins isolated by different milling techniques. *J. Wood Chem. Technol.* **2006**, *26*, 21−34.

(29) Sette, M.; Wechselberger, R.; Crestini, C. Elucidation of lignin structure by quantitative 2D NMR. *Chem.−Eur. J.* **2011**, *17* (34), 9529−9535.

(30) Crestini, C.; Melone, F.; Sette, M.; Saladino, R. Milled wood lignin: a linear oligomer. *Biomacromolecules* **2011**, *12* (11), 3928−3935.