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Perdeuterated Pyridinium Ionic Liquids for Direct Biomass Dissolution and Characterization

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1. Introduction

Fossil fuels, as the primary source of energy, chemicals, and materials for our modern society, are estimated to consist almost 90% of the energy consumption in the world in the resource such as petroleum, coal, and natural gas, particularly for the transportation sector (Simonetti & Dumesic, 2008). However, the use of fossil fuels for the generation of energy is associated with growing concerns related to energy security (the uneven geographical distribution of the fossil fuel reserves) and climate change. Therefore, ever-increasing societal demand for environmental and economic sustainability is placing a renewed focus on the agro-forest industry, due to, among others, the unquestionable environmental advantage associated with the reduction of the net emissions of CO_2 (a greenhouse gas) into the atmosphere (Clark et al., 2006; Ragauskas et al., 2006; Petrus & Noordermeer, 2006; Horvath and Anastas, 2007; Corma et al., 2007; Pu et al., 2008). Unlike fossil fuels, biofuels are widely considered to be "carbon neutral" because any CO_2 produced during fuel combustion can be consumed by subsequent biomass regrowth. In this sense, the aggressive government directives from U.S. and the European Union, are stimulating a gradual shift of our current fossil fuel-based economy towards a more renewable energy-based one to achieve the goals to produce 20% of fuels from renewable sources by 2030 (Serrano-Ruiz et al., 2010).

While 1st-generation biorefineries have successfully utilized readily precessable bioresources such as sucrose, starches and plant oils for bioethanol/biodiesel (Bothast & Schlicher, 2005; Marchetti et al., 2007; Huber & Corma, 2007), important moral and ethical questions have arisen from the use of these edible biomass (sugars, starches, and vegetable oils) for the large-scale production of biofuels. Therefore, more efficient utilization of renewable and low-cost nonedible biomass (lignocellulosic biomass) to maximize sustainable, economic societal development is of extreme significance.

However, intrinsic recalcitrance of biomass, known as resistance of the plant cell walls to break down due to its complicated structure (Cosgrove, 2005; Wyman et al., 2005; Himmel et al., 2007), has limited economical transformation of lignocellulosic biomass into biofuels. To address this challenge, extensive research has been directed towards a better understanding of plant cell wall structures and their constituents (Lewis & Paice, 1989; Jung et al., 1993), consisting of cellulose, hemicelluloses and lignin (Figure 1).
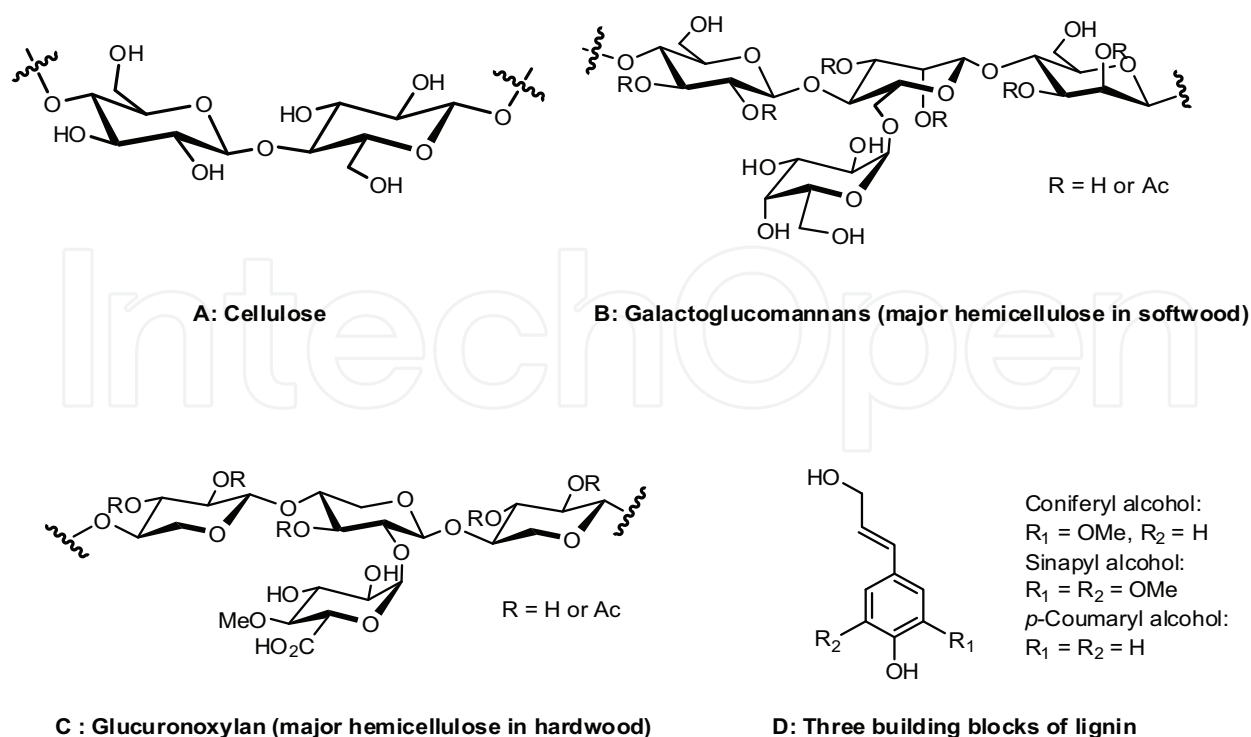
Fig. 1. The structures of three major biopolymers (**A-D**) of the plant cell walls

Among the cell walls' composite materials, lignin (see Fig. 1D), as the second most abundant natural polymer after cellulose and the natural glue for the plant cell walls, is produced by enzyme-mediated radical coupling of the three monolignols (Higuchi, 1985; Boerjan et al., 2003) and widely recognized as the major factor of biomass recalcitrance problem. With recent developments in the genetic engineering of lignin's biosynthetic pathway (Pena & Seguin, 2001; Baucher et al., 2003; Sticklen, 2006), new avenues to rationally designing bio-energy plants with reduced recalcitrance and thus improved processing properties has been opened for the next generation of biorefineries. As a large number of new plants with differing variations in lignin structure are developed the need for the efficient, economical and high-resolution analysis of the plant cell walls, particularly lignin (Davin & Lewis, 2005), becomes a pressing research issue.

Recently, 4:1 mixture of DMSO-d_6 and 1-methylimidazole-d_6 has been reported to dissolve non-derivatized ball-milled plant cell walls for a 2D NMR analysis, which provides a direct and accurate approach for the characterization of lignin and hemicellulose in plant biomass and was applied to samples of loblolly pine, quaking aspen and kenaf (Yelle et al., 2008). This approach is notable due to avoiding the need for lengthy, low-yielding lignin extraction protocols. Key to liquid NMR analysis of plant cell material is the efficient dissolution of ball-milled plant cell walls in a perdeuterated NMR solvent system.

Widely recognized as environmentally-friendly solvents, ionic liquid has been extensively documented for a variety of applications, due to its tunable physicochemical properties, including negligible vapor pressures and thermal/chemical stability over a wide range of temperatures (Welton, 1999). In addition to their role as green alternative reaction media (Wassercheid & Keim, 2000; Sheldon, 2001), Rogers and co-workers first reported that ionic liquid 1-*n*-butyl-3-methylimidazolium chloride ([Bmim]Cl, see 1a in Fig. 2) can dissolve cellulose without activation (Swatloski et al., 2002). We and others have shown that various

imidazolium-based ionic liquids can efficiently dissolve lignin (Pu et al., 2007), wood (Kilpelaeinen et al., 2007; Fort et al., 2007) and other biopolymers (Zhu et al., 2006; Fukaya et al., 2008; Zakrzewska et al., 2010). Despite wide applications of imidazolium-typed ionic liquids (see 1 in Fig. 2) as the solvents for biomass dissolution and functionalization, no perdueterated imidazolium ionic liquids has been reported in biomass NMR characterization due to their tedious multi-step synthesis (Hardacre et al., 2001; Giernoth et al., 2008).

R = Me, Allyl; **R¹** = Me, Et, Bu, Bz, Allyl
X = Cl, OAc, and $(MeO)_2PO_2$
1a : **R** = Me, **R¹** = Bu, **X** = Cl
2a : **R²** = Allyl, **X** = Cl
2b : **R²** = Allyl, **X** = Br
2c : **R²** = Bu, **X** = Cl
2d : **R²** = Cyanomethyl, **X** = Cl
2e : **R²** = H, **X** = Cl

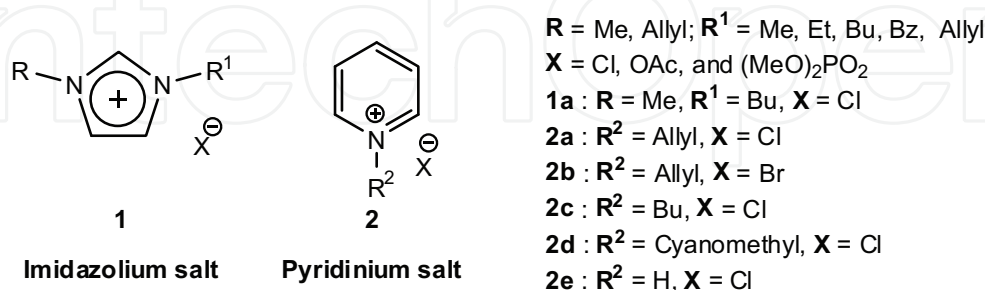**1**
Imidazolium salt

2
Pyridinium salt

Fig. 2. Structures of imidazolium and pyridinium salts for biomass dissolution

Hence, our research in this area is aimed at direct NMR analysis of the plant cell walls via readily accessible perdeuterated ionic liquids, maximizing accuracy and efficiency of characterization by dissolution of Wiley-milled biomass. In this chapter, we shall describe novel perdeuterated ionic liquid for direct dissolution and NMR analysis of the plant cell walls. Our starting point was the use of pyridinium-typed ionic liquids (2 in Fig. 2), which had not been reported for biomass dissolution, despite the easy accessibility of perdeuterated pyridinium salts via commercially available pyridine-d_5.

We started our search for a suitable perdeuterated pyridinium salt for direct biomass dissolution and NMR analysis by screening five pyridinium ionic liquids (see 2a-e in Fig. 2) with ball-milled poplar as the initial sample. Due to the pyridinium salt's high melting point, we also used DMSO-d_6 as the co-solvent to render the sample to be dissolved at a lower temperature and to reduce viscosity of the resulting biomass solution for NMR analysis. For example, 35 mg ball-milled poplar can be dissolved in 1.0 gram 1 : 2 [Apyr]Cl (1-allylpyridinium chloride, 2a) and DMSO-d_6 at 60 °C under nitrogen in 1 h as the suspended poplar particles disappeared and a homogeneous solution formed with increased viscosity and slightly coloration of the solution (entry 1 in Table 1). Further ^{1}H and ^{13}C NMR analysis of the dissolved poplar solution supported this claim as the spectra data contained siganls readily attributed to lignin and polysaccharides. However, the use of [Apyr]Br (1-allylpyridinium bromide, 2b) to replace [Apyr]Cl proved to be unsuccessful under identical conditions (<5 mg/g, entry 2 in Table 1). In contrast, [Bpyr]Cl (1-*n*-butylpyridinium chloride, 2c) showed similar biomass solubility as [Apyr]Cl (entry 3 in Table 1), and both [Cmpyr]Cl (cyanomethylpyridinium chloride, 2d) and [Hpyr]Cl (pyridinium chloride, 2e) led to excellent results (70 mg/g and 80 mg/g, entries 4 and 5 in Table 1), which is superior to traditional [Bmim]Cl (1-*n*-butyl-3-methylimidazolium chloride, 1a in Fig. 2) under identical conditions (entry 6 in Table 1).

It is therefore clear that pyridinium typed ionic liquids with chloride as the anion show good biomass solubility in the presence of DMSO-d_6 as co-solvent. Furthermore, chloride anion is crucial for our present system, which agrees with Rodgers' speculations that chloride is highly effective in interupting the extensive hydrogen-bonding cellulose network

Entry	Ionic Liquid (IL)	IL : DMSO-d_6	Biomass	Time (h)	Solubility [a] (mg/g)
1	[Apyr]Cl **2a**	1:2	Poplar[b]	1	35
2	[Apyr]Br **2b**	1:2	Poplar[b]	6	<5
3	[Bpyr]Cl **2c**	1:2	Poplar[b]	6	30
4	[Cmpyr]Cl **2d**	1:2	Poplar[b]	1	70
5	[Hpyr]Cl **2e**	1:2	Poplar[b,c]	1	80
6	[Bmim]Cl **1a**	1:2	Poplar[b]	1	40
7	None	-	Poplar[b,c]	6	-
8	[Hpyr]Cl **2e**	1:2	Poplar[d]	6	80
9	[Hpyr]Cl-d_6 **2e'**	1:2	Poplar[b]	1	80
10	[Hpyr]Cl-d_6 **2e'**	1:2	Poplar[d]	6	80
11	[Hpyr]Cl-d_6 **2e'**	1:2	Switchgrass[d]	6	100
12	[Hpyr]Cl-d_6 **2e'**	1:2	Pine[d]	6	60

[a] In all experiments dry biomass dispersed in 1.0 g bi-solvent system consisting of pyridinium salt and DMSO-d_6 was stirred at 60 °C under nitrogen for specific time except where indicated. [b] Sample was the ball-milled biomass. [c] The mixture was heated at 80 °C. [d] Sample was non-ball-milled biomass (20 mesh).

Table 1. Solubility of biomass in ionic liquid/DMSO-d_6 bi-solvent system.

presented in plant cell walls (Swatloski et al., 2002). Control experiments showed that the absence of ionic liquid led to no dissolution of poplar in DMSO-d_6 even under elevated temperature and prolonged heating, as confirmed by direct NMR analysis. Therefore, the presence of pyridinium chloride salt is crucial for the successful dissolution of the ball-milled sample.

A shortcoming of using ball-milled sample is that ball-milling processing has been reported to induce some chemical changes in the structure of lignin (Kondo et al., 1995; Ikeda et al., 2002;), which will affect the accuracy of native lignin characterization. Other shortcoming includes that ball-milled process takes up to 7 days and is less efficient if a large number of samples need to be characterized. In this context, we next investigated the non-ball-milled poplar sample (an average particle size of 20 mesh by Wiley mill, 1041 um) for direct dissolution and characterization in 1 : 2 [Hpyr]Cl/DMSO-d_6 bi-solvent system. Paralleled solubility of Wiley milled sample was obsvered through a prolonged time of 6 hours (entry 8 in Table 1). At this stage, perdeuterated [Hpyr]Cl-d_6 **2e'** was prepared from pyridine-d_5 (see Fig. 3a), and the perdeuterated bi-solvent system was tested for direct dissolution and NMR analysis of ball-milled and Wiley milled poplar sample (entries 9 and 10 in Table 1). Furthermore, besides hardwood poplar, both Wiley-milled grass sample (switchgrass, 20 mesh, average partical size : 1041 um) and Wiley milled softwood sample (pine, 20 mesh, average partical size : 1041 um) showed very good solubility in our current perdeuterated bi-solvent system (entries 11 and 12 in Table 1). Therefore, our current perdeuterated pyridinium chloride-d_6/DMSO-d_6 bi-solvent system proved to be effective for successful dissolution of hardwood (poplar), softwood (pine) and grass (swicthgrass) samples.

^{1}H NMR Spectra of both ball-milled and Wiley milled poplar solution in [Hpyr]Cl-d_6/DMSO-d_6 system, as shown in Fig. 3b and 3c, serve to illustrate signals from both lignin aromatic/olefinic protons (between 6.0 ppm and 8.0 ppm) and polysaccharides. Similar ^{1}H NMR spectrum was also found for Wiley-milled switchgrass sample in Figure 3d.

Both ball-milled and Wiley milled poplar samples were then subjected to ^{13}C NMR analysis as presented in Fig. 4a and 4b, which showed paralleled spectra. The signals at δ 61.5, 74.1, 75.8, 76.9, 80.1, and 103.0 ppm were attributed to cellulose and strong signals at δ 57.3 ppm were assigned as the lignin methoxy ^{13}C resonance. Some detailed aliphatic sub-lignin structures could also be identified as C_γ resonance in β-O-4 and β-5 (62.2 and 64.0 ppm), C_β in β-O-4 and C_α resonance in β-5 and β-β (between 80 and 86 ppm). At the same times, signals from sub-lignin aromatic rings, such as resonance of syringyl-like lignin structures at 106.0 ppm for C2/6 and C2, C5 and C6 resonance of guaiacyl-like lignin structures between 110.0 and 120.0 ppm can also be clearly identified in ^{13}C NMR spectra (cellulose, β-O-4, β-β, syringyl-like lignin structures, and guaiacyl-like lignin structures see Fig. 5). In addition, ^{13}C NMR analysis of Wiley milled switchgrass revealed similar spectral data as shown in Fig. 4c.
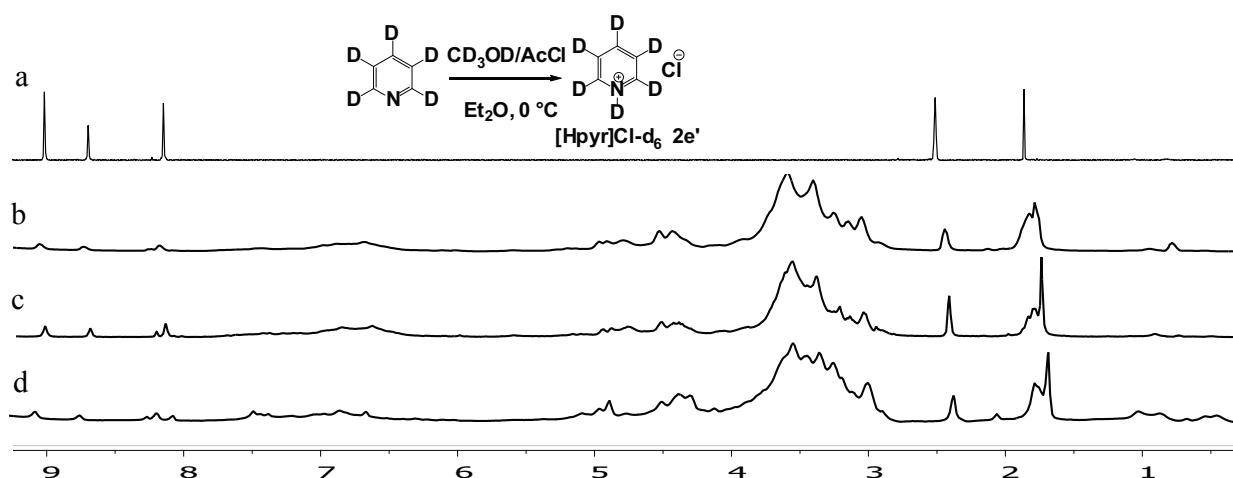
Fig. 3. (a) Preparation and ^{1}H NMR spectrum of [Hpyr]Cl-d$_6$ and ^{1}H NMR spectrum of biomass solution after dissolving biomass sample in 1:2 [Hpyr]Cl-d$_6$/DMSO-d$_6$ (1.0 g) at 60 °C: (b) Ball-milled poplar (80 mg) in 1:2 [Hpyr]Cl-d$_6$/ DMSO-d$_6$; (c) Wiley milled poplar (80 mg) in 1:2 [Hpyr]Cl-d$_6$/ DMSO-d$_6$; (d) Wiley milled switchgrass sample (100 mg).
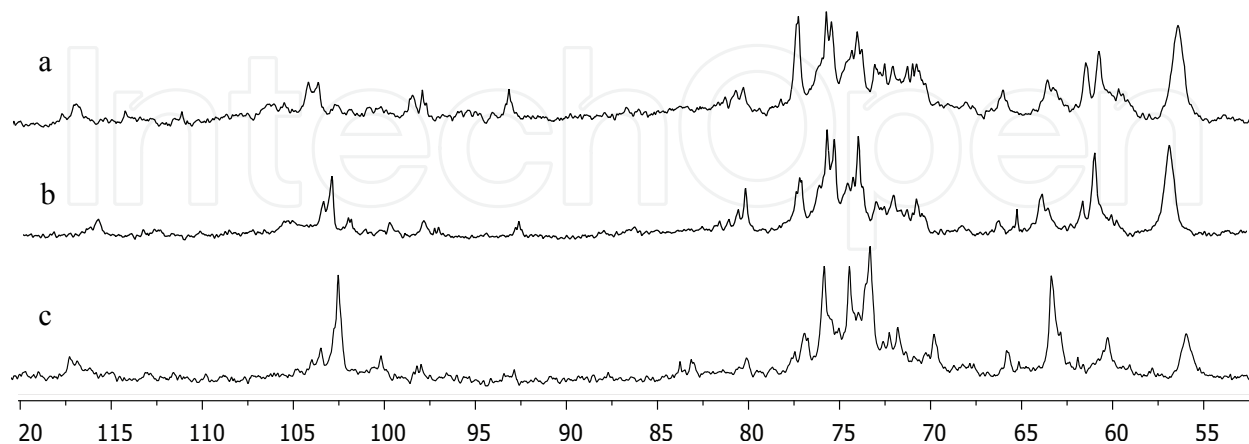

Fig. 4. ^{13}C NMR spectra of sample solution after dissolving biomass sample in 1:2 pyridinium molten salt/DMSO-d$_6$ (1.0 g) at 60 °C. (a) Ball-milled poplar (80 mg) in 1:2 [Hpyr]Cl-d$_6$/DMSO-d$_6$; (b) Wiley milled poplar (80 mg) in 1:2 [Hpyr]Cl-d$_6$/DMSO-d$_6$; (c) Wiley milled switchgrass (100 mg) in 1:2 [Hpyr]Cl-d$_6$/ DMSO-d$_6$.
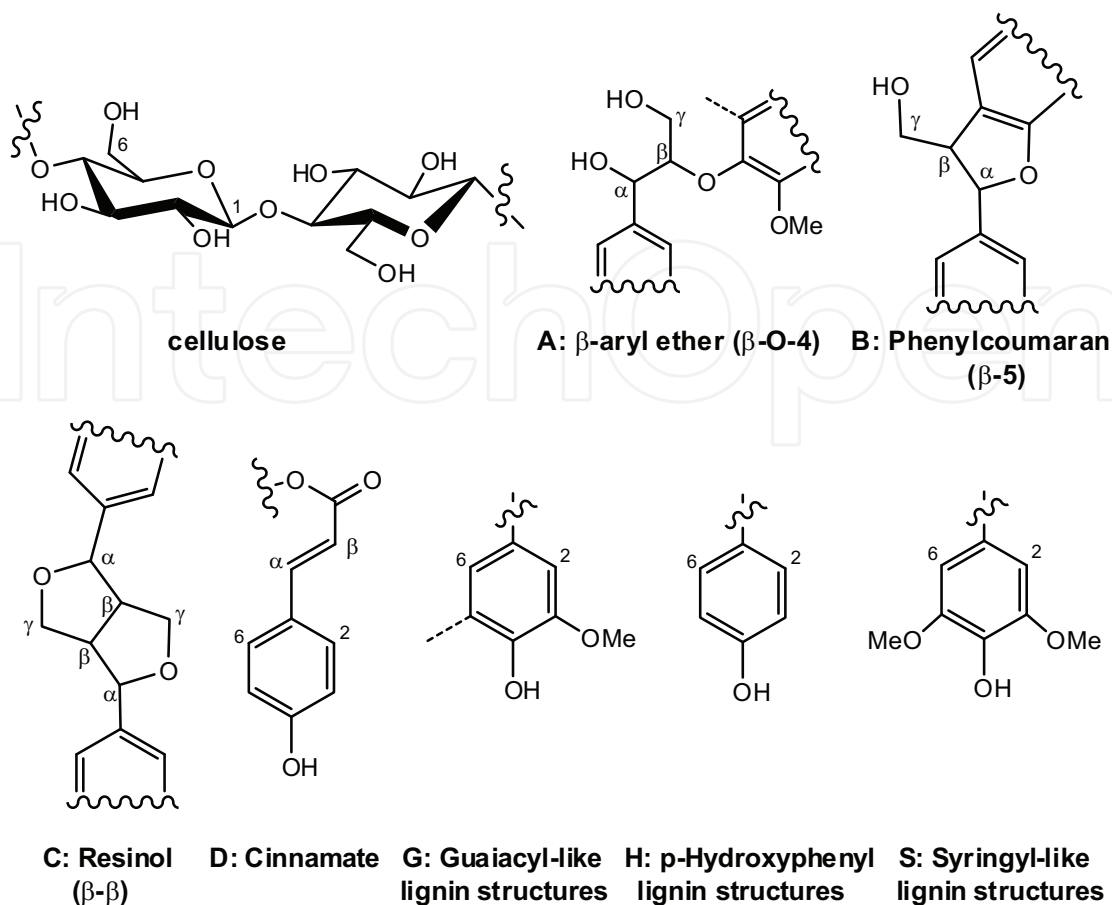
Fig. 5. Structures of cellulose and sub-lignin units

We next used 2D NMR (Heteronuclear Single Quantum Coherence, HSQC) analysis to investigate more detailed sub-lignin structures. As switchgrass lignin contains a typical HGS lignin (H: *p*-hydroxyphenyl, S: syringyl, G: guaiacyl) that can be readily analyzed by HSQC NMR, we summarized HSQC NMR of switchgrass in Fig. 6 to illustrate the aliphatic side chain and the aromatic ring ^{13}C-^1H correlations of the lignin component. The main cross signals in the aromatic region correspond to sub-lignin syringyl-like (S) and guaiacyl-like (G) units which appeared separately. The S unit cross peak for the C2,6/H2,6 ($S_{2/6}$) correlation appears at δC/δH 104.7/6.8 ppm. The G units showed different correlations at δC/δH 112.1/6.9, 115.8/6.7, 119.4/6.9 ppm for C2/H2 (G_2), C5/H5 (G_5) and C6/H6 (G_6), respectively, as shown in Figure 6. Furthermore, strong correlation signals at δC/δH 130.5/7.4 and 128.9/7.1 ppm reveal the presence of *p*-hydroxyphenyl (H structure in Fig. 5) unit. In addition, correlation signals at δC/δH 114.0/6.4 ppm ($D_β$) and 145.9/7.4 ppm ($D_α$) from *p*-coumarate and ferulate (see D in Fig. 5) can be clearly identified, which agree with the report that the grass lignin contains *p*-coumarate and ferulate units (Ralph et al., 1999). More importantly, the detailed sub-lignin side chain structure (aliphatic region) can be clearly assigned to the cross signal for lignin methoxyl group (δC/δH 57.3/3.7 ppm, the most prominent) and those for β-O-4 substructures at α-, β- and γ-C positions (δc/δH 74.5/4.5, 84.8/3.8, and 60.0/3.6 ppm, respectively, see A in Fig. 5). The presence of phenyl coumaran substructures (B in Fig. 5) can also be confirmed by C-H correlations at δC/δH 63.8/3.9 ppm. Similarly, the Wiley milled poplar sample also affords satisfactory HSQC spectra, as shown in Fig. 7.
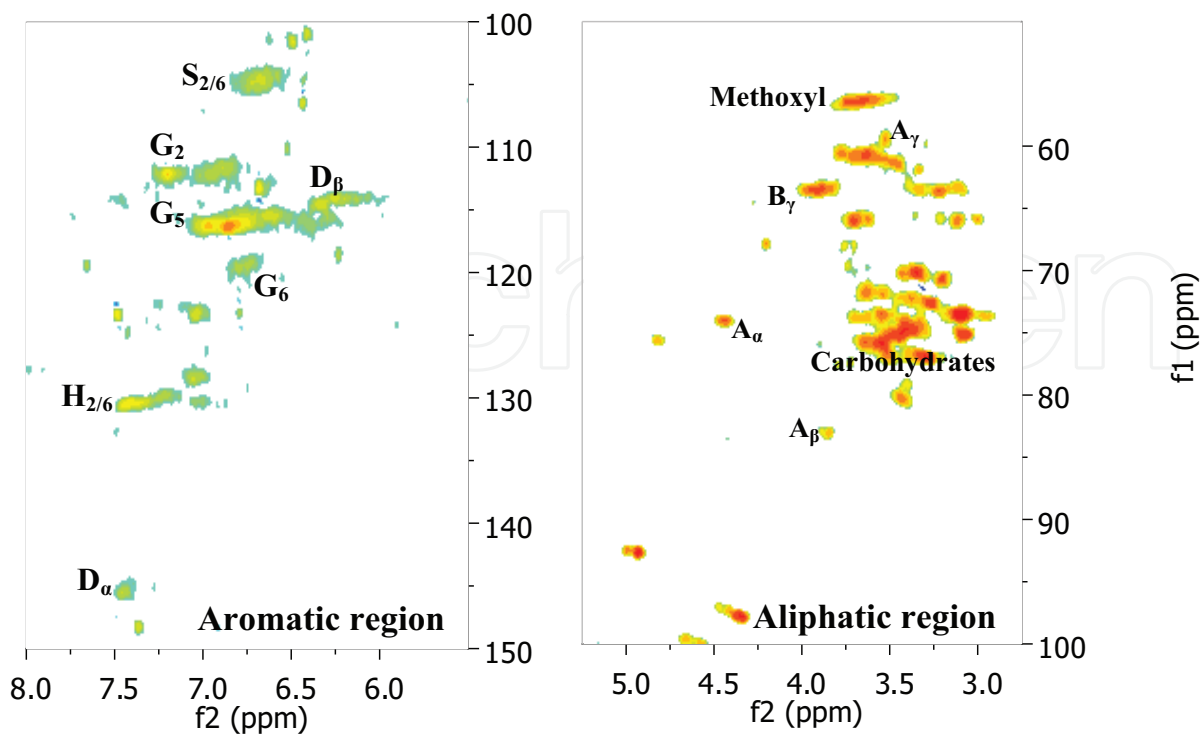
Fig. 6. (see correction 2) 2D HSQC spectra of switchgrass solution after dissolving 100 mg Wiley milled sample in 1:2 [Hpyr]Cl-d$_6$/DMSO-d$_6$ (1.0 g) at 60 °C.
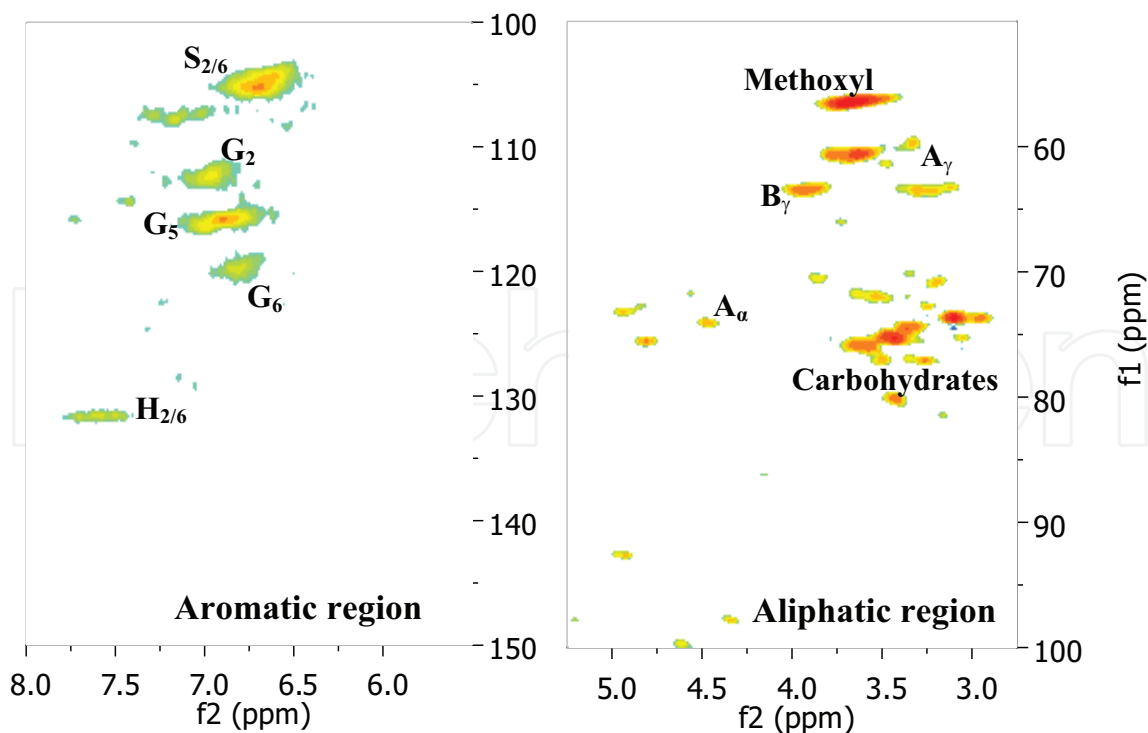
Fig. 7. (see correction 3) 2D HSQC spectra of poplar solution after dissolving 80 mg Wiley milled sample in 1:2 [Hpyr]Cl-d$_6$/DMSO-d$_6$ (1.00 g) at 60 °C.

Having successfully developed novel perdeuterated pyridinium chloride-d_6/DMSO-d_6 bi-solvent system for direct biomass dissolution and NMR characterization (Jiang et al., 2009), we further expanded our research to quantitative NMR analysis of the plant cell walls. Our initial efforts were directed toward biomass lignin content determination via ^{1}H NMR analysis.

While many methods based on gravimetric or spectrophotometric analysis (Iiyama & Wallis, 1989; Dence & Lin, 1992; Bose, 1998) have been traditionally used to analyze lignin content in plants with some disadvantages including a relatively large sample size, time-consuming procedure (gravimetric method) and the difficulty in finding an appropriate calibration standard (spectrophotometric methods). Very recently, near-infrared (NIR) spectroscopy method has been further modified to improve the precision of lignin content (Yeh et al., 2004; Hatfield & Fukushima, 2005; Chang et al., 2008; Yao et al., 2010), and high-throughput screening of plant cell wall composition via pyrolysis molecular beam mass spectroscopy can analyze lignin content and determine lignin S/G ratio (Sykes et al., 2009).

While there is a need for assessing a large numbers of "new" plants, precise analytic techniques for efficient lignin content assessment at a microscale are still a pressing research issue. In this sense, NMR methodologies to assess lignin content via dissolving native plant cell walls in perdeuterated ionic liquid system are attractive to serve as a supplemental to detailed structural information.

We started our initial search for a suitable procedure to measure the lignin content via direct biomass dissolution and quantitative ^{1}H NMR analysis with a ball-milled switchgrass sample dissolved in 1:2 [Hpyr]Cl-d_6/DMSO-d_6 system. DMSO-d_6 (99.9 atom%) was used as the co-solvent of [Hpyr]Cl-d_6 to reduce the solvent viscosity. Six dependent experiments were carried out to optimize the conditions for complete biomass dissolution with integration of DMSO as the internal standard, as shown in Table 2. Our experimental results show that the ratio of integration of signals of lignin aromatic/olefinic (signals between 6.0 ppm and 8.0 ppm) and integration of non-deuterated DMSO (2.5 ppm) increased with prolonging stirring at 25 °C, indicating incomplete biomass dissolution (Table 1, entries 1-3). In contrast, biomass dissolution carried out at higher temperature (60 °C) led to comparable ratios, which suggests complete biomass dissolution (Table 1, entries 4-6). Therefore, biomass dissolution carried out at 60 °C for 12 h was chosen as the standard condition for further analysis of lignin content.

Entry	Temperature (°C)	Time (h)	Ratio of integration[a,b] ($Intg_{6.0\text{-}8.0ppm} : Intg_{DMSO}$)
1	25	6	1.35
2	25	12	1.68
3	25	24	1.83
4	60	6	2.18
5	60	12	2.22
6	60	24	2.17

[a] Dried extract-free ball-milled switchgrass cell walls (40.0 mg), 600.0 mg 1 : 2 [Hpyr]Cl-d_6/DMSO-d_6 (DMSO-d_6, 99.9 atom % D) and spinbar (5 mm × 2 mm) in 5 mm NMR tube were stirred at specific temperature for the specific time for the optimization of biomass dissolution. [b] Direct ^{1}H NMR analysis of the resulting biomass solution afford the ratio of integration of signals (6.0 ppm-8.0 ppm, exclusively attributed to lignin aromatic signals) and integration of DMSO (2.5 ppm, non-deuterated DMSO as the internal standard).

Table 2. Optimization of ball-milled switchgrass complete dissolution.

After optimizing the conditions for completely dissolving biomass, we applied the linear extrapolation method for the measurement of lignin content by the addition of a specific amount of isolated switchgrass lignin to the biomass solution to examine integration ratio changes in the quantitative ^{1}H NMR spectra with non-deuterated DMSO as the internal standard. Four samples of 40.0 mg dried extractive-free ball-milled switchgrass samples and 2.0 mg, 4.0 mg, 6.0 mg, or 8.0 mg of isolated switchgrass lignin were added into a 5 mm NMR tube containing 600.0 mg 1 : 2 [Hpyr]Cl-d$_6$/DMSO-d$_6$, respectively. After 12 h stirring at 60 °C for complete biomass dissolution, the four mixtures were subject to quantitative ^{1}H NMR analysis with non-deuterated DMSO signal as the internal standard. As the signals between 6.0 ppm and 8.0 ppm can be exclusively attributed to lignin aromatic/olefinic region (see Fig. 8), we found a good linear relationship between the ratio of integrations and the added lignin amount (Scheme 1). Further data analysis via regression as modelling approach reveals that the lignin amount in 40 mg dry extractive-free switchgrass sample can be measured as 7.0 mg, and thus the lignin content in the switchgrass sample can be calculated to be 17.4%, which is comparable with traditional Klason lignin content of switchgrass as 17.1%. Therefore, our current method proves to give a comparable result with the traditional Klason lignin content.

Similarly, a ball-milled hardwood sample (poplar) was analyzed by direct dissolution and ^{1}H NMR analysis for the measurement of lignin content. A good linear relationship between the ratio of integrations and added lignin amount for ball-milled poplar sample, as shown in Scheme 2, was used to calculate the lignin amount in 40 mg poplar sample as 9.3 mg, which affords the poplar lignin content as 23.3%, which is consistent with Klason lignin content (24.1%).

Subsequently, we also investigated the use of Wiley milled samples to analyze biomass lignin content in order to improve efficiency without the complications associated with the ball-milling processes. Due to the incomplete dissolution of a Wiley milled sample in 5 mm
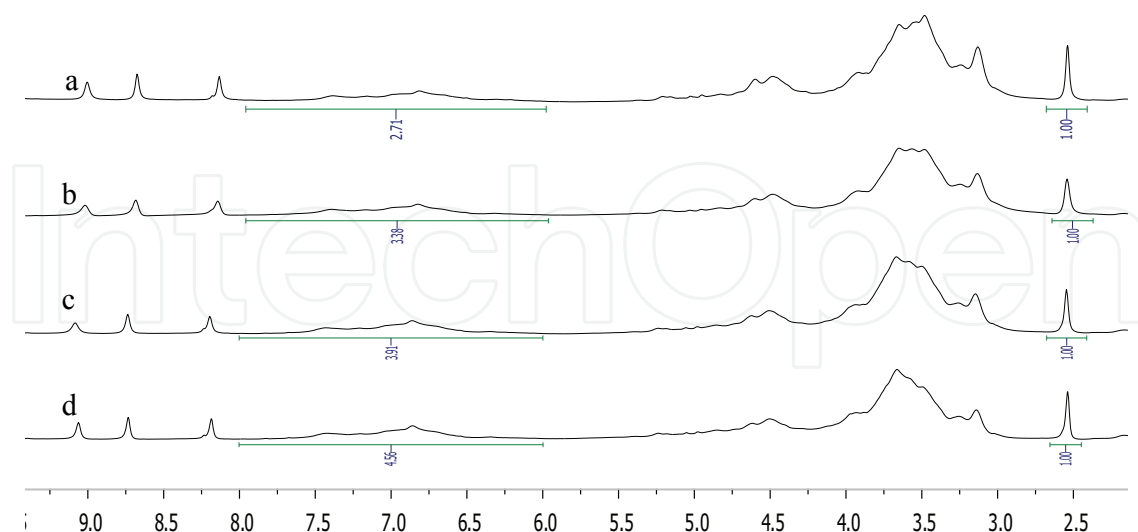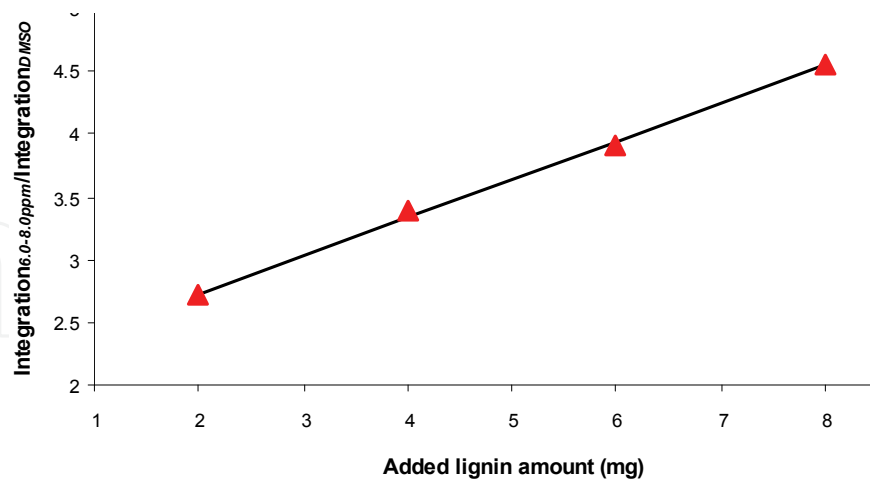

Fig. 8. ^{1}H NMR analysis of lignin content in ball-milled switchgrass cell walls: a) 40.0 mg dry switchgrass and 2.0 mg isolated switchgrass lignin; b) 40.0 mg dry switchgrass and 4.0 mg isolated switchgrass lignin; c) 40.0 mg dry switchgrass and 6.0 mg isolated switchgrass lignin; d) 40.0 mg dry switchgrass and 8.0 mg isolated switchgrass lignin.

Scheme 1. Linear relationship between ratios of integration of signals and added lignin amount (mg) in ball-milled swichgrass samples ($Y = 2.12 + 0.304 \times X$; lignin content = $0.025 \times 2.12 \div 0.304 = 17.4\%$)
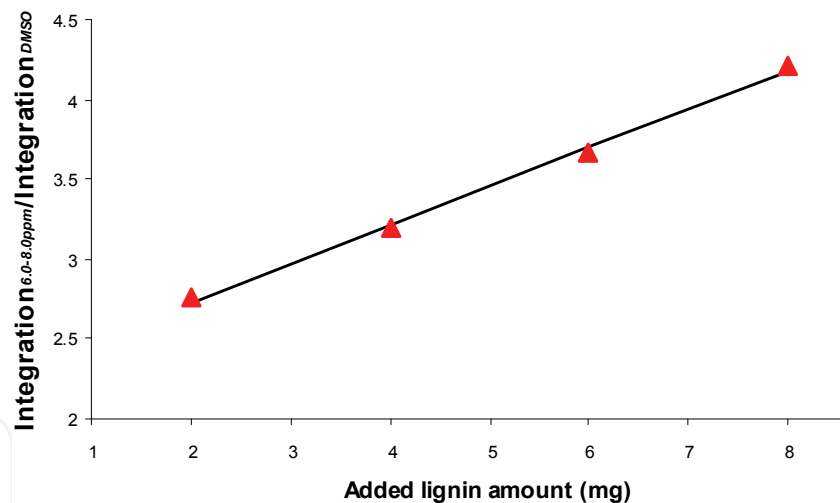
Scheme 2. Linear relationship between ratios of integration of signals and added lignin amount (mg) in 40 mg ball-milled poplar samples ($Y_1 = 2.76$; $Y_2 = 3.19$; $Y_3 = 3.67$; $Y_4 = 4.21$; $Y = 2.25 + 0.2415 \times X$; lignin content = $0.025 \times 2.25 \div 0.2415 = 23.3\%$)

NMR tube, we adopted a modified method involving the use of one signal of non-deuterated pyridinium cation (signals at 9.1 ppm) as the internal standard to analyze lignin content of Wiley milled poplar sample. Thus three samples of 30.0 mg dry extractive-free Wiley-milled poplar sample (20 mesh) and 1.0 mg, 2.0 mg, or 4.0 mg isolated poplar lignin were dissolved in 10 mL vial containing 500.0 mg 1 : 2 [Hpyr]Cl-d_6/DMSO-d_6 via vigorously stirring for 12 h at 60 °C, respectively. After the biomass solution was transferred

into 5 mm NMR tube, the vial was rinsed with 2×150 mg DMSO-d_6, which is also transferred into the NMR tube for ^{1}H NMR analysis (Figure 9). With data analysis of quantitative ^{1}H NMR spectra confirming a good linear relationship between ratio of integrations and the added lignin amount (see Scheme 3), the corresponding lignin content can be calculated to be 23.3%, which is consistent with Klason lignin content and lignin content analyzed from ball-milled poplar sample.
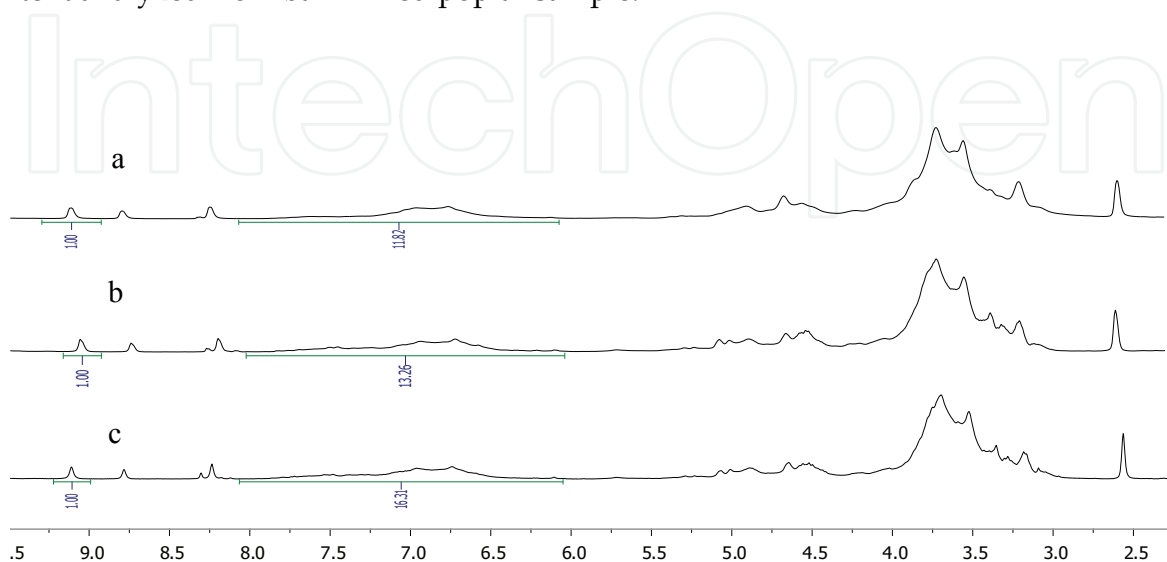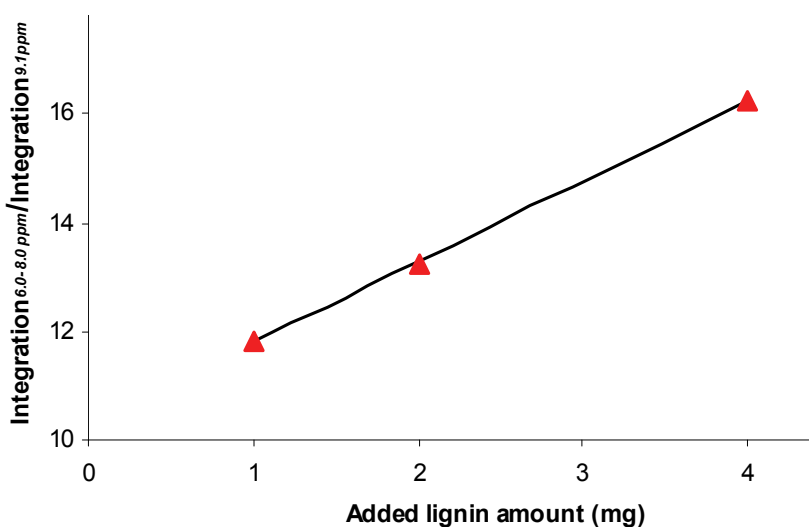

Fig. 9. ^{1}H NMR analysis of lignin content in Wiley milled poplar cell walls: a) 30.0 mg dry poplar and 1.0 mg isolated poplar lignin; b) 30.0 mg dry poplar and 2.0 mg isolated poplar lignin; c) 30.0 mg dry poplar and 4.0 mg isolated poplar lignin

Scheme 3. Linear relationship between ratios of integration of signals and added lignin amount (mg) in Wiley milled poplar samples ($Y = 10.325 + 1.4793 \times X$; lignin content = $0.0333 \times 10.325 \div 1.4793 = 23.3\%$)

Having analyzed Wiley milled poplar lignin content, we further analyzed softwood pine lignin content via three samples of 25.0 mg dry extractive-free Wiley-milled pine sample (20 mesh) with addition of 1.0 mg, 2.0 mg, or 4.0 mg isolated pine lignin, which were dissolved in 500.0 mg 1 : 2 [Hpyr]Cl-d_6/DMSO-d_6. After rinsed with 2 × 150 mg DMSO-d_6, the combined biomass solutions was analyzed by ^{1}H NMR. With data analysis of quantitative ^{1}H NMR spectra confirming a good linear relationship between ratio of integrations (signals at 9.1 ppm as the internal standard) and the added lignin amount (see Scheme 4), the corresponding lignin content can be calculated to be 28.9%, which is comparable with Klason lignin content (29.4%).
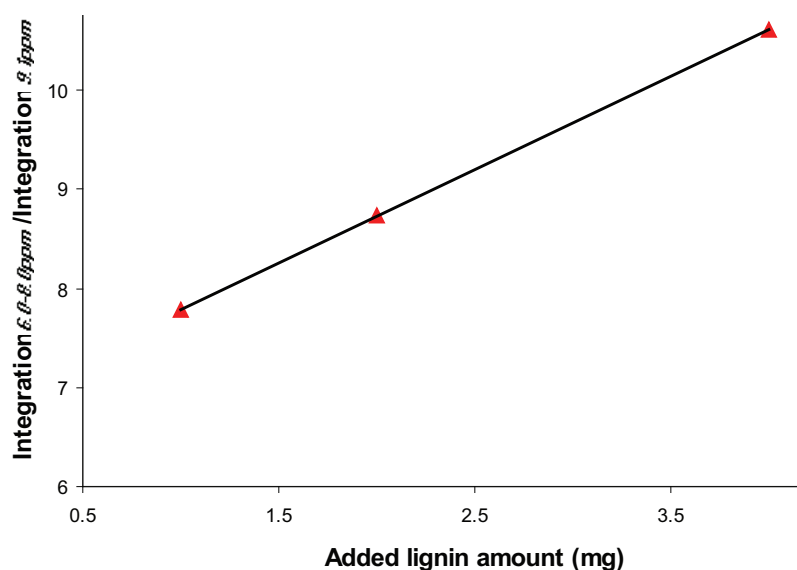
Scheme 4. Linear relationship between ratios of integration of signals and added lignin amount (mg) in 25 mg Wiley milled pine samples (Y_1 = 7.78; Y_2 = 8.74; Y_3 = 10.62; Y = 6.84 + 0.9457 × X; lignin content = 0.04 × 6.84 ÷ 0.9457 = 28.9%)

Therefore, we have applied perdeuterated pyridinium chloride for a novel methodology of efficient lignin content assessment of biomass samples (ball-milled or Wiley milled) at a microscale (Jiang, 2010), which showed comparable lignin contents as the traditional Klason lignin contents, providing a new venue for rapid assessing the lignin contents in large numbers of 'new' plants during biofuel research. This protocol significantly broadens the application of whole cell NMR analysis as the lignin content acquired by 1D ^{1}H NMR can serve as important supplemental information to the detailed structure of lignin ascertained by 2D HSQC NMR analysis.

In summary, we have successfully developed easy-accessible perdeuterated pyridinium chloride ionic liquid system for direct biomass dissolution and NMR characterization with improved efficiency of characterization by the use of Wiley milled biomass. With regard to future directions, there is still much room for increased applications of this system, such as

HSQC NMR analysis for evaluating biomass pretreatment, lignin HSG ratio determination via quantitative ^{13}C NMR and quantitative ^{31}P NMR analysis of in situ phosphitylated biomass. In addition, novel ammonium typed ionic liquids with good biomass solubility need to be developed due to strong ^{13}C NMR signals from both pyridinium cation and imidazolium cation (between 120.0 ppm and 150.0 ppm), which hinders detailed lignin aromatic ring structural analysis by ^{13}C NMR. Further developing novel ionic liquids and broadening of the scope of current perdeurated pyridinium chloride in whole cell NMR analyses, would similarly enhance better understanding of plant cell walls and recalcitrance.

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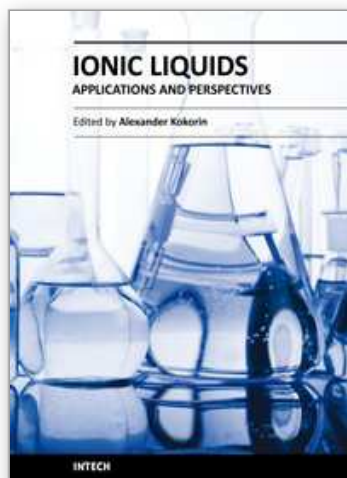
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