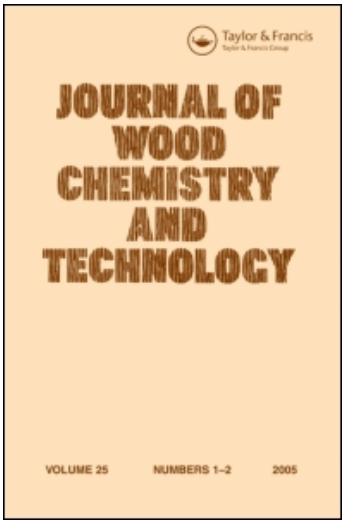

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Quantitative Phosphorus-31 NMR Analysis of Lignins, a New Tool for the Lignin Chemist

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QUANTITATIVE PHOSPHORUS-31 NMR ANALYSIS OF LIGNINS, A NEW TOOL FOR THE LIGNIN CHEMIST

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ABSTRACT

A novel quantitative method has been developed for the determination of the various types of hydroxyl groups present in lignins. The syringyl, guaiacyl and p-hydroxyphenyl free phenolic groups, as well as the primary, and the secondary hydroxyl groups (belonging to individual *erythro* and *threo* forms of the aryl-glycerol β-O-4 ether strctures) can be quantitatively determined from a ^{31}P NMR experiment. This is made possible by phosphitylating lignins with 1,3,2 dioxaphospholanyl chloride, followed by ^{31}P NMR spectroscopy, in the presence of a relaxation reagent (chromium acetylacetonate) and an internal standard. The various aspects leading to the development of this technique are discussed together with relevant statistical information pertaining to the reproducibility and quantitative validity of the method. This simple and novel form of spectroscopy may become a valuable resource to the lignin chemist, because it can supply detailed quantitative information about the structure of a soluble lignin sample.

INTRODUCTION

Lignin, the second most abundant biopolymer on the planet, binds the cellulose fibres together, imparting strength and rigidity to wood. The fundamental changes induced on this biopolymer during the pulping of wood, bleaching of pulp and yellowing of paper are of considerable complexity and industrial significance.

Any research endeavour that attempts to comprehend the nature of these changes requires the detailed knowledge of the chemical transformations that take place within lignin.

Wet methods of lignin analysis have been developed over a period of many years as a result of detailed investigations on the organic chemistry of lignin. These methods have contributed a great deal to our knowledge on the structure of lignins (1). In recent years, one and two dimensional proton and ^{13}C NMR spectroscopies have become indinspensible tools for the structural elucidation of lignins (2-4). These methods have confirmed the presence of the various functionalities present in lignin and have provided additional structural information (4).

Since magnetic resonance techniques of lignin analysis are showing considerable promise, with regard to their qualitative (2,4) and quantitative (5) reliability as analytical tools for the structural elucidation of these complex biopolymers, we have explored the development of novel magnetic resonance methods that may expand the frontiers of application of NMR to lignin analysis. To do this, we have recently developed solution phosphorus-31 based NMR methods to elucidate, fundamental structural details of lignocellulosic polymers (phosphorus-tagged).

In a series of papers with the general title *"^{31}P NMR in Wood Chemistry"* we have been examining the potential of ^{31}P NMR spectroscopy toward the characterization of model compounds (6), carbohydrates (7) and lignins (8,9), after derivatizing their labile protons with 1,3,2, dioxaphospholanyl chloride (I) (6-9). The reaction of (I) with functional groups bearing labile protons in lignins has proved to be a novel and powerful means to detect their three principal types of phenolic hydroxyls, i.e syringyl, guaiacyl and p-hydroxyphenyl groups. In addition, primary hydroxyls, carboxylic acids, and the *erythro* and *threo* forms of aryl-glycerol β-O-4 ether structures present in lignins were qualitatively detected (9). Some of these functional groups are ordinarily inaccessible while others can be determined by highly laborious wet chemical methods. The developed novel

spectroscopic technique was thus shown to have considerable potential for the structural elucidation of lignins and has been recommended as a simple and rapid method of qualitative analysis for this biopolymer (9).

In this paper, a detailed account of the aspects that induced the development of quantitative ^{31}P NMR spectroscopy of lignins is given.

EXPERIMENTAL

Lignin Preparation, Derivatizing Reagent (I)

Two hardwood (*populus tremuloides*) and one softwood (mixture of softwoods) lignin were used during this work. The hardwood samples were produced by the process of steam explosion and one of them was widely analyzed during a recent international round robin analytical effort (10). The softwood sample was a technical lignin (Indulin^{TM}) also examined during the international round robin effort. The details of preparation of the lignins examined have been described elsewhere (11). Prior to derivatization the lignins were placed in sample vials and dried under vacuum at 40-45^{0}C for 24 hours. This expelled any residual alcohols or ethers used during purification and ensured that their dry weight was accurately known for quantification purposes. All solvents and chemicals were of reagent or analytical grade. The derivatizing reagent 1,3,2 dioxaphospholanyl chloride (I) was synthesized from phosphorus trichloride (99.9%) and anhydrous ethylene glycol (99.0%) as described elsewhere (12) and purified by vacuum distillation. Analytical quality 1,3,2 dioxaphospholanyl chloride acceptable for these purposes was also obtained from Fluka and Aldrich chemicals.

Preparation of Solutions

Solvent Mixture (A) — A solvent mixture composed of pyridine (predistilled over NaOH) and deuterated chloroform in a 1.6:1.0 volume ratio was prepared. The mixture was protected from moisture with molecular sieves (prewashed with acetone and activated at 110^{0}C overnight).

Internal Standard — A stock solution of the internal standard was made by accurately weighing 0.055 moles of the standard in a 100 mL volumetric flask and diluting to the mark with the solvent mixture described above. When benzoic acid was used as internal standard, 6.71 grams were accurately weighed.

Relaxation Reagent & Internal Standard (B) — 1 mL aliquot of the internal standard solution was transferred to a 10 mL volumetric flask which contained about 25 mg of dry relaxation reagent (chromium acetylacetonate; $Cr(acac)_3$). The mixture was then diluted to the mark with the solvent mixture described above.

Derivatization Procedure

Depending on the number of quantitative experiments required to be carried out, the appropriate amount of dry lignin was weighed into a volumetric flask whose size is specified in Table I. The solvent mixture required (A) was then added together with a small magnetic stirring bar. The mixture was stirred until the lignin was fully dissolved. The required amount of derivatizing reagent (I) was added and the flask was sealed and shaken, to ensure thorough mixing. Finally the required amount of solution containing both the internal standard and the relaxation reagent was added (B).

NMR Spectroscopy

The ^{31}P NMR spectra were obtained by using inverse gated decoupling on a Varian XL-300 FT-NMR spectrometer at 121.5 MHz. The internal deuterium lock was provided by the deuterium atoms present in the deuterated chloroform, used as the solvent. The external standard was 85% H_3PO_4. All downfield shifts from H_3PO_4 were considered positive. A sweep width of 10000 Hz was observed and spectra were accumulated with a time delay of 2s between successive pulses, (unless otherwise specified). Pulse widths corresponding to a 45^0 flip angle were

Table I: The quantities of derivatization reagent (I), internal standard, weight of lignin, and total volume of solvent mixture to be used, depending on the number of ^{31}P NMR quantitative experiments required.

Number of experiments required	Size of volumetric flask required (mL)	Volume of reagent (I) to be used (μl)	Volume of solution containing relaxation reagent & internal standard (μL) (B)	Weight of internal standard received (mg)	Weight of lignin to be used (mg)	Volume of solvent mixture to use (μL)(A)
1	1	100	200	1.34	30	700
2-3	2	200	400	2.68	60	1400
7	5	500	1000	6.71	150	3500

used in order to ensure the preservation of uniform bandwidth over the spectral range examined. All chemical shifts reported are relative to the product of (I) with water which has been observed to give a sharp ^{31}P signal at 121.1 ppm (6-9). A line broadening of 4 Hz was used for the processing of the spectra. Baseline correction based on conventional commercial software was found to be adequate as demonstrated by the high degree of reproducibility obtained.

RESULTS AND DISCUSSION

In an effort to optimize the experimental protocol and spectral acquisition conditions, a variety of variables were first investigated and sources of error delineated.

Solvent Uniformity

Since in earlier reports (8) it was demonstrated that there is a significant dependency of the phosphorus chemical shift values with the solvent composition, a solvent mixture comprised of pyridine and deuterated chloroform at a volume

ratio of 1.6/1.0 was used throughout this work. Independent experiments showed that the amount of deuterium present in $CDCl_3$ was sufficient to provide a strong deuterium signal for locking the NMR instrument.

Internal Standard Selection

The choice of the compound to be used as an internal standard in quantitative ^{31}P NMR spectroscopy of lignins is based on the following definition. A pure crystalline solid possessing a reactive functional group whose ^{31}P NMR signal (after derivatization with (I)) will give a sharp signal in the outer limits of the region 137-126 ppm. A variety of compounds were examined as potential candidates. Dimethyl-L-tartrate (DMLT), is a crystalline solid possessing two vicinal hydroxyls next to two deshielding carbonyl groups. This gave a sharp ^{31}P NMR signal at 136.5 ppm. However, this was an unsuitable choice since it was found that the presence of the two vicinal hydroxyl groups resulted in incomplete derivatization. Detailed studies of the reactivity of this compound with 1,3,2 dioxaphospholanyl chloride showed the possibility of tautomerism being operating in the solvent mixture used throughout this work. Furthermore, the stability of DMLT phosphitylated with (I) was found to be questionable, since aged solutions were found to decompose. The dioxaphospholane ring structure was found to open, resulting in the formation of primary hydroxyls, when the solutions were aged or exposed to a mild thermal treatment.

Trialkyl phosphites were eliminated as potential internal standards since it has been demonstrated that they react with chromophores and carboxylic acids present in lignin (13,14). Aliphatic acids such as glacial acetic acid and adipic acid gave suitable signals at about 126.7 ppm. The only reservation for using aliphatic acids as internal standards is their extreme reactivity with (I), which in some cases may even cause decarboxylation reactions (6).

In an effort to minimize the reactivity of the phosphitylated derivatives of internal standards, aromatic acids were examined for their suitability. This choice was particularly useful since it was found that benzoic acid gave a sharp ^{31}P

NMR signal at 127.5 ppm after phosphitylation with (I). This is not, however, a universal internal standard since its ^{31}P NMR signal falls within the carboxylic acid region of the spectrum. During a search for alternative internal standards for use in quantitative ^{31}P NMR spectroscopy of lignins, the following compounds were also examined: 2,4,5 trimethoxybenzoic acid (126.9 ppm); 2,4,6 trimethoxybenzoic acid (127.2 ppm); 2,6 dimethoxybenzoic acid (127.5 ppm); 3,4,5 trimethoxybenzoic acid (127.5 ppm) and 2,3,4 trimethoxybenzoic acid (127.2 ppm); methyl-2-hydroxyisobutyrate (133.8 ppm). The most suitable choice for lignins which do not contain appreciable amounts of p-hydroxylphenyl moieties was found to be 4,4'-isopropylidenediphenol (Bisphenol-A), (128.3 ppm).

Delay Time Considerations

In order to obtain accurate signal areas under Fourier transform NMR conditions sufficient delay time between pulses should be used. In our earlier work (8) the phosphorus-31 spin-lattice relaxation times for a variety of model compounds were determined. It was reported that these values ranged between 5-10s for functional groups usually encountered in lignins. Such high T_1 values are not unusual in ^{31}P NMR spectroscopy (15). Therefore, a delay time of at least 50-60s needs to be applied between pulses if quantitative claims are to be made from the obtained spectra. Under conditions of relatively low lignin concentration such acquisition protocols will be unacceptable. Kasler and Tierney (16) have demonstrated that quantitative ^{31}P NMR spectra of organic compounds may be obtained in the presence of a compound bearing a paramagnetic metal centre (chromium acetylacetonate; $Cr(acac)_3$). Furthermore, Stanislawski and Van Wazer (17) and Schiff *et al.* (18) have shown that the^{31}P spin-lattice relaxation times can be considerably reduced when use of the 4-hydroxy and 4-amino derivatives of 2,2,6,6-tetramethylpiperidinyloxy free radicals was made. Both of these classes of compounds were examined for their suitability as relaxation reagents for the acquisition of quantitative ^{31}P NMR spectra of lignins. Chromium acetylacetonate was found to be a better choice for the purposes of this work. Figure 1 shows the

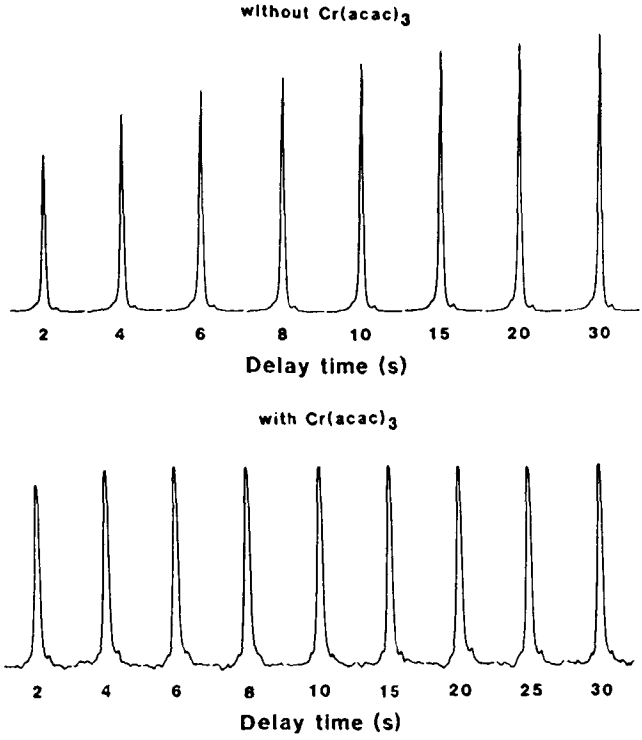

Figure 1: Relaxation time array experiments for phosphitylated dimethyl-L-tartrate; in the presence and absence of $Cr(acac)_3$. The presence of the relaxation reagent considerably reduces the delay time requirements for equilibrium magnetization.

relaxation time array experiments, in the presence and absence of $Cr(acac)_3$ for the two equivalent secondary hydroxyls of dimethyl-L-tartrate. In the absence of the relaxation reagent equilibrium magnetization was reached after about 30s of delay time between pulses. The presence of $Cr(acac)_3$ reduced this delay time to 2-4s.

The ^{31}P NMR signals of phosphitylated lignin produced from the steam explosion process of aspen wood were also examined in the presence of chromium

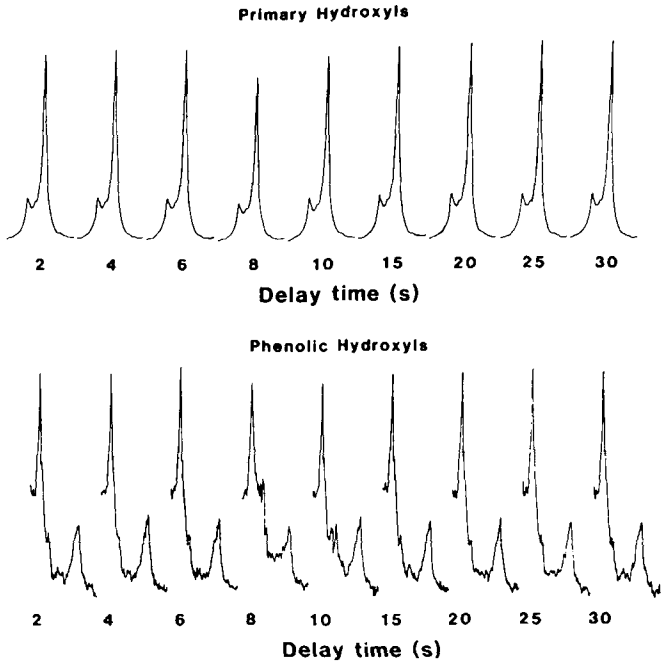

Figure 2: Relaxation time array experiments for the phosphitylated primary and phenolic hydroxyls of steam explosion lignin from aspen wood, in the presence of $Cr(acac)_3$. The relaxation reagent allows short delay times to be used since it ensures the complete recovery of magnetization.

acetylacetonate. The signals due to the phosphitylated primary and phenolic hydroxyls present in this lignin are shown in Figure 2 for a variety of delay times, ranging between 2 to 30 s. These signals were selected since their intensity was found to be most sensitive to delay time variations. The magnetization returns to equilibrium in the presence of chromium acetylacetonate within about 2s, as evidenced by no further increases in the intensities of these signals at higher delay times.

Independent determinations of the spin-lattice and spin-spin relaxation times of most lignin ^{31}P NMR signals in the absence and presence of chromium acetylacetonate confirmed our findings. In Figure 3 the actual measurements of

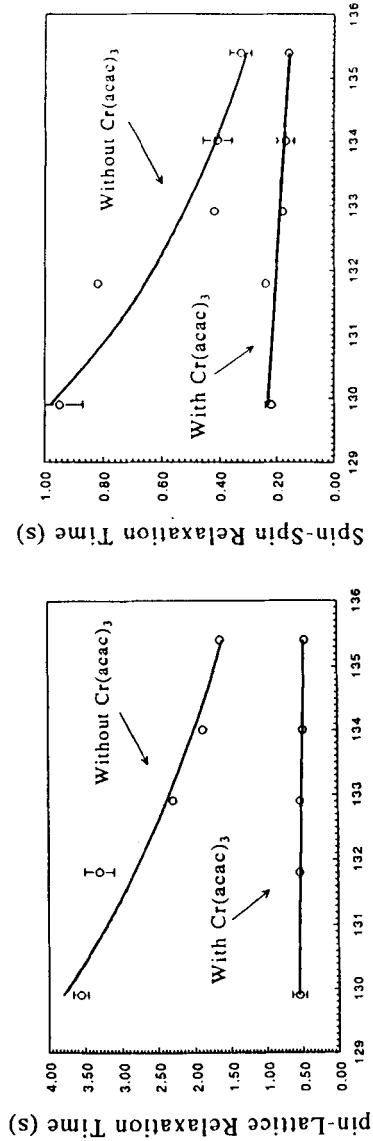

Figure 3: ^{31}P spin-lattice and spin-spin relaxation times for the main signals of a steam explosion lignin produced from aspen wood in the presence and absence of relaxation reagent, $Cr(acac)_3$.

the spin-lattice and spin-spin relaxation times for the main signals of lignin produced by the steam explosion of aspen wood are shown. The relaxation reagent reduced both relaxation parameters by about five fold.

Experimental data accumulated with this relaxation reagent showed that $Cr(acac)_3$ when present in high concentrations caused considerable line broadening in the acquired ^{31}P NMR spectra. Investigations on the concentration effects of $Cr(acac)_3$ on line broadening, showed that the optimum molar amount of relaxation reagent was about 800 times less than that of 1,3,2 dioxaphospholanyl chloride used for the phosphitylation reaction.

Reactivity Considerations

Issues of lignin reactivity toward 1,3,2 dioxaphospholanyl chloride were addressed by examining the functional group profiles of selected lignins after they had been derivatized with (I) and exposed to a mild thermal treatment (50^0C). At selected time intervals samples were withdrawn from the reaction mixture, internal standard (benzoic acid) and relaxation reagent were added and four quantitative ^{31}P NMR spectra were obtained. This allowed the question of quantitative derivatization of lignin to be addressed when it is phosphitylated with (I). Due to the large numbers of quantitative experiments carried out during this effort, the reproducibility and reliability of the results were also studied.

It is evident from the plots of Figure 4 that heating samples of phosphitylated lignins at 50^0C for variable periods of time had no significant effect. The amounts of phenolic and secondary hydroxyls belonging to the *erythro* and *threo* forms of the β-O-4 units in lignins were constant, irrespective of the amount of thermal energy supplied to the sample. At extended periods of reaction at 50^0C a slight decrease of the primary hydroxyl content was observed. When dimethylamino pyridine was used as a catalyst for the phosphitylation reaction no reactivity increases were observed between lignin and the phosphitylation reagent.

The errors and deviations of the data displayed in Figure 4 were found to be within acceptable limits, as evidenced by the statistical data of Table II. The

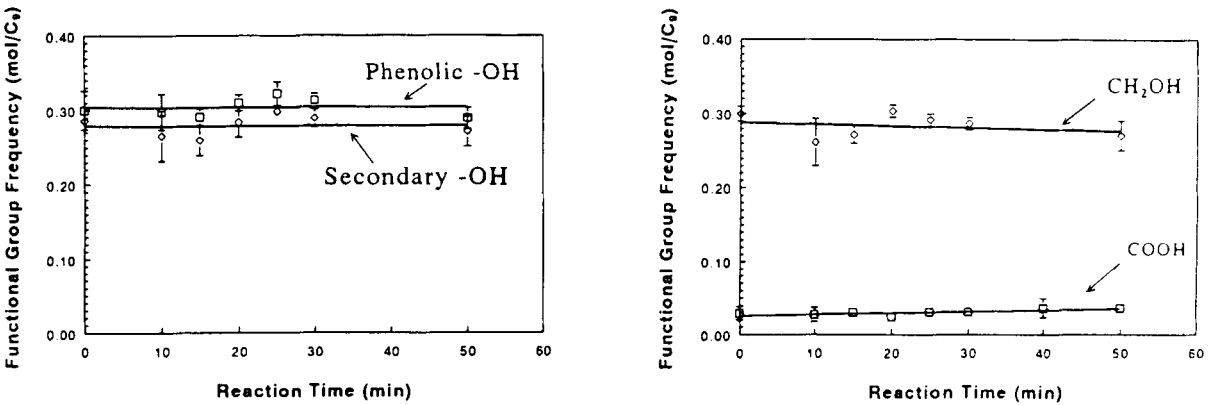

Figure 4: Functional group distributions of a hardwood lignin sample, as a function of time at 50^{0}C, after phosphitylation with (I). The error bars were calculated from four experiments carried out for each data point. The examined sample was a lignin produced by steam explosion of aspen wood.

Table II: Mean functional group distributions, standard errors, standard deviations and confidence limits obtained from the data of Figure 4.

Lignin Functional Group	Mean[1] Value (mol/C_9)	Standard Error	Standard Deviation	95% Confidence Limit	99 % Confidence Limit
CH_2OH	0.28	6.00×10^{-3}	1.6×10^{-2}	1.1×10^{-2}	1.5×10^{-2}
Secondary -OH	0.28	5.18×10^{-3}	1.3×10^{-2}	1.0×10^{-2}	1.3×10^{-2}
Phenolic -OH	0.30	$4.6\,0 \times 10^{-3}$	1.2×10^{-2}	8.9×10^{-3}	1.1×10^{-2}

[1] Mean of 30 measurements

results of quantitative ^{31}P NMR analyses are accurate and reproducible. The significant figures are subjected to the standard errors shown within the confidence limits specified in Table II. This series of experiments demonstrated that the relative stability of a phosphitylated hardwood lignin was adequate for quantification purposes. It was also shown that the reaction of (I) with aspen steam explosion lignin was quantitative at room temperature in the absence of a catalyst.

It is well known that the reactivity of a lignin sample depends significantly on the nature of the wood species. As such, one may argue that different lignins may posses different reactivities toward 1,3,2 dioxaphospholanyl chloride (I). Consequently, the universality of the proposed quantitative method of lignin analysis may be questioned. In an effort to address this issue, the reactivity of (I) with a sample of softwood lignin was also examined. The Indulin™ lignin produced from a mixture of softwoods during the kraft pulping process was the lignin chosen to be investigated. This was a particularly good choice, because it is a chemically modified lignin produced from softwoods, also investigated during the international round robin analytical effort (10,11).

In accord with the previous observations, the amounts of primary and those of secondary hydroxyl groups belonging to the *erythro* and *threo* forms of the β-O-4 units remaining were found to be unaffected by the thermal treatment (Figure 5). The reduction in the estimate of the total phenolic hydroxyl groups apparent in the data of Figure 5 represents a 10% decrease over a period of 30

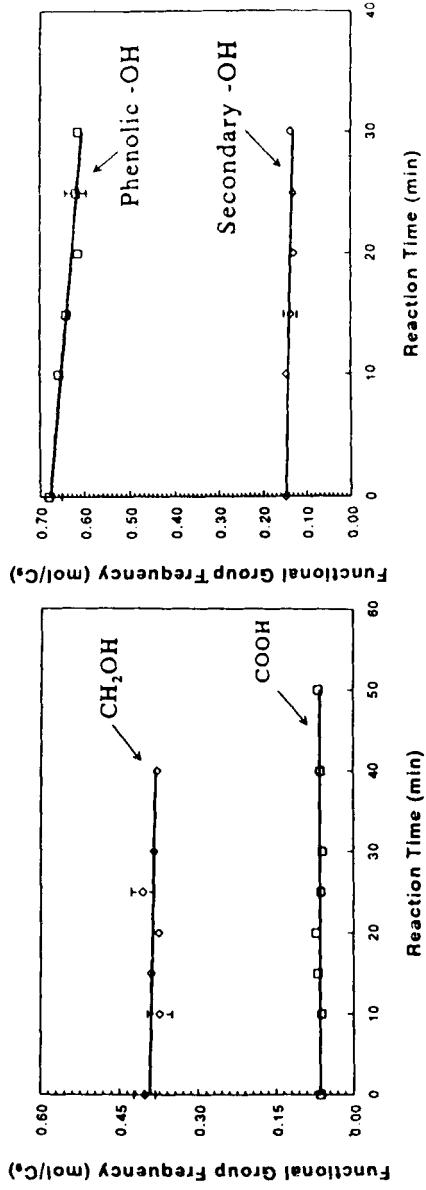

Figure 5: Functional group distributions of a softwood kraft lignin sample (Indulin™) as a function of time at 50°C, after phosphitylation with (I). The error bars were calculated from four experiments carried out for each data point. This sample was also examined during the international round robin analytical effort (10).

Table III: Mean functional group distributions, standard errors and standard deviations obtained from the data of Figure 5.

Lignin Functional Group	Mean[1],[2] Value (mol/C_9)	Standard Error	Standard Deviation	95% Confidence Limit	99 % Confidence Limit
CH_2OH	0.41	4.81×10^{-3}	1.2×10^{-2}	9.4×10^{-3}	1.2×10^{-2}
Secondary -OH	0.14	3.45×10^{-3}	8.4×10^{-3}	6.7×10^{-3}	8.9×10^{-3}
Phenolic -OH	0.57	1.10×10^{-2}	2.6×10^{-2}	2.1×10^{-2}	2.8×10^{-2}

[1] Mean of 28 measurements
[2] Total Phenolic -OH content : 0.57 mol/C_9; Total -OH content: 1.18 mol/C_9

minutes at 50^0C. Since quantitative ^{31}P NMR usually is carried out at room temperature on freshly phosphitylated samples of lignin this result is not alarming. Rate calculations based on the data of Figure 5 have shown that a 2% negative error may be obtained on the estimate of the total phenolic hydroxyl group, for a similar lignin sample, if the spectra were obtained after one hour from the onset of derivatization at room temperature.

Table III shows the statistical analysis of the data of Figure 5 carried out in a manner similar to that described in Table II. For the case of the total phenolic hydroxyl content the figures are subject to greater variations compared to those of the other functional groups.

The mean values of total phenolic hydroxyl and of total hydroxyl contents, reported for this lignin sample, during the international round robin analytical effort, were 0.67 mol/C_9 and 1.23 mol/C_9 respectively (10). These figures compare favourably with those of Table III. The somewhat lower figure of phenolic hydroxyl content obtained by ^{31}P NMR is most likely due to the reactivity considerations observed for this lignin.

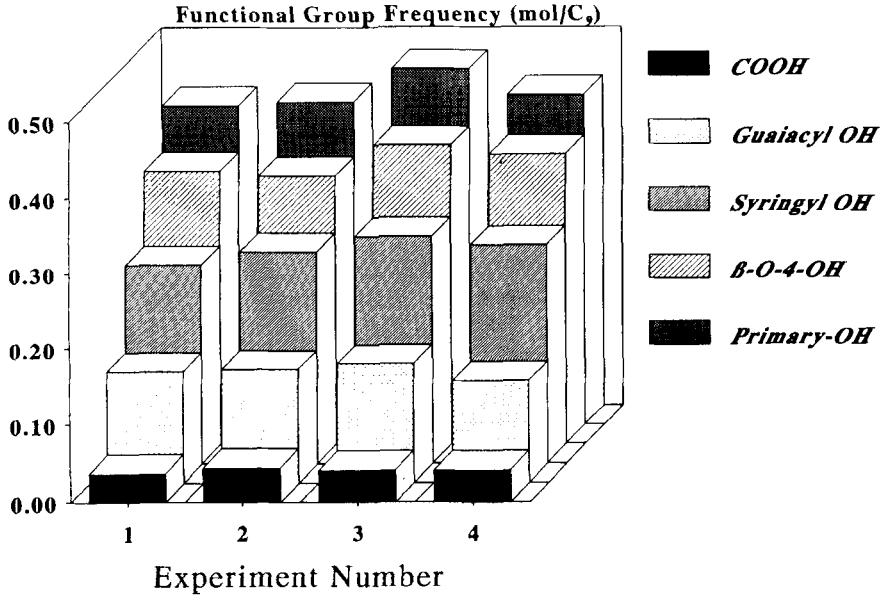

Figure 6. The reproducibility of four quantitative ^{31}P NMR experiments carried out on the sample of the steam explosion lignin from aspen, used during the international round robin analytical effort (10).

Table IV Standard deviations and mean values for the reproducibility experiments displayed in Figure 6.

Lignin Functional Group	Mean[1],[2] Value (mol/C_9)	Standard Error	Standard Deviation	95% Confidence Limit	99 % Confidence Limit
Carboxylic Acids	0.04	1.4×10^{-3}	2.8×10^{-3}	2.8×10^{-3}	3.7×10^{-3}
Syringyl Hydroxyls	0.27	7.9×10^{-3}	$1.6. \times 10^{-2}$	1.5×10^{-2}	2.0×10^{-2}
Guaiacyl Hydroxyls	0.14	4.7×10^{-3}	9.6×10^{-3}	9.3×10^{-3}	1.2×10^{-2}
ß-O-4 *alpha* Hydroxyls	0.37	9.1×10^{-3}	1.8×10^{-2}	1.8×10^{-2}	2.3×10^{-2}
Primary Hydroxyls	0.44	1.0×10^{-2}	2.15×10^{-2}	2.1×10^{-2}	2.7×10^{-2}

(1) Average values of 4 experiments on about 30 mg of lignin

(2) Total Phenolic -OH content : 0.42 mol/C_9; Total -OH content: 1.27 mol/C_9

Reproducibility of the Technique

In an effort to examine the reproducibility and consequently the quantitative validity of our results, at room temperature derivatization conditions, the sample of steam explosion lignin from aspen wood (*populus tremuloides*) used during the international round robin effort was subjected to four quantitative ^{31}P NMR analyses. It was thus possible to compare the results furnished by ^{31}P NMR with those produced by independent methods in other laboratories for the same sample. The reproducibility of the four experiments is shown in the histogram of Figure 6. The mean values, standard deviations, standard errors and confidence limits calculated for each of the five functional groups displayed in Figure 6 are shown in Table IV. A comparison of the total phenolic (0.45 mol/C_9) and the total hydroxyl groups (1.26mol/C_9), reported for this lignin during the international round robin analytical effort (10), is very favourable with the values determined by quantitative ^{31}P NMR spectroscopy (0.42 and 1.27 mol/C_9 respectively).

Reproducible results were obtained by derivatizing about 30 mg of lignin followed by a set of four quantitative ^{31}P NMR spectra. It is, however, preferable to derivatize about 60 mg of lignin in order to obtain two sets of four quantitative ^{31}P NMR spectra. This procedure ensures the averaging of possible variations which may occur during sampling and acquisition. Using 2s delay times and acquiring 8 quantitative ^{31}P NMR spectra (200 transients each) requires approximately 1 hour of instrument time.

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