



The application of ^{13}C -labeled tetramethylammonium hydroxide (^{13}C -TMAH) thermochemolysis to the study of fungal degradation of wood

T.R. Filley ^{a,*}, P.G. Hatcher ^b, W.C. Shortle ^c, R.T. Praseuth ^a

^a*Geophysical Laboratory, Carnegie Institution of Washington, 5251 Broad Branch Road NW, Washington DC, 20015, USA*

^b*Department of Chemistry, Ohio State University, Columbus OH, 43210, USA*

^c*USDA Forest Service, Northeastern Forest Experiment Station, PO Box 640, Durham, NH 03824, USA*

Received 29 July 1999; accepted 1 December 1999

(Returned to author for revision 25 October 1999)

Abstract

This paper presents the results from an assessment of the application of a new molecular analytical procedure, ^{13}C -TMAH thermochemolysis, to study the chemical modification of lignin by white-rot and brown-rot fungi. This technique differs from other molecular chemolysis procedures (e.g. TMAH thermochemolysis and CuO alkaline oxidation) as it enables one to determine the amount of hydroxylated aromatic components in degraded lignin residues through a selective lignin depolymerization and ^{13}C -labeled methylation reaction. Major differences were observed in the chemical composition and yield of lignin monomers released from a limited sample set of field and laboratory inoculation brown-rot and white-rot degraded residues when analyzed by ^{13}C -TMAH thermochemolysis. The brown-rot residues were characterized by high yields of 3,4-dihydroxy phenyl compounds, presumably due to fungal demethylation of methoxyl groups on guaiacyl lignin, and relatively low yields of aromatic acids that result from microbial side chain oxidation. The white-rot residues were characterized by low yields of demethylated lignin monomers but relatively high yields of monomers exhibiting side chain oxidation. If generally applicable, this distinct chemical functionality has important implications for the chemical reactivity and solubility of degraded wood residues and consequently the cycling of terrestrial carbon in the geosphere. The ^{13}C -TMAH thermochemolysis procedure provides a rapid and sensitive tool for tracking microbial modifications of lignin in terrestrial environments including coastal sediments, forest soils and waters receiving terrestrial organic matter. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: ^{13}C -Labeled tetramethylammonium hydroxide; Demethylation; Phenols; Basidiomycetes; Lignin; White-rot fungal degradation; Brown-rot fungal degradation

1. Introduction

Lignin, a biomacromolecule unique to vascular land plants, is distributed widely in the hydrosphere and geosphere in various states of degradation (e.g. Prahl et al., 1994; Bianchi et al., 1997; Opsahl and Benner, 1997). It is composed of methoxy-substituted phenyl propanoid

monomers linked together by a variety of ether and carbon-carbon bonds (Fengal and Wegner, 1984) and can comprise up to 33 wt% of wood cells (Higuchi, 1981). Many fungi have developed specialized chemical and enzymatic means for removing and even metabolizing the lignin barrier that encloses the cellulose and hemicellulose of woody tissue (see reviews in Higuchi, 1985; Eriksson et al., 1990; Blanchette, 1991), and are thought to be the main agents responsible for the biological cycling of lignin. One broad class of fungi, the basidiomycetes, contain wood degrading members that

* Corresponding author. Tel.: +1-202-686-2410; Fax: +1-202-686-2419.

E-mail address: tfilley@gl.ciw.edu (T.R. Filley).

can, under a given set of environmental conditions, selectively decompose either cellulose or lignin or both simultaneously (Eriksson et al., 1990). In general, white-rot basidiomycetous fungi delignify wood without significant depolymerization or oxidization of the wood cellulose. The basidiomycetous fungi characterized as brown-rot fungi are capable of oxidizing the cellulosic components of woody tissue while presumably imparting little chemical alteration to lignin. Exceptions to the exclusive nature of either lignin or cellulose degradation do occur in both white-rot and brown-rot fungi (e.g. Enoki et al., 1988).

The mechanisms of wood degradation by fungi are complicated and the chemical pathways have been investigated in decay experiments using isolated ligninase enzymes (e.g. Tien and Kirk 1983; Srebotnik et al., 1997), Fenton-type chemistry experiments (e.g. Goodell et al., 1997; Jellison et al., 1997), and fungal inoculation studies (e.g. Otjen et al., 1987; Enoki et al., 1988; Robert and Chen, 1989; Blanchette et al., 1994). Fig. 1 illustrates the principal types of chemical changes imparted to lignin during fungal alteration: side chain oxidation, demethylation and demethoxylation, and aromatic ring cleavage. The model guaiacyl lignin dimer shown in the center of Fig. 1 contains what is thought to be the dominant structural linkage in lignin, the β -O-4 aryl ether bond (Adler, 1977). The residues remaining after white-rot degradation of model compounds or woods are more frequently characterized as having undergone extensive side chain oxidation and aromatic ring cleavage (Umezawa and Higuchi, 1987; Srebotnik et al., 1997), whereas brown-rot residues are enriched in

hydroxylated phenyl (catechol derivatives) substituents as a result of methoxyl demethylation or demethoxylation (referred to hereafter as only demethylation), with only a small degree of side chain oxidation (Ander et al., 1988; Enoki et al., 1988). The chemical properties (i.e. pH, solubility, metal binding capacity, redox capacity, microbial toxicity, and chemical reactivity toward sugars, amines and other phenols) of the degraded residues will vary significantly depending upon these chemical modifications. Therefore, wood decaying fungi are important factors in the geochemical fate of lignin.

In light of the potential influence that the chemical properties of degraded wood residues could impart to soils, waters and sediments, the ability to characterize the overall functionality and degradative state of lignin detritus is important. Tetramethylammonium hydroxide (TMAH) thermochemolysis is an analytical method that can be applied to the analysis of lignin residues in geochemical samples (e.g. Challinor, 1995; Hatcher et al., 1995; Hatcher and Minard, 1996; McKinney and Hatcher, 1996). It effectively depolymerizes those β -O-4 linkages with adjacent hydroxyl groups, the principal linkage in the lignin polymer, via a base catalyzed mechanism (Filley et al., 1999). The selectivity of this procedure for the β -O-4 bond makes it possible to monitor degradative alteration of the lignin side chain. Additionally, acidic oxygen functionalities (e.g. aromatic and aliphatic alcohols and acids) are derivatized during the depolymerization reaction with a methyl group from the TMAH, making the monomers amenable to gas chromatography. Upon the TMAH thermochemolysis of lignin, a suite of derivatized lignin

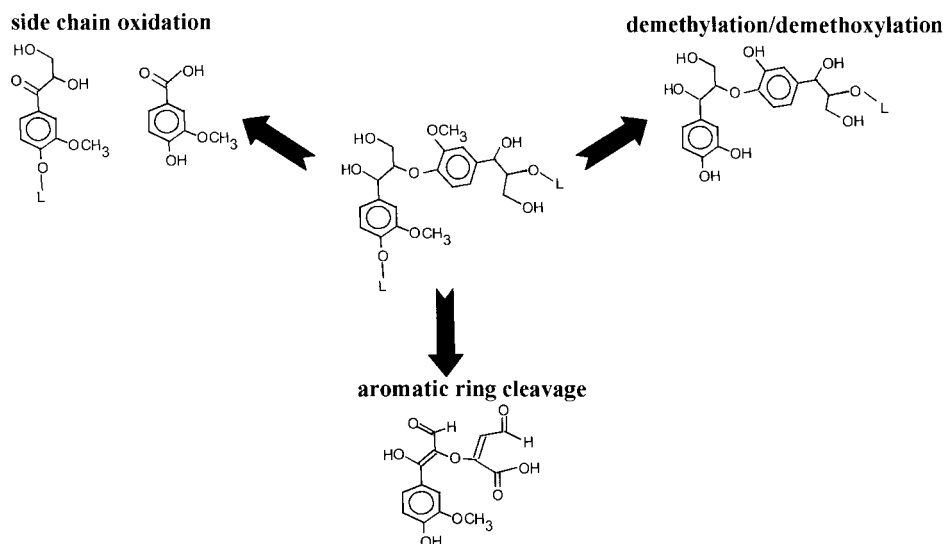


Fig. 1. Examples of the principal chemical modifications to a guaiacyl-based lignin by wood-rot fungal degradation (side chain oxidation, aromatic ring cleavage and demethylation or demethoxylation of the 3-methoxyl carbon). See text for references concerning the fungal alteration of lignin and lignin components. L = lignin biomacromolecule.

monomers are released, the composition and yield of which are characteristic of taxonomic source and relative degradation state of the lignin (e.g. Hatcher et al., 1995; Martin et al., 1995). The Appendix shows the eight most common monomers identified from fresh and degraded gymnosperm woods, as referred to in this paper.

The ability to infer the relative degradation state of the propyl side chain is achieved through the use of molecular ratios such as the acid/aldehyde (Ad/Al) or G6/G4 (see Appendix for explanation of notations) parameter. Previous investigations using TMAH thermochemolysis on lignin-containing samples have demonstrated that the (Ad/Al) ratio is indicative of the extent of degradation of the lignin (Hatcher et al., 1995; McKinney and Hatcher, 1996). A similar relationship has also been demonstrated with the use of the cupric oxide (CuO) alkaline oxidation technique for lignin analysis (e.g. Goñi et al., 1993). Additionally, the release of permethylated lignin monomers containing the intact glycerol side chain (compounds G14 and G15 in the Appendix) is a major advantage of the TMAH thermochemolysis procedure not shared by CuO oxidation or analytical pyrolysis. The presence of the full monomers may provide the closest indication of the diagenetic state (degree of degradation) and structure of lignin in the sample under investigation.

A comparison between Fig. 1 and the Appendix illustrates, however, one of the problems of the TMAH thermochemolysis procedure. In the analysis of TMAH thermochemolysis products one cannot distinguish between the original methoxyl groups on lignin and those added during the derivatization. This is a significant drawback when lignin residues are suspected to have undergone demethylation during microbial decay. A similar bias is present in the alkaline CuO oxidation procedure as all aromatic ortho hydroxyl structures are oxidatively cleaved to diacids and lost from the analytical window. Analytical pyrolysis has the capability of discerning demethylation (van der Heijden and Boon,

1994; Klap et al., 1998) but not in highly acidic lignin fragments and, as stated, not on structures that represent the full lignin monomer, such as G14 and G15.

In this paper we demonstrate the application of a new technique, ^{13}C -labeled TMAH (^{13}C -TMAH) thermochemolysis (Filley et al., 1999), to the study of the chemical alteration of lignin. ^{13}C -TMAH thermochemolysis uses a ^{13}C -labeled methyl group as the methylating agent that permits one to determine the yield of phenolic groups (i.e. monitor microbial demethylation) in the lignin residues by analysis with structural mass spectrometry. Fig. 2 illustrates this point with the example of the ^{13}C -TMAH methylation of a guaiacyl lignin unit. The molecular weight of the permethylated product exhibits an increase of one mass unit from 274 to 275 when the starting material is the 3,4-dihydroxy functionality ($R=\text{H}$) rather than the 3-methoxy, 4-hydroxy ($R=\text{CH}_3$) arrangement found in undegraded lignin. ^{13}C -TMAH thermochemolysis maintains the same depolymerization properties and derivatization efficiency as standard TMAH but adds this additional dimension of information concerning the yield of hydroxylated phenyl groups in the residue. A detailed description of the synthesis of ^{13}C -TMAH and the mechanism by which it depolymerizes lignin is provided in Filley et al. (1999).

Three gymnosperm woods which have been degraded by white-rot and brown-rot fungi are examined to test the application of the ^{13}C -TMAH thermochemolysis technique. Two of the sample sets, those degraded in controlled laboratory inoculation experiments, were previously analyzed by the alkaline CuO oxidation procedure (see Goñi et al., 1993) and a brief comparison of the two studies is made here. The third set contains field samples of balsam fir identified to have undergone white-rot and brown-rot fungal degradation. The results presented herein, although performed on a small sample set, demonstrate that this procedure is a rapid and facile method for determining molecular changes to lignin that are not readily apparent using other molecular techniques.

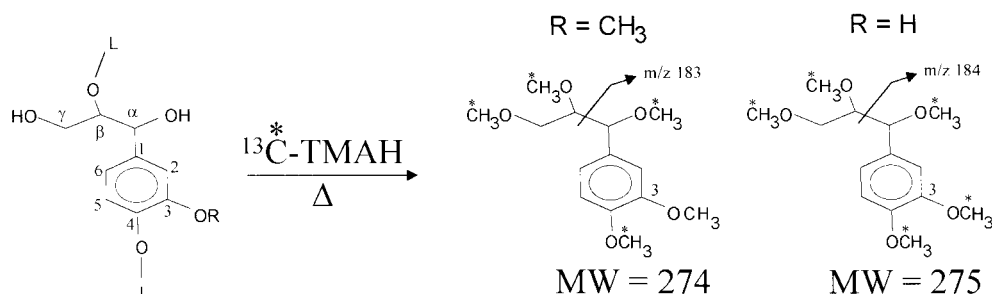


Fig. 2. ^{13}C -TMAH methylation of a guaiacyl-lignin monomer with and without a methoxyl carbon at position 3 on the aromatic ring. Subunit with a dihydroxy substitution ($R=\text{H}$) exhibits a molecular ion 1 mass unit higher, at $m/z=275$, than the subunit with a methoxyl carbon ($R=\text{CH}_3$) at position 3. L =lignin biomacromolecule. Roman numeral and Greek letters refer to positions on the lignin monomer.

2. Samples and methods

2.1. White-rot and brown-rot degraded woods (laboratory and field samples)

The chemical alterations affected by the fungal degradation of lignin in three gymnosperm woods, balsam fir (*Abies balsamea*), white spruce (*Picea glauca*), and loblolly pine (*Pinus taeda*), were assessed by the ^{13}C -TMAH thermochemolysis technique. The spruce and pine samples were provided by Dr. Miguel Goñi of the Marine Science Program, University of South Carolina and Dr. John Hedges of the School of Oceanography, University of Washington. These samples included undegraded controls of pine and spruce as well as those degraded 12 weeks by the white rot fungus, *Phlebia tremellosa*, and the brown-rot fungus, *Fomitopsis pinicola*. Details of the fungal inoculation experiments are provided in Goñi et al. (1993) as well as the detailed elemental and molecular analysis of the fungally-degraded residues by the CuO alkaline oxidation method. Field samples of balsam fir with white-rot typically caused by *Armillaria* sp. and cubical brown-rot typically caused by *Fomitopsis pinicola* were collected along with sound wood from the White Mountains region, New Hampshire. The sound and degraded samples of fir were identified and collected by Dr. Walter Shortle (USDA Forest Service).

2.2. ^{13}C -TMAH thermochemolysis

^{13}C -TMAH was synthesized as described in Filley et al. (1999). The ^{13}C -TMAH thermochemolysis of standards as well as fresh and degraded wood samples was performed as outlined below. Approximately 15 mg of dry ^{13}C -TMAH was weighed into glass ampoules with approximately 1 mg (weighed to the nearest hundredth of a milligram) of either dry wood sample (oven dried at 70°C) or standard mixture. The ampoules were evacuated on a vacuum line and flame sealed, after which they were agitated to physically mix the solids. The ampoules were then heated to 250°C in a convection oven for 30 min. Care was taken to properly anneal the sealed end of the ampoule to prevent explosion upon heating to 250°C. After removal from the oven the ampoules were allowed to cool to room temperature, scored, frozen in liquid nitrogen to condense methanol and gases, and cracked open. The products were extracted from the ampoules with methylene chloride and added to a 4 ml vial. The products were washed with a NaCl/water solution where the aqueous layer was extracted three times with 500 μl of methylene chloride. The organic fractions were combined in a vial and blown to near dryness under a gentle stream of N_2 (gas). 2,4-Dimethoxy toluene was added as a quantification standard prior to analysis by gas chromatography–mass spectrometry (GC–MS).

This procedure differs from previously published off-line TMAH thermochemolysis procedures (e.g. McKinney et al., 1995; Filley et al., 1999) in that no solvent (i.e. methanol) is added to the ampoules prior to evacuation and heating. As the present experiments are concerned with the quantification of the number of ^{13}C -labeled methyl groups in the derivatized monomers no unlabeled methanol solvent could be added to the ampoules.

Mixtures of vanillic acid (VA) and 3,4-dihydroxybenzoic acid (3,4-DHBA) in a gradient between 100% of each compound, were made to determine if the % of aromatic alcohol at the 3-position on the aromatic ring in known mixtures can be determined based upon analysis of mass spectra (see the following sections). The TMAH thermochemolysis of these two compounds produces methyl, 3,4-dimethoxybenzoate (G6, see Appendix) where the methoxyl group in the 4-position is completely ^{13}C -labeled and the ^{13}C content of the methoxyl in the 3-position is proportional to the amount of 3,4-DHBA in the VA/3,4-DHBA mixture.

2.3. Product analysis

The products from the ^{13}C -TMAH thermochemolysis were analyzed by capillary GC–MS on a HP 6890 GC interfaced to an HP 5972A quadrupole scanning mass spectrometer. A 5% phenyl methylsilicon bonded phase (J&W DB-5MS) fused silica capillary column (30 m $\times$ 0.25 mm i.d. $\times$ 0.25 μm film thickness) was used for the separation. The GC oven was temperature programmed from 50 to 140°C at a rate of 10°C/min and then from 140 to 300°C at 5°C/min. Identification of many of the compounds are based upon comparison of mass spectra of products from reaction with unlabeled TMAH to the Wiley spectral library as well as a detailed analysis of the individual spectra. The compounds identified are listed in Appendix. Individual standards were not available for some of the compounds, making a number of identifications tentative. Molar response factors for individual compounds including G4, G5, and G6 were determined from commercially available standards. The quantities of the remainder of the identified compounds were determined using an average response factor of the three standards.

Organic carbon compositions (% OC) of the fresh and degraded fir samples were determined on a Carlo Erba 1106 CHN analyzer. No pretreatment with acid was performed because of the possibility of removing soluble organics in the degraded woods. The % OC of the pine and spruce samples was taken from Goñi et al. (1993, Table 1). The % OC was used to normalize monomer yields to units of $\mu\text{g}/\text{mg}$ OC. The average mean deviation for duplicate analysis of individual monomers was 11.9%.

Structural mass spectrometry was used to determine ^{13}C -enrichment levels (% ^{13}C) of individual monomers

due to ^{13}C -methylation of aromatic hydroxyl at position 3 on the ring. The following equation (Filley et al., 1999) was used for % ^{13}C determination.

$$\% ^{13}\text{C} = \left(\frac{(M_L + 1) - (n_L/n)(M_L + 1)_{\text{calc}}}{M_L + (M_L + 1) - (n_L/n)(M_L + 1)_{\text{calc}}} \right) \times 100 \quad (1)$$

The term (M_L) is the abundance of the molecular ion of the labeled monomer or fragment ion that contains the aryl methoxyl groups while ($M_L + 1$) is the abundance of the molecular ion or appropriate fragment that is +1 mass unit higher than M_L . ($M_L + 1$)_{calc} is the calculated intensity of the molecular ion or appropriate fragment which is +1 mass unit higher than M_L . The value for ($M_L + 1$)_{calc} is determined by multiplication of (M_L) by the ratio ($M + 1$)/ M where M represents the abundance of the molecular ion or appropriate fragment ion of the same compound obtained from unlabeled TMAH thermochemolysis. Any increase in $M_L + 1$ above ($M_L + 1$)_{calc} is due to an aromatic hydroxyl group at position 3 on the ring that is subsequently methylated by ^{13}C -TMAH. The terms n_L and n represent the number of carbon atoms in a specific ^{13}C -labeled and unlabeled methylated lignin monomer, respectively, that contribute to $M_L + 1$ and $M + 1$. For example, unlabeled 3,4-dimethoxybenzophenone (G5) has 10 carbon atoms that can contribute $\sim 1.1\%$ ^{13}C to the abundance of $M + 1$ while the same compound produced from the ^{13}C -TMAH thermochemolysis of 3-methoxy, 4-hydroxybenzophenone has only 9 carbon atoms that can contribute to the $M_L + 1$. The ratio of n_L/n accounts for this difference and its effect on the abundance of $M_L + 1$. The ^{13}C content of 6 monomers from each of the wood samples was calculated in this fashion. The % demethylation of the 3-methoxyl carbon in the lignin remaining in the degraded residues was determined by subtraction of the ^{13}C content of monomers in the degraded samples by the ^{13}C content of the respective monomers in the control (fresh) samples. Positive deviations from the ^{13}C content in the control are attributed to demethylation by the fungi. The average analytical precision in these calculations was 3.2%.

The mass spectrum of the product (G6) obtained from the ^{13}C -TMAH thermochemolysis of mixtures of VA and 3,4-DHBA was also analyzed with Eq. 1. The calculated ^{13}C content for G6 produced in these experiments was used to generate a calibration curve to assess the accuracy and precision of this technique. The purity of the standards purchased from Aldrich (VA at 97% pure) limited the determination of accuracy as it is suspected that a small portion of the VA contamination contained dihydroxy functionalities (see Results and discussion section).

2.4. ^{13}C -TMAH thermochemolysis of calibration mixtures

Analysis of mixtures of VA and 3,4-DHBA were investigated to determine the accuracy and precision of the ^{13}C -TMAH thermochemolysis procedure for determining aromatic hydroxyl content as it would apply to the microbial demethylation of the 3-methoxyl carbon of guaiacyl lignin. TMAH methylation of a mixture of VA and 3,4-DHBA produces methyl, 3,4-dimethoxy benzoate or G6 (see Appendix). The ^{13}C -TMAH thermochemolysis of a mixture of VA and 3,4-DHBA produces G6 with a ^{13}C -labeled methyl group in the ester and in the methoxyl group at position 4 on the ring. The methyl of the original methoxyl group on VA (at position 3 on the aromatic ring) undergoes minimal exchange with a ^{13}C -labeled methyl (Filley et al., 1999) while the hydroxyl group at position 3 on the ring of 3,4-DHBA will be completely methylated by a ^{13}C -labeled methyl group. Analysis of the mass spectrum of the ^{13}C -labeled G6 using Eq. (1) determines the amount of 3,4-DHBA in a given VA:3,4-DHBA mixture as the % ^{13}C of G6. G6 produced from the ^{13}C -TMAH thermochemolysis of only VA is analyzed to determine the baseline exchange between original aryl methoxyl groups and the ^{13}C -TMAH.

Fig. 3 shows the mass spectra of G6 that results from the unlabeled TMAH thermochemolysis of VA (upper spectrum), the ^{13}C -TMAH thermochemolysis of VA (middle spectrum), and ^{13}C -TMAH thermochemolysis of a mixture of 38.0 mol% 3,4-DHBA in VA (lower spectrum). The molecular ion of G6 produced by the ^{13}C -TMAH thermochemolysis of VA increases 2 mass units from 196 to 198. Quantification of the amount of ^{13}C -labeled methyl added to the 3-methoxyl position indicates that there is 3.1% ($\pm 0.2\%$ ^{13}C) exchange between the original 3-methoxyl methyl group and the ^{13}C -labeled methyl groups on the ^{13}C -TMAH. The mechanism of this exchange is discussed in Filley et al. (1999). Because the VA is only 97% pure as obtained from the supplier it is not known if some of this increase in ^{13}C content is due to the presence of dihydroxy impurities. This low level of exchange ($\sim 3.0\%$) is consistent with our previous report (Filley et al., 1999) showing an increase of 1.7% ^{13}C after the ^{13}C -TMAH thermochemolysis of eugenol (Aldrich Chemicals, 98% pure). Analysis of the lower spectrum in Fig. 3 with Eq. (1) demonstrates that the ^{13}C -TMAH thermochemolysis procedure accurately determines the % DHBA to be 39.0 ± 0.3 . The slightly higher calculated ^{13}C content with respect to the known value at 38.0 mol% may be due to exchange at the methoxyl carbon or to impurities.

Fig. 4 shows the plot of a calibration curve generated for the ^{13}C -TMAH thermochemolysis of the VA/3,4-DHBA mixtures ranging from 0 to 65 mol% 3,4-DHBA. All determined % ^{13}C have a standard deviation of less than 0.3% ^{13}C for triplicate analyses. All of

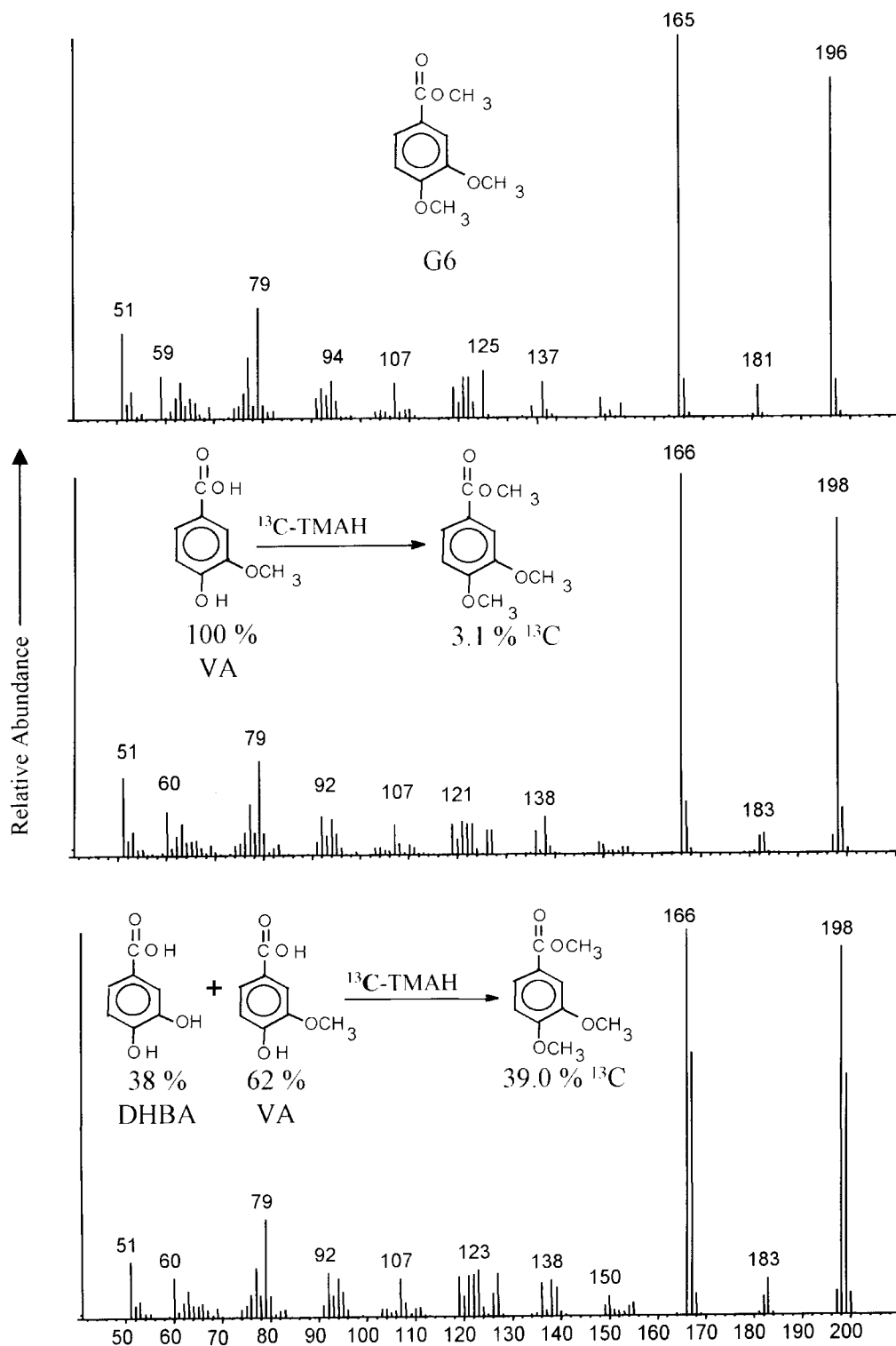


Fig. 3. Mass spectra showing G6 from the unlabeled TMAH thermochemolysis of vanillic acid (VA) (upper spectrum), G6 from the ^{13}C -TMAH thermochemolysis of VA (middle spectrum) and G6 from the ^{13}C -TMAH thermochemolysis of a mixture of VA and 3,4-dihydroxybenzoic acid (3,4-DHBA) 62:38 (lower spectrum).

the calculated values show excellent agreement with the known quantity of 3,4-DHBA. At higher yield of 3,4-DHBA, the amount of baseline exchange or contamination associated with VA is diluted to an insignificant amount. The high accuracy and precision associated with the determination of the hydroxyl content on the aromatic ring ensures that the ^{13}C -TMAH thermochemolysis procedure is capable of accurately representing the extent of fungal demethylation associated with wood decomposition investigated in this study.

3. Results and discussion

3.1. Composition of lignin monomers from undegraded and degraded woods

Table 1 shows the OC-normalized yields for selected lignin monomers produced in the ^{13}C -TMAH thermochemolysis of the undegraded and degraded woods investigated in this study. The compound notations correspond to the Appendix. Deviations from the mean

of duplicate analysis are presented in the table. Expanded chromatograms for the TMAH reaction products from the loblolly pine series (fresh, white-rot and brown-rot) are shown in Fig. 5. Higher molecular weight compounds of lignin origin, presumably dimers, elute at longer retention times but their structures were not determined and they are not the subject of this work. This study's focus is to investigate the production of derivatized and ^{13}C -labeled lignin monomers and their potential use as indicators of botanical source and the degree and type of microbial alteration of the parent lignin biomacromolecule.

3.1.1. Undegraded (control) woods

^{13}C -TMAH thermochemolysis of the three undegraded samples generates primarily guaiacyl-based lignin monomers indicative of gymnosperm woods. The product yield and distribution of methylated lignin monomers are similar to that previously reported in the TMAH thermochemolysis analysis of fresh gymnosperms (e.g. Challinor, 1995; Hatcher and Minard, 1995; Martin et al., 1995). The dominant compound in each

Table 1

Yields of the major compounds released by ^{13}C -TMAH thermochemolysis of undegraded controls, white-rot degraded and brown-rot degraded loblolly pine, white spruce and balsam fir samples. Yields are reported in $\mu\text{g}/\text{mg}$ organic carbon. The delta notation refers to the difference in yields between the control sample and the degraded sample

Compound	Control	Avg. dev.	B-rot	Avg. dev.	$\Delta\text{B-rot}$	% $\Delta\text{B-rot}$	W-rot	Avg. dev.	$\Delta\text{W-rot}$	% $\Delta\text{W-rot}$
Fir										
G4	12.4	0.3	9.6	0.5	-2.8	-22.3	2.1	0.1	-10.3	-82.9
G5	6.2	0.2	4.3	0.4	-1.9	-30.8	2.8	0.1	-3.4	-54.8
G6	5.0	0.3	5.0	0.9	0.0	0.2	15.4	1.6	10.4	208.0
G7+G24	7.5	0.1	5.4	0.6	-2.1	-28.6	3.5	0.3	-4.0	-53.3
G8	4.2	0.0	3.1	0.3	-1.1	-26.7	1.9	0.2	-2.3	-54.8
G14	3.7	0.3	3.1	0.6	-0.6	-15.6	1.8	0.2	-1.9	-51.4
G15	5.4	0.1	3.5	0.6	-1.9	-35.2	2.2	0.4	-3.2	-59.3
Sum	44.5	0.6	34.0	1.5	-10.4	-23.4	29.7	1.7	-14.7	-33.0
Spruce										
G4	8.48	0.85	6.02	0.98	-2.47	-29.1	3.25	0.21	-5.23	-61.7
G5	4.00	0.68	5.74	0.85	1.74	43.4	3.46	0.10	-0.55	-13.7
G6	2.02	0.09	6.94	1.03	4.92	244.3	16.63	1.67	14.62	725.3
G7+G24	6.25	0.50	8.37	1.23	2.12	33.9	4.04	0.18	-2.21	-35.3
G8	3.48	0.23	3.88	0.53	0.40	11.4	1.87	0.22	-1.61	-46.3
G14	3.43	0.60	1.62	0.38	-1.81	-52.8	0.91	0.16	-2.52	-73.4
G15	4.66	1.02	3.11	0.69	-1.55	-33.3	1.51	0.09	-3.15	-67.6
Sum	32.32	1.70	35.67	2.27	3.34	10.3	31.67	1.72	-0.66	-2.0
Pine										
G4	11.25	1.90	4.57	1.00	-6.68	-59.4	5.01	1.20	-6.24	-55.4
G5	4.93	0.62	4.54	0.57	-0.39	-7.8	4.78	1.09	-0.15	-3.0
G6	4.00	0.41	5.90	0.73	1.89	47.3	13.34	1.27	9.33	233.2
G7+G24	7.64	0.46	7.94	0.97	0.31	4.0	5.62	1.28	-2.02	-26.4
G8	3.56	0.01	2.72	0.58	-0.84	-23.7	2.61	0.65	-0.95	-26.6
G14	2.04	0.02	1.90	0.28	-0.15	-7.1	1.26	0.30	-0.78	-38.4
G15	2.58	0.01	2.70	0.49	0.12	4.5	1.68	0.36	-0.91	-35.1
Sum	36.00	2.09	30.26	1.86	-5.74	-15.9	34.29	2.55	-1.70	-4.7

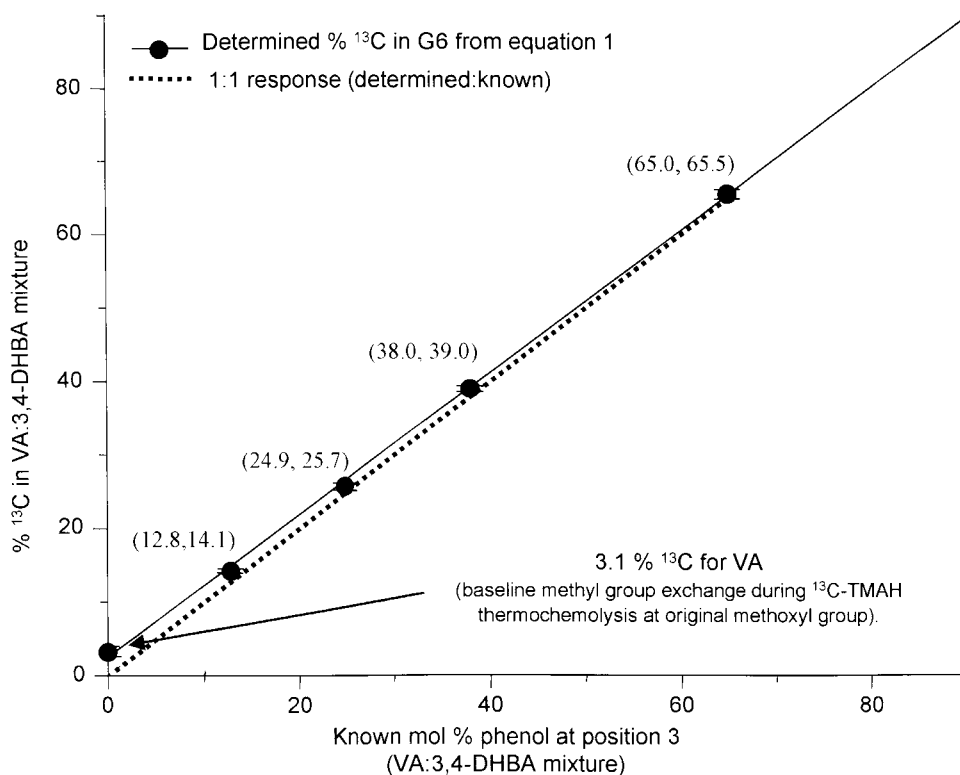


Fig. 4. Plot comparing the % of 3,4-DHBA determined by the analysis of the mass spectra of the ^{13}C -TMAH products (G6) in a VA/3,4-DHBA mixture and the known molar ratio of VA and 3,4-DHBA in that mixture. Eq. (1) from the text was used to calculate the amounts based upon integration of the mass fragment of 166 and 167 from the ^{13}C -labeled G6.

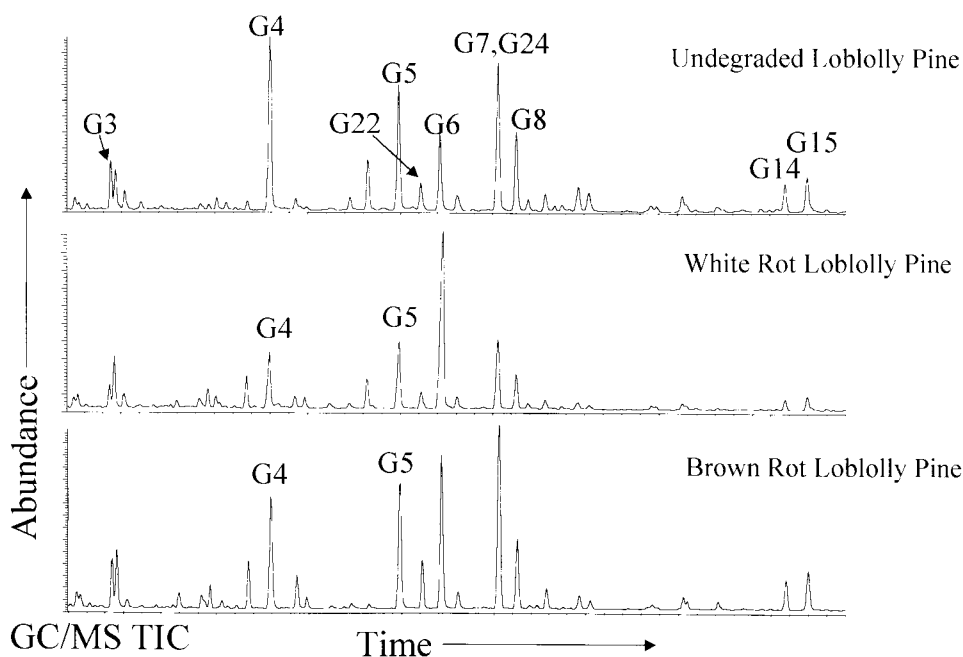


Fig. 5. Partial total ion count for GC-MS analysis of the products from the ^{13}C -TMAH thermochemolysis of fresh (upper trace), white-rot fungal degraded (middle trace) and brown-rot fungal degraded (lower trace) loblolly pine sample. Identifications associated with peaks refer to structures in the Appendix.

fresh sample is G4, 3,4-dimethoxy benzaldehyde, which is a characteristic feature of the TMAH thermochemolysis of undegraded guaiacyl lignin. The compound G4 is proposed to result from the TMAH thermochemolysis of those β -O-4 linkages that contain adjacent hydroxyl groups on the propyl side chain (Filley et al., 1999), the bonding illustrated in the undegraded lignin dimer in Fig. 1. As such, the relative yield of G4 may be indicative of the degree of alteration of those β -O-4 linkages (Filley et al., 1999). Also present in significant yield in the thermochemolysis products are the diastereomeric pairs of the enantiomers in peaks G14 and G15. These compounds contain the full methylated glycerol side chain and are near to the structure of the complete methylated lignin monomer. These compounds are also thought to be indicative of undegraded β -O-4 linkages with adjacent hydroxyl groups in the sample (Hatcher and Minard, 1995; Filley et al., 1999). Their presence in the TMAH thermochemolysis products of natural lignin-containing samples indicates that some portion of the sample contains lignin fragments that have not undergone complete side chain oxidation.

Even though the three control woods were all undegraded the sum total yield of lignin monomers differed among the samples; measured at 32.3, 36.0 and 44.5 $\mu\text{g}/\text{mg}$ OC for white spruce, loblolly pine and balsam fir, respectively. Significant differences in the relative yields of the individual compounds in the undegraded woods are also evident, most notably differences in yield of G4, G6 and G14 and G15. These features may reflect differences in the nature of the linkages of the lignin macromolecule among the samples or in the association of lignin within the lignocellulosic network of the wood cells.

3.1.2. Degraded woods

Table 1 and Fig. 5 indicate that upon white-rot and brown-rot decay the principal changes (as evidenced by TMAH thermochemolysis) occur in the relative yields of lignin monomers while the overall distribution of compounds remains the same. The yield of lignin monomers from the three sample sets (two sets of laboratory inoculations and one set of field samples) of degraded woods were compared to their respective undegraded (control) woods not subjected to fungal inoculation or fungal decay in the field. Fig. 6a and b plots the change in lignin monomer yield, with respect to undegraded woods, after white-rot and brown-rot fungal degradation, respectively. White-rot fungal decay of these samples results in changes primarily to the yield of G4 and G6. The carbon-normalized yield of G6 increases significantly in the white-rot residues with respect to the controls (ranging from approximately 2.1 to 7.3 times) with degraded spruce exhibiting the greatest increase. All other quantified compounds decrease in yield relative to the controls.

G4 exhibits the largest % decrease (87% for the white-rot balsam fir) in yield for any individual compound. Here the term % decrease refers to decrease in relative yield ($\mu\text{g}/\text{mg}$ OC of remaining sample) that is not corrected for mass loss. In this study as well as other published TMAH thermochemolysis reports (Hatcher et al., 1995), G4 is always the dominant compound formed upon TMAH thermochemolysis of fresh woods and is the major monomer formed upon thermochemolysis of a β -O-4 linked lignin dimer (Hatcher and Minard, 1995).

The yields of lignin monomers from the brown-rot residues differ significantly from the white-rot woods particularly in the magnitude of the change in yields and in a lack of systematic direction of change in yield. The carbon-normalized yields of G6 show only modest gains. The largest increase in G6, at 2.4 times the control yield, is for the white spruce sample while G6 in the

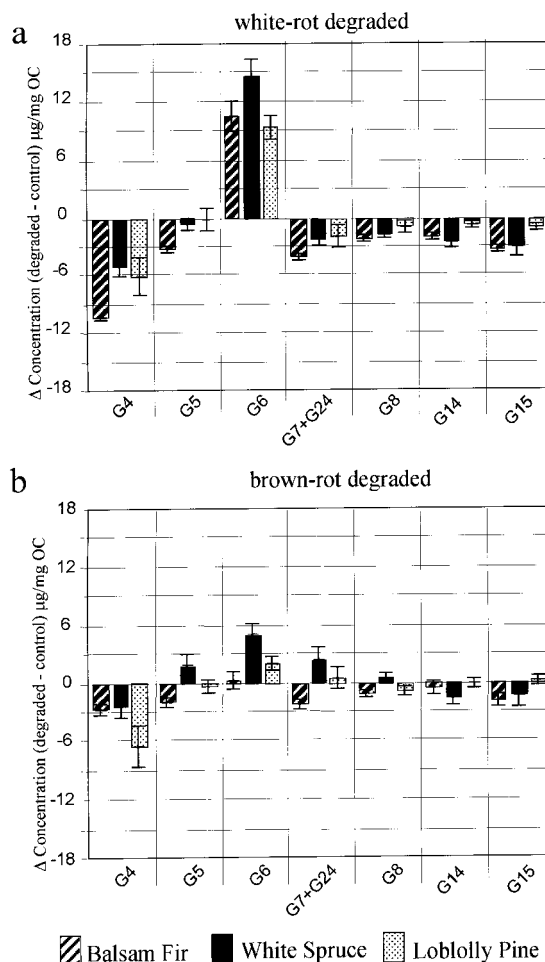


Fig. 6. Graph of the change in concentration (degraded-control), in $\mu\text{g}/\text{mg}$ OC, of the ^{13}C -TMAH thermochemolysis lignin monomer products after degradation of fir, spruce and pine woods by white-rot fungi (a) and brown-rot fungi (b). See Table 1 for concentrations and Appendix for monomer structures.

fir sample was essentially invariant. The yield of G4 decreased in all three brown-rot residues. The decrease in G4 yield ranged from 22.3 to 59.4% of the control sample, a smaller overall decrease than that observed in the three white-rot residues.

G6 obtained after TMAH thermochemolysis of fresh and degraded wood could have many potential sources. It can be derived from the methylation of free vanillic acid, cleavage of an ether-bound vanillic acid residue, cleavage of a lignin subunit in an intermediate oxidation state, or, as shown previously (Hatcher and Minard 1995; Filley et al., 1999), as a reaction by-product from fresh lignin. It is also possible that G6 may be derived from flavanoids such as epicatechin or tannins, however this has not been tested. The high yield of G6 obtained from the white-rot wood is most likely derived from bound and free vanillic acid subunits as white-rot degradation is known to increase aromatic carboxyl content during the ligninase mediated oxidation of lignin and model compounds (e.g. Robert and Chen, 1989; Srebotnik et al., 1997). Molecular analyses using alkaline CuO oxidation (e.g. see Hedges et al., 1988), of woods degraded by white-rot fungi indicate that phenolic acids, such as vanillic acid, are in higher yield relative to the corresponding aldehydes (e.g. vanillin). The dramatic increase in the yield of the permethylated vanillic acid (G6) upon ^{13}C -TMAH thermochemolysis of white-rot residues is consistent with these previous findings. A likely source for the production of G4 during the TMAH thermochemolysis of fresh woods is thought to be a retro-aldol reaction of β -hydroxy ketones which are formed in the base catalyzed decomposition of the β -O-4 linkage with adjacent hydroxyl groups in lignin (Filley et al., 1999). As such, the pronounced decrease in yield of G4 during white-rot fungal degradation is indicative of the loss of lignin structure containing the β -O-4 aryl ether-hydroxy subunit.

The compounds G14 and G15 exhibit a large decrease in yield (ranging from 35 to 73%) in the white-rot degraded residues. The pine sample shows the smallest decrease in G14 and G15 yields at 38.4 and 35.1%, respectively, whereas the spruce sample shows the greatest decrease in G14 and G15 yields at 73.4 and 67.6%, respectively. The brown-rot residues of balsam fir and white spruce show more modest changes in G14 and G15 yields, exhibiting declines from 15.6 and 35.2% to 52.8 and 33.3%, respectively. The brown-rot pine exhibits a decrease in G14 of only 7% and an increase in G15 yield by 4.5%, but these changes are near the analytical variability for the analysis.

Changes in the yields of G14 and G15 are important to track because they are the monomers that are the closest in structure to the average lignin superstructure. The complete loss of these compounds from the product distribution would indicate loss of the dominant linkage in lignin, the β -O-4 bond. Unlike other oxidative or

pyrolytic techniques applied to lignin analysis TMAH thermochemolysis primarily releases lignin monomers that are bound by those β -O-4 bond with adjacent hydroxyl groups. The procedure cannot, to any appreciable degree, break aliphatic-aryl bonds (McKinney et al., 1995). It is, therefore, highly sensitive to any changes that occur on the propyl side chain due to oxidation or hydrolysis.

In general, both brown-rot and white-rot woods exhibit increases in the yield of G6 associated with systematic decreases in the yield of G4, G14, and G15. The cross plot of the yield of G6 vs. G4 and G14 + G15 in Fig. 7 demonstrates this relationship. The undegraded woods are characterized by relatively high G4, G14 and G15 yields with very low G6 yields. White-rot woods all plot at high G6 yields and low G4, G14 + G15 values indicating extensive fungal alteration of the β -O-4 bonds and the production of bound or free vanillic acid structures (i.e. oxidation of the α -carbon on the lignin side chain to a carboxyl group as shown in Fig. 1). Brown-rot woods plot at intermediate values exhibiting decreases in G4, G14 and G15 with relatively modest increases in G6. These changes in lignin monomer yield, due to the brown-rot, suggest side chain alteration at a level sufficient to decrease the G4 yields, such as hydrolysis or oxidation of side chain hydroxyl groups, but without the full oxidative cleavage of the α - β carbon bond which would produce bound and free vanillic acid structures.

No clear trend is evident in the summed yield of lignin monomers in each sample set (see Table 1). For the two laboratory degradation studies, the total monomer yield decreases slightly but with differences near analytical uncertainty. Variations in overall monomer yields are primarily governed by the changes in G4 and G6 yields. The balsam fir field sample exhibits significantly greater decrease in lignin monomer yield with white-rot residues exhibiting a 33% decrease and brown-rot residues exhibiting a 10.4% decrease. The greater decrease in lignin monomer yields for the field samples as compared to the laboratory inoculations may be due to the leaching of degraded lignin fragments by rain water, degradation over a longer period of time or degradation by a more aggressive fungi or microbial community.

3.2. The demethylation of lignin by white-rot and brown-rot fungi

In this section the % demethylation of the 3-methoxyl carbon in lignin monomers from the ^{13}C -TMAH thermochemolysis of the undegraded white-rot and brown-rot woods is investigated by analysis of the mass spectra using Eq. (1). Only those compounds that do not suffer from co-elution of important mass fragments are shown. Table 2 presents this data. Any increase in the % ^{13}C with respect to the control is presumed to be the

result of fungal demethylation and so is referred to as the % demethylation of the 3-methoxyl carbon in the following text.

3.2.1. % ^{13}C of lignin monomers in undegraded and degraded woods

Fig. 8(a)–(c) shows the % ^{13}C values for each of the lignin monomers from the undegraded woods, white-rot and brown-rot residues, respectively. For the undegraded samples, all of the lignin monomers with the exception of G6, exhibit a % ^{13}C at, or slightly higher, than the values anticipated based upon the methyl exchange at the 3-methoxy position demonstrated for vanillic acid and eugenol, 3.1 and 1.7%, respectively. Excluding G6, the average ^{13}C content for the five lignin monomers is measured at 4.5, 4.6 and 4.7% for the undegraded fir, spruce and pine samples (see Table 2). The % ^{13}C values for G6 in each of the woods show anomalous ^{13}C -enrichments with respect to the other lignin monomers, measured at 7.4, 13.5 and 11.4% ^{13}C for the undegraded fir, spruce and pine samples, respectively. This relatively high ^{13}C content upon ^{13}C -TMAH of

fresh woods indicates that there are structures in the woods that contain the 3,4-dihydroxy aromatic unit which may or may not be part of the lignin structure. Some aromatic wood extractives (e.g. flavanoids and lignans) contain 3,4-dihydroxy units (e.g. reviews in Higuchi, 1985) that may form completely ^{13}C -methylated G6 upon ^{13}C -TMAH thermochemolysis. The reactivity of these extractives toward TMAH thermochemolysis has not been tested in our laboratories.

Fig. 8(b) and (c) indicates that brown-rot and white-rot fungal degradation impart very different chemical signatures to the residual lignin. The white-rot woods [Fig. 8(b)] exhibit minor increases in % ^{13}C , on average less than 1.0% ^{13}C , with respect to the undegraded samples. For the fir and spruce white-rot samples G6 shows a decreased ^{13}C content, with respect to the controls, indicating that the dihydroxy precursors are removed, possibly metabolized, in the white-rot fungal decay process. All of the lignin monomers, including G6, from the brown-rot woods show dramatic increases in their % ^{13}C indicating that a demethylation of the 3-methoxyl carbon has occurred during decay, generating

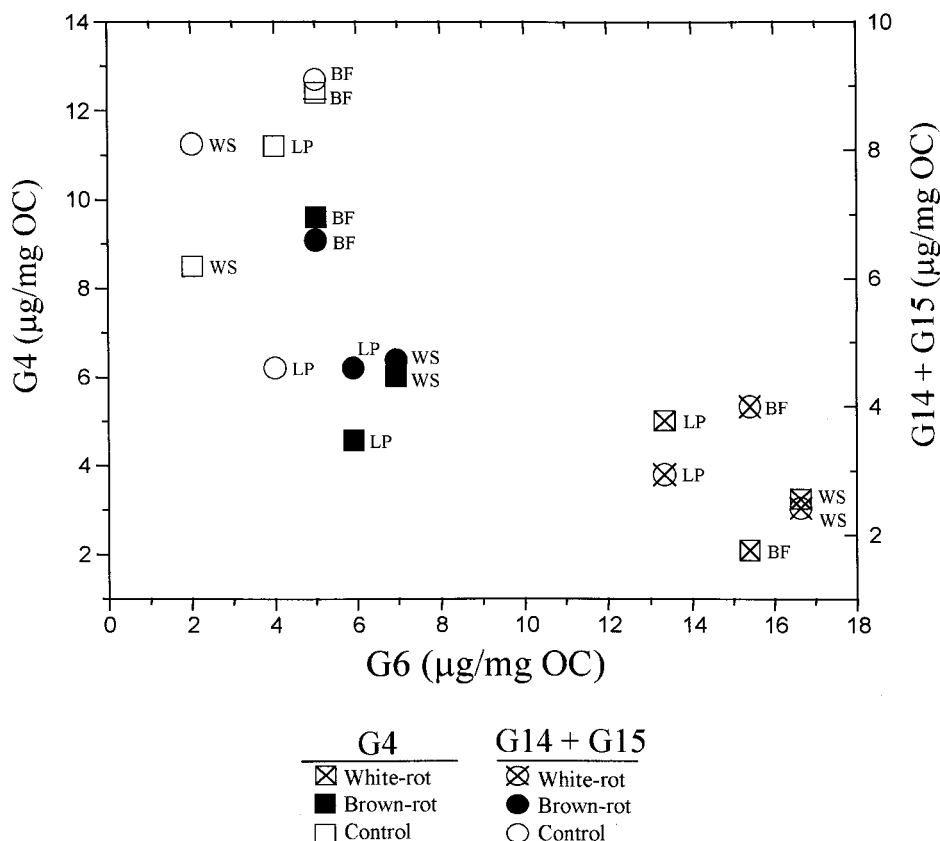


Fig. 7. Double Y cross plot of the concentrations (μg/mg OC) of G4 and the sum of G14 + G15 vs. G6 in undegraded, white-rot and brown-rot fir, spruce and pine samples. Concentration values are given in Table 1 and monomer structures are shown in Appendix. (BF = balsam fir, WS = white spruce, LP = loblolly pine).

3,4-dihydroxy phenyl structures in the lignin residue. No systematic trend in ^{13}C depletion or enrichment can be found with respect to the length of the carbon side chain.

3.2.2. % Demethylation in degraded residues

Fig. 9 shows the % demethylation of the 3-methoxyl carbon after white-rot [Fig. 9(a)] and brown-rot [Fig. 9(b)] fungal degradation of the woods for each lignin monomer. All of the monomers generated after ^{13}C -TMAH thermochemolysis of the white-rot residue exhibit only minor increases in demethylation relative to the undegraded control sample. The highest degree of white-rot fungal demethylation, at 2.7%, is found for G5 released from the fir. The average % demethylation of the lignin monomers is 0.49, 0.91 and 0.82% for fir, spruce and pine residues.

Fungal degradation studies of model lignin compounds and woods, some synthesized with ^{14}C -labeled methoxyl groups, illustrate that white-rot fungi can indeed demethylate lignin (Ander et al., 1985; Ander and Eriksson, 1985) but the extent of reaction is highly dependent on experimental conditions and the species of fungi used. Such studies monitor the evolution of

methanol or formaldehyde from the demethylation of the compounds under investigation. Robert and Chen (1989) estimated a 5–8% demethylation of spruce lignin by the white-rot fungus, *Phanerochaete chrysosporium* using ^{13}C -NMR analysis. A general conclusion of most of these studies, however, is that white-rot fungi do not enrich the degraded residue in hydroxylated phenyl compounds due to demethylation (Eriksson et al, 1990). Additionally, it is not clear whether demethylation occurs while the lignin superstructure is intact or after side chain oxidation and depolymerization has occurred.

With regard to the present study any such difference in the order of reaction (i.e. side chain oxidation then demethylation) should be manifest in a difference between the relative % demethylation values of the oxidized monomers and G14 and G15. For example, if vanillic acid structures are generated prior to demethylation of the methoxyl carbon we would expect to find G6 with very high % demethylation values and G4, G14 and G15 with virtually no measurable demethylation. No dramatic difference of this kind was observed in the three samples investigated in this study. The small degree of measured overall demethylation by the white-rot

Table 2

^{13}C content determinations based upon analysis of mass spectra of compounds released by ^{13}C -TMAH thermochemolysis using Eq. (1). The numbers in parentheses are the masses used in Eq. (1) to calculate ^{13}C content. % Demethylation values, noted by the Δ symbol, are determined by subtraction of the control values from the degraded samples. The average (arithmetic mean) ^{13}C content of each sample, based upon the six compounds quantified, is also presented along with average % demethylation (Δ) values of each sample which are based upon the difference of the average ^{13}C contents

Compound	Control	Avg. dev.	B-rot	Avg. dev.	Δ B-rot	W-rot	Avg. dev.	Δ W-rot
Fir (% ^{13}C)								
G4 (166/167/168)	3.00	0.14	10.09	0.05	7.09	4.09	0.50	1.09
G5 (181/182)	4.26	0.05	12.17	0.11	7.91	5.85	0.04	1.59
G6 (166/167)	7.44	0.15	16.20	0.58	8.76	7.08	0.52	-0.36
G8 (194/195)	5.30	0.43	12.06	0.16	6.76	6.32	1.30	1.02
G14 (183/184)	5.25	0.30	15.93	0.66	10.68	5.16	0.02	-0.09
G15 (183/184)	4.73	0.15	14.31	0.53	9.58	4.44	0.17	-0.29
Average	5.00	0.58	13.46	1.05	8.46	5.49	1.50	0.49
Spruce (% ^{13}C)								
G4 (166/167/168)	3.38	0.21	10.29	0.46	6.91	5.40	0.21	2.02
G5 (181/182)	3.98	0.09	10.76	0.03	6.78	6.66	0.10	2.68
G6 (166/167)	13.51	0.18	19.40	0.20	5.89	10.60	1.67	-2.91
G8 (194/195)	4.99	0.23	10.57	0.35	5.58	6.67	0.22	1.68
G14 (183/184)	6.05	0.50	15.45	0.14	9.40	6.62	0.16	0.57
G15 (183/184)	4.81	0.10	12.59	0.04	7.78	6.23	0.09	1.42
Average	6.12	0.63	13.18	0.63	7.06	7.03	1.71	0.91
Pine (% ^{13}C)								
G4 (166/167/168)	3.59	0.13	9.29	0.27	5.70	5.12	0.14	1.53
G5 (181/182)	4.22	0.10	9.83	0.14	5.61	5.60	0.02	1.38
G6 (166/167)	11.47	0.33	19.09	0.43	7.62	12.80	0.60	1.33
G8 (194/195)	4.94	0.15	9.07	0.08	4.13	5.70	0.04	0.76
G14 (183/184)	5.72	0.01	11.31	0.40	5.59	6.50	0.05	0.78
G15 (183/184)	5.11	0.03	10.21	0.24	5.10	5.80	0.19	0.69
Average	5.84	0.40	9.92	0.71	4.68	6.07	0.65	0.82

fungi may in fact be due to rapid metabolism of the 3,4-dihydroxy phenyl demethylation products. The results presented herein agree well with the general relationships ascribed to white-rot degradation in that there is extensive side chain oxidation and little or no demethylation evident in the residue.

The lignin monomers released from the brown-rot residues all exhibit extensive demethylation [Fig. 9(b)], in sharp contrast to what was observed in the white-rot residues. The average demethylation in each sample set is 8.5, 7.1 and 4.7% for the fir, spruce and pine residues, respectively. The field degraded fir sample exhibits the highest % demethylation for all individual compounds among the three sample sets. These results are consistent with degradation studies using model compounds that indicate methoxyl demethylation is a principal initial reaction in the brown-rot degradation of wood (e.g. Ander et al., 1988).

For the fir and spruce brown-rot samples the % demethylation for G14 and G15 is higher than the %

demethylation values of the oxidized monomers. This implies that demethylation by these brown-rot fungi occurs while the lignin superstructure is still intact. Therefore lignin residues from brown-rot woods should be enriched in high molecular weight polyphenolic structures. The build up of these 3,4-dihydroxy phenyl units in the lignin residue from brown-rot degraded woods is consistent with previous degradation studies that determined bulk functional group speciation in degraded residues (e.g. Kirk, 1984).

3.3. Chemical indicators of white-rot and brown-rot fungal degradation

Interpretation of the yield data for the lignin monomers suggests that the relative yields of G4 and G6 are dependent upon the mechanism by which different fungi degrade the lignin side chain. The ratio of G6/G4 has been employed as an indicator of the relative state of degradation of sedimentary lignin where an increased

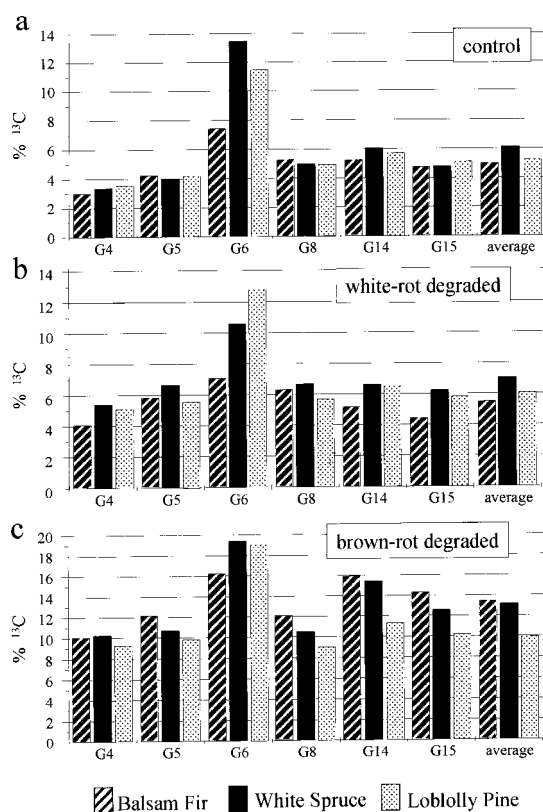


Fig. 8. Graph of the ^{13}C content of the 3-methoxy group of individual guaiacyl lignin monomers as well as the average (arithmetic mean) ^{13}C content for each sample which is based upon the six monomers released after ^{13}C -TMAH thermochemolysis of undegraded controls (a), white-rot (b) and brown-rot (c) fir, spruce and pine.

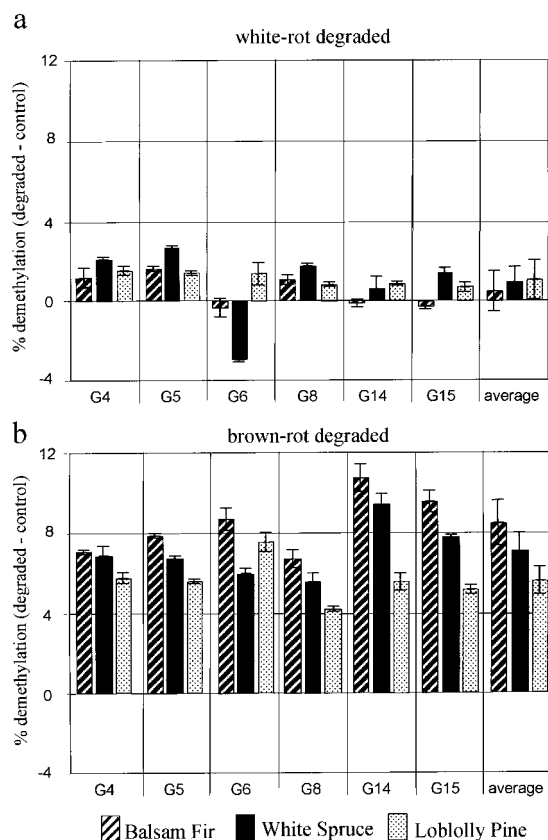


Fig. 9. Graph of the % demethylation ($\% ^{13}\text{C}$ degraded – $\% ^{13}\text{C}$ control) of the 3-methoxyl group of individual guaiacyl lignin monomers as well as the average % demethylation of each sample which is based upon the difference of the average ^{13}C content of the undegraded controls, white-rot (a) and brown-rot (b) fir, spruce and pine.

ratio suggests a higher degree of degradation (Hatcher et al., 1995). Fig. 10 plots the G6/G4 ratio for each of the woods investigated in this study. There is a clear differentiation, based upon the magnitude in the G6/G4 ratio, between the white-rot (high G6/G4), brown-rot (intermediate G6/G4), and fresh woods (low G6/G4) lending credence to the use of this ratio to infer the relative degradation state of sedimentary lignin.

From the data presented thus far it can be determined these white-rot and brown-rot fungal degraded woods have very distinct and characteristic residues. The brown-rot residues exhibit a high level of demethylation and a low level of acidic functionalities (e.g. G6). These white-rot residues show low levels of demethylated structures and have a significant amount of oxidized aromatic units. We can use these relationships to differentiate between white-rot and brown-rot residues by constructing a cross plot of the G6/G4 ratio (a proxy for side chain oxidation) for a particular wood residue and the % demethylation for any particular lignin monomer from that same sample. Fig. 11(a)–(d) demonstrates the robust nature of this technique for differentiating the chemical manifestations brown-rot and white-rot woods. The % demethylation (Table 2) of four lignin monomers, G14, G5, G15 and G6 from each sample are plotted against the G6/G4 ratio for that sample. The cross plots of brown-rot degraded wood and white-rot degraded wood completely separate permitting a clear indicator of which fungal type acted upon the wood. All of the monomers shown in Table 2 exhibit this same general relationship. Importantly, the field balsam fir samples agree with the trend established by the laboratory degradation study.

One must be cautious to generalize this limited data set but it appears from plots in Fig. 11 that the ^{13}C -TMAH thermochemolysis procedure affords a rapid and clear distinction between the two fungal groups.

The clear separation of variables in these plots suggests that it would be easy to distinguish between the comparative roles of each group of fungi in soil production or in the dissolved organic matter leached from humic soils. More field and laboratory studies are required to further test these relationships. Research on cores from forest soils that have undergone a variety of stresses from drought to acid rain may yield important information about the structure of lignin in the soils and the roles of different microbial groups as a result of such environmental stresses. For example, application of ^{13}C -TMAH thermochemolysis to soils of different pH and mineral composition may help to shed light on the disparate preservation potential that has been documented for the lignocellulose complex in high and low pH soils (e.g. van Bergen et al., 1998; Nierop, 1998). One may envisage that the chemistry that fungi impart to the lignin prior to and after entering the soil complex may “chemically prime” the wood residue for selective leaching, bacterial alteration or pH-controlled polymerization reactions with other catechol moieties, sugars or organic nitrogen.

3.4. Comparison between ^{13}C -TMAH and CuO analysis of pine and spruce samples

Data from the analysis of the pine and spruce samples by ^{13}C -TMAH thermochemolysis and alkaline CuO oxidation (see data and discussion in Goñi et al., 1993) are briefly compared in the following section. It is instructional to observe how the two procedures track the chemical alterations imparted to the residues by the white-rot and brown-rot fungi.

The overall yield of lignin monomers reported in Goñi et al. (1993) for the CuO analysis of the control, brown-rot and white-rot pine were 151, 168 and 125 $\mu\text{g}/\text{mg}$ OC. The yields for the control, brown-rot and

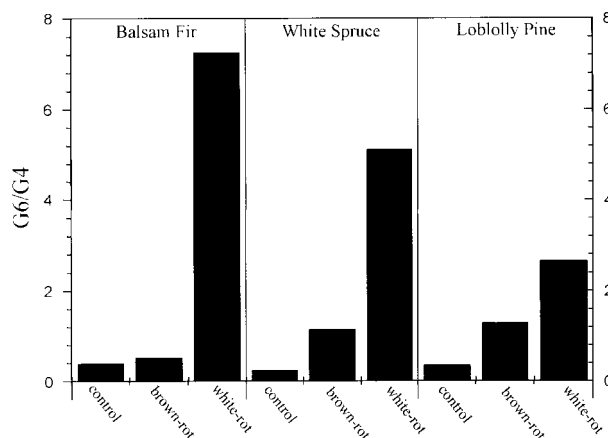


Fig. 10. Graph of the ratio of the concentration of G6 to the concentration of G4 (Ad/Al or G6/G4 ratio) for the undegraded controls, white-rot and brown-rot samples of fir, spruce and pine investigated in this study.

white-rot spruce samples were 150, 167 and 102 $\mu\text{g}/\text{mg}$ OC in that study. These values are on average more than four times greater than the yield of monomers by ^{13}C -TMAH thermochemolysis of the same samples. The difference in yields between the CuO method and the ^{13}C -TMAH method most likely results from the different chemical mechanism each method employs for lignin depolymerization. CuO is a base catalyzed hydrolysis with a one-electron oxidation that results in aliphatic–aliphatic and aliphatic–aromatic carbon bond oxidation (Goñi and Hedges, 1992). The oxidation of C–C bonds during CuO oxidation is evidenced by the liberation of lignin monomers with carboxyl and formyl groups at the 5- and 6-position on the aromatic ring. These compounds are not detected in ^{13}C -TMAH products. ^{13}C -TMAH is a base-catalyzed cleavage with in situ derivatization that only cleaves the β -O-4 bonds that have hydroxyls in an adjacent position on the side chain (Filley et al., 1999). ^{13}C -TMAH thermochemolysis can then be expected to depolymerize less of the overall lignin

structure but at the same time it can be expected to be more sensitive to chemical alterations to the β -O-4 bond.

This latter point is evident when comparing the Ad/Al ratios between the two techniques. In general, increases in the Ad/Al ratio in the CuO products, measured as the ratio of the yields of vanillic acid to vanillin, are demonstrated to coincide with fungal oxidation of lignin (Hedges et al., 1988). Goñi et al. (1993) reported Ad/Al ratios for the control, brown-rot and white-rot pine at 0.25, 0.25 and 0.38, respectively, while the control, brown-rot and white-rot of the spruce samples are reported at 0.21, 0.22 and 0.46, respectively. Comparing the G6/G4 (Ad/Al) values in Table 1 to those from Goñi et al. (1993) illustrates major differences that can also be attributed to the different chemical mechanisms of the two procedures. CuO oxidation demonstrated very little change in the Ad/Al ratio of white-rotted woods and no change in the brown-rot residues. The ^{13}C -TMAH values showed increases in Ad/Al for both brown-rot and white-rot residues with the white-rot residues exhibiting

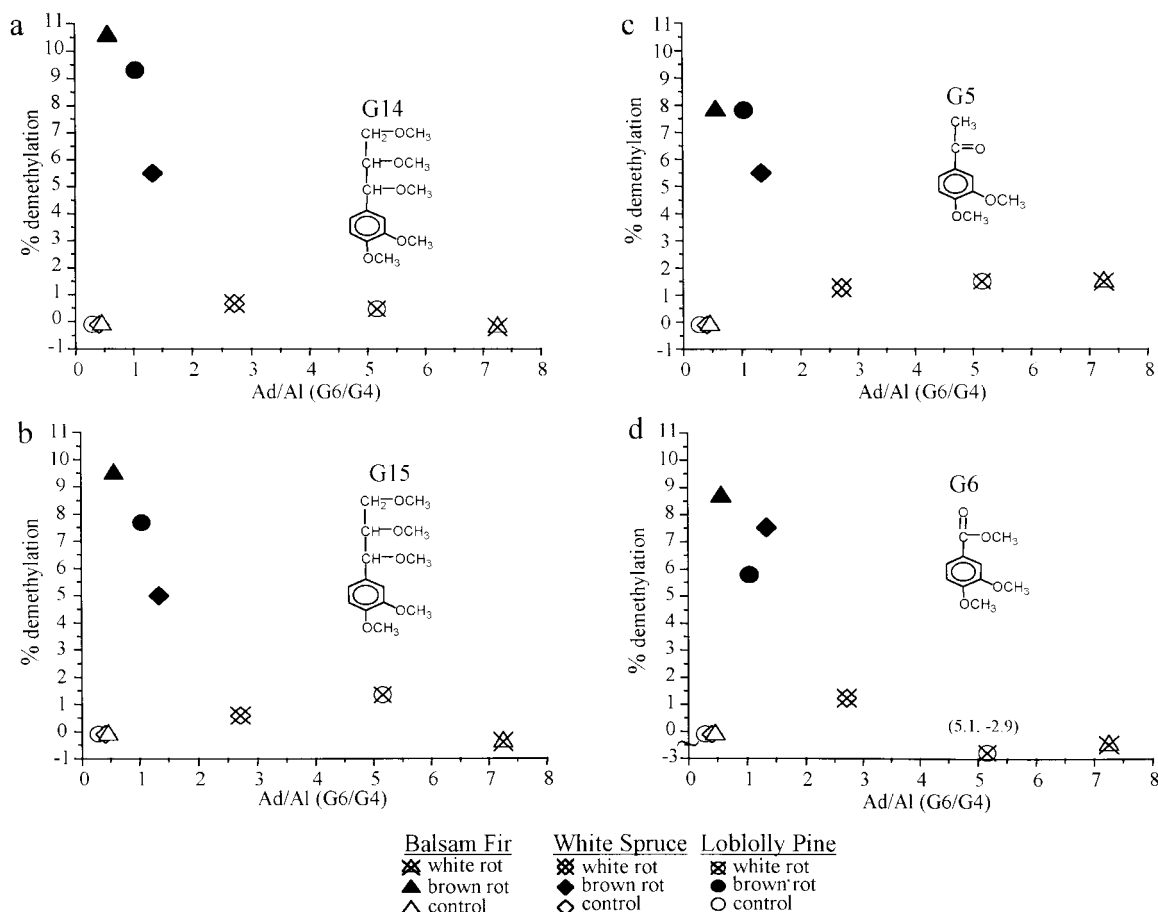


Fig. 11. Cross plot of the Ad/Al or G6/G4 ratio for the undegraded and degraded wood samples investigated in this study (see Table 1) and the % demethylation calculated for the lignin monomers G14 (a), G15 (b), G5 (c) and G6 (d). See Table 2 for % demethylation values.

extremely large increases, up to a 25-fold increase in the case of spruce wood. The comparison of the Ad/Al ratios underscores the sensitivity of the ^{13}C -TMAH method to changes at the β -O-4 bond that appear to be masked in the alkaline CuO oxidation technique.

The detection of the demethylated lignin monomers in the brown-rot residues adds an additional dimension of mechanistic information concerning microbial decay not readily available in molecular studies until now. Just under 10% of G14 exhibits demethylation in the brown-rot degraded spruce sample. This yields important mechanistic information concerning the order in which characteristic decomposition reactions are performed by fungi as well as important information concerning chemical functionality of vascular plant detritus that will ultimately have a large impact on soil organic matter and dissolved organic matter composition.

The ^{13}C -TMAH thermochemolysis and alkaline CuO oxidation procedures seem to yield different but complementary information. The CuO technique is well suited for an overall mass accounting of lignin, excluding demethylated structures, in fresh and highly degraded geochemical samples whereas the ^{13}C -TMAH thermochemolysis procedure may be more sensitive to specific biogeochemical processes (e.g. side chain hydroxyl alteration and demethylation) that alter lignin during the early stages of wood decay and mobilization.

4. Conclusions

Application of ^{13}C -TMAH thermochemolysis to the analysis of lignin residues offers a number of advantages that permit highly sensitive analyses of lignin composition and diagenetic state in sediments and waters. These advantages include:

1. A chemolytic mechanism that is selective to the β -O-4 linkage in lignin, the principal linkage in the lignin polymer.
2. The ability to infer the relative oxidation state of the propyl side chain through the use of molecular ratios such as the Ad/Al (G6/G4) parameter.
3. The ability to calculate the % of lignin structures that contain aromatic hydroxyl functional groups. This allows one to determine the extent of demethylation of the lignin polymer during fungal/microbial degradation.
4. The release of the complete permethylated lignin monomer containing the intact glycerol side chain. The presence of the complete monomers provides the closest indication of the diagenetic state of the structure of lignin in the sample under investigation.
5. Major differences were observed in the chemical composition and yield of monomers released from

these brown-rot and white-rot degraded residues when analyzed by the ^{13}C -TMAH thermochemolysis procedure that were not apparent when investigated by CuO oxidation. The brown-rot residues were characterized by high yields of 3,4-dihydroxy phenyl groups, due to the demethylation of the 3-methoxyl carbon, but relatively low yields of aromatic acids from side chain oxidation. Low yields of demethylated lignin monomers but relatively high yields of monomers indicative of side chain oxidation characterized white-rot degraded residues.

This distinct chemistry, if generally applicable, has important implications for the cycling of terrestrial detrital carbon in the geosphere which may provide insight into the wide variations in stability observed for the lignocellulose complex in soils of different pH and mineral composition.

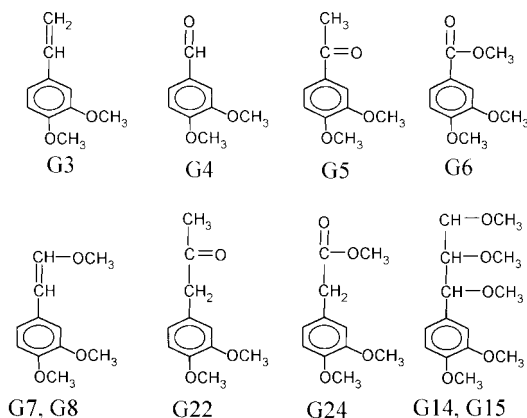
Acknowledgements

Funding for this study was provided by the Geophysical Laboratory, Carnegie Institution of Washington. We wish to thank Robert Blanchette, Miguel Goñi and John Hedges for providing wood samples for this study. Also, Pim van Bergen, George Cody, Rose Filley, Marilyn Fogel, John Hedges, Sue Ziegler as well as two anonymous reviewers are thanked for their constructive comments and reviews.

Associate Editor—C. Largeau

Appendix

Structure Appendix



G3 = 3,4-dimethoxystyrene

G4 = 3,4-dimethoxybenzaldehyde

G5 = 3,4-dimethoxyacetophenone
 G6 = 3,4-dimethoxybenzoic acid, methyl ester
 G7 and G8 = *cis* and *trans*-1-(3,4-dimethoxyphenyl)-2-methoxyethylene
 G22 = 1-(3,4-dimethoxyphenyl)-2-propanone
 G24 = 3,4-dimethoxyphenol acetic acid, methyl ester
 G14 and G15 = threo/erythro 1-(3,4-dimethoxyphenyl)1,2,3-trimethoxypropane

References

- Adler, E., 1977. Lignin-past, present and future. *Wood Science Technology* 11, 169–218.
- Ander, P., Eriksson, K.-E., 1985. Methanol formation during lignin degradation by *Phanerochaete chrysosporium*. *Applied Microbiology and Biotechnology* 21, 96–102.
- Ander, P., Eriksson, M.E.R., Eriksson, K.-E., 1985. Methanol production from lignin related substances by *Phanerochaete chrysosporium*. *Physiol Plant* 65, 317–321.
- Ander, P., Stoytschev, I., Eriksson, K.-E., 1988. Cleavage and metabolism of methoxyl groups from vanillic and ferulic acids by brown-rot and soft-rot fungi. *Cellulose Chemical Technology* 22, 255–266.
- van Bergen, P.F., Nott, C.J., Bull, I.D., Poulton, P.R., Evershed, R.P., 1998. Organic geochemical studies of soils from the Rothamsted classical experiments — IV. Preliminary results from a study of the effect of soil pH on organic matter decay. *Organic Geochemistry* 29, 1779–1795.
- Bianchi, T.S., Lambert, C.D., Santschi, P.H., Guo, L., 1997. Sources and transport of land-derived particulate and dissolved organic matter in the gulf of Mexico (Texas Shelf/Slope): the use of lignin phenols and loliolides as biomarkers. *Organic Geochemistry* 27, 65–78.
- Blanchette, R.A., 1991. Delignification by wood-decay fungi. *Annual Reviews of Phytopathology* 29, 381–398.
- Blanchette, R.A., Obst, J.R., Tore, E.T., 1994. Biodegradation of compression wood and tension wood by white and brown rot fungi. *Holzforschung* 48, 34–42.
- Challinor, J.M., 1995. Characterisation of wood by pyrolysis derivatization-gas chromatography/mass spectrometry. *Journal of Analytical and Applied Pyrolysis* 35, 93–107.
- Enoki, A., Tanaka, H., Fuse, G., 1988. Degradation of lignin-related compounds, pure cellulose, and wood components by white-rot and brown-rot fungi. *Holzforschung* 42, 85–93.
- Eriksson, K.E.L., Blanchette, R.A., Ander, P., 1990. *Microbial and Enzymatic Degradation of Wood and Wood Components*. Springer-Verlag, Berlin (397 pp.).
- Fengel, D., Wegner, G., 1984. *Wood. Chemistry Ultrastructure, Reactions*. Walter de Gruyter, Berlin (613 pp.).
- Filley, T.R., Minard, R.D., Hatcher, P.G., 1999. Tetramethylammonium hydroxide (TMAH) thermochemolysis: proposed mechanisms based upon the application of ^{13}C -labeled TMAH to a synthetic model lignin dimer. *Organic Geochemistry* 30, 607–621.
- Goodell, B., Jellison, J., Liu, J., Danial, G., Paszczynski, A., Fekete, E. et al., 1997. Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood. *Journal of Biotechnology* 53, 133–162.
- Goñi, M.A., Nelson, B., Blanchette, R.A., Hedges, J., 1993. Fungal degradation of wood lignins: geochemical perspectives from CuO-derived phenolic dimers and monomers. *Geochimica et Cosmochimica Acta* 57, 3985–4002.
- Goñi, M.A., Hedges, J.H., 1992. Lignin dimers: structure, distribution and potential geochemical applications. *Geochimica et Cosmochimica Acta* 56, 4025–4043.
- Hatcher, P.G., Nanny, M.A., Minard, R.D., Dible, S.D., Carson, D.M., 1995. Comparison of two thermochemolytic methods for the analysis of lignin in decomposing gymnosperm wood: the CuO oxidation method and the method of thermochemolysis with tetramethylammonium hydroxide (TMAH). *Organic Geochemistry* 23, 881–888.
- Hatcher, P.G., Minard, R.D., 1995. Comment on the origin of benzenecarboxylic acids in pyrolysis methylation studies. *Organic Geochemistry* 23, 991–994.
- Hatcher, P.G., Minard, R.D., 1996. Comparison of dehydrogenase polymer (DHP) lignin with native lignin from gymnosperm wood by thermochemolysis using tetramethylammonium hydroxide (TMAH). *Organic Geochemistry* 24, 593–600.
- Hedges, J.I., Blanchette, R.A., Weliky, K., Devol, A.H., 1988. Effects of fungal degradation on the CuO-products of lignin: a controlled laboratory study. *Geochimica et Cosmochimica Acta* 52, 2717–2726.
- van der Heijden, E., Boon, J.J., 1994. A combined pyrolysis mass spectrometric and light microscopic study of peatified Calluna wood isolated from raised bog peat deposits. *Organic Geochemistry* 22, 903–919.
- Higuchi, T., 1981. Lignin structure and morphological distribution in plant cell walls. In: Kirk, T., Higuchi, T., Chang, H. (Eds.), *Lignin Biodegradation: Microbiology, Chemistry, and Potential Applications*, Vol. 1. CRC Press, Boca Raton, pp. 1–19.
- Higuchi, T. (Ed.), 1985. *Biosynthesis and Biodegradation of Wood Component*. Academic Press, Orlando, San Diego, New York, London, Toronto, Montreal, Sydney, Tokyo.
- Jellison, J., Connolly, J., Goodell, B., Doyle, B., Illman, B., Fekete, F. et al., 1997. The role of cations in the biodegradation of wood by the brown rot fungi. *International Biodeterioration and Biodegradation* 39, 165–179.
- Kirk, T.K., 1984. Degradation of lignin. In: Gibson, D. (Ed.), *Microbial Degradation of Organic Compounds*. Marcel Dekker, New York.
- Klap, V.A., Boon, J.J., Marten, A.H., Soelen, J.V., 1998. Chemical characterization of lignin preparations of fresh and decomposing *Spartina angelica* by pyrolysis mass spectroscopy. *Organic Geochemistry* 28, 707–727.
- Martin, F., del Rio, J.C., Gortzalez-Vila, F.J., Verdejo, T., 1995. Thermally assisted hydrolysis and alkylation of lignins in the presence of tetra-alkylammonium hydroxides. *Journal of Analytical and Applied Pyrolysis* 35, 1–13.
- McKinney, D.E., Carson, D.M., Clifford, D.J., Minard, R.D., Hatcher, P.G., 1995. Off-line thermochemolysis versus flash pyrolysis for the in situ methylation of lignin: is pyrolysis necessary? *Journal of Analytical and Applied Pyrolysis* 34, 41–46.
- McKinney, D.E., Hatcher, P.G., 1996. Characterization of peatified and coalified wood by tetramethylammonium hydroxide (TMAH) thermochemolysis. *International Journal of Coal Geology* 32, 217–228.

- Nierop, K.G.J., 1998. Origin of aliphatic compounds in a forest soil. *Organic Geochemistry* 29, 1009–1016.
- Opsahl, S., Benner, R., 1997. Distribution and cycling of terrigenous dissolved organic matter in the ocean. *Nature* 386, 480–482.
- Otjen, L., Blanchette, R., Effland, M., Leatham, G., 1987. Assessment of 30 white rot basidiomycetes for selective lignin degradation. *Holzforschung* 41, 343–349.
- Prahl, F.G., Ertel, J.R., Goñi, M.A., Sparrow, M.A., Eversmeyer, B., 1994. An assessment of terrestrial organic carbon contributions to Washington coastal sediments. *Geochimica et Cosmochimica Acta* 58, 3035–3048.
- Robert, D., Chen, C.L., 1989. Biodegradation of lignin in spruce wood by *Phanerochaete chrysosporium*: quantitative analysis of biodegraded spruce lignins by ^{13}C NMR spectroscopy. *Holzforschung* 43, 323–332.
- Srebotnik, E., Jensen, K.A., Jensen, A., Kawai, S., Hammel, K., 1997. Evidence the *Ceriporiopsis subvermispora* degrades nonphenolic lignin structures by a one-electron-oxidation mechanism. *Applied Environmental Microbiology* 63, 4435–4440.
- Tien, D., Kirk, T.K., 1983. Lignin-degrading enzyme from the Hymenomycete *Phanerochaete chrysosporium* Burds. *Science* 221, 661–663.
- Umezawa, T., Higuchi, T., 1987. Mechanism of aromatic ring cleavage of β -O-4 lignin substructure models by lignin peroxidase. *Federation of European Biochemical Societies Letters* 218, 255–260.