Quantitative ^{13}C NMR Analysis of Lignins with Internal Standards

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Novel protocols for acquiring quantitative ^{13}C NMR spectra of lignins have been developed using the internal reference compounds 1,3,5-trioxane and pentafluorobenzene. Trioxane offers a convenient internal standard for collecting inverse gated proton decoupled ^{13}C NMR spectra for lignins, whereas pentafluorobenzene can be used to provide information on the amount of methine carbon using the DEPT experiment. In each case, the internal reference compounds provide single, un-overlapped sharp signals in the middle of the spectral region, permitting facile integration. These integrals could be used to determine the amounts of different structural features of lignins, expressed in absolute units of millimoles per gram. The optimum parameters for these experiments were validated for a variety of spectrometer platforms, and standard errors were determined for different spectral areas using lignin model compounds and "standard" lignins. In addition, the data derived for the International Round Robin "standard" lignins showed good agreement with the data from quantitative ^{31}P NMR spectroscopy and published data, obtained by independent laboratories using independent methods of analysis.

Keywords: *Lignin; nuclear magnetic resonance spectroscopy (NMR); inverse gated proton decoupled carbon-13 NMR spectroscopy; distortionless enhancement by polarization transfer (DEPT); internal standard; acquisition protocol; International Round Robin lignins; relaxation reagent*

INTRODUCTION

^{13}C NMR spectroscopy has been shown to be of significant potential in providing detailed structural information for lignins. In particular, the advent of multidimensional NMR techniques has extended the prospect of lignin structural analysis considerably (*1*). Structures of even minor or unknown components have been elucidated with applications of a combination of 2D HMQC and 3D HMQC-HOHAHA experiments (*2*). In addition, ^{13}C NMR has been indispensable in the quantitative determination of the amounts of different structural units in lignin (*3*−*13*). Whereas the broad proton NMR signals that occur over a narrow frequency range render limited quantitative information, ^{13}C NMR spectroscopy provides an elegant alternative, mainly due to its significantly larger chemical shift dispersion.

Current practices in the use of quantitative ^{13}C NMR spectroscopy for the study of lignin are confined to using the aromatic and methoxyl signals as internal standards in expressing the various functional groups per C9 or per methoxyl unit, respectively (*12*, *13*). Such a practice, although applicable to native lignins, may be a source of serious errors if analyses of technical lignins are to be undertaken because technical lignins contain degraded side chains and fragmented aromatic rings (*14*). Similar problems may arise when one attempts to study structurally altered mutants (*15*) or genetically modified lignins (*16*). For such samples, where the C9 content is seriously altered, functional group content expressed in the form of "per 100 phenylpropane units" could be confusing and misleading.

For these reasons it is imperative that a standard technique be developed that will permit the various carbon atoms, present on heavily modified and technical lignins, to be determined with accuracy. The technique should also allow such moieties to be expressed in absolute units of millimoles per gram of sample. As an example, the successful application of internal standards for the quantitative ^{31}P NMR analysis of hydroxyl-containing functional groups within different lignins (*17*, *18*) may be cited.

In the present effort, various lignin samples were examined with two quantitative ^{13}C NMR techniques: distortionless enhancement by polarization transfer (DEPT) and inverse gated ^{1}H decoupling. Quantitation was achieved by using carefully chosen internal standards that displayed clear, un-overlapped signals in the middle of the ^{13}C NMR spectra. Consequently, the various environments that comprise the carbon skeleton in lignins have been reliably quantified. Our data were derived by applying the developed ^{13}C NMR spectroscopic protocols on a set of International Round Robin lignins (*19*). Our analyses were then compared with data obtained by using quantitative ^{31}P NMR spectroscopy and other techniques, reported elsewhere, using independent methods of analysis.

MATERIALS AND METHODS

Materials and Sample Preparation. 1,3,5-Trioxane and pentafluorobenzene were purchased from Aldrich and used without additional purification. The lignin model compounds used in this paper are shown in Figure 1, namely, 4-hydroxyl-3-methoxybenzyl alcohol (vanillyl alcohol) (**1**), 1-(3,4-dimethoxyphenyl)-1-ethoxy-2-(2-methoxyphenoxy)ethane (**2**), and 2-(2-methoxyphenoxy)-3-(3,4-dimethoxyphenyl)-1,3-propanediol (**3**).

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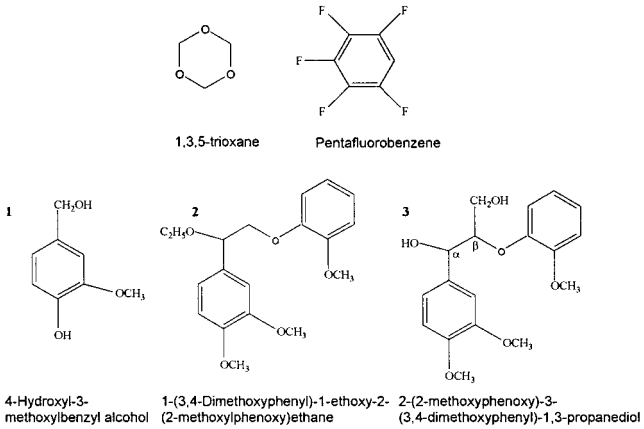

Figure 1. Structures of internal standards and lignin model compounds used in this work.

Compound **1** was purchased from Aldrich, and compounds **2** and **3** were synthesized in our laboratory according to published procedures. The source and preparation of the three Round Robin lignins used in our experiments have been described by Milne et al. (*19*). The lignins can be requested for analytical purposes from the National Renewable Energy Laboratory, Golden, CO. Indulin kraft pulping lignin was nonacetylated, whereas steam explosion yellow poplar lignin and steam explosion aspen lignin were used in both their nonacetylated and acetylated forms.

Samples for recording NMR spectra were prepared by accurately weighing predried lignin samples and the internal reference compounds (either 1,3,5-trioxane or pentafluorobenzene), and dissolving them in DMSO-d_6. Typical sample concentrations were of the magnitude of ~200 mg/mL for lignin samples or model compounds and ~1 M for the internal reference compound, although the amount of the internal reference can be substantially reduced. A minute amount of $Cr(acac)_3$ (~2 mg) was added to the samples to facilitate the relaxation of the magnetization.

Quantitative Analysis of ^{13}C NMR Data. ^{1}H and ^{13}C NMR spectra were acquired on a Varian Unity-500 (125.7 MHz for ^{13}C observation), a Mercury-400 (100.6 MHz for ^{13}C observation), and a Mercury 300 (75.5 MHz for ^{13}C observation) spectrometers. ^{31}P NMR spectra were recorded with a Varian Mercury-200 operated at 81.0 MHz. Broadband probes were used to observe directly ^{13}C or ^{31}P resonance signals with Waltz-16 proton decoupling. On the Varian Unity 500, a 10 mm probe was installed for directly observing the low band nuclei, and the other spectrometers were equipped with 5 mm probes. A 10 mm probe is preferred for collecting inverse gated proton decoupled ^{13}C spectra due to sensitivity reasons. However, this is not essential provided that long time acquisition is possible. Proton and carbon spin−lattice relaxation times (T_1) were measured with the standard inversion−recovery method. Quantitative ^{13}C NMR spectra were collected with inverse gated proton decoupling (*20*) or with the DEPT sequence (*21, 22*). Typical spectral widths for ^{13}C spectra were 30 kHz at 500 MHz, 25 kHz at 400 MHz, and 18 kHz at 300 MHz, and the acquisition time was 0.5 s on all of the spectrometer platforms. Relaxation delays were set to be ~5 times the T_1 valuess of either carbon signals (for inverse gated proton decoupled ^{13}C NMR spectra) or proton signals (for the DEPT experiments) with the longest T_1 values. Specifically, for recording the inverse gated proton decoupled ^{13}C spectra, 12.5 s was used, and for recording the DEPT spectra 1.2 s was used. For an inverse gated proton decoupled ^{13}C spectrum, typically at least 36 h was required at our 500 MHz equipped with a 10 mm broadband probe, and this period could be as long as 80 h with a 5 mm switchable probe at 400 MHz. DEPT experiments, however, can be completed within 12−24 h at our 400 MHz. To ensure a flat baseline, the sampling delay and the receiver gain were optimized before acquisition, and the dc offset was corrected during data processing. No further postacquisition baseline correction was needed. The baseline

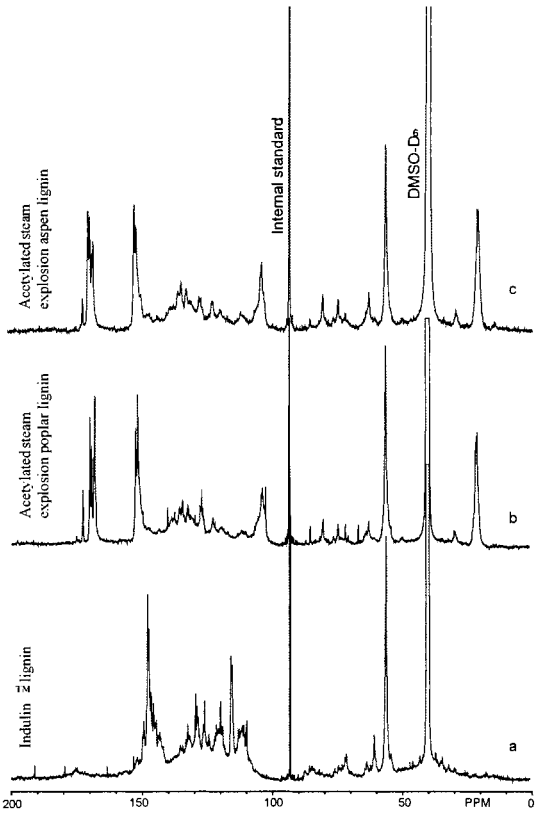

Figure 2. Quantitative ^{13}C NMR spectra of Round Robin lignins.

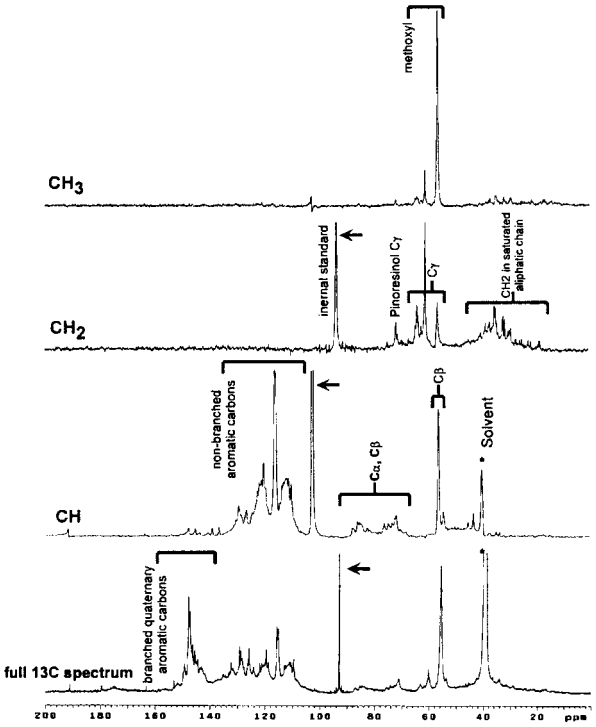

Figure 3. Quantitatively edited ^{13}C spectra using the DEPT pulse sequence.

quality of the spectra collected for lignin samples can be seen in both Figures 2 and 3. The acquired FIDs were processed typically with a 10 Hz line broadening. Spectra were referenced indirectly with the solvent deuterium signals. Integration of signals was carried out using Varian software VNMR 6.1B. The amounts of different functional groups/spectral areas were then readily calculated on the basis of the relative integrals to that of the internal reference.

Table 1. Standard Errors Calculated for Different Areas of the Spectra

spectral area (ppm)	assignment[a]	standard error (%) compd **I**	standard error (%) compd **II**	standard error (%) Indulin	values for Indulin (mmol/g)
154.6−140.7	Ar[b] C-3, 4	2.2	3.3	4.3	11.38 (0.22)[c]
140.7−123.4	ArC-1, C-5 condensed	2.0	4.3	3.8	12.1 (0.20)
123.4−105.3	ArC-2, 5, 6	2.2	3.6	1.6	13.58 (0.10)
89.8−58.6	C-α, β, γ	6.4	5.7	8.1	4.48 (0.16)
58.1−52.5	OCH_3, C-β in β-β	0.7	1.8	2.0	5.26 (0.05)

[a] The assignment for lignin signals serves only as a rough guide due to signal overlapping. Demixing of signals is resolved by DEPT experiments. [b] Aromatic carbons. [c] Standard deviation in parentheses.

Quantitative Analysis of ^{31}P NMR Data. Quantitative ^{31}P NMR spectra of nonacetylated steam explosion yellow poplar and steam explosion aspen lignins were analyzed according to the method described by Granata and Argyropoulos (*18*). To phosphitylate the lignin prior to the analysis, 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane was used and cholesterol was employed as an internal standard.

RESULTS AND DISCUSSION

Internal Standards. Our search for useful internal standards was defined by two major requirements. Primarily, the desired compound should give a single peak in the spectral region free of lignin signals. Second, the proposed internal standard should be stable, soluble in the solvents usually used in the NMR spectroscopy of lignins, and inert toward the lignin functional groups. During the present effort we have identified two low molecular weight organic compounds, 1,3,5-trioxane and pentafluorobenzene, that meet these requirements. There is also anecdotal evidence that tetramethylthiourea disulfide could also function in this respect. Trioxane provides a sharp single peak at 93 ppm in both inverse gated proton decoupled ^{13}C spectra and the DEPT CH_2 subspectra, whereas pentafluorobenzene shows a single carbon signal at ∼102 ppm in the DEPT CH subspectra. Strictly speaking, pentafluorobenzene shows a triplet at ∼102 ppm due to the couplings to the nearby fluorine atoms (∼12 Hz). Native or technical lignins do not show any signals in the region between 90 and 105 ppm, providing an opportunity for the accommodation of internal standards (Figure 1).

The single major disadvantage of using internal standards in ^{13}C NMR acquisitions is their relatively long spin−lattice relaxation time constants (∼6 s), which would render the total acquisition time very long. Given that the relaxation delay must be set ∼5 times the longest T_1, the total time for recording inverse gated proton decoupled spectra could easily exceed 80 h. By using paramagnetic relaxation agents, however, one can easily circumvent this, since paramagnetic species can substantially shorten the T_1 values through electron−nucleus dipolar−dipolar interactions. A minute amount of chromium acetylacetonate [$Cr(acac)_3$] was found to substantially reduce the carbon and proton longitudinal relaxation times with acceptable carbon line widths, whereas the use of minute amounts did not introduce appreciable $Cr(acac)_3$ signals. The amount of paramagnetic relaxation agent was adjusted so that the longest ^{13}C longitudinal relaxation time (which invariably is of the internal standard) had a value of ∼2.5 s for the acquisition of the inverse gated proton decoupled ^{13}C spectra, and the proton T_1 value was ∼0.2 s for the acquisition of the DEPT experiments. The total data acquisition time for the inverse gated proton decoupled ^{13}C spectra can be further reduced by optimizing the pulse angle and the repetition rate according to the work of Traficante (*23*, *24*). In our work, the maximum signal/noise ratio was obtained at an 83° tip angle and a pulse−repetition period of 4.5 multiplied by the longest T_1 value.

Inverse Gated Proton Decoupled ^{13}C NMR. ^{13}C NMR spectroscopy has long been more suitable than proton NMR spectroscopy for characterizing lignin due to the following reasons: (i) in a ^{13}C NMR spectrum, the spectral data obtained arise from the "backbone" of the molecule, providing information about the nature of all carbons in the molecule; (ii) ^{13}C NMR spectra are not complicated by spin−spin coupling effects, giving rise to single lines for each carbon environment when proton decoupling is applied during the ^{13}C acquisition; and (iii) the ^{13}C NMR chemical shift range spans a much wider region than its proton counterpart at 500 MHz. However, routine ^{13}C NMR spectra do not lead to quantitative information. This is because when proton decoupling is applied during both the relaxation delay and the acquisition period, the signal intensities do not correspond to the actual number of atoms due to nuclear Overhauser effects (nOe). To obtain a quantitative ^{13}C NMR spectrum, an inverse gated proton decoupling needs to be applied to minimize the nOe effect. In addition, the relaxation delay must be set at least 5 times longer than the ^{13}C longitudinal relaxation time.

During this work we examined the accuracy and precision of quantifying lignin signals with an internal standard by inverse gated proton decoupled ^{13}C NMR spectroscopy using two lignin model compounds and Indulin lignin. The errors associated with the technique were defined by acquiring multiple replicates for the lignin model compounds and for the lignin sample. The two lignin model compounds used were a "mononuclear", compound **1**, and a "dinuclear", compound **2** (Figure 1). The standard errors determined, based on the comparison of the data from the recorded spectra for each compound, are shown in Table 1. The application of the chosen parameters to the acquisition of the ^{13}C NMR spectra for compound **1** showed excellent reproducibility. The standard error for all spectral areas (with the exception of the 58−90 ppm region) was below 5.0%. The 58−90 ppm region, assigned to signals from the aliphatic chain carbons, showed the highest standard error (6.4%) due to the lowest signal to noise ratio (lowest sensitivity) in this region. Compound **2** showed a slightly and systematically higher level of standard error due to lower sample concentration and hence worst signal to noise ratios. The lignin chosen for our assessment was the lower molecular weight Indulin lignin, because its solution in DMSO-d_6 is of the lowest viscosity, and hence the resolution of the acquired ^{13}C NMR spectra was excellent. This sample was recorded three times under the same conditions. The standard errors were all below 5% for different spectral regions. These were calculated from the standard deviation values, shown in Table 1. The aliphatic region (58−90 ppm), however,

Table 2. Comparison of Data between Quantitative ^{13}C and ^{31}P NMR Techniques and Literature Values

functional group [integration range (ppm)]	steam explosion yellow poplar lignin (acetylated)			steam explosion aspen lignin			Indulin lignin	
	^{13}C NMR[a]	^{31}P NMR[b]	literature	^{13}C NMR[c]	^{31}P NMR[d]	literature	^{13}C NMR	literature
OH_{total} [171.0−168.9]	4.28 mmol/g or 1.04/PPU[e]	4.54 mmol/g	1.06−1.16/PPU[f]	4.65 mmol/g or 1.26/PPU	4.76 mmol/g	1.16−1.26/PPU[f]		
$OH_{phenolic}$ [168.9−167.1]	2.16 mmol/g or 0.51/PPU	2.29 mmol/g	0.48−0.54/PPU[f]	1.72 mmol/g or 0.46/PPU	1.75 mmol/g	0.42−0.43/PPU[f]		
$OH_{phenolic}$ to $OH_{aliphatic}$ ratio	1.01	1.02	0.83−0.95[f]	0.57	0.58	0.53−0.58[f]		
OCH_3 [58.6−54.1]	4.97 mmol/g or 15.4% (wt)		15.3 ± 0.6%[g]	4.25 mmol/g or 13.1% (wt)		12.7 ± 0.8%[g]	4.68 mmol/g or 14.5% (wt)	14.0 ± 0.8%[g]

[a] Derived for acetylated lignin. [b] Derived for nonacetylated lignin. [c] Derived for acetylated lignin. [d] Derived for nonacetylated lignin. [e] PPU, phenylpropane unit. [f] Data from Faix et al. (*26*). [g] Data from Milne et al. (*19*).

again had a standard error of ~8.0%, which was anticipated due to poor signal to noise ratio (Figure 2).

The accuracy and precision of quantification for lignin samples with internal standards were assessed. The data derived with the inverse gated proton decoupled ^{13}C NMR spectra were compared with those derived in independent laboratories using independent methods of analysis (Table 2) (*19*, *25*). As an additional reference, quantitative ^{31}P NMR of nonacetylated steam explosion lignins derivatized with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane was employed. This method supplies reliable data about the content of the various hydroxyl groups present in lignin, and the data are expressed in millimoles per gram (*17*, *18*). As such, it is relatively easy to compare such sets of data based on the acquired quantitative ^{13}C NMR spectra of the acetylated lignins, and the comparison could reliably prove if the developed protocols supply reproducible and reliable results. In addition, we examined and compared data for methoxyl group content (*19*, *25*, *26*).

The functional group distribution data for the different hydroxyl groups calculated from the signals of the acetyl carbons for two acetylated steam explosion hardwood lignins (Table 2) show excellent correlation with published results obtained for the same lignins using quantitative ^{1}H, ^{13}C, and ^{31}P NMR spectroscopic and wet-chemical methods (*25*). The difference between the experimental and published data is within the variability of the method as reported by Faix et al. (*26*). Quantitative ^{31}P NMR data also showed very good correlation with the quantitative ^{13}C NMR data. These data are somewhat higher than those obtained by quantitative ^{13}C NMR during this work (Table 2). However, it should be noted that Faix et al. (*26*) also reported that ^{31}P NMR supplies slightly higher values than ^{13}C NMR spectroscopy.

Methoxyl group analyses revealed that for all lignins the data obtained by quantitative ^{13}C NMR spectroscopy using the selected internal standard correlate well with earlier published data from wet-chemical methods (*19*). Samples of acetylated steam explosion yellow poplar lignin also showed excellent correlation (Table 2), whereas the aspen steam explosion and Indulin kraft lignin data were within standard deviation margins determined for wet-chemical methods (*19*). In general, data comparison on the hydroxyl group contents proved that the inverse gated proton decoupled ^{13}C NMR with internal standard provides results with good reliability.

Distortionless Enhancement by Polarization Transfer. Although the inverse gated proton decoupled ^{13}C NMR spectral results are very reliable for the quantification of lignin, the method suffers from two major disadvantages. Primarily, the sensitivity of this technique is significantly lower than that of the routine 1D ^{13}C NMR experiment due to the loss of the nOe effect. Second, extensive signal overlapping occurs, and hence signal assignments are difficult; therefore, quantitative structural information should be treated with caution. These difficulties can be somewhat circumvented by the DEPT experiment. The DEPT experiment edits the ^{13}C NMR spectra by proton multiplicities. Not only can clean observations be obtained for the CH, CH_2, and CH_3 signals, but signal intensities can be greatly improved (*22*). The sensitivity enhancement is achieved by polarization transfer from proton magnetization (which is much more sensitive to observe by NMR due to the large gyromagnetic ratio of protons) to ^{13}C magnetization and by taking advantage of the fact that proton relaxation time is faster than that of ^{13}C. The spectral editing is accomplished by the application of various flip angles of the final proton pulse. The magnitude of the observable coherence for each of the CH, CH_2, and CH_3 carbon signals depends on this flip angle due to their different precession frequencies, resulting from the different C−H J couplings. By running simple algebra for a suitable linear combination of spectra, obtained at different flip angles, carbon subspectra of methyl, methylene, and methine groups can be obtained. The theoretical basis of the quantitative nature of the DEPT experiments has been thoroughly discussed, and sources of error and such analyses have been assessed (*21*). The most common source for error may result from setting one J value for a wide range of single-bond $^{13}C-^{1}H$ J coupling constants, present in a given system. Furthermore, in the case of polymers, possible signal loss due to efficient T_2* mechanisms may also occur.

We have compared the results of the quantitative analysis of a lignin model compound (compound **3**) with both the DEPT and inverse gated proton decoupled ^{13}C NMR techniques. With pentafluorobenzene as an internal standard, two CH signals (Cα and Cβ) have been quantified with both methods and compared to the amount calculated from carefully weighing the sample. These results are given in Table 3. The slightly poorer results from inverse gated proton decoupled ^{13}C spectra may be due to a poorer signal to noise ratio. Nevertheless, the accuracy of these two quantitative techniques is remarkable.

Quantitative analysis of Indulin lignin with DEPT techniques was then attempted. The edited subspectra of the CH, CH_2, and CH_3 spectra for the lignin sample are shown in Figure 3, together with the full ^{13}C spectrum. Clear overlapping between the quaternary carbon (condensed aromatic C-1 and C-5) and aromatic C-2,5,6 signals is observed in the inverse gated proton decoupled ^{13}C spectra in the region between 100 and 160 ppm. The precise discrimination between tertiary

Table 3. Comparison of Quantitative Analytical Data of Inverse Gated Proton Decoupled ^{13}C NMR and DEPT Techniques for Lignin Model Compound 3

	C_α (84.3 ppm) (mmol)	C_β (74.2 ppm) (mmol)
amount calculated from the sample's weight	0.201	
amount obtained from inverse gated proton decoupled ^{13}C NMR spectra	0.177	0.178
amount obtained from DEPT experiments	0.197	0.197

Table 4. Quantitative Data Obtained with the DEPT Sequence for the Most Significant Regions of Indulin Lignin

	region between 65 and 90 ppm (mostly Cα, Cβ, Cγs)		region between 105 and 160 ppm (mostly aromatic carbons)
CH	2.02 mmol/g		10.3 mmol/g
CH_2	0.88 mmol/g	1.04 mmol/g[a]	
C_9			28.6[a] mmol/g

[a] This value is obtained by subtracting the amount of methine carbon measured by the DEPT experiment from the total amount of carbon determined from the inverse gated proton decoupled spectrum.

and quaternary aromatic carbons is of considerable importance in structural analysis of lignins by ^{13}C NMR spectroscopy, because it provides information about the substitution patterns of its aromatic moieties. This information, together with the chemical shifts of the carbons, could lead to the elucidation of the branch points of the three-dimensional lignin macromolecules and an estimation of the degree of condensation of the aromatic moieties. With the DEPT pulse sequence, the discrimination of these NMR signals seems to be satisfactorily achieved. In addition, the signals at ~71.6 ppm from $C\gamma H_2$ in pinoresinol substructures (*27*) can also be well demixed in the edited subspectra from the Cαs and Cβs in the region between 65 and 90 ppm. The third advantage of using the DEPT sequence is clean suppression of solvent DMSO signals, which leads to a much smaller dynamic range of the data acquisition and a flatter spectral baseline, which further increases the sensitivity of the analysis.

The quantitative results obtained by using the DEPT sequence are given in Table 4. The quaternary carbons in the region between 105 and 160 ppm were calculated by subtracting the amount of methine carbons (determined from the DEPT experiments using pentafluorobenzene as an internal standard) from the total amount of carbons measured by the inverse gated proton decoupled ^{13}C NMR techniques. This was possible because this region is free of methyl and methylene signals. The amount of methylene carbons in the region between 65 and 90 ppm was obtained in two ways: (i) by subtracting the amount of methine carbons (determined from DEPT experiments using pentafluorobenzene as an internal standard) from the total amount determined from the inverse gated proton decoupling technique, because this region is free of quaternary and methyl carbons; and (ii) from the DEPT experiment using trioxane as an internal standard. As a matter of fact, both of these methods gave very similar values for the amount of methylene carbons in this region, and therefore validate each other.

Potential problems encountered during the quantitative DEPT analysis of lignin are twofold. The DEPT sequence contains three J modulation delay periods set to $(2J)^{-1}$; consequently, signal intensities could be altered by mis-setting these periods relative to actual $^1J_{C_{13}-H_1}$ values, and editing errors could result. The errors involved in using DEPT over the normal range of aliphatic and aromatic $^1J_{C_{13}-H_1}$ are assessed by analyzing DEPT with variable time delay periods using the Heisenberg quantum picture approach (*21*). Our analysis shows that a 10% deviation of ^1J relative to the real J value reduces a CH peak by 2%, a CH_2 peak by 5%, and a CH_3 peak by 7%, all being fairly modest errors. Furthermore, signals with T_2* values shorter than $3/(2J)$ (~10 ms) will disappear before the final reading pulse, resulting in intensity losses. To our knowledge, there have been no estimates of carbon T_2* values for lignin. We roughly estimated T_2* values from the line widths of the ^{13}C slices of a 2D HSQC spectrum of Indulin lignin (data not shown). For such lignin samples, prepared according to our conditions, the typical line width of ^{13}C is ~12 Hz, which will lead to T_2* values of ~25 ms, permitting an accurate quantification.

In a similar context we have varied the J value settings from 130 to 155 Hz at two spectrometer frequencies (300 and 400 MHz) covering a normal range of aliphatic and aromatic $^1J_{C_{13}-H_1}$ for lignin. Sample relaxation conditions were also varied by adjusting the amount of relaxation reagent. Typical T_1 values for the internal standards span from 0.1 to 1 s. Despite the range of conditions used in our experiments, consistent analytical results were obtained (data not shown).

This paper demonstrates that internal standards may be used for the quantification of ^{13}C signals in lignins, expressed in absolute units of millimoles per gram of sample. Such units could be very useful when stoichiometric calculations are required. Such quantification protocols become particularly important for severely altered lignin samples. The DEPT sequence, when used for the quantification of ^{13}C NMR signals, can provide more detailed and accurate data to the time-consuming inverse gated proton decoupled spectra. For example, using the DEPT sequence and pentafluorobenzene as internal standard, the pinoresinol units in lignin can be quantified. Despite the fact that a number of parameters may contribute to errors in quantifying DEPT experiments, compared to inverse gated proton decoupled ^{13}C acquisition, the higher sensitivity and selectivity of the DEPT sequence warrant closer attention for its role in lignin structure elucidation.

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