Application of new methods for the investigation of lignin structure

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Chapter 6

Application of New Methods
for the Investigation of Lignin Structure

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The main cell wall phenolics, detrimental to forage digestibility (Jung, 1989), have been classified into core and "noncore" lignins, on the basis of their relative susceptibility towards hydrolysis. Noncore lignin consists of low-molecular weight phenolics, released from cell walls by mild hydrolyses, and is chiefly represented by ester-linked *p*-hydroxycinnamic acids (Hartley & Ford, 1989). As these noncore lignin components are discussed elsewhere (see chapter 9 by Ralph and Helm in this book), the focus here will be on core lignins. Core lignins are the highly condensed phenylpropanoid cell wall polymers that are largely resistant to mild degradation procedures. They are composed of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) building units, in various proportions according to their origin. Gymnosperm lignins are chiefly constituted of G units. Angiosperm lignins are a mixture of G and S units. In addition, grass lignins are composed of H, G, and S units. These phenylpropane units are interconnected by a series of ether and carbon-carbon linkages (Adler, 1977; Tanahashi & Higuchi, 1989), in various bonding patterns (Fig. 6–1). The most frequent interunit β-O-4 bonds are the targets of lignin depolymerization processes. In contrast, the other β–5, β–1, β–β, 5–5, and 5–O–4 interunit bonds (Fig. 6–1), referred to as the condensed bonds, are more resistant towards degradation. Their relative amounts are not yet clearly established. A lignin-building unit may be involved in one, two, or more bonding patterns, such as those depicted in Fig. 6–1. Besides these main substructures, there are other minor ones.

    The objective of this chapter is to present the structural information gained from three analytical strategies recently developed in the field of lignin research or which have significantly benefited from instrumental strides. Structural information discussed herein concerns the monomer composition of lignins, their key functionalities, and their main interunit linkages. These are relevant with regard to forage research as it has been frequently emphasized that not only the lignin amount, but also the lignin structure influences the forage nutritional value (Jung, 1989). These structural features should
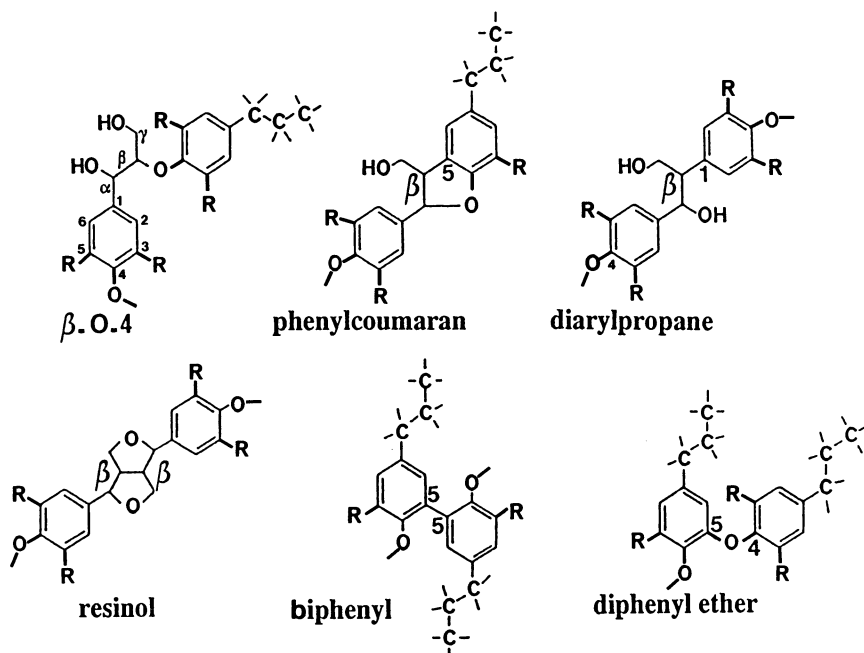
Fig. 6–1. Principal bonding patterns between the para hydroxyphenyl (R = H), guaiacyl (R at C-3 = OMe; R at C-5 = H), and syringyl (R = OMe) C_6C_3 core lignin units. The phenolic group at C-4 may be free or etherified. The pino-, medio-, and syringa-resinol structures involve 2 G, 1G/1S, and 2S units. Structures are outlined with their usual C numbering and symbols.

be complemented by data on the macromolecular characteristics of lignins, their complex association with other cell wall components, and their large structural heterogeneity with cellular and subcellular localization.

The methods evaluated here are analytical pyrolysis, solution state carbon-13 (^{13}C) nuclear magnetic resonance (NMR) spectroscopy, and chemical degradation methods, chiefly illustrated by thioacidolysis. Such a patchwork survey, which does not compile all the available methods, underlines the complementary performances of these tools. Owing to its high energy input, pyrolysis degrades in situ lignins to small phenolics that retain information merely on lignin content and monomer composition. This restricted informative character is counterbalanced with high sensitivity and screening capabilities. On the contrary, the nondestructive and poorly sensitive ^{13}C NMR spectroscopy of soluble lignin samples preserves all the structural features of these polymers within a myriad of specific signals. The difficulty then lies in the assignment of these signals and how representative the soluble lignins are when compared to in situ lignins. Finally, thioacidolysis provides some information about in situ lignin units and interunit bonds, with high sensitivity and easy quantitation, but only for the less-condensed moieties of the polymers, degradable to monomers and dimers. Obviously, the lack of a single analytical tool providing a complete and unequivocal picture

of core lignins makes the syngergistic combination of such methods the only realistic approach to unravel the complex and heterogeneous lignin networks.

The evaluation of in situ core lignins is usually performed from extractive-free cell walls, which have been subjected to solvent extractions to eliminate soluble components. This is important to minimize the interference problem between core lignins and other soluble phenolics. When the analytical method calls for a preliminary lignin isolation step, another problem is that there is no method for isolating core lignins in their intact state. Lignin preparations free of contaminants can only be obtained by vigorous treatments, which modify lignin structure. The most frequent soluble lignin fractions used for analytical purposes are the lignins prepared from ball-milled samples (Björkman, 1954). Owing to the heterogeneous distribution of lignin structures in cell walls, to the modifications introduced by the milling step, and to the moderate recovery yield of milled lignins, these isolated lignin fractions are not always representative of the whole polymer, either qualitatively or quantitatively.

I. STRUCTURAL SCREENING AND MOLECULAR PROFILING OF LIGNINS AND LIGNOCELLULOSICS BY ANALYTICAL PYROLYSIS

In recent years, analytical pyrolysis has been applied to the structural analysis of lignocellulosics. The major attribute of pyrolytic methods is their screening capability: one pyrolysis run only requires a few micrograms of sample, without necessity of lignin isolation, and is very rapid. On the other hand, most of the limitations of the method concern the extent to which the results may be qualitatively and quantitatively discussed, in relation with the close examination of core lignin structure.

A. Principle and Practical Considerations

As with chemical degradations, analytical pyrolysis allows characterization of isolated or in situ lignins, by first involving the polymer breakdown to low-molecular weight phenolics and secondly determining these degradation compounds.

The main pyrolytic sequences of production and analysis of degradation products are pyrolysis-mass spectrometry (Py-MS), pyrolysis-gas chromatography (Py-GC), and pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS). These production and analysis steps are often performed without interruption, by direct coupling of the pyrolyzer to the analytical device (on-line analysis).

In Py-MS, all the Py compounds are introduced without separation in a mass spectrometer, to yield a complex mass spectrum. In Py-GC or Py-GC-MS, the volatile Py adducts are first separated in a capillary column, so that they ideally arrive separately in the nondiagnostic flame ionization detector or in the mass spectrometer, respectively. The pyrograms (chromato-

grams of Py compounds) are discussed with reference to standard compounds (Faix & Meier, 1989).

Detailed descriptions of the Py step have been performed (Boon, 1989; Meier & Faix, 1992). The rapid heating of a microquantity of sample to several hundred degrees Celsius in the absence of oxygen, within a very short time, and the immediate removal of the primary Py products from the heating zone are a prerequisite to reliable Py results. The most frequent heating system is the flash Py, within about 0.1 s, performed either by the inductive heating of a ferromagnetic wire to its Curie joint temperature or by the resistive heating of a platinum ribbon or coil (Meier & Faix, 1992). In the case of Py-MS, the resistive heating may be accomplished in the ion source of the mass spectrometer, with the filament of the direct introduction probe (Schulten, 1984; Boon, 1989).

In Py-MS, which yields one mass spectrum for all the Py products, soft ionization methods are used to avoid an excessive fragmentation of molecular ions to less diagnostic daughter ions. Low voltage electronic impact (El) is most commonly employed and less frequently chemical ionization (CI) or field ionization (FI) (Boon, 1989). The explored mass range goes up to m/z 2000 in CI mode. This allows detection of monomer, dimer, and oligomer ions. As Py-MS is aimed at recovering many data sets from large series of samples, data processing by discriminant analysis is used to extract the most discriminating mass peaks between samples (Boon, 1989; Moers et al., 1990).

In on-line Py-GC-MS, the volatile Py products are flushed by helium onto a GC capillary column, generally coated with an apolar silicone phase. The mass spectra of the Py products are then commonly obtained by electronic impact (70 eV), in the 20 to 250 mass range. This narrow mass range exploration is assigned to the GC-filtering effect, which would prevent dimers from reaching the mass spectrometer. It may also be purposely restricted to the m/z 110 to 250 area, to specifically observe the peaks of lignin-derived products, in reconstructed ion chromatograms, or "ligninograms," as sugar-derived products mainly fragment to lower mass values in the ion source (Boon, 1989).

B. Pyrolysis-Mass Spectrometry Structural Screening of Lignocellulosics

The complex Py-MS spectra of cell walls will not be discussed in detail. Almost every mass peak of the Py-MS fingerprint originates from several compounds. For example, the mass peak at m/z 180 can be assigned to molecular ions of Z or E coniferyl alcohol and of 4-vinylsyringol (Genuit et al., 1987). Monomer ion markers for various cell wall components have been identified (Table 6-1). Ion markers for the sugar components are typically in the low mass range. Ion markers of the G lignin units correspond to a sequence of molecular ions, from guaiacol gradually substituted by propenoid sidechains to coniferyl alcohol (Haider & Schulten, 1985). Analogous ion markers for the lignin S units are found at +30 atomic mass units (a.m.u.) (Table 6-1).

Table 6-1. Specific monomer ion markers of lignocellulosics Py-MS spectra (electronic impact). Data from Hartley and Haverkamp (1984), Haider and Schulten (1985), Boon (1989), Moers et al. (1990), and Niemann et al. (1990).

Hexosan and hexose ion markers: 32, 43, 55, 58, 60, 72, 73, 74, 82, 84, 85, 96, 98, 110, 112, 124, **126, 144**†

Pentosan and pentose ion markers: 32, 43, 55, 58, 60, 61, 72, 73, 74, 85, 86, 96. 98, 112, **114**

Guaiacyl lignin units ion markers: 124, 137, 138, 150, 152, 164, 166, 178, **180**
Syringyl lignin units ion markers: 154, 168, 194, **210**

P-coumaric units ion markers: **120,** 165
Ferulic units ion markers: **150,** 194

Protein ion markers: 34, 48, 56, 64, 69, 80, 81, 83, 92, 97, 100, 117

† The most specific ions within each series are in bold characters.

Besides ion markers for core lignin, the ions at m/z 120 and 150, assigned to 4-vinylphenol and 4-vinylguaiacol, respectively, were shown to chiefly stem from the pyrolytic decarboxylation of *p*-coumaric and ferulic units. Their noticeable presence in the Py-MS spectrum of a plant material is typical of grass cell walls (Hartley & Haverkamp, 1984).

In addition, Py-MS spectra present phenolic dimer ions, in the m/z 240 to 420 range, particularly when milder ionization procedures are used (Schulten, 1984; Haider & Schulten, 1985; Pouwels, 1989). In agreement with the literature (Nimz, 1974), the high relative intensity of the m/z 418 dimer ion in the time-integrated-Py-FI mass spectrum of a poplar milled wood lignin fraction (Fig. 6–2) might reflect the significant occurrence of syringaresinol structures in this hardwood lignin sample.

Recent papers have illustrated the potential of Py-MS to structurally screen lignocellulosic samples, in a few minutes per sample and from microgram amounts of material. The following selected examples, presented in a nonexhaustive way, illustrate this.

Tissue fractions from carnation (*Dianthus caryophyllus* L.) cultivars were classified according to their relative lignin and polysaccharide contents, esti-
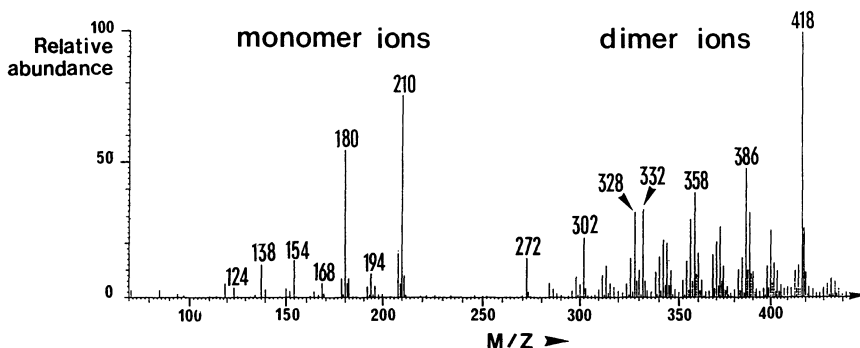

Fig. 6–2. Time-integrated Py-Fl mass spectrum of a poplar milled wood lignin pyrolyzed in the 50 to 500 °C range. Monomers ions at m/z [210, 194, 168, 154] and [180, 138, 124] are markers of the syringyl and guaiacyl units respectively. Dimer ion at m/z 418 may be indicative of syringaresinol structures and the [272, 302, 332] series may be assigned to stilbenes formed from diarylpropane structures (Pouwels, 1989).

mated from specific ion markers. In agreement with the literature (Terashima, 1990), it was found that lignin from the xylem in the early stages of lignification gave rise mainly to G ion markers, whereas G-S lignin type was found in older tissues (Niemann et al., 1990).

In a similar microcharacterization, the first steps of elicited lignification in petunia (*Petunia hybrida* cv. Violet 30) cell cultures were studied. The occurrence of G units, after a few days of incubation with the elicitor, was thus established (Hagendoorn et al., 1990).

In the field of forage research, Hartley and Haverkamp (1984) hypothesized that *p*-coumaric and ferulic acids were partly associated to pentosan components, from discriminant analyses of Py-MS data. They further observed a good correlation between the relative intensity of the m/z 120 4-vinylphenol ion and the *p*-coumaric acid content of grass cell walls. On the contrary, discrepancies between the ion intensity at m/z 150 and the ferulic acid content were reported (Wallace, 1989). This emphasizes that the relative intensity of some ion markers cannot be directly related to cell wall composition. Kivaisi et al. (1990) demonstrated, by Py-MS, that carbohydrate-lignin complexes were spontaneously released after incubation of barley straw in an artificial rumen. The Py-MS method, however, was not capable of giving any evidence of lignin structural modification during this incubation.

In summary, Py-MS is well adapted to the compositional screening of cell walls. In the field of lignins and lignocellulosics, the main Py-MS studies concern the evaluation of lignocellulosics for their lignin content and monomer composition, the tracing out of natural or pathological lignification, and the survey of biodegradation and peatification processes. No information about lignin functionality or interunit bonds can be clearly obtained.

C. Pyrolysis-Gas Chromatography-Mass Spectrometry Molecular Profiling of Lignins and Lignocellulosics

The GC separation of the volatile degradation products before their mass spectrometric analysis allows identification of components by comparison of their retention times and mass spectra to those of appropriate models or to literature data (Obst, 1983; Boon, 1989; Faix & Meier, 1989; Pouwels, 1989; Ralph & Hatfield, 1991; Meier & Faix, 1992).

A few papers pointed out some problems met in Py-GC-MS quantitations. According to Ralph and Hatfield (1991), some lignin-derived monomers may be recovered only in low yield due to the decomposition of these phenolics in the glass insert at the head of the capillary column. A closer study of this experimental difficulty was performed for *p*-hydroxycinnamic acids. It was shown that the careful silylation of the glass insert was a prerequisite to the quantitative capillary GC analysis of these underivatized acids (Fritz & Moore, 1987).

From G lignins, the main identified phenolics were guaiacol, 4-methylguaiacol, 4-ethylguaiacol, 4-vinylguaiacol, eugenol, isoeugenol, vanillin, coniferaldehyde, and coniferyl alcohol. Their S analogues were additionally

found from angiosperm lignins. In this case, about 75 phenolics were identified (Faix & Meier, 1989; Ralph & Hatfield, 1991; Meier & Faix, 1992). The presence of catechol derivatives was assigned to demethylation reactions occurring during the thermal breakdown of lignins (Faix & Meier, 1989; Masuku, 1991).

Besides the lignin-derived peaks, the pyrograms present a few diagnostic peaks from cellulose and xylans, but their relative importance is not a close reflection of the polysaccharide content, owing to a low Py yield (Pouwels, 1989; Ralph & Hatfield, 1991). Nevertheless, Py-GC-MS was used to comparatively estimate the relative proportions of lignins and polysaccharides in cell walls. By summing the peaks specific for lignins, hexosans, and pentosans, Faix et al. (1990) reported cell wall evaluations satisfactorily correlated with the results of standard chemical methods. While such quantitative use may require a prolonged practice, the qualitative examination of pyrograms at least allows the detection of residual lignins in carbohydrate fractions, and conversely, the detection of residual carbohydrates in isolated lignins.

The Py-GC-MS has been often used to classify lignins according to their H/G/S monomer ratio (see chapter 13 by Jung and Deetz in this book). The S/G Py ratio of angiosperm lignins was calculated, from the summed area of the S peaks to the one of the G peaks. It should be kept in mind, however, that the Py monomers represent < 20% of core lignins (Faix & Meier, 1989). As pyrolytic depolymerization proceeds by cleavage of ether bonds, this S/G pyrolytic ratio was shown to be higher than the actual value, because the S units are more often involved in ether-bonding pattern than the G ones. In addition, the H units incorporated in H/G/S synthetic lignins were poorly recovered among the Py monomers, owing to their involvement in condensed bonding patterns (Faix & Schweers, 1975). Such reservations on the representativity of the H/G/S analytical ratio are common to the pyrolytic and chemical degradative methods. Provided that they are taken into account, the S/G pyrolytic ratio may be valuably discussed. For example, Pouwels (1989) obtained results indicative that beech (*Fagus sylvatica* L.) root lignins had a lower S content than stem lignins and that the S units might be more easily biodegraded than the G ones. Another valuable use of the Py-GC-MS method, allowed by its high sensitivity, was the estimation of the low S content of softwood lignins (Fullerton & Franich, 1983; Obst & Landucci, 1986a). More difficult is the unequivocal estimation of the H units in grass core lignins, owing to the interference of *p*-coumaric and *p*-hydroxyphenylpropane units degraded to common products. On the contrary, Py-GC-MS gave clearcut evidence of unusual chlorinated phenolics in chlorite-delignified cell walls (Pouwels, 1989).

Besides the lignin amount and type, little other structural information has been obtained by Py-GC-MS. The observations of different lignin pyrograms were generally interpreted as an evidence of different structures, but without detailed explanations. In addition, the evaluation of the pyrograms from acetylated lignin samples gave an estimation of the acetylatable hydroxyl groups (Sjöstrom & Reunanen, 1990).

This survey of analytical Py indicates that its main application in lignin research is the evaluation of the lignin amount and monomer composition, with screening capabilities for large series of samples. In the case where the quantity of sample is in the micro- or even nanogram range (Obst, 1983), this is probably the most suited method to readily obtain information on lignins, even if present as trace components. By comparison with standard pyrograms, the pyrolytic profiles may also be used as fingerprints to establish the origin of a lignin sample, without any detailed chemical interpretation. The severe pyrolytic depolymerization causes intense modifications in the sidechains of the various cell wall phenolics. Hence, the detection of the aforementioned phenols should be considered as an indicative, but not definitive, core lignin signature.

Future improvements of the pyrolytic techniques will concern a better control of the quantitative reproducibility, a better basic knowledge of the relations between pyrograms and lignin structure to go further in their interpretation (Meier & Faix, 1992), and the introduction of new techniques to circumvent the GC-filtering problem, with maintenance of product identification by Py-Liquid Chromatography-MS or Py-MS-MS (Boon, 1989).

II. THE LIGNIN CARBON SKELETON TRACED OUT BY CARBON-13 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Fourier transform ^{13}C-NMR spectroscopy of lignins allows the observation of resonance signals spread over a wide spectral range and assignable to the various carbons of the lignin skeleton. This nondestructive method is thus attractive to obtain information about lignin units, interunit bonds, and key functionalities. Solution and solid-state NMR methods have both contributed to the structural investigation of lignins, since 1973. This traceable to the rapid advances in NMR instrumentation, which permitted new NMR strategies to unravel the complex spectra of lignins.

Lüdemann and Nimz (1973) introduced solution ^{13}C-NMR to the structural investigation of lignins. Their pioneering work, performed on softwood (conifer), hardwood (deciduous), and grass lignins, as well as on synthetic lignins, before and after acetylation (Lüdemann & Nimz, 1973; Nimz & Lüdemann, 1976; Nimz et al., 1982, 1984), was followed with the quantitative use of ^{13}C-NMR spectra and the application of one (1-D)- and two (2-D)-dimensional multipulse techniques.

Solid-state ^{13}C-NMR started a few years later in the field of lignocellulosic research (Bartuska et al., 1980). Various applications of this technique have been recently reviewed (Newman, 1989). Like solution NMR, it affords information about lignin units and interunit bonds. Compared to solution NMR, it offers the major advantage of characterizing insoluble or in situ lignins, but its main drawback is the severe peak overlap resulting from inherently broad linewidths and interferences with carbohydrates. A considerable amount of structural information is thus lost. With future advances in solid-state NMR spectroscopy, it is likely that this problem will be partly

alleviated. With regard to the objective of this chapter, the presentation will focus on the more informative solution ^{13}C-NMR spectroscopy of soluble lignin samples, despite the lack of quantitative and qualitative representativity of these soluble lignins.

A. Assignments of Lignin Spectra: Qualitative and Quantitative Aspects

Carbon-13-NMR spectrometers operating at ^{13}C-NMR frequency higher than 50 MHz have allowed a considerable increase on both the signal resolution and the signal-to-noise ratio of the lignin spectra (Chen & Robert, 1989; Landucci, 1991a). This is exemplified in Fig. 6–3, comparatively showing the ^{13}C-NMR spectra of a poplar lignin sample recorded with spectrometers operating at 22.63 and 100.6 MHz. A high field ^{13}C-NMR lignin spectrum may contain more than 50 resolved lines. This represents an immense amount of information, as each of these lines is related to a specific C type. Per contra, the assignment of such spectra constitutes a real challenge. This was primarily done by comparison with the spectra of many monomer
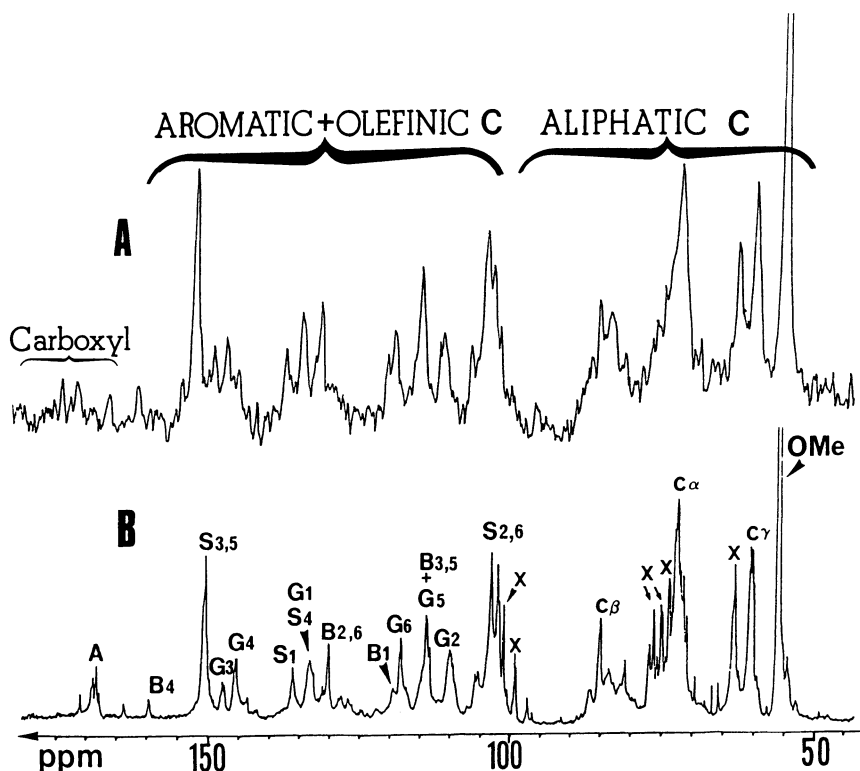

Fig. 6–3. Carbon-13-NMR spectra of a poplar milled wood lignin recorded at (A) 22.63, and (B) 100.6 MHz, in DMSO-d$_6$. Sample concentration and recording time are similar. Main quoted assignments correspond to G: guaiacyl, S: syringyl core lignin units and to B: *p*-hydroxybenzoic esters, with the usual carbon numbering. Cα, Cβ, Cγ : aliphatic carbons in arylglycerol-β-aryl ethers. A: acetyl groups in xylans. X: xylose signals.

and dimer models (Lüdemann & Nimz, 1974; Kringstad & Mörck, 1983). Besides this empirical approach, a few additivity rules about the substituent effects on aromatic chemical shifts (i.e., the spectral position of the signals) could be drawn (Hassi et al., 1987). Facing this myriad of model compound data, it will be possible to fruitfully consult a ^{13}C-NMR database of lignin models (Landucci, 1991a).

The difficulty in assigning the overcrowded ^{13}C-NMR spectrum of soluble lignins can be seen from the data of Table 6–2. It is, however, beyond the scope of this chapter to give an exhaustive list of possible assignments. Rather, the objective of Table 6–2 is to report a few typical core lignin signals and emphasize that many of these peaks cannot be related to a unique lignin structure. This table also points to the signal interferences which happen between core lignins and noncore lignin or carbohydrate components.

Interferences from noncore lignin components are essentially met in Gramineae lignin samples in the case of the p-coumaric and ferulic units, and in Salicaceae lignin samples in the case of p-hydroxybenzoic esters. These noncore lignin components, present in relatively low amounts, give rise to sharp peaks distinctly visible above the broader signals of aromatic carbons from core lignins (see Fig. 6–3 and 6–4). Interferences from carbohydrates vary according to the lignin isolation procedure used and sample origin. The most frequently used lignin fractions, those isolated by the relatively mild procedures of ball milling and cellulase hydrolysis, typically contain a few percent of sugars when isolated from normal softwoods, 5 to 10% in the case of hardwoods, and 10 to 20% in the case of grasses (Lapierre, 1986; Scalbert et al., 1986). The presence of sugars can be seen from the signals of anomeric C-1 carbons in the 92 to 102 ppm range. These signals are sharp, allowing their easy detection. Mild purification procedures have been recommended to alleviate the sugar interferences (Chen & Robert, 1989). However, they may cause selective losses of some lignin moieties and are often ineffective in the case of grass samples. It is beyond the scope of this section to discuss ^{13}C-NMR analysis of carbohydrates (see chapter 5 by Nevins in this book) and only a few specific and diagnostic signals have been reported in Table 6–2. The main sugar component of poplar and wheat lignin fractions corresponds to xylose internal units of polysaccharides. By examining the other sugar signals, it was shown that the polysaccharides accompanying poplar and wheat lignin fractions were acetylated 4-O-methyl-glucuronoxylans and arabino-glucuronoxylans, respectively (Lapierre et al., 1984; Scalbert et al., 1986). In comparable lignin samples from gymnosperm woods, the contamination is usually lower and sugar signals are hardly discernible, except in the spectra of compression wood lignins showing clearcut galactose signals (Fig. 6–5). Hence, the characteristic galactan of compression wood (Timell, 1981) was closely associated with lignins. While such observations may enlighten the lignin-polysaccharide relationships, sugar signals are an impediment to detailed discussion of the aliphatic lignin signals and may lead to misinterpretations.

One (1-D)- or 2-D multipulse NMR techniques (Schilling, 1985) have been used to aid the assignment of lignin spectra. Among the 1-D experi-

Table 6-2. Assignments of some signals from core lignin, carbohydrate and noncore lignin components present in the ^{13}C-NMR spectra of wood and grass lignin fractions (in DMSOd$_6$). Data from Himmelsbach and Barton (1980), Lapierre et al., (1984, 1987), Scalbert et al. (1986), Chen and Robert (1989).

Shift range and C type	Chemical shift, ppm	Core lignin	Carbohydrates	Phenolic acids
200-165 ppm carbonyl and carboxyl	194	CO in Ar-CH = CH-CHO and in Ar-COR	--	--
	192-191.5	Ar-CHO	--	--
	172.1	--	Aliphatic R-COOH	--
	170.1	--	CO in uronic esters	--
	169.8-169.1	--	acetyl CO in xylans	--
	167.7	--	--	COOH in Fe ethers
	167.2	Ar-COOH	--	--
	166.3	--	--	COOR in Pc and Fe esters
	165.4	--	--	COOR in B esters
165-103 aromatic and olefinic C	162.0	--	--	C-4 in B esters
	161.4	--	--	C-4 in Pc ethers
	159.9	--	--	C-4 in Pc esters
	156.3	C-4 in H	--	--
	153-152	C-3,5 in S; C-3 in G diphenyl; Cα in Ar-CH = CH-CHO	--	--
	149.2-149.0	C-3 in G	--	--
	147.5-147	C-4 in G, C-3 in G', C-3,5 in S'	--	C-4 in Fe esters/ethers
	145.6-145	C-4 in G'	--	C-3 in Fe esters/ethers
	144		--	Cα in Pc, Fe esters
	143.8-143	C-4 in phenylcoumaran; C-4 in diphenyl	--	Cα in Pc, Fe ethers
	137.9-137.7	C-1 in S, in syringaresinol	--	--
	135.2-134.7	C-4 in S, S', C-1 in G	--	--
	133.3-133	C-1 in G', S', H	--	--
	131.4	--	--	C-2,6 in B esters
	130.1	--	--	C-2,6 in Pc esters/ethers
	129.1-128	Cβ in olefinic C	--	--

Table 6-2. Continued.

Shift range and C type	Chemical shift, ppm	Core lignin	Carbohydrates	Phenolic acids
	133.3–133	C-1 in G', S', H	--	--
	131.4	--	--	C-2,6 in B esters
	130.1	--	--	C-2,6 in Pc esters/ethers
	129.1–128	Cβ in olefinic C	--	--
	127.9	C-2,6 in H	--	--
	125.2–124.9	--	--	C-1 in Pc, Fe esters/ethers
	122.8	--	--	C-6 in Fe esters
	122.2	--	--	C-6 in Fe ethers
	120.5	--	--	C-1 in B esters
	119.6–119.3	C-6, in G, G'	--	--
	116.7	--	--	⎡ Cβ in Pc, Fe ethers
	115.5–114	C-5 in G, G' / C-3,5 in H	--	C-3,5, in PC, B esters / C-5 in Fe esters / ⎣ Cβ Pc, Fe esters
	111.7–111.2	C-2 in G, G'	--	--
	106.9–106.6	C-2,6 in S with αCO	--	--
	104.7–104.4	C-2,6 in S, S'	--	--
108–20 ppm aliphatic carbons	107.4	--	C-1 Ara	--
	101.8	--	C-1 Xyl	--
	97.7 and 92.4	--	C-1 Xyl' β and α	--
	97.3	--	C-1 4-O-Me Glu	--
	87.1–85	⎡ Cβ in β-O-4 / ⎣ Cα in resinols	--	--
	81.7–81	--	C-4 4-O-Me Glu, Glu	--
	76.2–75.8	--	C-4 in Xyl, $Xyl_9\beta$	--

Table 6-2. Continued.

Shift range and C type	Chemical shift, ppm	Core lignin	Carbohydrates	Phenolic acids
	74.8–74.0	--	C-2, C-3 Xyl', C-3 Xyl	--
	72.6	--	C-2 Xyl	--
	72.3–71.7	Cα in β-O-4	--	--
	71.3	Cα in resinols	C-2 in 4-O-Me Glu	--
	71	--	C-3 Xyl'	--
	63.2–62.7	⎡ Cγ in phenylcoum. ⎢ Cβ in diarylpropanes ⎢ Cγ in β-O-4 with αCO ⎣	C-5 in Xyl and Xyl'β	--
	60.1–59.7	Cγ in β-O-4	C-5 in 4-O-Me Glu, C-6 Glu	--
	58.8	--	C-5 in Xyl'α, OMe in 4-O-Me Glu	--
	55.9	OCH$_3$ in H, G, S	--	OCH$_3$ in Pc, Fe
	54.5	Cβ in resinols	--	--
	53.3	Cβ in phenylcoumaran.	--	--
	20.8–20.2	--	Acetyl CH$_3$ in Xylans	--

† Usual carbon numbering and symbols for lignins and phenolic acids are used.

‡ Core lignin: p-hydroxyphenyl units = H; etherified and nonetherified at C-4 guaiacyl units = G and G'; etherified and nonetherified at C-4 syringyl units = S and S'.

§ Carbohydrates: internal and terminal reducing end xylose units = Xyl and Xyl'; arabinose units = Ara; glucose units = Glu; 4-O-Methyl glucuronic acid units = 4-O-Me Glu.

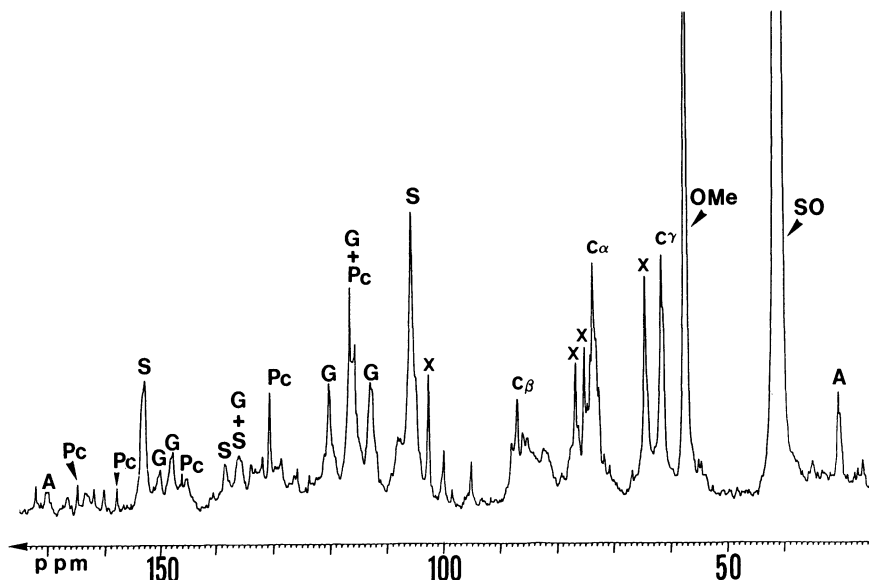¶ Noncore lignin: p-hydroxybenzoic acid units = B; ferulic and p-coumaric acid units = Pc and Fe.

Fig. 6-4. Carbon-13-NMR spectrum of a wheat-milled straw lignin in DMSO-d$_6$ (SO). Only the major contributors to signals are quoted. G, S, Cα, Cβ, Cγ, A, and X: same as in Fig. 6-3. Pc: *p*-coumaric esters.

ments, those involving polarization transfer, such as the DEPT experiment, were used to separately observe the lignin CH_3, CH_2, and CH on different subspectra (Lapierre et al., 1984; Bardet et al., 1985). Two-dimensional lignin spectra were recorded from ^{13}C-enriched poplar lignins, prepared to alleviate the poor sensitivity of the homonuclear 2-D ^{13}C INADEQUATE NMR experiment (Bax et al., 1981). These ^{13}C-enriched poplar lignins were obtained by growing poplars in a [13]CO_2-enriched atmosphere (Lapierre et al., 1984). The lignin enrichment, at 11% [13]C, was sufficient for the success of the INADEQUATE experiment applied for the first time to a macromolecule (Guittet et al., 1985; Bardet et al., 1986; Lapierre et al., 1987). The examination of these 2-D INADEQUATE poplar lignin spectra revealed most of the C connectivities of the lignin and sugar skeletons from unequivocally separated signals. It confirmed most of the assignments previously obtained from 1-D spectra and corrected a few important errors. Other types of 2-D experiments, such as those revealing the [13]C-^{1}H connectivities, have been recently applied to lignins (Ede, 1991).

One approach used to partly alleviate the problem of peak overlap in a more routine way relies on the manipulation of the digitized data, which are a collection of exponentially decaying sine waves before Fourier transformation. Mathematical treatments of this composite-free induction decay (FID) signal have provided a resolution enhancement of the resulting spectrum. These simple procedures, which do not require separate data acquisition, aim at the reduction of signals in the initial part of the FID where the fast-relaxing components, giving rise to the broader linewidths, essentially reside. Such a technique has been used to discriminate fast relaxing and slowly
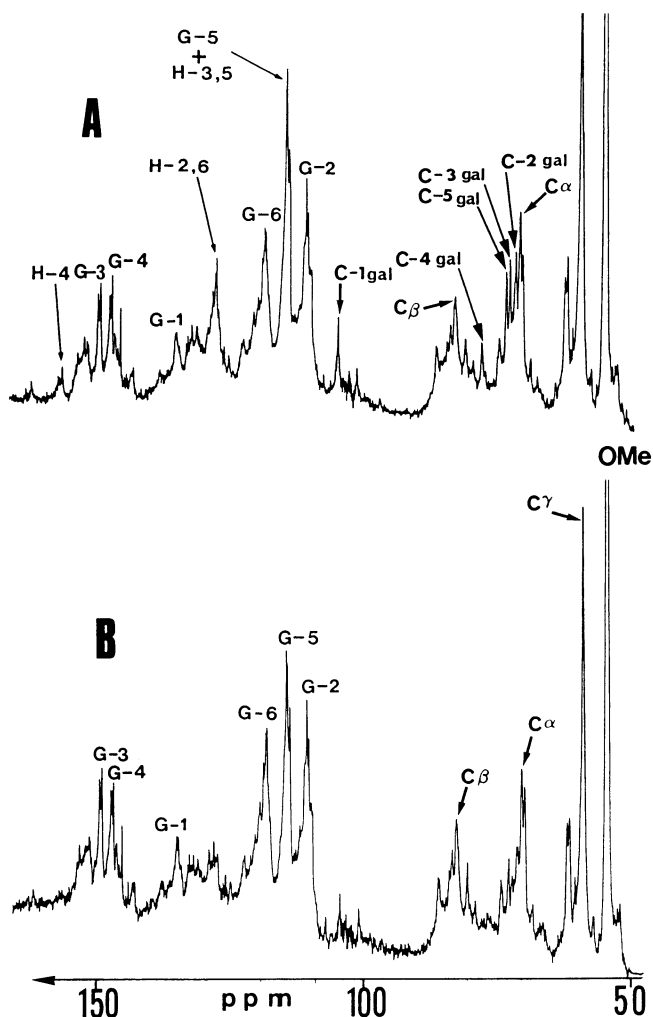
Fig. 6-5. Carbon-13-NMR spectra of pine milled wood lignins from (A) compression wood and (B) opposite wood. Besides the usual G signals, the spectrum of compression wood lignin shows signals from p-hydroxyphenylpropane units H and from galactose gal. residues.

relaxing components of lignin samples and localize the signals from the mobile xylan present in a willow-milled wood lignin sample (Landucci, 1991b).

Besides these strategies used to qualitatively assign the lignin spectra, a major contribution of ^{13}C-NMR in lignin research was afforded by its quantitative use. When recorded in routine conditions, the areas of the signals are not proportional to the number of corresponding ^{13}C nuclei. With special recording conditions, however, the quantitation of lignin spectra is meaningful. This upgrading of the ^{13}C-NMR method was first applied to lignins by Robert and Gagnaire (1981). Using long relaxation delay between pulses and the inverse gated proton decoupling (IGD) experiment, these authors obtained quantitative ^{13}C-NMR lignin spectra. The requirements to

do this have been elucidated by Landucci (1985). Later, it was shown that the DEPT subspectra could also provide quantitative information (Chen & Robert, 1989). Peak clusters, corresponding to the main C types, have been defined and the principle of the calculations and some assumptions have been reported (Landucci, 1985; Chen & Robert, 1989). The aromatic region of the spectrum is frequently chosen as the standard for relative quantitations of the various C types.

B. Structural Features Accessible from Solution Carbon-13 Nuclear Magnetic Resonance Spectroscopy of Lignins

The main structural features accessible from solution ^{13}C-NMR of lignins are the following ones.

1. Type and Amount of Monomer Building Units

The signals from G and S aromatic carbons have been considered for lignin classification. In addition, the methoxyl/aryl (R) ratio, calculated from the areas of the methoxyl peak (59–54 ppm) and the aromatic peak cluster (156–103 ppm), was used to estimate the S/G ratio from the formula S/G = (6 − R)/(2R − 6) (Landucci, 1985; Obst & Landucci, 1986a). It was found that S/G ratios determined from ^{13}C-NMR of hardwood lignins were lower than those determined from chemical degradation procedures, as the latter essentially characterize the uncondensed and S-rich lignin moiety (Obst et al., 1987). For heartwood lignins such calculations might be erroneous, owing to the occurrence of nonlignin aromatic signals (Obst & Landucci, 1986b). The unequivocal estimation of H core lignin units in grass lignins has not been performed by ^{13}C-NMR essentially for lack of specific signals, devoid of interferences. The H signals could be localized but not quantified from the spectrum of compression wood lignin (Fig. 6–5), which was reported to contain up to 30% of H units (Timell, 1981).

2. Lignin Functionality

Several key functional groups in lignins were quantified from the IGD or DEPT spectra. The prominent ones are the methoxyl groups used to classify lignins from their S/G ratio, as previously mentioned. Low methoxyl contents may give an indirect support to the presence of H units in native lignins and occurrence of demethoxylation and demethylation reactions in degraded lignins (Obst et al., 1989). In the same context, the aliphatic/aromatic C ratio, provided that there are no interferences from nonlignin components, affords information on the average length of lignin sidechains. Thus, it could be possible to estimate the extent of degradative sidechain cleavage as met in biodegradation, which causes the Cα-Cβ cleavage, or in drastic alkaline hydrolyses, which split off Cγ groups.

Primary and secondary alcoholic hydroxyl groups and phenolic hydroxyls, important reactive sites in lignins, were quantified from the integration of their specific acetate CO signals in acetylated lignin spectra, centered at 170, 169.4, and 168.5 to 168.1 ppm, respectively (Robert et al., 1984).

In addition, minor functional groups, as aldehydic, ketonic, and carboxylic groups, were characterized as a reflection of lignin oxidative degradation reactions (Hemmingson & Wong, 1989).

The evaluation of olefinic carbons, however, could not be directly performed from ^{13}C-NMR. For example, whereas many literature data have proven the significant formation of vinylic carbons during kraft cooking, their indirect estimation from ^{13}C-NMR led to values close to zero (Robert et al., 1984). This may be related to interferences between olefinic and aromatic C signals.

3. Lignin Interunit Bonds

The β–O–4, β–5, β–1, and β–β interunit bonds were traced from their specific aliphatic signals and their frequencies qualitatively compared (Nimz et al., 1982, 1984). Quantitative estimations of β–O–4 and [β–β + β–5] bonding patterns were also performed (Chen & Robert, 1989). The amounts of other lignin interunit linkages could not be easily determined from ^{13}C-NMR lignin spectra. Estimation of the content of quaternary aromatic carbons, other than those at C–1, C–4, and methoxyl-substituted positions, gives an indication of the content of condensed aromatic units in lignin, provided one assumes that olefinic carbons are negligible constituents (Chen & Robert, 1989).

From this nonexhaustive survey of lignin solution ^{13}C-NMR spectroscopy, the following conclusions may be drawn. The basic advantage of this nondestructive method is that it provides a wealth of qualitative and quantitative structural information on lignin, that would otherwise require the use of several analytical methods. It can further provide indications about the nature of contaminants closely associated to lignin samples, thereby enlightening the relationships between core lignins and other cell wall components. The main practical limitation, at present, is the relatively low sensitivity, which involves an important sample demand (100–300 mg) and long recording times (5–10 h). The most severe drawback of lignin solution state NMR is the method used to isolate soluble lignin: this method may alter the structure of in situ lignin in some way. Furthermore, soluble lignin preparations represent a fraction of total lignin and may not give a true picture of the total lignin in the plant. Even though this is not a specific ^{13}C-NMR problem, care must be taken to avoid misinterpretation of the results linked to interference problem or incorrect assignments. As these assignments primarily rely on comparisons with model compounds, the choice of the most appropriate models requires an a priori structural knowledge of lignins. In that respect, ^{13}C-NMR spectroscopy is most valuable when used in combination with other techniques.

III. CLOSE EXPLORATION OF LIGNIN STRUCTURE BY CHEMICAL DEGRADATION METHODS: THE CASE OF THIOACIDOLYSIS

In a common strategy applied to polymer study, the chemical degradation methods for core lignins call for the depolymerization to low molecular weight compounds. These compounds are then analyzed to yield some structural information about the starting polymer.

In the case of lignins, however, the repeatedly invoked limitation of such a strategy is that only moderate yields of degradation products are obtained, owing to the occurrence of the condensed bonds. In that respect, lignins differ from other biopolymers such as proteins, polysaccharides, and nucleic acids that contain interunit bonds susceptible to both chemical and enzymic hydrolyses. Hence, the chemical degradation methods for lignin characterize only the less condensed portion of the polymer. Furthermore, the phenolic degradation products may participate in secondary reactions, which decrease their recovery yield or create artifacts. Nevertheless, degradative methods have played a key role in the elaboration of our current knowledge on lignin structure (Adler, 1977). Ever since the comprehensive work of Freudenberg, who initiated many of the commonly applied degradation methods such as the alkaline oxidations, extensive efforts have continued to optimize existing methods and develop new ones.

Since 1982, we have been interested in acid solvolyses of lignins as a way to provide unequivocal information on core lignin structure. These acid solvolyses are less familiar to lignin chemists than the alkaline oxidations. Nitrobenzene or cupric oxide alkaline oxidations have been so universally used that their results constituted the basis of the H/G/S lignin classification (Sarkanen & Hergert, 1971; Logan & Thomas, 1985). These routine methods are thus supported by abundant literature data and have the major attributes of providing in satisfactory yield a few lignin-derived monomers. Their main drawback, however, is the interference between core lignins and other cell wall phenolics. This is particularly severe in the case of the nitrobenzene oxidation of grass samples, where p-coumaric and ferulic acids are degraded to the same benzaldehydes as H and G core lignin units, respectively, and with an approximately 60% degradation yield (Iiyama & Lam, 1990). To better examine core lignins, we thus chose to develop acid solvolyses, even though these methods were less documented and led to more complex mixtures than the routine alkaline oxidations. Thioacidolysis was conceived from the acidolysis method, performed according to Lundquist (1976) and practiced for a few years in our laboratory (Lapierre, 1986). As emphasized by Lundquist (1976), secondary reactions occur during acidolysis, which lower the recovery of the monomer degradation products. In addition, rearrangement reactions make the monomer mixture fairly complex. Hence, new experimental conditions were employed both to minimize secondary reactions and simplify the mixture of lignin-derived monomers. Whereas acidolysis was performed in dioxane/water 9/1 v/v, 0.2 M HCl, at 100 °C, and for 4 h, thioacidolysis was conceived by replacing water with ethanethiol

and HCl with BF_3 etherate. These simple changes had outstanding conse-
quences on the performance of the method and its subsequent development.

A. Principle of the Thioacidolysis Procedure

Thioacidolysis has proved to be an evolutive procedure. The type and
amount of information it provides can be varied with additional treatments
performed before or after the key depolymerization step. This is summa-
rized in Table 6-3.

The first procedure, involving simply the analysis of lignin-derived
monomers, provides an estimation of those lignin units only linked by β-
O-4 bonds. For the sake of conciseness, these units are herein referred to
as the uncondensed C_6C_3 lignin units, which means that they are not in-
volved in carbon-carbon or diphenylether bonds. Such units are probably
the most typical structures of core lignins. In addition, their content may
be viewed as a reflection of the polymer degradability. It is worthy to under-
line that thioacidolysis, as well as the other available chemical degradation
methods, does not provide an estimation of the total amount of lignin β-
O-4 bonds.

The second procedure, involving the CH_2N_2 methylation of lignin-free
phenolic groups prior to thioacidolysis, was developed to specifically charac-
terize the terminal β-O-4 linked lignin units that contain free-phenolic groups.
These groups exert a pronounced effect on the solubility and reactivity proper-
ties of the polymer.

The third procedure, involving a desulfurization step after the depoly-
merization one, seems to significantly upgrade the basic thioacidolysis proce-
dure. Using this method, it is possible to estimate the amounts of the
lignin-derived dimers involving various carbon-carbon and diphenylether
bonds that impart resistance to core lignins.

Thioacidolysis is an acidic depolymerization method that proceeds by
cleavage of lignin β-O-4 bonds. The ether-cleaving reagent combines BF_3
etherate and EtSH. It efficiently cleaves ether bonds, except those of methoxyl
groups and diphenylether structures, without concomitant recondensation
reactions. This was shown from model compound studies and the gel-
permeation analysis of the completely soluble reaction product from poplar-
milled wood lignins. The resulting profile mainly corresponded to monomeric

Table 6-3. Evolution of the tioacidolysis method (Lapierre, 1986; Lapierre & Rolando, 1988; Lapierre et al., 1991a, b, c).

| Year	Chemical procedure	Analysis step	Final information
1984	Thioacidolytic depoly- merization only	GC and GC-MS of lignin- derived monomers	Type and amount of uncon- densed β-O-4 units
1988	CH_2N_2 permethylation, then thioacidolysis	GC and GC-MS of monomers with C_4OH and C_4OMe	Percentage of free phenolic groups in β-O-4 uncon- densed units
1991	Thioacidolysis, then Raney nickel desul- furization	GC and GC-MS of lignin- derived monomers and dimers	Indication of the frequencies of various lignin interunit bonds
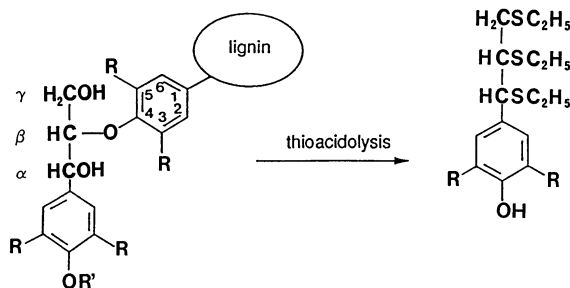
Fig. 6-6. Main thioethylated monomers recovered from the thioacidolysis of arylgylcerol-β-aryl ether structures of core lignins (R = H or OMe; R' = H or Cβ).

and dimeric products (Lapierre, 1986). The main lignin-derived monomers are those depicted in Fig. 6-6. They chiefly originate from arylglycerol C_6C_3 units interconnected only by β-O-4 bonds and are recovered in high yield as a 50/50 mixture of diastereoisomers, for the H, G, and S series. In addition, uncondensed structures of the catechol type, met in industrial lignins, can be characterized. Structural information is also obtained for other types of sidechain substitution patterns, as described in Table 6-4. This table shows that β-O-4 linked benzaldehyde, cinnamaldehyde, and cinnamyl alcohol end groups are converted to specific monomers. This table also illustrates that p-coumaric and ferulic units are degraded to the corresponding free acids and their Michael addition products, compounds distinct from those derived from core lignin.

Compared to alkaline oxidation, thioacidolysis provides more information on core lignin structure, without interference from other cell wall phenolics. The monomer yield from alkaline nitrobenzene oxidation, however, is higher than from thioacidolysis. In the former case, monomers originate not

Table 6-4. Specific monomers obtained by thioacidolysis of various phenolic structures in plant cell walls. Their phenolic rings are not involved in carbon-carbon or diphenylether interunit bonds. R = p-hydroxyphenyl, guaiacyl, or syringyl ring.

| Initial structure and sidechain	Recovered monomers
Core lignin	
C_6C_3 arylglycerol-β-arylethers -CHOH-CHOAr-CH_2OH	R-CHSEt-CHSEt-CH_2SEt erythro/threo, 50/50
C_6C_3 β-arylethers with αCO -CO-CHOAr-CH_2OH	R-CSEt=CHSEt Z and E
p-hydroxybenzaldehyde end groups -CHO	R-CH(SEt)$_2$
p-hydroxycinnamyl alcohol end groups -CH=CH-CH_2OH	R-CH=CH-CH_2SEt and R-CHSEt-CH_2-CH_2SEt
Noncore lignin	
p-hydroxycinnamic acid units ester-or ether-linked	R-CH=CH-COOH and R-CHSEt-CH_2-COOH
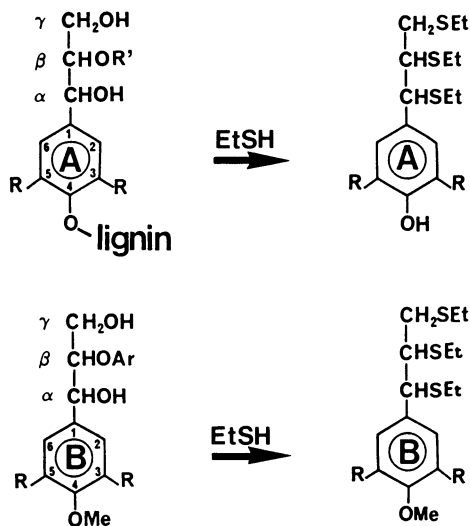
Fig. 6-7. Main thioethylated monomers recovered from the thioacidolysis of CH_2N_2 methylated core lignins (R = H or OMe; R' = H or Ar). The methyl group of C-4 in rings B is introduced by the methylation step. The relative percentage [B/(A + B)]% thus represents the amount of free phenolic groups in uncondensed lignin structures and is calculated for each H, G, and S series (Table 6-6).

only from β-O-4 uncondensed C_6C_3 units but also partly from those involved in β-5, β-1, and β-β interunit bonding patterns (Chang & Allan, 1971; Iiyama & Lam, 1990).

When thioacidolysis is performed from CH_2N_2 methylated lignins, additional monomers with methylated phenolic groups (Fig. 6-7) are recovered. Their relative content, compared to the overall monomer yield, indicates the proportion of free and methylatable phenolic groups within the uncondensed C_6C_3 units. The overall yield in thioacidolysis monomers is not affected by the methylation step. In addition, this CH_2N_2 methylation was shown to be complete and it was not impeded by neighboring methoxyl groups.

The determination of these various lignin-derived monomers is performed by capillary GC of their trimethylsilylated derivatives (TMSi) and their identity checked by GC-MS and comparison with authentic compounds purified from preparative thioacidolyses (Lapierre, 1986; Lapierre et al., 1986; Rolando et al., 1992).

The thioacidolysis-desulfurization procedure aims at the determination of the lignin-derived dimers. In the thioacidolysis mixture, besides the thioethylated monomers, there are thioethylated dimers. Their direct GC determination is impeded by the thioethyl substituents that make their molecular weight and number of isomeric forms excessive. To quantify these dimers, a desulfurization step is performed following the depolymerization. Thioethyl substituents are completely removed by a mild treatment employing commercial grade Raney nickel in methanol, 4 h at 80 °C or overnight at 30 °C. The easiness by which a total desulfurization is obtained makes unlikely the concomitant occurrence of side reactions leading to artifacts. The main dimers

are shown in Fig. 6–8, for the pure G, mixed G-S M, and pure S series (Lapierre et al., 1991a, b, c). Their relative importance varies according to the lignin origin. In addition, minor dimers representative of the β–6 and tetrahydrofuran-bonding patterns were found. In the case of compression wood and grass samples, low amounts of the mixed H-G dimers were observed. By subjecting various dimer models to the whole procedure, it was verified that no additional artifact interunit bonds were formed, except in the case of the resinol structures. In this case and during thioacidolysis, an additional Cα–C6 bond appeared besides the unaffected Cβ–Cβ bond, while the Cα–O–Cγ bonds were cleaved. Hence, the tetralin-type dimers depicted in Fig. 6–8 chiefly stem from resinol structures. The relative frequencies of the series of spruce lignin-derived dimers representative of the 5–5, β–5, β–1, 4–O–5, and β–β interunit bonds were similar between 2 and 6 h of thioacidolysis, which suggests that these dimers are released from core lignin with similar yields. The elimination of Cγ groups neighboring the β–1 and β–5 bonds, however, increased with prolonged thioacidolyses. Similar to the lignin-derived monomers, the lignin-derived dimers were determined by capillary GC of their TMSi derivatives and identified by GC-MS (Lapierre et al., 1991a, b).

The sensitivity of these various procedures is high, owing to satisfactory reaction yields and subsequent capillary GC analyses. Only the weighing accuracy governs the sample demand. The reproducibility is not affected by small experimental changes, such as the reaction duration or BF₃ nor-
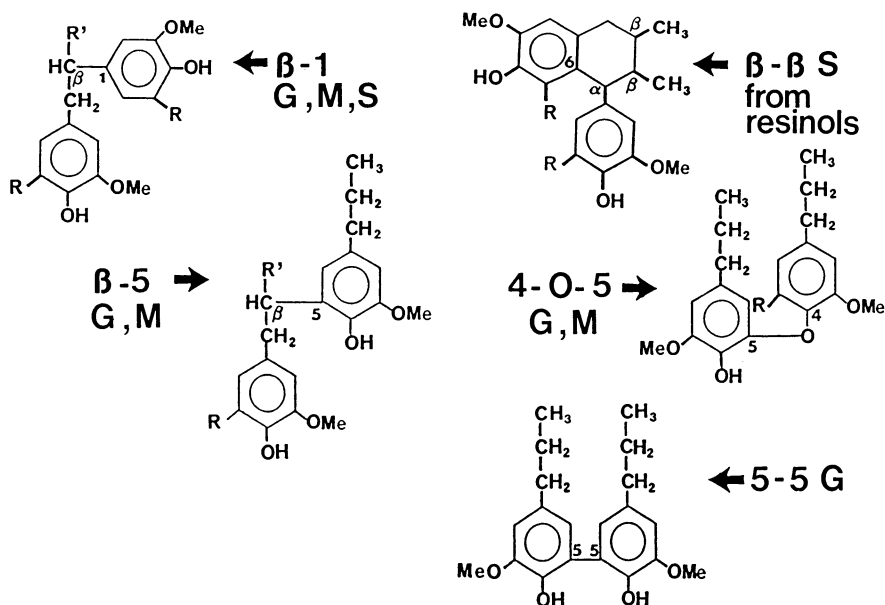

Fig. 6–8. Main dimers recovered from the thioacidolysis, then Raney nickel desulfurization of core lignins. Pure guaiacyl G dimers are obtained from gymnosperm lignins. In addition, mixed guaiacyl-syringyl M, and pure syringyl S dimers are recovered from angiosperm lignins. R = H or OMe. R′ = H or CH₂OH.

mality. The mean standard errors between duplicate analyses are in the 5 to 10% range. The main limitation is the lack of commercially available authentic compounds for calibration and identification. The monomer and dimer analysis enables about 50 to 70% of the woody gymnosperm and angiosperm native lignins, respectively, to be characterized.

B. Structural Features Revealed by the Thioacidolysis of Lignins

The total yield and relative composition of the thioacidolysis main monomers closely reflects the amount and ring type of uncondensed lignin C_6C_3 structures. The results shown in Table 6-5 indicate that lignins of woody angiosperms analyzed so far contain twice as many β-O-4 uncondensed units as the lignins from woody gymnosperms and grass straws. It can be seen that the yields from woody species are more uniform than those from graminaceous plants, emphasizing the genetic or tissular variability of the latter samples. Among the angiosperm species, wood lignins present large variations in S/G thioacidolytic ratios, on the whole higher than those oberved for grass straw lignins. H thioacidolysis monomers were systematically found in all the grass species examined so far, but in low amount. This means that p-hydroxyphenylpropane units are typically incorporated in grass core lignins, but are present in low amount in their uncondensed moiety. Preliminary results for grass lignin-derived dimers do not indicate a higher relative abundance of H units.

With the aim of more closely examining lignin structure, the functional group analysis of lignin sidechains in the uncondensed lignin structures was performed, on the basis of Table 6-4, to decipher the structural modifications introduced in lignins during a photodegradation treatment, simulating

Table 6-5. Yield of the main H, G, and S thioethylated monomers recovered from the thioacidolysis of various extractive-free cell walls (in micromoles per gram of Klason lignin); tr = traces; nd = not detected.

Sample	Total yield H + G + S	Relative distribution H : G : S
Woods		
Spruce [*Picea abies* (L.) Karsten]	1230	2 : 98 : tr
Pine (*Pinus pinaster* Ait.)—compression wood	1140	18 : 82 : tr
—opposite wood	1020	2 : 98 : tr
Poplar (*Populus trichocarpa* Hook.)	2390	nd : 41 : 59
Birch (*Betula verrucosa* Ehrh.)	2460	nd : 24 : 76
Oak (*Quercus robur* L.)	1970	nd : 32 : 68
Nothofagus dombeyi (Mirb.) Oerst.	2355	nd : 14 : 86
Laurelia phillipiana Looser	1860	nd : 66 : 44
Straws		
Wheat (*Triticum aestivum* L., cv. Champlein)	1040	5 : 49 : 46
Triticale (*Secalotriticum*, cv. Montcalm)	1610	3 : 42 : 55
Rye (*Secale cereale* L., cv. dominant)	1670	2 : 44 : 54
Corn (*Zea mays* L.)	610	4 : 35 : 61
Rice (*Oryza sativa* L., cv. Balilla)	630	15 : 45 : 60

Fig. 6-9. Gas chromatography analysis (as their TMSi derivatives) of the monomers recovered from the thioacidolysis of (A) original and (B) photodegraded spruce lignin fraction. Pairs of diastereoisomers 1 and 6 stem from arylglycerol-β-aryl ether structures of the guaiacyl and catechol series, respectively. Compounds [2 + 3], 4 and 5 originate from coniferyl alcohol, vanillic acid, and vanillin end groups, respectively. I.S.: internal standard.
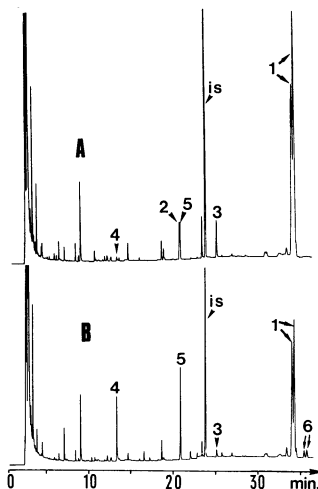
solar irradiation. Figure 6-9 shows the GC analysis of the lignin-derived monomers recovered from the thioacidolysis of original and photodegraded spruce lignin samples. The main photodegradative events were directly read from these chromatograms and also quantified (Pan et al., 1991). The lower relative importance of peak 1, assigned to the major thioacidolysis monomers, revealed that uncondensed arylglycerol lignin structure are fewer after irradiation, which suggests the photoformation of condensed linkages. The drastic decrease in peaks 2 and 3, assigned to coniferyl alcohol end groups, indicated that these end groups were highly susceptible to photodegradation. Conversely, the relative increase in peaks 4 and 5, derived from vanillic acid and vanillin end groups, showed that $C\alpha$–$C\beta$ photooxidative cleavages occurred in lignins. Finally, the appearance of peak 6, assigned to the catechol analogues of peak 1, reflected the occurrence of demethylation reactions during phototreatment.

The methylation-thioacidolysis procedure afforded original information about natural lignins. It was observed that the H, G, and S lignin units were not evenly distributed at the periphery or inside the core lignin network, among the uncondensed lignin structures, it was found that about 90% of the H units met in grass or compression wood lignins were terminal units with free phenolic groups. In contrast, the syringyl units in natural lignins were shown to be extensively etherified at their phenolic groups, which was not any more the case with industrial kraft lignins. The behavior of the G uncondensed structures was intermediate between the extreme behaviors of the H and S units (Lapierre & Rolando, 1988). The reason for these puzzling differences is not yet clearly established.

Employing the same strategy, the molecular origin of the characteristic alkali solubility of grass lignins was investigated (Lapierre et al., 1989). A systematic difference was observed between grass and wood lignins, about 40 to 55% of the uncondensed G units were terminal units with free phenolic groups in grass lignins, whereas the levels were approximately one-half

Table 6-6. Percentages of the free phenolic groups within the uncondensed guaiacyl or syringyl lignin structures, as shown from thioacidolyses of CH_2N_2 methylated extractive-free cell walls.

Samples	G%	S%
Wood		
Pine compression wood	22	--
Pine opposite wood	23	--
Poplar tension wood	25	3.5
Poplar opposite wood	28	3.6
Straw		
Wheat	38	4
Triticale	38	4
Rye	38	4
Barley	38	5
Rice	53	6
Corn	50	5
Oilseed rape	29	3

this in wood lignins (Table 6-6). This difference was not specific for annual plants, as the value for oilseed rape straw resembled that of wood lignins. In addition, it was evidenced that grass lignins solubilized by a mild alkali treatment were enriched in G-free phenolic groups, compared to the total or residual lignin fraction (Table 6-7). A clue to this structural investigation was provided by the observation that the CH_2N_2 exhaustive methylation of free phenolic groups suppressed, to a large extent, the solubilization of grass lignins in $2N$ NaOH solution and at 40 °C, even after prolonged treatment (Lapierre et al., 1989). Accordingly, the molecular origin of the NaOH solubility of in situ grass lignins could be related to their high content in G-free phenolic groups.

The determination of lignin-derived dimers revealed that the main dilignols recovered from conifer lignin (Table 6-8) were representative of the 5-5, β-5, β-1, and 4-O-5 bonds, whereas the pinoresinol-derived dimer was present in negligible amount (Lapierre et al., 1991a, b). This was in accordance with the data of Sakakibara (1980), obtained by the hydrogenolysis procedure. In addition, the heterogeneous structure of core lignins was evidenced in the case of the β-1 bonding pattern. These substructures were much more frequent in the easily solubilized lignin fragments, associated with polysaccharides in the course of milled wood lignin purification steps (Table 6-9). Even if these β-1 linkages are still the matter of conflicting results, the diarylpropane structures of natural lignins were shown to be easily solubilized,

Table 6-7. Percentages of free phenolic groups in the uncondensed guaiacyl structures of grass straw samples. Alkali extractions of straw lignins have been performed by $2 N$ NaOH at 40 °C, and for 2 h.

Samples	Wheat	Rye	Triticale
Original straw in situ lignins	38	38	37
Alkali solubilized then purified lignins	44	44	43
Residual lignins in alkali-extracted straw	29	31	28

Table 6-8. Absolute and relative yields of thioacidolysis products issued from natural and synthetic guaiacyl lignins.

Samples	Spruce in situ lignin	Synthetic DHP
Total yield in main G products (micromoles per gram lignin)		
− monomers	1260	620
− dimers	310	202
Relative frequencies (% molar) of the G dimers provided with following interunit bonds:		
− 5–5	35	17
− β--5	33	53
− β–1	26	6
− 4–O–5	6	2
− β–β†	Traces	22

† Tetralin dimer originates from pinoresinols.

highly reactive, and mainly peripheral lignin end groups (Gellerstedt & Zhang, 1991; Lapierre et al., 1991c; Pan et al., 1991). Another support to the intriguing role of the diarylpropane structures in natural lignins relied on the observation that synthetic G lignins or DHPs, prepared in various conditions, afforded generally low levels of β–1 dimers, when subjected to the whole procedure (Table 6-8 and Tollier et al.,1991). Conversely, these synthetic G lignins gave rise to significant amounts of pinoresinol-derived dimer and β–5 ones. These results emphasized that DHPs, which proved to be outstandingly useful models, do not adequately represent the structure of natural lignins. Current investigations are in progress to optimize the polymerization conditions of DHPs, prepared by peroxidasic dehydrogenation of coniferyl alcohol, so that the final synthetic polymers more closely resemble natural lignins, as revealed by the present methodology.

The analysis of the angiosperm lignin-derived dimers is far more complex than in the case of the conifer G lignins. This complexity is underlined in the chromatogram of Fig. 6-10, showing the GC separation of the degradation products recovered from poplar in situ lignins. The results obtained for angiosperm wood lignins may be summarized as follows (Table 6-10).

The main recovered dimers were representative of the β–1 and syringaresinol structures, in accordance with the results reported by Nimz (1974) and obtained from the alkaline depolymerization of thioacetylated lignins (thioacetolysis). The medioresinol- and pinoresinol-derived dimers were only recovered as trace components. The S units were less involved in the dimer than in the monomer fraction. Preliminary results obtained for grass in situ

Table 6-9. Relative frequencies of the main dimers recovered from the thioacidolysis of water-precipitable and water-soluble spruce-milled wood lignin fractions. The water-soluble lignin fraction is heavily contaminated with carbohydrates.

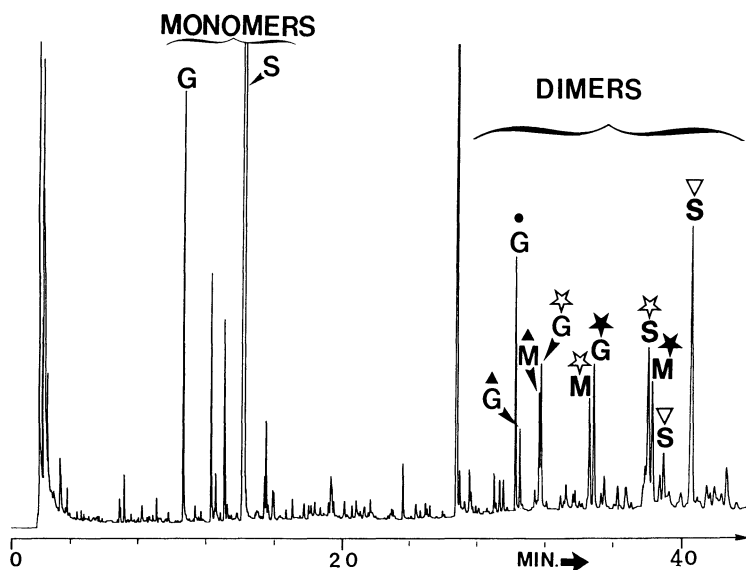| Sample	H_2O-precipitable lignin	H_2O-soluble lignin
Interunit linkage type		
− 5–5	34.2	20.6
− β–5	29.2	19.4
− β–1	29.1	56.2
− 4–O–5	7.5	3.8

Fig. 6-10. Gas chromatography analysis of the monomers and dimers recovered from poplar in situ lignin. G, M, S: see Fig. 6-9. The dimers are representative of the ●:5-5, ★: β-5, ☆: β-1, ▲: 4-O-5, and ▽: β-β (from resinols) interunit bonds.

than in the monomer fraction. Preliminary results obtained for grass in situ lignins have revealed that the relative frequencies of the various recovered dimers is similar to the figures presented in Table 6-10. The main ones are representative of the β-1 and syringaresinol bonding patterns, followed with the β-5 dimers and then by the 4-O-5 and 5-5 ones. Only small amounts of H-G dimers could be observed, mainly for the 5-5 series.

Table 6-10. Total yields and relative frequencies in syringyl (S), mixed guaiacyl-syringyl (for dimers) (M), and guaiacyl (G) lignin-derived thioacidolysis products (in micromoles per gram of Klason lignin), recovered from hardwood samples.†

Sample	Poplar	Birch
Total yield and relative frequency of S, G monomers and S, M, G dimers		
Monomers: S + G	2390	2460
S/G	63/37	76/24
Dimers: S + M + G	259	325
S/M/G	42/20/38	49/25/26
Relative frequencies of the S, M, and G dimers with the following interunit bonds. Values between brackets indicate their proportions within each dimer series.		
β-1, (S/M/G)	31 (42/22/36)	34 (56/23/21)
β-β, S‡	29	29
β-5, (M/G)	17 (48/52)	19 (57/43)
4-O-5, (M/G)	10 (54/46)	11 (58/42)
5-5, G	13	7

† With the assumption that the reaction yield of each step is about 80%, and that the average C_6C_3 molecular weight is 200, these data indicate that about 70% of core lignins have been characterized.
‡ Tetralin-type dimer from syringaresinols.

These various thioacidolysis results were purposely presented in a more descriptive than explanatory way to show the potentialities of the method. Its main attribute is an ability to reveal detailed and original information, by a procedure that is routine since it is convenient and quick to perform. Its main limitation relies on the fact that the extrapolation of the results obtained for the lignin-derived monomers and dimers to the whole polymer is questionable. Future investigations will aim at examining whether the oligomer fraction structurally differs to a large extent from the monomer and dimer fractions.

IV. CONCLUSION

In spite of considerable instrumental advances, the problem inherent in the structural investigation of insoluble and cross-linked core lignins are still severe. Nevertheless, it was shown herein that the development of new methodologies has improved our understanding of the complex structure of these polymers. The conclusion will illustrate the complementary information obtained from the destructive and nondestructive strategies, as applied to core lignins from brown midrib (bmr) corn mutants.

These bmr corn mutants have received repeated attention in the area of lignin and animal nutrition research, due to their depressed lignin content and improved digestibility (Kuc & Nelson, 1964; Grand et al., 1985; see chapter 13 by Jung and Deetz in this book). Grand et al. demonstrated that the catechol O-methyltransferase activity was lower in bmr mutant corn seedlings, compared to the normal lines. We have investigated the structure of lignins from mature internodes of bmr corn mutant and compared them to the analogous normal line lignins by ^{13}C-NMR spectroscopy (Tollier et al., 1992, unpublished data) and thioacidolysis (Lapierre et al., 1988).

The comparisons of the ^{13}C-NMR spectra of milled straw lignin fractions isolated from the mutant and normal corn internodes (Fig. 6–11) showed that both lignins are associated with arabino-glucuronoxylans. The presence of noncore lignin components in these lignin fractions was clearly evidenced from the specific and sharp signals primarily corresponding to p-coumaric esters. The difference in monomer composition of the mutant and normal core lignins was demonstrated from the relative comparisons of the C-2 and C-6 syringyl and G signals, which unequivocally revealed that mutant lignins have lower content of syringylpropane units, in accordance with the results of Kuc and Nelson (1964), obtained by nitrobenzene oxidation. All this structural information was obtained in a nondestructive way and concerned the whole soluble lignin samples.

The analysis of the thioacidolysis monomers recovered from the mutant and normal in situ lignins afforded original information. While the lower content of S units in mutant lignins was confirmed (Table 6–11), the occurrence of additional 5-hydroxyguaiacyl units in the uncondensed lignin moiety was unequivocally demonstrated from the GC-MS analysis of the main lignin-derived monomers (Fig. 6–12). In addition, thioacidolysis performed
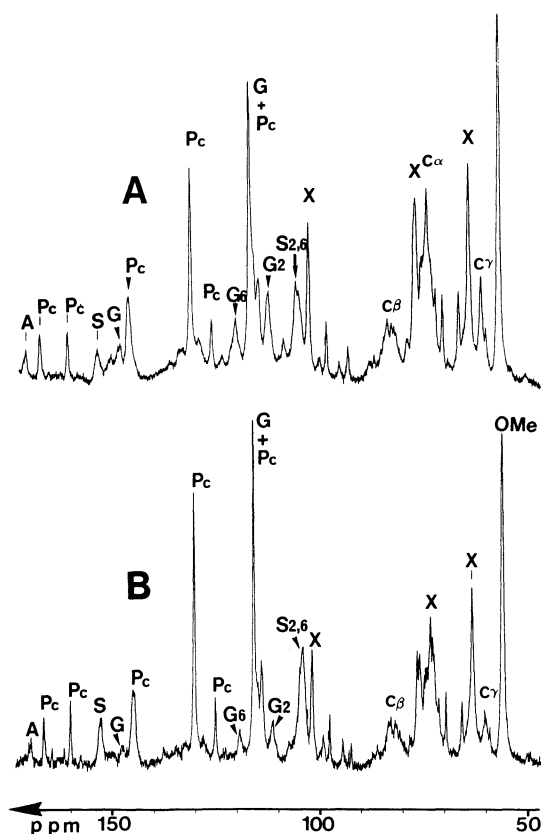
Fig. 6-11. Carbon-13-NMR spectra of milled lignins isolated from the corn internodes of (A) mutant bmr and (B) normal lines. For peak quotations, see Fig. 6-3 and 6-4.

after CH_2N_2 methylation showed that these 5-hydroxyl phenolic groups were etherified in the native lignins. These various results supported the hypothesis of β-O-4 linked 5-hydroxyguaiacyl units, incorporated in the mutant lignin according to Fig. 6-13. Noteworthy is the fact that the incorporation of additional as yet undetected monomer units in the core of mutant lignins has been suggested by Kuc and Nelson (1964), without any experimental data to support this hypothesis, as nitrobenzene oxidation did not allow the characterization of orthodiphenolic rings.

Table 6-11. Thioacidolysis yields in p-hydroxyphenyl (H), Guaiacyl (G), syringyl (S), and 5-hydroxyguaiacyl (5-OH G) monomers recovered from internodes of mutant brown midrib (bmr) and normal corn plants (in micromoles per gram of Klason lignin).

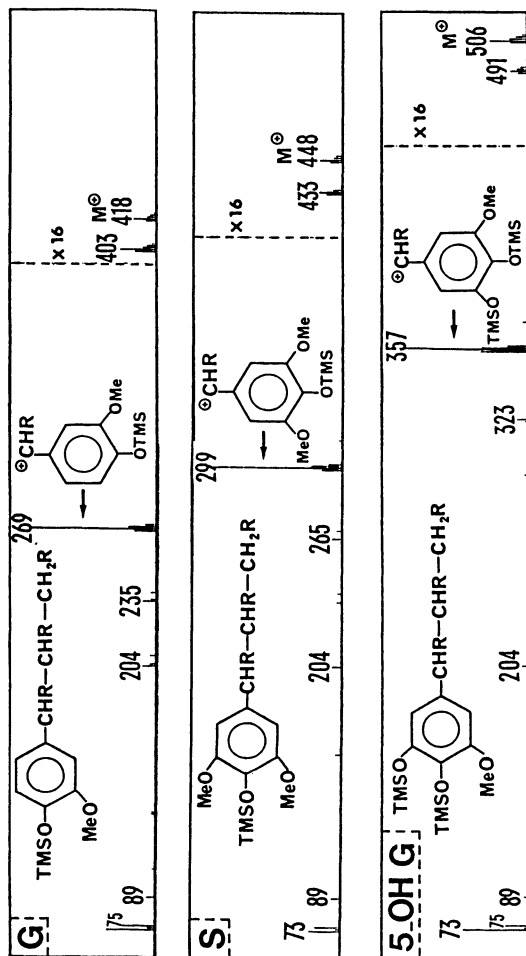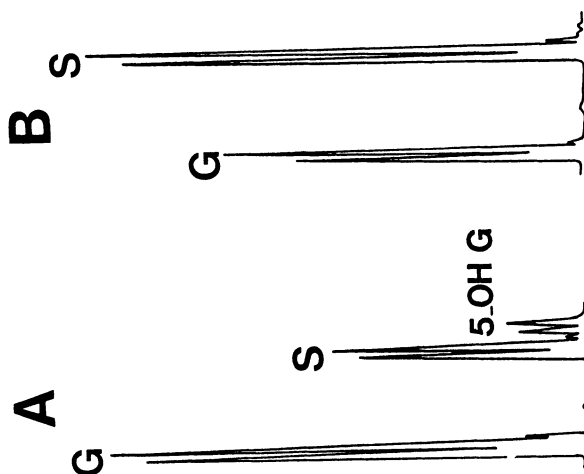| Plant	H	G	S	5-OH G
Mutant bmr line	19	305	118	32
Normal line	23	214	369	--

Fig. 6-12. Partial GC chromatogram showing the separation of the main thioacidolysis diastereoisomers (analyzed as their TMSi derivatives) recovered from the corn internodes of (A) mutant bmr and (B) normal lines. The mass spectra of the guaiacyl G, Syringyl S, and 5-hydroxy guaiacyl 5-OH G TMSi compounds allowed their unequivocal identification. R = SEt.
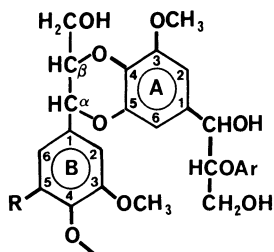
Fig. 6-13. Mode of incorporation of the unusual 5-OH guaiacyl units in the abnormal lignins of bmr mutant corn plants. Thioacidolysis of A units from original or permethylated in situ bmr corn lignins provides the 5-OH guaiacyl monomers identified in Fig. 6-12.

The present chapter was purposely restricted to a few routine methodologies that scientists can readily apply to obtain and interpret reproducible results with a critical eye. These techniques, however, are less suited to accurately and unequivocally account for the spatial and temporal heterogeneity of core lignins. In that respect, the elegant and sophisticated methodology developed by Terashima (Terashima, 1990; see chapter 10 by Terashima et al. in this book) is outstandingly fruitful to examine native lignins in various cellular and subcellular areas.

ACKNOWLEDGMENT

REFERENCES

Adler, E. 1977. Lignin chemistry—Past, present and future. Wood Sci. Technol. 11:169–218.

Bardet, M., M.F. Foray, and D. Robert. 1985. Use of the DEPT sequence to facilitate the ^{13}C NMR structural analysis of lignins. Makromol. Chem. 186:1495–1504.

Bardet, M., D. Gagnaire, R. Nardin, D. Robert, and M. Vincendon. 1986. Use of ^{13}C enriched wood for structural NMR investigation of wood and wood components, cellulose and lignin, in solid and solution. Holzforschung 40(Suppl.):17–24.

Bartuska, V.J., G.E. Maciel, H.I. Bolkers, and B.I. Fleming. 1980. Structural studies of lignin isolation procedures by ^{13}C NMR. Holzforschung 34:214–217.

Bax, A., R. Freeman, and T.A. Frenkiel. 1981. An NMR technique for tracing out the carbon skeleton of an organic molecule. J. Am. Chem. Soc. 103:2102–2104.

Björkman, A. 1954. Isolation of lignin from finely divided wood with neutral solvents. Nature (London) 4440:1057–1058.

Boon, J.J. 1989. An introduction to pyrolysis mass spectrometry of lignocellulosic materials: case studies on barley straw, corn stem and Agropyron. p. 25–50. In A. Chesson and E.R. Orskov (ed.) Physico-chemical characterization of plant residues for animal feed and industrial use. Elsevier Appl. Sci. Publ. Co., New York.

Chang, H.m., and G.G. Allan. 1971. Oxidation. p. 433–485. In K.V. Sarkanen and C.H. Ludwig (ed.) Lignins: Occurrence, formation, structure and reactions. Wiley-Interscience, New York.

Chen, C.-L., and D. Robert. 1989. ^{1}H and ^{13}C NMR spectroscopy of lignin. p. 138–174. In W.A. Wood and S.T. Kellogg (ed.) Methods in enzymology. Vol. 161. Academic Press, San Diego.

Ede, R.M. 1991. Lignin analyses by two-dimensional NMR techniques. 1. p. 367–374. *In* Proc. Symp. on Wood and Pulping Chemistry, Melbourne. 30 April–3 May 1991. Australian Pulp and Paper Ind. Tech. Assoc. (APPITA), Parkville, Victoria.

Faix, O., J. Bremer, D. Meier, and I. Fortmann. 1990. Qualitative and quantitative characterization of lignins and polysaccharides from lignocellulosics by analytical pyrolysis. p. 4. *In* Proc. Seminaire Lignine, Nancy, France. 15–16 Oct. 1990. ARBOLOR, Nancy, France.

Faix, O., and D. Meier. 1989. Pyrolytic and hydrogenolytic degradation studies on lignocellulosic pulps and lignins. Holz Roh-Werkst. 47:67–72.

Faix, O., and W. Schweers. 1975. Vergleichende Untersuchugen an Polymermodellen des Lignins (DHPs) verschiedner Zusammensetzungen. 7. Mitt Pyrolyse. Holzforschung 29:224–229.

Fritz, J., and K.J. Moore. 1987. Separation and quantification of lignin-derived phenolic monomers using high resolution gas chromatography. J. Agric. Food Chem. 35:710–713.

Fullerton, T.J., and R.A. Franich. 1983. Lignin analysis by pyrolysis-GC-MS. Characterization of ethanol lignin pyrolysates and identification of syringyl units in *Pinus radiata* milled wood lignin. Holzforschung 37:267–269.

Gellerstedt, G., and L. Zhang. 1991. On the behavior of reactive lignin structures in high yield pulping. 1. p.81–84. *In* Proc. Int. Symp. on Wood and Pulping Chemistry, Melbourne. 30 Apr.–3 May 1991. Australian Pulp and Paper Ind. Tech. Assoc. (APPITA), Parkville, Victoria.

Genuit, W., O. Faix, and J.J. Boon. 1987. Characterization of beech milled wood lignin by pyrolysis-gas chromatography-photoionization mass spectrometry. Anal. Chem. 59:508–512.

Grand, C., P. Parmentier, A. Boudet, and A.M. Boudet. 1985. Comparison of lignins and of enzymes involved in lignification in normal and brown midrib (*bm₃*) mutant corn seedlings. Physiol. Vég. 23:905–911.

Guittet, E., J.Y. Lallemand, C. Lapierre, and B. Monties. 1985. Applicability of the ^{13}C NMR INADEQUATE experiment to lignin, a natural polymer. Tetrahedron Lett. 22:2671–2674.

Hagendoorn, M.J.M., T.P. Traas, J.J. Boon, and L.H.W. Van Der Plas. 1990. Orthovanadate induced lignin production, in batch and continuous cultures of *Petunia hybrida*. J. Plant Physiol. 137:72–80.

Haider, K., and H.R. Schulten. 1985. Pyrolysis field ionization mass spectrometry of lignins, soil humic compounds and whole soil. J. Anal. Appl. Pyrolysis 9:317–331.

Hartley, R.D., and C.W. Ford. 1989. Phenolic constituents of plant cell walls and cell walls biodegradability. p. 137–145. *In* N.G. Lewis and M.G. Paice (ed.) Plant cell wall polymers. Biogenesis and biodegradation. ACS Symp. series, Vol. 399. Am. Chem. Soc., Washington, DC.

Hartley, R.D., and J. Haverkamp. 1984. Pyrolysis-mass spectrometry of the phenolic constituents of plant cell walls. J. Sci. Food Agric. 35:14–20.

Hassi, H.Y., M. Aoyama, D. Tai, C.-L. Chen, and J.S. Gratzl. 1987. Substituents effects on ^{13}C chemical shifts of aromatic carbons in β–O–4 and β–5 type lignin model compounds. J. Wood Chem. Technol. 7:555–581.

Hemmingson, J., and H. Wong. 1989. Characterization of photochemically degraded newsprint solubles by ^{13}C NMR and IR spectroscopy. Holzforschung 43:141–147.

Himmelsbach, D.S., and F.E. Barton II. 1980. ^{13}C nuclear magnetic resonance of grass lignins. J. Agric. Food Chem. 28:1203–1208.

Iiyama, K., and T.B.T. Lam. 1990. Lignin in wheat internodes. Part 1 : The reactivities of lignin units during alkaline nitrobenzene oxidation. J. Sci. Food Agric. 51:481–491.

Jung, H.G. 1989. Forage lignins and their effects on fiber digestibility. Agron. J. 81:33–38.

Kivaisi, A.M., H.J.M. Op den Camp, H.J. Lubberding, J.J. Boon, and G.D. Vogels. 1990. Generation of soluble lignin-derived compounds during degradation of barley straw in an artificial rumen reactor. Appl. Microbiol. Biotechnol. 33:93–98.

Kringstad, K.P., and R. Mörck. 1983. ^{13}C NMR spectra of kraft lignins. Kolzforschung 37:237–244.

Kuc, J., and O.E. Nelson. 1964. The abnormal lignins produced by the brown-midrib mutants of maize. Arch. Biochem. Biophys. 105:103–113.

Landucci, L.L. 1985. Quantitative ^{13}C NMR characterization of lignins. 1. A methodology for high precision. Holzforschung 39:355–359.

Landucci, L.L. 1991a. Application of modern liquid-state NMR techniques to lignin characterization. 1. p. 375–380. *In* Proc. Int. Symp. on Wood and Pulping Chemistry, Melbourne. 30 Apr.–3 May 1991. Australian Pulp and Paper Ind. Tech. Assoc. (APPITA), Parkville, Victoria.

Landucci, L.L. 1991b. ^{13}C NMR technique for discriminating high and low molecular weight components. 2. p. 341–344. *In* Proc. Int. Symp. on Wood and Pulping Chemistry, Melbourne. 30 Apr.–3 May 1991. Australian Pulp and Paper Ind. Tech. Assoc. (APPITA), Parkville, Victoria.

Lapierre, C. 1986. Hétérogénéité des lignines de peuplier : mise en évidence systématique. Thèse de Doctorat d'Etat, Paris-sud Univ.

Lapierre, C., D. Jouin, and B. Monties. 1989. On the molecular origin of the alkali solubility of gramineae lignins. Phytochemistry 28:1401–1403.

Lapierre, C., B. Monties, E. Guittet, and J.Y. Lallemand. 1984. Photosynthetically ^{13}C-labelled poplar lignins: ^{13}C NMR experiments. Holzforschung 38:333–342.

Lapierre, C., B. Monties, E. Guittet, and J.Y. Lallemand. 1987. RMN ^{13}C bidimensionnelle des lignines de peuplier : étude des corrélations entre atomes de carbone et réexamen par la méthode INADEQUATE des attributions des signaux du spectre. Holzforschung 41:51–58.

Lapierre, C., B. Monties, and C. Rolando. 1986. Thioacidolysis of poplar lignins. Holzforschung 40:113–118.

Lapierre, C., B. Pollet, B. Monties, and C. Rolando. 1991a. Thioacidolysis of spruce lignins : GC-MS analysis of the main dimers recovered after Raney nickel desulphuration. Holzforschung 45:61–68.

Lapierre, C., B. Pollet, and B. Monties. 1991b. Lignin structural fingerprint by thioacidolysis followed with Raney nickel desulphuration. 1. p. 543–549. *In* Proc. Int. Symp. on Wood and Pulping Chemistry, Melbourne. 30 Apr.–3 May 1991. Australian Pulp and Paper Ind. Tech. Assoc. (APPITA), Parkville, Victoria.

Lapierre, C., B. Pollet, and B. Monties. 1991c. Heterogeneous distribution of diarylpropane structures in spruce lignins. Phytochemistry 30:659–662.

Lapierre, C., and C. Rolando. 1988. Thioacidolyses of pre-methylated lignin samples from pine compression and poplar woods. Holzforschung 42:1–4.

Lapierre, C., M.T. Tollier, and B. Monties. 1988. Mise en évidence d'un nouveau type d'unité constitutive dans les lignines d'un mutant de Maïs bm3. C.R. Acad. Sci. Paris, Sér. III 307:723–728.

Logan, K.J., and B.A. Thomas. 1985. Distribution of lignin derivatives in plants. New Phytol. 99:571–585.

Lüdemann, H.D., and H.H. Nimz. 1973. Carbon-13 nuclear magnetic resonance spectra of lignins. Biochem. Biophys. Res. Commun. 52:1162–1169.

Lüdemann, H.D., and H.H. Nimz. 1974. ^{13}C-Kernresonanzspektren von Ligninen, 1.Chemische Verschiebungen bei Monomeren und Dimeren Modell substanzen. Makrom. Chem. 175:2393–2407.

Lundquist, K. 1976. Low-molecular weight lignin hydrolysis products. Appl. Polym. Symp. 28:1393–1407.

Masuku, C. 1991. Thermal reactions of the C-O and alkyl C-C bonds in lignin model compounds. Ph.D. diss., Helsinki Univ. Technol., Finland.

Meier, D., and O. Faix. 1992. Pyrolysis-gas chromatography-mass spectrometry. p. 177–179. *In* C. Dence and S. Lin (ed.) Methods in lignin chemistry. Springer-Verlag, Berlin.

Moers, M.E.C., M. Baas, J.J. Boon, and J.W. De Leeuw. 1990. Molecular characterization of total organic matter and carbohydrates in peat samples from a cypress swamp by pyrolysis-mass spectrometry and wet chemical methods. Biogeochemistry 11:251–277.

Newman, R.H. 1989. Carbon-13 N.M.R. studies of lignin in solid samples—A review. DSIR Rep.CD 2396. Private Bag, Petone, New Zealand.

Niemann, G.J., R.P. Baayen, and J.J. Boon. 1990. Differentiation between tissues from carnation (*Dianthus caryophyllus*) stems by pyrolysis-mass spectrometry. Ann. Bot. 65:461–472.

Nimz, H.H. 1974. Beech lignin: Proposal of a constitutional scheme. Angew. Chem. Int. Ed. 13:313–321.

Nimz, H.H., and H.D. Lüdemann. 1976. Kohlenstoff-13-NMR Spektren von Ligninen 6. Lignin und DHP Acetate. Holzforschung 30:33–40.

Nimz, H.H., M. Nemr, P. Schmidt, C. Margot, B. Schaub, and M. Schlosser. 1982. Carbon-13 NMR spectra of lignins, 9. spin-lattice relaxation times (T$_1$) and determination of interunit linkages in three hardwood lignins (*Alnus glutinosa, Corylus avellanus* and *Acer pseudoplantanus*). J. Wood Chem. Technol. 2:371–382.

Nimz, H.H., U. Tschirner, M. Stähle, R. Lehmann, and M. Schlosser. 1984. Carbon-13 NMR spectra of lignins, 10. Comparison of structural units in spruce and beech lignin. J. Wood Chem. Technol. 4:265–284.

Obst, J.R. 1983. Analytical pyrolysis of hardwood and softwood lignins and its use in lignin-type determination of hardwood vessel elements. J. Wood Chem. Technol. 3:377–397.

Obst, J.R., and L.L. Landucci. 1986a. The syringyl content of softwood lignin. J. Wood Chem. Technol. 6:311–327.

Obst, J.R., and L.L. Landucci. 1986b. Quantitative ^{13}C NMR of lignins—Methoxyl:aryl ratio. Holzforschung 40(Suppl):87–92.

Obst, J.R., L.L. Landucci, and W. Manders. 1987. Characterization of hardwood lignins: Solution and solid state ^{13}C NMR. 1:p.273–276. *In* Proc. Int. Symp. on Wood and Pulping Chemistry, Paris.

Obst, J.R., N.J. McMillan, R.A. Blanchette, D.J. Christensen, O. Faix, J.S. Han, T.A. Kuster, R.H. Newman, and R.C. Pettersen. 1989. Characterization of Canadian artic fossil woods. 2. p.289–308. *In* Proc. Int. Symp. on Wood and Pulping Chemistry, Raleigh, NC. Tech. Assoc. for Pulp and Paper Ind. (TAPPI), Atlanta.

Pan, X., D. Lachenal, C. Lapierre, and B. Monties. 1991. Analysis of spruce photodegraded lignins by thioacidolysis. 1:p.451–455. *In* Proc. Int. Symp. on Wood and Pulping Chemistry, Melbourne. 30 Apr.–3 May 1991. Australian Pulp and Paper Ind. Tech. Assoc. (APPI-TA), Parkville, Victoria.

Pouwels, A. 1989. Analytical pyrolysis mass spectrometry of wood derived polymer fractions. Ph.D. diss. Amsterdam Univ., the Netherlands.

Ralph, J., and R.D. Hatfield. 1991. Pyrolysis-GC-MS characterization of forage maaterials. J. Agric. Food Chem. 39:1426–1437.

Robert, D., M. Bardet, G. Gellerstedt, and E.L. Lindfors. 1984. Structural changes in lignin during kraft cooking. Part 3. On the structure of dissolved lignins. J. Wood Chem. Technol. 4:239–263.

Robert, D., and D. Gagnaire. 1981. Quantitative analysis of lignins by ^{13}C NMR. 1. p. 86–88. *In* Proc. Int. Symp. on Wood and Pulping Chemistry, Stockholm. 9–12 June 1981.

Rolando, C., B. Monties, and C. Lapierre. 1992. Thioacidolysis. p. 334–349. *In* C. Dence and S. Lin (ed.) Methods in lignin chemistry. Springer-Verlag, Berlin.

Sarkanen, K.V., and H.L. Hergert. 1971. Classification and distribution. p. 43–94. *In* K.V. Sarkanen and C.H. Ludwig (ed.) Lignins: Occurrence, formation, structure, and reactions. Wiley-Interscience, New York.

Sakakibara, A. 1980. A structural model of softwood lignin. Wood Sci. Technol. 14:89–100.

Scalbert, A., B. Monties, E. Guittet, and J.Y. Lallemand. 1986. Comparison of wheat straw lignin preparations. I. Chemical and spectroscopic characterizations. Holzforschung 40:119–127.

Schilling, G. 1985. Recent advances in NMR spectroscopy of plant phenolics. Ann. Proc. Phytochemical Soc. Europe 25:57–74.

Schulten, H.R. 1984. Relevance of analytical pyrolysis studies to biomass conversion. J. Anal. Appl. Pyrolysis 6:251–272.

Sjöstrom, J., and M. Reunanen. 1990. Characterization of lignin and carbohydrates dissolved in groundwood pulping by pyrolysis-gas chromatography/mass spectrometry. J. Anal. Appl. Pyrolysis 17:305–318.

Tanahashi, M., and T. Higuchi. 1989. Chemical degradation methods for characterization of lignins. p. 101–110. *In* W.A. Wood and S.T. Kellog (ed.) Methods in enzymology. Vol. 161. Academic Press, San Diego.

Terashima, N. 1990. A new mechanism for formation of a structurally ordered protolignin macromolecule in the cell wall of tree xylem. J. Pulp Paper Sci. 16:J150–J155.

Timell, T.E. 1981. Recent progress in the chemistry, ultrastructure and formation of compression wood. 1:99–147. *In* Proc. Int. Symp. on Wood and Pulping Chemistry, Stockholm. 9–12 June 1981. SPCI, Stockholm, Sweden.

Tollier, M.T., C. Lapierre, B. Monties, C. Francesch, and C. Rolando. 1991. Structural variations in synthetic lignins (DHPs) according to the conditions of their preparation. 2. p. 35–40. *In* Proc. Int. Symp. on Wood and Pulping Chemistry, Melbourne. 30 Apr.–3 May 1991. Australian Pulp and Paper Ind. Tech. Assoc. (APPITA), Parkville, Victoria.

Wallace, G. 1989. A study of phenolic-carbohydrate linkages in the Gramineae. Ph.D. diss. Univ. of Glasgow, UK.