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Understanding the Degree of Condensation of Phenolic and Etherified C-9 Units of in Situ Lignins

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ABSTRACT: A novel approach for the quantification of the degree of condensation at the C_5 position of etherified and phenolic phenylpropane (C-9) units of in situ lignin is described. This is achieved by degrading unmethylated and methylated wood by thioacidolysis and analyzing the resultant product mixtures by quantitative ^{31}P NMR spectroscopy. Applying this new method to compression wood and normal wood from *Pinus radiata* showed that, whereas 41−47% of etherified guaiacyl C-9 units are condensed at the C_5 position, almost all phenolic guaiacyl C-9 units exist as uncondensed moieties. Analysis of milled wood lignin (MWL) isolated from the same wood by ^{31}P NMR spectroscopy before and after thioacidolysis showed that the phenolic guaiacyl C-9 units were more condensed than those in the in situ lignin. This is likely due to partial cleavage of the more condensed etherified linkages during the lignin isolation, leading to a relative increase in condensed phenolic guaiacyl C-9 units.

KEYWORDS: β-aryl ether linkages, compression wood, degree of condensation, in situ lignin, methylated wood, milled wood lignin, ^{31}P NMR spectroscopy, phenolic C-9 units, *Pinus radiata*, etherified C-9 units, thioacidolysis

INTRODUCTION

Lignin is a biopolymer composed of phenylpropane (C-9) units linked to each other by a number of different types of ether and carbon−carbon interunit linkages.[1] Some interunit linkages, such as α- and β-aryl ether bonds, are easily cleaved during pulping, whereas others such as carbon−carbon linkages (5-5 biphenyl linkages) are less easily cleaved.[2,3] For practical reasons it is useful to know how extensively a lignin is broken down under a given set of conditions. This concept has been extended to distinguish between "uncondensed" C-9 units, which are linked via easily cleaved linkages and are therefore easily broken down to monomers, and the less readily degraded "condensed" C-9 units. Although a practically useful concept, it is difficult to define a condensed C-9 unit, with the definition often depending on the method used for its analysis. Bose et al.[4] suggest a condensed C-9 unit to be one containing a C−C bond at any ring position except C_1 or one connected to another C-9 unit via a diaryl ether linkage. Note that, according to this definition, condensed units are produced both by oxidative coupling reactions, which are a natural part of lignification, and by "condensation reactions", which can occur during pulping.

Furthermore, the aromatic groups in lignin exist in either phenolic or etherified form. Phenolic units constitute only a minor portion of the C-9 units in the lignin, typically 13% for softwoods and 7−11% in hardwoods.[5] Such phenolic units tend to be more reactive than etherified units during alkaline pulping,[3] bleaching using oxygen and chlorine dioxide,[6] chemimechanical pulping,[7] and photodegradation.[8] Accordingly, C-9 units in lignin can be classified into four categories based on the substitution at C_5 as shown in Table 1.

A range of methods have been used to determine the relative proportions of uncondensed to condensed C-9 units in in situ lignins. One approach is to first isolate the lignin from the wood and then analyze it by a technique such as NMR spectroscopy. Lignin is most commonly isolated from wood by ball-milling followed by extraction of the solubilized lignin with a solvent, commonly a dioxane−water mixture, and a subsequent purification step.[9,10] The yields of lignin may be increased by combining this treatment with enzymes to degrade the carbohydrates[11] or including a mild acid treatment in the process.[12,13] The proportion of condensed units in the resulting isolated lignin can be determined by ^{1}H NMR spectroscopy from the number of aromatic protons per aromatic ring[14] or by quantitative ^{13}C NMR spectroscopy from the number of protonated carbons per aromatic ring.[15,16] In addition, specific interunit linkages can be quantified by NMR spectroscopy.[15] The major limitation of this approach is that the isolated lignins such as milled wood lignins (MWLs) are not necessarily representative of the native lignin, because they represent only a small proportion of the lignin in the original wood, and lignins may be structurally modified during their isolation.[17]

Alternatively, one can chemically degrade the in situ lignin and measure specific degradation products associated with the reactive C-9 units. In this case, the higher the level of degradation products, the less condensed the lignin is considered to be. Such degradation methods are valuable because structural information can be obtained without isolating the lignin. Commonly used methods include thioacidolysis[18] or derivatization followed by reductive cleavage (DFRC)[19] to measure the levels of releasable β-ether units, nitrobenzene oxidation to measure the levels of hydroxybenzaldehydes,[20] and nucleus exchange to measure the levels of released phenols.[21] Other degradative methods afford specific products from condensed C-9 units, which can be analyzed. Examples include oxidative degradation to measure the ratio of degradation products from condensed and uncondensed

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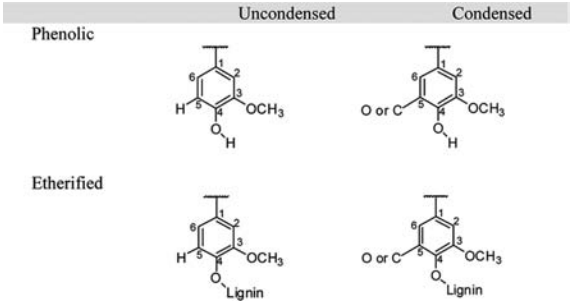
Table 1. Different Types of Guaiacyl C-9 Units



phenolic units,[22] oxidative degradation in combination with an alkaline pretreatment to measure the ratio of degradation products from condensed to uncondensed phenolic units plus etherified units,[4,23] analysis of dimers and oligomers produced during thioacidolysis by electrospray mass spectroscopy[24] or by GC-MS following desulfurization,[25] and hydrogenolysis.[26] The molecular weight of the lignin thioacidolysis product can also be used as an indication of the degree of condensation of the lignin, with a more condensed lignin giving a greater proportion of dimeric and oligomeric products in the thioacidolysis mixture.[27,28] In addition, uncondensed (at C_5) phenolic guaiacyl units have been measured via the Mannich reaction[29,30] or by oxidation with Fremy's salt.[31]

^{31}P NMR spectroscopy is a useful technique for quantifying condensed and uncondensed free phenolic moieties in isolated lignins.[32] However, the method does not provide information on etherified moieties in lignin. Smit[33] has extended this technique to quantify condensed and uncondensed moieties in both phenolic and etherified C-9 units in in situ lignins by coupling this technique with thioacidolysis. When lignin is subjected to thioacidolysis, α- and β-aryl ether linkages are cleaved to produce equimolar amounts of new phenolic hydroxyl moieties. Unlike GC-MS quantification of thioacidolysis monomer yield, thioacidolysis/quantitative ^{31}P NMR spectroscopy quantifies the total amount of C-9 units (etherified and phenolic Table 1). Hence, thioacidolysis/^{31}P NMR spectroscopy offers new insights into the lignin structure without having to isolate lignin. A similar approach using DFRC and ^{31}P NMR has subsequently been reported but is applicable only to isolated lignins.[34,35]

It is well established that phenolic units in isolated and in situ lignins may be methylated by exhaustive treatment with diazomethane.[36−40] This reaction has proved to be very useful for the study of the behavior of phenolic units in lignins. For example, if in situ wood lignin is methylated prior to kraft pulping,[37] reactions attributable to phenolic units are suppressed, allowing the influence of phenolic units to be probed under these conditions. Similarly, if in situ wood lignin is methylated prior to thioacidolysis[39] or DFRC analysis,[41] then it is possible to distinguish between phenolic and etherified β-aryl ether linked C-9 units.

In this paper we report a novel analytical method for determining the degree of condensation of phenolic and etherified C-9 units in in situ lignins. The method used is based on degradation of lignin in both the original wood and methylated wood by thioacidolysis and then analysis of the resultant thioacidolysis products by quantitative ^{31}P NMR spectroscopy. Novel lignin structural information obtained from the application of this method to in situ lignins is reported and compared with results obtained from the isolated lignins.

Table 2. Chemical Composition of *Pinus radiata* Extractive Free Normal Wood and Compression Wood Samples

	amount, % of extracted wood	
component	normal wood	compression wood
lignin	27.0	37.0
arabinan	1.4	1.1
galactan	2.0	9.2
glucan	45.7	34.7
mannan	10.4	6.5
xylan	5.2	4.5

MATERIALS AND METHODS

Reagents. All reagents were of analytical grade and purchased from Sigma-Aldrich (Auckland, New Zealand). Deionized water was used throughout.

Samples and Preparation. Samples of severe compression wood were collected from a disk cut 5 m up the trunk of a *Pinus radiata* tree just above the point at which the tree had lost its terminal leader and an adjacent branch had grown to become the main stem. The disk contained 20 growth rings. Normal wood was collected from the same disk diametrically opposite to the compression wood. Such opposite wood is chemically very similar to normal wood[42] and so is referred to as normal wood here.

The wood samples were air-dried, ground in a Wiley mill to pass a 20 mesh screen, extracted with dichloromethane overnight in a Soxhlet extractor, and reground to pass a 60 mesh screen. The total lignin content was determined in duplicate as the sum of the Klason lignin and acid-soluble lignin following standard methods (Tappi Standard T222 om-88 and Tappi Standard UM 250) scaled down to analyze 0.25 g of wood. Monomeric carbohydrates in the filtrates were determined by ion chromatography[43] and results expressed as anhydrosugar units. The chemical compositions of the two samples are shown in Table 2.

Milled Wood Lignin. MWLs were prepared following the procedure described by Björkman,[9] with the exception that the extracted wood was milled for 4 days in a porcelain ball mill equipped with porcelain balls under dry N_2. The lignins from compression wood and normal wood were isolated in yields of 11 and 15% (of lignin) and had carbohydrate contents of 1.2 and 1.3%, respectively.

Methylation of Wood. Following the method of Gierer and Norén,[37] compression wood and normal wood samples (200 mg, extractives free) were swollen in dry dioxane (20 mL) for 1 week. An ethereal solution of diazomethane, prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, was added 10 times over a 1 week period. Persistence of the yellow color of diazomethane for at least 24 h was taken as an indication that methylation was complete. The methylated wood meal was separated with the aid of a centrifuge, washed successively with diethyl ether and methanol, and then dried under vacuum.

Methylation of Milled Wood Lignin. Compression wood MWL (23.3 mg) and normal wood MWL (21.2 mg) were dissolved in dioxane/methanol (2:1, 3 mL), and an excess of ethereal diazomethane (~5 mL) was added. The resulting yellow solution was stored at 4 °C. Addition of ethereal diazomethane (~1 mL) was continued daily over the next 3 days, and the mixture was stored for a further 24 days at 4 °C. The resulting solution was treated with a stream of nitrogen to remove remaining diazomethane, concentrated under reduced pressure, redissolved in dioxane, and added dropwise to diethyl ether (1 mL). The resultant precipitate was separated with the aid of a centrifuge,

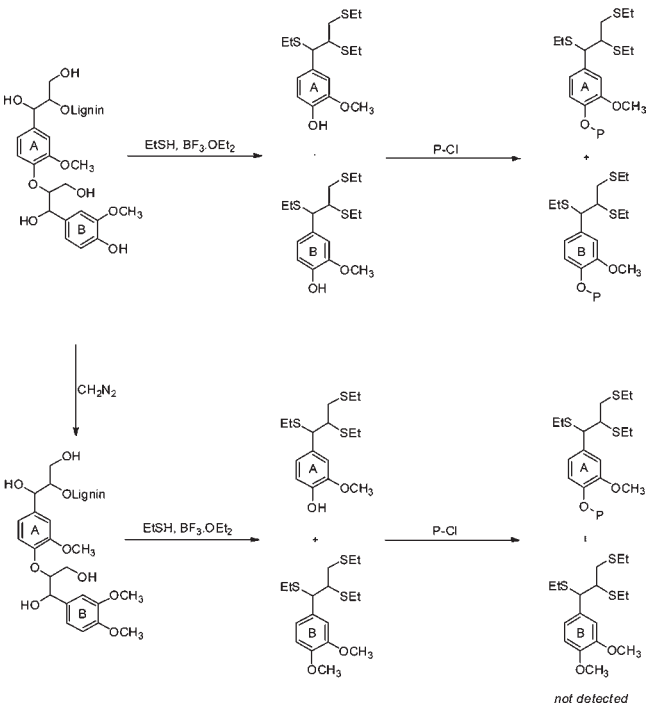


Figure 1. Schematic showing the response of etherified C-9 units (A) and phenolic C-9 units (B) in the original and methylated wood lignins to thioacidolysis/^{31}P NMR analysis. **P** = 4,4,5,5-tetramethyl-1,3,2-dioxaphospholane.

redissolved in dioxane, and concentrated under reduced pressure to yield a solid (18.8 and 16.7 mg, respectively). The absence of a bathochromic shift in the ionization difference ($\Delta\varepsilon_i$) spectra[44] of these samples was taken as evidence for complete methylation.

Releasable *β*-Aryl Ethers. Releasable β-aryl ethers were determined in duplicate following the method of Pasco and Suckling,[45] and the silylated monomeric products were determined by gas chromatography−mass spectrometry. The amounts of releasable guaiacyl and *p*-hydroxyphenol β-aryl ethers are calculated from the levels of *erythro*- plus *threo*-isomers of 1-(4-hydroxy-3-methoxyphenyl)-1,2,3-(tristhioethyl)propane and 1-(4-hydroxyphenyl)-1,2,3-(tristhioethyl)propane, respectively, in the thioacidolysis mixture.[18]

Thioacidolysis/^{31}P NMR Spectroscopy. Thioacidolysis of the duplicate wood samples (100 mg), methylated and unmethylated, was carried out using dioxane (27 mL), ethanethiol (3 mL), and boron trifluoride etherate (0.75 mL) in PTFE-lined stainless steel reactors at 110 °C for 4 h.[33] The cooled reaction mixture was transferred to a separating funnel, and a known amount of internal standard (cholesterol, ca. 10 mg) was added. The solution was adjusted to pH 5 and extracted with dichloromethane, and the resulting extract was dried with $MgSO_4$ and concentrated under reduced pressure.

A portion of the dry thioacidolysis product (ca. 30 mg) of the wood as prepared above or of the MWL was dissolved in pyridine/chloroform-*d* (0.5 mL, 1.6:1 v/v). Quantitative ^{31}P NMR and signal assignment were carried out following the method of Granata and Argyropoulos.[32] For lignins not subjected to thioacidolysis, the internal standard was added at this point. The relaxation reagent (100 μL, chromium(III) acetylacetonate, 5.0 mg/mL in the same solvent) was then added followed by 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (**1**) (100 μL, Sigma-Aldrich). The solution was made up to 1 mL with the same solvent and analyzed by quantitative ^{31}P NMR spectroscopy. Spectra were collected on a Bruker Avance NMR spectrometer operating at 161 MHz using a 5 mm probe. At least 750 scans were collected via an inverse

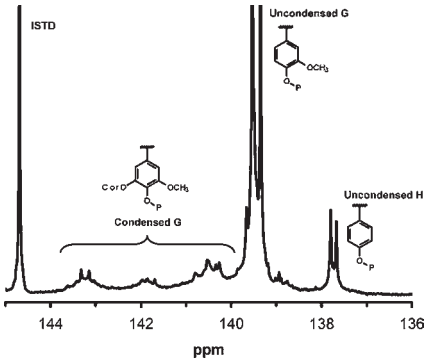


Figure 2. Quantitative ^{31}P NMR spectrum of phosphitylated thioacidolysis products from compression wood.

gated decoupled sequence using a 90° pulse, a sweep width of 25 kHz, and a delay of 20 s. Chemical shifts were referenced to the reaction product of **1** with water at 132.2 ppm.

RESULTS AND DISCUSSION

Method Principles. On thioacidolysis of the original unmethylated wood, both etherified (A) and phenolic C-9 units (B) in the original lignin are converted to phenolic C-9 units (Figure 1). The resultant phenols can then be derivatized with the phospholane **1** and analyzed by quantitative ^{31}P NMR spectroscopy. In this way it is possible to determine the levels of uncondensed C-9 units, which are protonated at C_5, and condensed C-9 units, which are linked at C_5 to another unit via an ether or carbon−carbon linkage (Figure 2). Approximately 75% of the total C-9 units in softwood lignin are analyzed by this method. Levels of uncondensed *p*-hydroxyphenol (H) C-9 units are also quantified.

Alternatively, if the wood is first pretreated with diazomethane to methylate any phenolic units, then all peaks associated with the phenolic region in the ^{31}P NMR spectra after thioacidolysis will be attributable to C-9 units which were etherified in the original wood (Figure 1). The levels of phenolic C-9 units can be calculated by the difference between the original wood and the methylated wood.

Degree of Condensation in in Situ Lignin. Table 3 shows the results of applying this method to in situ lignins from both normal and compression wood from *P. radiata*. Undoubtedly the most surprising observation here is the remarkable difference in the degree of condensation of the phenolic and etherified C-9 units. For guaiacyl C-9 units, only 1−5% of the phenolic units were condensed at C_5, whereas for the etherified units, 40−45% of the C-9 units were condensed. Nearly all of the uncondensed H C-9 units were present as phenolic units. Condensed H C-9 units cannot be detected by this method.

The fact that the phenolic C-9 units in in situ lignins are less condensed than the etherified C-9 units has been reported previously by using different methodologies, although the magnitude of the difference seen in these studies was much less than reported here. Chen et al.[46] reported the yield of nitrobenzene oxidation products, which are formed only from uncondensed C-9 units, to be 78% for the phenolic and 28% for the etherified components of in situ spruce wood lignin by combining periodate oxidation with nitrobenzene oxidation. Bose et al.,[4] using the permanganate oxidation method, showed that 25% of the phenolic C-9 units were condensed at the C_5 position versus

Table 3. Thioacidolysis/^{31}P NMR Spectroscopic Analysis of in Situ Lignins and the Corresponding Milled Wood Lignins

		C-9 units (mmol/g lignin)					
		uncond H	uncond G	cond G	total	OH/100 C-9[a]	% cond G
in situ lignins							
normal wood	total C-9 units	0.06	2.63	1.35	4.04	76	34 ± 4
	etherified units	trace	1.96	1.34	3.30	62	41 ± 4
	phenolic units[b]	0.06	0.67	0.01	0.74	14	1 ± 4
compression wood	total C-9 units	0.45	2.34	1.48	4.27	80	39 ± 4
	etherified units	trace	1.65	1.44	3.09	58	47 ± 4
	phenolic units[b]	0.45	0.69	0.04	1.18	22	5 ± 4
MWLs							
normal wood	total C-9 units	0.12	2.48	1.38	3.98		36 ± 4
	etherified units[b]	0.02	1.80	0.96	2.78		35 ± 4
	phenolic units	0.10	0.68	0.42	1.20	22	35 ± 4
compression wood	total C-9 units	0.41	2.34	1.31	4.06		36 ± 4
	etherified units[b]	0.07	1.67	0.97	2.71		37 ± 4
	phenolic units	0.34	0.67	0.34	1.35	25	34 ± 4
	SD[c]	0.010	0.062	0.069			
	LSD[c]	0.010	0.065	0.073			

[a] Assuming a C-9 unit molecular weight of 183 g/mol. [b] Calculated by difference. [c] Least significant difference (LSD = 0.05) values are for comparing differences between the means of duplicates of each sample. LSD and standard deviations (SD) were calculated by repeated analysis of six *P. radiata* normal wood samples over 2 weeks.

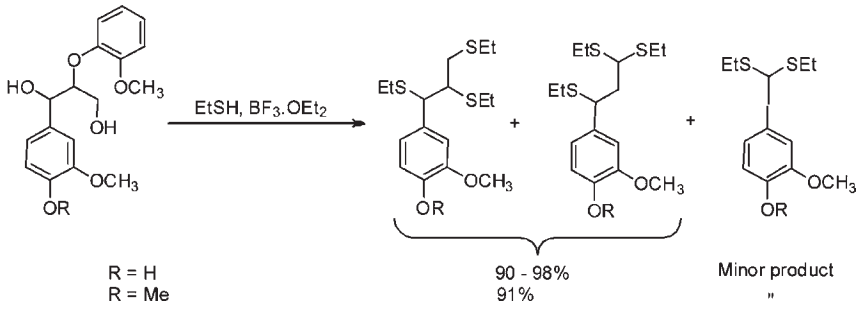


Figure 3. Thioacidolysis of β-aryl ether model compounds.

32% when the in situ lignin was pretreated with alkaline CuO to measure both etherified and phenolic units. They also suggested that the etherified fraction of the lignin was richer in 5-5 units than the phenolic fraction.

A key assumption inherent in the method described here is that during thioacidolysis, which occurs at elevated temperatures in the presence of the Lewis acid BF_3, the bonding pattern at C_5 in the C-9 units remains unchanged. This assumption is also inherent in all methods that use thioacidolysis to provide lignin structural information.[18,24,25,28] Consistent with this hypothesis, studies reported in the literature have provided no evidence for significant condensation occurring at C_5 during thioacidolysis. For example, Figure 3 shows that thioacidolysis of the β-aryl ether model compounds affords high yields of identified compounds, without any evidence of condensation at C_5 (see also ref 18). Furthermore, model compound studies have shown that although side chains are modified, interunit linkages involving the C_5 position, for example, 5-5 and β-5, are not cleaved during thioacidolysis.[25,33] Of particular relevance to the results described here, analysis of a mixture of known amounts of three model compounds representing uncondensed guaiacyl, 5-5 biphenyl, and β-5 phenylcoumaran structures by thioacidolysis/^{31}P NMR spectroscopy afforded the expected phenolic units in yields of between 94 and 97%.[33]

Complete methylation of phenolic C-9 units is crucial to the success of this method. Although it was difficult to unequivocally demonstrate complete methylation of the in situ lignin, a well-established methylation procedure was followed.[36−40] The analysis of an isolated MWL methylated by the same method using ionization difference ($\Delta\varepsilon_i$) spectroscopy[44] showed, in line with an earlier paper,[41] that no phenolic units remained in the lignin following methylation. Furthermore, the level of phenolic units in *P. radiata* normal wood, 14%, is similar to values determined for other softwoods by periodate oxidation or UV methods,[47] again consistent with complete methylation of the phenolic units.

Our results show that the only significant difference between the two wood types was that the compression wood had a higher proportion of free phenolic units (22%) than the normal wood (14%) (Table 3). The higher proportion of phenolic C-9 units in

Table 4. Phenolic and Etherified Releasable β Ethers (Micromoles per Gram of Lignin)

		normal wood		compression wood	
		original	methylated	original	methylated
H	etherified	24	trace	180	6
	phenolic		23		140
G	etherified	1098	844	843	686
	phenolic		277		270
H + G	total β-ethers	1122	1144	993	1102

compression wood has been seen previously[27,48] and can be attributed to the elevated levels of H units, which mainly exist as phenolic C-9 units in compression wood.[40] As shown in Table 3, 34 and 39% of the total C-9 units were present as condensed guaiacyl C-9 units in normal wood and compression wood, respectively. These observations confirm previous findings where 36% of the total C-9 units were condensed in both wood types.[33] It was originally thought that compression wood lignin had an increased proportion of condensed type linkages, particularly 5-5 type linkages, compared with normal wood lignin.[48,49] However, consistent with our results, recent studies reported no significant differences.[50,51]

Phenolic and Etherified Releasable β-Aryl Ethers. Analysis of the levels of phenolic and etherified releasable β-aryl ethers in the same two methylated wood samples following the method of Lapierre et al.[39] showed that, for lignins in both normal wood and compression wood, essentially all of the *p*-hydroxyphenyl β-aryl ether units were phenolic, whereas only approximately 25% of the guaiacyl β-aryl ether units were phenolic (Table 4). The results were in full agreement with published results for *Pinus pinaster* compression wood,[39] further assuring the complete methylation of samples.

Comparison with Milled Wood Lignin. It was also of interest to compare the degree of condensation of etherified and phenolic C-9 units in the in situ lignins with that of MWLs isolated from the same woods. The results in Table 3 show that MWLs have a much higher proportion of condensed phenolic guaiacyl units than the corresponding in situ wood lignins. For example, in normal wood MWL, 35% of the phenolic guaiacyl units were condensed, versus 1% in in situ wood lignin. Isolated normal wood lignins also had a considerably higher free phenolic content than the in situ lignin, 22 versus 14% (Table 3), but fewer releasable β-ether units, 1041 versus 1122 μmol/g lignin, suggesting that the elevated level of condensation of phenolic units in the isolated MWL is due to cleavage of the more condensed etherified interunit linkages during ball-milling. Cleavage of β-ether linkages during ball-milling has been reported by a number of authors.[17,52] Overall, these findings show that caution is required when using MWL to provide information on the nature of the phenolic units in in situ wood lignins.

A similar proportion of condensed C-9 units has been reported previously for black spruce MWL using DFRC/^{31}P NMR spectroscopy.[35] However, the number of C-9 units analyzed by thioacidolysis/^{31}P NMR spectroscopy is more than twice that reported by DFRC/^{31}P NMR spectroscopy (3.98 vs 1.62 mmol/g lignin). This is likely due to the incomplete cleavage of β-aryl ether bonds during DFRC.[34,53]

In conclusion, analysis of both the original wood and the methylated wood by thioacidolysis/quantitative ^{31}P NMR spectroscopy provided new information on the degree of condensation of C-9 units in in situ lignins. In particular, we have found that phenolic guaiacyl units are much less condensed at C_5 than those present as etherified units in the in situ wood, 1 versus 41% for normal wood. Phenolic guaiacyl units in MWL were more condensed than those in the in situ lignin. Overall, these findings show that caution is required when using MWL to provide information on phenolic units in in situ wood lignins.

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