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TECHNICAL ADVANCE

Non-degradative dissolution and acetylation of ball-milled plant cell walls: high-resolution solution-state NMR

Fachuang Lu[1,2] and John Ralph[1,2,*]
[1]*U.S. Dairy Forage Research Center, USDA-Agricultural Research Service, Madison, WI 53706, USA, and*
[2]*Department of Forestry, University of Wisconsin, Madison, WI 53706, USA*

Received 17 February 2003; revised 31 March 2003; accepted 13 May 2003.
*For correspondence (fax +1 608 264 5147; e-mail jralph@wisc.edu).

Summary

Two solvent systems for fully dissolving, and optionally derivatizing, finely ground plant cell wall material at room temperature are described: dimethylsulfoxide (DMSO) and tetrabutylammonium fluoride (TBAF) or *N*-methylimidazole (NMI). *In situ* acetylation produces acetylated cell walls (Ac-CWs) that are fully soluble in chloroform. Lignin structures tested remain fully intact. The dispersion of ^{13}C–^{1}H correlations afforded by two-dimensional (2D) nuclear magnetic resonance (NMR) experiments reveals the major lignin units, allowing the whole lignin fraction to be analyzed by high-resolution solution-state NMR methods for the first time. Non-degradative cell wall dissolution offers the potential to analyze polysaccharide components, and improve current cell wall analytical methods by using standard homogeneous solution-state chemistry.

Keywords: lignin, lignin model compound, cellulose, polysaccharide, hemicellulose, 2D NMR.

Introduction

The ability to dissolve plant cell walls without degradation would provide significantly improved methods for cell wall structural analysis and allow standard solution-state derivatization and reaction chemistries to be more effectively applied.

Plant cell walls are composite materials with complex structures. In higher plants, the walls are mainly composed of polysaccharides (cellulose and various hemicelluloses) and lignins (Jung *et al.*, 1993; Lewis and Paice, 1989). Derived primarily from *p*-hydroxycinnamyl alcohols, lignins result from radical-coupling polymerization reactions in the wall after the polysaccharides have been laid down. They provide a matrix in which the polysaccharides become embedded and possibly cross-linked, forming an extensive three-dimensional (3D) polymer network. The diversity of polymers in the wall and their chemical and physical associations make it difficult to isolate the component polymers in pure form. Because of its highly regular and crystalline form, cellulose is most readily separated, yet freeing cellulose from hemicelluloses and lignin remains an energy-demanding process; cellulose is purified industrially in the kraft-pulping processes by typically treating the cell walls in approximately 2 M caustic soda and sodium sulfide at 170°C for 2 h. Hemicelluloses comprise a class of polysaccharides with considerable structural complexity. Fractions can be isolated by various extraction procedures, but these are usually accompanied by degradation (Aspinall, 1982). The enigmatic lignins remain best described as phenylpropanoid combinatorial polymers formed under simple chemical control without regular defined structures (Baucher *et al.,* 2003). Lignins are difficult to extract intact. Fractions, usually still associated with polysaccharides, can be solvent-extracted following fine milling, notably ball-milling (Björkman, 1954); larger fractions can be obtained by enzymatically digesting polysaccharides using crude polysaccharidases before solvent extraction (Chang *et al.*, 1975).

A great deal of interest has focused on the lignin component. While fulfilling crucial support, water conduction, and defense roles for the plant, it is this component that limits polysaccharide utilization in various natural and industrial processes; for example, lignins inhibit cell wall digestibility in ruminant animals (Jung and Deetz, 1993), and must be degraded to liberate cellulose fibers for making fine paper. There has been considerable effort recently on utilizing biogenetic methods targeting genes in the

monolignol biosynthetic pathway to reduce lignin amounts or alter lignin structure in the wall for improved processing potential (Baucher *et al.*, 2003; Boudet and Grima-Pettenati, 1996; Chiang, 2002; Dixon and Ni, 1996; Whetten and Sederoff, 1991). Currently, however, analysis of the lignin component is via destructive methods, or via nuclear magnetic resonance (NMR) of isolated lignins (Ralph *et al.*, 1999) — solid-state NMR does not have the resolution required to provide sufficient structural detail. But lignin isolation as a 'milled wood lignin' (MWL) may yield as little as 15% of the lignin in a form suitable for solution-state NMR (Ralph *et al.*, 1997).

Researchers have therefore been anxious to develop methods that might allow the entire lignin fraction, and indeed the whole cell wall, to be analyzed by NMR. In the past, we and others have tried to apply the various cellulose solvent systems to the whole cell wall, but with little success. Other dissolution methods are simply too destructive on the components of the wall. Here we describe two solvent systems that fully dissolve finely divided (vibratory ball-milled) cell walls, apparently non-degradatively. Ball-milling is commonly used in lignin isolation procedures. *In situ* acetylation is demonstrated for its value in providing derivatized cell walls that are completely soluble in chloroform. The derivatized cell wall can therefore be analyzed by high-resolution solution-state NMR methods, providing considerable insight into wall structure without the need for polymer fractionation.

Results and discussion

Cell wall dissolution in DMSO–TBAF

Recently, a combination of dimethylsulfoxide (DMSO) and tetrabutylammonium fluoride (TBAF) has been used for dissolution of cellulose and as a system for its efficient acetylation (Heinze *et al.*, 2000). This report attracted our attention because DMSO is also a good solvent for isolated lignins. We thought that DMSO–TBAF might have a potential to dissolve the whole cell wall. Although a preliminary trial with the ground wood cell wall material was discouraging, ball-milled walls were readily and completely dissolved in the system at room temperature. Moreover, many reactions, including acylation and etherification, could be performed in the binary solvent system, resulting in derivatized cell walls soluble in many organic solvents. For example, complete methylation of phenolic hydroxyls of *p*-coumarates and ferulates in maize was effected within 30 min by simply adding methyl iodide.

Fully acetylated cell wall material (made simply by adding acetic anhydride without additional catalysts, following or during the dissolution process) was completely soluble in solvents such as methylene chloride or chloroform, and largely soluble in acetone, all solvents ideal for NMR. NMR showed that the cellulose, as had been reported previously by Heinze *et al.* (2000), and the lignin component remained uncompromised. Reactions with various lignin model compounds confirmed that the primary structures in lignins remained unaltered in this system. Models included dimeric phenylcoumaran (β–β), pinoresinol (β-5), and β-ether (β–O−4) compounds. The free-phenolic phenylcoumaran model is the most sensitive to base as the phenylcoumaran ring can open. TBAF is a strong base, but the model remained completely intact after the procedures.

Solution-state 2D NMR of the acetylated whole cell walls was able to resolve the major lignin structures from the dominant polysaccharides. However, the polysaccharide-derived NMR spectral regions were excessively complex. It appeared that hemicelluloses were undergoing oxidation or structural alteration of some kind; DMSO–acetic anhydride is a powerful oxidizing system (Albright and Goldman, 1965). The excess acetic anhydride required in this system is responsible. If samples were instead acetylated by addition of excess *N*-methylimidazole (NMI) and acetic anhydride, the products became indistinguishable from those resulting from the DMSO–NMI system described below. This dissolution system, therefore, is particularly useful for etherification reactions and will continue to be pursued for analytical applications. However, despite this being the best system encountered for dissolving the entire cell wall, for the purpose of obtaining derivatized cell walls for high-resolution solution-state NMR spectroscopy, it has been superceded by the following more direct alternative.

Cell wall dissolution in DMSO–NMI

A system not previously described as a cell wall or even a cellulose solvent system has the properties required to produce cell wall materials in a form suitable for NMR; it rapidly and completely dissolves the wall, allows for facile derivatization reactions such as acetylation, and can be used to generate products ideal for solution-state NMR. The system is NMI in DMSO (DMSO–NMI). Complete dissolution of a variety of ball-milled cell wall materials can be effected within 1–3 h at room temperature. The solutions are homogeneous and clear. *N*-methylimidazole is an excellent acylation catalyst, so *in situ* acetylation (for example) is readily accomplished. Oxidation reactions (of the DMSO-acetic anhydride system) are not evidenced in the presence of NMI. The acetylated cell wall (Ac-CW) is readily isolated, essentially quantitatively, by precipitating into water. It is completely soluble in chloroform or methylene chloride.

NMR of acetylated cell walls

Figure 1 shows a picture of NMR tubes of Ac-CWs from pine and aspen woods, *c.* 150 mg ml^{-1} in deuterochloroform

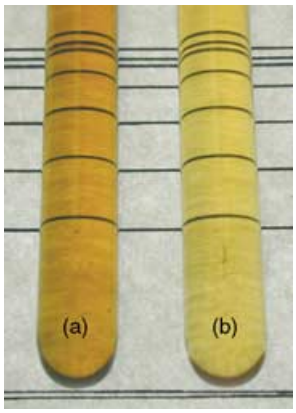


Figure 1. Plant cell walls can be solubilized for NMR.
The picture shows NMR tubes containing solutions of Ac-CWs from solvent-extracted ball-milled (a) pine wood and (b) aspen wood from the DMSO–NMI system. Over 100 mg is dissolved in $CDCl_3$ (0.5 ml) in 5-mm tubes. The homogeneous solutions are viscous, but clear, as evident from the transmitted lines (from a paper-plot placed behind the tubes).

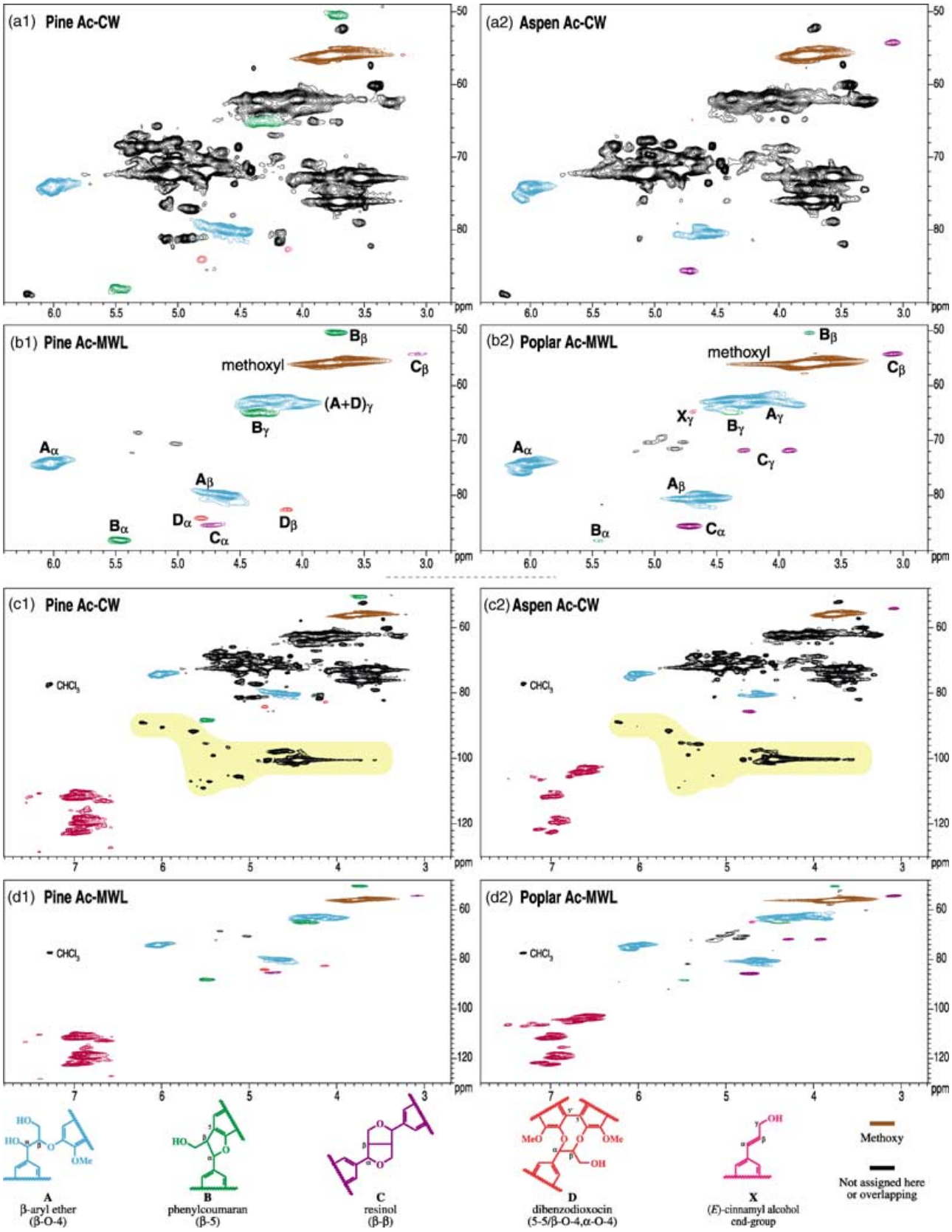
($CDCl_3$); the clarity of the solutions is demonstrated by the background (part of a plotted proton NMR spectrum) showing through the tubes. The solutions are rather viscous at over about 200 mg ml^{-1}, but quite mobile at less than 150 mg ml^{-1}. Such concentrations are quite suitable for NMR. However, the lignin component of interest, typically only approximately 20% of the cell wall, is now considerably more dilute than in samples of comparable total concentration prepared directly from isolated lignins. The fivefold sensitivity decrease can be offset by utilizing higher-field instruments and/or cryogenically cooled probes (see later in Figure 4). Although most of the metals associated with the cell walls (CWs) are removed following the acetylation and precipitation, washing of the chloroform solutions with EDTA (6 mM, pH = 8) helps remove additional traces and improves the NMR relaxation behavior (Ralph *et al.*, 1994, 1999).

Although the spectra were expected to be dominated by polysaccharide peaks, the various (acetylated) cellulose, hemicelluloses, and lignin resonances dispersed well in 2D NMR, allowing substantive interpretation. Here we concentrate on the lignin assignments. Figure 2 shows 1-bond ^{13}C–^{1}H correlation spectra taken on a 360-MHz spectrometer. The upper plots show the lignin side-chain regions of the Ac-CW spectra and, for comparison and aid in spectral assignment, the same regions from spectra of isolated lignins from similar (but not identical) samples.

As seen from the color coding in Figure 2(b1) (Ralph *et al.*, 1999), the diagnostic lignin methoxyl, major β-ether units **A**, substantial phenylcoumaran units **B**, and more minor resinol **C** and dibenzodioxocin **D** units are well resolved in the guaiacyl-acetylated MWL (Ac-MWL) from pine. The predominantly guaiacyl (4-hydroxy-3-methoxyphenyl) lignins in pine (and softwoods in general) derive from the mono-methoxylated monomer coniferyl (4-hydroxy-3-methoxycinnamyl) alcohol. At least some of the side-chain correlations for all of these structures are also remarkably well resolved in the pine Ac-CW spectrum (Figure 2a1).

Hardwood lignins, including the poplar lignin shown on the right of Figure 2, derive more substantially from sinapyl (3,5-dimethoxy-4-hydroxycinnamyl) alcohol and have syringyl/guaiacyl lignins — see Figure 5 below for distinctions and structures. The additional methoxyl at the 5-position of aromatic ring precludes the involvement of syringyl units in 5-linked structures such as the phenylcoumaran **B** and the 5–5-linked moiety of dibenzodioxocins **D**. The lignins are characterized by a higher β-ether **A** content, as well as by having more prominent β–β-coupled (resinol) units **C**. Also, cinnamyl alcohol endgroups **X** may be more prominent. The poplar Ac-MWL shows the prominent **A**, and **C** units and minor amounts of **B** and **X** (Figure 2b2). Again, these structures and the methoxyl are revealed in the related aspen Ac-CW spectrum (Figure 2a2) (although viewing at lower contour levels is needed to clearly see the **B** and **X** units).

The lower section of Figure 2 shows the more complete aromatic and aliphatic regions from the same experiments. Particularly noteworthy is that the aromatic components appear to be entirely because of the lignins as seen by the comparison of the aromatic profile between the Ac-CW (c) and Ac-MWL (d) spectra. Also highlighted in spectra c, with yellow shading, are the anomeric C/H regions for the polysaccharide components. The huge contour centered at approximately 100.4/4.40 p.p.m. is logically from cellulose acetate (see later in Figure 3) (although regrettable overlap with the analogous xylosyl anomeric is anticipated). The other anomerics from both pyranosyl and furanosyl hexa- and pentasaccharide units are extremely well dispersed, suggesting that substantive interpretation of these components will be possible. To date, there does not appear to have been any attempt to use the well-dispersed anomeric region in 2D ^{13}C–^{1}H correlation spectra for an analysis of peracetylated polysaccharides. Future work will attempt to assign all the peaks in this region using conventionally acetylated known polysaccharides and oligosaccharide models, which will also reveal whether all carbohydrate components remain structurally intact under the dissolution conditions. Reduced polysaccharide degree of polymerization (DP) caused by ball-milling is strongly evident. Cursory integration of the cellulose internal anomeric peak to the presumed acetylated α- and β-anomers of the reducing end units (at 88.9/6.22 and 91.5/5.63 p.p.m.) suggests molar ratios of only 20–25 : 1. This DP estimate of 20–25 is considerably lower than the DP of cellulose reported *in planta*, in the 7–10 000 range (Goring and Timell, 1962), suggesting that ball-milling has cleaved the long cellulose chains hundreds of times. There is no evidence to suggest that the depolymerization is coming from the


(a1) Pine Ac-CW
(a2) Aspen Ac-CW
(b1) Pine Ac-MWL
(b2) Poplar Ac-MWL
methoxyl
Bβ
Cβ
(A+D)γ
Bγ
Aα
Aβ
Dα
Dβ
Bα
Cα
Xγ
Aγ
Cγ
(c1) Pine Ac-CW
(c2) Aspen Ac-CW
(d1) Pine Ac-MWL
(d2) Poplar Ac-MWL
CHCl3
ppm
A
β-aryl ether
(β-O-4)
B
phenylcoumaran
(β-5)
C
resinol
(β-β)
D
dibenzodioxocin
(5-5/β-O-4,α-O-4)
X
(E)-cinnamyl alcohol
end-group
Methoxy
Not assigned here
or overlapping

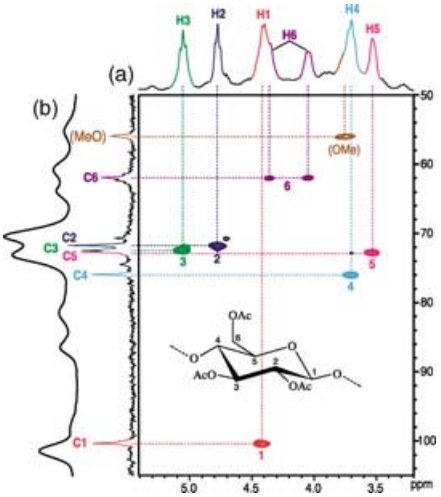


Figure 3. The cellulose component.
(a) The cellulose (and methoxyl) region of the 2D-HSQC spectrum of aspen Ac-CW showing the advantage of solution-state NMR in completely resolving all C–H correlations.
(b) A solid-state NMR spectrum of pine wood for comparison of line widths.

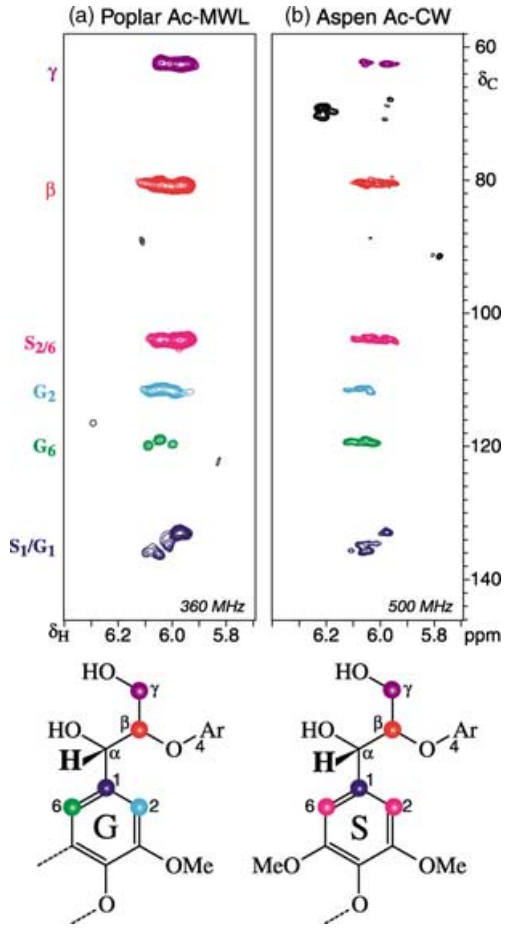


Figure 4. Long-range correlation spectra.
Regions of HMBC (long-range ^{13}C–^{1}H correlation) spectra showing α-proton correlations in important β-ether units, essentially unencumbered by competing correlations even in the complex Ac-CW sample. This experiment is particularly valuable as the long-range correlations provide information about a unit's derivation from coniferyl versus sinapyl alcohol (Ralph *et al.*, 1999). A proton will correlate with any carbon 2- or 3-bonds away. Thus, proton Hα should correlate with carbons Cγ (3-bonds away) and Cβ (2-bonds) in the side-chain, and C1 (2-bonds), C2 (3-bonds), and C6 (3-bonds) in the aromatic ring. The symmetry of the syringyl unit results in both C2 and C6 resonances being at about 103.5 p.p.m., whereas in the unsymmetrical guaiacyl units, the C2 resonance is at about 111 p.p.m. and C6 is at about 119 p.p.m. (a) Poplar Ac-MWL at 360 MHz. (b) Higher-resolution aspen Ac-CW at 500 MHz using a cryogenic probe for increased sensitivity; G = guaiacyl, S = syringyl.

dissolution processes, but the findings heighten the need to evaluate less severe milling conditions for future work. There are a great variety of ball-milling methods. The steel ball-mill used here is extremely efficient, requiring only 1.5 h for effecting particle-size reduction. Others use much longer time, and Ikeda *et al.* (2002) have strongly recommended ball-milling in toluene to avoid lignin structural alteration.

Figure 3(a) further illustrates the value of being able to apply solution-state NMR methods. The 2D section is from the same aspen HSQC experiment used in Figure 2(c2), but plotted at much higher contour levels so that only the most intense peaks, essentially just the cellulose and methoxy contours, are seen. The spectrum at this level is almost identical for the pine Ac-CW (not shown). A short one-dimensional (1D) ^{13}C experiment is shown on the left projection, with the 1D ^{1}H experiment on the top. Although there is some overlap of the carbons (notably carbons 5 and 3) and the protons (notably proton-1 and one of the 6-protons, as well as lignin methoxyl with proton-4) in their respective 1D spectra (projections), all six carbon/proton pairs are beautifully resolved in the 2D experiment allowing all the data to be readily determined. Also shown is a solid-state ^{13}C spectrum of pine wood (Figure 3b). Although chemical shift comparisons are not valid for this sample compared to the Ac-CW in the remainder of the figure, the spectrum is included for comparison of line widths and resolution. The solid-state spectrum provides additional

Figure 2. 2D-HSQC–NMR spectra of Ac-CWs and isolated lignins.
Spectra from samples in $CDCl_3$ show how readily the lignin components can be seen even in complex whole cell wall mixtures of pine (left column, 1) and aspen/poplar (right column, 2). (a, b) Zoomed-in lignin side-chain region. Note how well resolved at least one correlation is for each major lignin structure. (c, d) Spectral range covering the aromatics and aliphatics. (a) and (c) are from whole cell walls, and (b) and (d) from isolated lignins for comparison. The predominant lignin structures **A–D**, **X**, and the methoxyl are colored in CW samples (a and c) where they can be clearly defined. The aromatic regions are also colored in (c) and (d) to facilitate the identification and comparison of lignin-derived aromatic peaks. The anomeric polysaccharide correlations are highlighted by pale yellow background shading (c) to show how well dispersed the peaks are, suggesting that polysaccharide components should be readily identifiable from analysis of this region.

information regarding the two allomorphic forms of crystalline cellulose (Kono *et al.*, 2002), but the greater carbon line widths (and the extreme line widths of proton NMR data in solid-state spectra) preclude the acquisition of informative 2D spectra.

Obviously, the Ac-CW material can be subjected to the whole range of solution-state NMR methods, including homonuclear correlation experiments such as COSY and TOCSY, and heteronuclear experiments such as the HSQC illustrated in Figures 2 and 3, and also long-range ^{13}C–^{1}H correlation experiments. Even 3D experiments are perfectly viable, and should provide a way to obtain complete side-chain data for the individual lignin units because each unit is likely to be reasonably well isolated onto its own plane in a 3D TOCSY–HSQC experiment (Marita *et al.,* 2001; Ralph *et al.*, 1999), for example. One of the more demanding experiments for viscous solutions of polymeric materials is the long-range ^{13}C–^{1}H correlation HMBC experiment (Willker *et al.*, 1992). The experiment involves a delay of typically 80–100 msec to allow for long-range coupling interactions to evolve. If the sample relaxes too rapidly, as can happen with large polymers, particularly with metal contamination, there will be little-to-no signal left to be acquired (Ralph *et al.*, 1999). As shown in Figure 4, such experiments on our Ac-CW samples are quite successful. The small sections of the HMBC spectra highlight the α-proton correlations in the important β-ether units on a whole aspen Ac-CW sample compared with the same region from a poplar acetylated lignin isolate (Ac-MWL). This experiment elegantly reveals that the β-ether units are both syringyl and guaiacyl in the aspen lignin. This syringyl/guaiacyl information, previously revealed in comparisons of laboriously isolated lignins from mutant and transgenic (Marita *et al.*, 1999), as well as more recently on transgenic aspens (Li *et al.*, 2002), appears to now be possible without having to isolate the lignins. It must continue to be emphasized that a key benefit is that this analysis is on the whole lignin, not just the fraction that could previously be isolated for NMR, where partitioning of the lignin is a potential issue (Marita *et al.*, 2003a).

Simple syringyl:guaiacyl (S:G) comparisons on whole cell wall material are readily possible from the straightforward 1-bond ^{13}C–^{1}H correlation experiments (HMQC, HSQC), as illustrated in Figure 5. Distinguishing aromatic syringyl from guaiacyl correlations is qualitatively trivial because the more electron-rich syringyl rings have their protonated carbons (and with lesser distinction, their protons) at a lower chemical shift than their guaiacyl counterparts (Figure 5b). Furthermore, the guaiacyl correlations profile is remarkably similar to that in a guaiacyl-only lignin isolate (Figure 5a); chemical shifts of guaiacyl moieties in guaiacyl–guaiacyl structures are not significantly different from those in guaiacyl–syringyl moieties (and no different types of linkages are involved). Volume integrals on this spectrum predict an S:G of 1.8. Thioacidolysis S:G-values for aspen are approximately 2.2 (Li *et al.*, 2002); of course, this S:G reflects only releasable monomeric units, and syringyl units are more releasable (having no possible 5-condensation). Adoption of more quantitative methods for these 2D experiments (Heikkinen *et al.*, 2003; Zhang and Gellerstedt, 2000) should allow determination of quite accurate S:G ratios for the whole cell wall. Current methods provide estimates only from quantifiable releasable units following various types of degradation (Lin and Dence, 1992), or by methoxyl analysis (Obst, 1982); the latter method requires the exact lignin content to be known, and assumes that the lignins are composed only of S and G units.

Another feature of the lignins in aspen and poplar, the *p*-hydroxybenzoates acylating the γ-OHs of lignin side-chains, is as readily revealed in the whole cell wall spectrum, Figure 5(b), as in the isolated lignins themselves (Marita *et al.*, 2001; Ralph *et al.*, 1999).

Perspectives and conclusions

Although a total interpretation of all the contours in the congested regions of the spectra in Figure 2 is unlikely, it should be obvious that high-resolution solution-state NMR of (acetylated) whole cell wall material will be extremely valuable in assessing the structural components of the wall. The dispersion is sufficient to identify most lignin structures, and will be potentially quantifiable by more quantitative versions of HMQC or HSQC spectroscopy (Heikkinen *et al.*, 2003; Zhang and Gellerstedt, 2000). The degree of detail discernable is far higher than can be accomplished by solid-state NMR on solid material, and yet there is every indication that we are looking at the whole cell wall (that has admittedly been degraded by the ball-milling step). Sensitivity reduction caused by utilizing whole cell walls instead of isolated fractions can be largely offset by using cryogenically cooled probes that are becoming common in modern NMR instruments.

For the many valuable samples accumulated from previous studies of lignin-biosynthetic-pathway mutants and transgenics, we shall be applying these new methods to both the whole cell walls and the residues remaining following the dioxane-water extraction of 'MWL' from polysaccharidase-treated walls—often this material still contains the bulk of the lignin. It should be possible to observe the diagnostic signatures of gene downregulation that have, to date, been noted only on the soluble component. For example, COMT-deficient angiosperms (poplar, aspen, corn, *Arabidopsis*, and alfalfa) have been shown to incorporate substantial amounts of the novel monolignol 5-hydroxyconiferyl alcohol into their lignins when the pathway to sinapyl alcohol is downregulated (Marita *et al.,* 2003a,b; Ralph *et al.,* 2001a,b); this incorporation produces

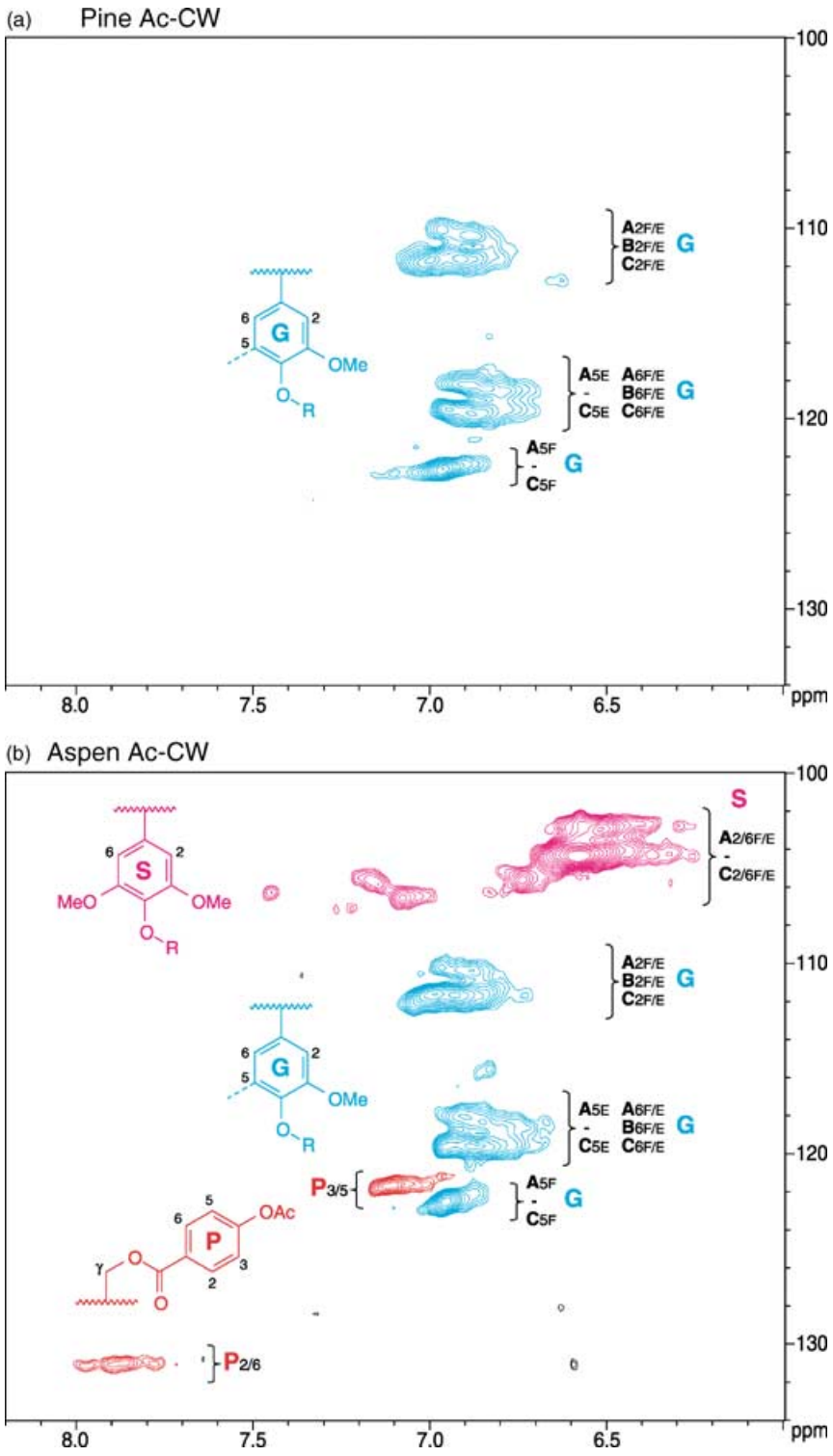


Figure 5. Distinguishing syringyl, guaiacyl, and *p*-hydroxybenzoate components.
Aromatic C–H correlation regions of the HSQC spectra of (a) the pine and (b) the aspen whole cell wall samples showing how readily syringyl **S** and guaiacyl **G** (and the pendant γ-*p*-hydroxybenzoyl **P**) components can be delineated. Assignments of the major contours for the major lignin units (**A–C**, see Figure 2) only are given, based on data in the NMR database of Lignin and Cell Wall Model Compounds (Ralph *et al.*, 2002); F = free-phenolic unit, E = etherified.

benzodioxane structures in the lignins. The diagnostic α- and β-C/H correlations appear at approximately 77/5.0 and 76/4.4 p.p.m., the clear regions in the spectra in Figure 2, suggesting that their detection will be straightforward.

At present, either dissolution method works well on the woody plant and maize samples examined so far. The DMSO–NMI solvent is preferred for generating Ac-CWs suitable for high-resolution NMR, and is likely to be the

choice for other applications where acylation is required. The DMSO–TBAF dissolution will be more valuable if etherification reactions are sought, such as for phenol methylation; phenol methylation in a heterogeneous whole cell wall is a tedious affair (Lapierre *et al.*, 1988). Acylation can still be accomplished directly *in situ*. However, oxidation needs to be averted by swamping the system with, for example, NMI before adding the acylating reagent.

A significant limitation at present is the need to finely grind the material. Fine grinding, by a method such as vibratory ball-milling, which is already required to isolate lignin fractions, does of course break bonds, and therefore reduces polymer DP (as shown here with the cellulose component) and causes structural alteration. We are assessing just how much milling is required. Cryogenic milling to a less fine state also appears to provide material that can be fully dissolved, although material mechanically ground to pass through a 1-mm mesh in a mechanical Wiley mill will not. Although we shall endeavor to find methods that work for less finely ground material, it is already extraordinarily useful to be able to prepare samples of the whole cell wall (and particularly the entire lignin component), without fractionation or partitioning the polymers, in days instead of in weeks, for analysis by high-resolution solution-state NMR.

In conclusion, the ability to fully dissolve ball-milled plant cell walls, without further apparent degradation, portends enormous potential for applying high-resolution solution-state NMR to elucidate structural details without having to resort to the laborious isolation methods of the past. It is also expected that the systems will allow the application of standard chemical derivatization and reaction methods, significantly improving the old heterogeneous methods.

Experimental procedures

General

Solvents used were AR grade and supplied by Fisher Scientific, Atlanta, USA. Reagents, including TBAF and NMI, were from Aldrich, Milwaukee, WI, USA. NMR spectra were acquired on a Bruker DRX-360 instrument fitted with a 5-mm ^{1}H/broadband gradient probe with inverse geometry (proton coils closest to the sample). The central chloroform solvent peak was used as internal reference (δ_C 77.0, δ_H 7.27 p.p.m.). The standard Bruker implementation (invietgssi) of the gradient-selected sensitivity-improved inverse (^{1}H-detected) HSQC experiment (Willker *et al.*, 1992) was used for the spectra in Figures 2, 3, and 5; the standard (inv4gslplrnd) gradient-selected inverse-detected HMBC experiment with an 80-msec long-range coupling delay was used for the spectra in Figure 4. The 500-MHz spectrum in Figure 4(b) was acquired on a 500-MHz Bruker DMX-500 equipped with an inverse-gradient ^{1}H/^{13}C/^{15}N cyroprobe for higher sensitivity (Marita *et al.*, 2001). These experiments and their applications to lignins are detailed in a recent book's chapter on 'Solution-state NMR of Lignins' (Ralph *et al.*, 1999).

Lignin isolation for NMR

Briefly, cell wall material is isolated by exhaustive solvent extraction, dried, and then ball-milled. We typically use stainless steel ball-milling (7-kg jars, 2.3 kg of 6-mm diameter stainless steel ball bearings, in a home-built, vigorously shaking and rotating assembly) for 1.5 h; others use ceramics for up to 1 week (Ikeda *et al.,* 2002). An MWL can be isolated by extraction into 96 : 4 dioxane:-water (Björkman, 1954), followed by freeze-drying and water-washing to remove small sugars. Higher yields are obtained following treatment with crude polysaccharidases for several days to remove most of the polysaccharides and produce an enzyme lignin which is then extracted with dioxane:water to give an MWL and an enzyme-lignin residue (Chang *et al.*, 1975). Our full procedures can be found in several references (Marita *et al.*, 1999, 2001, 2003b; Ralph *et al.*, 1997, 1999, 2001a). The actual pine lignin used here was isolated in 12.5% yield as described previously by Ralph *et al.* (1997); the poplar lignin was isolated in 65% yield (Marita *et al.,* 2001; Ralph *et al.,* 2001a).

Dissolution and derivatization in DMSO–TBAF

The ball-milled cell wall sample (600 mg) was suspended in DMSO (16 ml), and TBAF (2.6 g) was added. A clear solution was formed in 15 min−1.5 h, depending on the sample.

Phenol methylation, if desired, could be carried out by simply adding methyl iodide (MeI, 0.7 ml). After reaction (typically for just 30 min), excess MeI is removed under vacuum.

Alternatively, or as the next step, acetylation is accomplished by adding excess NMI (5 ml) and acetic anhydride (7.5 ml), and the mixture is stirred for 1.5 h. The precipitate is recovered as described below in the DMSO–NMI procedure.

Acetylation can be carried out by adding acetic anhydride (8 ml) using the TBAF as catalyst, but this acetylation procedure produces some oxidation artifacts. Typically the weight of Ac-CWs was approximately 120% of the weight of the original cell wall.

Dissolution and derivatization of lignin model compounds

Dimeric β–β-, β−5-, and β–O−4-lignin model compounds were tested to determine if they retained their structural integrity throughout the dissolution and derivatization process. The compound (10 mg) was dissolved in DMSO (2 ml) followed by the addition of TBAF (50 mg). The solution was stirred for 1 h, and acetic anhydride (1 ml) was added. After 5 h, the products were isolated by extraction into ethyl acetate, and were characterized by NMR.

To monitor reactions occurring during dissolution directly in the NMR tube, the following example is given for the most sensitive free-phenolic phenylcoumaran model. The model (5 mg) was dissolved in DMSO-d_6 (0.5 ml) in a 5-mm NMR tube, and NMR spectra (^{1}H and ^{13}C) were recorded. TBAF (25 mg) was added, and the NMR spectra were recorded over 1 h. After 16 h, spectra were again recorded. Acetic anhydride (0.25 ml) was added to the tube. Spectra recorded after 5 h showed that acetylation was complete, and that the phenylcoumaran structure remained intact.

Dissolution and derivatization in DMSO–NMI

The ball-milled cell wall sample (600 mg) was suspended in DMSO (10 ml), and NMI (5 ml) was added. A clear solution was formed in 3 h or less, depending on the sample.

Excess acetic anhydride (3 ml) was added, and the mixture was stirred for 1.5 h. The clear brown solution was transferred into water (2000 ml), and the mixture was allowed to stand overnight. The precipitate was recovered by filtration through a nylon membrane (0.2 μm). The product was washed with water (250 ml) and dried under vacuum at room temperature. If necessary, EDTA washing was as described previously by Ralph *et al.* (1994). The weight of Ac-CWs was typically 137–140% of the weight of the original cell wall.

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References

Albright, J.D. and Goldman, L. (1965) Dimethyl sulfoxide-acid anhydride mixtures. New reagents for oxidation of alcohols. *J. Am. Chem. Soc.* **87**, 4214–4216.

Aspinall, G.O. (1982) Isolation and fractionation of polysaccharides. In *The Polysaccharides* (Aspinall, G.O., ed.), **Vol. 1**, New York: Academic Press, pp. 19–35.

Baucher, M., Ralph, J. and Boerjan, W. (2003) Lignin biosynthesis. In *Annual Reviews of Plant Biology*, **Vol. 54**, Stanford, CA: Highwire Press, pp. 519–546.

Björkman, A. (1954) Isolation of lignin from finely divided wood with neutral solvents. *Nature*, **174**, 1057–1058.

Boudet, A.M. and Grima-Pettenati, J. (1996) Lignin genetic engineering. *Mol. Breed.* **2**, 25–39.

Chang, H.-M., Cowling, E.B., Brown, W., Adler, E. and Miksche, G. (1975) Comparative studies on cellulolytic enzyme lignin and milled wood lignin of sweetgum and spruce. *Holzforschung*, **29**, 153–159.

Chiang, V.L. (2002) From rags to riches. *Nat. Biotechnol.* **20**, 557–558.

Dixon, R.A. and Ni, W. (1996) Genetic manipulation of the phenylpropanoid pathway in transgenic tobacco: new fundamental insights and prospects for crop improvement. *Biotechn. Biotechnol. Equipment*, **10**, 45–51.

Goring, D.A.I. and Timell, T.E. (1962) Molecular weight of native celluloses. *Tappi*, **45**, 454–460.

Heikkinen, S., Toikka, M.M., Karhunen, P.T. and Kilpeläinen, I.A. (2003 J-dependence of polarization transfer in NMR spectroscopy. Application to wood lignin. *J. Am. Chem. Soc.* **125**, 4362–4367.

Heinze, T., Dicke, R., Koschella, A., Kull, A.H., Klohr, E.A. and Koch, W. (2000) Effective preparation of cellulose derivatives in a new simple cellulose solvent. *Macromol. Chem. Phys.* **201**, 627–631.

Ikeda, T., Holtman, K., Kadla, J.F., Chang, H.M. and Jameel, H. (2002) Studies on the effect of ball milling on lignin structure using a modified DFRC method. *J. Agric. Food Chem.*, **50**, 129–135.

Jung, H.G. and Deetz, D.A. (1993) Cell wall lignification and degradability. In *Forage Cell Wall Structure and Digestibility* (Jung, H.G., Buxton, D.R., Hatfield, R.D. and Ralph, J., eds), Madison, WI: ASA-CSSA-SSSA, pp. 315–346.

Jung, H.G., Buxton, D.R., Hatfield, R.D. and Ralph, J., eds (1993) *Forage Cell Wall Structure and Digestibility*, Madison, WI: ASA-CSSA-SSSA, pp. 1–794.

Kono, H., Yunoki, S., Shikano, T., Fujiwara, M., Erata, T. and Takai, M. (2002) CP/MAS C-13 NMR study of cellulose and cellulose derivatives. Part 1. Complete assignment of the CP/MAS C-13 NMR spectrum of the native cellulose. *J. Am. Chem. Soc.* **124**, 7506–7511.

Lapierre, C., Monties, B. and Rolando, C. (1988) Thioacidolyses of diazomethane-methylated pine compression wood and wheat straw in situ lignins. *Holzforschung*, **42**, 409–411.

Lewis, N.G. and Paice, M.G., eds (1989) *Plant Cell Wall Polymers. Biogenesis and Biodegradation*, **Vol. 399**, Washington, DC: Am. Chem. Soc. pp. 1–676.

Li, L., Zhou, Y., Cheng, X., Sun, J., Marita, J.M., Ralph, J. and Chiang, V.L. (2002) Combinatorial modification of multiple lignin traits in trees through multigene cotransformation. *Proc. Natl. Acad. Sci. USA*, in press.

Lin, S.Y. and Dence, C.W. (1992) *Methods in Lignin Chemistry*. Heidelberg: Springer-Verlag.

Marita, J., Ralph, J., Hatfield, R.D. and Chapple, C. (1999) NMR characterization of lignins in *Arabidopsis* altered in the activity of ferulate-5-hydroxylase. *Proc. Natl. Acad. Sci. USA*, **96**, 12328–12332.

Marita, J.M., Ralph, J., Lapierre, C., Jouanin, L. and Boerjan, W. (2001) NMR characterization of lignins from transgenic poplars with suppressed caffeic acid *O*-methyltransferase activity. *J. Chem. Soc. Perkin Trans.* **1**, 2939–2945.

Marita, J.M., Ralph, J., Hatfield, R.D., Guo, D., Chen, F. and Dixon, R.A. (2003a) Structural and compositional modifications in lignin of transgenic alfalfa downregulated in caffeic acid *O*-methyltransferase (COMT) and caffeoyl-coenzyme A *O*-methyltransferase (CCOMT). *Phytochemistry*, **62**, 53–65.

Marita, J.M., Vermerris, W., Ralph, J. and Hatfield, R.D. (2003b) Variations in the cell wall composition of maize *brown midrib* mutants. *J. Agric. Food Chem.* **51**, 1313–1321.

Obst, J.R. (1982) Guaiacyl and syringyl lignin composition in hardwood cell components. *Holzforschung*, **36**, 143–152.

Ralph, J., Hatfield, R.D., Quideau, S., Helm, R.F., Grabber, J.H. and Jung, H.-J.G. (1994) Pathway of *p*-coumaric acid incorporation into maize lignin as revealed by NMR. *J. Am. Chem. Soc.* **116**, 9448–9456.

Ralph, J., MacKay, J.J., Hatfield, R.D., O'Malley, D.M., Whetten, R.W. and Sederoff, R.R. (1997) Abnormal lignin in a loblolly pine mutant. *Science*, **277**, 235–239.

Ralph, J., Marita, J.M., Ralph, S.A. *et al.* (1999) Solution-state NMR of lignins. In *Advances in Lignocellulosics Characterization* (Argyropoulos, D.S. and Rials, T., eds). Atlanta, GA: TAPPI Press, pp. 55–108.

Ralph, J., Lapierre, C., Lu, F., Marita, J.M., Pilate, G., Van Doorsselaere, J., Boerjan, W. and Jouanin, L. (2001a) NMR evidence for benzodioxane structures resulting from incorporation of 5-hydroxyconiferyl alcohol into lignins of *O*-methyl-transferase-deficient poplars. *J. Agric. Food Chem.* **49**, 86–91.

Ralph, J., Lapierre, C., Marita, J. *et al.* (2001b) Elucidation of new structures in lignins of CAD- and COMT-deficient plants by NMR. *Phytochemistry*, **57**, 993–1003.

Ralph, S.A., Landucci, L.L. and Ralph, J. (2002) NMR database of lignin and cell wall model compounds. Available over Internet at http://www.dfrc.ars.usda.gov/software.html.

Whetten, R. and Sederoff, R. (1991) Genetic-engineering of wood. *For. Ecol. Manage.* **43**, 301–316.

Willker, W., Leibfritz, D., Kerssebaum, R. and Bermel, W. (1992) Gradient selection in inverse heteronuclear correlation spectroscopy. *Magn. Reson. Chem.* **31**, 287–292.

Zhang, L. and Gellerstedt, G. (2000) Achieving quantitative assignment of lignin structure by combining 13C and HSQC NMR techniques. In *Sixth European Workshop on Lignocellulosics and Pulp*, Bordeaux, France: University of Bordeaux 1, pp. 7–10.