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IMPROVED METHODOLOGIES FOR BIOMASS WET CHEMICAL ANALYSIS

A Thesis

Presented for the

Master of Science Degree

The University of Tennessee, Knoxville

Lindsey McCulloch Kline

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ABSTRACT

The purpose of this thesis study was to further the development of lignocellulosic biomass as a potential renewable energy source by investigating new wet chemical compositional analysis techniques to be used to monitor changes in biomass composition resulting from size reduction and separation processes such grinding and sieving. Numerous disadvantages to the standard wet chemical analysis procedure as developed by US Dept of Energy and the National Renewable Energy Laboratory (NREL) were identified as targets for possible improvements. The overall objective was utilization of ionic liquids as a "green" alternative to the use of aqueous acidic solvents employed in the NREL protocol. These experiments included direct spectral analyses to quantify the lignin constituent, and successive enzymatic hydrolysis for quantification of the cellulose constituent.

Results contained herein revealed that solubilization of biomass occurred in ionic liquids, which allowed for rapid spectroscopic determination of its lignin composition. The enzymatic hydrolysis of cellulose occurred in an ionic liquid-rich solvent system, and quantification of the cellulolytic monosaccharide products was achieved using high performance liquid chromatography.

Motivated by the disadvantages associated with the NREL biomass compositional analysis procedure, a new analysis procedure utilizing ionic liquids was proposed and developed as an approach aimed towards improving laboratory safety and analysis time. The study was approached by first quantifying the solubility of biomass in ionic liquids. Direct quantification of the lignin content was conducted by two methods, UV-visible spectrophotometric analyses after the addition of a dilution agent, acetonitrile, and Fourier Transform Infrared Spectroscopy. The cellulose component of yellow poplar was then completely hydrolyzed using a cellulolytic enzyme in the ionic liquid-rich reaction media, and the hydrolysate was then analyzed by high performance liquid chromatography for the quantification of glucose monomeric units.

Success was achieved in the design of the analysis procedure, and it was employed for the quantification of lignin and cellulose in yellow poplar. There was also

a highly predictable conversion of cellulose to glucose and cellobiose by the cellulase in the ionic liquid-rich reaction media. A biomass compositional analysis procedure for the quantification of lignin and cellulose was created and was observed to be consistent in comparison with the results from the NREL protocol. The total lignin content as a percent of dry mass in yellow poplar was found to be 25.1% ± 0.8 using the NREL protocol, and 21.5% ± 0.4 and 25.6% ± 0.1 by the UV-visible and Fourier Transform Infrared Spectroscopy approaches, respectively, in the methods described herein. The glucan component was quantified as 43.5% ± 0.5 utilizing the NREL protocol and 43.6% ± 0.3 through analysis of the enzymatic hydrolysate as part of these methodologies.

Keywords: lignocellulosic biomass, feedstock analysis, ionic liquids, cellulase

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NOMENCLATURE

g	grams
min	minutes
mL	milliliter
M	molar
ºC	degree Celsius
wt%	weight percent by mass
v/v	volume to volume
g/g	gram to gram

Abbreviations

[Bmim][Cl]	1-n-butyl-3-methylimidazolium chloride
[Bmim][Br]	1-n-butyl-3-methylimidazolium bromide
[Bmim][Ac]	1-n-butyl-3-methylimidazolium acetate
ILs	Ionic liquids
NREL/DOE	National Renewable Energy Laboratory, US Department of Energy (Golden, CO)
HPLC	High performance liquid chromatography
FTIR	Fourier Transform Infrared spectroscopy
UV-VIS	Ultraviolet – visible (light) spectroscopy
TFPC	Tennessee Forest Products Center at the University of Tennessee

CHAPTER 1

INTRODUCTION

1.1 - Overview

As crude oil prices continue to increase and supplies waver, the development of lignocellulosic biomass as a potential renewable energy source is becoming more urgent. By 2006 bioethanol only contributed approximately 2% to the total transportation fuels used in the United States.[26] It is the goal of the Biomass R&D Technical Advisory Committee, established by the US Congress, to replace thirty percent of the US petroleum consumption with biofuels by 2030. Identifying and utilizing alternative, renewable feedstocks for the development of biofuels as alternatives to conventional petroleum fuels will play an important role in meeting the increasing US energy demand. However, to make cellulosic ethanol a cost-competitive alternative fuel, it is imperative to determine the composition of various biomass species and their anatomical components to identify which feedstocks have the most potential value in terms of available fermentable sugars (Table 1). Renewable feedstocks may include large scale crops such as corn, as well as agricultural and forest residues including tobacco stalks, corn stover, wheat straw, aspen, poplar, and municipal waste.[25]

Table 1. Percent dry weight composition of lignocellulosic feedstocks (Mosier, et al. 2005).

Feedstock	*Glucan (Cellulose)*	*Xylan (Hemicellulose)*	*Lignin*	*Others*[1.]
Corn Stover	37.5	22.4	17.6	22.5
Corn Fiber	14.28	16.8	8.4	60.52
Pine Wood	46.4	8.8	29.4	15.4
Poplar	49.9	17.4	18.1	14.6
Wheat Straw	38.2	21.2	23.4	17.2
Switch Grass	31	20.4	17.6	31
Office Paper	68.6	12.4	11.3	7.7

1. Includes ash, protein, extractives, other unknown soluble solids, and acetyl content.

1.2 - Proposed Work and Thesis Objectives

Research still needs to be conducted to make the production of ethanol from lignocellulosic feedstocks cost-competitive. Of relevance are advances in biomass integrated size reduction via chopping and grinding, which may lead to dry separation of plant components by anatomical and chemical properties. Rapid analyses of chemical constituents are needed for the proper treatment and utilization of biomass in a biorefinery and to analyze the composition of particulates resulting from the size reduction of biomass via grinding and sieving. Moreover, research by Womac and co-workers has demonstrated that partial separation of particulates can occur due to differences in either aerodynamic or gravimetric forces between the different anatomical parts of the same biomass source, which often reflects differences in biomass chemical composition.[15] Traditional wet chemical analysis is needed to produce standards for the new methodologies including spectroscopic and chemical analyses. Near Infrared spectroscopy (NIR) has been used as a rapid analysis tool for estimating the composition of lignocellulose and other organic compounds in various biomass feedstocks.[17, 30] However, the chemical composition of each woody and herbaceous feedstock must be previously determined using wet chemical analysis to calibrate an NIR spectrometer. Therefore enhancement of current compositional analysis methodologies is of urgency for improving efficiency and costs of processing lignocellulosic biomass. The focus of this research is to improve the simplicity and to reduce the labor required for the chemical analysis of lignin and cellulose in biomass fractions created by new size reduction and separation systems utilizing ionic liquids as an environmentally-friendly solvent.

Chapter 2 of this thesis presents background information into the conventional processing methods for biomass and biomass constituents. The chapter then introduces ionic liquids as a new designer solvent system, and concludes with a brief description of the cellulase enzymes as employed for the hydrolysis of biomass.

Chapter 3 discusses the current standard for biomass compositional analysis, *Determination of Structural Carbohydrates and Lignin in Biomass*, as developed by US Dept of Energy, National Renewable Energy Laboratory (NREL).[32] A sample matrix of

corn stover anatomical fractions was tested using these methods for determination of glucan, xylan, acid-soluble lignin, acid-insoluble lignin, and ash as a percent of dry mass, in collaboration with Ms. Lu Liu and Drs. Philip Ye and Alvin Womac of the Biosystems Engineering Department at the University of Tennessee. As a result of this study, the major disadvantages to this analysis procedure were identified as areas for improvement within the new protocols designated by this research. Chapters 3 through 5 focus upon research performed by the author of this thesis.

Chapter 4 focuses on the direct solubilization of the biomass and its constituents into ionic liquids. The changes in physical morphology are discussed, along with the possibility of selective precipitation of the biomass and its constituents from the solution.

Chapter 5 presents experimental work completed to study the direct quantification of biomass constituents after solubilization in ionic liquid. Direct analysis of lignin content was studied using UV-Visible (UV-VIS) spectroscopic analysis and Fourier Transform Infrared (FTIR) spectroscopy. Enzymatic hydrolysis of the solubilized biomass was completed using cellulase for rapid quantification of the cellulose content.

The thesis culminates in Chapter 6 with a summary of the conclusions and recommendations for future work.

The objectives of this research are:

1) To reproduce the standard NREL wet chemical analysis procedure and identify its shortcomings as an environmentally-conscious method for biomass analysis (Chapter 3 of thesis)

2) To verify the solubility of biomass and its constituents in alternative solvents systems based on ionic liquids (Chapter 4)

3) To determine if total lignin content can be rapidly quantified using either UV-VIS and/or FTIR spectroscopic analysis of biomass samples solubilized in ionic

liquids, and develop a universal model for prediction of total lignin content for varying lignin types and biomass species (Chapter 5)

4) To replace the hazardous and time-consuming acid-catalyzed hydrolysis of polysaccharides in lignocellulosic biomass with an operationally simple and environmentally preferable enzymatic hydrolysis of cellulose used to quantify glucan (Chapter 4)

5) To validate the quantification of lignin and cellulose in yellow poplar through comparison of results utilizing the new methodologies presented by the author and those employed in the NREL protocol. (Chapter 6)

CHAPTER 2

BACKGROUND

2.1 - Structure of Lignocellulosic Biomass

The general structure of lignocellulosic biomass is a network of biopolymers; cellulose and hemicellulose are surrounded by the lignin constituent (Fig. 1). The word saccharide is a derivative of the Greek word *sakcharon*, meaning sugar. The three major classes of carbohydrates are monosaccharides, oligosaccharides, and polysaccharides. Monosaccharides are simple sugars, with one polyhydroxy aldehyde or ketone unit and two or more hydroxyl groups. Glucose is the most abundant monosaccharide in nature. Monosaccharides such as glucose can be oxidized; the carbonyl carbon is oxidized to a carboxyl group, and therefore are called reducing sugars. Oligosaccharides are short-chain oligomers of monosaccharide units covalently attached through glycosidic bonds, which are formed when a hydroxyl group of one sugar reacts with the anomeric carbon of another. These include disaccharides, such as cellobiose, which is obtained by partial hydrolysis of cellulose (Figs. 2-4). Polysaccharides, also called glycans, are polymers of approximately 20 or more monosaccharide units.

Both cellulose and hemicellulose are long-chain polymers, or polysaccharides, making up the bulk of the plant material, up to approximately 90% in some forms of biomass. Cellulose is either highly crystalline or amorphous, and comprised of linear chains of 10,000 to 15,000 β-1,4' glycosidic-linked D-glucose units. This bonding arrangement is rigid and very stable. The long cellulose molecules, otherwise called microfibrils, give strength and rigidity to plant cell walls. The microfibrils are held in bundles by hydrogen bonding between the hydroxyl groups of the glucose rings. Hemicellulose is a biopolymer of mostly five-carbon sugars such as xylose, mannose, and arabinose. This heteropolymer has an amorphous crystallinity.

While cellulose is the most common biopolymer on earth, lignin is the next most abundant. Lignin further acts as a structural and protective element in the cell wall around the carbohydrate constituents, and is to some extent covalently bonded to

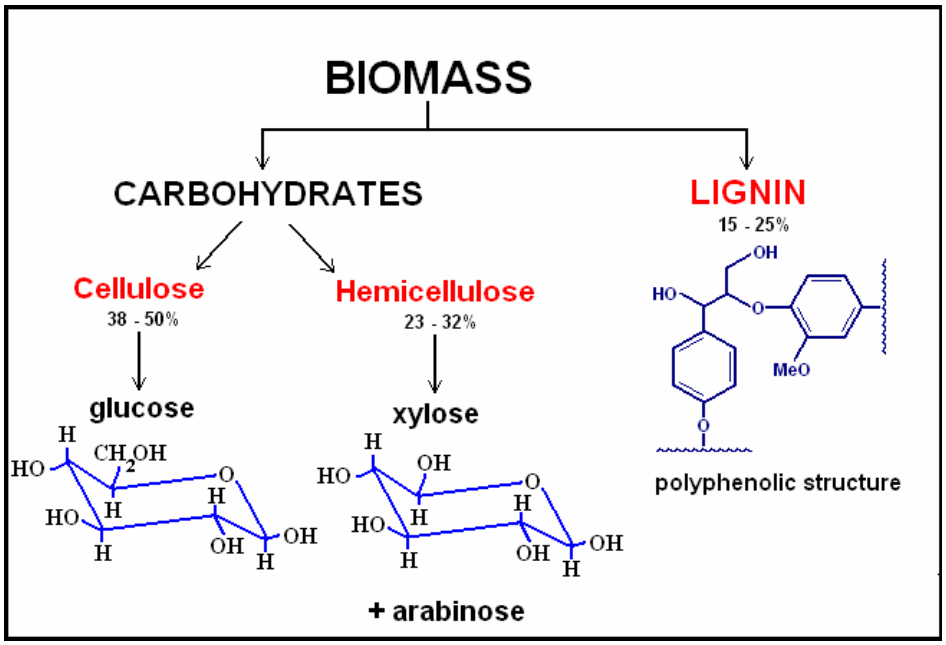

Figure 1. Complex composition and structure of biomass requires new technologies.

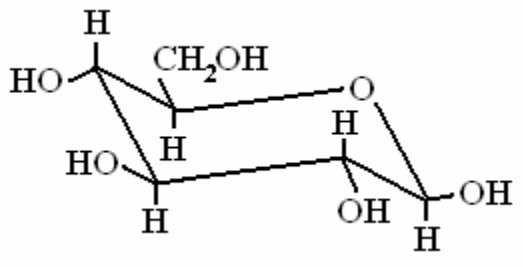

Figure 2. Monosaccharide D-glucose.

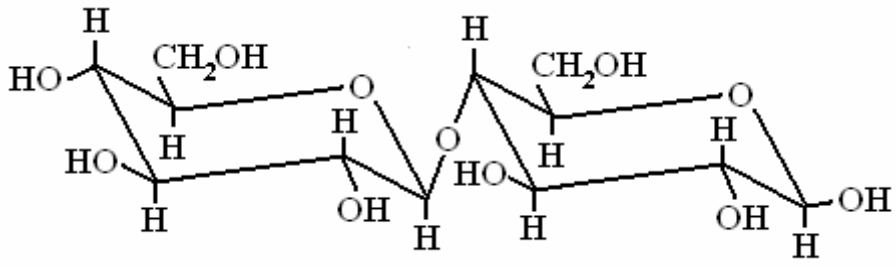

Figure 3. The disaccharide cellobiose is obtained by a partial hydrolysis of cellulose.

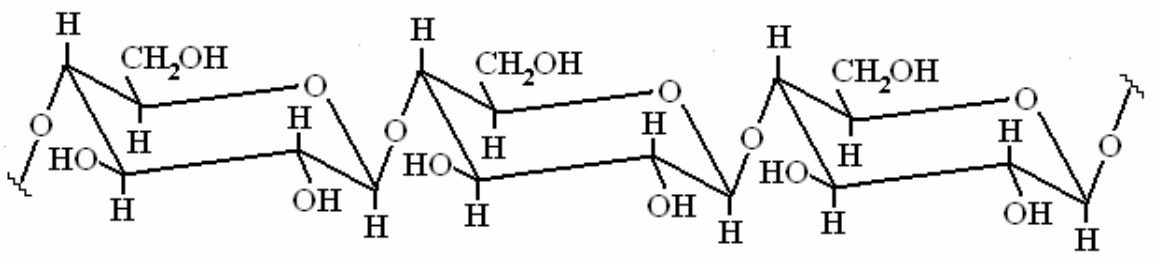

Figure 4. Cellulose consists of D-glucose units linked by β-1,4' glycosidic bonds.

cell wall hemicelluloses. Lignin is a complex aromatic polymer consisting of phenylpropane units bonded together by ether and carbon-carbon linkages. Lignin makes up approximately 20% of hardwoods and 30% of softwoods.[4] However, little is known of the natural structure of lignin and its complex network of bonds. The structural features of lignin make it difficult to measure quantitatively as they differ among plant species, plant parts, and within plant cell walls.[14] The three major groups for classification of lignin are based on the structural monomeric units: guiaicyl-lignin, found in conifers, lycopods, ferns, and horsetails; guaiacylsyringyl-lignin, found in angiosperms; and guiaicylsyringyl-*p*-hydroxyphenyllignin, found in grasses.[34]

2.2 - Processing and Analysis of Lignocellulosic Biomass

The complex structure of biomass is resistant to hydrolysis, so pretreatment processes are essential to allow for efficient processing of lignocellulosic biomass. The recalcitrance of biomass to hydrolysis is due to the enclosure of glucan and xylan by lignin and the high crystallinity of cellulose, which further increases resistance to conversion processes. As seen in Figure 5, pretreatment processes remove these structural barriers that impede hydrolysis and allows for separation and quantification of the chemical constituents, or to produce higher yields of fermentable sugars for conversion to ethanol.[23]

An initial step in pretreatment processes is cleaning and grinding the incoming biomass feedstocks to increase the available surface area by reducing the particle size.

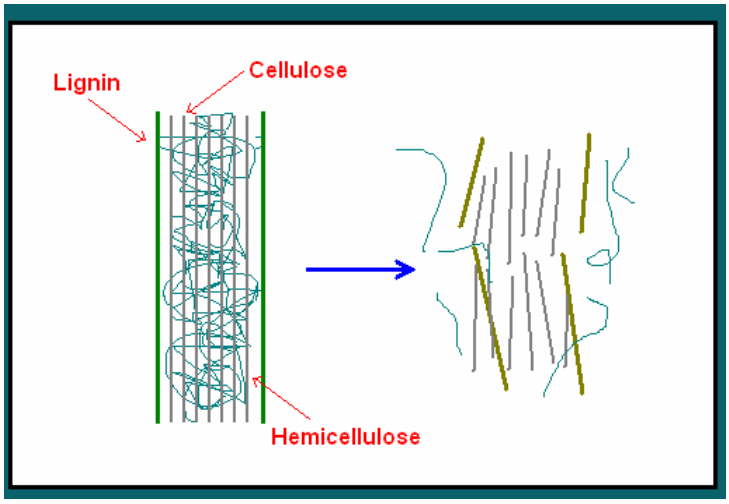

Figure 5. Pretreatment processes disrupt the complex structure of biomass.

During hydrolysis the chemical bonds of the long cellulose and hemicellulose chains are broken and simple sugars are formed in high concentrations necessary for fermentation. Subsequently, glycosidic bonds are readily hydrolyzed by acid, so oligosaccharides can be hydrolyzed to their monosaccharide units by boiling with dilute acid such as sulfuric acid. Other methods incorporate enzymatic hydrolysis as an eco-friendly alternative to dilute or concentrated acid.[22]

Delignification, separation of the cellulose and hemicellulose from lignin, is necessary for any pretreatment method for the complex structure of biomass to open and allow access to cellulose. Chemical delignification results in lignin whose properties may vary from the original biomass sample. It has been observed that the molecular weights of the isolated lignin becomes progressively lower during delignification process.[4] Lignin in wood is made soluble by chemical treatments such as sulfuric acid. Larger molecules remain insoluble and can be filtered from the hydrolysate.

Physical and chemical pretreatment methods currently utilized include: steam explosion, hot water, lime, ammonia fiber/freeze explosion, and dilute acid.[23] Steam explosion involves applying high pressure steam over a few minutes to hydrolyze hemicellulose by acetic acid resulting from acetyl groups released by hydrolysis. The steam is rapidly vented to reduce pressure, and the biomass is flash cooled. Elevated temperatures are used to maintain water in a liquid state for a hot water pretreatment.

Otherwise known as hydrothermolysis, hot water pretreatment involves applying compressed hot water to biomass for up to 15 minutes at 200 - 230°C. Alkali or lime pretreatment allows for ambient temperatures and lowers pressures, but requires longer application times compared to other pretreatment methods. Application of the alkali pretreatment removes lignin from the biomass and acetyl groups from hemicellulose. The ammonia fiber/freeze explosion (AFEX) pretreatment involves application of an aqueous ammonia solution through a column reactor packed with biomass at 160 – 180ºC for approximately 15 minutes. This process removes lignin and hemicellulose, and decrystallizes cellulose with swelling of the cellulose molecules.[23]

Dilute acid has been used in industrial settings for many years. Dilute sulfuric acid is applied to ground biomass at 160-220°C to increase digestibility of cellulose and to yield desirable monosaccharides from hemicellulose. Once glucose is formed under these conditions, it can dehydrate and form the furan 5-hydroxylmethylyfurfural (HMF) in the presence of aqueous acidic solution. Similarly, the sugars released from the hydrolysis of xylan can also undergo dehydration to form furfural.[10] These compounds can inhibit microbial fermentation and must be removed. The dilute acid treatment is desirable for the production of bioethanol as the hydrolysis is selective toward xylan while the cellulose fraction remains in a solid phase, successfully separating it from xylanose.[10] If fermentation is desired, hexoses are readily fermented to ethanol, while pentoses such as xylose and arabinose are utilized by a few native microorganisms. However, there is current research involving the genetic modification of certain yeast strains with the ability to co-ferment both pentoses and hexoses to ethanol and other value-added products.[5] The use of acid to remove hemicellulose performs well with a wide range of feedstocks from hardwoods to agricultural residues. Corn cobs and stover were found to be particularly well suited to this method of pretreatment.[23]

2.3 - Ionic Liquids as Solvents for Biomass and Its Biopolymer Constituents

In recent years there has been an increasing interest in the use of ionic liquids (ILs) as novel solvent systems for chemical and biological processes. ILs are molten

organic salts that form ions are near ambient temperatures. With a wide range of beneficial characteristics including low volatility and flammability, ILs should be considered as eco-friendly replacement for more volatile organic solvents in chemical reaction media.[13, 24] ILs have also shown good thermal stability, high heat capacity, and high thermal conductivity. This attractiveness has led to the development of several new commercial processes involving ILs. BASF's Biphasic Acid Scavaging using Ionic Liquids (BASIL) process uses ILs to remove acids from reaction mixtures. Ionic liquids with wide electrochemical windows have cations and anions resistant to oxidation and reduction, making them potential media for electrochemical applications. New applications within the petroleum and chemical industries including desulfurization of transportation fuels, reaction solvents and others are emerging.[3] Some of the areas developing new technologies using ILs can be seen in Table 2.[31]

ILs have recently been called "designer solvents" because numerous types have already been selectively synthesized using different ion pairs, making ILs extremely versatile solvents. The ability of ILs to remain liquid at near-ambient temperatures is possible by the combination of cations and anions affecting the salt's normal crystalline structure. Work by Deetlefs et. al. involves the ability to choose a suitable IL for a given

Table 2. Wide variety of applications being developing for ionic liquids.

Analytics	**Electrochemistry**
▪ Gas chromatography columns ▪ Stationary phase for high performance liquid chromatography ▪ Matrices for mass spectrometry	▪ Electrolyte in batteries ▪ Electrolyte in sensors ▪ Metal plating
Synthesis	**Performance Additives**
▪ Solvents ▪ Catalysis ▪ Biphasic reactions ▪ Manufacturing of nanomaterials ▪ Microwave chemistry	▪ Plasticizers ▪ Dispersing agents ▪ Compatibilizers ▪ Solubilizers
Separation	**Engineering Fields**
▪ Gas absorption ▪ Extraction	▪ Lubricants ▪ Thermodynamic fluids

function by predicting the physical properties of density and surface tension of ILs from their structure.[9] The ability to design ILs for desirable physical properties such as density, melting point, and viscosity has lead to an increasing interest in synthesizing custom ILs to act as novel solvent systems for processing of biomass.[11, 21]

Most traditional solvents are incapable of solubilizing carbohydrates and lignin; thus new solvent systems are needed for the processing of lignocellulosic biomass. One exception is N-methylmorpholin-N-oxide (NMMO), a crystalline compound that can form several stable and crystalline hydrates with water. The anhydrous and monohydrated NMMO have been shown to be good solvents for cellulose. It is now used commercially in the preparation of homogeneous cellulose-NMMO-water solutions for manufacturing cellulose fibers and films.[6, 12] Recent studies have shown that dissolution and subsequent hydrolysis of complex macromolecules and polymeric materials is possible in some hydrophilic ILs.[27, 33, 39] In particular, 1-n-butyl-3-methylimidazolium chloride, [Bmim][Cl], was shown to solubilize cellulose from a variety of sources, both natural and refined, with no degradation up to 22 wt%.[11] This dissolution is believed to be due to the high chloride concentration in [Bmim][Cl] which is highly effective in breaking the network of hydrogen bonds within cellulose.[39] Fort and co-workers obtained 5 wt% suspensions of dried and finely ground wood chips in a solution of [Bmim][Cl] at 100ºC through stirring for 2 to 24 hours. The color and viscosity of the solutions intensified with time. Visual inspection of the suspended particles showed that remaining particulates were smaller as time progressed, again indicating dissolution of the biomass.[11]

Solubilization of biopolymers is often evaluated using visual observation of the solutions; however, recent studies have employed nuclear magnetic resonance (NMR) relaxation measurements of the IL ^{13}C and $^{35/37}C$ nuclei at varying temperature and concentrations of the dissolved carbohydrate to better quantify the solvation of cellulose and other polysaccharides. Variations in relaxation times can yield information on the dynamics of both moieties making up the solvent, providing quantitative data regarding their interaction with the solutes. These relaxation measurements demonstrated that the

solvation of cellulose by the IL [Bmim][Cl] involves hydrogen bonding between the carbohydrate hydroxyl protons and the IL chloride ions.[12, 19, 27]

It is believed that IL preparation techniques greatly influence the degree of solubilization of biopolymers, in particular the temperature of the solution.[19] Heinze and co-workers found that the amount of dissolved cellulose in IL is also limited by the degree of polymerization and type of IL.[12] It has been observed that ILs, particularly those based on 1-butyl-3-methylimidazolium, are hydroscopic and can quickly absorb water when exposed to air (Fig. 6). This is a considerable limitation as the absorbance of water greatly decreases the ability of the IL to solubilized the biopolymer cellulose as the moisture competitively links to the cellulose microfibrils through hydrogen bonding. Some physical properties such as polarity and viscosity of the ILs as well as the structure of the IL, are dependent on the concentration of absorbed water.[35] In summary, preparation and experimental conditions must be closely investigated to optimize the utilization of ILs as novel solvents for the quantification of biomass composition. The development of new ILs would also allow for improvements in dissolution of lignocellulosics biomass in IL and would allow for further applications within these environmentally preferable solvents.

2.4 - Cellulase in Ionic Liquid

There are multiple pretreatment methods currently available to partially or completely hydrolyze lignocellulosic biomass for fermentation. Many require a high

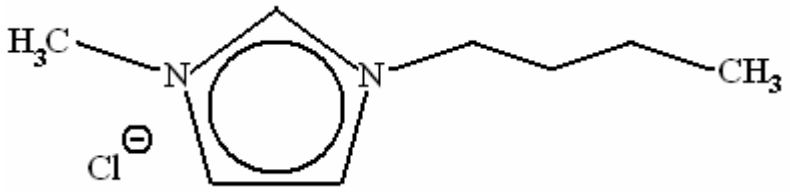

Figure 6. Structure of [Bmim][Cl].

chemical usage (up to 3%v H_2SO_4) and produce large waste streams (i.e. gympsum).[23]Excluding dilute acid, none of the pretreatment methods presented produce free pentosan sugars. However, the conditions used with acid-catalyzed hydrolysis are severe, utilizing high heat (up to 121°C) and volatile chemicals, which presents hazardous lab conditions as well the potential degradation of some pentosans and glucose. Sugar degradation within aqueous acidic solutions results in the dehydration of glucose to produce 5-hydroxylmethlyfurfural (HMF), and xylose is largely converted to furfural, both microbial inhibitors. An enzymatic pretreatment would present the most specific and eco-friendly pretreatment method.[10]

Cellulose hydrolysis to glucose can also be catalyzed using the enzyme system known as cellulase. Commonly produced by aerobic and anaerobic bacteria and fungi, cellulase enzymes from *Trichoderma* and *Aspergillus* are frequently used. The cellulase enzyme system is a mixture of three enzymes: Endo (1,4-β) glucanases, which make free chain ends available; exo (1,4-β) glucanases, which hydrolyze cellulose substrate to cellobiose units; and β-glucosidase, which hydrolyzes cellobiose to glucose. Cellulases adsorb to the insoluble cellulose, and the biocatalytic depolymerization of the cellulose microfibrils is followed by the desorption of the enzymes. Unlike acid-catalyzed hydrolysis, the employment of cellulases produce practically no glucose degradation products.[7] However, commercial cellulases and hemicellulases are too costly and yield too low of a turnover to be cost competitive compared to other pretreatment methods.[10]

Other cellulases have been reported to effectively hydrolyze cellulose in an IL and water reaction medium. "Celluzyme 0,7T", a commercially available cellulase from *Humicola insolens* from Novozymes, Inc (Franklinton, NC), was recently shown to successfully hydrolyze 0.15 g/L carboxymethyl cellulose in IL and water (1:10 v/v). Celluzyme 0,7T was incubated in IL for 24 hours before being exposed to the substrate. ILs included in the study were [Bmim][Cl], [Bmim][BF_4], and [Bmim][PF_6]. Although not surpassing reaction rates obtained in aqueous media, the hydrolysis rates within the IL systems were similar to that of an aqueous control.[24] Varying the reaction media to a higher IL composition resulted in rates nearly equal to that of water with [Bmim][PF_6]

and [Bmim][BF_4]. [Bmim][Cl] differs from the other treatments as it has hygroscopic characteristics. The nonpolar IL [Bmim][PF_6] is completely immiscible with water and [Bmim][BF_4] is also known for its hydrophobicity. The Cl^- anion was believed to inactivate the enzyme through denaturation and unfolding of the enzyme.[24, 38]

The employment of ILs as a pretreatment step has also shown success for enzymatic biocatalysis. Dadi and co-workers reported that cellulose precipitated from [Bmim][Cl] through the addition of water was hydrolyzed in an aqueous system catalyzed by the *Trichoderma reesei* cellulase. This resulted in hydrolysis rates approximately fifty times higher when compared to untreated cellulose due to the IL's ability to disrupt the cellulose structure.[7] Liu and co-workers saw that pretreated wheat straw in [Bmim][Cl] again resulted in higher hydrolysis rates.[18]

Dissolution of lignocellulosic biomass in ILs allows for further applications within these environmentally preferable solvents. Because [Bmim][Cl] has been shown to disrupt the cellulose morphology within lignocellulosic biomass from crystalline to amorphous, it is believed that the effect of the IL will be to accelerate biocatalysis in ILs. However, Turner and co-workers reported an inactivation and unfolding of *Trichoderma reesei* cellulase in [Bmim][Cl] and [Bmim][BF_4] with pH 4.8 at 50ºC in varied concentrations of both substrate and enzyme. Hydrolysis rates were not enhanced in [Bmim][Cl] due to the irreversible inactivation of the cellulase by the Cl^- ion.[36] Some improvements have been examined to allow for an enhanced cellulase activity within IL. One such method involved freeze drying the cellulase in the presence of the lyoprotectant PEG.[38]

In summary, early examinations have shown that ILs have the ability to achieve solubilization of biomass. Future research will increase understanding of the application of ILs in the pretreatment of biomass and utilizing these solvents as medium for enzymatic hydrolysis of the cellulose component within lignocellulosic biomass.

CHAPTER 3

ASSESSMENT OF NREL PROTOCOLS

3.1 - Introduction

The laboratory analytical procedure *Determination of Structural Carbohydrates and Lignin in Biomass*, as developed by US Dept of Energy, National Renewable Energy Laboratory (NREL) has become the accepted standard for biomass compositional analysis.[32] Carbohydrates and lignin make up the major portion of most lignocellulosic biomass, so these constituents must be measured as part of a comprehensive biomass analysis to assess the potential value of each feedstock. The procedure is suitable for samples that do not contain extractives. A two-step acid-catalyzed hydrolysis is used to fractionate biomass into quantifiable forms.

The NREL standard wet chemical analysis procedure requires volatile chemicals to penetrate the complex structure of biomass that is resistant to hydrolysis. The resistance of biomass to hydrolysis and further conversion processes is due to the enclosure of glucan and xylan by lignin and the high crystallinity of cellulose. The NREL acid-catalyzed hydrolysis removes these structural barriers that impede hydrolysis and allows for separation and quantification of the chemical constituents. The use of these hazardous chemicals is a great disadvantage to this procedure as they pose a risk to laboratory safety. The NREL protocol is also extremely time-consuming, uses significant amounts of labor and energy, and is subjected to error because the biomass sample and its components are transferred numerous times to multiple vessels.

In order to proceed with the objectives within this research, familiarization of the DOE/NREL wet chemistry analysis was necessary, as this protocol will act as the standard for validation of any new methodologies. This is the first objective of this thesis. This chapter presents a description of the NREL protocol for biomass compositional analysis. A test matrix of approximately thirty corn stover samples was analyzed with duplicates to test repeatability. The analysis of the samples of various corn stover anatomical parts was completed in February 2006, in collaboration with Drs. X.

Philip Ye and Alvin Womac, and Ms. Lu Liu. The results for quantification of glucan, xylan, acid-soluble lignin, acid-insoluble lignin, and ash are presented for these samples. As a result of this study, the major disadvantages to this analysis procedure are identified for areas of possible improvement.

3.2 - Experimental Work

3.2.1 - Materials

The corn stover samples were provided by the Biosystems Engineering and Soil Science department at the University of Tennessee, which were first finely-ground via a Wiley knife mill (size No. 40 mesh) and dried at 105°C for 24 hours. Reagents included 72% w/w sulfuric acid (purity ≥ 98%), calcium carbonate (ACS reagent grade), and acetonitrile (purum ≥ 99%, HPLC grade), all from Fisher Scientific (Suwanee, GA). Standard sugar samples cellobiose, glucose, xylose, galactose, arabinose, and mannose (purum ≥ 98%) were acquired from Sigma-Aldrich (St. Louis, MO). Water was purified using a 0.2 μm filter. Glass pressure tubes with 90 mL capacity, screw on Teflon caps, and o-ring seals, were available from Ace Glass (Louisville, KY). Teflon stir rods approximately 5 cm longer than the pressure tubes were obtained from Fisher. Coors porcelain, medium porosity #60531 filtering crucibles from Sigma were used with various filtration and Erlenmeyer flasks from Fisher. The hydrolysate solutions were filtered using 0.2 μm, nylon syringe filters (#09-720-5) from Fisher.

3.2.2 - Methods

Analysis of moisture, ash, acid-soluble and insoluble lignin, and two major saccharide types was conducted according to procedures published by NREL.[32] These methods excluded measurements for protein, extractives, acetate, and other soluble solids. The method of high performance liquid chromatography sugar analysis of the hydrolysate departed from the protocol with the utilization of a Prevail carbohydrate ES

column (250 x 4.6 mm x 5 μm) and precolumn (7.5 x 4.6 mm cartridge) by Alltech Associates, a division of WR Grace (Deerfield, IL) at 25°C. Employment of the column and operating conditions were similar to those published recently.[2] The Alltech Varex MKIII evaporative light scattering detector (ELSD) was used for compound detection, utilizing a drift tube temperature of 90°C. Both monomeric and dimeric sugars were detected with a shortened analysis time in gradient mode. For the gradient analysis, the sample injection volume was 20 μL of solution from a microcentrifuge tube, and the mobile phase flow rate was 1 mL min^{-1}. The initial solvent was 85% acetonitrile, 15% water to 30 minutes, with gradual increase in water content to 25% over 10 minutes.

The following is a chronological summary of the protocol methodologies (Fig. 7):

1. Biomass samples are measured out to approximately 300 ± 10.0 mg and ground into small particles (size No. 40 mesh, estimated 0.425 mm particle size) using a Wiley knife mill, located in the Food Engineering Laboratory of the Biosystems Engineering Department at the University of Tennessee, then dried at 105ºC to obtain the oven dry weight.

2. The ash content is found gravimetrically after combustion of non-ash materials at 575 6ºC for 24 hours in the Fisher Isotemp programmable muffle furnace, located in the Water Quality lab in the Biosystems Engineering and Soil Science Department at the University of Tennessee.

3. A two-step hydrolysis is used to fractionate the biomass into forms that are easily quantified. First, 3.00 ± 0.01 mL of sulfuric acid is applied to the biomass in a 90 mL glass screw-top pressure tube with Teflon cap and o-ring seal. The tubes are incubated in a water bath at 30 ± 3 °C for one hour. The sample is stirred with Teflon rods to ensure even acid to particle content and uniform hydrolysis. The acid is then diluted to a 4% concentration through the addition of 84.0 ± 0.04 mL deionized water. The tube is then placed in the Napco 8000-DST bench top autoclave (Winchester, VA) for one hour at 121ºC.

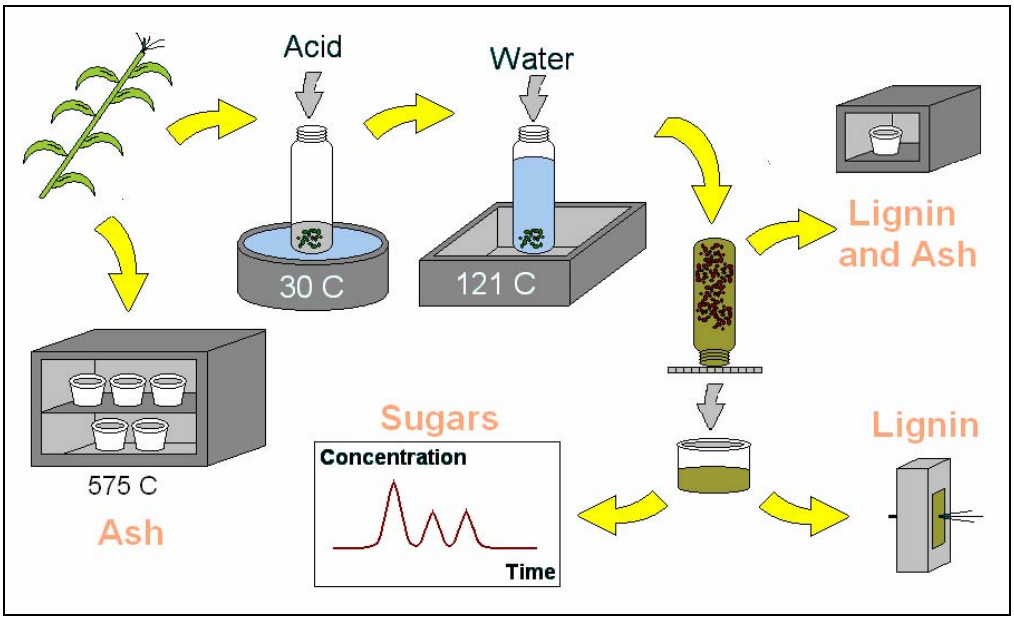

Figure 7. Flowchart of DOE/NREL wet chemistry analysis.

4. A set of sugar recovery standards is used to correct for losses due to deconstruction of sugars during dilute acid hydrolysis. These standards included glucose, xylose, galactose, arabinose, and mannose in concentrations closely resembling the concentrations of sugars in the test samples (approximately 0.1-4 mg mL^{-1}). The required amounts of each standard sugar is weighed out and added to 10.0 mL deionized water and 348 µL of 72% sulfuric acid in a glass pressure tube and hydrolyzed with the test samples as described above.

5. This application of dilute sulfuric acid results in soluble and insoluble matter, and the two fractions are separated with filtering crucibles. Additional deionized water is used to transfer all remaining solids out of the pressure tubes into the filtering crucibles. Approximately 50 mL of the filtrate is set aside for analysis of acid-soluble lignin and structural carbohydrates.

6. The insoluble solids fraction contains ash, protein, and acid-insoluble lignin. The total mass of the insoluble matter is found gravimetrically after drying at 105ºC

for a minimum of four hours. The ash content must be accounted for as described in Step 2. The remainder of the insoluble fraction mass is assumed to be acid-insoluble lignin.

7. The soluble liquid fraction contains reducing sugars from cellulose and hemicellulose, acid-soluble lignin, as well as some protein and acetate. The acid soluble lignin is measured by UV-VIS spectroscopy with the UV-1700 spectrophotometer by Schimadzu Scientific Instruments (Columbia, MD). The background and reference sample are both deionized water. The sample was diluted as necessary to bring the absorbance at 320 nm (recommended wavelength for quantification of acid-soluble lignin from corn stover) into the range of 0.7-1.0, and this absorbance was recorded in duplicate.

8. During hydrolysis the polymeric carbohydrates are hydrolyzed into monomeric forms which are soluble in the hydrolysis liquid. These sugars (glucose, xylose, mannose, arabinose, and galactose) are then quantified using HPLC. Glucan is proportional to the percentage of glucose found. A series of calibration standards of concentrations 0.1-4 mg mL^{-1} in water are created for the compounds that are to be quantified. Each sample is prepared for HPLC analysis by transferring approximately 20 mL of each filtrate reserved in Step 5 to a 50 mL Erlenmeyer flask. Calcium carbonate is used to neutralize each sample to pH 5-6. The sample is then allowed to settle, and the supernatant is decanted. The decanted liquid is passed through a 0.2 μm syringe filter into a microcentrifuge tube.

3.3 - Results and Discussion

As seen in Table 3, the NREL protocols for compositional analyses have been completed for six corn stover anatomical components. These compositions are similar to those found in NREL published values for corn stover.[1] The results established

Table 3. Average chemical composition of various anatomical parts of corn stover. Values reflect the average of two measurements [1, 2, 3]

Component	*% Dry Basis*						
	Pith	*Husk*	*Rind*	*Node*	*Leaf*	*Sheath*	Published Values - Whole corn stover[5]
Cellulose and Hemicellulose[4.]	78.4 ± 2.6	86.7 ± 0.9	76.6 ± 2.3	74.9 ± 0.8	74.5 ± 4.1	77.6 ± 4.0	76.8
Glucan	38.8 ± 0.2	44.6 ± 0.1	48.5 ± 0.1	22.5 ± 01	26.6 ± 1.3	26.6 ± 1.1	37.4
Xylan	20.4 ± 2.4	34.5 ± 0.8	26.7 ± 0.1	13.3 ± 0.7	13.8 ± 2.8	15.5 ± 2.8	21.1
Lignin	17.4	10.7	19.9	20	19.7	16.6	20.1
Acid Insoluble Lignin	15.7	8.8	18.9	18.3	17.9	14.7	18.4
Acid Soluble Lignin	1.7	1.9	1	1.7	1.8	1.9	1.7
Ash	4.2	2.7	3.5	5.1	5.8	5.8	5.0
Moisture	8	7.5	7.4	8.9	15.1	10.8	18.0

1. Analysis methodology conducted according to those dictated by DOE/NREL protocols [32], excluding measurements for protein, extractives, acetate, and other soluble solids.
2. Sugar analysis based on alternative operating conditions (Agblevor et al., 2004)
3. Arabinan, galactan, and mannan excluded as these sugars were below detection limit
4. Total cellulose and hemicellulose content calculated using mass balance of all other quantified components
5. (Aden et.al, 2002)

compositional differences between anatomical components, with the husk of corn stover having the highest sugar content (glucan plus xylan), while the node had the lowest.

According to the author's assessment, the NREL protocols need improvements in the following areas to achieve a more rapidity and robustness:

1. Reduction of analysis time: The actual acid hydrolysis requires several hours of incubation, but the most time consuming step involves the quantification of ash. The combustion of the whole biomass, as well as the remaining insoluble solids after hydrolysis, requires the use of the 575°C furnace for 48 cumulative hours.

2. Improvement of laboratory safety of the acid-catalyzed hydrolysis procedure and the HPLC analyses of carbohydrates: The dilute acid hydrolysis is evidently hazardous with the use of sulfuric acid, and conditions of high heat and pressure are also required. Large amounts of volatile solvents (a minimum of approximately 1 liter of acetonitrile needed per sample set) are used for analysis of sugars.

3. Reduction of the number of transfer steps of the biomass sample between vessels to minimize error: Each constituent is quantified in a separate vessel from that in which the hydrolysis originally took place. As many of the quantifications are based on gravimetric measurements, there is error for every constituent's calculated amounts due to the multiple transfers.

3.4 - Conclusions

Based on the assessment given above, the idea of using ionic liquids (ILs), salt-like organic materials consisting of ions that form a liquid at near-ambient temperatures, as a new solvent system for this type of analysis was explored. Utilization of ILs, solvents capable of solubilizing biomass and its constituent biopolymers, addresses many of the

above problems. It is believed that solubilization of biomass in ILs will allow for rapid spectroscopic determination of biomass composition and reduce the number of transfer steps for biomass samples during the analysis. Due to the low flammability and volatility and high thermal stability of ILs, an improvement of laboratory safety is anticipated.

CHAPTER 4

UTILIZING IONIC LIQUIDS FOR SOLUBILIZING BIOMASS

4.1 - Introduction

It has recently been shown that ILs are capable of efficiently dissolving complex macromolecules and polymeric materials. In particular, 1-n-butyl-3-methylimidazolium chloride, [Bmim][Cl], was shown to solubilize cellulose from a variety of sources, both natural and refined, with no degradation.[27, 39] This dissolution is believed to be due to the high chloride concentration in [Bmim][Cl] which is highly effective in breaking the network of hydrogen bonds within cellulose.[39] Also of importance was the fact that a variety of biological compounds can be dissolved simultaneously with polymeric solutes such as cellulose in [Bmim][Cl]. However, a considerable limitation of ILs is the fact that most are extremely sensitive to moisture and need to be protected. This hygroscopic character greatly decreases the ability of the IL to solubilize cellulose as the moisture competitively links to the cellulose microfibrils through hydrogen bonding.

Fort and co-workers obtained 5 wt% solutions of dried and finely ground wood chips in a solution of [Bmim][Cl] at 100ºC through stirring for 2 to 24 hours. The color and viscosity of the solutions intensified with time. Visual inspection of the suspended particles showed that remaining particulates were smaller as time progressed, again indicating dissolution of the biomass.[11] While the majority of previous work utilized stirring at temperatures at least 10°C above the melting point of the IL, Swatloski and co-workers found that microwave heating can significantly accelerate the dissolution of cellulose in IL. Solutions containing up to 25 wt% cellulose can be prepared in [Bmim][Cl] under microwave heating with frequency of microwave energy of 2.45 GHz with 3-5 second pulses.[33]

Cellulose is easily reconstituted from an IL-cellulose solution by the addition of a precipitant such as water, acetone, methanol, or acetonitrile.[11, 39] The yield of recovered

solute has ranged from 30 to 60 wt%.[11] It has also been shown that delignification of lignocellulosic biomass is possible through dissolution into and recovery from ILs. Biomass such as wood and straw can be partially dissolved in [Bmim][Cl] as ILs have the ability to dissolve both lignin and polysaccharides simultaneously.[39] The cellulose component can be precipitated from the IL solution by the addition of water, and the other organic compounds such as lignin and extractives, remain in solution. This method of delignification results in a higher decrystallization of cellulose than other refined celluloses from steam explosion or chemical pretreatment.[39]

The following chapter presents research on the direct solubilization of biomass and its constituents into three ILs of interest. The changes in physical morphology are discussed, along with the possibility of selective precipitation of the biopolymers from the solution.

4.2 - Experimental Work

4.2.1 - Materials

The three ILs of interest included 1-butyl-3-methylimidazolium chloride ([Bmim][Cl]), 1-Butyl-3-methylimidazolium bromide ([Bmim][Br]), and 1-Butyl-3-methylimidazolium acetate ([Bmim][Ac]), all HPLC grade (purum ≥ 98.0%) from Fluka Chemicals (St. Louis, MO). Commercially available cellulose (60-65% crystalline, purum ≥ 98%), organosolv lignin, and xylan (from wheat) powders were obtained from Sigma-Aldrich (St. Louis, MO). Whole yellow poplar was provided by the Tennessee Forest Products Center at the University of Tennessee, which was first finely-ground to an approximate particle size of 0.425 mm via a Wiley knife mill (size No. 40 mesh) and dried at 105°C for 24 hours. Various other solvents included methanol, acetonitrile, hexane, acetone, acetonitrile, and methylene chloride (all purum ≥ 99%, HPLC grade) from Fisher Scientific (Suwanee, GA). Water was purified using a 0.2 μm filter.

4.2.2 - Methods

4.2.2.1 - Solubilization of Biomass and Biopolymers in IL

The desired concentrations of lignin, cellulose, xylan, and yellow poplar in ILs ranged from 1 – 22 wt% to test for maximum solubility. All sample solutions were incubated and agitated at 400 rpm using 0.5 inch magnetic stir bars for 24 hours at 60°C (Fig. 8). After the 24 hour incubation period, the samples were compared through visual inspection for evidence of solubilization or other physical changes to the solutions. Subsequently, the solubilized samples were examined with an Olympus BX51 light microscope at 100x magnification (Center Valley, PA), located in the Wood Chemistry Lab as part of the Tennessee Forest Products Center at the University of Tennessee. The solutions were examined for evidence of suspended particles to determine a maximum solubility for each biomass constituent and ground biomass. These experiments involved recording images of dissolved biomass and its constituents in the three ILs of interest, with and without agitation or incubation.

4.2.2.2 - Precipitation of Biopolymers from ILs

Experiments were completed exploring the selective precipitation of xylan, cellulose, lignin, and yellow poplar from [Bmim][Cl] through the use of water and

Figure 8. Samples were incubated in stirred batch mode for 24 hours.

polar organic solvents. Samples were prepared at 1-2 wt% of each biopolymer in [Bmim][Cl] using 0.5 inch magnetic stir bars at 60°C for 24 hours of incubation. A series of co-solvents were added at equal volume proportions to each sample. This was followed by a series of centrifugation cycles at 13,000 rpm at 25°C for five minutes each. Visual observation was used to evaluate the presence of precipitation of the individual solubilized constituent. The threshold for visual observation was estimated for approximate particle size of 10 microns. The co-solvents utilized were water, acetonitrile, methanol, hexane, acetone, and methylene chloride.

4.3 - Results and Discussion

4.3.1 - Dissolution of Biopolymers and Biomass in IL

After the 24 hour incubation period, a color and viscosity change and a decrease in size of suspended particles indicated at least partial solubilization of purified samples of cellulose (~10%), lignin (~22% for four different sources), and finely-ground biomass (~2% corn stover and yellow poplar) in [Bmim]][Cl] at 60°C (Fig. 9). Visual inspection for translucence and visible suspended particles before and after centrifugation of xylan in IL indicates poor solubilization (<1 wt% in [Bmim][Cl]), suggesting delignification through partial solubilization of the biomass constituents within the IL may be possible, lending to improved hydrolysis of the biomass.

As seen in Figure 10, cellulose can be solubilized in [Bmim][Cl] up to approximately 10 wt% with the given conditions. This is significantly lower than the concentration of 22 wt% found in previous studies.[11] However, Liu and co-workers observed that temperature has an influence on solubilization in ILs, so this is believed to be the limiting factor in this work.[19] The cellulose solutions are assumed saturated as the concentration in [Bmim][Cl] increases as visible particles are observed in solutions over10 wt%. It is noted, however, that the size of the suspended particles is significantly smaller than that found in the non-incubated sample at 20 microns, indicating at least partial solubilization of the cellulose powder (Fig. 10a). Solutions of cellulose in IL at

Figure 9. Solubilization of lignin, cellulose, and xylan in [Bmim][Cl], respectively.

2 wt% were prepared for all three of the available ILs. Comparison of the non-incubated sample versus that of the incubated sample for [Bmim][Br] and [Bmim][Ac] show dissolution after 24 hours at 60°C as the solutions appeared completely clear of any suspended particles (Fig. 11). In addition to the IL's ability to solubilize the biopolymers, it was believed that the shearing effect from the stir bars contributed to the decrease in particle size in solution. Therefore if this work was to be scaled up, it is recommended that the conditions of shearing and energy input be considered.

It was impossible to determine solubility using visual observation of organosolv lignin in IL due to the dark brown color of the solutions. However, microscopic images of organosolv commercial lignin powder in [Bmim][Cl] reveal a higher maximum solubility concentration when compared to cellulose. Lignin added at 2 wt% to this IL shows immediate evidence of solubilization even without incubation (Fig. 12a). The maximum concentration of lignin in [Bmim][Cl] is approximated at 22 wt% by examination of the microscope images for suspended particles less than 1 micron. Concentrations higher than 22 wt% were not examined as the IL solution becomes extremely viscous and no longer capable of mixing, so the lignin solution was assumed to be saturated at 22 wt%. Solutions of organosolv lignin in [Bmim][Br] and [Bmim][Ac] at 2 wt% were also examined using light microscopy. Solubilization was achieved at 2 wt% for [Bmim][Cl] at 60°C, but there was small particulates remaining in the

[Bmim][Ac] solution (Fig. 13). This particular IL most likely requires a higher incubation temperature or additional agitation time for additional solubilization of the lignin powder.

Microscopic analysis of xylan solubilized in IL agrees with the conclusions made from visual observation of the samples before and after centrifugation. Maximum solubility of xylan in [Bmim][Cl] was approximated at 1 wt% from the translucence of the sample and lack of suspended particles. As the concentration of xylan in [Bmim][Cl] is increased to 2 wt%, the number and size of particles remaining in suspension increases and shows very little solubilization (Fig. 14). The inability of ILs to dissolve xylan may be beneficial in future work involving the physical separation of biomass as delignification may be possible, allowing for isolation of xylan. As seen in Figure 15, the other ILs of interest were more successful in solubilizing xylan particles of approximately 50 microns.

The microscopic analysis of ground yellow poplar in the ILs was completed to determine the appropriate concentration to be utilized in this biomass compositional analysis procedure. As seen in Figure 16, the maximum solubility in [Bmim][Cl] was estimated at approximately 2 wt% based on the translucence and lack of suspended particles visible with 100x magnification. The other ILs of interest were also able to at least partially solubilize yellow poplar at this concentration (Fig. 17). It is the author's assessment that this concentration is low due to the xylan component within the biomass, which limits its solubility. Of interest is that as the concentration reaches 5 wt%, the ionic liquid [Bmim][Cl] achieves only partial solubilization of the biomass, but there is visual evidence of fibrous material remaining in suspension, thus reinforcing the idea of delignification and decrystallization of the biomass structure.

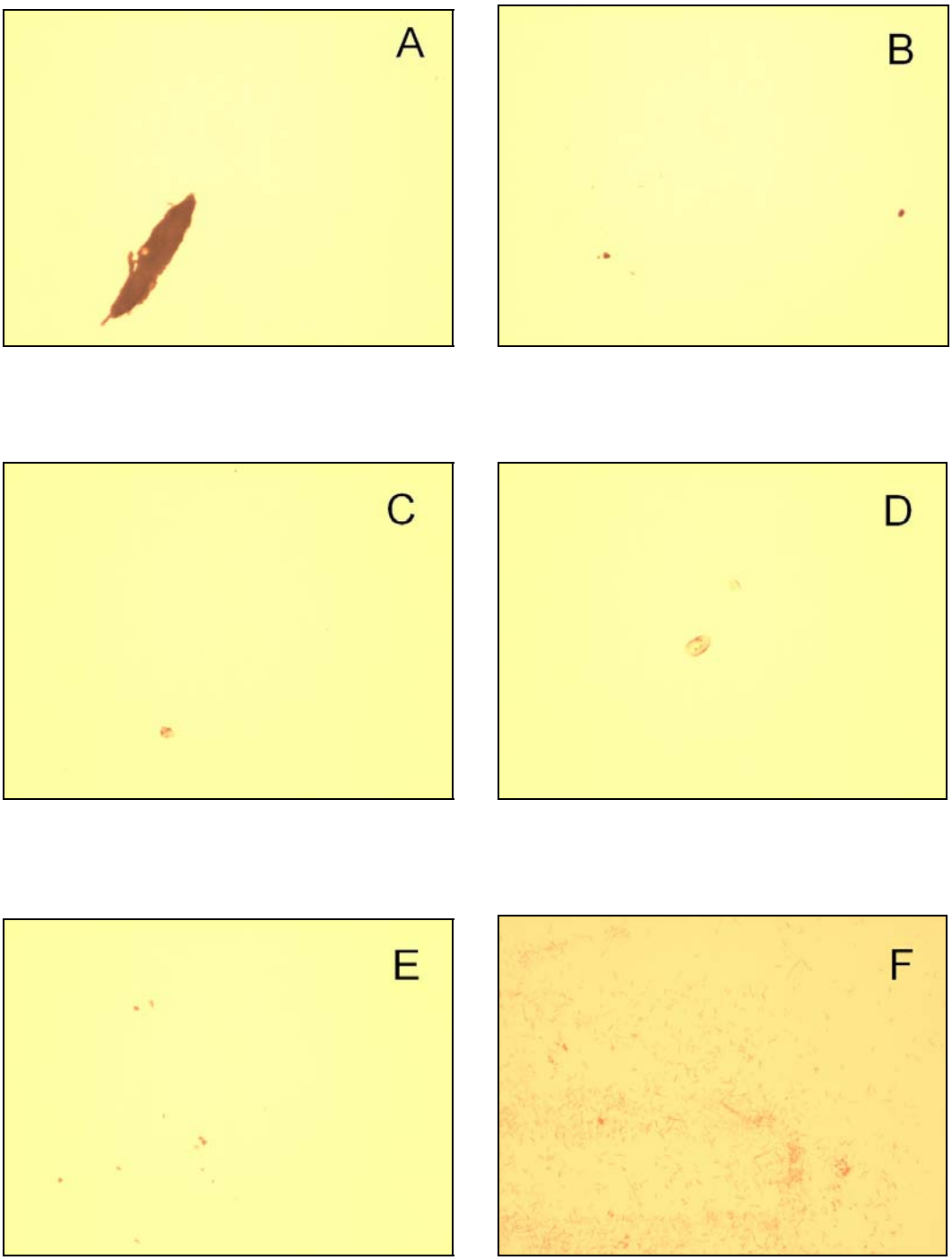

Figure 10. Cellulose solubilized in [Bmim][Cl] at 60°C at varying concentrations. (a) 2 wt% with no incubation, particle size estimated at 20 microns; (b) 1 wt%, visible particles estimated at less than 1 micron; (c) 2 wt%; (d) 5 wt%; (e) 10 wt%; (f) 15 wt%.

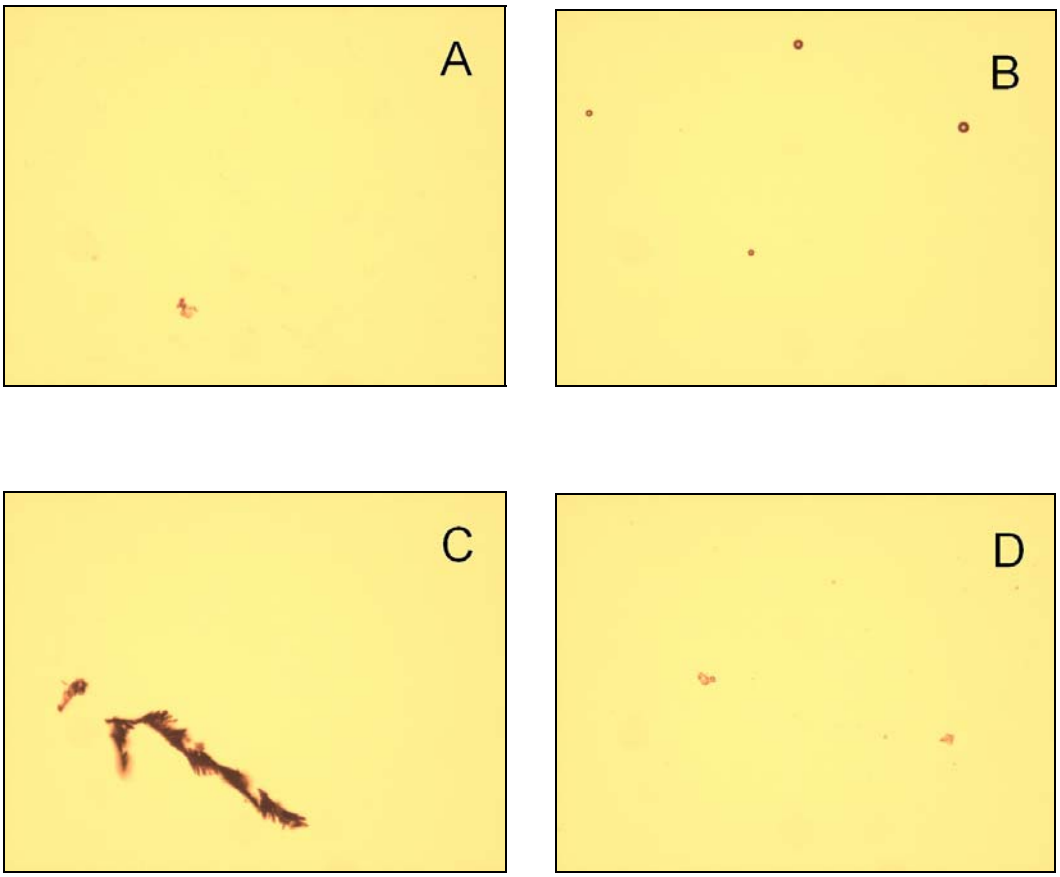

Figure 11. Cellulose solubilized at 2 wt% in (a, b) [Bmim][Br] and (c,d) [Bmim][Ac] at 60°C. Figures (a) and (c): no incubation, particle size estimated at 20 microns; Figures (c) and (d): 24 hours incubation time allowed; particle size estimated at less than 1 micron.

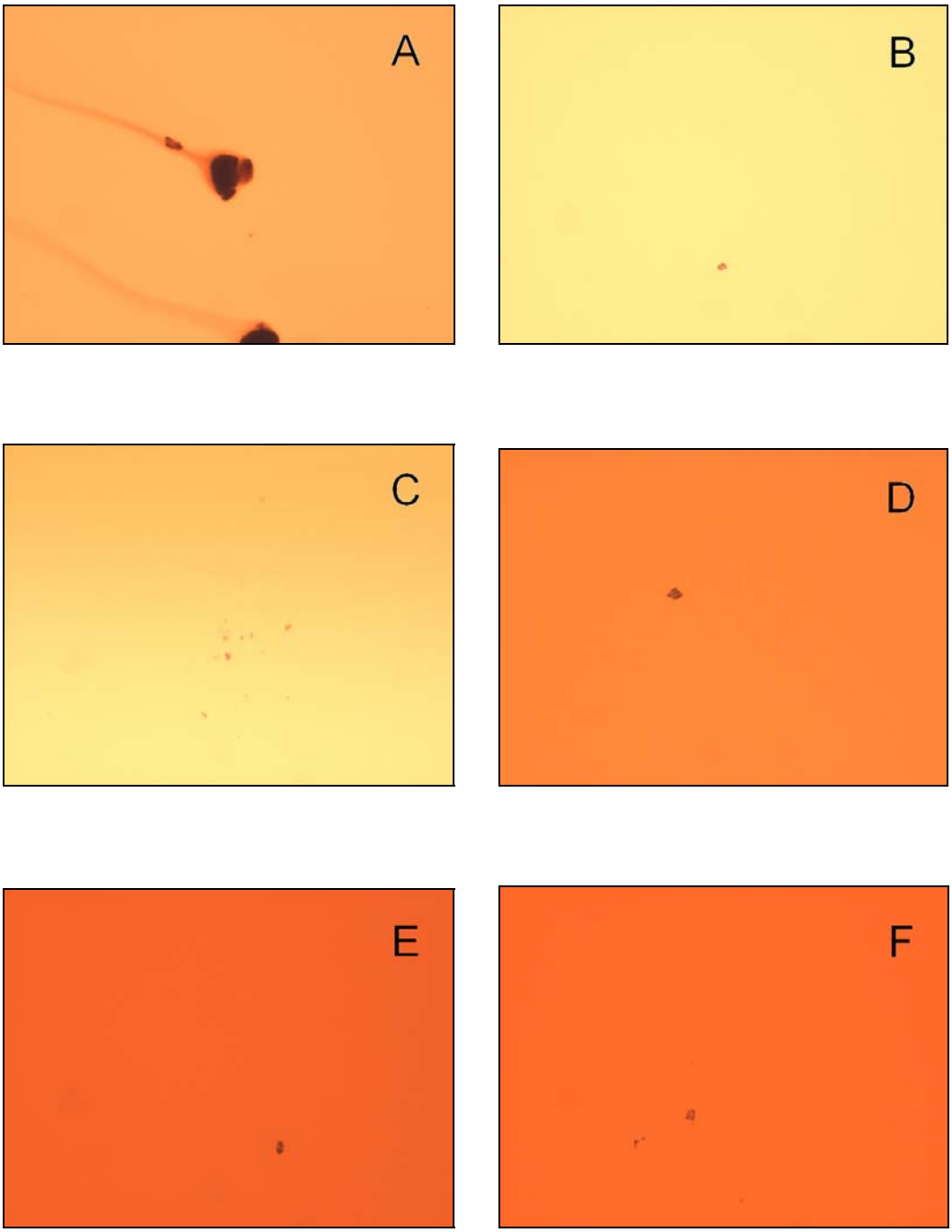

Figure 12. Organosolv lignin solubilized in [Bmim][Cl] at 60°C at varying concentrations. (a) 2 wt% with no incubation, particle size estimated as 10 microns; (b) 2 wt%, visible particle size estimated at less than 1 micron; (c) 5 wt%; (d) 10 wt%; (e) 20 wt%; (f) 22 wt%.

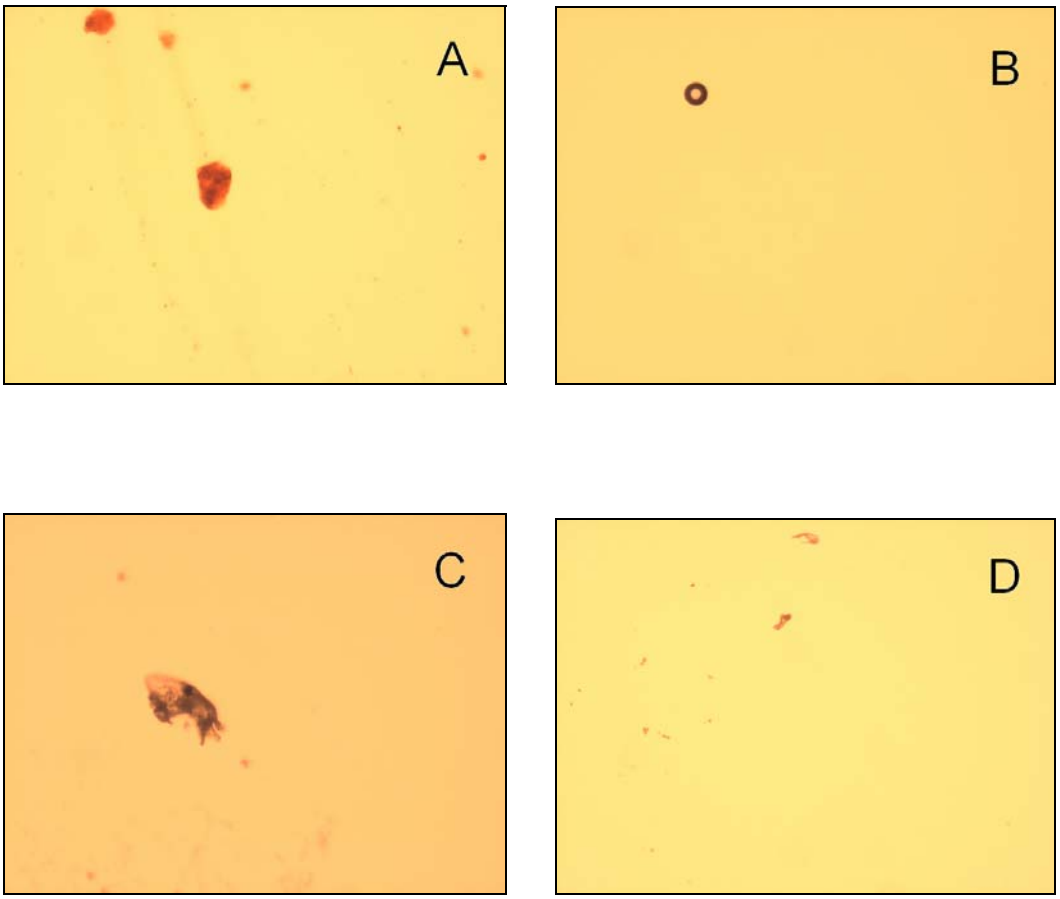

Figure 13. Organosolv lignin solubilized at 2 wt% in (a, b) [Bmim][Br] and (c,d) [Bmim][Ac] at 60°C. Figures (a) and (c): no incubation, particle size estimated at 10 microns; Figures (c) and (d): 24 hours incubation time allowed; particle size estimated at less than 1 micron.

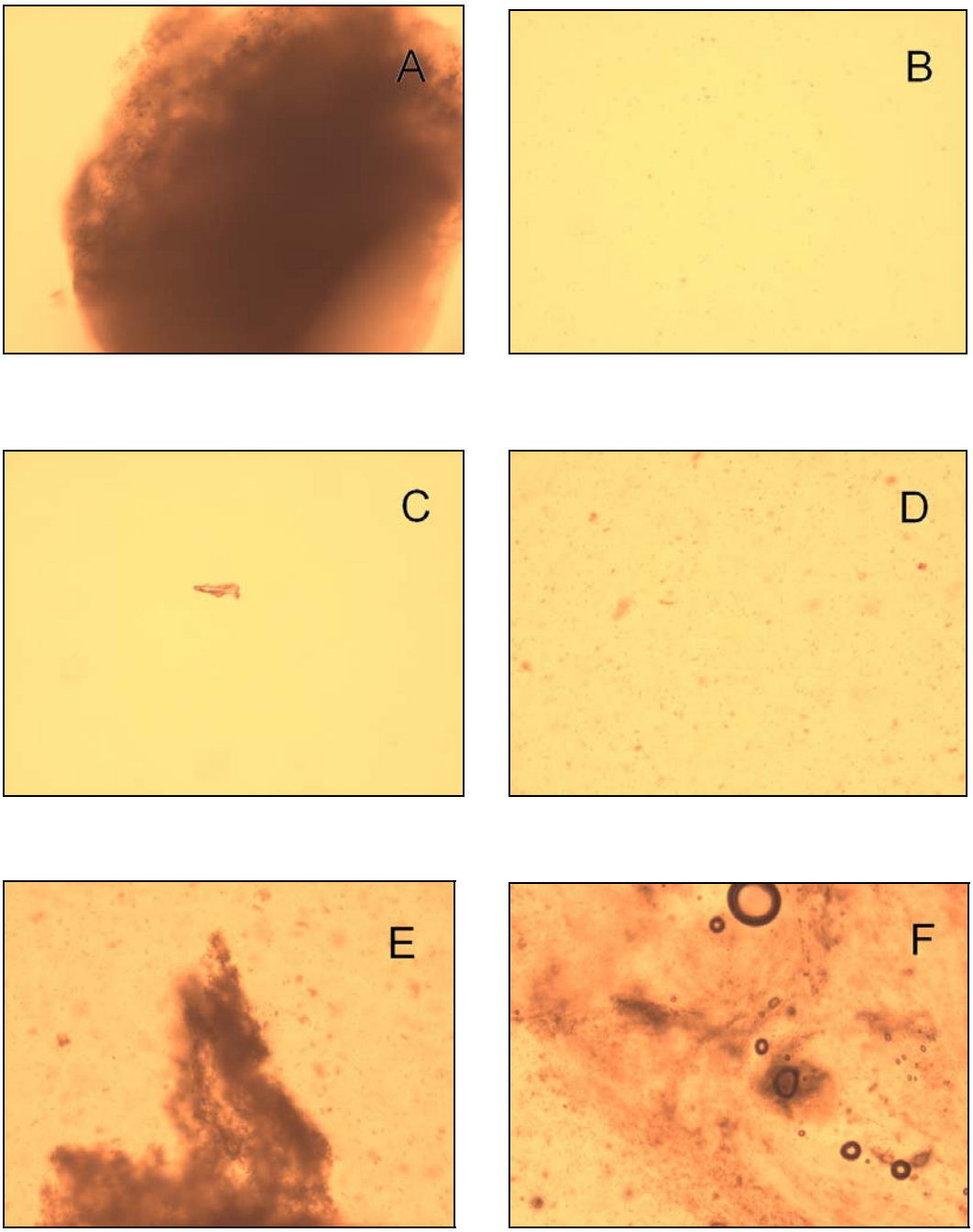

Figure 14. Xylan solubilized in [Bmim][Cl] at 60°C at varying concentrations. (a) 1 wt% with no incubation, particle size estimated as 50 microns; (b) 1 wt%, particle size estimated as less than 1 micron; (c) 2 wt%; (d) 5 wt%; (e) 10 wt%; (f) 15 wt%.

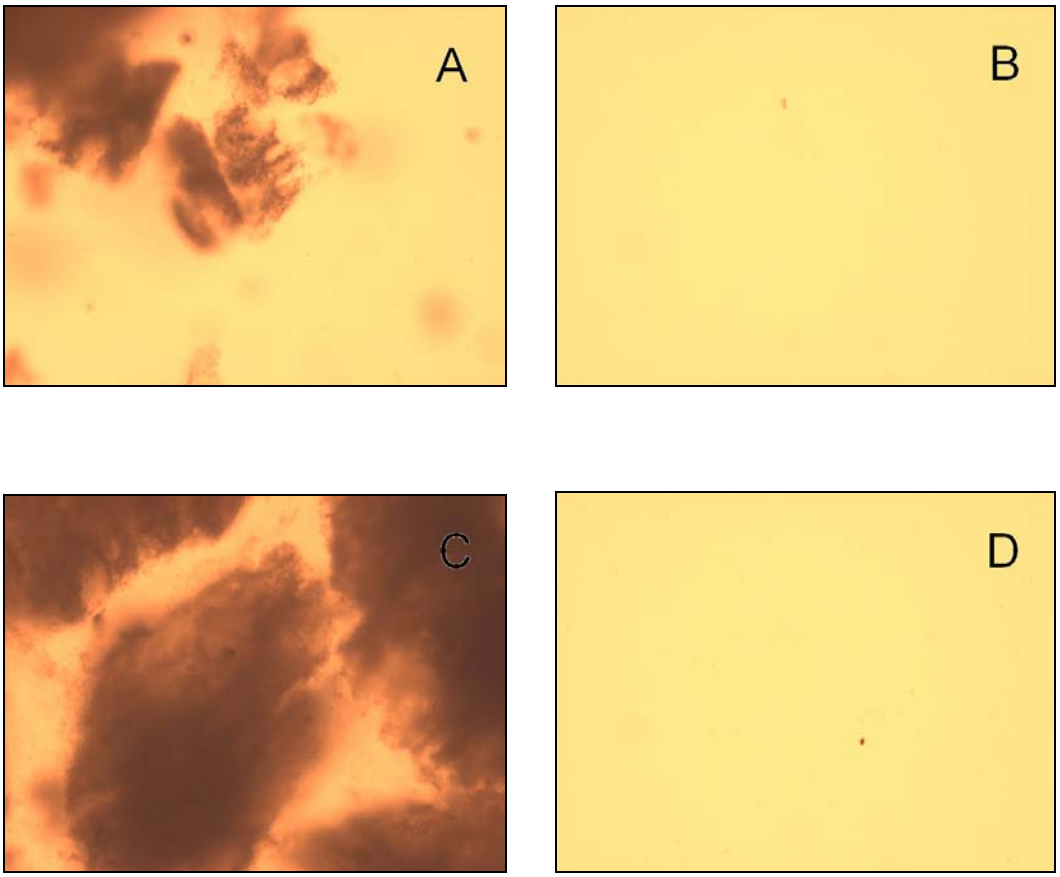

Figure 15. Xylan solubilized at 2 wt% in (a, b) [Bmim][Br] and (c,d) [Bmim][Ac] at 60°C. Figures (a) and (c): no incubation, particle size estimated at 50 microns; Figures (c) and (d): 24 hours incubation time allowed; particle size estimated at less than 1 micron.

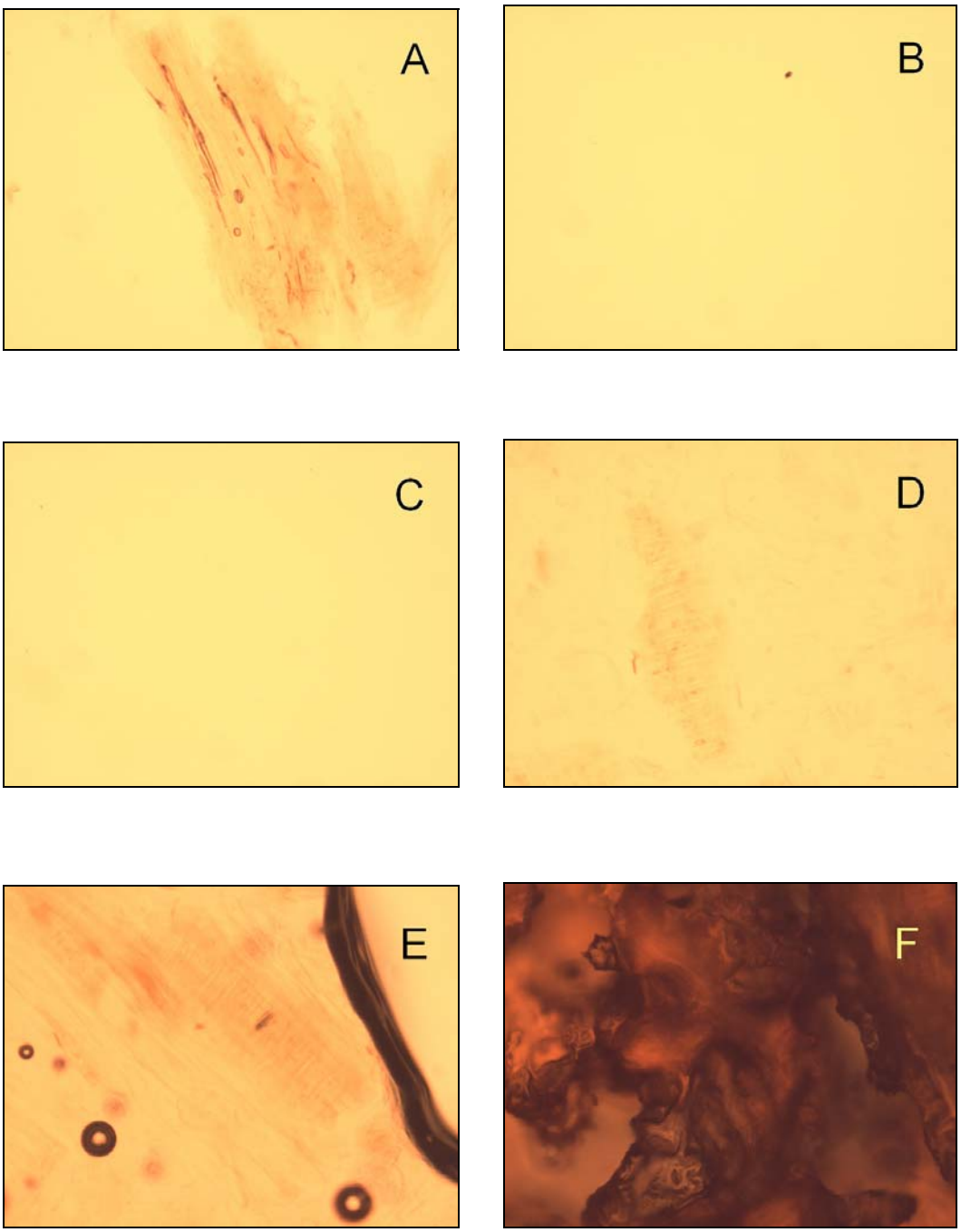

Figure 16. Yellow Poplar with approximate particle size of 0.4 mm solubilized in [Bmim][Cl] at 60°C at varying concentrations. (a) 2 wt% with no incubation; (b) 1 wt%, particle size estimated as less than 1 micron; (c) 2 wt%; (d) 5 wt%; (e) 10 wt%; (f) 20 wt%.

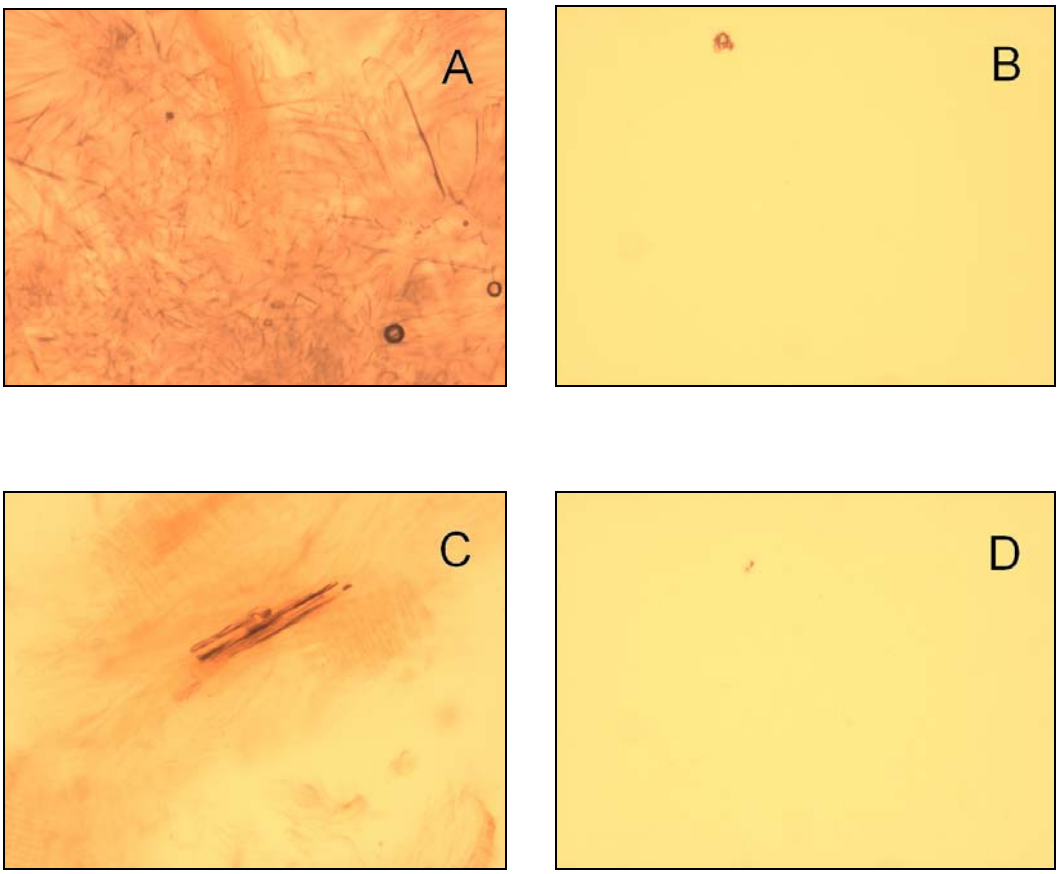

Figure 17. Yellow poplar solubilized at 2 wt% in (a, b) [Bmim][Br] and (c,d) [Bmim][Ac] at 60°C. Figures (a) and (c): no incubation, particle size estimated at 0.4 mm; Figures (c) and (d): 24 hours incubation time allowed; particle size estimated at less than 1 micron.

4.3.2 - Precipitation of Biopolymers and Biomass Solubilized in ILs

Precipitating the biomass and its constituents from the IL solutions through the use of a co-solvent was successful. During the experiments, the behavior of the co-solvent when added to pure IL was observed to be either miscible or immiscible. Those solutions that were miscible were then centrifuged and observed for any visual evidence of precipitation of the biomass constituent. Hexane and acetone were immiscible with [Bmim][Cl] and could not be used for precipitation of any constituent (Fig. 18).

As seen in Table 4, precipitation of biomass constituents and whole yellow poplar were possible with at least one of the co-solvents used. The individual cellulose and xylan constituents were easily precipitated with all miscible co-solvents used. However, lignin was only precipitated with water. There was evidence of at least partial precipitation using all co-solvents with the yellow poplar solutions.

4.4 - Conclusions

As a result of the solubility experiments described above, there is evidence that ionic liquids are a suitable host for the processing of biomass. There is solubilization of all constituents within whole finely-ground biomass at concentrations of 2 wt% or

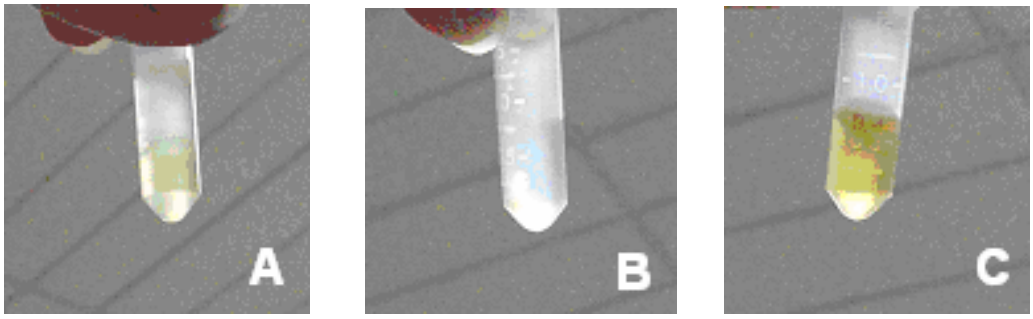

Figure 18. Visual observation of precipitation of biopolymers and biomass solubilized in [Bmim][Cl] with the addition of co-solvents at equal volume proportions at 25°C. (a) Acetone is immiscible with IL (no biomass present in system); (b) Precipitation of yellow poplar with water; (c) Immiscible methanol does not precipitate lignin.

Table 4. Precipitation behavior with the addition of several co-solvents to ionic liquid solutions.[1.]

Yellow Poplar		
Co-Solvent	***Behavior***	***Precipitation after Centrifugation?***
Methanol	Dissolves	Yes
Water	Dissolves	Yes
Hexane	Immiscible	-
Acetone	Immiscible	-
Acetonitrile	Dissolves	Yes
Methylene Chloride	Dissolves	Yes
Lignin		
Co-Solvent	***Behavior***	***Precipitation after Centrifugation?***
Methanol	Dissolves	No
Water	Dissolves	Yes
Hexane	Immiscible	-
Acetone	Immiscible	-
Acetonitrile	Dissolves	No
Methylene Chloride	Dissolves	No
Cellulose		
Co-Solvent	***Behavior***	***Precipitation after Centrifugation?***
Methanol	Dissolves	Yes
Water	Dissolves	Yes
Hexane	Immiscible	-
Acetone	Immiscible	-
Acetonitrile	Dissolves	Yes
Methylene Chloride	Dissolves	Yes
Xylan		
Co-Solvent	***Behavior***	***Precipitation after Centrifugation?***
Methanol	Dissolves	Yes
Water	Dissolves	Yes
Hexane	Immiscible	Yes
Acetone	Immiscible	-
Acetonitrile	Dissolves	-
Methylene Chloride	Dissolves	Yes

1. All samples prepared at 1 wt% in [Bmim][Cl] at 60°C with magnetic stirring at 400 rpm, then cooled to room temperature. Each co-solvent was added in equal amounts to that of the IL (v/v) and vortexed. Miscible solutions were then centrifuged at 13,000 rpm at 25°C in five minute cycles.

lower. [Bmim][Cl] was chosen for further inspection in this research as there seemed to be a greater degree of solubility of the constituents in this ionic liquid by visual inspection of suspended particles. Yellow poplar was dissolved up to 2 wt% in [Bmim][Cl], so this was chosen as the recommended concentration for use in the biomass compositional analysis procedure as developed in this research.

As a result of the precipitation experiments, it appears that ILs can be reused through the addition of a co-solvent. As shown above, several co-solvents including water effectively precipitate all biomass constituents from the solution. If the water can then be removed by drying, the IL is essentially recycled for use with additional preparations. Future experiments could explore these co-solvents or various other co-solvents and their efficiency (i.e. amount of precipitate recovery) for selective precipitation of biomass constituents from IL solutions. After repeated washings with these co-solvents, any remaining IL could be detected using FTIR analysis to ensure complete removal. The amount of recovered biomass could be measured gravimetrically, allowing for the selection of the best co-solvent for various steps in the biomass compositional analysis.

Selective precipitation was not investigated during the course of this research as the effect of the ILs on the complex structure and bonding between the various biomass constituents is unknown. However, it is recommended that future work involve the study of acetonitrile, methanol, or methylene chloride as potential co-solvents for selective precipitation experiments and subsequent fractionation of biomass constituents. This may be possible as these co-solvents have been shown to precipitate xylan and cellulose while lignin is dissolved in the IL system.

Because lignin is not precipitated with the addition of acetonitrile, this particular co-solvent was chosen for further inspection in this research. The IL solutions become viscous as lignin is solubilized, and this co-solvent acted as a potential dilution agent. Incorporating this solvent as a dilution agent is fitting as part of the biomass compositional analysis as acetonitrile is used as the mobile phase for subsequent HPLC analysis of the biomass samples in the compositional analysis procedure.

CHAPTER 5

REVISED WET CHEMICAL ANALYSIS PROTOCOL TO QUANTIFY OF CELLULOSE, HEMICELLULOSE, AND LIGNIN IN BIOMASS

5.1 - Introduction

The laboratory analytical procedure *Determination of Structural Carbohydrates and Lignin in Biomass*, as developed by US Dept of Energy, National Renewable Energy Laboratory (NREL) has become the accepted standard for biomass compositional analysis.[32] However, the author's assessment as described in Chapter 3 is that the NREL protocol need improvements in the following areas to achieve more rapidity and robustness: reduction in analysis time, improvement in laboratory safety of the acid-catalyzed hydrolysis procedure and the HPLC analyses of carbohydrates, and reduction of the number of transfer steps of the biomass sample between vessels to minimize error. Based on this assessment, the idea of using ILs as a new solvent system for this type of analysis was explored due to their ability to solubilize many of the components of biomass. Mirroring the NREL protocols, any new biomass compositional analysis methodologies will focus on the composition of lignocellulosics, i.e., the weight fraction of glucan, xylan, lignin, and ash. Alternative techniques based on chemical and spectral analysis methodologies that utilized ILs were investigated.

Spectroscopic methods have been shown successful in quantifying dissolved constituents of biomass. Recent studies by Fort and co-workers have shown that the use of ^{13}C NMR spectra of the [Bmim][Cl] solutions over time confirms dissolution of the cellulosic material and lignin. Progressive improvement of the signal-to-noise ratio of the cellulose and lignin resonances revealed an increase in dissolution of these polymers. The cellulose material was quantified using the C-1 and C-6 signals in the 105-92 and 63-59 ppm range at 90ºC. The integral of the lignin aromatic OMe ^{13}C resonance at 57 ppm was used to quantify lignin.[11]

Infrared spectroscopy has been shown to help reveal impurities in ILs. Complete removal of water and other –OH-rich species was confirmed by observing the absence of a band in the 3400 - 3800 cm^{-1} region of the infrared spectra.[16] The combination of excellent dissolution properties and a large spectral transparency make ILs suitable solvents for other spectroscopic measurements, including the ultraviolet-visible (US-VIS) region. UV-VIS spectra for [Bmim][Cl] result in an extremely weak band at 360 nm. As the chloride concentration is increased, a shift to shorter wavelengths for the UV cut-off is observed.[16]

Both of Infrared and UV-VIS spectroscopic methods were investigated in the included research as both have shown potential ability for quantification of lignocellulosic biomass constituents dissolved in ILs. Cellulose hydrolysis to glucose can be catalyzed using the enzyme system known as cellulase. Because the employment of cellulases has shown to produce practically no glucose degradation products, the hydrolysis of the cellulose component within biomass solubilized in IL through utilization of cellulase from *Aspergillus niger* was investigated.

5.2 - Theory: Beer-Lambert Law

One of the most widely used applications of spectroscopy is for the quantitative determination of the concentration of substances in solution. When light is irradiated on a certain substance and then is transmitted, a relational formula can be established between light and sample concentration (Fig. 19).

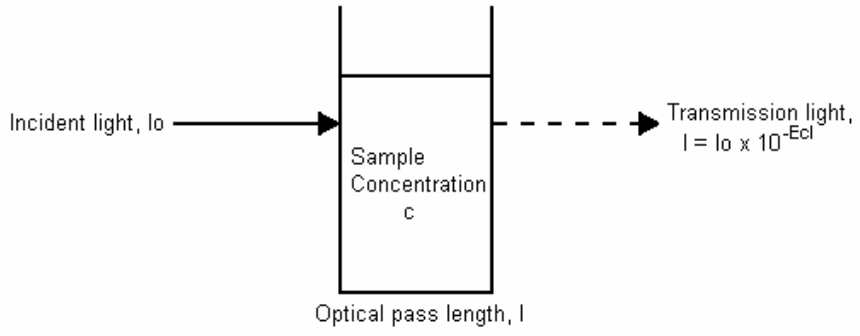

Figure 19. Beer-Lambert law describes how absorbance is proportional to the concentration of the sample and the light pathlength.

As seen in Figure 19,

$$T = \frac{I}{I_0} = 10^{-\varepsilon cl} \qquad \text{(Eqn 1)}$$

$$Abs = \log(\frac{1}{T}) = \log(\frac{I_0}{I}) = \varepsilon cl \qquad \text{(Eqn 2)}$$

where

T = transmittance

Abs = absorbance or optical density

ε = absorptivity constant for the substance under specific conditions (extinction coefficient) ($conc^{-1}$ cm^{-1})

c = sample concentration

l = optical pass length (cm)

From the above equations, it can be seen that transmittance is not proportional to the concentration of the sample, but absorbance is proportional to the concentration of the sample and is proportional to light path length. However, the optical path length was held constant for this project. The absorbance and the extinction coefficient depend on wavelength at which the measurement is made. As seen in Figure 20, the plot of

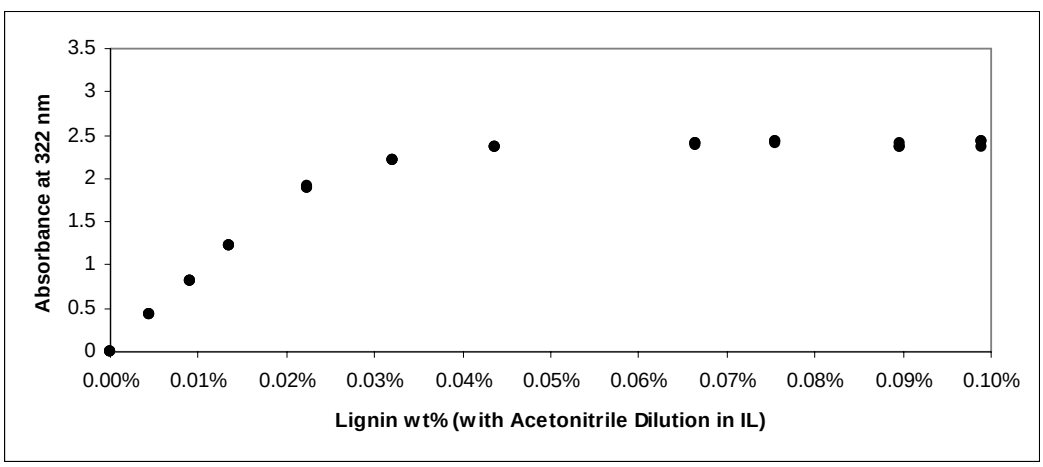

Figure 20. Plot of absorbance versus concentration of the substrate of interest reveals the linear relationship described by the Beer-Lambert law.

absorbance as a function of concentration reveals a linear relationship, but the absorbance range for which this holds true is limited. Therefore only data with absorbances ≤1.0 within the linear range described by the Beer-Lambert law will be accepted for application within this research.

5.3 - Experimental Work

5.3.1 - Materials

Commercially available lignin samples were obtained from Sigma and included hydrolytic, organosolv, and alkali lignin (Fig. 21). One additional lignin sample was obtained from the Tennessee Forest Products Center at University of Tennessee to be referred to as "TFPC lignin" in this thesis. The lignin structure of this wood-based sample was unknown. This lignin was included in the model as a fourth lignin type representing other more exotic sources compared to the three commercial powders. Solvents utilized included [Bmim][Cl] (purum ≥ 98.0%, HPLC grade) from Fluka Chemicals and acetonitrile (purum ≥ 99.0%, HPLC grade) from Sigma. This IL was chosen for this research as a result of the solubility experiments described in Chapter 4.

Cellulose powder (60-65% crystalline structure) from Sigma was used in initial enzymatic hydrolysis experiments in ILs. In addition to [Bmim][Cl] the ILs of interest included [Bmim][Br] and [Bmim][Ac], all from Fluka Chemicals (purum ≥ 98.0%, HPLC grade). Finely ground yellow poplar was obtained from Tennessee Forest Products Center (TFPC) at the University of Tennessee. The sample was dried at 105°C before use. Cellulase from *Aspergillus niger* was obtained from Sigma and was utilized in all experiments. The enzyme was stored in a 4°C freezer. The specific activity of the cellulase is defined as 1.92 units of glucose produced per mg of solid. High performance liquid chromatography (HPLC) mobile phase consisted of deionized water and acetonitrile from Sigma (purum ≥ 99.0%, HPLC grade).

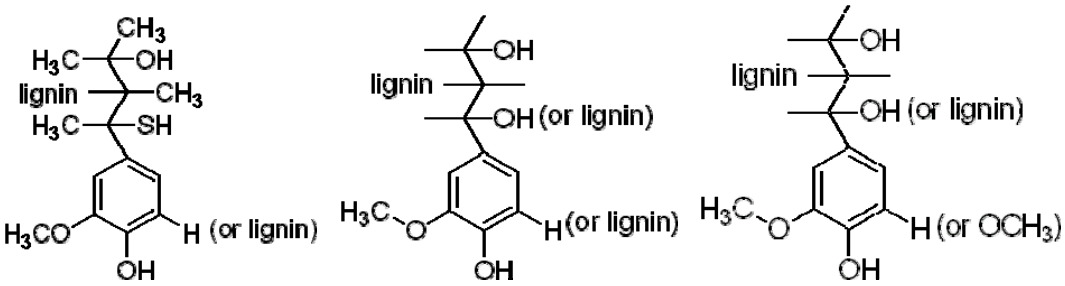

Figure 21. Chemical structures of three lignin types. (a) Alkali lignin; (b) Hydrolytic lignin; (c) Organosolv lignin.

5.3.2 - Methods

5.3.2.1 - Lignin Quantification through a UV-VIS Spectrophotometric Analysis

Analysis of lignin content in an IL solution was completed through a UV-VIS approach with the UV-1700 spectrophotometer by Shimadzu Scientific Instruments (Columbia, MD). To construct the lignin quantification model, the lignin samples were prepared in concentrations ranging from 0 – 22 wt% in 0.5 mL solutions of [Bmim][Cl] at 60°C in glass scintillation vials and agitated at 400 rpm for 24 hours. Two sets of approximately 30 samples were desired for each of the four lignin types for construction of the prediction model. To collect the spectra, the IL solutions were heated to 60°C to reduce viscosity to aid in data collection and transfer to the cuvettes. Three UV-VIS spectra from 190 – 1100 nm were then collected at 25°C for each sample using quartz cuvettes with a one cm-pathlength with a absorption reference of [Bmim][Cl]. The spectral data were written to a Microsoft Excel spreadsheet for subsequent data analysis.

To construct a mathematical model based on the Beer-Lambert Law using UV-VIS absorbance, the spectra were analyzed using the Unscrambler® statistical software (version 8.05) from CAMO Software (Woodbridge, NJ) to determine the optimal wavelength for lignin prediction. The method for modeling several response variables, Y, by means of a set of predictor variables, X, is traditionally completed using multiple linear regression. This method works well as long as the X-variables are fairly few and uncorrelated. With modern measuring instrumentation including spectrometers, the X-variables tend to be many and also strongly correlated.[37]

Partial least squares (PLS) regression is a multivariate statistical procedure that creates a model for the relationship between a set of correlated predictor variables and a set of response variables.[8, 20] In this study, the predictor variables are the UV spectra and the response variable is the lignin concentration within the IL, which is independently measured for each sample. A PLS-1 algorithm is extremely powerful in dealing with interferences associated with the spectral data because it focuses on those spectral features that correlate with the parameter of interest while simultaneously minimizing the effect of spectral features that do no correlate with the parameter of interest.[8] The mathematical calibration model takes the form of a regression vector made up of regression coefficients that are determined by the PLS-1 algorithm.

This regression vector can be represented by

$$\hat{y} = b_0 + b_1x_1 + b_2x_2 + \ldots + b_nx_n \qquad \text{(Eqn. 3)}$$

where

$\hat{y}$ = the response variable (lignin concentration in the IL solution) (wt%)

b values = regression coefficients determined by the PLS-1 algorithm during the calibration phase

x values = the predictor variables (measured absorbances at the different wavelengths that make up the spectral range)

Models were generated using full cross-validation procedure, where the Unscrambler® statistical software extracted one of the samples for validation of the regression. This procedure ensures that the predictive capabilities of the model are reliable. Partial-least-squares regression with cross-validation and removal of outliers were repeated until reasonable calibration results were obtained. A summary description of the PLS technique can be found in recently published work.[28] The optimal wavelength for lignin quantification in IL was identified as the maximum regression coefficient found by the analysis. The corresponding absorbance at this wavelength was recorded for each sample and was plotted as a function of the concentration of lignin in the sample.

5.3.2.2 - Lignin Quantification through a FTIR Approach

Fourier Transform Infrared (FTIR) spectroscopy was also used to analyze the concentration of lignin solubilized in ILs with the Spectrum One FTIR spectrometer from Perkin Elmer (Waltham, MA). Three mid-range infrared spectra from 650-4000 cm^{-1} were collected for each sample with 16 scans per sample at 25°C, followed by multivariate analysis using all of the spectra collected. First, the spectra were transformed from transmittance to absorbance values, and the spectral resolution of the wavelength variable was reduced one to four cm^{-1}. The data was then normalized to account for the fact that the applied sample volume was not equal for all samples. Principal component analysis (PCA) is a multivariate analysis technique that removes the inter-correlation within a data set, and describes it into a series of components, or loadings, that contain most of the valuable spectral information.[8] PCA was utilized in this analysis in an attempt to identify any apparent clusters in the sample set, and to indicate the number of components that should be used in the analysis for optimal prediction ability of the model.

The result of the PLS-1 regression calibration procedure is a plot of the recovery function with predicted versus measured values. This plot reveals the model's calibration correlation, a measure of the degree of linear relationship between two sets of data (i.e. area under the infrared spectra and concentration of lignin in the IL solutions). A perfect model would have a slope of 1, an offset of 0, and a correlation coefficient of 1. The corresponding root mean square error of calibration ($RMSE_{Cal}$) was recorded for each sample set as this indicates the modeling error in the concentration data. The model's ability to predict future samples correctly is evaluated using the root mean square error of validation ($RMSE_{Val}$), a result of the cross-validation procedure included in the statistical analysis. $RMSE_{Val}$ expresses the average error to be expected associated with future predictions.

To validate the model, five solutions of yellow poplar in [Bmim][Cl] were prepared as described above. The samples ranged in biomass concentration from 0.5-2.0 wt%, and three spectra were collected for each.

5.3.2.3 – Enzymatic Hydrolysis of IL-Solubilized Biomass for the Quantification of Glucan

Experiments involving the enzymatic hydrolysis of cellulose and ground biomass were completed at 40°C in stirred batch mode. Completing this initial work proved difficult due to the hygroscopic nature of the ILs. Therefore each sample vial was wrapped and sealed with parafilm to minimize exposure to the atmosphere. The hydrolysis must be carried out in a sealed environment with a carefully controlled temperature. The samples were prepared as described in above chapters with the cellulose powder solubilized in the ILs at 60°C in 7 mL scintillation vials by magnetic stirring at 400 rpm for 24 hours. The enzymes were allowed to warm to room temperature. The solutions were then cooled to 40°C, and water was added to create the desired IL/water reaction media composition. The enzyme required a constant temperature of 40ºC, so the sample vials were placed in a heated silicone oil bath during the duration of the reaction. The enzyme powder was added directly to the media and immediately mixed into the solution. The amount of hydrolysis was expected to vary considerably with the composition of the reaction media, so multiple sample preparations of varying compositions (for the reaction media, substrate, and enzyme) were investigated.

The experimental method involved measuring the extent of hydrolysis over time by analyzing aliquots of reaction mixture by high performance liquid chromatography (HPLC), with an aminopropysilyl column for saccharides (Prevail™ Carbohydrate ES) by Alltech Associates, a division of WR Grace (Deerfield, IL). The aliquots of the reaction media were removed and dipped in a 90°C water bath to stop the reaction before further analysis. The detection of cellobiose and glucose for the quantification of cellulose was used to calculate the progress of the hydrolysis by the enzyme. Analysis of the sugar platform was completed with operating conditions similar to those published recently,[2] (See Section 2.2).

5.4 - Results and Discussion

5.4.1 - Lignin Quantification through UV-VIS Spectrophotometric Analysis

The PLS regression analysis of the UV-VIS spectra for lignin dissolved in [Bmim][Cl]at 25°C determined an optimal wavelength of 532 nm for lignin quantification in [Bmim][Cl]. A linear relationship was revealed between the concentration of the four lignin powders in [Bmim][Cl] and their corresponding absorbance at 532 nm. However, it was found that the concentration range of lignin in IL must be extremely small to be described by the Beer-Lambert law. While the correlation coefficients were very high for two of the lignin types, the concentration of lignin in IL must be approximately ≤ 0.15 wt% in the solution for absorbances ≤ 1.0. This limitation revealed that a dilution agent was needed to allow the UV-VIS lignin determination model to be applicable to a larger concentration range for the biomass compositional analysis.

As described in Chapter 3, acetonitrile was revealed to be a possible dilution agent with lignin solubilized in IL due to its inability to precipitate lignin from [Bmim][Cl]. When utilizing this solvent the viscosity of the IL samples was lowered, also facilitating in data collection for the UV-VIS approach. A color intensity change was observed with increasing concentrations of lignin in the acetonitrile-diluted samples (Fig. 22). The appropriate dilution for all samples was chosen to be 1100x by mass, allowing the most concentrated samples to be applied to the linear range of the lignin model. However, it was found that samples with lignin concentrations ≤1 wt% were too dilute in concentration to be accurately measured. Therefore, it was determined that a lesser dilution with acetonitrile would be needed for samples within this concentration range. A lignin-IL solution set of 15 samples was created with concentrations ranging from 0-1 wt% with a dilution of 50x by mass. A second set of fifteen samples with concentrations from 1-22 wt% were diluted by 1100x (g/g). Duplicates were created for both sample sets, totaling to 60 samples for each lignin type. Three spectra were then collected for each sample with a reference of IL with the appropriate dilution for each sample set. All UV spectra exhibited two peaks, regardless of dilution (Fig. 23). The

Figure 22. Acetonitrile Dilutions (1100:1) of (0 – 0.22 w/w) hydrolytic lignin.

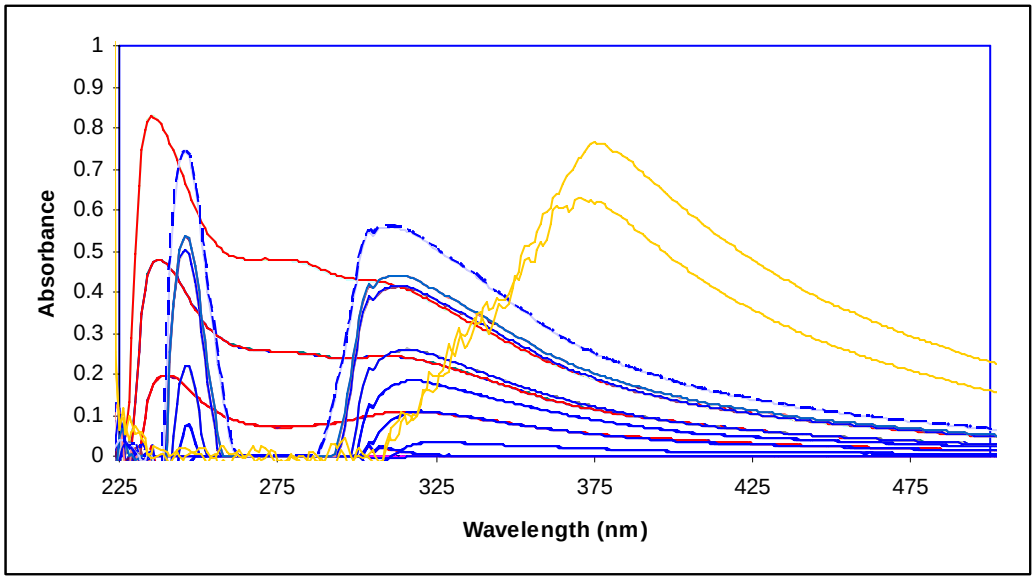

Figure 23. Spectra of organosolv lignin powder solubilized in [Bmim][Cl] in a 1 cm-pathlength cell; gold spectra are lignin in pure [Bmim][Cl] at 60°C; red spectra are concentrations ≥1 wt% diluted by 1100x (g/g) acetonitrile at 25°C; blue spectra are concentrations ≤ 1 wt% diluted by 50x (g/g) acetonitrile at 25°C.

visible absorption maximum for acetonitrile is approximated in the range of 235 – 245 nm. Therefore the peaks of each spectrum were attributed to acetonitrile and the lignin component, respectively. Statistical analysis was again performed as described above to determine the optimal wavelengths for each sample set. It was noted that the inclusion of

acetonitrile in the solvent systems led to a spectral shift compared to that for samples dissolved in pure IL. Because two acetonitrile dilutions were utilized, the visible absorption maximum differed for each sample set in addition to lignin type. The optimal wavelengths found by statistical analyses for four lignin types by sample set (i.e. concentration range and dilution amount) can be found in Table 5.

The optimal wavelength for prediction of all four lignin types (i.e. all spectra combined) was determined to be 322 nm with the addition of the dilution agent. Incorporating acetonitrile allowed for a wider concentration range of measurable samples, resulting in correlation values of at least 0.99 for the organosolv and TFPC lignin types (Fig. 24). The maximum applicable lignin concentration ranges from 0.008 wt% for hydrolytic lignin to approximately 0.014 wt% for the alkali type.

It is believed that the large percentage of acetonitrile in the sample solvent systems had great influence over the optimal wavelengths found by statistical analyses of the absorbance spectra. Therefore a wavelength of 360 nm was investigated for improvement upon the lignin determination model so the acetonitrile has less influence on the absorbance for predicting lignin content. Utilizing absorbance readings at 360 nm resulted in correlation coefficients of ≤0.99 for organosolv, hydrolytic, and the TFPC lignin (Fig. 25).

Table 5. Optimal wavelengths for four lignin types in [Bmim][Cl] with acetonitrile dilution.[1.]

Sample Set	***Hydrolytic***	***Organosolv***	***Alkali***	***TFPC***	***All Types Combined***
Concentrations ≤ 1 wt% lignin in IL	322 nm	334 nm	289 nm	302 nm	357 nm
Concentrations 1-22 wt% lignin in IL	323 nm	322 nm	277 nm	324 nm	323 nm
All Concentrations	356 nm	324 nm	356 nm	304 nm	322 nm

1. Samples prepared in two sets: concentrations ≤ 1 wt% in [Bmim][Cl], and concentrations from 1-22 wt%. The sets were diluted by 50x and 1100x (g/g), respectively. Optimal wavelengths determined as the maximum regression coefficients of multivariable analysis with partial-least-squares regression utilizing the Unscrambler® statistical software.

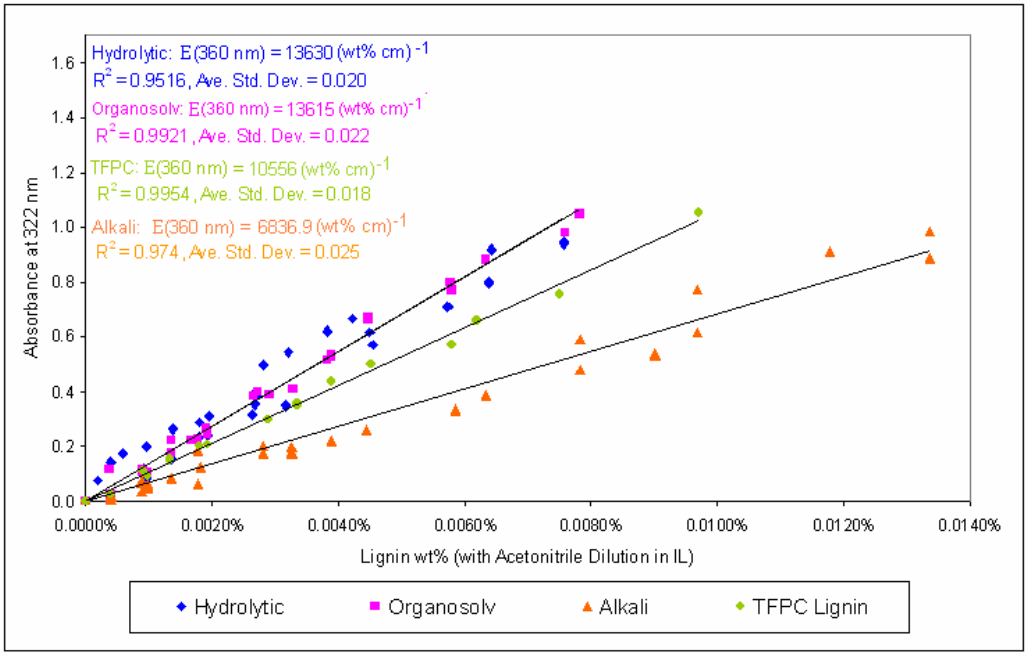

Figure 24. UV-VIS absorbance at 322 nm of four lignin types solubilized in [Bmim][Cl] (0-22 wt%) with acetonitrile dilutions; samples with lignin concentrations ≤ 1 wt% diluted 50x (g/g) and samples 1-22 wt% diluted 1100x (g/g).

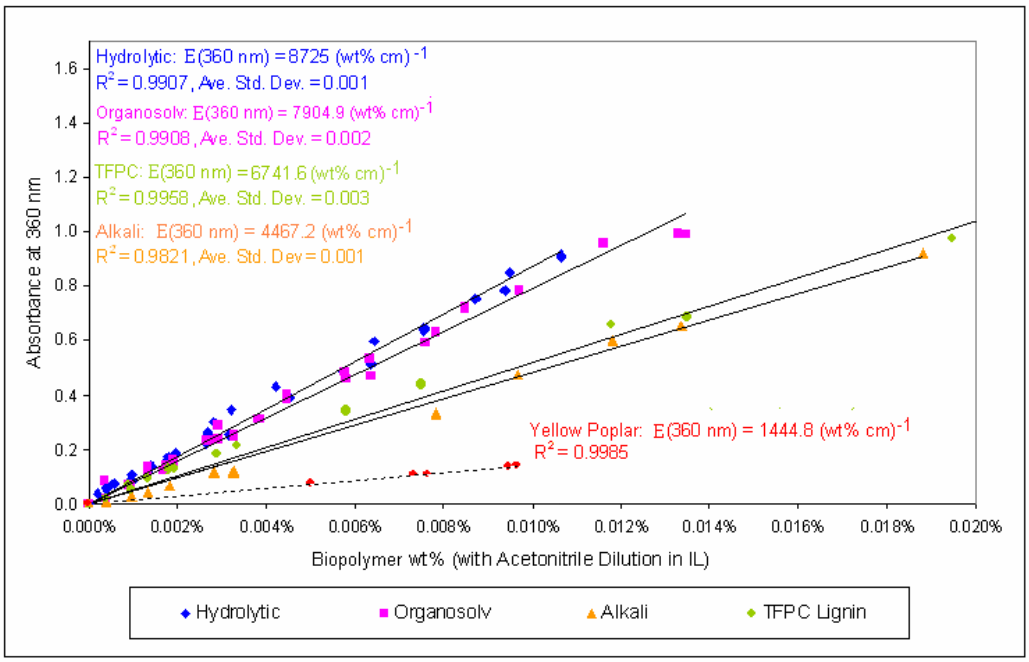

Figure 25. UV-VIS absorbance at 360 nm of four lignin types solubilized in [Bmim][Cl] (0-22 wt%) with appropriate acetonitrile dilutions; and yellow poplar samples with concentrations ≤ 1 wt% diluted 25x (g/g) and samples >1wt% diluted 50x (g/g).

These subsequent tests showed that different lignin sources resulted in unique extinction coefficients; due to either a difference in the chemical structure of lignin or the amount of impurity between the samples. The extinction coefficients were approximately 8730, 7900, 6740 and 4470 $wt\%^{-1}$ cm^{-1} for hydrolytic, organosolv, unknown, and alkali lignin, respectively. The purity of the lignin samples was investigated by collaboration with Dr. Nicole Labbé of the University of Tennessee Forest Products Center, using Laser Induced Breakdown Spectroscopy (LIBS). LIBS technology uses a highly energized laser to superheat and break down the lignin samples while a spectrometer collects characteristic electromagnetic radiation for elements contained within each sample.[20] For each lignin powder, in particular alkali lignin, impurities in the form of elemental content were detected, explaining some of its variance from the other lignin powder extinction coefficients and lowest correlation coefficient of 0.98 for alkali lignin. The maximum applicable lignin concentration range is improved for this model compared to the 322 nm model as The Beer-Lambert law is maintained from 0.010 wt% for hydrolytic lignin to 0.020 wt% for the alkali type.

To predict the concentration of lignin in [Bmim][Cl] the sample should be diluted 1100x (g/g) for concentrations over 1 wt% lignin, and by 50x (g/g) for samples with concentrations less than 1 wt% lignin. It is believed that ILs have the ability to solubilize biomass without significant chemical changes to the natural structure of it components.[11] Therefore fitting the absorbance reading at 360 nm of an unknown biomass sample to this model will reveal the lignin structure most similar to that of the unknown lignin as it is found in nature without alteration from processing or extraction. A series of yellow poplar samples were prepared in [Bmim][Cl] at concentrations of 2.0, 1.5, 1.0, 0.75, and 0.5 wt%. To remain within concentration range that is described by the Beer-Lambert Law the concentration of the unknown lignin should be 0.01 wt%. It is known from the solubility experiments described in Chapter 4 that the maximum biomass concentration for complete solubilization is 2 wt% or less. It is also known that the lignin content in lignocellulosic biomass is approximately 25 wt%. Therefore the dilution amount for prediction of lignin content in yellow poplar using this model should be set at 50x (g/g)

by mass for samples with biomass concentrations of approximately 2 wt%, and 25x (g/g) for biomass concentrations less than or equal to 1 wt%.

As seen in Figure 25, the five yellow poplar samples ranging from 0.5 – 2.0 wt% lignin were analyzed using the UV-VIS approach. The samples were diluted and their respective absorbances were recorded at 360 nm. The extinction coefficient for the yellow poplar was found to be 1444.8 $wt\%^{-1}$ cm^{-1}. It was determined experimentally through wet chemical analysis procedures following that of the NREL protocol as described in Chapter 3 that the yellow poplar had a total lignin mass fraction of 25.1%.[32] Therefore the predicted lignin content in the yellow poplar is calculated by dividing this extinction coefficient by the corresponding coefficient for each lignin type. The error was assumed as standard calibration error calculated by the "linest" function in Microsoft Excel. The results of the analysis of the yellow poplar samples can be found in Table 6.

It was observed during the analysis of the yellow poplar samples that there was some precipitation caused by the addition of the acetonitrile dilution agent. It was also noted that the error for predicted lignin content using the TFPC, hydrolytic, and organosolv lignin models was negative. The difference in the experimental versus calculated concentrations of lignin in the yellow poplar is probably due to the partial

Table 6. Predicted lignin content in yellow poplar utilizing the UV-VIS approach.[1, 2.]

Lignin Type	*Extinction Coefficient ($wt\%^{-1}$ cm^{-1})*	*Predicted wt% Lignin*	*% Difference from NREL Lignin Value*
Hydrolytic	8730	16.6% ± 0.3	-34.0%
Organosolv	7900	18.3% ± 0.3	-27.2%
TFPC	6740	21.4% ± 0.4	-14.6%
Alkali	4470	32.3% ± 0.6	28.8%
Experimental	1440	-	-

1. Finely ground yellow poplar was dried at 105°C overnight before solubilization to in [Bmim][Cl] at 60°C with magnetic stirring at 400 rpm for 24 hours. Samples with lignin concentrations ≥ 1 wt% in IL are diluted by 50x (g/g) acetonitrile; samples ≤ 1 wt% lignin are diluted by 25x (g/g)

2. The actual lignin content in yellow poplar was measured as 25.1 wt% using wet chemical analysis.

precipitation of lignin. Acetonitrile precipitates all biomass components except lignin when solubilized individually in IL. However, the intermolecular bonding between the lignin and the other constituents within biomass may be causing some of the lignin to be partially precipitated rather than remaining solubilized in the diluted solution. However, using the extinction coefficient for the TFPC lignin calibration gave the most accurate results. Therefore it is assumed that the lignin structure of yellow poplar is closest to the wood-based lignin type provided by the University of Tennessee Forest Products Center. The error for prediction of lignin content in biomass through the use of TFPC lignin calibration model was 14.6%.

5.4.2 – Lignin Quantification through a FTIR Approach

An advantage to using FTIR analysis over UV-VIS is the ability to directly analyze a small sample volume (approximately 10-20uL) of the IL solutions without the need for a dilution agent. The disadvantage to a lignin determination model constructed from FTIR spectra is that infrared spectra data sets are large and complex representations of the samples, and must undergo the statistical analyses as described above before being applied to this model. The result of the PLS-1 regression calibration procedure is a plot of the recovery function with predicted versus measured values (Fig. 26). The correlation

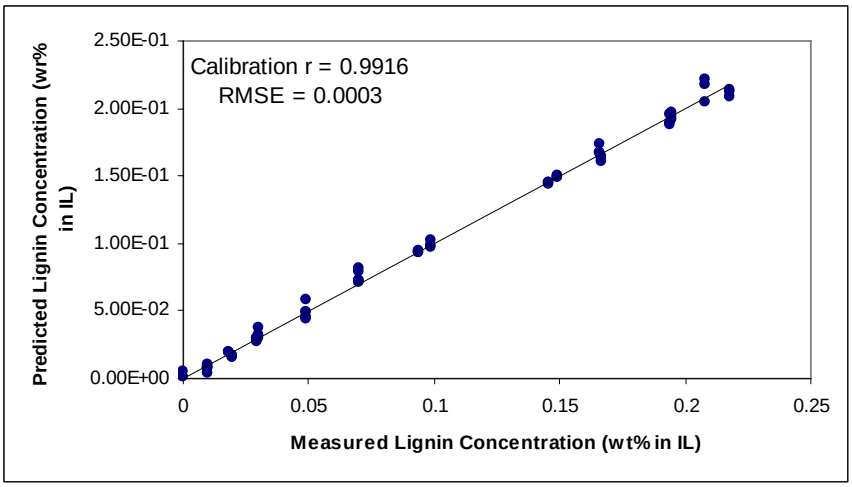

Figure 26. Recovery function for partial least squares regression of mid-range infrared spectra for organosolv lignin in [Bmim][Cl] with concentrations ranging from (0-0.22 w/w) at 25°C.

coefficient (R) and root mean square error of calibration ($RMSE_{Cal}$) or root mean square error of validation ($RMSE_{Val}$) for each lignin type were used as a way to compare the PLS models and can be found in Table 7.

Based on the calibration correlation values found with the recovery functions for each lignin type, the FTIR model for prediction of lignin concentration in ILs did not perform well for the samples with hydrolytic lignin. The low calibration correlation value is most likely explained by the variance within the sample set and unrepresentative sample volumes. Multiple spectra of the same sample resulted in a large amount of variance, demonstrating that the small droplets were not representative of the IL sample. The alkali lignin type seems to give the lowest coefficient of correlation with values of 0.98 and 0.88 for the 1-22 wt% and 0-1 wt% sample sets, respectively. This performance agrees with the results obtained using the UV-VIS approach, and is most likely explained by the impurity of the sample used in the calibration. The model calibration parameters appear quite good for the other lignin types, in particular the TFPC lignin with correlation values of 0.9983 and 0.9916. In almost all cases the correlation values are lower for thevalidation compared to the calibration due to the fact the validation test set has fewer data points than the calibration set. In most cases these two measures of the accuracy of the models are comparable.

To validate the model, five solutions of yellow poplar in [Bmim][Cl] were prepared as described above. The samples ranged in biomass concentration from 0.5-2.0 wt%, and three mid-range infrared spectra were collected for each. The mid-range infrared spectra collected for the five yellow poplar samples were normalized as described above for the calibration of the model. The validation spectra were applied to the models for each lignin type and concentration range to produce prediction recovery functions. The correlation values and root mean square errors of prediction ($RMSE_{Pred}$) were recorded for each lignin type. The TFPC lignin performs the best with R_{Pred} values of 0.9257 and 0.9914 for the 1-22 wt% and 0-1 wt% sample sets, respectively. It is assumed that the lignin structure of the yellow poplar samples most resembled this wood-based lignin sample from the TFPC. The $RMSE_{Pred}$ value obtained for these samples was 0.1169% and 0.0903%, respectively; suggesting that the regression model prepared from

Table 7. Performance of the various lignin types for prediction of lignin in yellow poplar by FTIR.[1,2].

Sample Set	*No. of PC's*	R_{cal}	$RMSE_{cal}$	R_{val}	$RMSE_{val}$	R_{pred}	$RMSE_{pred}$
Hydrolytic 1-22 wt%	3	0.9883	0.0102%	0.9727	0.0161%	-0.8108	0.6803%
Hydrolytic 0-1 wt%	5	0.9905	0.0271%	0.8329	0.0440%	0.9015	0.0715%
Organosolv 1-22 wt%	2	0.9983	0.0043%	0.9980	0.0046%	-0.1218	0.1019%
Organosolv 0-1 wt%	4	0.9916	0.0003%	0.9755	0.0005%	-0.6891	0.1077%
TFPC 1-22 wt%	4	0.9989	0.0001%	0.9674	0.0005%	0.9257	0.1169%
TFPC 0-1 wt%	2	0.9934	0.0062%	0.8838	0.0263%	0.9914	0.0903%
Alkali 1-22 wt%	4	0.9863	0.0118%	0.9767	0.0154%	-0.5825	0.7747%
Alkali 0-1 wt%	5	0.8757	0.0004%	0.9796	0.0005%	0.5684	0.5663%

1. Three mid infrared spectra from 650 to 4000 cm^{-1} with 16 scans for each sample were recorded for each sample volume of one droplet (approximately 10-20 uL) at 25°C; the absorbance data was reduced to a spectral resolution of four cm^{-1}, normalized, and analyzed using PLS regression after the removal of outliers with the Unscrambler® statistical software.

2. Abbreviations: PC = principal component, R_i = correlation coefficient, $RMSE_i$ = root mean square error, cal = calibration, val = validation, pred = prediction concentrations $\leq$1 wt%.

the mid-range infrared spectral data is quite stable over time. Utilizing the TFPC lignin prediction model resulted in a predicted lignin content of 25.6% in the 2 wt% yellow poplar sample.

5.4.3 – Enzymatic Hydrolysis of IL-Solubilized Biomass for the Quantification of Glucan

Initial experiments exploring the enzymatic hydrolysis of commercial cellulose powder proved successful in media containing water, but not pure ILs. The reaction media consisted of a 90:10 and 10:90 (v/v) [Bmim][Cl]/water solutions, an aqueous control, and a sample in which the enzyme was incubated in IL for several hours prior to the addition of water and substrate. Aliquots of each sample were taken at 1, 3, 15, and 47 hours and dipped in a 90°C water bath to stop the reaction. HPLC chromatograms of the aliquots show the formation of glucose and the disaccharide cellobiose (Fig. 27). The percent hydrolysis of cellulose hydrolysis by *A. niger* at 40ºC over two days can be seen in Table 8.

It was observed from this experiment that the reactions carried out in media comprised of primarily IL resulted in very little conversion to cellobiose or glucose. Agreeing with recently published work, the Cl^- anion was believed to inactivate the

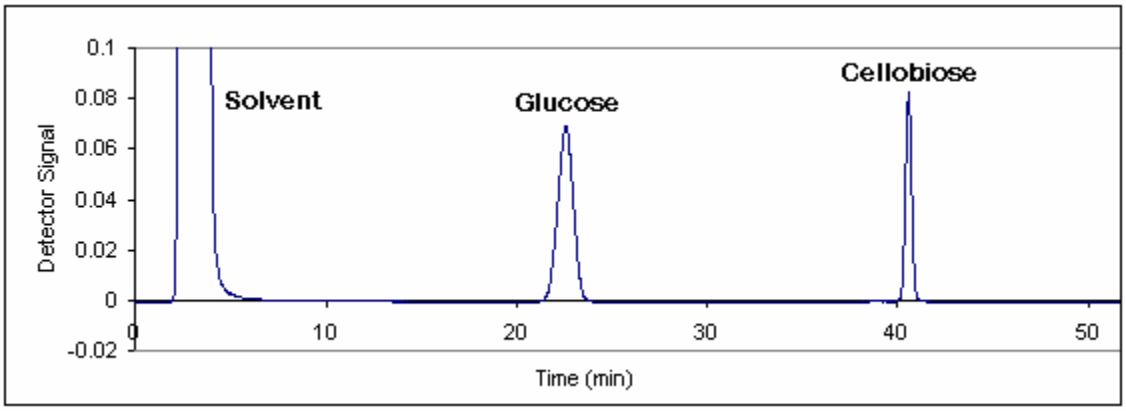

Figure 27. HPLC Chromatograph of cellulose hydrolysate (2 wt% in [Bmim][Cl] and water 90:10 (v/v)) after 24 hours by *Aspergillus niger* (6.5 wt%) at 40ºC.

Table 8. Hydrolysis of cellulose (2 wt%) by cellulase (6 wt%) in [Bmim][Cl]/water reaction media at 40°C.[1.]

		Sample Composition (wt%)		
Reaction Media	*Time (hr)*	*Glucose*	*Cellobiose*	*Total*
IL and Water (90:10 v/v)	1	4.0% ± 0.6	9.2% ± 0.4	13.3% ± 1.0
	3	4.2% ± 0.2	10.1% ± 0.4	14.3% ± 0.6
	18	4.2% ± 0.2	10.1% ± 0.2	14.3% ± 0.4
	47	4.5% ± 0.2	10.0% ± 0.4	14.5% ± 0.6
IL and Water) (90:10 v/v) with Incubated Cellulase	1	0.0% ± 0	1.3% ± 0.1	1.3% ± 0.1
	3	0.0% ± 0	0.9% ± 0.1	0.9% ± 0.1
	18	0.0% ± 0	3.2% ± 0.2	3.2% ± 0.2
	47	0.0% ± 0	3.2% ± 0.1	3.2% ± 0.1
Water	1	30.4% ± 0.6	28.3% ± 0.7	58.6% ± 1.3
	3	40.8% ± 0.6	19.4% ± 0.6	60.2% ± 1.2
	18	77.4% ± 0.5	0.5% ± 0.1	77.9% ± 0.6
	47	84.4% ± 0.5	0.0% ± 0	84.4% ± 0.5
IL and Water (10:90 v/v)	1	14.2% ± 0.4	32.1% ± 0.7	46.3% ± 1.1
	3	15.0% ± 0.4	30.2% ± 0.7	45.2% ± 1.1
	18	31.1% ± 0.5	13.8% ± 0.5	44.8% ± 1.0
	47	39.4% ± 0.6	10.3% ± 0.4	49.7% ± 1.0

1. Cellulose first solubilized to 2 wt% in [Bmim][Cl]/water at 60°C using magnetic stirring at 400 rpm for 24 hours.

2. Aliquots at specified reaction times analyzed using HPLC analysis with utilization of an aminopropysilyl column for saccharides (Prevail™ Carbohydrate ES) by Alltech Associates, a division of WR Grace (Deerfield, IL) and operating conditions similar to those published recently[2]. Hydrolysis error calculated using the standard error for the cellulose and cellobiose calibration models

enzyme.[36] It was also recently shown that incubation of cellulase from *Humicola insolens* (Celluzyme 0,7T) in IL at room temperature maintained its activity in [Bmim][Cl].[24] Therefore samples were also analyzed where the enzyme was incubated in the IL for 24 hours prior to addition of the biomass. These samples produced almost no hydrolysis of the cellulose powder, again indicating the inactivation of the enzyme by the IL. It is believed that the enzyme is inactivated within the first hour of the reaction as the total conversion rates plateau for each of the systems containing IL; only the aqueous reaction media continues to yield a mixture of cellobiose and glucose. However, there is a decrease in the composition of cellobiose and an increase in the amount of glucose with little or no increase in the overall extent of hydrolysis, indicating that the glucosidase component of the cellulase system are not inactivated by the IL. Important to note was that the sample with 10% IL by volume produced total conversion rates closest to that of the aqueous control for the first hour.

Subsequent experiments explored the use of alternative ILs, [Bmim][Br] and [Bmim][Ac], in addition to [Bmim][Cl]. The hydrolysis of finely-ground yellow poplar solubilized in IL was explored with the progress of the reaction quantified through HPLC analysis after 30 minutes of reaction time. The reaction media consisted of each IL and water at ratios of 100:0, 90:10, and 10:90 (v/v), and an aqueous control The biomass was dried at 105ºC before being solubilized to 2 wt% at 60ºC in stirred batch mode (Table 9).

The enzymatic hydrolysis reactions carried out in samples with 100% IL had very low yields compared to the other media compositions. Again this is believed to be due to the inactivation of *A. niger* cellulase by the IL. It was also noted that low yields may be due to the poor mixing of these solutions, as the high viscosity at 40°C allowed little agitation or initial mixing of the enzyme into the solution. The 100% [Bmim][Br] sample was not analyzed at all due to this fact. The addition of water for an IL/water media at 90:10 (v/v) was also not successful in high yields of cellobiose and glucose. The samples with media with 10:90 (v/v) IL/water media produced the highest yields of cellobiose and glucose. The [Bmim][Br] sample performed the worst with only 17.7% conversion. It was observed that [Bmim][Cl] displayed the most potential ability for hosting enzymatic

Table 9. Conversion rates for IL-hosted enzymatic hydrolysis of yellow poplar.[1,2]

	Reaction Media	*Total*	*Glucose*	*Cellobiose*
Water	100% Water	105.8% ± 2.5	101.3% ± 2.0	4.4% ± 0.7
[Bmim][Cl]	100% [Bmim][Cl]	3.4% ± 0.5	1.2% ± 0.2	2.1% ± 0.4
	90% [Bmim][Cl] 10% Water	7.9% ± 0.6	7.3% ± 0.5	0.6% ± 0.2
	10% [Bmim][Cl] 90% Water	64.5% ± 8.2	27.5% ± 3.0	36.9% ± 5.3
[Bmim][Br]	100% [Bmim][Br][3]	-	-	-
	90% [Bmim][Br] 10% Water	16.6% ± 2.6	4.0% ± 0.7	12.6% ± 2.0
	10% [Bmim][Br] 90% Water	17.7% ± 3.5	3.4% ± 2.8	14.3% ± 0.7
[Bmim][Ac]	100% [Bmim][Ac]	3.6% ± 0.6	0.6% ± 0.1	3.0% ± 0.4
	90% [Bmim][Ac] 10% Water	15.3% ± 2.4	2.1% ± 0.4	13.2% ± 2.0
	10% [Bmim][Ac] 90% Water	48.4% ± 5.7	48.4% ± 5.7	0.0 ± 0.2%

1. Finely ground yellow poplar was dried at 105°C overnight before solubilization to in each IL at 60°C with magnetic stirring at 400 rpm for 24 hours; water was added to the solutions to produce the desired IL/water media composition.

2. At 30 minutes an aliquot of each sample was analyzed using HPLC analysis with utilization of an aminopropysilyl column for saccharides (Prevail™ Carbohydrate ES) by Alltech Associates, a division of WR Grace (Deerfield, IL) and operating conditions similar to those published recently.[2] Hydrolysis error calculated using the standard error for the cellulose and cellobiose calibration models for the HPLC column and operating conditions.

3. Sample would not stir due to its high viscosity.

hydrolysis reactions as the cellulose was converted to 64.5% cellobiose and glucose after 30 minutes.

It was believed that the employment of ILs as a pretreatment step would result in successful enzymatic activity by altering the physical morphology of the biomass to increase the accessible area for the cellulase.[7] Yellow poplar was solubilized in the three ILs of interest by the solubilization methods as described above. Yellow poplar was also incubated in water for 24 hours under agitation as a control. The biomass was regenerated from the IL solutions with the addition of water as a precipitating co-solvent. The solution was vacuumed filtered using fine porosity porcelain filtering crucibles, washing with approximately 500 mL of water over three hours. The recovered biomass was then dried at 105°C for a minimum of four hours. The dried biomass was hydrolyzed at 2 wt% in a 10:90 (v/v) IL/water solution for 30 minutes and catalyzed by cellulase at 40°C (Table 10). These preliminary tests revealed that the [Bmim][Cl] ionic liquid again performed the best for enzymatic hydrolysis of the cellulose content with total conversion to cellobiose and glucose approaching 76.3%. However, this conversion was significantly lower than that of the aqueous control, reaching 96.2% conversion within the 30 minutes of reaction time. Therefore all further experiments involving the enzymatic hydrolysis of biomass were completed using [Bmim][Cl].

These tests show that [Bmim][Cl] may act as a promising pretreatment method before enzymatic hydrolysis of biomass in water, as the hydrolysis rate of [Bmim][Cl]-incubated of yellow poplar was closest to that of water compared to other pretreatment methods (Table 10). The rate of conversion is much higher using this methodology compared to those experiments with the enzyme acting in reaction media containing only IL. However, adoption of this step in the wet chemical analysis procedure would greatly increase the number of biomass transfer steps and total analysis time and will therefore be disregarded in this research.

Subsequent experiments for the analysis of glucan content in yellow poplar focused on establishing optimal conditions suitable for a biomass compositional analysis procedure with high repeatability. Based on the assessments discussed above, 2 wt%

Table 10. Conversion rates for precipitated yellow poplar in aqueous solutions.[1, 2.]

Hydrolysis (30 minutes)	100% Water Pretreatment	[Bmim][Cl] Pretreatment	[Bmim][Br] Pretreatment	[Bmim][Ac] Pretreatment
Total	96.2% ± 5.5	76.3% ± 3.8	5.0% ± 0.5	12.9% ± 0.6
Glucose	41.5% ± 2.6	45.0% ± 3.0	2.5% ± 0.4	6.2% ± 0.3
Cellobiose	54.7% ± 2.9	31.2% ± 0.9	2.5% ± 0.2	6.8% ± 0.3

1. Using a magnetic stir plate and 7mL glass scintillation vials, dried biomass was solubilized to 2 wt% in 1 mL [Bmim][Cl] at 60ºC for 24 hours; the solution was vacuumed filtered with fine porosity filtering crucibles with 500 mL of water over 3 hours; the recovered biomass was dried at 105°C for a minimum of 4 hours and then added to 2 mL of 10:90 (v/v) IL/water solution at 2 wt% equal mass of *Aspergillus niger* cellulase; the biomass was hydrolyzed at 40ºC in stirred batch mode.

2. At 30 minutes an aliquot of each sample was analyzed using HPLC analysis with utilization of an aminopropysilyl column for saccharides (Prevail™ Carbohydrate ES) by Alltech Associates, a division of WR Grace (Deerfield, IL) and operating conditions similar to those published recently.[2] Hydrolysis error calculated using the standard error for the cellulose and cellobiose calibration models

yellow poplar was solubilized in [Bmim][Cl] at 60°C for 24 hours using magnetic stirring at 400 rpm. An equal amount bymass of enzyme to biomass was added once the solutions had cooled to 40°C. Since previous experiments demonstrated the inactivation of the enzyme by one hour of reaction, fresh enzyme equal by mass to the biomass was added to the samples every 30 minutes up to two hours, at which point HPLC analysis revealed 100% conversion to glucose by the cellulase. The conversion results for four samples can be seen in Table 11.

The extent of hydrolysis was calculated to exceed 100% for each sample analyzed due to standard calibration error. The calculations were also only based on the glucan component, so any glucose produced from hydrolysis of the hemicellulose could skew the hydrolysis calculations (<5 wt%).[1, 29] As repeatability was high in this series of hydrolysis samples, a 2 wt% validation sample as described above was used to test this

Table 11. Hydrolysis of 2 wt% yellow poplar in [Bmim][Cl] and water (10:90 v/v) at 37°C.[1.]

	Sample 1	Sample 2	Sample 3	Sample 4	Average
30 minutes reaction time					
% Hydrolysis	13.7%	20.1%	17.7%	14.8%	16.6% ± 1.5
Glucose (wt%)	11.5%	16.9%	13.9%	11.8%	13.5%
Cellobiose (wt%)	1.8%	3.2%	3.8%	3.0%	2.9%
1 hour reaction time					
% Hydrolysis	35.5%	37.2%	42.6%	38.2%	38.4% ± 0.8
Glucose (wt%)	30.5%	30.9%	35.3%	30.9%	31.9%
Cellobiose (wt%)	5.0%	6.3%	7.3%	7.3%	6.5%
1.5 hours reaction time					
% Hydrolysis	72.7%	72.5%	75.1%	75.1%	73.8% ± 1.0
Glucose (wt%)	64.0%	61.4%	61.9%	62.6%	62.4%
Cellobiose (wt%)	8.7%	11.1%	13.1%	12.5%	11.4%
2 hours reaction time					
% Hydrolysis	106.6%	105.9%	109.6%	109.3%	107.8% ± 1.5
Glucose (wt%)	96.5%	94.3%	97.7%	96.5%	96.2%
Cellobiose (wt%)	10.2%	11.6%	11.9%	12.8%	11.6%

1. Finely ground yellow poplar was dried at 105°C overnight before solubilization to 2 wt% in of each IL at 60°C with magnetic stirring at 400 rpm for 24 hours; water was directly added to the solutions to produce the desired 10:90 (v/v) IL/water media composition; an equal amount of enzyme by mass to biomass was added once the solutions had cooled to 40°C; the samples were agitated at 400 rpm in a silicone oil bath.

2. Every 30 minutes fresh enzyme was added equal to that of the initial amount and an aliquot of each sample was analyzed using HPLC analysis with utilization of an aminopropysilyl column for saccharides (Prevail™ Carbohydrate ES) by Alltech Associates, a division of WR Grace (Deerfield, IL) and operating conditions similar to those published recently.[2] Hydrolysis error calculated using the standard error for the cellulose and cellobiose calibration models

methodology as part of a compositional analysis procedure. It was known that the actual glucan content within the sample was 43.5 wt% by wet chemical analysis. The progress of the enzymatic hydrolysis was analyzed at the specified reaction times using HPLC. As seen in Table 12, the glucan fraction was calculated as only 24.5% at 30 minutes of reaction, falling short of the experimental value. However, by the second hour, 43.6% of conversion was achieved, resulting in a prediction error of less than one percent.

5.5 - Conclusions

Solubilization of biomass in ILs allowed for rapid spectroscopic determination of lignin composition and reduced the number of transfer steps for biomass samples during the analysis. UV-VIS spectrophotometric analysis demonstrated that the Beer-Lambert Law was applicable to the four lignin types when absorbance readings were measured at 360 nm. The advantage to this approach is that it visually demonstrates which lignin types have similar chemical structures as plots of visible absorbance versus lignin concentration in solution showed that each lignin structure tested has a unique extinction coefficient. The main disadvantage of the UV-VIS approach is the need for the acetonitrile dilution agent, thus preventing a direct analysis of the biomass sample after solubilization in IL. The difference in the experimental versus calculated concentrations of lignin in the yellow poplar is probably due to the partial precipitation of lignin. Acetonitrile precipitates all biomass components except lignin when solubilized individually in IL. However, the intermolecular bonding between the lignin and the other constituents within biomass may be causing some of the lignin to be partially precipitated rather than remaining solubilized in the diluted solution. Incorporation of an additional hydrolysis step (i.e. enzymatic hydrolysis) or other pretreatment step might improve upon this source of error by breaking the inter-molecular bonds between cellulose, hemicellulose, and lignin to prevent the precipitation of the lignin fraction. The model was used for analysis of yellow poplar dissolved in IL. Utilization of the extinction coefficient for the TFPC lignin calibration gave the most accurate results. Therefore it is

Table 12. Prediction of percent glucan in yellow poplar based on enzymatic hydrolysis in IL.[1, 2, 3.]

Reaction Time	Average Conversion to Glucose and Cellobiose with Specified Protocol Conditions	Predicted Glucan of Biomass Sample (wt%)	% Error
0.5 hours	16.6%	24.5%	-43.7%
1 hour	38.4%	41.3%	-5.0%
1.5 hours	73.8%	42.6%	-1.9%
2 hours	107.8%	43.6%	0.3%

1. Finely ground yellow poplar was dried at 105°C overnight before solubilization to 2 wt% in [Bmim][Cl] at 60°C with magnetic stirring at 400 rpm for 24 hours; water was directly added to the solution to produce the desired 10:90 (v/v) IL/water media composition; an equal amount of enzyme by mass to biomass was added once the solution had cooled to 40°C; the samples were hydrolyzed in a silicone oil bath to maintain temperature control.

2. Every 30 minutes fresh enzyme was added equal to that of the initial amount and an aliquot of each sample was analyzed using HPLC analysis with utilization of an aminopropysilyl column for saccharides (Prevail™ Carbohydrate ES) by Alltech Associates, a division of WR Grace (Deerfield, IL) and operating conditions similar to those published recently.[2] Hydrolysis error calculated using the standard error for the cellulose and cellobiose calibration models

3. Actual glucan mass fraction found at 43.5% through wet chemical analysis; values of average conversion to glucose found in Table 11; sample calculations for predicted glucan and percent error found in the Appendix of this work.

assumed that the lignin structure of yellow poplar is closest to the wood-based lignin type provided by the University of Tennessee Forest Products Center.

The FTIR approach also resulted in a high correlation (0.99) for some lignin types between the area underneath the mid-range infrared spectra from 650-4000 cm^{-1} and the lignin concentration in [Bmim][Cl]. It was observed that the model did not perform well for lignin concentrations lower than 1 wt% in [Bmim][Cl]. The disadvantage to a lignin determination model constructed from FTIR spectra is the small sample volume. Multiple spectra of the same sample resulted in a large variance, demonstrating that the small droplet aliquots were not homogeneous. It was also noted that the infrared spectra data sets are large and complex representations of the samples, and must undergo statistical analyses before being utilized in the lignin determination model. The mid-range infrared spectra for yellow poplar solubilized in [Bmim][Cl] were analyzed using multivariate analysis in the Unscrambler® statistical software. The resulting correlation coefficients and root mean square errors of prediction revealed that the spectra for this biomass species most closely matched the calibration models for the TFPC lignin. Therefore both spectral approaches agree that the yellow poplar has a lignin structure that closely matches the TFPC lignin source. It is recommended that additional lignin types and sources would better the model and overall lignin prediction in both approaches.

Preliminary experiments have demonstrated that cellulase partially hydrolyzed cellulose and yellow poplar solubilized in reaction media at 10:90 (v/v) [Bmim][Cl]/water in stirred batch mode. The *A. niger* cellulase most probably undergoes inactivation in the IL and IL/water mixtures. Therefore, it is suggested to solubilize 2 wt% biomass in a 10:90 (v/v) [Bmim][Cl]/water media with the addition of equal amounts of cellulase to biomass by mass every 30 minutes for a total reaction time of two hours. The reaction should be carried out in carefully sealed vials within a silicone oil bath at 40°C. Complete conversion of the cellulose content in the yellow poplar to glucose was observed, and the repeatability of these methods was high with very small error of prediction when compared to results from the NREL analysis procedure.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

The goal of developing the new methodologies presented in this research is to use ILs to dissolve biomass and its components and to allow the IL to act as a media for eco-friendly analysis of biomass. It has been shown here that lignocellulosic biomass such as yellow poplar can be solubilized in IL at 2 wt% or less. This was achieved using magnetic stirring at 60°C, the optimal temperature observed to aid in solubilization without degradation. Once solubilized, the biomass sample remains in this same vial for the entire analysis procedure. First, the lignin content can be quantified using either UV-VIS or FTIR spectral analyses. Using a visible wavelength of 360 nm, the Beer-Lambert Law is observed up to 0.01 wt% for all lignin types explored, with a 0.99 correlation achieved in most cases. A large range of lignin concentrations is applied to the model through the use of an acetonitrile dilution agent. FTIR analysis is beneficial as there is no need for a dilution agent, simplifying the procedure even further. UV-VIS and FTIR spectroscopic analysis should be used in a side-by-side comparison for rapid quantification of lignin content for biomass sources and identification of similar lignin structures.

Next, utilizing equal amounts of cellulase and biomass at 2 wt% in a 10:90 (v/v) [Bmim][Cl]/water media at 40°C for two hours resulted in very small prediction errors of glucan content when compared to values found through the NREL protocol. [Bmim][Cl] was chosen as the optimal IL for this biomass compositional analysis procedure due to its ability to easily solubilize all biomass constituents, as well as having the highest reactions rates when used as a host to enzymatic hydrolysis. Adopting this step into the biomass compositional analysis procedure would improve upon the laboratory safety issues found in the NREL protocol, as well as fulfilling the need for an eco-friendly hydrolysis of biomass. However, alternative analysis techniques should be explored when quantifying the mixture of sugars being produced from the hydrolysis. Supercritical fluid chromatography (SFC) is a relatively new technique that has been applied to polar

solvents such as carbohydrates for several years. This technique employs a mobile phase consisting of a highly compressed gas just above its critical temperature and pressure. The exploration of this technique could highly benefit the research and methodologies presented in this thesis. The elimination of organic solvents used for the mobile phase of HPLC would increase laboratory safety and environmental impacts of this analysis procedure.

It must be noted that this study only included utilization and analysis of cellulase. Incorporating another enzyme such as xylanase or an enzyme cocktail better suiting the hydrolysis of lignocellulosic biomass could greatly benefit the conversation rates, thus shortening total analysis time. Utilization of a more specific enzyme could also allow for selective precipitation of the biopolymers. Recent studies suggest that cellulase from *Humicola insolens* (Celluzyme 0,7T) may be a more active and stable cellulase preparation for use in ILs.[24]

The ash content of the biomass sample should be calculated by mirroring the methodologies outlined in the NREL protocols. The biomass is combusted at 575°C, and ash is quantified gravimetrically. Having already quantified cellulose, lignin, and ash, the hemicellulose mass fraction is calculated by mass balance. On the basis of all above findings, the main constituents of biomass (cellulose, hemicellulose, lignin, and ash) can be easily and safely quantified through the use of ionic liquids (Fig. 28).

A validation sample was used to test whether the new methodologies involving the enzymatic hydrolysis of yellow poplar could replace the acid-catalyzed hydrolysis as described in the NREL protocol. The sample was prepared and analyzed as described above. In summary, the results of the predicted mass fractions of lignin, cellulose, hemicellulose, and ash can be found in Table 13. It is the author's assessment that the prediction errors for the lignin and glucan mass fractions are reasonable for the recently developed methodologies included in this research. Again, it is recommended that the hydrolysis of hemicellulose within an IL/water reaction media be studied to improve upon the predicted xylan mass fraction. This current protocol excludes the quantification of other components such as arabinan, galactan, mannan, acetate, and protein as they

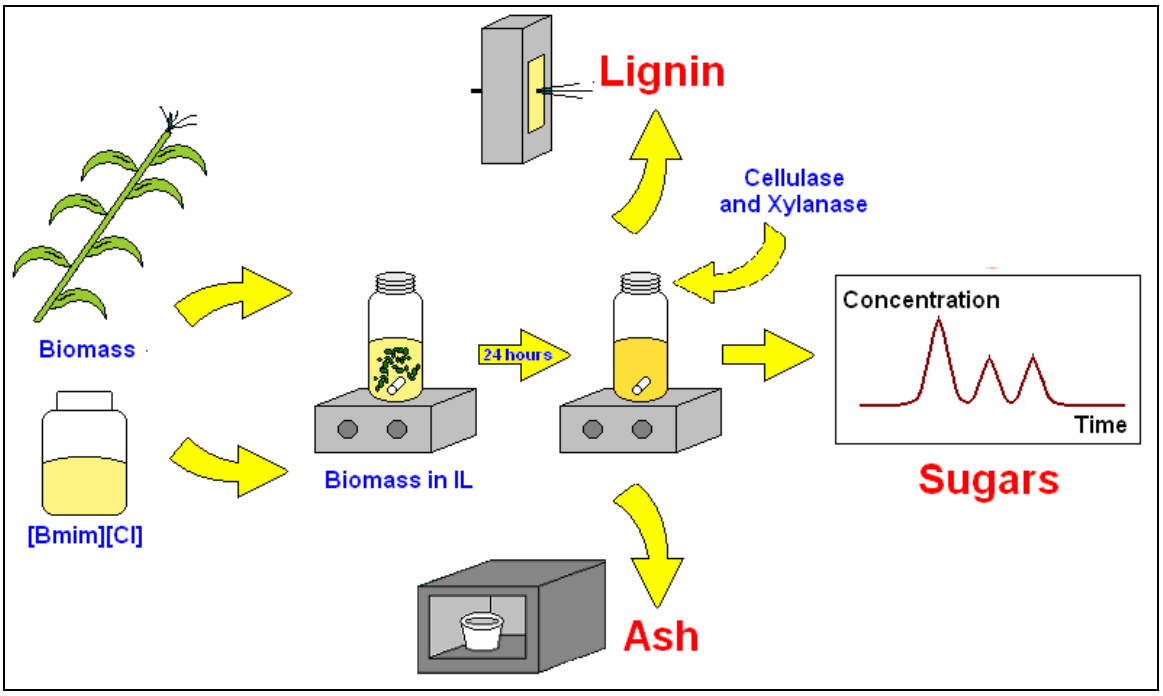

Figure 28. Flowchart of the new methodologies for biomass wet chemical analysis.

Table 13. Comparison of analyses of a yellow poplar utilizing the NREL protocol and the new methodologies for biomass wet chemical analysis.[1,2,3,4.]

Overall Analysis with New Methodologies			
Component	**NREL Mass Fraction[5.] (% dry basis)**	**Kline Mass Fraction (% dry basis)**	**% Difference**
Lignin (UV-VIS) Approach	25.1% ± 0.8	21.5% ± 0.4	-14.3%
Lignin (FTIR) Approach	25.1% ± 0.8	25.6% ± 0.1	2.0%
Ash	3.5% ± 0.1	3.5% ± 0.1	-
Glucan	43.5% ± 0.5	43.6% ± 0.3	0.2%
Xylan	15.9% ± 0.6	29.4% ± 0.8	84.9%

1. Finely ground yellow poplar was dried at 105°C before solubilization to 2 wt% in of each IL at 60°C with magnetic stirring at 400 rpm for 24 hours; water was directly added to the solutions to produce the desired 10:90 (v/v) IL/water media composition; an equal amount of enzyme by mass to biomass was added once the solutions had cooled to 40°C; the samples were agitated at 400 rpm in a silicone oil bath.

2. Three UV spectra were collected from 190-1100 nm at 25°C, and the absorbances at 360 nm were recorded for prediction using the UV-VIS approach. Sample calculation can be found in the Appendix.

3. Three mid infrared spectra from 650-4000 cm^{-1} with 16 scans for each sample were recorded for each sample volume of one droplet (approximately 10-20 uL); the absorbance data was reduced to a spectral resolution of four cm^{-1}, normalized, and analyzed using PLS regression after the removal of outliers with the Unscrambler® statistical software.

4. Every 30 minutes fresh enzyme was added equal to that of the initial biomass; the samples were agitated at 400 rpm in a silicone oil bath and an aliquot of each sample was analyzed using HPLC analysis with utilization of an aminopropysilyl column for saccharides (Prevail™ Carbohydrate ES) by Alltech Associates, a division of WR Grace (Deerfield, IL) and operating conditions similar to those published recently.[2] Hydrolysis error calculated using the standard error for the glucose and cellobiose calibration models

5. The NREL mass fractions for the components found with utilization of the laboratory analytical procedure *Determination of Structural Carbohydrates and Lignin in Biomass* [32], as developed by US Dept of Energy, National Renewable Energy Laboratory (NREL).

are considered negligible and are therefore accounted for in the calculation for the hemicellulose component. Other improvements, including the reduction in analysis time, use of hazardous chemicals, and number of transfer steps, are summarized in Table 14. This protocol results in a reduction of transfer steps by 80%, analysis time by 40%, and there is a complete elimination of sulfuric acid.

This analysis procedure improves upon the drawbacks of the NREL biomass compositional analysis protocols and fulfills the objectives of the research: A reduction in overall analysis time, improvement in laboratory safety of the acid-catalyzed hydrolysis procedure, and the HPLC analyses of carbohydrates, and reduction of the number of transfer steps of the biomass sample between vessels to minimize error. Utilization of ILs, solvents capable of solubilizing biomass its constituent biopolymers, addresses many of the above problems. Future experiments are recommended to improve upon the total enzymatic hydrolysis of the biomass, leading to the possibility of component fractionation and recovery through selective precipitation for further analysis or processing.

Utilizing and further developing this analysis procedure will aid in the overall goal of identifying alternative, renewable feedstocks for the development of biofuels as alternatives to conventional petroleum fuels. To make cellulosic ethanol a cost-competitive alternative fuel, it is imperative to study the composition of various biomass species and their anatomical components to identify which feedstocks have the most potential in terms of available fermentable sugars. As crude oil prices continue to increase, the development of fuels from lignocellulosic biomass will play an important role in meeting the increasing US energy demand.

Table 14. Quantification of improved methodologies for biomass compositional analysis.[1,2.]

Overall Improvements (for one sample) with New Methodologies			
Approximate Required Component	**NREL Protocol**	**Kline Protocol Utilizing ILs**	**% Savings**
Analysis Time	48 hours	29 hours	40.0%
Sulfuric Acid	3 mL	0	100%
Other Hazardous Solvents	1 L	1 L	0
Transfer Steps	5	1	80%

1. Approximations made through the utilization of the laboratory analytical procedure *Determination of Structural Carbohydrates and Lignin in Biomass* [32], as developed by US Dept of Energy, National Renewable Energy Laboratory (NREL) and methodologies presented in this thesis (Kline Protocol).

2. The approximation of "Other hazardous solvents" for the Kline Protocol based on the utilization of the UV-VIS spectrophotometric approach

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Appendix

Appendix: Sample Calculation of Quantification of Glucan in Biomass Solubilized in IL

Sample 1: Hydrolysis of yellow poplar in 10:90 (v/v) [Bmim][Cl]/water reaction media (Initial Data found in Table A-1). Hydrolysis data presented is one of the samples used to create protocol for hydrolysis of biomass in an IL/water reaction medium.

Finely ground yellow poplar was dried at 105°C overnight before solubilization to 2 wt% in of each IL at 60°C with magnetic stirring at 400 rpm for 24 hours; water was directly added to the solutions to produce the desired 10:90 (v/v) IL/water media composition; an equal amount of enzyme by mass to biomass was added once the solutions had cooled to 40°C; the samples were agitated at 400 rpm in a silicone oil bath.

Table A-1. Initial sample composition and other collected data.

Time = 0		
Reaction Vial		wt%
Biomass (g)	0.0110	0.21%
Cellulase (g)	0.0147	0.28%
Water (mL)	4.7496	88.89%
Ionic Liquid (g)	0.5562	10.41%
(mL)	0.5283	
Total Sample Volume (mL)	5.2779	
Cellulose Mass Fraction of Biomass sample (g)	0.0048	
Sugars (mol-glu/L)	0.0056	
Water (v/v)	89.99%	
IL (v/v)	10.01%	
Total Sample (g)	5.3433	
Total Sample Vial (g)	15.4246	

The amount of hydrolysis in each experiment is determined through HPLC analysis with utilization of an aminopropysilyl column for saccharides (Prevail™ Carbohydrate ES) by

Alltech Associates, a division of WR Grace (Deerfield, IL) and operating conditions similar to those published recently.[2] Hydrolysis error calculated using the standard error for the cellulose and cellobiose calibration models. This results in a concentration of each carbohydrate (mg/mL). The amount hydrolysis achieved is calculated using moles of glucose units per volume, as the β-1,4' glycosidic bonds are broken during hydrolysis of the cellulose. The molecules molecular weights are 162, 171, and 180 g/mol for cellulose, cellobiose, and glucose, respectively.

From earlier biomass wet chemistry analysis dictated by the NREL protocol, the yellow poplar sample has a theoretical glucan content of 43.5 wt%. Therefore the cellulose available for hydrolysis is,

$$Cellulose(g) = 0.435(0.0110g) = 0.0048g \qquad \text{(Eqn. 1)}$$

$$Cellulose\ (mol\text{-}glu\ units/L) = \frac{0.0048g}{5.2779mL} \cdot \frac{1000mL}{L} \cdot \frac{mol-glu}{162g} \qquad \text{(Eqn. 2)}$$

$$= 0.0056\ mol\text{-}glucose\ unit/L$$

An aliquot equal to 0.1545g (approximated as 0.1545mL) of the reaction media was taken at 30 minutes and diluted with 0.1545mL of water. After HPLC analysis of the reaction media, there is 0.0630 mg/mL of glucose and 0.0119 mg/mL of cellobiose present after 30 minutes. An internal standard was used to create a correction factor used in the quantification calculations. Converting these concentrations to moles of glucose units and taking into consideration the dilution gives,

$$Glu\cos e = \frac{0.0630g}{L} \cdot \frac{mol-glu}{180g} \cdot \frac{(0.1545+0.1545)mL}{0.1545mL} \cdot Correction \qquad \text{(Eqns. 3 and 4)}$$

$$= 0.0006 mol\text{-}glucose\ units/L$$

$$Cellobiose = \frac{0.0119g}{L} \cdot \frac{L}{1000mL} \cdot \frac{mol-glu}{342g} \cdot \frac{2cellobiose}{1glu\cos e} \cdot \frac{(0.1545+0.1545)mL}{0.1545mL}$$

$$= 0.0001\ mol\text{-}glucose\ units/L$$

The conversion can also be divided into hydrolysis to either cellobiose or glucose. This could also be thought as the composition of sugars in the reaction media after 30 minutes of

hydrolysis. Therefore the amount of conversion (hydrolysis) of cellulose to cellobiose and glucose is,

$$\%Glu\cos e = \frac{Glu\cos e}{Cellulose} = \frac{0.0006\,{}^{mol-glu}\!/_{L}}{0.0056\,{}^{mol-glu}\!/_{L}}(100) = 11.5\% \qquad \text{(Eqn. 5)}$$

$$\%Cellobiose = \frac{Cellobiose}{Cellulose} = \frac{0.0001\,{}^{mol-glu}\!/_{L}}{0.0056\,{}^{mol-glu}\!/_{L}}(100) = 2.3\% \qquad \text{(Eqn. 6)}$$

$$\text{Total Hydrolysis} = \%Glu\cos e + \%Cellobiose = 13.7\% \qquad \text{(Eqn. 7)}$$

The hydrolysis values were then calculated for each specified time for the remaining samples to calculate the average amount of hydrolysis with these conditions.

To utilize the enzymatic hydrolysis protocol, aliquots were collected for the yellow poplar sample as described above and analyzed them with HPLC to determine the amount of cellobiose and glucose produced. This data is summarized in Table A-2.

Table A-2. Initial sample composition and other collected data.

Time = 0		
Reaction Vial		wt%
Biomass (g)	0.0109	0.20%
Cellulase (g)	0.0111	0.21%
Water (mL)	4.9309	91.89%
Ionic Liquid (g)	0.5826	10.86%
(mL)	0.5534	
Total Sample Volume (mL)	5.4843	
Cellulose Mass Fraction in the Biomass Sample (g)	0.0047	
Sugars (mol-glu/L)	0.0053	
Water (v/v)	89.91%	
IL (v/v)	10.09%	
Total Sample (g)	5.3660	
Total Sample Vial (g)	15.9529	

At 30 minutes of hydrolysis, the concentrations of glucose and cellobiose were found to be 0.0369 and 0.0110 mg/mL, respectively. The sample aliquot was 0.1501mL, diluted with 0.1536mL of water. Using Equations 3 and 4, the concentrations of the detected sugars was calculated as 0.0004 and 0.0001 mol-glucose units per liter, respectively. The average hydrolysis of the yellow poplar under the given conditions is 16.6%. Therefore the predicted glucan content in the biomass sample after 30 minutes of reaction time if found,

$$Glucan_{\Pr edicted} = \frac{Sugars_{Measured} \times Volume_{Sample}}{Hydrolysis_{Average} \times Biomass} \qquad \text{(Eqns. 8 and 9)}$$

$$= \frac{0.0005 \frac{mol-glu}{L} \cdot \frac{162g}{mol-glu} \frac{5.4843mL}{1000}}{16.6\% \times 0.0109g}(100) = 24.5\%$$

$$\%Error = \frac{Glucan_{\Pr edicted} - Glucan_{Actual}}{Glucan_{actual}} = \frac{24.5 - 43.5\%}{43.5\%} = -43.5\%$$

Vita

Lindsey McCulloch Kline was born in Maryville, Tennessee. She graduated from Virginia Polytechnic Institute and State University in Blacksburg, Virginia in 2005 with a Bachelor of Science degree in Biosystems Engineering. In the summer of 2005, she married her husband Aaron Kline and moved to Knoxville, Tennessee, where she then began a Master of Science program at the University of Tennessee-Knoxville Biosystems Engineering Department with research in the Bioprocess Engineering discipline.