2-Chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane, a Reagent for the Accurate Determination of the Uncondensed and Condensed Phenolic Moieties in Lignins

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The use of 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane as a phosphitylation reagent in quantitative ^{31}P NMR analysis of the hydroxyl groups in lignins has been thoroughly examined, and an experimental protocol recommended for spectra acquisition has been developed. Quantitative analysis of six "*standard lignins*" gave results comparable to those obtained by other methods of analysis. Excellent resolution of the various phenolic hydroxyl environments including those present in condensed moieties was observed. However, this was at the expense of resolution in the aliphatic hydroxyl region, where no distinction between primary, secondary, and the *erythro* and *threo* forms of the secondary hydroxyls of the β-O-4 bonds can be made.

Keywords: *Analysis; aromatic groups; hydroxyls; lignins; nuclear magnetic resonance (NMR); phenols; phosphorus; quantitative*

INTRODUCTION

Magnetic resonance techniques when applied to lignins have proved to be excellent analytical tools for the structural elucidation of these complex biopolymers. Accordingly, the work of our laboratory has been focused on the development of novel magnetic resonance methods aimed at expanding 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 phosphorus-tagged lignocellulosic polymers. In recent years, we have been examining the potential of ^{31}P NMR spectroscopy for the quantitative characterization of lignins, after derivatizing their labile protons with 2-chloro-1,3,2-dioxaphospholane (**I**) (Argyropoulos *et al.*, 1993a,b; Argyropoulos, 1994a,b). This reagent allows the overall distribution of hydroxyl groups present in a lignin sample to be determined. It has been particularly useful for quantifying the carboxylic and guaiacyl phenolic hydroxyls as well as the primary and secondary hydroxyl groups belonging to the *erythro* and *threo* forms of the arylglycerol β-O-4 ether structure (Argyropoulos, 1994a,b; Jiang and Argyropoulos, 1994). However, for hardwood lignins and lignins resulting from various pulping and bleaching processes, signal overlap occurred between the syringyl phenolic structures and those belonging to primary and condensed phenolic groups. Another phosphitylation reagent, namely 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (**II**), has been found to be particularly good at resolving this region at the expense of fine resolution between the primary and secondary hydroxyls (Fillppov *et al.*, 1991; Kostukevich *et al.*, 1993; Sun and Argyropoulos, 1994; Faix *et al.*, 1994). This work examines the potential of 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (**II**) as another phosphitylation reagent that can provide quantitative structural information for lignins. In this paper we describe our efforts to fully develop this reagent for use in the quantitative analysis of lignins. More specifically, we have examined stability, internal standard, and spin–lattice relaxation issues for lignins phosphitylated with **II** and have arrived at a set of recommended spectral acquisition conditions. We then applied this reagent toward the quantitative analysis of six "*standard lignins*" (Chum *et al.*, 1993; Milne *et al.*, 1992) and have compared the functional group distributions obtained to those reported previously (Argyropoulos, 1994b; Faix *et al.*, 1993; Milne *et al.*, 1992). In an effort to demonstrate the potential of this reagent toward the detection of condensed phenolic structures in lignins, we have also analyzed a series of kraft lignins (isolated at various degrees of delignification) and two milled wood lignins from softwood and hardwood species.

EXPERIMENTAL PROCEDURES

Lignins and Reagents. The details of preparation for the six standard lignins examined during an international round robin analytical effort have been described elsewhere (Milne *et al.*, 1992; Bjorkman, 1956). Also, the details of isolation and characterization of the kraft lignins examined in this work have been reported by Jiang and Argyropoulos (1994). Two milled wood lignins, from black spruce (*Picea mariana*) and aspen (*Populus tremuloides*) wood chips, were isolated according to the method of Bjorkman (1956). Prior to derivatization, all lignins were dried for 24 h under vacuum at 30 °C in the presence of silica gel (Sigma, type III). All solvents and chemicals were of reagent grade. Pyridine, reagent grade, was further dried by heating it under reflux with potassium hydroxide. It was then distilled at atmospheric pressure and stored over molecular sieves (type 4A) under nitrogen.

2-Chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (**II**) was prepared from pinacol (Aldrich) and phosphorus trichloride (Aldrich) in the presence of triethylamine (Aldrich), according to the method of Zwierzak (1967). The yield after distillation was 34%: ^{31}P NMR ($CDCl_3$) 176.0 ppm [175.9 ppm (Zwierzak, 1967)].

Solution Preparation. A solvent mixture composed of pyridine and deuterated chloroform (Isotec) in a 1.6/1 v/v ratio was prepared, on the basis of considerations outlined previously (Argyropoulos *et al.*, 1993b). The solution was protected from moisture with molecular sieves (3A) and kept in a sealed container under nitrogen. A solution was then prepared by utilizing the above preparation; chromium(III) acetylacetonate (Aldrich, 5.0 mg/mL) and cyclohexanol (Aldrich, 10.85 mg/mL), served as relaxation reagent and internal standard, respectively.

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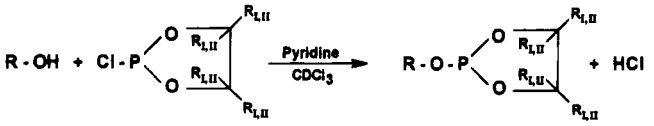
Scheme 1



[a] ROH represents the labile protons of model compounds and lignins; R_I = H [i.e. 2-chloro-1,3,2-dioxaphospholane (**I**)], R_{II} = CH_3 [i.e. 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (**II**)].

Lignin Phosphitylation Procedure. Thirty milligrams of lignin was accurately weighed into a 1 mL volumetric flask. The sample was then dissolved in 0.5 mL of the above solvent mixture. The tetramethylphospholane (**II**) (50 μL) was then added, followed by the internal standard and the relaxation reagent solution (100 μL each). Finally, the solution was made up to the 1 mL mark with more solvent mixture. The volumetric flask was tightly closed and shaken to ensure thorough mixing.

NMR Spectroscopy. The ^{31}P NMR spectra were obtained on a Varian XL-300 NMR spectrometer by using methods essentially identical to those described by Argyropoulos (1994a,b) with minor modifications as dictated by the nature of the tetramethylphospholane (**II**) and our measurements of the spin–lattice relaxation time profiles. More specifically, an observation sweep width of 6600 Hz was used, and the spectra were accumulated with a delay time of 25 s between successive pulses. All chemical shifts reported are relative to the reaction product of water with **II**, which has been observed to give a sharp signal in pyridine/$CDCl_3$ at 132.2 ppm.

RESULTS AND DISCUSSION

The phosphitylation reaction of labile protons with reagents **I** and **II** in model compounds and lignins used during our work is depicted in Scheme 1.

In an effort to map the correlation of structure with the phosphorus chemical shift values obtained, after phosphitylation with **II**, a detailed model compound investigation (described elsewhere) was undertaken (Jiang *et al.*, 1995). This work also examined the structure/chemical shift correlation of substituted phenols, β-O-4 models, and compounds resembling diarylmethane, biphenolic and stilbene structures. The information, thus obtained, was used for assigning the signals for the lignins presented in this work. Furthermore, this effort showed that a clear separation between phenolic hydroxyl groups and between primary and secondary aliphatic groups is possible after phosphitylation of their labile protons with **II** (Jiang *et al.*, 1995). However, no distinction between the primary and the secondary aliphatic hydroxyls can be made with reagent **II**, in sharp contrast to 1,3,2-dioxaphospholanyl chloride, reagent **I** (Argyropoulos *et al.*, 1993a; Argyropoulos, 1994a,b; Jiang and Argyropoulos, 1994; Saake *et al.*, 1995).

As far as the analysis of lignins is concerned, the use of 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (**II**) as the phosphitylation reagent presents a distinct advantage compared to the use of 1,3,2-dioxaphospholanyl chloride (**I**). Its use allows for a better resolution between the signals due to the syringyl phenolic hydroxyls from those of the aliphatic hydroxyls, an issue of concern when hardwood lignins are to be examined (Faix *et al.*, 1994; Saake *et al.*, 1995). An additional distinct advantage of reagent **II** is also apparent when one examines technical lignins, where condensed aromatic structures bearing phenolic hydroxyl groups are present in significant amounts. Finally, the presence of the four methyl groups (Scheme 1) in reagent **II** also offered a somewhat reduced reactivity compared to reagent **I**, thus increasing the stability of the phosphitylated compounds and lignins. The evidence pertaining to these points will be presented throughout the following discussion.

The use of an internal standard has become an indispensable means to quantify the various hydroxylic functional groups present in lignin (Argyropoulos, 1994a). Our work also showed that when 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (**II**) is used for such a purpose, cyclohexanol is an ideal internal standard to use. Its selection is based on the following considerations: it is a stable, readily available compound that reacts quantitatively with **II** (Sun and Argyropoulos, 1994); most significantly, however, its phosphorus derivative gives rise to a phosphorus signal within our working range, and it does not overlap with any signals derived from lignin (Sun and Argyropoulos, 1994).

The presence of the four methyl groups within the structure of 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (**II**) significantly affected the spin–lattice relaxation profiles of the phosphorus atoms attached on lignin compared to those obtained with reagent **I** (Argyropoulos, 1994a; Argyropoulos *et al.*, 1993b). Such measurements on samples of milled wood lignin from aspen wood and from kraft lignin (Indulin) obtained from softwood showed that the range of the spin–lattice relaxation times of the phosphorus NMR signals obtained was between 4.0 and 1.0 s. In a manner similar to that described in our previous efforts (Argyropoulos, 1994a), the presence of a relaxation reagent (chromium acetylacetonate) reduced the above range to about 2.0–0.5 s. However, the spin–lattice relaxation times of the phosphorus nuclei in phosphitylated cyclohexanol (internal standard in the presence of chromium acetylacetonate) were found to be about 4.6 s. As such, the experimental protocol for spectral acquisition on lignins phosphitylated with **II** dictates the use of considerably more extended pulse delays, even in the presence of chromium acetylacetonate. Notably, our work with 1,3,2-dioxaphospholanyl chloride has demonstrated that (in the presence of relaxation reagent) pulse delays of 2 s are adequate. This is clearly a disadvantage of reagent **II**, since longer acquisition protocols are required (25 s delay times are suggested).

In an effort to examine the stability of lignins phosphitylated with **II**, solutions of a softwood kraft lignin (Indulin) were quantitatively analyzed after 1 h from the onset of the phosphitylation reaction and after 1 week. This work demonstrated that there was no difference in the results obtained between the freshly derivatized solutions and those that were allowed to age.

Subsequently, our efforts were focused at fully documenting the quantitative validity of the choices of phosphitylating reagent, acquisition profiles, and internal standard (enumerated above). To do this, we phosphitylated six standard lignins (Chum *et al.*, 1993; Milne *et al.*, 1992). In a manner identical to that described previously (Argyropoulos, 1994b), we compared these results with those obtained during a round robin effort applying independent methods of analysis (Milne *et al.*, 1992). The results of this comparison are presented in Table 1. In general, the data presented in Table 1 demonstrate that both phosphitylating reagents give approximately equivalent results [with the exception of the syringyl phenolic hydroxyl values, which may result in a lower estimate when 1,3,2-dioxaphospholanyl chloride (**I**) is used as the phosphit-

Table 1. Functional Group Distribution (Moles/C_9) Obtained by Quantitative ^{31}P NMR Analysis of Six Standard Lignins Using 4,4,5,5-Tetramethyl-1,3,2-dioxaphospholane (II) as the Phosphitylating Reagent[a]

lignin sample	COOH	guaiacyl −OH	syringyl −OH	total phenolic −OH	total aliphatic −OH	total hydroxyl content
steam explosion	0.06	0.13	0.23	0.47	0.67	1.20
(aspen)	NA[b]	NA	NA	(0.45)	NA	1.26
	[0.04]	[0.14]	[0.27]	[0.42]	[0.81]	[1.27]
steam explosion	0.08	0.18	0.34	0.57	0.53	1.18
(yellow poplar)	NA	NA	NA	(0.59)	NA	(1.20)
	[0.00]	[0.15]	[0.24]	[0.48]	[0.58]	[1.06]
ball-milled enzyme	0.02	0.07	0.05	0.20	1.18	1.41
(cottonwood)	NA	NA	NA	(0.18)	NA	(1.47)
	[0.00]	[0.04]	[0.05]	[0.15]	[1.05]	[1.20]
Alcell organosolv	0.06	0.26	0.49	0.82	0.33	1.22
(mixed hardwood)	NA	NA	NA	(0.73)	NA	(1.20)
	[0.06]	[0.25]	[0.45]	[0.70]	0.48	[1.24]
Indulin kraft	0.077	0.35		0.65	0.46	1.19
(mixed softwood)	NA	NA		(0.67)	NA	(1.23)
	[0.06]	[0.21]		[0.57]	[0.55]	[1.18]
sucrolin acid hydrolyzed	0.005	0.13	0.17	0.50	0.140	0.76
(bagasse)	NA	NA	NA	(0.44)	NA	(0.78)
	NA	NA	NA	NA	NA	NA

[a] Data in parentheses are the averages obtained by the round robin analytical effort (Milne *et al.*, 1992), while data in square brackets were those obtained when 1,3,2-dioxaphospholanyl chloride (**I**) was used as the phosphitylating reagent (Argyropoulos, 1994b). Calculations were carried out as described in Argyropoulos (1994b). [b] NA, not applicable.

ylating reagent]. This is not a surprising finding, since reagent **II** offers a considerably better resolved signal for the syringyl hydroxyl groups compared to those obtained when 1,3,2-dioxaphospholanyl chloride (**I**) is used. Since the syringyl signals in hardwood lignins obtained by the use of reagent **II** somewhat overlapped with those of the primary aliphatic hydroxyls, the estimates of the latter are now lower when reagent **II** is used. Finally, the essentially identical values obtained for the total hydroxyl contents for most of the lignins when use of either phosphitylating reagent was made further demonstrate and verify the validity of the above statements. The maximum errors of the method based on reproducibility measurements were found to be as follows for the various functional groups: ±1.9% for aliphatic hydroxyl groups, ±1.4% for condensed phenolic units, ±1.3% for guaiacyl phenolic units, and ±1.7% for carboxylic acids.

A detailed discussion of other salient features of the results given in Table 1 will follow, based on the spectral data of Figures 1 and 2.

Detection of Phenolic and Condensed Phenolic Structures in Steam Explosion and Milled Wood Lignins. The quantitative ^{31}P NMR spectrum of the round robin steam explosion lignin from aspen wood is shown in Figure 1. The syringyl to guaiacyl free phenolic hydroxyl group ratio, as derived from the signals centered at 142.9/139.8 ppm, was found to be about 1.8, while our previous efforts with reagent **I** showed this ratio to be 1.9 (Argyropoulos, 1994b). The fact the more syringyl units are produced during the steam explosion treatment, most likely as a result of scission of β-O-4 bonds, has already been reported by Robert *et al.* (1988) and supported by the work of Obst and Landucci (1986). Indeed, when a milled wood lignin sample from aspen wood was quantitatively analyzed after phosphitylation with **II**, it gave a syringyl to guaiacyl free phenolic hydroxyl group ratio of 0.78. The total phenolic hydroxyl content for the same sample was found to be 0.47 mol/C_9, in satisfactory agreement with the average value obtained during the round robin effort (0.45 mol/C_9).

Other signals in the quantitative ^{31}P NMR spectrum of the aspen steam explosion lignin merit discussion (Figure 1). The small signal centered at 141.9 ppm represents an amount of 0.07 mol/C_9 or about 15% of the total phenolic −OH. Our previous work has shown that model compounds bearing 5,5′-biphenolic structures would show a signal at this position (Jiang *et al.*, 1995). Furthermore, our work with a sample of milled wood lignin from aspen wood showed the same signal, accounting for about 8% of the total free phenolic −OH groups. The fact that milled wood lignin from the same wood species showed a somewhat reduced content of this signal is in agreement with the work of Robert *et al.* (1987), where it was shown that steam explosion may induce condensation reactions in lignin. As such, it is likely that this signal originates from 5,5′-biphenyl structures known to be present in lignins (Aulin-Erdtman, 1952). Similar conclusions were also reached during our previous effort (Argyropoulos, 1994b), where 1,3,2-dioxaphospholanyl chloride (**I**) was used as the phosphitylating reagent. However, this work demonstrates that the use of **II** considerably aids the detection of such structures present in relatively low amounts. The shape of the signal centered at about 137.9 ppm presented difficulties in being integrated by itself. Our model compound work, however, showed that in this region phenolic compounds which possess no substituents in either the 2 or the 6 position of the aromatic ring can be found. It is thus likely that this signal belongs to *p*-hydroxyphenols known to be present in hardwood lignins (Jiang *et al.*, 1995).

The quantitative ^{31}P NMR spectrum of the steam explosion lignin from yellow poplar (Figure 1) also suggests the presence of 5,5′-biphenolic structures as evidenced from the small signal at about 141.9 ppm. This signal amounts to 0.05 mol/C_9 or about 9% of the total phenolic −OH. In the same context, the quantitative ^{31}P NMR spectrum of ball-milled enzyme lignin from cottonwood (Figure 1) showed a very small signal at about 141.9 ppm, also suggesting the detection of 5,5′-biphenolic hydroxyl groups. In this sample 0.02 mol/C_9 of such moieties was detected. However, the weak

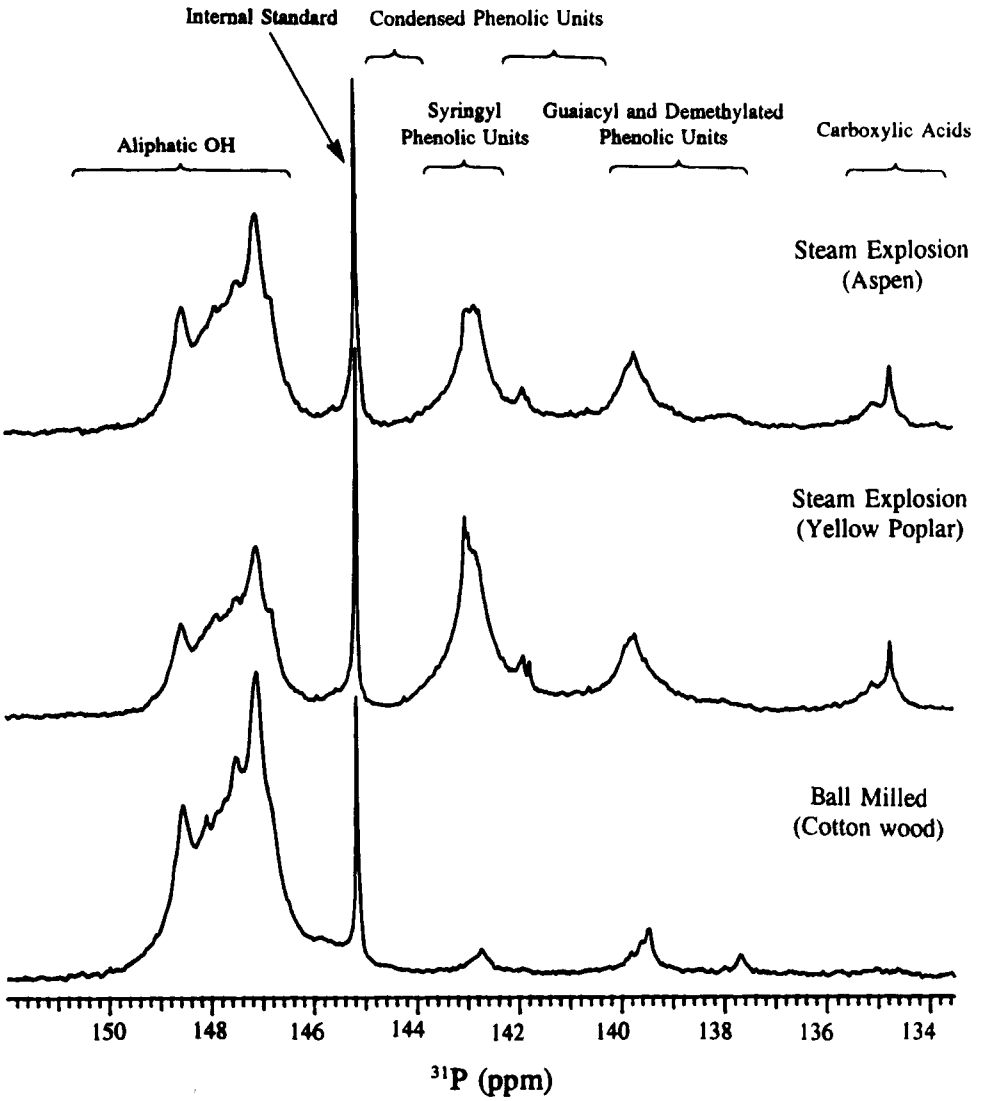


Figure 1. Quantitative ^{31}P NMR spectra and signal assignment of steam explosion lignins from aspen and yellow poplar and of a ball-milled enzyme lignin from cottonwood (Milne *et al.*, 1992). The phosphitylation was carried out using 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (**II**).

nature of the signal presented integration difficulties, thus limiting the accuracy of the estimate. For the same lignin sample, the signal centered at about 137.6 ppm once again may be assigned at *p*-hydroxyphenylpropane units possessing a free phenolic −OH in an amount of 0.003 mol/C_9.

Carboxylic acids can accurately be detected with the use of reagent **II**. The signals of such phosphitylated moieties were found to be somewhat more stable than those produced when **I** was used.

Detection of Phenolic and Condensed Phenolic Structures in Technical Lignins. In Figure 2 the quantitative ^{31}P NMR spectra of the three round robin lignins produced by various technical processes are shown.

The spectrum of Alcell organosolv lignin produced from a mixture of hardwood species shows a signal at about 141.9 ppm, most likely due to 5,5′-biphenolic structures. Once again, small amounts of *p*-hydroxyphenylpropane moieties bearing free phenolic hydroxyls are apparent from the signal at about 137.8 ppm. Their amount was found to be 0.07 mol/C_9.

The quantitative ^{31}P NMR spectrum of Induline (kraft lignin from softwood) presents convincing evidence for the potential of reagent **II** in accurately quantifying condensed phenolic structures in such lignins (Figure 2). The region between 140.3 and 144.4 ppm contains this significant information (Jiang *et al.*, 1995). The total amount of condensed structures including diphenylmethanes, diphenyl ethers, and 5,5′-biphenolic moieties was found to be 0.27 mol/C_9 or 41% of the total phenolic −OH content. One may actually distinguish four types of free phenolic −OH groups in the condensed region from this spectrum. Their resolution, however, at this stage is insufficient, precluding detailed assignment. The tentative assignment of the signal centered at 143.5 ppm, as being due to diarylmethane condensed structures, can be made on the basis of our model compound work (Jiang *et al.*, 1995). Alkaline pulping processes are known to induce such condensed structures in lignin (Robert and Bardet, 1984; Gierer, 1970; Marton, 1971; Chiang and Funaoka, 1988). On a tentative basis, the amount of diphenylmethane condensed structures bearing free phenolic hydroxyl groups in this sample was found to be 9.8% of the total free phenolic −OH. The signal centered at 137.8 ppm, which amounts to 0.036 mol/C_9, is likely to be due to *p*-hydroxyphenylpropane moieties, possibly resulting from demethylation reactions occurring during the kraft pulping process.

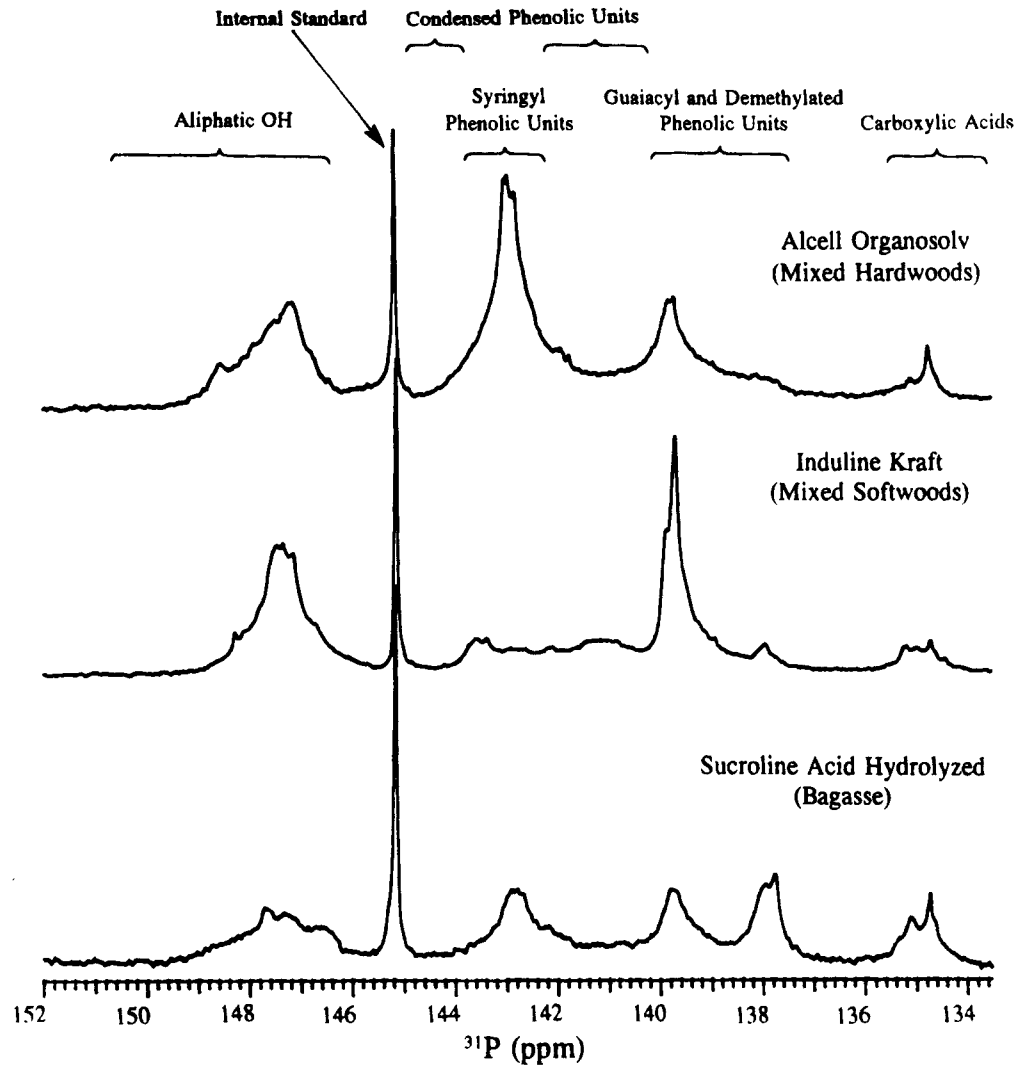


Figure 2. Quantitative ^{31}P NMR spectra and signal assignment of three commercial lignins (Milne *et al.*, 1992). The phosphitylation was carried out using 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (**II**).

The quantitative ^{31}P NMR spectrum of acid-hydrolyzed lignin from bagasse (Sucrolin) is also shown in Figure 2. A large amount of carboxylic acid groups was found be present in this sample (0.095 mol/C_9, representing about 12% of the total hydroxyl content in this sample). The total hydroxyl content of this sample was found to be 0.76 mol/C_9, in good agreement with the value of 0.78 mol/C_9 obtained by ^1H NMR (Faix *et al.*, 1994). The total free phenolic content was found to be 0.50 mol/C_9, once again close to the value of 0.44 mol/C_9 detected by ^1H NMR (Faix *et al.*, 1994). Our methodology actually allowed the determination of the guaiacyl and syringyl moieties, which were found to be 0.13 and 0.17 mol/C_9, respectively. Not surprisingly, this lignin showed a strong signal centered at 137.9 ppm due to *p*-hydroxyphenylpropane units bearing a free phenolic hydroxyl group. Their amount was detected to be 0.125 mol/C_9. Such moieties are known to be present in large amounts in grass lignins (Nimz *et al.*, 1981; Erickson *et al.*, 1973).

Quantification of Condensed Structures during Kraft Pulping. The potential of this reagent in detecting condensed structures in technical lignins was further explored by examining a sample of milled wood lignin and a series of kraft lignins isolated at different degrees of delignification from the same softwood species (*Picea mariana*) (Jiang and Argyropoulos, 1994). Our efforts are thus documented in Figure 3, while Table 2 attempts to present in detail our findings. This effort permitted the actual visualization of the development of condensed structures to be obtained at three different degrees of delignification, i.e. at 16.5, 22.1, and 39.1%.

Evidently, a significant amount of condensed structures accumulates within solubilized kraft lignins at about 16% delignification. These species seem to further accumulate at subsequent levels of delignification.

When the total amounts of condensed structures are expressed as a percentage of the total phenolic hydroxyls, one may compare these results with those obtained by oxidative degradation. Gellerstedt *et al.* (1987), using oxidative degradation, have studied the kraft lignins obtained from pine wood. Among their results, three samples isolated at 15, 28, and 37% delignification levels are closest to our experiments. Oxidative degradation showed that they contained 36.6, 41.1, and 43.5% condensed structures expressed as percent of the total phenolic hydroxyl. Our samples, isolated at 16.5, 22.1, and 39.1% delignification levels, contained 38, 38.9, and 43.7% condensed structures based on the total phenolic hydroxyl. In addition, a comparison of the sums of phenolic hydroxyls belonging at guaiacyl and catechol moieties can be made for the same sets of samples. Once again, when these moieties are expressed in percent terms of the total phenolic hydroxyl groups, Gellerstedt *et al.* (1987) obtained 61,

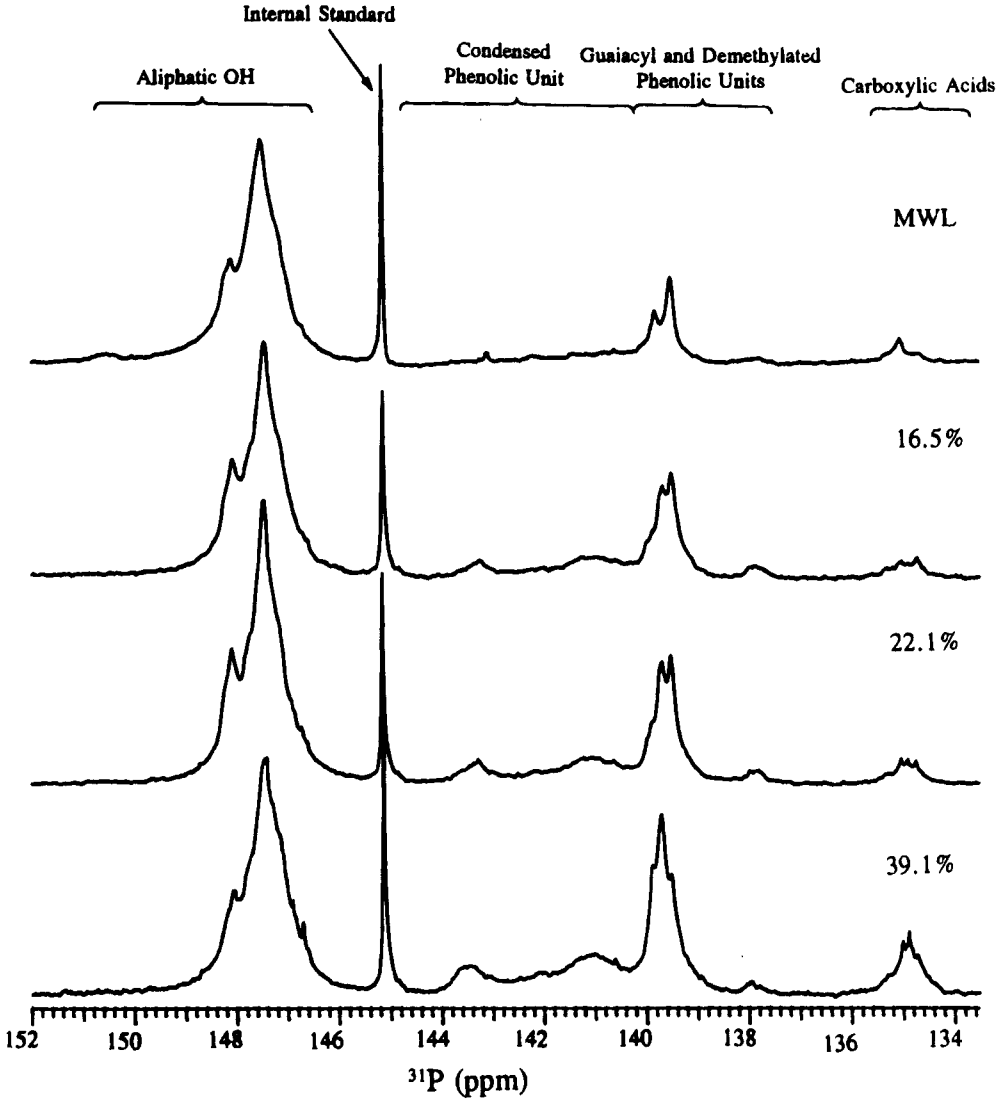


Figure 3. Quantitative ^{31}P NMR spectra and signal assignment of a milled wood lignin obtained from black spruce (*P. mariana*) and of three kraft lignins for which details of isolation at the various degrees of delignification (percent) have been described elsewhere (Jiang and Argyropoulos, 1994).

Table 2. Functional Group Distribution (Millimoles/Gram) As Derived by Quantitative ^{31}P NMR Analyses of the Samples for Which Spectra Are Displayed in Figure 3[a]

lignin sample[b]	degree of delignification (%)	carboxylic acids	aliphatic −OH	guaiacyl and catechol −OH	condensed phenolic −OH	total phenolic −OH	total hydroxyl content
MWL	0.0	0.21	4.27	0.77 (64.3)	0.36 (30.0)	1.2	5.6
kraft soluble lignin	16.5	0.25	5.09	1.31 (57.2)	0.87 (38.0)	2.2	7.5
	22.1	0.21	4.78	1.34 (57.2)	0.91 (38.9)	2.3	7.2
	39.1	0.51	3.79	1.58 (52.7)	1.31 (43.7)	2.9	7.2

[a] Data in parentheses are expressed as percent of the total phenolic −OH content. [b] Wood species: black spruce (*P. mariana*).

56.8, and 52.7%, while during the present effort we obtained 57.2, 57.2, and 52.7%. As such, a reasonable agreement between the two sets of results is apparent, bearing in mind that different species, somewhat different degrees of delignification, and different techniques were used to detect these moieties.

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