

HSQC-NMR analysis of lignin in woody (*Eucalyptus globulus* and *Picea abies*) and non-woody (*Agave sisalana*) ball-milled plant materials at the gel state

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Abstract

In situ analysis of lignin by 2D NMR of whole plant material was carried out by swelling finely ball-milled samples in deuterated dimethylsulfoxide (DMSO-*d*₆) and sonicated so that a gel was formed in the NMR analysis tube. Solution HSQC NMR spectra of different plant materials representative for hardwood (*Eucalyptus globulus*), softwood (*Picea abies*), and non-woody plants (*Agave sisalana*) are presented here. The spectra show signals corresponding to those of the main plant constituents, such as lignin and polysaccharides. The lignin signals were assigned by comparing the HSQC spectra of the whole plant materials with the HSQC spectra of their respective milled-wood lignins (MWLs). In general terms, the major lignin structural features, such as the relative abundances of the main lignin substructures, the syringyl/guaiacyl ratios and the extent of γ -acetylation of the lignin side-chain observed in the HSQC spectra of the whole plant materials, matched those obtained from the HSQC spectra of the isolated MWLs. Therefore, this technique, which needs only minor amounts of lignocellulosic material and minimal sample preparation, can be useful for the rapid screening of plant lignins without the need for tedious and time-consuming lignin isolation procedures.

Keywords: 2D NMR; acylation; eucalypt; heteronuclear single quantum correlation (HSQC); lignin structure; milled-wood lignin (MWL); sisal; spruce; wood.

Introduction

A main challenge in elucidating the structure of lignins is their isolation from wood or other lignocellulosic materi-

als in a chemically unaltered form. Several lignin isolation procedures have been developed. The classical procedure (Björkman 1956) consists of aqueous dioxane extraction from ball-milled wood. The yield of the resulting milled-wood lignin (MWL) is usually low (around 33% related to the Klason lignin content), although this can be increased to some extent by extension of milling time (Lai and Sarkanen 1971). Nevertheless, MWL is considered in the first instance to be approximately representative of native lignin.

Other approaches are: 1) subjecting the milled wood to enzymatic treatment with cellulolytic enzymes to remove most of the carbohydrate components (Chang et al. 1975; Wu and Argyropoulos 2003; Holtman et al. 2004; Hu et al. 2006); 2) completely dissolving ball-milled wood in a solvent system (dimethylsulfoxide, DMSO, and N-methylimidazol, NMI) followed by precipitation in dioxane/water in the course of which lignin and carbohydrate fractions are separated (Fasching et al. 2008); or 3) isolating most of the lignin as lignin-carbohydrate complexes after endoglucanase treatment (Henriksson et al. 2007). However, it is well recognized that these lignin preparations, in particular the MWL due to its low yield, represent only a part of the native lignin in the wood cell wall and may not be representative of the whole lignin present.

Indeed, it has also been demonstrated that MWL can undergo some structural modifications during isolation, especially during the milling process (Fujimoto et al. 2005; Guerra et al. 2006; Hu et al. 2006; Balakshin et al. 2008). Because lignin is intimately interpenetrating the other major components (cellulose and hemicelluloses), it is obvious that its truly native form can only be studied by analytical methods applicable directly on the whole plant material.

Nowadays, the best information on lignin structure related to phenylpropanoid units and inter-unit linkages may be derived from 2D NMR spectroscopy obtained by preparations according to the MWL method (Ralph et al. 1999, 2004; Capanema et al. 2004, 2005; Zhang et al. 2006; del Río et al. 2008; Rencoret et al. 2008a). To obtain more information about the unaltered native lignin, some other methods have recently been proposed for the “in situ” analysis of lignins by 2D NMR of whole plant materials. The application of solvent systems (including DMSO and ionic liquids, such as NMI) is the first successful attempt for fully dissolving finely ground wood that is then acetylated for purification (Lu and Ralph 2003; Kilpeläinen et al. 2007). The mechanically disintegrated and acetylated cell wall is then fully soluble in chloroform allowing the whole lignin to be analyzed

directly (in the environment of dissolved polysaccharides) by NMR. Acetylation has the advantage to make readily visible the various OH groups; however, it can result in loss of important structural information since different lignins, mainly from grasses and other herbaceous plants, are known to be naturally acetylated to a high extent (Ralph 1996; Lu and Ralph 2002; del Río et al. 2004, 2007, 2008), most probably as a mechanism for regulating lignin structure at the monolignol level (Martínez et al. 2008).

The original procedure of Lu and Ralph (2003) was later modified and a perdeuterated solvent system was applied to allow a direct in-tube dissolution and solution-state NMR of the whole cell wall without derivatization (Yelle et al. 2008). The solvent system contains NMI- d_6 , which needs to be synthesized. Other solvent systems (DMSO/LiCl) have also been proposed that dissolve whole milled wood prepared by as short as 2 h milling with no significant lignin structural change (Wang et al. 2008). Another recent and simpler approach consists of swelling finely ground plant material in DMSO- d_6 and forming a gel directly in the NMR tube, which is readily amenable to NMR analysis (Kim et al. 2007, 2008; Rencoret et al. 2008b). This method also needs finely milled plant material, but the milling time (1–4 h depending on the sample) is far lower than that needed for MWL isolation, thus structural changes of lignin due to milling is kept to a minimum.

The aim of the present work is to contribute to this topical research field. Heteronuclear single quantum correlation (HSQC) solution NMR spectra of different plant materials are presented and the spectral data are semi-quantitatively evaluated. Finely ball-milled hardwood (*Eucalyptus globulus* Labill.), softwood (*Picea abies* (L.) Karst.), and sisal (*Agave sisalana* Perrine) as a non-woody plant, were suspended in DMSO- d_6 in the NMR tube and sonicated for 10–30 min. This treatment leads to the formation of a gel, which is amenable to HSQC-NMR spectroscopy. The spectra will be compared with those of respectively isolated MWL preparations.

Experimental

Samples

E. globulus and *P. abies* wood and *A. sisalana* fibers were grounded in a Retsch cutting mill to pass through a 100-mesh screen and then successively extracted with acetone for 8 h in a Soxhlet extractor and with hot water (100°C) for 3 h. Klason lignin content was estimated according to Tappi standard T 222 om-88 (Tappi 2004). The extracted sawdust was finely ball-milled in a Retsch S100 centrifugal ball at 400 rpm (around 10 h) in an agate jar and the balls were from the same material.

Gel formation

Approximately 100 mg of finely ball-milled wood was suspended in 0.75 ml of DMSO- d_6 in the NMR tube and sonicated for 10–30 min, depending on the sample, in an ultrasonic cleaning bath (Ultrasons JS 3000513 from Selecta, with a frequency of 40 kHz and 150 W power consumption), until a homogeneous gel was formed, and HSQC spectra were recorded.

MWL

Plant material (free of extractives and hot water solubles) was ball milled in toluene during 150 h. Lignin was extracted by dioxane/water (9:1, v/v), followed by evaporation of the solvent, and purified as described previously (Björkman 1956). The final yields ranged from 10% to 15% based on the Klason lignin content. Around 40 mg of MWL was dissolved in 0.75 ml of DMSO- d_6 for the HSQC experiments.

NMR

2D NMR spectra were recorded at 25°C on a Bruker AVANCE 500 MHz equipped with a z-gradient triple resonance probe in HSQC experiments. The spectral widths were 5000 Hz and 25 000 Hz for the ^1H - and ^{13}C -dimensions, respectively. The number of collected complex points was 2048 for ^1H -dimension with a recycle delay of 5 s. The number of transients was 64, and 256 time increments were always recorded in ^{13}C -dimension. The $^1J_{\text{CH}}$ used was 140 Hz. The J -coupling evolution delay was set to 3.2 ms. Squared cosine-bell apodization function was applied in both dimensions. Prior to Fourier transform, the data matrixes were zero filled up to 1024 points in the ^{13}C -dimension. The central solvent (DMSO) peak was used as an internal chemical shift reference point (δ_{C} 40.1; δ_{H} 2.50 ppm).

A semiquantitative analysis of the intensities of the HSQC cross-signal was performed (Heikkinen et al. 2003; Liitiä et al. 2003; Zhang and Gellerstedt 2007). Since the cross-signal intensity depends on the particular $^1J_{\text{CH}}$ value, as well on the T_2 relaxation time, a direct analysis of the intensities is elusive. Thus, the integration of the cross-signals was performed separately for the different regions of the HSQC spectra, which contain signals that correspond to chemically analogous carbon-proton pairs. For these signals, the $^1J_{\text{CH}}$ coupling value is relatively similar, their chemical shifts are also similar to each other, hence the error of off-resonance effect is small, and therefore can be used semiquantitatively to estimate the relative abundance of the different species. In the aliphatic oxygenated region, the relative abundance of the different inter-unit linkages were estimated from $\text{C}_\alpha\text{-H}_\alpha$ correlations (except for cinnamyl alcohol end-groups where $\text{C}_\gamma\text{-H}_\gamma$ correlations were used) to avoid possible interference from homonuclear $^1\text{H}\text{-}^1\text{H}$ couplings, and the relative abundance of side-chains involved in the different inter-unit linkages were calculated. In the aromatic region, $\text{C}_{2,6}\text{-H}_{2,6}$ correlations from S units and $\text{C}_2\text{-H}_2$ plus $\text{C}_6\text{-H}_6$ correlations from G units were used to estimate the S/G ratio of lignin. The percentage of lignin acylation in sisal was obtained by integrating the signals from $\text{C}_\beta\text{-H}_\beta$ correlations in acylated and non-acylated $\beta\text{-O-4'}$ structures.

Results and discussion

The HSQC-NMR spectra of the whole plant materials selected for this study, together with the spectra of their respective MWL, are presented in Figure 1. Carbohydrate signals were predominant. They included correlations in the range $\delta_{\text{C}}/\delta_{\text{H}}$ 60–85/2.5–5.5 ppm that partially overlapped with some lignin signals, and the well-resolved anomeric correlations in the range $\delta_{\text{C}}/\delta_{\text{H}}$ 95–105/4.2–5.5 ppm. Gel spectra were compared with spectra obtained from the same samples after complete acetylation as described by Lu and Ralph (2003) (data not shown). The comparison showed lower intensities of the cellulose signals (compared with those of the hemicelluloses and lignin signals) when the spectra were

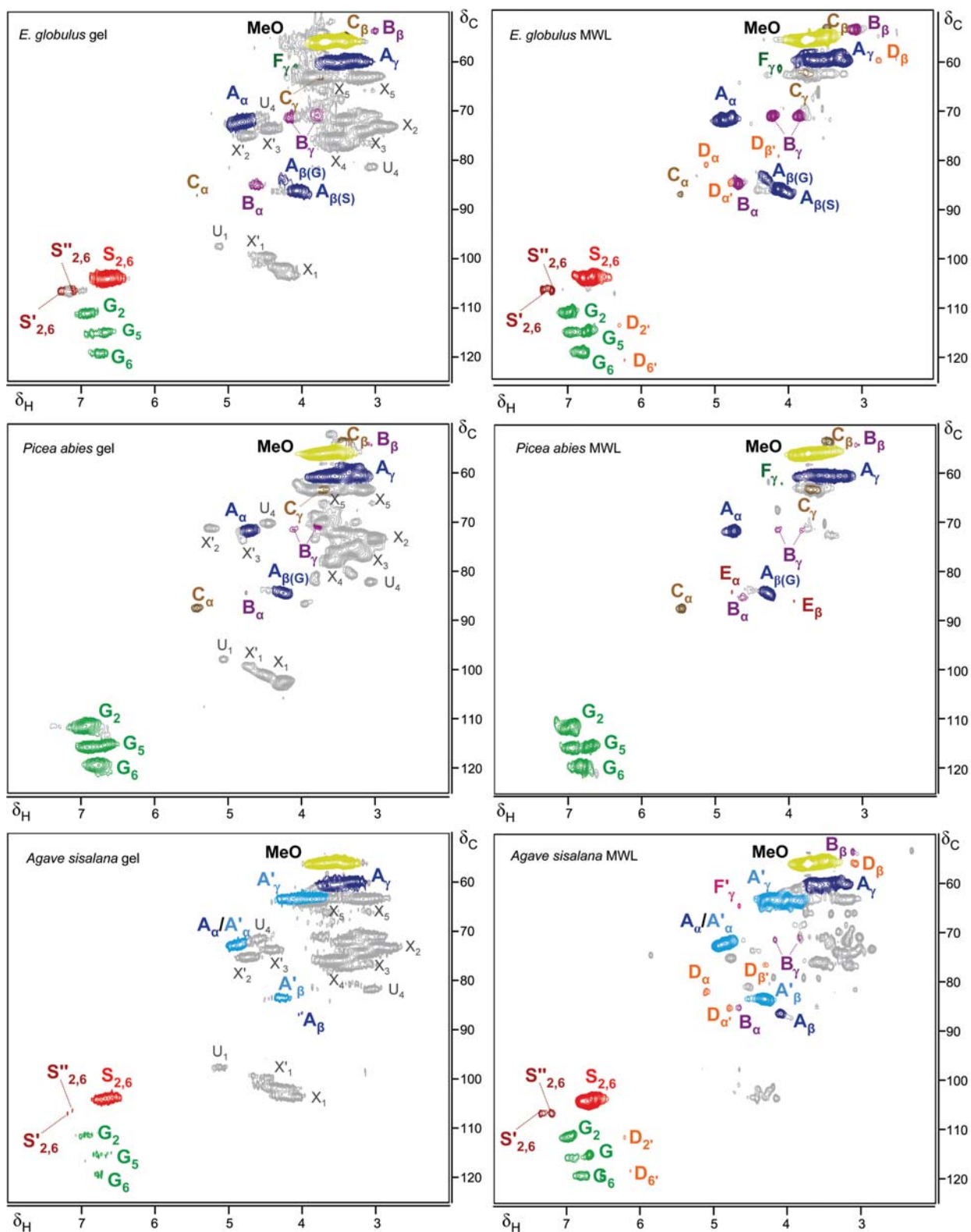


Figure 1 Expanded HSQC NMR (δ_C/δ_H 50–125/2.0–8.0 ppm) spectra of eucalypt wood, spruce wood and sisal fibers, and of their respective MWL preparations. The lignin structures identified, and the main polysaccharide units, are presented in Figure 2. See Table 1 for assignment of the different lignin cross-signals.

acquired at the gel state. This fact, which is probably related to the crystalline moiety of cellulose, is an advantage for the analysis of lignins as already pointed out by Kim et al. (2008). Therefore, hemicelluloses cross-signals were predominant in the gel NMR spectra obtained,

which were tentatively assigned to ‘normal’ (X) and partially acetylated (X’) neutral sugars, and 4-methyluronic acid (U) units.

Cellulose signals were also observed being partially overlapping with some of the signals of lignin and hemi-

celluloses. The hemicelluloses signals would correspond to 4-methylglucuronic-substituted and partially-acetylated xylan in eucalypt and sisal, and partially-acetylated glucomannan (and some pectin) in spruce, in agreement with previous studies (Evtuguin et al. 2003; Hannuksela and Hervé du Penhoat 2004; Marques et al. 2008). Xylose acetylation (at C2/C3) would also affect the chemical shifts of contiguous carbons, and the main effect on the acetate-bearing C is discussed below. The existence of these two types of hemicelluloses was evidenced by differences in the anomeric cross-signals, as shown for similar samples (Yelle et al. 2008), but especially by fingerprint C₂-H₂ correlations for the acetylated xylose (δ_C/δ_H 75.4/4.74 ppm) and mannose units (δ_C/δ_H 71.1/5.23 ppm). A similar acetylation degree at the xylose C₃-position of both eucalypt and sisal xylan was evidenced by the corresponding signal (δ_C/δ_H 73.7/4.43 ppm). Interestingly, lignin signals could also be clearly observed in all the HSQC spectra of whole plant materials, despite the fact that lignin is not their main constituent (27% and 18% Klason lignin content for spruce and eucalypt, respectively, and only 11% Klason lignin for sisal). Unequivocal assignment of the lignin signals was obtained by comparing the HSQC spectra of the whole plant materials with those of their respective MWL. Other 2D NMR studies on lignin reported in the literature were helpful for signal assignment (Ämmälähti et al. 1998; Ralph et al. 1999, 2004; Liitiä et al. 2003; Capanema et al. 2004, 2005; Ibarra et al. 2007a,b; del Río et al. 2008; Rencoret et al. 2008a). The main lignin cross-signals observed in the HSQC spectra are listed in Table 1, and the main lignin substructures identified in the plant materials are presented in Figure 2.

The HSQC spectra presented in Figure 1 show only the region δ_C/δ_H 50–125/2.0–8.0 ppm, where the main oxygenated aliphatic and aromatic lignin signals appear. All the spectra from whole plant materials showed prominent signals corresponding to β -O-4' alkyl-aryl ether linkages (substructures **A** and **A'**), as also observed in the HSQC of their respective MWL. The C _{γ} -H _{γ} correlations in β -O-4' substructures in eucalypt and spruce woods were observed at δ_C/δ_H 60.0/3.38 and 3.71 ppm, partially overlapped with cellulose C₆-H₆ cross-signals. The HSQC spectrum of sisal fibers clearly shows the presence of intense signals corresponding to acylated γ -carbon in the range between δ_C/δ_H 63.5/3.83 and 4.30 ppm, together with the presence of signals from normal hydroxylated γ -carbon (at δ_C/δ_H 60.0/3.38 and 3.71 ppm), as can also be observed in the HSQC of sisal MWL. The HSQC spectrum of sisal fibers, therefore, undoubtedly demonstrates that this lignin is extensively acylated and that acylation takes place only at the γ -position of the lignin side-chain. Previous studies have indicated that acylation in sisal occurs exclusively with acetate groups and predominantly over syringyl units (del Río et al. 2004, 2007, 2008). The presence of acetylated lignin units in the HSQC of sisal fibers excludes the possibility of acetylation as an artifact produced during the MWL isolation protocol.

The C _{α} -H _{α} correlations in β -O-4' substructures (**A** and **A'**) were observed at δ_C/δ_H 72.3/4.86 ppm for β -O-4' substructures linked to S units (in eucalypt wood and sisal fibers) and at δ_C/δ_H 71.6/4.73 ppm for β -O-4' substructures linked to G units (in spruce wood). The C _{β} -H _{β} correlations were observed in the range at δ_C/δ_H 83.6–86.5/4.10–4.64 ppm, and also differed among the samples. In

Table 1 Assignment of main lignin ¹³C-¹H cross-signals in the HSQC spectra shown in Figure 1.

Labels	δ_C/δ_H (ppm)	Assignment
B _{β}	53.7/3.12	C _{β} -H _{β} in β - β' (resinol) substructures (B)
C _{β}	53.7/3.44	C _{β} -H _{β} in β -5' (phenylcoumaran) substructures (C)
A _{γ}	60.0/3.38–3.71	C _{γ} -H _{γ} in β -O-4' substructures (A) and others
D _{β}	60.3/2.75	C _{β} -H _{β} in β -1' (spirodienone) substructures (D)
F _{γ}	61.9/4.09	C _{γ} -H _{γ} in cinnamyl alcohol end-groups (F)
C _{γ}	63.3/3.66	C _{γ} -H _{γ} in β -5' (phenylcoumaran) substructures (C)
A' _{γ}	63.8/3.83–4.30	C _{γ} -H _{γ} in γ -acetylated β -O-4' substructures (A') and others
F' _{γ}	64.7/4.67	C _{γ} -H _{γ} in cinnamyl acetate end-groups (F')
B _{γ}	71.7/3.81 and 4.17	C _{γ} -H _{γ} in β - β' (resinol) substructures (B)
A _{α} /A' _{α}	72.3/4.86 and 71.6/4.73	C _{α} -H _{α} in β -O-4' substructures (A and A')
D _{β'}	79.8/4.11	C _{β} -H _{β} in β -1' (spirodienone) substructures (D)
D _{α}	81.7/5.09	C _{α} -H _{α} in β -1' (spirodienone) substructures (D)
A' _{β}	83.6/4.32	C _{β} -H _{β} in γ -acetylated β -O-4' substructures (A')
E _{α}	83.6/4.83	C _{α} -H _{α} in 5-5' (dibenzodioxocin) substructures (E)
A _{β} (G)	84.1/4.28	C _{β} -H _{β} in β -O-4' substructures (A) linked to a G unit
B _{α}	85.4/4.64	C _{α} -H _{α} in β - β' (resinol) substructures (B)
E _{β}	85.9/3.88	C _{β} -H _{β} in 5-5' (dibenzodioxocin) substructures (E)
A _{β} (S)	86.5/4.10	C _{β} -H _{β} in β -O-4' substructures (A) linked to a S unit
D _{α'}	86.6/4.39	C _{α} -H _{α} in β -1' (spirodienone) substructures (D)
C _{α}	87.7/5.45	C _{α} -H _{α} in β -5' (phenylcoumaran) substructures (C)
S _{2,6}	103.8/6.68	C ₂ -H ₂ and C ₆ -H ₆ in syringyl units (S)
S' _{2,6} S'' _{2,6}	106.7/7.36 and 7.21	C ₂ -H ₂ and C ₆ -H ₆ in C _{α} -oxidized syringyl units (S' and S'')
G ₂	111.5/6.99	C ₂ -H ₂ in guaiacyl units (G)
D _{2'}	114.1/6.26	C ₂ -H ₂ in β -1' (spirodienone) substructures (D)
G ₅	115.2/6.71 and 6.94	C ₅ -H ₅ in guaiacyl units (G)
D _{6'}	119.5/6.08	C ₆ -H ₆ in β -1' (spirodienone) substructures (D)
G ₆	119.5/6.83	C ₆ -H ₆ in guaiacyl units (G)

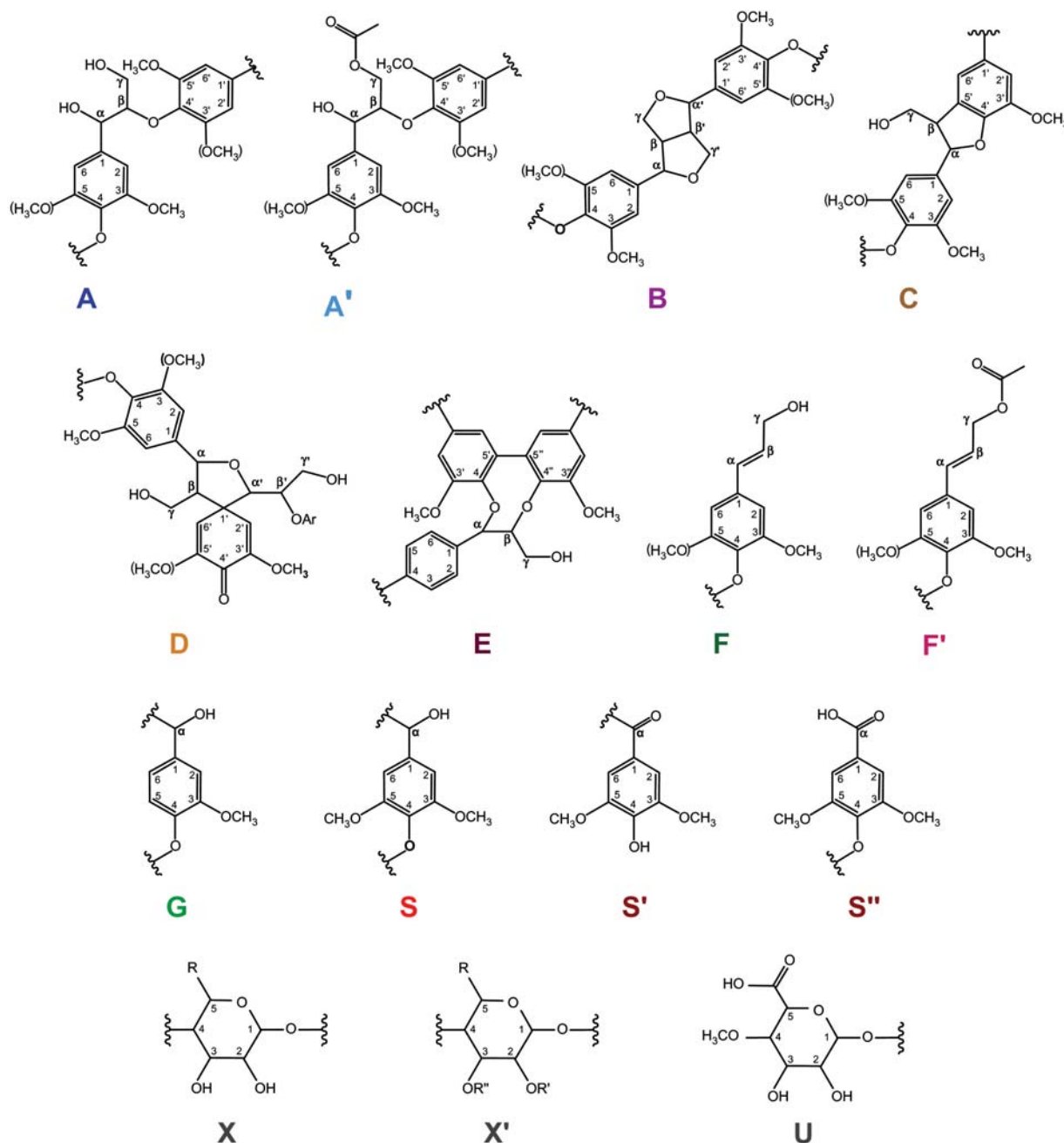


Figure 2 Lignin substructures, and main polysaccharide units, observed in the HSQC spectra: (A) β -O-4' linkages; (A') β -O-4' linkages with γ -acetyl groups; (B) resinol structures formed by β - β' , α -O- γ' , and γ -O- α' linkages; (C) phenylcoumaran structures formed by β -5' and α -O-4' linkages; (D) spirodienone structures formed by β -1', α -O- α' linkages; (E) dibenzodioxocin structures formed by 5'-5'', α -O-4' and β -O-4'' linkages; (F) cinnamyl alcohol end-groups; (F') cinnamyl acetate end-groups; (G) guaiacyl unit; (S) syringyl unit; (S') oxidized syringyl unit bearing a carbonyl group at C $_{\alpha}$ (phenolic); (S'') oxidized syringyl unit bearing a carboxyl group at C $_{\alpha}$; (X) xylopyranose (R, OH) or gluco/mannopyranose (R, CH $_2$ OH) units; (X') acetylated xylopyranose (R, OH) or acetylated gluco/mannopyranose (R, CH $_2$ OH) units (R' or R'', CH $_3$ -C=O); and (U) 4-O-methylglucuronic/4-O-methylgalacturonic acid.

eucalypt wood, the C $_{\beta}$ -H $_{\beta}$ correlations were observed at δ_C/δ_H 86.5/4.10 in β -O-4' substructures linked to an S unit and at δ_C/δ_H 84.1/4.28 in β -O-4' substructures linked to a G unit. In spruce, only the latest C $_{\beta}$ -H $_{\beta}$ correlation (typical of a G-lignin) was observed, while in sisal the C $_{\beta}$ -H $_{\beta}$ correlations were observed at δ_C/δ_H 86.5/4.10 ppm in normal γ -hydroxylated β -O-4' aryl ether substructures (A) but shifted to δ_C/δ_H 83.6/4.32 ppm in γ -acylated β -O-4' substructures (A'). Therefore, it should be possible to use these differentiating C $_{\beta}$ -H $_{\beta}$ correlations in acylated and

non-acylated β -O-4' structures to estimate the percentage of lignin acylation in sisal.

Unfortunately, the signal for the C $_{\beta}$ -H $_{\beta}$ correlations in acylated β -O-4' structures (δ_C/δ_H 83.6/4.32 ppm) could overlap with the C $_{\beta}$ -H $_{\beta}$ correlation signal in those of non-acylated β -O-4' substructures including a second G unit, as found in eucalypt gel and MWL (δ_C/δ_H 84.1/4.28 ppm) and special attention must therefore be given when estimating the extent of lignin acylation. However, the presence of γ -acylating groups in sisal β -O-4' structures is

confirmed by the strong C_γ - H_γ correlation signal (δ_C/δ_H 64/3.8–4.3 ppm). This conclusion is also supported by modified DFRC analyses (del Río et al. 2008).

Signals for other lignin substructures were also observed in the HSQC spectra of the plant materials. Some of these signals correspond to minor structures, which can only be clearly observed in the HSQC of the MWLs. Strong signals for resinol (β - β' / α - O - γ' / γ - O - α') substructures (**B**) were observed in the HSQC spectrum of eucalypt wood, with their C_α - H_α , C_β - H_β and the double C_γ - H_γ correlations at δ_C/δ_H 85.5/4.64, 53.7/3.12 and 71.7/3.83 and 4.30 ppm, respectively. Resinol substructures (**B**) were also observed, although in lower amounts, in the HSQC spectrum of spruce wood. Resinols (**B**) could not be detected in the HSQC spectrum of sisal fibers due to its very low lignin content and the low abundance of these substructures owing to blocking of the γ -carbon in acetylated monolignols (del Río et al. 2008; Martínez et al. 2008); nevertheless, small signals could be seen in the HSQC spectrum of its MWL.

Phenylcoumaran (β -5'/ α - O -4) substructures (**C**) were observed in the HSQC spectrum of spruce and eucalypt woods, the signals for their C_α - H_α and C_β - H_β correlations being observed at δ_C/δ_H 87.7/5.45 and 53.7/3.44 ppm, respectively, and that of C_γ - H_γ correlation overlapping with xylan C_5 - H_5 cross-signals around δ_C/δ_H 63.3/3.66 ppm. Signals for spirodienone substructures (**D**) were clearly seen in the HSQC spectra of the MWL of eucalypt and sisal, with the C_α - H_α , C_α - $H_{\alpha'}$, C_β - H_β , and C_β - $H_{\beta'}$ correlations at δ_C/δ_H 81.7/5.09, 86.6/4.39, 60.3/2.75, and 79.8/4.11 ppm, respectively. Spirodienones could only be observed in the HSQC of eucalypt wood with low intensity. Likewise, dibenzodioxocin structures (**E**), which were clearly observed in the HSQC of the MWL of spruce, with the C_α - H_α and C_β - H_β correlations at δ_C/δ_H 83.6/4.83 and 85.9/3.88, respectively, were observed with low intensity in the HSQC spectrum of spruce wood.

Finally, cinnamyl alcohol end-groups (**F**) were only observed in the HSQC of eucalypt wood, with the C_γ - H_γ correlation at δ_C/δ_H 61.9/4.09 ppm, but were not detected in the HSQC of spruce wood due to their low abundance. Moreover, in sisal MWL, the cinnamyl alcohol end-groups (**F'**) were found to be acetylated at the γ -carbon and the signal of the C_γ - H_γ correlation shifted to δ_C/δ_H 64.0/4.69 ppm, though this signal could not be observed in the HSQC spectrum of sisal fibers.

The main lignin cross-signals present in the aromatic region of the HSQC spectra correspond to the aromatic rings of the different lignin units. Signals from syringyl (S) and guaiacyl (G) units could be clearly observed in all the spectra of whole plant materials matching those of their respective signals in MWLs (Figure 1). Eucalypt and sisal (two angiosperms) contained S and G units, while spruce (a gymnosperm) had only G units. No traces of *p*-hydroxyphenyl (H) units could be observed in any of the spectra of the plant materials, in accordance with their absence in the HSQC spectra of their MWL preparations.

The S units showed a prominent signal for the $C_{2,6}$ - $H_{2,6}$ correlation at δ_C/δ_H 103.8/6.68 ppm, while the G units showed different correlations for C_2 - H_2 (δ_C/δ_H 111.5/6.99 ppm), C_5 - H_5 (δ_C/δ_H 115.2/6.71 and 6.94), and C_6 - H_6 (δ_C/δ_H 119.5/6.83 ppm). The double C_5 - H_5 signal revealed some heterogeneity among the G units, especially affecting the C_5 - H_5 correlation, probably because it is due to different substituents at C_4 (e.g., phenolic or etherified in different substructures). The strong displacement of the cross-signal at δ_C/δ_H 115.2/6.94 after *in vitro* acetylation (not shown here) indicates that this signal corresponds to phenolic G units. Signals corresponding to $C_{2,6}$ - $H_{2,6}$ correlations in C_α -oxidized S units (**S'** and **S''**) (δ_C/δ_H 106.7/7.36 and 7.21 ppm, respectively) (Ralph et al. 2004; Ibarra et al. 2007b) were present in the HSQC spectra of the eucalypt wood and sisal fiber, as also observed in their MWL. The relative abundance of these oxidized moieties estimated from the HSQC is similar (approximately 10% of total S-lignin units) in the whole eucalypt wood and in its MWL. This fact indicates that these moieties might exist as such in the native eucalypt lignin, although their provenance from the milling process cannot be completely ruled out. In the case of sisal fibers, the signals were very weak in the HSQC spectrum.

The major lignin structural features are listed in Table 2 for both plant material and MWL, such as the percentage of various side-chains, the S/G ratios, and the percentage of γ -acylation (estimated from the C_β - H_β correlations in acylated and non-acylated β - O -4' structures). The relative abundance of various types of side-chains were calculated and referred to the amount of total side-chains. As expected, the main lignin substructures were the β - O -4'-linked ones, followed by β - β' resinol substructures in the case of eucalypt wood and β -5' phenylcoumaran and β - β' resinol substructures in spruce

Table 2 Relative abundance of main inter-unit linkages (as percentages of side-chains involved), percentage of γ -acylation of the side-chain, and S/G ratio of lignin in the selected lignocellulosic materials.

	Eucalypt		Spruce		Sisal	
	Wood	MWL	Wood	MWL	Fiber	MWL
Linkage abundance (%)						
β - O -4' aryl ether (A,A')	80	76	69	65	100	85
β - β' resinols (B)	14	17	10	11	0	4
β -5' phenylcoumarans (C)	2	2	18	18	0	2
β -1' spirodienones (D)	2	2	0	0	0	7
5-5' dibenzodioxocins (E)	0	0	3	5	0	0
Cinnamyl alcohol end-groups (F,F')	2	3	0	1	0	2
Percentage of γ -acylation ^a	0	0	0	0	70	72
S/G ratio	2.8	2.9	0	0	3.6	3.9

^aEstimated from the C_β - H_β correlations in acylated and non-acylated β - O -4' structures.

wood. In sisal fibers, the β -O-4' aryl ether linkage was also the most predominant with only traces of other structures that could only be observed in its MWL. Structures present in low abundances (i.e., spirodienones and dibenzodioxocins) could hardly be seen in the HSQC spectra of whole plant materials. This is especially true for sisal due to its low lignin content. Moreover, the lignin from sisal is extensively acetylated at the γ -carbon of the side-chain, being very different from the other two lignins. The percentage of lignin acetylation (70%) of sisal matched that calculated from the HSQC of its MWL (del Río et al. 2008). Finally, the lignin S/G ratios calculated from the HSQC spectra of the whole plant materials also closely matched those calculated from the MWL preparations.

Conclusions

HSQC-NMR of DMSO- d_6 gels of ball-milled plant materials seems to be an efficient method for the rapid "in situ" analysis of lignin in plants, without the need of prior lignin isolation. The method requires only very low amounts of sample and can be used for rapid characterization of the major structural features of plant lignins (i.e., inter-unit linkages, S/G ratios and presence and extent of γ -acylation of the lignin side-chain). The main lignin structures as well as the S/G ratios and the extent of lignin acetylation closely matched those obtained from the MWL. The similarities between the HSQC of whole plant materials and the isolated MWL indicate that MWL is still a reliable and representative preparation of the native lignin that can be used for comprehensive lignin structural analysis. Although ball-milling and sonication would induce some structural changes, the low milling and sonication times used keeps the possibilities of lignin structural modification to the minimum. However, a major drawback of the method is the inability to perform HMBC experiments and to detect minor lignin structures in plant materials with low lignin contents.

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