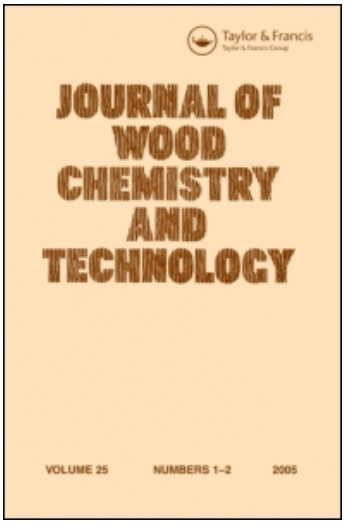

Journal of Wood Chemistry and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597282

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Online publication date: 28 February 2001

To cite this Article MacKay, J. , Dimmel, D. R. and Boon, J. J.(2001) 'PYROLYSIS MASS SPECTRAL CHARACTERIZATION OF WOOD FROM CAD-DEFICIENT PINE', Journal of Wood Chemistry and Technology, 21: 1, 19 — 29
To link to this Article: DOI: 10.1081/WCT-100102652
URL: http://dx.doi.org/10.1081/WCT-100102652

JOURNAL OF WOOD CHEMISTRY AND TECHNOLOGY, 21(1), 19–29 (2001)

PYROLYSIS MASS SPECTRAL CHARACTERIZATION OF WOOD FROM CAD-DEFICIENT PINE

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ABSTRACT

An extremely low level of the cinnamyl alcohol dehydrogenase (CAD) enzyme activity in a mutant loblolly pine tree leads to a different pool of precursors for lignin production. Characterization of CAD-deficient wood by pyrolysis mass spectroscopy indicates significant increased levels of dihydroconiferyl alcohol, not usually considered a lignin subunit. Also, in comparison to normal pine lignin, the CAD-deficient lignin has increased levels of coniferaldehyde, the substrate of CAD, and of *p*-coumaryl alcohol, along with greatly decreased levels of coniferyl alcohol. These findings are consistent with trees of different ages and confirm that there is considerable plasticity in the biosynthesis of lignin. Trees are able to utilize structures beyond the traditional definition of precursors to make lignin.

INTRODUCTION

Loblolly pines with unusual lignin can be obtained through crosses of trees that have a mutant gene, the *cad-nl* allele, found in breeding stocks.[1,2] The *cad* gene encodes an enzyme, cinnamyl alcohol dehydrogenase (CAD), that is normally required in lignin biosynthesis.[3,4] The *cad-nl* allele can be used in well-defined crosses to produce trees that are almost totally CAD-deficient (homozygous for the *cad-nl* allele) or partially CAD-deficient (heterozygous for the *cad-nl* allele). Totally CAD-deficient (referred to hereafter as CAD-) trees are more easily pulped than normal trees.[5] The reasons behind this increased reactivity are of high interest to scientists attempting to develop superior sources of pulpwood.

Lignins from completely CAD- trees are built up from unusual monomers. Analysis of a CAD- milled wood lignin (MWL) by NMR indicated the presence of three unusual monomers in significant amounts. The monomers are coniferaldehyde (**1**), vanillin (**2**), and dihydroconiferyl alcohol (**3**) (Fig. 1).[2] The increase in dihydroconiferyl alcohol (DHCA) was dramatic and unexpected. While solution NMR is a powerful characterization tool, it requires isolation of lignin and, thus, may give an inadequate representation of the entire lignin fraction in its native state. For, example the NMR characterization of pine CAD- lignin used an MWL sample that represented less than 20% of the total lignin.[2] In addition, scientists disagree on whether the prolonged ball-milling required to isolate MWL lignin alters the structure of the lignin.[6] Finally, the results obtained by NMR were based upon the analysis of a single tree. Given the significance of the NMR conclusion and the noted limitations, we sought to verify the high levels of DHCA in whole-wood samples, and with more than one tree, by other analyses.

Thioacidolysis analysis of whole-wood samples has shown that the lignin in totally CAD-trees contains much higher levels of DHCA units

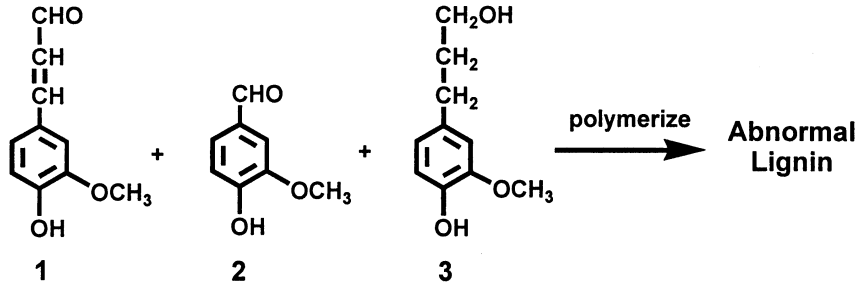


Figure 1. Building blocks for lignin production in CAD-deficient trees.

and C_5-linkages, and correspondingly less C_β-O_4 linkages, than normal wood.[7] Such linkage distribution is consistent with lignin biosynthesis theory, in which lignin is produced by coupling delocalized phenolate radicals. With the type of monomers available in the CAD-case, there are greatly reduced opportunities to couple at a C_β-site. We have applied pyrolysis mass spectroscopy (PYMS) to the same wood samples studied by the thioacidolysis techniques. PYMS can provide fingerprinting information on lignin in the wood matrix.[8] Molecular-induced information is obtained on the polysaccharide and lignin distribution in microgram size wood particles in a single analytical run. The temperature-resolved data-acquisition process separates loosely bound from more condensed cross-linked fractions. Specific information is obtained on the monomeric and oligomeric units in lignin.[9,10]

Mass Spectral Techniques

PYMS is a powerful technique for studying whole-wood samples. Homogenized particulate samples are placed on a resistively heatable Pt/Rh filament mounted on a direct-insertion probe inside the ion source of a mass spectrometer. The filament is heated rapidly and the sample vaporized and pyrolyzed over a discrete time period. Spectra are continuously recorded as the sample vaporizes. The heating and subsequent ionization cause polymeric compounds to liberate (at least some of) their monomeric components; in essence, we see the mass spectra of the low-molecular-weight components. Several modes of sample ionization are available; we employed electron ionization (EI) and chemical ionization (CI). If a relatively low (i.e., 16 eV) EI voltage is employed, molecular ion fagmentaton is minimized. This is an important consideration when dealing with samples that are mixtures of substances. The spectra become very complex when fragment ions of one compound overlap with molecular ions of another.

Chemical ionization has the advantage of providing a "soft" molecular ionization, one that typically does not provide much fragmentation. The CI gas employed in our studies was ammonia; the NH_3 molecules are ionized and collide with other NH_3 molecules to generate NH_4^+ ions that then add directly or deliver H^+ to the compound to give $[M + 18\ (NH_4)]^+$ and/or $[M + 1\ (H)]^+$ ions. Significant amounts of fragment ions can result, for example, when a protonated molecule can lose a simple molecule, such as H_2O, and generate very stable cations in the process. This process is more prominent when the internal energy of the neutral molecule is relatively high due to thermal processes on the filament.

Another technique employed in this study was PYMS/MS. Here again, a low-voltage EI ionization of the vaporized sample generates abundant amounts of molecular ions. The magnet is set to pass one ion, i.e., m/z 180, into a chamber containing helium gas. The ion is accelerated from the ion source at 8 kV and undergoes high-energy collisions with the helium gas to generate fragment ions that are then passed through an energy-sector analyzer. The latter is scanned to show the distribution of the fragment ions. In essence, we are recording mass specta of MS-separated ions and confirming component structures present in the mixture. For example, the m/z 180 of coniferyl alcohol ($ArCH{=}CHCH_2OH$) will show characteristic fragmentation ions that will be different from the m/z 180 of dihydroconiferyl aldehyde ($ArCH_2CH_2CHO$). Knowing these fagmentation patterns in advance allows for accurate assignment of the m/z 180 ion in a spectrum.

RESULTS AND DISCUSSION

EI-PYMS Characterization

Wood samples from totally CAD- and normal loblolly pine seedlings and from one 12-year-old CAD-deficient tree were vaporized/pyrolyzed over a temperature range of 160-640°C and subjected to 16 eV EI ionization. The respective mass scans were time-integrated to provide the partial spectra data shown in Fig. 2. The normal wood samples had a dominant signal at m/z 180 that corresponds to coniferyl alcohol (CA).[10,11] The CAD- samples showed m/z 178, 180, and 182 signals of comparable intensities. The former signal corresponds to coniferaldehyde and the latter to DHCA. Several other lignin-related signals are also observed, including m/z 124 (fragment ion of CA), 137 (fragment ion of CA), 150 (ethenyl-methoxyphenol), 152 (vanillin), and 164 (propenyl-methoxyphenols).

The relative ratios of the signals of interest (Table 1) were calculated from the two spectra shown in Fig. 2. The relative signal intensities probably do not represent the actual ratios of the components in the samples due to differences in mass-spectral response. The only condition where intensities equal component ratios is when each component ionizes and fragments to the same extent; this is not likely, except for close related isomers, such as *cis*/*trans* alkenes, and *m*/*p*-substituted benzenes, etc. If authentic samples were available, known mixtures could be prepared and subjected to the PYMS conditions to develop relative responses. This was not done in our study. Therefore, all that can be concluded from the data in Table 1 is that the ratios of components are significantly different for the normal and CAD-

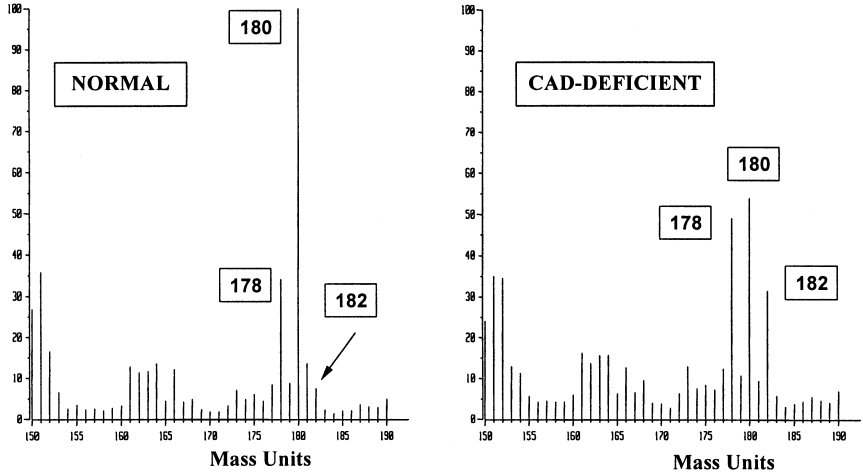
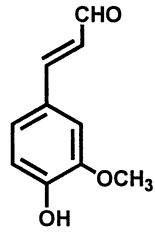
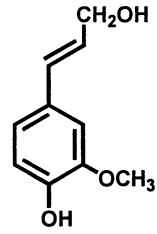
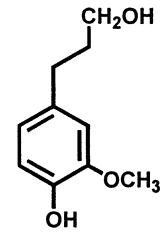


Figure 2. Partial PYMS EI mass spectra representative of results obtained with normal and CAD- wood. The Y-axis is the relative abundance; 100 represents the most abundant signal in the spectrum.

Table 1. Relative EI-PYMS Area Ratios for Normal and CAD-Deficient Woods.

Wood	Relative Area Ratios of Select Ions		
			
	178	**180**	**182**
Normal	0.33	1	0.05
CAD-	0.90	1	0.60

woods. Much lower coniferyl alcohol and more dihydroconiferyl alcohol (DHCA) are present in the CAD- wood.

We observed the same significant increase in DHCA m/z 182 signal both in the CAD-deficient seedlings and the 12-year-old tree. This finding indicates that high levels of DHCA are not specific to one individual or to a developmental stage of the tree. A multivariate analysis (not shown) clearly

discriminated between the normal and CAD- wood types. Coniferyl and dihydroconiferyl alcohol concentrations were the strongest and practically the only important changes. This analysis also revealed a smaller increase of m/z 150, 107, and 94 ion the CAD- woods, usually associated with *p*-coumaryl alcohol subunits (H units). A similar finding regarding H units was detected by thioacidolysis in CAD- pine seedlings of this same family.[7]

The incorporation of coniferaldehyde residues into the lignin of plants or trees with a low-level CAD activity has been well documented and demonstrated with several techniques, including PYMS.[12-15] In contrast, the utilization of DHCA as a lignin precursor appears, so far, unique to CAD- pine and may be the result of a more severe decrease in enzyme activity than in other studies.

Ammonia CI-PYMS Characterization

Samples of totally CAD- and normal loblolly pine wood were also analyzed by CI-PYMS to verify the m/z 182 signal assignment is, indeed, due to DHCA. The vaporized samples were subjected to ammonia chemical ionization and the mass spectra time-integrated over the temperature range 160-640°C to provide the partial spectral data shown in Fig. 3. Both wood

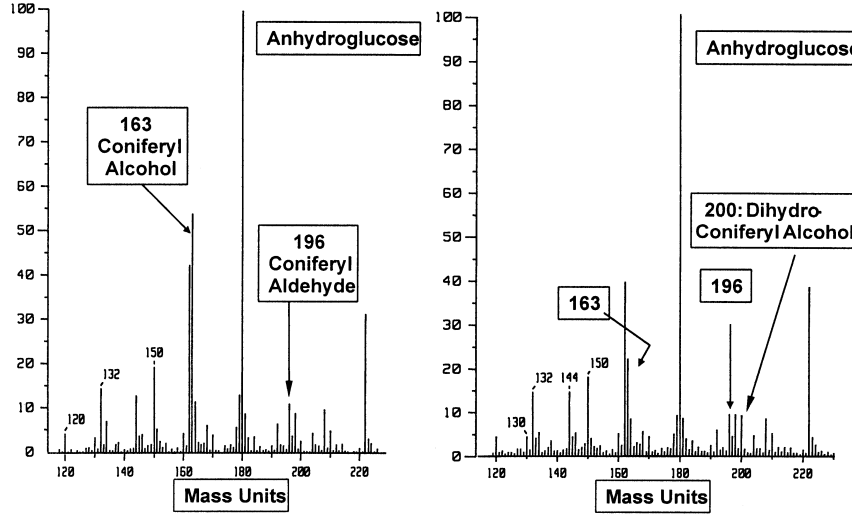


Figure 3. PYMS ammonia CI mass spectra of normal and CAD- wood. The Y-axis is the relative abundance; 100 represents the most abundant signal in the spectrum.

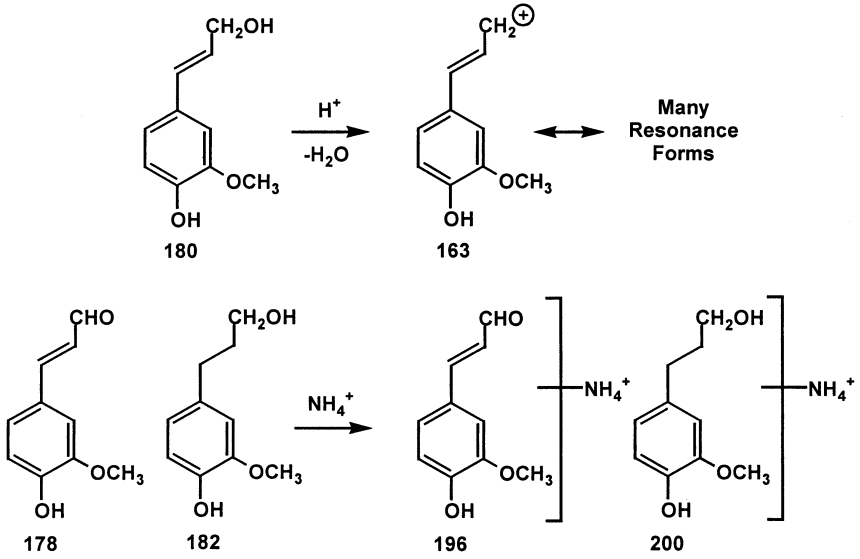


Figure 4. Ammonia CI reactoins with lignin structures of interest.

samples have a large signal from 1,6-anhydroglucose, a cellulose fragment.[8] As for lignin-related signals, the normal wood shows a strong m/z 163 signal that corresponds to protonation of coniferyl alcohol, followed by loss of water (Fig. 4).[11] The CAD- wood shows a much lower m/z 163 signal and more intense m/z 196 and 200 signals. The latter signals correspond to ammonium ion addition to coniferaldehyde and DHCA, respectively (Fig. 4). Here again, the data provide qualitative, rather than quantitative information.

PYMS/MS Characterization

Structure assignments for the ions observed in the EI-PYMS were confirmed by PYMS/MS. The m/z 180 ion was shown to produce m/z 152, 137 (strong), and 124 ions that are characteristic of coniferyl alcohol. The m/z 182 ion was shown to produce m/z 164, 152, 137 (strong), and 122 ions that are characteristic of dihydroconiferyl alcohol. Figure 5 presents some possible structures for these ions.

The important point of the PYMS/MS data is that the fragment ions from the m/z 178, 180, and 182 ions are the expected ions from the proposed lignin structures and not related to carbohydrate or other component struc-

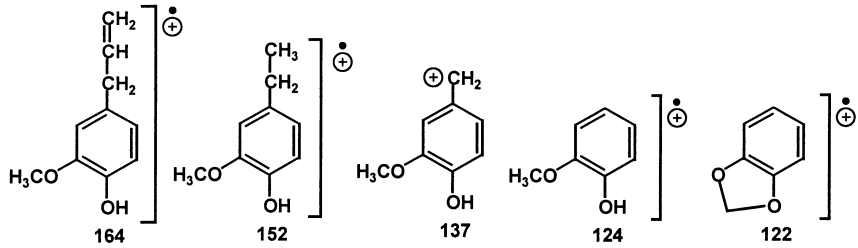


Figure 5. Possible ion structures observed in the mass spectra of normal and CAD-wood.

tures. In addition, the ions are the same in both normal and CAD- wood, meaning that there was a shift in relative distributions of lignin monomeric compounds, rather than new (non lignin) compounds being formed by the CAD- plant.

CONCLUSIONS

Whole-sample pyrolysis mass spectra of normal and totally CAD-deficient loblolly pines indicate that the latter has a much higher relative level of dihydroconiferyl alcohol, and somewhat higher level of coniferaldehyde. The extremely low-level CAD enzyme in pine trees that carry the *cad-nl* allele clearly lead to important changes in the subunit composition and structure of lignin. These changes, first observed in an isolated MWL by FTIR,[1] NMR,[2] and UV,[16] have now been confirmed by analyses that do not require lignin isolation. The NMR analysis of MWL showed that DHCA is a significant lignin subunit in the CAD- trees. This finding has now been confirmed by both thioacidolysis[7] and PYMS of wood samples. These later studies have furthermore demonstrated that this interesting finding is consistent among CAD-deficient trees and that it does not appear to be dependent on age or growth conditions. Similarly, the dominant features of the FTIR and UV spectra of MWL from a CAD- tree were also observed in the spectra obtained with samples from several seedlings or trees using finely ground wood (FTIR[1]) or thin wood sections (UV-microphotospectrometry[17]). Overall, results obtained with wood samples have been in strong agreement with those from isolated lignin samples.

The utilization of DHCA as a lignin precursor constitutes one more example of the considerable metabolic plasticity of lignin biosynthesis in plants.[11,13] Interestingly, the increased incorporation of DHCA has only been reported in pine, so far; a mechanism for its formation has yet to be

demonstrated. However, the various analyses of totally CAD- trees clearly demonstrate that DHCA is intimately linked to other lignin subunits and, therefore, functions as a *bona fide* lignin precursor in this case.[2,7,18] Although unexpected, the incorporation of DHCA points to greater opportunity to manipulate lignin structure than previously anticipated.

EXPERIMENTAL

Plant material

Loblolly pine seedlings of a selfed family obtained from a *cad-nl* heterozygous mother-tree (7-56) were grown for 10 months under standard greenhouse conditions. The *cad* genotype (CAD- or wild type) of each seedling was determined using RAPD markers as a fingerprinting method.[1] Wood was isolated from randomly selected seedlings related to two wild-type (30.4% and 31% lignin) and two CAD- (24.9% and 27.5% lignin). Identification and characterization of a 12-year-old CAD- tree (28% lignin) were described.[2,16] Wood samples were air dried and extracted with water, followed with acetone. Aliquots of the samples were homogenized in a glass minimortar using water as a suspension fluid.

Instrumentation

Pyrolysis-MS. PYMS analyses (in direct temperature-resolved MS mode) were performed according to Pillonel et al.[19] Between 5 and 10 μg of an aqueous wood particle suspension was applied to the wire and dried *in vacuo*. The pyrolysis was carried out using an insertion probe equipped with a resistively heated Pt/Rh (9/1) filament loop (100 μm diameter) inserted inside the ion source of a JEOL SX-102A double-focusing mass spectrometer (reversed geometry B/E). Ionization was performed under electron ionization (16 eV EI) or ammonia chemical ionization conditions (NH_3 CI). The mass spectrometer was operated at 10 kV. The mass range was scanned from m/z 20-1000 (16 eV EI) or m/z 60-1000 (NH_3 CI) with a cycle time of 1 second. Data were acquired and processed using the JEOL MP7000 data system. Data were exported to a high-speed computer for further data processing by multivariate analysis. Discriminant analysis (double-stage principal-component analysis[20]) was performed on the data to identify sets of mass peaks whose intensity was significantly different between the various samples. Both FOMpyroMAP and Chemometriks programs[21] were used for this analysis.

Pyrolysis MS/MS. The pyrolysis conditions were similar to the PYMS described above. A home-built collision cell behind the magnet just before the electron sector allowed the performance of MIKES (Mass analyzed Ion Kinetic Energy Spectroscopy) studies of select ions. The analysis had to be performed in a multichannel-averaging mode of the data acquisiton for reasons of sensitivity. Helium was used as the collision gas.The analysis was performed on m/z 178, 180, and 182 obtained by low voltage EI.

ACKNOWLEDGEMENTS

This work was partially supported by the United States Department of Agriculture, Grant No. 97-35103-4796. The mass spectrometric work is part of the approved research program FOM 28 of the Stichting FOM (Foundation of Fundamental Research of Matter) which is subsidized by the Netherlands Organization for Scientific Research (NWO). Mr. Jerre van der Horst and Mr. Gert Eijkel are acknowledged for their technical assistance with the MS and multivariate analysis work.

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