# SIMPLIFIED DETERMINATION OF LIGNIN CONTENT IN HARD AND SOFT WOODS VIA UV-SPECTROPHOTOMETRIC ANALYSIS OF BIOMASS DISSOLVED IN IONIC LIQUIDS

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A new simple and safe method for quantifying lignin content in lignocellulosic biomass is described. The approach consists of measuring the absorbance of a solution of whole biomass dissolved in the ionic liquid 1-$n$-butyl-3-methyl imidazolium chloride, [Bmim][Cl], at 440 nm via ultraviolet- (UV-) visible spectrophotometry. An extinction coefficient for a lignin standard, highly pure lignin isolated from biomass through an organosolv process, is used in conjunction with the Beer-Lambert Law to calculate the lignin concentration. Principal component analysis (PCA) of Fourier Transform-Infrared (FTIR) spectra collected for several different lignin standards was performed to understand the differences in their chemical structure and composition (e.g., the relative amounts of syringyl and guaiacyl units). A rapid FTIR analysis of the whole biomass sample with unknown lignin content is required to assist in the proper selection of the lignin standard for the subsequent spectrophotometric analysis. The proposed method was tested and validated on two biomass types: Yellow poplar and Southern pine. The spectrophotometric approach yielded lignin contents for Yellow poplar and Southern pine of 25.7 ± 1.1% and 26.7 ± 0.7%, respectively, which are comparable to the values obtained by a standard wet chemical protocol, 25.1% ± 0.7 and 26.6 ± 0.4%, respectively.

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## INTRODUCTION

Compositional analysis of lignocellulosic feedstocks, e.g., corn stover, sugar cane, switchgrass, tobacco stalks, wheat straw, aspen, poplar, other agricultural and forest residues, and municipal wastes is imperative for their utilization in the manufacture of biofuels, cellulosic products (paper), sugars and other food additives, and associated co-products in a biorefinery. Lignin, typically present at 15-25% in lignocellulosics, is the second most abundant source of renewable and sustainable carbon behind cellulose (Argyropoulos and Menachem 1998; Bozell 2008). Since the emphasis of biorefinery processes applied to lignocellulosics has been the isolation and conversion of polysaccharides into biofuels, lignin is often regarded as a low-value by-product. Therefore, a partially hydrolyzed, heterogeneous, and impure lignin stream is produced,

which typically is incinerated to produce energy for the heating of process streams (Xu et al. 2006). However, a higher quality lignin can be potentially processed and utilized for value-added products such as aromatics or valuable phenolic by-products, carbon fiber, activated carbon filters, adhesives, monomeric units for polyurethane, polyesters, bioplastics, and bio-oils, non-viscous organic liquids suitable for blending with conventional fossil fuels (Bonini et al. 2005; Kleinert and Barth 2008; Thielemans et al. 2002; Xu et al. 2006).

Therefore, quantification of lignin content in lignocellulosic feedstocks is important for the operation of a biorefinery. A laboratory analytical procedure developed by the US Department of Energy, National Renewable Energy Laboratory (NREL) is the most commonly employed method to determine the lignin content in biomass (NREL 2008b). The approach, a modification of the Klason process (Kirk and Obst 1988), consists of treating biomass with strong (72%) sulfuric acid, followed by a dilute acid step, to hydrolyze the polysaccharides, leaving the majority of the lignin as an insoluble residue that is quantified gravimetrically, after its isolation via filtration. A non-negligible proportion of the lignin is solubilized in the acidic solution, quantified using ultraviolet- (UV-) visible spectrophotometry through the Beer-Lambert law, with similar progressive methods (Parasuraman et al. 2007). Although accurate and widely employed, this protocol possesses disadvantages, such as laboratory safety concerns of handling concentrated acids, numerous transfer steps of the biomass sample between vessels, which can lead to experimental error, and it is laborious. A more robust and safe analytical approach is desired to assess biomass composition and allow for high-throughput monitoring of process control within a commercial biorefinery. Noninvasive methods such as near-infrared (NIR) spectroscopy have been used to rapidly and reliably determine lignin and saccharide content of lignocellulosics; however, those approaches require a wet chemical analysis of a set of standards to be performed (Hatfield and Fukushima 2005; Kelley et al. 2004; Kiefer et al. 2009; Sanderson et al. 1996; Schultz et al. 1985).

Since lignin absorbs more strongly in the UV and visible regions than cellulose or hemicellulose, spectrophotometry is an appealing method for lignin quantification; however, this approach is hindered by the insolubility of the biomass in most common solvents (Mongeau and Brooks 2001). In addition, most solvents used in lignin and wood chemistry to achieve partial solubilization, such as dimethyl sulfoxide (DMSO), dimethylformamide (DMF), and pyridine, can yield environmental and laboratory safety hazards. A second hindrance is the difficult identification of appropriate calibration standards, due to the differences in absorbance of guaiacyl (G), syringyl (S), and p-hydroxyphenyl (H) monomeric phenylpropanoid units (Fergus and Goring 1970; Hatfield and Fukushima 2005), which vary significantly between biomass feedstocks and are highly dependent upon experimental factors. The most successfully performed spectroscopic approach, the "acetyl bromide method," measures absorbance at 280 nm for solutions of finely ground biomass dissolved in acetic acid through conjugation of lignin's free OH groups and acetic acid, followed by bromine replacement of α-carbon OH groups (Hatfield and Fukushima 2005). However, Mongeau and Brooks (2001) determined that this method overestimates the lignin concentration due to the interference of polysaccharides.

Ionic liquids (ILs), molten organic salts that are liquid at temperatures below 100°C, are potentially useful solvents for the dissolution of biomass that may yield improved environmental friendliness due to their low volatility and lack of flammability (Jain et al. 2005; Paljevac et al. 2006; Pu et al. 2007; Zavrel et al. 2009). However, there is still uncertainty regarding their toxicity and resulting practicality for applications relating to biomass characterization (Zhao et al. 2007). Imidazolium-based ILs have been used to solubilize cellulose, lignin, and lignocelluloses at levels of 22, 13.9, and 5.0 wt %, respectively (Fort et al. 2006; Kilpelainen et al. 2007; Pu et al. 2007; Remsing et al. 2006; Sun et al. 2009; Swatloski et al. 2002; Zhu et al. 2006). It was found through ^{13}C cross-polarization (CP) magic angle spinning (MAS) NMR that dissolution in [Bmim][Cl] resulted in little chemical modification in lignin from loblolly pine wood (Sievers et al. 2009). Fort and co-workers have employed ^{13}C-NMR spectral analysis of [Bmim][Cl] solutions to quantify cellulose and lignin (Fort et al. 2006).

In this paper, the lignin content of hard and soft woods (Yellow poplar and Southern pine, respectively) was measured using UV-visible spectrophotometric analysis of whole biomass in IL making use of appropriate lignin standards. The standards were analyzed by Fourier Transform Infrared Spectroscopy (FTIR) to identify characteristic chemical groups and hence determine their utility as standards for creation of a prediction model.

## EXPERIMENTAL

### Materials and Methods

The IL 1-*n*-butyl-3-methyl-imidazolium chloride [Bmim][Cl] (purum $\geq$ 98.0%, HPLC grade) was obtained from Sigma-Aldrich (St. Louis, MO). To control its hygroscopic behavior (Tran et al. 2003), the IL was stored with desiccant and under vacuum. Lignins from sugar cane (isolated by a hydrolytic method), hardwood (isolated via an organosolv method), and Norway spruce (prepared by alkali treatment, as a by-product from a commercial pulp mill) were obtained from Sigma-Aldrich. Organosolv lignins from mixed hardwood, bagasse (sugar cane post soluble sugar extraction by repeated washing and mechanical size reduction), Southern pine, and Yellow tulip poplar were provided by the US Department of Energy, National Renewable Energy Laboratory (NREL). Whole biomasses, Yellow poplar (*Liriodendron tulipfera*), and Southern pine (*Pinus taeda*), ground using a Wiley mill to a particle size of 40 mesh (0.425 mm), were obtained from the Tennessee Forest Products Center at the University of Tennessee. The biomasses were purified via extraction using the laboratory analytical procedure developed by NREL, to remove non-structural material called extractives (e.g. resin, tannins, soil, chlorophyll, waxes, and other minor components) prior to analysis to prevent interference with later analytical steps (NREL 2008a). The purity of each lignin was assessed using a standard wet chemical analysis protocol, and it was found that most of the lignin standards were >90% pure, with the major impurities being moisture and ash.

**FTIR Analysis of Lignin Standards**

FTIR spectroscopy was employed using the Spectrum One FTIR spectrometer from Perkin Elmer (Waltham, MA) with a diamond attenuated total reflectance (ATR) attachment. Spectra from 4000 to 600 cm$^{-1}$ were collected for the lignin and biomass powders in absorbance mode with 8 scans per spectrum at 1 cm$^{-1}$ resolution. The spectra underwent an ATR correction to account for less than optimal sample/crystal interface contact, were transformed by reducing the spectral resolution to 4 cm$^{-1}$, and normalized. Full multiplicative scatter correction (MSC) was applied to remove light scatter variations found in reflectance spectroscopy, which yield large interference effects. In order to better assess the differences and similarities that exist between the lignin samples, multivariate analysis was performed on the FTIR lignin standard spectra, as described below.

**Wet Chemical Analysis of Various Biomasses and Lignin Standards**

The determination of percent moisture, ash, and saccharides (from hydrolysis of cellulose and hemicelluloses) was achieved by the protocol developed by the US-DOE-NREL, using three replicates (NREL 2008b). The procedure is suitable for samples that do not contain extractives. A two-step acid-catalyzed hydrolysis was performed to fractionate the sample into soluble and insoluble matter, and the two fractions were separated through filtration. The insoluble solids fraction, which consists primarily of acid-insoluble lignin, was quantified gravimetrically. The monomeric units of poly-saccharides within the soluble liquid fraction were quantified using an HPLC system equipped with an Aminex HPX-87P column (300 x 7.8 mm ID, 9 μm particle size) from Bio-Rad (Hercules, CA) and a model 410 refractive index detector from Waters (Milford, MA). The acid-soluble lignin content of the soluble liquid fraction was measured by UV spectrophotometry with a two-beam UV-1700 series spectrophotometer from Shimadzu Scientific Instruments (Columbia, MA). The ash content of the sample was determined gravimetrically after combustion of non-ash materials at 575ºC for 24 hours in a muffle furnace.

**Solubilization of Lignin, Hard and Soft Woods in Ionic Liquid**

Lignin standard or biomass (0 to 0.1 wt%) was added to [Bmim][Cl], and the mixture underwent magnetic stirring at 400 rpm and 80°C for 24 hours. The samples were then visually inspected for evidence of solubilization or physical changes. Subsequently, the solubilized samples were examined with an Olympus BX51 light microscope (Center Valley, PA) at 100x magnification, for evidence of suspended particles, i.e., non-solubilized biomass. The complex viscosity of pure [Bmim][Cl] and solutions of 0.025 wt% hardwood lignin, and 0.05 wt% Yellow poplar in IL, which are representative biomass concentrations employed in this investigation, was measured utilizing the Advanced Rheometer Expansion System (ARES) rheometer from TA Instruments (New Castle, DE) (Sammons et al. 2008). Analysis of variance (ANOVA) was conducted using SAS$^{©}$ statistical software and least squares means were compared (ANOVA mixed procedure, 2008, SAS Institute. Inc., Cary, NC). Treatment means were compared using Fisher's least significant difference method where P ≤ 0.05 to determine if there is a statistical difference between the samples being compared.

## Ultraviolet (UV)-Visible Spectrophotometric Determination of Lignin Content in Hard and Soft Woods

Analysis of lignin content in an IL solution was achieved using UV-visible spectrophotometry with the same instrument described above. Absorbance within the 190-1100 nm spectral range was measured using a 1 nm spectral resolution. Samples were referenced to pure [Bmim][Cl]. A large amount of noise was observed in the UV spectral region due to the strong absorbance of the IL (Fig. 3 inset); therefore, absorbance data for 190-380 nm were not used in the analysis. To determine extinction coefficient values for the lignin standards, stock solutions of 0 to 0.1 wt% lignin in [Bmim][Cl] were prepared as described in the previous subsection and transferred while hot ($80^{o}$C) to small-volume (0.5 mL) 1.0 cm pathlength quartz cuvettes and allowed to cool to room temperature. It was determined that samples must have lignin concentrations in IL of $\leq$ 0.02 wt% in order for their absorbance values to be $\leq$ 1.0, within the linear range described by the Beer-Lambert law. Extinction coefficients for lignin standards dissolved in [Bmim][Cl] were corrected for impurities present in the lignins. To test the effect of temperature on the UV-visible absorbance of the IL solutions, three spectra were collected at 25°C and 80°C for samples of pure [Bmim][Cl], 0.025 wt% hardwood lignin, and 0.05 wt% Yellow poplar in IL. ANOVA was conducted on the absorbance measurements at the optimal wavelength of 440 nm for each solution using SAS$^{©}$ statistical software, and least mean squares were compared at 5% significance level.

To determine the optimal wavelength for lignin quantification, the UV-visible spectra were analyzed by Partial Least Squares (PLS), a multivariate statistical method which relates independent variables (**X** values) and dependent variables (**Y** values) (Martens and Naes 1989; Rials et al. 2002). PLS determines a set of latent variables that maximizes the covariance between **X** (**X** = *Mn*) and **Y** (**Y** = *MN*), where *M* represents the number of samples, *n* is the number of independent variables, and *N* stands for the number of dependent variables. It decomposes **X** and **Y** into the forms:

$$\mathbf{X} = \mathbf{TP^T} + \mathbf{E} \tag{1}$$
$$\mathbf{Y} = \mathbf{UQ^T} + \mathbf{F} \tag{2}$$

where **T** and **U** (= *MA*) are the matrices of the extracted *A* score vectors, **P** (= *nA*) and **Q** (= *MA*) are the loading matrices, and **E** (= *Mn*) and **F** (= *MN*) are the residual matrices. The PLS method searches for weight vectors (**W** and **C**) that maximize the sample covariance between **t** and **u**, where **t** and **u** are X-scores and Y-scores of a component, respectively. By regressing **X**(**Y**) on **t**(**u**), the loading vector **p**(**q**) can be computed:

$$\mathbf{p} = (\mathbf{t^T t})^{-1} \mathbf{X^T t} \tag{3}$$
$$\mathbf{q} = (\mathbf{u^T u})^{-1} \mathbf{Y^T u} \tag{4}$$

Then, the PLS regression model can be expressed as **Y** = **XB** + **G**, where **G** is the residual vector. Here **B** represents the regression coefficient, which is given by:

$$\mathbf{B} = \mathbf{W(P^T W)^{-1} C^T} = \mathbf{X^T U(T^T X X^T U)^{-1} T^T Y} \tag{5}$$

In this case, the UV-visible spectral data with many variables, (**X**), is related to the one property of interest (**Y**), the lignin concentration in solution, allowing the entire spectrum (380-1100 nm) to be used for the prediction of lignin content. The optimal wavelength for lignin quantification in IL via absorbance at a single UV-visible wavelength was identified as the wavelength with the highest regression coefficient (**B***)* found by the analysis (Equ. 5). Models were generated using a full cross-validation procedure with the Unscrambler v.9.8 statistical software (CAMO Software Inc., Woodbridge, NJ). With this technique, a sample is left out from the data set and the model is calculated based on the remaining data. The value of the left-out data point is used for prediction. The process is repeated with another sample from the calibration set, until every sample has been left out one time. This procedure ensures that the predictive capabilities of the model are reliable and the model is not over-fitted. An overview of the PLS technique is given elsewhere (Martens and Naes 1989; Rials et al. 2002).

To test the utility of the spectrophotometric approach, solutions of 0.05 wt% Yellow poplar and Southern pine in [Bmim][Cl] were prepared as described above, and subsequently diluted with additional [Bmim][Cl] as needed to lower absorbance at the optimal wavelength of 440 nm to <1.0 with three absorbance spectra collected per solution. The mass percentage of lignin in the IL-based solution, *%Lignin$_{sol'n}$*, was calculated by the Beer-Lambert Law:

$$\%Lignin_{sol'n} = \frac{Abs_{440}}{\varepsilon_{440} \cdot l} \times 100\% \qquad (6)$$

where *Abs$_{440}$* is an average absorbance for lignin standard in IL at 440 nm, $\varepsilon_{440}$ is the extinction coefficient for lignin standard at 440 nm and *l* is the pathlength (1 cm). Subsequently, the lignin weight percent in biomass is readily calculated:

$$\%Lignin = \frac{\%Lignin_{sol'n}}{\%Biomass_{sol'n}} \times 100\% \qquad (7)$$

where *%Biomass$_{sol'n}$* is the concentration of biomass in the IL-based solution, wt%.


## RESULTS AND DISCUSSION

### Chemical Structure and Composition of Lignin Standards

FTIR spectroscopy is valuable for analyzing the chemical structure of lignins through the probing of their distinct chemical bonds. The spectra of the various lignin standards, depicted in Fig. 1a, show typical patterns for lignin with bands at 1600, 1515, 1459, and 1423 cm$^{-1}$ (Table 1). Differences between spectra are observed in several regions. For example, lignin derived from Norway spruce lignin via an alkali-based extraction process lacks a spectral band at ~1703 cm$^{-1}$ attributable to the carbonyl moieties of aldehyde/ketone groups (Fig. 1a), suggesting the chemical modification of most carbonyl groups under the high pH conditions (Orton et al. 2004). The other lignin

standards contain this band, suggesting that the organosolv and hydrolytic isolation methods modify the aldehyde and ketone groups to a much lesser extent. In addition, differences occur within the 1100-1400 cm$^{-1}$ region, particularly for the weak signals at 1327 and 1263 cm$^{-1}$ and strong signals at ~1223 and 1123 cm$^{-1}$ assigned to guaiacyl (G) and syringyl (S) units, respectively (Table 1), suggesting differences in the relative amounts of G and S between lignin standards. A strong signal is also observed for the bagasse lignin at 1167 cm$^{-1}$, reflecting the prominence of the ester bonds associated with p-hydroxyphenyl propane units in the lignin of grasses.  FTIR spectra of hard and soft woods (Yellow poplar and Southern pine, respectively), presented in Fig. 1b, contain the bands associated with lignin listed above and those attributed to cellulose and hemicelluloses (e.g., 899 cm$^{-1}$ attributable to the C-H deformation vibration, and 1031-1015 cm$^{-1}$, attributable to the C-O stretch of primary –OH groups.)

**Table 1**.  Assignment of Infrared Spectral Bands for Lignin and Biomass in Solution (Faix 1991; Holomb et al. 2008; Kiefer et al. 2008; Popescu et al. 2007; Schultz and Glasser 1986; Tejado et al. 2007).

| Observed Peaks (cm$^{-1}$) | Peak Assignment |
|---|---|
| 1707-1691 | Unconjugated carbonyl stretching of aldehyde/ketone groups |
| 1615-1600 | C=C stretching of aromatic ring in lignin |
| 1567 | C=O stretching (ionic compounds only) |
| 1515-1511 | C=C stretching of aromatic ring in lignin |
| 1460-1459 | C-H bending of methyl and methylene groups |
| 1425-1423 | C-H deformation in lignin |
| 1327-1323 | C=O stretching of the syringyl unit |
| 1267 | C-O stretching of guaiacyl unit |
| 1219-1211 | C-C, C-O, and C=O stretching of guaiacyl unit |
| 1167 | C-O stretching of ester group |
| 1123-1110 | Aromatic C-H deformation of syringyl units |
| 1031-1015 | C-O stretching of primary alcohols |
| 983 | CH=CH bending |
| 911 | C-H bending of syringyl units, aromatic ring |
| 899 | C-H deformation vibration of cellulose |
| 835-815 | C-H bending of syringyl units |
| 755 | Asymmetric bending of HCCH group |

Principal component analysis (PCA) is a useful mathematical technique that visually depicts the degree of correlation that exists within multivariable data.  It is a powerful tool in the analysis of FTIR spectral datasets to assess major and minor differences between spectra, thus reflecting differences in chemical structure and composition of the samples (Chen et al. 1998; Labbé et al. 2006).  As occurs with the PLS technique described above, PCA mathematically treats each infrared spectrum of the lignin standards as a dataset with each wavenumber considered to be a separate variable.
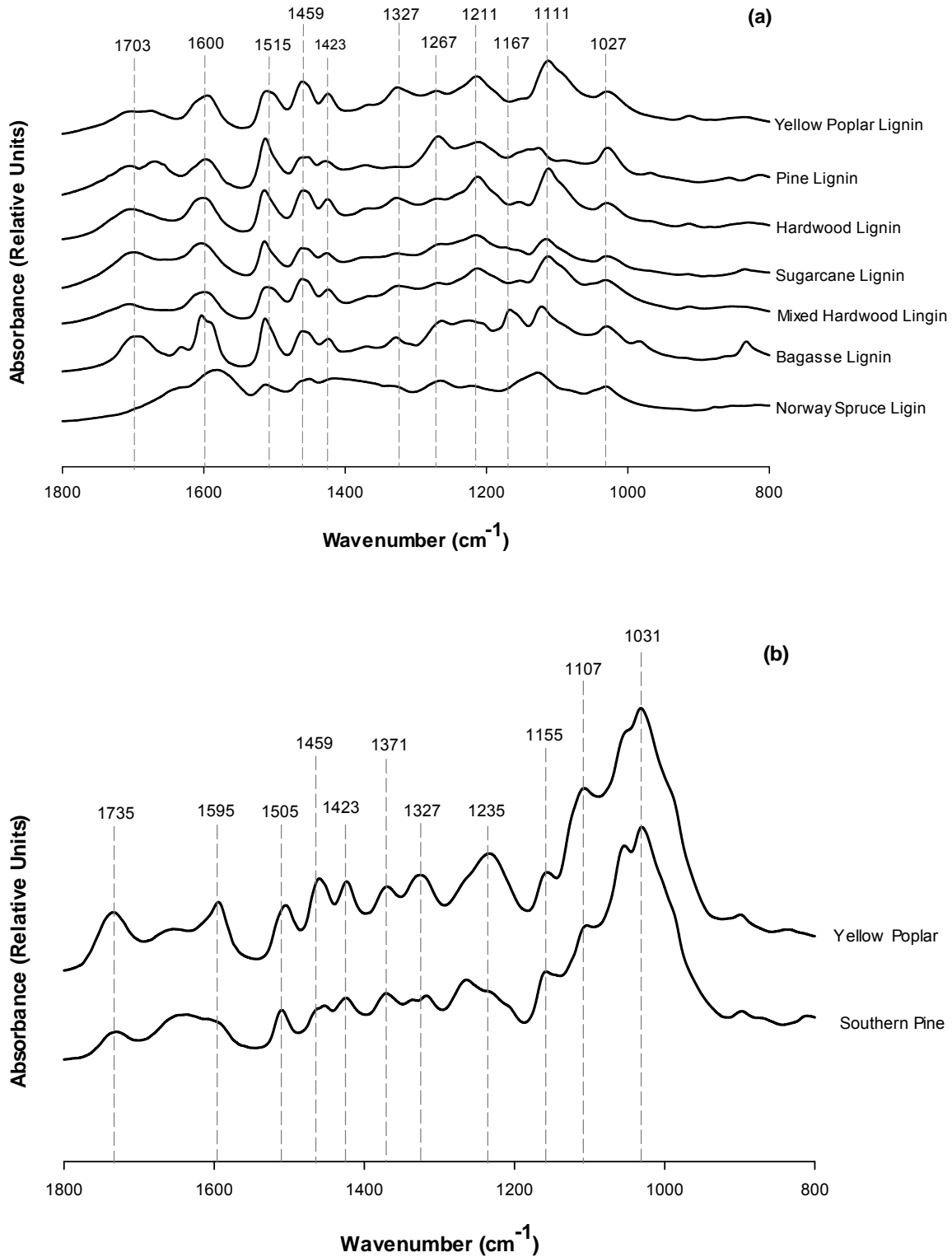
**Fig. 1.** (a) FTIR spectra of various lignin standards and (b) Yellow poplar and Southern pine

**Table 2.** Chemical Properties and Extinction Coefficients of Lignin Standards and Whole Woody Biomass on a Dry Basis

| Lignin Standard or Woody Biomass Type[a] | % $H_2O$ & Volatiles [b,c] | % Ash [b] | % Glucan + Xylan [b,d] | % Lignin | S/G Ratio[e] | $E^f_{440\,nm}$ [g], Wt%$^{-1}$ cm$^{-1}$ |
|---|---|---|---|---|---|---|
| Yellow Tulip Poplar Lignin | 6.1 ± 0.19 | 0.4 ± 0.2 | < DL | 93.5 ± 0.2 [g] | 1.073 | 48.7 ± 1.3 |
| Mixed Hardwood Lignin | 4.3 ± 0.08 | 0.4 ± 0.3 | < DL | 95.3 ± 0.2 [g] | 1.017 | 22.7 ± 0.6 |
| Hardwood Lignin | 2.0 ± 0.20 | 0.1 ± 0.1 | < DL | 97.9 ± 0.2 [g] | 0.957 | 35.7 ± 1.5 |
| Pine Lignin | 8.4 ± 0.16 | 0.5 ± 0.2 | < DL | 91.1 ± 0.2 [g] | 0.867 | 20.3 ± 0.5 |
| Sugar Cane Lignin [h] | 4.2 ± 0.20 | 0.3 ± 0.1 | < DL | 95.5 ± 0.2 [g] | 0.821 | 39.7 ± 0.6 |
| Bagasse Lignin | 2.3 ± 0.15 | 0.3 ± 0.0 | < DL | 97.4 ± 0.2 [g] | 0.704 | 3.2 ± 0.1 |
| Norway Spruce Lignin [i] | 7.9 ± 0.20 | 8.4 ± 0.3 | < DL | 83.4 ± 0.3 [g] | 0.534 | 12.4 ± 0.3 |
| Yellow Poplar | 3.3 ± 0.1 | 3.2 ± 0.1% | 53.3± 0.7% [j] | 25.1 ± 0.7%[b] | 0.980 | ND[k] |
| Southern Pine | 6.4 ± 0.3 | 6.4 ± 0.3%[i] | 49.4± 0.5% [,l] | 26.6 ± 0.4% [b] | 0.852 | ND[k] |

a. Isolated by an organosolv method unless noted otherwise
b. Obtained by standard wet chemical protocols as developed by the US Dept Energy, National Renewable Energy Laboratory (NREL 2008b).
c. Wet basis
d. DL indicates component was present at trace levels but unquantifiable as concentration remained below detection limits (<1 wt%).
e. Syringyl/guaiacyl ratio calculated using normalized FTIR spectra with intensity of peaks at 1327 and 1267 cm$^{-1}$, respectively.
f. Extinction coefficient for lignin or biomass dissolved in [Bmim][Cl]. Measurements performed at 25°C.
g. 440 nm was identified as the universal optimum wavelength that yielded the highest regression coefficient from the PLS analysis of UV-visible spectra from 380-1100 nm with three replicates, 1 cm pathlength, at 25°C for all lignin standards in [Bmim][Cl].
h. Isolated using a hydrolytic method.
i. Isolated using an alkali process.
j. *41.6* ± 0.7% glucose, 11.7 ± 0.6% xylose
k. Not determined
l. 44.6 glucose, 4.8 ± 0.4% xylose

PCA transforms this multivariable dataset to a new dataset that is dependent on typically 2 to 4 variables, known as principal components. The first principal component is associated with the majority of the variability between the spectra, with each successive principal component associated with a decreasing proportion of the variability. Each principal component is associated to a set of "loadings" which are directly related to the contributing wavenumbers. Each spectrum is "scored" according to their degree of correlation with the loading of each principal component. Therefore spectra that share similar score values will cluster together on the scores plot, showing that they possess similar spectral features and hence have similar chemical composition and structure. A summary description of the PCA technique is given elsewhere (Martens and Naes 1989).

A preliminary employment of PCA for the seven lignin standards listed in Table 2 showed that the lignin standards isolated via hydrolytic and alkali methods (sugar cane

and Norway spruce, respectively) differed significantly from the other five lignin standards, which were isolated through an organosolv method (data not shown). In addition, the wet chemical analysis of the lignin standards, performed according to a standard protocol (NREL 2008b), demonstrates that Norway spruce lignin is unique in its relatively high concentration of ash, $8.4 \pm 0.3\%$, compared to 0.1-0.5% for the other lignin standards (Table 2), presumably due to residual alkali resulting from the isolation procedure. Hence it was concluded that the chemical composition and structure of the hydrolytic and alkali standards differed greatly from the other lignin standards, perhaps due to differences methods used to isolate them. Therefore, only the five selected organosolv lignin standards were considered for further use. This is in agreement with previous work showing that an organosolv process produces a relatively pure, unaltered, and high-quality lignin of low molecular mass (El Hage et al. 2009). FTIR spectra for the five organosolv lignin standards were analyzed by PCA. The score plot for this dataset as functions of the first two principal components, PC1 (accounting for 54% of the variance) versus PC2 (27%), is shown in Fig. 2a. Three distinct clusters are observed. On the right (positive along PC1) are the spectra for the hardwood lignin standards. A second cluster (negative along PC1 and positive along PC2), consists of spectra for the grass (bagasse) lignin. The third cluster (negative along PC1 and PC2) represents softwood (pine) lignin. The loadings plot for PC1 indicates the key differences that are responsible for grouping the samples along PC1, more specifically the chemical features that distinguish the hardwood lignins from the softwood/grass lignin standards (Fig. 2b). The variables (wavenumbers) with high peak intensity (positive or negative) contribute the most to the separation. The bands responsible for separating hardwood from the other lignin standards are 1512, 1459, 1323, 1265, 1167, 1111, 1028, and 911 cm$^{-1}$ (Fig. 2b). The assignment of these bands is given in Table 1. Positive bands at 1323, 1111, and 911 cm$^{-1}$ for PC1 and a positive score for hardwood lignins confirm that this type of lignin has higher syringyl content than the other lignins. Grass lignin is segregated from the softwood lignin mostly due to the chemical band at 1167 cm$^{-1}$ for PC2, reflective of the lower concentration of ester groups (associated with p-hydroxyphenyl propane units) and G units for pine lignin, respectively.

Because the relative concentration of S and G structural units has been shown by the PCA assessment of the FTIR data to reflect key differences between the lignin standards, quantitative evaluation of absorbance occurring at specific bands associated with S and G (1327 cm$^{-1}$ and 1267 cm$^{-1}$, reflecting the C-O stretching of S and G, respectively) has been employed to calculate the S/G ratio as described in the literature (Faix 1991; Owen and Thomas 1989; Popescu et al. 2007; Rutkowska et al. 2009; Schultz and Glasser 1986). Prior to the calculation the spectra were ATR corrected and normalized using the intensity at 1505 cm$^{-1}$, corresponding to the C=C stretching of lignin's aromatic rings (Faix 1991). Although it is a comparative measure, when applied to all lignin standards and biomass types, this method allows for a quick and easy classification of the lignins based on their S/G ratio. As expected, the highest S/G ratios are obtained for Yellow tulip poplar, mixed hardwood, and hardwood lignin (Table 2), agreeing with the higher S/G ratio of lignins from hardwood relative to softwoods and grasses (Argyropoulos and Menachem 1998).
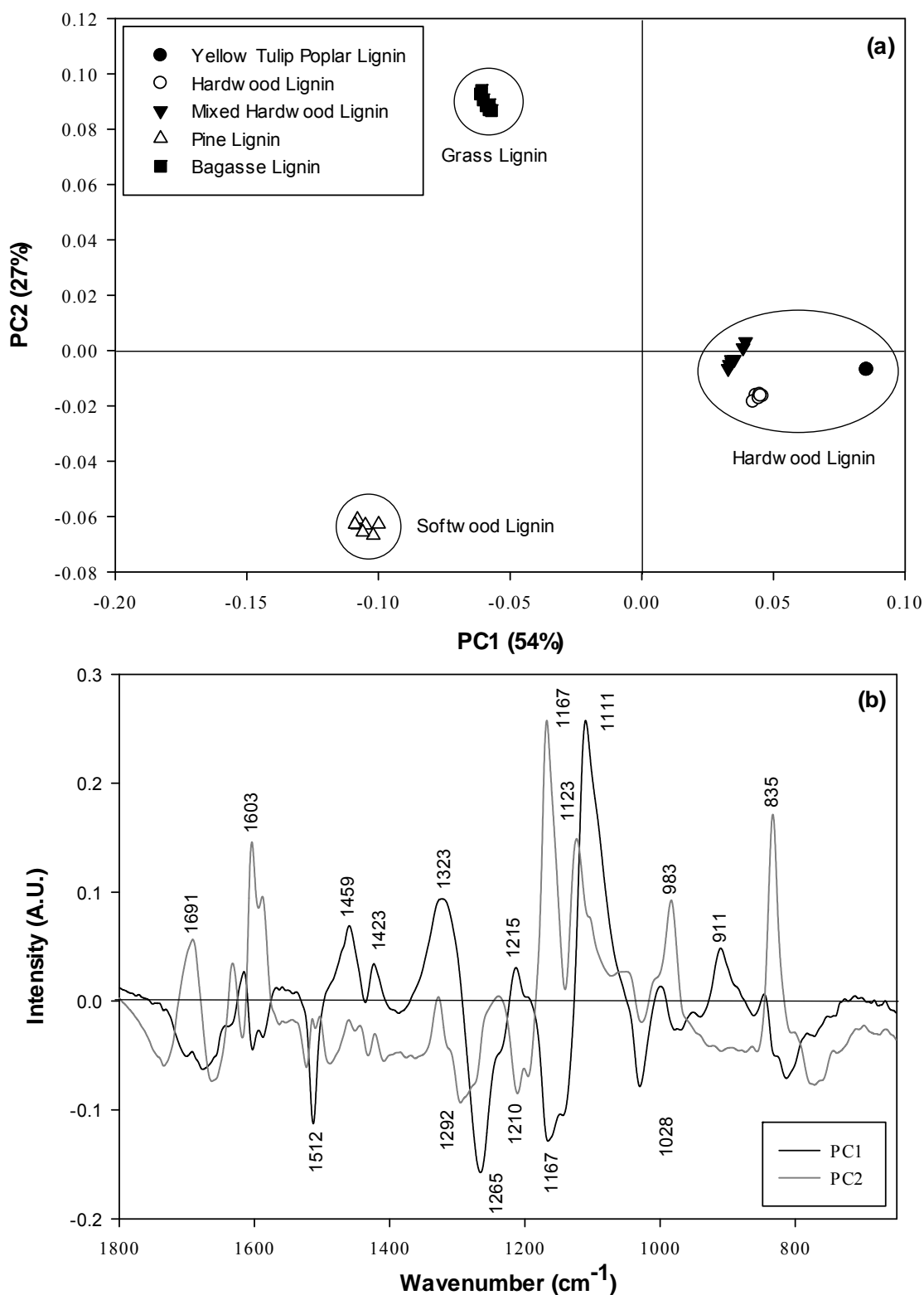
**Fig. 2.** Results from the principal component analysis for the FTIR spectra of the 5 lignin standards isolated via an organosolv method. (a) Score plot as a function of the first and second principal components, PC1 and PC2. (b) Loadings for PC1 and PC2.

## Quantification of Lignin through UV-VIS Spectrophotometric Analysis

In the experimental protocol, lignin and whole biomass solutions in IL were prepared at 80°C, then transferred quickly to the cuvettes while remaining hot, and allowed to cool to near room temperature prior to spectrophotometric measurement.   The solutions were more easily transferable at 80°C than at room temperature due to the lower viscosity of the IL solutions at the higher temperature.  Moreover, the complex viscosity of 0.025 wt% lignin and 0.05 wt% Yellow poplar solutions at 80°C (0.11-0.14 Pa·s) was 50-fold lower than at 25°C (6.1-7.8 Pa·s).   For this protocol to be effective, the methodology employed for preparing IL solutions must not cause any change in the physicochemical structure of the lignin standards or whole biomasses relative to each other from that which is encountered in nature.  To address the effect of temperature on the IL solutions, an ANOVA mean separation test was performed upon complex viscosity measurements of whole biomass and lignins dissolved in IL.  No significant differences were found between complex viscosity measurements at a 5% significance level, suggesting that dissolution affected the physicochemical properties similarly.  It also needs to be shown that the cooling of the solution to 25°C did not induce any major physicochemical changes in the IL solutions that would produce artifacts in the spectrophotometric analysis.  This is particularly important since the sample temperature for spectrophotometric analysis was not carefully controlled.   An ANOVA means separation test applied to UV-visible absorbance of lignin standards and whole biomass at 440 nm demonstrated that no significant differences occurred between 25°C and 80°C for a given lignin or biomass sample, suggesting the protocol employed for transferring solutions to the cuvettes and measuring absorbance did not introduce any artifacts in the data.

The UV-Vis spectra of the various lignin standards at 0.05 wt% as well as pure [Bmim][Cl] are depicted in Fig. 3.  Partial Least Squares analysis of the data from the visible spectral region, 380-1100 nm, demonstrated that a wavelength of 440 nm was optimal for lignin quantification.   Plots of absorbance at this wavelength versus concentration yielded a linear relationship for all lignin standards, with coefficients of correlation being >0.995 (Fig. 4).  As shown in Table 2, the extinction coefficients at 440 nm ranged from 3.2 to 48.7 wt%$^{-1}$ cm$^{-1}$.

The lignin content of a hard and soft wood, Yellow poplar, and Southern pine, respectively, was estimated using UV-visible spectrophotometry.  Samples of 0.05 wt % whole biomass (on a dry basis) in [Bmim][Cl] were prepared and then diluted with additional IL to lower absorbance values at 440 nm to $\leq 1.0$ (for a pathlength of 1 cm). From this measurement, the lignin concentration was calculated using Eqs. 6 and 7 according to each of the hardwood and softwood lignin standards (Table 3).

The question to answer is which of the standards is the most appropriate to employ for the calculations.  It is believed that [Bmim][Cl] has the ability to solubilize biomass without significant chemical changes to the natural structure of its components at low temperature (Fort et al. 2006).  Therefore, estimation of lignin content should employ the lignin standard most similar in chemical structure to that of the biomass.  Based on this principle, the choice of lignin standard was narrowed to the hardwoods lignins for analysis of Yellow poplar.  To  identify the  best  standard, the  S/G ratio of the standards
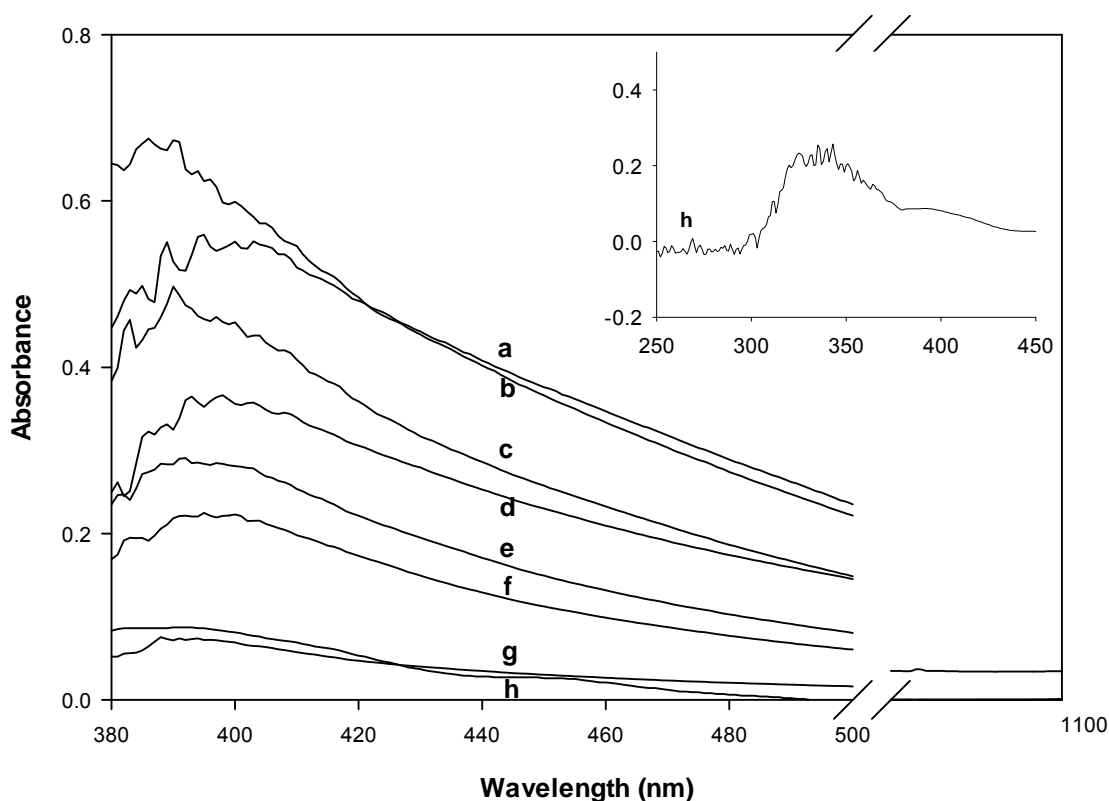
**Fig. 3.** UV-visible absorbance spectra of the various lignin standards at 0.01 wt% in [Bmim][Cl]: (a) Yellow poplar lignin, (b) sugar cane lignin, (c) hardwood lignin, (d) mixed hardwood lignin, (e) pine lignin, (f) Norway spruce lignin, (g) bagasse lignin, (h) [Bmim][Cl]
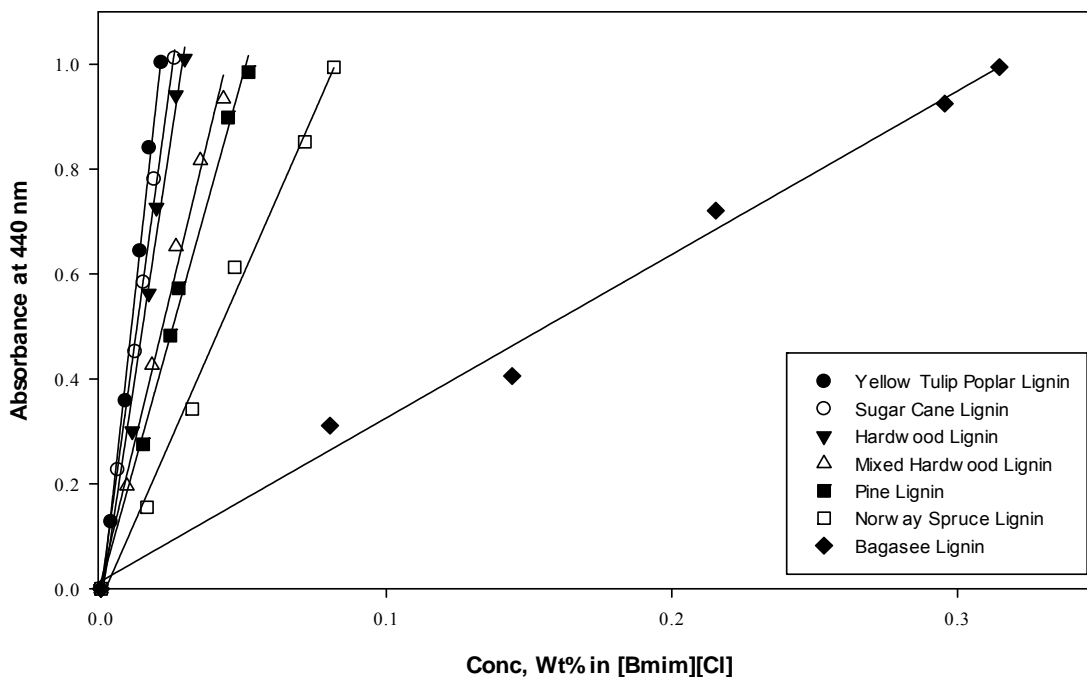


**Fig. 4**. Absorbance at 440 nm versus concentration for solution of lignin standards dissolved in [Bmim][Cl].

was compared to that of Yellow poplar. The "hardwood" (organosolv) lignin provided the closest match (Table 2). It was surprisingly shown that the Yellow poplar lignin standard possessed an S/G ratio that significantly differed from that of the Yellow poplar sample analyzed, most likely due to the extraction process, emphasizing the need for a rapid classification method to choose the appropriate standard to be used with the spectrophotometric approach. Employment of the extinction coefficient for hardwood lignin yielded a lignin concentration of 25.7 ± 1.1 % for Yellow poplar, which nearly matches the value obtained by wet chemical analysis, of 25.1 ± 0.7% (Table 3). By the same approach, the "pine" (organosolv) lignin standard was found to be the best lignin standard for Southern pine. The use of this standard's extinction coefficient in Eqs. 1 and 2 yielded a lignin content of 26.7 ± 0.7% for Southern pine, which is very similar to the value obtained using wet chemical analysis, 26.6 ± 0.4% (Table 3).

**Table 3**. Predicted Lignin Content of Yellow Poplar (Wt%, on a Dry Basis) Determined by Standard and Newly-developed IL-based Protocols.

| | Yellow Poplar | Southern Pine |
|---|---|---|
| Traditional Wet Chemistry[a] | 25.1 ± 0.7 | 26.6 ± 0.4 |
| Lignin Standard Used and Corresponding Lignin Content[b] | | |
| Yellow Tulip Poplar Lignin | 18.8 ± 0.5 | 11.1 ± 0.3 |
| Mixed Hardwood Lignin | 40.5 ± 1.0 | 23.9 ± 0.6 |
| Hardwood Lignin | **25.7 ± 1.1[c]** | 15.2 ± 0.6 |
| Pine Lignin | 45.2 ± 1.1 | **26.7 ± 0.7 [c]** |

a. National Renewable Energy Laboratory (NREL 2008b).
b. Values derived from 3 replicate UV-visible spectra of whole biomass dissolved in [Bmim][Cl] taken at wavelengths between 380-1100 nm using 1 cm pathlength cells and sample volume of approximate 0.5 mL at 25°C. The average absorbance value at 440 nm for the three replicates was used with the extinction coefficient of the indicated lignin standard, given in Table 2, to calculate the lignin concentration via Equations 1 and 2.
c. Derived value using the lignin standard possessing the most similar syringyl / guaiacyl ratio to the woody biomass being analyzed (Table 2)

## CONCLUSIONS

1. Using [Bmim][Cl], an imidazolium-type ionic liquid as a solvent for dissolution of hard and soft wood, enabled the rapid and simple determination of the lignin mass fraction through employment of ultraviolet-visible spectrophotometry. Twenty-four hours was chosen as duration for biomass dissolution to ensure that biomass was fully dissolved. Although a smaller duration and/or slightly higher temperature could be used to achieve complete dissolution, optimization of the dissolution time and conditions was not conducted. Importantly, the sample preparation required for this method is minimal when compared to alternative approaches such as Klason methods, with the major benefit being that this method is applicable to whole biomass. This method is overall less labor intensive and

does not lead to the degradation or fractionation of lignins between multiple phases, in contrast to Klason lignin quantification, which via degradation divides lignin between two phases. i.e., acid-soluble and -insoluble lignin.

2. The accuracy of this approach requires the proper selection of a lignin standard, obtained through isolation by an organosolv process.  A similar choice between standards is required for traditional wet chemistry methods, which also utilize spectrophotometry for quantification acid-soluble lignin (NREL 2008b).  For the traditional approach, extinction coefficients are determined *a priori* for biomass hydrolysates as calibration standards obtained via the same wet chemical protocol.  For the approach described herein, it is believed the major issue for selecting an appropriate lignin standard is a match of its ratio of syringyl (S) and guaiacyl (G) units relative to that of the whole biomass being analyzed.  Lignins isolated using an organosolv process are preferred, since this process does not change the S/G ratio from that of the native lignin.  Measurement of the S/G ratio was estimated by FTIR spectroscopy because this approach is rapid, requires minimal sample preparation, and can be incorporated into automated instrumentation for high throughput analysis of samples.

3. Because ionic liquids are already being implemented as part of new methodologies for lignocellulosic pretreatment processes, we envision that the quantitative methodology described herein can be extended further for processing of the plant material for uses in the bioenergy industry. A larger set of lignin standards is required to improve the model's accuracy, and to extend its use to other lignocellulosic biomass sources, such as grasses.  However, as evidenced by the accuracy of the prediction of lignin in Yellow poplar and Southern pine as described above, the potential value of this method has been established.  As a standard library is created, the amount of necessary FTIR analysis will be little or none as it would just act as an initial classification method for choosing the appropriate standard for the spectrophotometric approach.

## ACKNOWLEDGMENTS

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