

Abiotic Degradation of Lignified Cell Walls by Carbonate and Copper Salt

NATHALIE DUROT,[†] BRIGITTE POLLET,[‡] CATHERINE LAPIERRE,[‡] AND
BERNARD KUREK^{*,†}

Equipe Parois Végétales et Matériaux Fibreux, UMR de Fractionnement des Agro-Ressources et Emballages, Institut National de la Recherche Agronomique (INRA), 2 Esplanade Roland Garros, F-51686 Reims Cedex 2, France, and Laboratoire de Chimie Biologique, UMR INRA-INA-PG, Centre de Grignon, 78850 Thiverval Grignon, France

This study reports on the destructure of Wheat straw and Spruce wood cell walls after maceration in potassium carbonate or sodium hydroxide at pH = 10 in the presence of copper acetate. The alkaline treatments had a predominant impact on the wheat straw cell wall components over copper acetate. Either K-carbonate or Na-hydroxide extracted from wheat straw a particular lignin fraction rich in condensed C–C linkages, leading to the unmasking of new ether-linked sub-structures in the cell wall. This unmasking was increased in the presence of copper salt but only in the nonextracted Wheat straw sample incubated in carbonate and not in the corresponding extractive-free sample. This difference was related to the leaching of compounds from the nonextracted cell wall, which could sustain oxidative activity of copper by hindering its precipitation into inactive hydroxide and/or carbonate species. In Spruce wood samples, copper salt was the principal factor impacting on the lignin structure over alkali alone. Its effect was, however, only detected at the level of C–C linked dimers. These results confirmed that unmasking of lignin sub-structures also occurred in Spruce wood, but probably through mechanisms different from that evidenced in Wheat straw.

KEYWORDS: *Triticum aestivum*; *Picea abies*; calcareous soils; lignin; polysaccharides; pectins; phenolic acids; plant cell wall; transition metal

INTRODUCTION

The transformation of plant material into humic substances is a complex process involving various microorganism species as well as several types of abiotic reactions (1). Among these, it has been suggested that oxidation by transition metals such as Fe, Cu, and Mn may participate to the incorporation of phenolic and lignin related compounds into humus (2, 3). This phenomenon would be particularly relevant in soils amended with urban compost or in soils where agricultural practices involves the use of metal based pesticides (4). As an example, long-term accumulation as high as 250–1000 mg copper per kg of calcareous soils has been reported in vineyards (5).

To the best of our knowledge, abiotic processes involving metal cations that may participate in plant material decomposition were not reported to occur in calcareous soils. Indeed, at first glance, due to the local pH, which can range from pH = 8–10, the soluble transition metals are expected to precipitate into carbonates or hydroxides species, leading to the hindering of their potential oxidative activity (6).

Several facts, however, suggest that abiotic degradation of plant cell wall could still take place under these particular conditions. Indeed, the effect of strong alkali on plant cell wall and particularly Wheat straw, is well documented. These include swelling of polysaccharides, delignification, extraction of low molecular weight constituents of the cell wall (7–9). Also, the ability of several metals to complex to lignocellulosic materials under various conditions is well-known. This leads to chelation of the metal, oxidations, enhancement of polysaccharide dissolution, or precipitation (10–12).

The objective of this study is to demonstrate that a significant abiotic modification of the plant cell wall can occur under conditions that mimic incubation in alkaline soils contaminated by transition metal. We have therefore investigated the impact of mild alkali treatments on the polymers constituting the plant cell wall, first without copper salt, and then with this component widely used in the formulation of pesticides (copper acetate, copper sulfate, and copper oxychlorides) (13).

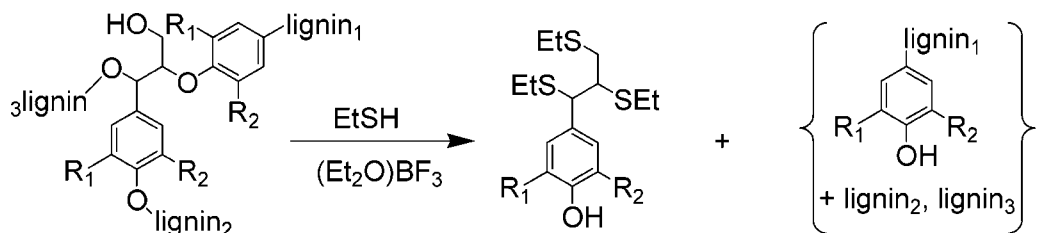
MATERIALS AND METHODS

Plant Materials and Substrates. Wheat straw (*Triticum aestivum* L; var Scipion) was harvested by hand at full maturity and air-dried. Internodes were separated, collected, and used throughout this study after reduction into ~0.1–0.5 mm particle size. This nonextracted straw

* To whom correspondence should be addressed. Tel.: 33 3 2677 3593. Fax: 33 3 2677 3599. E-mail: kurek@reims.inra.fr.

[†] Institut National de la Recherche Agronomique.

[‡] Centre de Grignon.

G units: R₁ = H, R₂ = OCH₃S units: R₁ = R₂ = OCH₃

monomers

dimers (see below)

+ trimers + oligomers

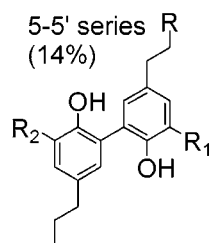
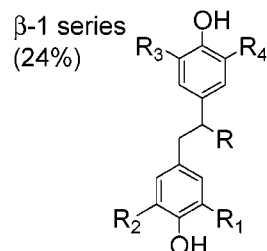
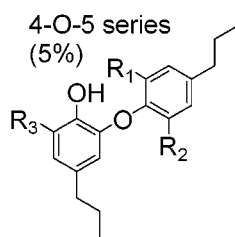
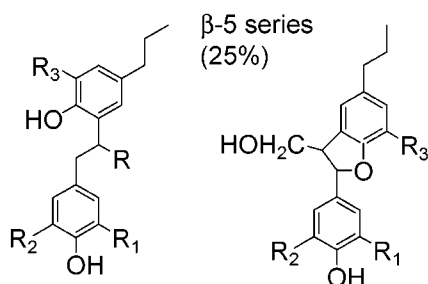
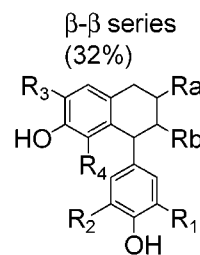
R = H or CH₃R = CH₂OH or HR = H, CH₃ or CH₂OHRa = Rb = CH₃ orRa = CH₃, Rb = H orRa = CH₂OH, Rb = CH₃for all structures: R_n (n=1, 2, 3, 4) = OCH₃ or H

Figure 1. Degradation pathway of lignin during the thioacidolysis reaction and structure of the main monomers and dimers recovered therefrom. G, S = Guaiacyl and Syringyl moieties in lignins, respectively.

is named hereafter NE-STRAW. Alternatively, Spruce (*Picea abies*) wood sawdust and Wheat straw internodes were exhaustively and successively extracted in a Soxhlet apparatus with toluene-ethanol mixture, ethanol, and then water as described previously (14). The extractive-free wood and Wheat straw (designated hereafter EF-WOOD and EF-STRAW, respectively) were freeze-dried and ball milled to a particle size of ~0.1–0.5 mm.

Treatments of Straw and Wood by Alkali and Copper Salts. Wheat straw or Spruce wood (NE-STRAW, EF-STRAW, and EF-WOOD; 50 mg) were incubated for 24 h with stirring at room temperature (20–25 °C) in alkaline solution (10 mL). To avoid artifactual abiotic oxidations by transition metals, the potassium carbonate, sodium hydroxide, and hydrochloric acid used in the experiments contained less than 0.05 ppm of transition metal salt (Suprapur quality from Merck).

The following reaction conditions 1–4 were tested: 1, potassium carbonate 100 mM, pH = 10, adjusted with hydrochloric acid, referenced as K₂CO₃ treatment; 2, potassium carbonate 100 mM, pH = 10, plus copper (II) acetate monohydrate (10 mM), referenced as K₂CO₃ + CuAc₂ treatment; 3, sodium hydroxide 100 mM, carefully titrated to pH = 10 with dilute HCl solution, and readjusted, if necessary, after addition of NE-STRAW, EF-STRAW, or EF-WOOD,

referenced as NaOH treatment; 4, sodium hydroxide 100 mM, pH = 10, plus copper(II) acetate (10 mM), referenced as NaOH + CuAc₂ treatment.

The control experiments consisted in NE-STRAW, EF-STRAW, or EF-WOOD incubated for 24 h at room temperature in 10 mL of deionized water. The NE-STRAW, EF-STRAW and EF-WOOD treated by water are named hereafter in the text and tables ref-NE-STRAW (or reference samples), ref-EF-STRAW, and ref-EF-WOOD, respectively.

At the end of the reaction period, the NE-STRAW, EF-STRAW, and EF-WOOD were separated from the alkali medium by filtration. The reaction medium was kept for further purification of the lignins extracted from the cell walls, as described below. The treated samples were then extensively washed with deionized water and freeze-dried.

Determination of the Catalytic Activity of Copper Acetate in Alkaline Medium. The oxidative activity of copper acetate in the reaction medium was followed by the formation of polymeric tetraguaiacol from guaiacol (15). For this purpose, copper acetate (10 mM) was incubated in conditions 1–4 in the presence or absence of NE-STRAW or EF-STRAW (50 mg). At different incubation time, the medium was centrifuged, and 1 mL of the clear solution was mixed with 2 mL of 50 mM guaiacol in MeOH. In parallel, guaiacol was

incubated in medium 1 and 3, with or without NE-STRAW or EF-STRAW, to determine either the background noise of a nonspecific autoxidation process of guaiacol by alkali or the influence of cations present within the straw which could interfere with the measurements. The formation rate of tetraguaiacol was followed spectrophotometrically at 436 nm and the activity (specific or background noise), was calculated with a molar absorptivity of $\epsilon = 6.39 \text{ cm}^2/\mu\text{mol}$ (15, 16).

Distribution of Copper in the Medium and in Wheat Straw After the Reaction. The different substrates were incubated in the various reaction media 1–4. At the end of the reaction time, the straw was recovered on a Gooch crucible, and the filtrate was acidified with H_2SO_4 to $\text{pH} = 1$. The copper in the filtrate was then determined quantitatively by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP/AES) relative to standard copper solutions of known concentration (17). The copper remaining “inside” or “on” Wheat straw after extensive washing with water was determined by atomic absorption spectrometry (AAS). For this purpose, solubilization from the cell wall was performed with 6 M HCl at room temperature and the extracts were further diluted for analysis (17). The amount of copper precipitated in solution was determined indirectly by subtracting from the initial copper content added to the medium the measured solubilized and adsorbed fractions.

Lignin Recovery from Alkali Soluble Fraction. The filtrated reaction medium was first acidified with 6 M HCl to $\text{pH} = 1$ and kept at 4 °C during 1 h. The precipitate formed was recovered by centrifugation and dissolved in dioxane/water mixture (9:1; vol/vol). Further purification was done by precipitation overnight at 4 °C in 1 M acetic acid. The lignin was then recovered by centrifugation, washed twice with a minimum of cold water and then freeze-dried.

Lignin Content Determination. The lignin content in samples was estimated gravimetrically according to a procedure similar to that described by Effland (18) and modified by Monties (19).

Lignin Characterization. The determination of the amount of structures linked by alkyl–aryl ether bonds in lignins was performed by thioacidolysis according to the published procedure (20, 21) (Figure 1). The main lignin-derived monomers recovered were separated by capillary gas chromatography as trimethylsilyl derivatives on a DB-5 column (Hewlett-Packard) and detected with FID, as described previously (22). The yield of monomers of the Guaiacyl and Syringyl units measured reflects then the amount of such units only involved in α or β -O-4 bonds (Figure 1). The C–C linked dimers released during the procedure were further desulfurized and analyzed by GC and GC-MS, as described elsewhere (23, 24); they are representative of the different carbon–carbon and diaryl ether linkages in the polymer and referred to as the “condensed bond” (Figure 1).

Carbohydrate Analysis. Neutral Sugars. Hydrolysis of Wheat straw polysaccharides was performed by H_2SO_4 according to Blakeney et al. (25). After hydrolysis, the reaction medium was diluted with water, and the monosaccharides released were separated by high performance anion-exchange chromatography (HPAEC) on a PA-1 Column (Dionex) in NaOH (22). The products were detected by pulsed amperometry (PAD), using a gold electrode coupled to an ED-40 device (Dionex).

Uronic Acids Content. Uronic acids were released from the cell wall after the combination of acid methanolysis and hydrolysis, according to de Ruiter (26). The reactant for methanolysis (2 M HCl in anhydrous MeOH) was prepared with an Alltech Kit (ref 18503) according to the Aldrich technical notice. The HCl–MeOH reactant was then added to the cell wall residue and heated at 80 °C for 16 h. The samples were dried under N_2 gas flow and subjected to 4M tri-fluoro acetic acid (TFA) (27). TFA was eliminated by evaporation under vacuum. The solid residue obtained was dissolved in water, filtered, and injected on a CarboPac PA-1 anion exchange column (Dionex) for HPAEC analysis. Acidic sugars were separated with a gradient of NaOH and sodium acetate as described before (22). The detection of uronic acids was performed by PAD, as for neutral monosaccharides (see above).

Phenolic Acids Content Determination. The content in ester and ether linked *p*-coumaric (pCA) and ferulic (FA) acids of Wheat straw sample after successive alkaline hydrolysis and acidolysis, respectively, according to Scalbert (28). Total cinnamic acids were determined after one step strong alkaline hydrolysis at 170 °C (29). The phenolic acids

released were separated by HPLC and detected by UV spectrophotometry, as described elsewhere (22).

Statistical Analysis of Datas. Two types of analyses were performed. As a first approach, to determine the predominant effects of each treatment on the different cell wall structures, multivariate analysis and mean comparison tests were performed through the analysis of variance (ANOVA). The analysis matrix consisted of treatments (water (reference), K_2CO_3 , and NaOH), the presence of copper (without, with), and when relevant, the type of substrate used (NE-STRAW, EF-STRAW, and EF-WOOD). The rough analytical data were computed with the SPSS software (SPSS, Inc., Chicago, IL). The *F* factor, significance, and association (ϵ^2) factors were calculated and interpreted. In a second period, selected paired observations were analyzed by student tests for small populations ($n < 30$) to sharpen the previous interpretations.

RESULTS AND DISCUSSION

The impact of alkali and of copper acetate treatments on the cell wall polymers of Wheat straw and Spruce wood was investigated. For this purpose, the content and structure of lignin, cellulose, hemicelluloses, and pectins was determined.

Fate and Oxidative Activity of Copper Acetate in Alkaline Media in the Presence of Wheat Straw. The incubation of copper acetate in NaOH or K_2CO_3 at $\text{pH} = 10$ induced its precipitation into insoluble carbonate and/or hydroxide species (6). Without straw added to the reaction medium, the precipitation was almost quantitative in NaOH and K_2CO_3 after 30 min (data not shown) (30). The presence of NE-STRAW, however, allowed the recovery of 10–15% (w/w) of the initial copper in solution after 24 h incubation time in K-carbonate or Na-hydroxide but only 6–9% when EF-STRAW was used instead (30). This suggests that lignin plus other components that are extracted from the NE- or EF-STRAW could stabilize the copper into solution, therefore hindering its precipitation.

The stabilization of copper into soluble form in alkali by wheat straw extractives was proposed here to sustain a metal-based oxidative catalysis. Indeed, in the absence of straw, copper was almost fully precipitated, and a very low oxidation rate of guaiacol in solution was measured (less than 1% of the reference

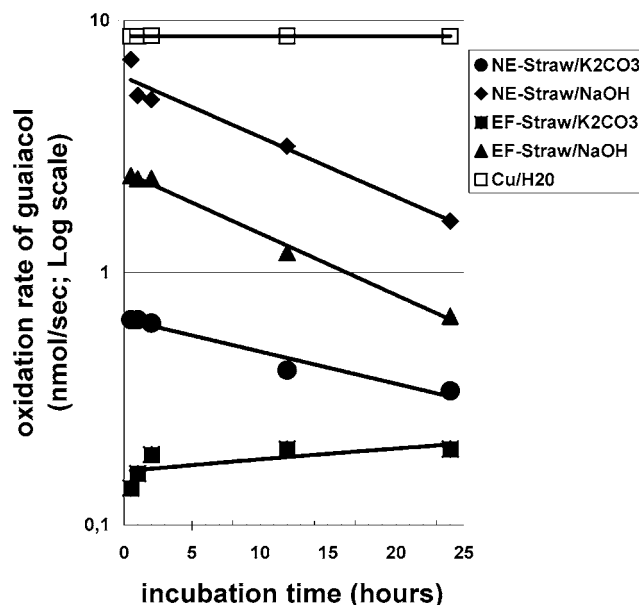


Figure 2. Oxidative activity of copper acetate in water only ($\text{Cu}/\text{H}_2\text{O}$) and in carbonate (K_2CO_3) or hydroxide (NaOH) media in the presence of Wheat straw (NE-STRAW and EF-STRAW). The activity of CuAc_2 in K_2CO_3 or NaOH at $\text{pH} 10$, in the absence of Wheat straw is equal to 1% of the activity of CuAc_2 in water ($\text{Cu}/\text{H}_2\text{O}$ sample).

Table 1. Lignin Content of Wheat Straw and Spruce Wood^a

	H ₂ O	treatment (1) ^b K ₂ CO ₃	treatment (2) K ₂ CO ₃ + CuAc ₂	treatment (#3) NaOH	treatment (4) NaOH + CuAc ₂
NE-STRAW ^c	23.1 ± 1.3 (a) ^d	18.7 ± 1.4 (b) ^d	19.7 ± 1.3 (b)	18.2 ± 1 (b)	NM ^e
EF-STRAW	21.8 ± 0.3 (c)	14.7 ± 0.3 (d)	15.9 ± 0.1 (d)	18.2 ± 0.2 (e)	18.6 ± 0.2 (e)
EF-WOOD	27.8 ± 0.3 (f)	28.8 ± 0.3 (f)	28.6 ± 0.4 (f)	29 ± 0.4 (f)	29 ± 0.5 (f)

^a Klason lignin content, expressed in g/100 g of material. ^b Refer to material and methods for reaction condition. ^c Caution note: for NE-STRAW samples, overestimation of the Klason lignin contents maybe possible, due to the presence of extractives. ^d Different lettering in lines for NE-STRAW, EF-STRAW, and EF-WOOD series indicates a significant difference between the samples at a 95% confidence level, for $n > 6$. ^e NM = not measured.

Table 2. Yields of Monomers Released by Thioacidolysis from Lignin Extracted from EF-STRAW by Alkali

	treatment (1) ^a EF-STRAW + K ₂ CO ₃	treatment (2) EF-STRAW + K ₂ CO ₃ + CuAc ₂	treatment (3) EF-STRAW + NaOH	treatment (4) EF-STRAW + NaOH + CuAc ₂
G structures ^b	133 ± 4.4	104 ± 3	336 ± 13	133 ± 9
S structures ^b	101 ± 5	107 ± 3	364 ± 11	128 ± 9

^a Refer to material and methods for reaction condition. ^b In $\mu\text{mol/g}$ of extracted lignin. G, S = Guaiacyl and Syringyl, respectively. Refer to **Figure 1** for structures.

activity of CuAc₂ incubated in water only at pH ~6.5; **Figure 2**). In the presence of EF-STRAW or NE-STRAW, however, the residual activities of guaiacol oxidation in NaOH solution increased up to 8 and 19%, respectively. When K₂CO₃ was used, the same impact of straw on oxidative activity of the remaining copper was observed, but the rates measured were lower (2 and 4% of the reference activity of CuAc₂ in water, see **Figure 2**).

The nature of the products extracted by alkali is probably an important factor governing either the stabilization of copper in solution and its oxidative activity. We have indeed reported recently that NaOH at pH = 10 extracts a lower amount of lignins from EF-STRAW than K₂CO₃ and that the structures of the solubilized lignins are different (see also **Tables**

3 and **4**) (17). On the other hand, the lignins extracted from NE-STRAW and EF-STRAW could directly compete with guaiacol for copper oxidation, as shown for example during veratryl alcohol oxidation by peroxidase in the presence of lignins (31). Such guaiacol/lignin competition could also then explain the (apparent) lower activity of copper measured in K₂CO₃, where a higher amount of lignin is available for oxidation (17).

Modification of the Structure and Content of the Cell Wall Polymers by Alkali and Copper Acetate. *Mass Balance and Products Recovery After Maceration in Alkali.* The incubation of Wheat straw and Spruce wood in potassium carbonate and sodium hydroxide at room temperature and at pH = 10 induced a loss of weight that represents less than 10% of the starting material in 24 h. This was mainly due to the technical procedure used in the experiments, such as washing and filtering stages, and not only to the leaching of molecules or to the lignin solubilization in alkali (see text above and following).

Content and Structure of Aromatic Constituents. Several distinctive effects of alkali and of the copper acetate on cell wall could be evidenced, according to the type of plant considered (straw or wood).

Wheat Straw. First of all, the lignin from Wheat straw is characterized by a fraction that is highly soluble in alkali, as opposed to the residual core lignin or to the lignin in wood (32). The extraction yields of such lignins ranged from 13% for NaOH-treated EF-STRAW to 30% for K₂CO₃ treated EF-STRAW (**Table 1**). In all cases, the presence of CuAc₂ did not significantly increase the extraction yields (**Table 1**). Biochemi-

Table 3. Yields of Monomers Released by Thioacidolysis from Lignin in NE-STRAW and in EF-STRAW

	G structure ^a	S structure ^a
ref-EF-STRAW	411 ± 29 (a) ^b	448 ± 15 (d) ^b
treatment (1) ^c EF-STRAW + K ₂ CO ₃	664 ± 59 (b)	718 ± 54 (e)
treatment (2) EF-STRAW + K ₂ CO ₃ + CuAc ₂	693 ± 91 (b)	748 ± 83 (e)
treatment (3) EF-STRAW + NaOH	614 ± 51 (b)	655 ± 88 (e)
treatment (4) EF-STRAW + NaOH + CuAc ₂	521 ± 94 (c)	539 ± 52 (e)
ref-NE-STRAW	453 ± 78 (f) ^b	473 ± 69 (j) ^b
treatment (1) ^c NE-STRAW + K ₂ CO ₃	601 ± 62 (g)	682 ± 57 (j)
treatment (2) NE-STRAW + K ₂ CO ₃ + CuAc ₂	896 ± 88 (h)	911 ± 10 (k)

^a Results in $\mu\text{mol/g}$ of Klason lignin. G, S = Guaiacyl and Syringyl, respectively. Refer to **Figure 1** for structure. ^b Paired mean comparisons; different lettering in columns for EF-STRAW and NE-STRAW series indicates a significant difference between the samples at a 95% confidence level, for $n > 6$ samples. ^c Refer to materials and methods for reaction condition.

Table 4. Variations in the Yields of C–C Linked Dimers Released by Thioacidolysis from Lignin in NE-STRAW

dimer structure ^a	treatment (1) ^b NE-STRAW + K ₂ CO ₃	treatment (2) NE-STRAW + K ₂ CO ₃ + CuAc ₂ ^c
β - β	+ 16% ^c	+ 8%
β -5	+ 30%	+ 15%
β -1	+ 14%	+ 21%
4-O-5	+ 71%	+ 74%
5-5'	+ 53%	+ 62%

^a Refer to **Figure 1** for structures. ^b Refer to materials and methods for reaction condition. ^c Relative to ref NE-STRAW. Determined from specific ion count for each dimer type in GC-MS. Variations relative to control $\leq 15\%$ are not considered to be significant.

cal analysis of the extracted NE- and EF-STRAW showed that no modifications of their contents in etherified and esterified phenolic acids occurred. This suggested that that these reticulating structures in the cell wall were not involved in the solubilization process of lignins (total content of phenolic acids in Ref-EF-STRAW = 0.25 ± 0.05% weight, full data not shown, refer also to refs 32 and 30).

The structure of the solubilized lignins from EF-STRAW was determined by thioacidolysis (**Table 2**). When copper salt is present in NaOH medium during the extraction, the lignin solubilized was more condensed (= with lower amount of ether-linked structures; **Figure 1**) than the corresponding lignin extracted by NaOH alone. Considering that oxidation of model compounds such as coniferyl alcohol and β -O-4 lignin-like

Table 5. Yields and Variations after Treatments of Monomers and Dimers Released by Thioacidolysis from Lignin in EF-WOOD

	Ref-EF-WOOD	treatment (1) ^a EF-WOOD + K ₂ CO ₃	treatment (2) EF-WOOD + K ₂ CO ₃ + CuAc ₂	treatment (3) EF-WOOD + NaOH	treatment (4) EF-WOOD + NaOH + CuAc ₂
monomer ^b	1384 ± 62	128.3 ± 131 ^c	1571 ± 141 (S) ^c	1302 ± 65	1300 ± 90
		dimer structure ^b			
5–5'	50.9 ± 6.3 ^b	–12.25% ^d (S) ^c	+17.7% (S, s) ^c	–8%	–10%
4–O–5	9.9 ± 1.5	–21% (S)	+59.2% (S, s)	–5%	–5% (s)
β–6	20.7 ± 2.8	+25% (S)	+33.7% (S)	+11%	+32% (S, s)
β–1	41 ± 10.7	–47% (S)	+28% (S, s)	–42% (S)	–53% (S)
β–5	45 ± 11.2	–28.6% (S)	+61% (S, s)	+12%	–3%
β–β	16.2 ± 5.2	–24% (S)	–3% (s)	–52% (S)	–55% (S)
total dimers	184 ± 23.5	–24.6% (S)	+33% (S, s)	–11%	–17% (S)

^a Refer to materials and methods for reaction condition. ^b Mainly Guaiacyl structures are present in Spruce wood. Results in μmol/g of Klason lignin. Refer to **Figure 1** for structures. ^c In parentheses, paired mean comparisons: S, data significantly different from Ref-EF-WOOD at a 95% confidence level for $n > 6$ samples; s, the same, but relative to the carbonate (1) or hydroxide (3) treated samples. ^d In percent of structure in ref-EF-WOOD.

Table 6. Composition of Wheat Straw in Polysaccharides

	arabinose ^a	galactose ^a	glucose ^a	xylose ^a	galacturonic acid ^b	glucuronic acid ^b
ref NE-STRAW	2.4 ± 0.3	0.55 ± 0.09	38 ± 5	23.5 ± 4	0.22 ± 0.07	0.73 ± 0.03
treatment (1) ^c NE-STRAW +K ₂ CO ₃	2.4 ± 0.2	0.31 ± 0.08 (S) ^d	41 ± 5	26 ± 3	0.19 ± 0.02 (S)	0.45 ± 0.5 (S)
treatment (2) NE-STRAW +K ₂ CO ₃ + CuAc ₂	2.4 ± 0.1	0.27 ± 0.2 (S)	38 ± 2	25 ± 1	0.18 ± 0.03 (S)	0.38 ± 0.5 (S)
ref-EF-STRAW	2.6 ± 0.1	0.49 ± 0.06	38.1 ± 1.2	23.4 ± 0.6	0.19 ± 0.08	0.47 ± 0.03
treatment (1) ^c EF-STRAW +K ₂ CO ₃	2.7 ± 0.1	0.37 ± 0.01 (S)	41 ± 1.0	24 ± 0.8	0.06 ± 0.01 (S)	0.15 ± 0.03 (S)
treatment (2) EF-STRAW +K ₂ CO ₃ + CuAc ₂	2.3 ± 0.1	0.27 ± 0.02 (S)	38 ± 2	23 ± 0.9	0.08 ± 0.01 (S)	0.23 ± 0.7 (S)
treatment (3) EF-STRAW +NaOH	2.7 ± 0.2	0.46 ± 0.06	38 ± 3.9	25.2 ± 1.3	0.04 ± 0.01 (S)	0.14 ± 0.03 (S)
treatment (4) EF-STRAW +NaOH + CuAc ₂	2.5 ± 0.05	0.45 ± 0.05	38 ± 3	21.4 ± 1.7	0.08 ± 0.01 (S)	0.21 ± 0.01 (S)

^a In g/100 g of material. ^b In mg/100 g of material. ^c Refer to materials and methods for reaction condition. ^d (S), paired comparisons of mean; data significantly different from Ref-NE-STRAW or Ref-EF-STRAW at a 95% confidence level for $n > 6$ samples.

model compounds occur in alkali media (unpublished; see ref 30), the extracted lignins could follow the same fate, with subsequent structural modifications. Nevertheless, the lignin fraction extracted by K₂CO₃ and NaOH were initially different, even though copper addition to the reaction medium finally yielded a quite similar lignin type (**Table 1**).

The monomers and dimers pattern structures of lignin remaining in EF-STRAW and NE-STRAW samples after incubation in alkali was determined (**Figure 1**). The case study of Wheat straw and that of Spruce wood were considered separately, as the specific delignification of NE-STRAW and EF-STRAW by alkalis does not occur in EF-WOOD (**Table 1**).

The treatment of Wheat straw by hydroxide and carbonate increased the amount of monomers linked by alkyl–aryl ether bonds in NE- and EF-STRAW and released by thioacidolysis (**Table 3**). This phenomenon was designated as the unmaking of some lignin structures not accessible to the reactants used for analysis, probably through opening of the cell wall structure (17). Mean comparisons by the ANOVA method of the rough data from **Table 3** showed that carbonate or hydroxide treatments could explain 72% of all variations recorded in all samples analyzed ($\eta^2 = 0.72$ for cumulated G plus S structures). The

impact of copper was also significant, but less predominant than alkali ($\eta^2 = 0.29$). Nevertheless, in paired comparison, the unmasking of lignin structures by copper in NE-STRAW incubated in carbonate was shown to be important (+40%; see **Table 3**). As discussed above, this could be related to the increased stability of copper complexes formed with specific extractives from NE-STRAW that allow better oxidative catalysis of the substrate. In this case, a lignin depolymerization by copper could be at the origin of the apparent unmasking of lignin structures.

The modification of the lignin structure in NE-STRAW by carbonate and copper was further confirmed semiquantitatively through GC-MS analysis of dimeric structures released by thioacidolysis from the polymer (**Table 4**, see also **Figure 1** for structures). The yield in dimers increased similarly in NE-STRAW treated by carbonate or carbonate plus copper, relative to the control Ref-NE-STRAW.

Spruce Wood. The results obtained on Spruce wood were different from NE-STRAW and EF-STRAW. First of all, as expected, no lignin was removed from Spruce wood by alkali under these conditions, even in the presence of copper (**Table 1**). The carbonate buffer or sodium hydroxide had no statistically significant impact on the amount of alkyl–aryl ethers structures

Table 7. Composition of Spruce Wood in Polysaccharides

	arabinose ^a	galactose ^a	glucose ^a	xylose ^a	mannose ^a	galacturonic acid ^b	glucuronic acid ^b
ref EF-WOOD	1.1 ± 0.1	1.6 ± 0.4	51.0 ± 7.1	3.9 ± 0.7	12.5 ± 1.7	19 ± 1	39 ± 0.3
treatment (1) ^c EF-WOOD+K ₂ CO ₃	1.1 ± 0.2	1.8 ± 0.4	52.4 ± 6.3	3.9 ± 0.2	13.7 ± 1.6	11 ± 1	21 ± 0.4
treatment (2) EF-WOOD+K ₂ CO ₃ + CuAc ₂	1.0 ± 0.2	1.6 ± 0.3	50.2 ± 5.3	3.0 ± 0.5	11.3 ± 1.1	15 ± 3	29 ± 0.3
treatment (3) EF-WOOD+NaOH	1.3 ± 0.02	1.5 ± 0.05	60.1 ± 4	3.5 ± 0.2	11.4 ± 0.4	15 ± 1	31 ± 0.6
treatment (4) EF-WOOD+ NaOH+ CuAc ₂	1.2 ± 0.2	1.5 ± 0.2	56.9 ± 1.6	3.4 ± 0.4	10.9 ± 1.7	16 ± 3	29 ± 0.4

^a In g/100 g of material. ^b In mg/100 g of material. ^c Refer to material and methods for reaction condition.

released by thioacidolysis (**Table 5**). However, copper acetate treatment in carbonate (condition 2) increased this recovery yields by 13% (**Table 5**). As a confirmation of the specific action of copper in carbonate, the total amount of C–C linked dimers recovered after thioacidolysis increased also by 33% (**Table 5**, condition 2), whereas a decrease was obtained in all other conditions used for reaction. Multivariate analysis from rough data of **Table 5** confirmed that copper was the predominant factor that explains structure variations in lignins, particularly at the level of 4–O–5, β -1 and β -5 structures ($F = 9.675$, $p = 0.006$). Indeed, the alkali treatment alone (K₂CO₃ and NaOH) could not be significantly associated with dimer structure variation ($F < 0.050$, $p > 0.80$). This suggests that the unmasking phenomenon in Spruce wood could be related to the partial depolymerization of lignin by copper in alkali, at the level of C–C linked structures.

Content of Cellulosic, Hemicellulosic, and Pectic Polysaccharides. The multivariate analysis of the raw data from **Tables 6** and **7** indicated that K₂CO₃ or NaOH treatments had no significant impact on the main neutral sugar composition of all of the substrates analyzed (wheat straw and spruce wood; $F < 1$, $p > 0.5$). The only exception was Glucose in EF-WOOD, where a higher recovery of cellulose monomers after analysis was measured (**Table 7**; $F = 3.445$, $p = 0.045$). At the opposite, a clear impact of the alkali on Glucuronic acid structures was observed, either in straw and wood (**Tables 6** and **7**). Finally, in all cases, the presence of copper in the reaction media was not a discriminatory factor that induced a variation of the mean compositions in sugars significantly above the random background noise (F value not significant).

Thus, Glucuronic acid removal from wheat straw was the predominant effect of hydroxide or carbonate treatments on polysaccharides in all samples analyzed. This could be the consequence of the delignification of the EF-STRAW and NE-STRAW by alkali (see above and ref 17), but not in the case of Spruce wood, where no delignification was obtained. Therefore, it seems that this class of molecules, probably coming from hemicellulose in wheat straw and from pectinic material in wood, were dissolved independently from the other classes of cell wall constituents.

CONCLUSIONS

This study has demonstrated that abiotic modification of cell wall structures occurred by maceration in mild alkali, mainly at the level of the lignin polymer. The unmasking of some lignin structures in Wheat straw was confirmed both at the level of β -O-4 linked monomers and of C–C linked dimers. This

effect was not observed in Spruce wood in the absence of copper salts and where no delignification occurred in alkali. This lead to the conclusion that the extraction of the alkali soluble lignin fraction in Wheat straw is at the origin (or is, at least, a prerequisite) of this phenomenon.

Copper acetate was shown to have primarily an impact on the lignin structures of Spruce wood, as evidenced both at the level of β -O-4 linked monomers and of C–C linked dimers. In Wheat straw, no supplementary effect over alkali could be obtained with copper salts, indicating that lignin in situ was not a target for the oxidative copper species. The repolymerization of the lignin extracted from Wheat straw was, however, strongly suggested.

These oxidative copper species, only characterized through their ability to oxidize guaiacol in vitro, were assumed to be formed through associations of the metal with extractives from the cell walls. How these complexes are stabilizing copper in alkali to oxidize solubilized Wheat straw lignins and how they can diffuse in plant cell wall to oxidize Spruce lignin structures in situ remains to be discovered.

In conclusion, the data presented suggested that plant residues buried in calcareous soils contaminated by copper salts could be abiotically destructured, provided maceration in a water or a damp phase can occur. The occurrence of this phenomenon evidenced in vitro remains now to be demonstrated and validated in field or in soil tests.

ACKNOWLEDGMENT

We wish to thank O. Vitrac, from the UMR FARE EMOA Team, for useful information on the handling of data for multivariate analysis.

LITERATURE CITED

- Sevchenko, S. M.; Bailey, G. Life after death: lignin–humic relationship reexamined. *Crit. Rev. Environm. Sci. Technol.* **1996**, *26*, 95–153.
- Shindo, H.; Huang, P. M. Role of MnIV oxide in abiotic formation of humic substances in the environment. *Nature* **1982**, *298*, 363–365.
- Miltner, A.; Zech, W. Beech Leaf Litter Lignin Degradation and Transformation As Influenced By Mineral Phases. *Org. Geochem.* **1998**, *28*, 457–463.
- Kaschl, A.; Römhelda, V.; Chenb, Y. The influence of soluble organic matter from municipal solid waste compost on trace metal leaching in calcareous soils. *Sci. Total Environ.* **2002**, *291*, 45–57.

- (5) Delas, J.; Dartigues, A. Exemples de problèmes régionaux. II Le sud-ouest. *Ann. Agronom.* **1970**, *18*, 17–29.
- (6) Feitknecht, W.; Schindler, P. Solubility constant of metal oxides, metal hydroxides, and metal hydroxide salts in aqueous solution. *Pure Appl. Chem.* **1963**, *6*, 130–199.
- (7) Hultom, T.; Lonnberg, B.; Laxen, T.; Elsakhawy, M. Alkaline pulping of cereal straw. *Cellul. Chem. Technol.* **1997**, *31*, 65–75.
- (8) Bjerre, A. B.; Olesen, A. B.; Fernqvist, T.; Ploger, A.; Schmidt, A. S. Pretreatment of wheat straw using combined wet oxidation and alkaline hydrolysis resulting in convertible cellulose and hemicellulose. *Biotechnol. Bioeng.* **1996**, *49*, 568–577.
- (9) Sun, R.; Lawther, J. M.; Banks, W. B. Influence of alkaline pretreatments on the cell wall components of wheat straw. *Ind. Crop Prod.* **1995**, *4*, 127–145.
- (10) Merdy, P.; Guillon, E.; Aplincourt, M.; Dumonceau, J.; Vezin, H. Copper Sorption on a Straw Lignin: Experiments and EPR Characterization. *J. Colloid Interface Sci.* **2002**, *245*, 24–31.
- (11) Bryant, P. S.; Edwards, L. L. Cation exchange of metals on kraft pulp. *J. Pulp Pap. Sci.* **1996**, *22*, J37–J42.
- (12) Lapierre, L.; Paleologou, M.; Berry, R. M.; Bouchard, J. The limits of metal removal from kraft pulp by acid treatment. *J. Pulp Pap. Sci.* **1997**, *23*, J539–J542.
- (13) Schramel, O.; Michalke, U. B.; Kettrup, A. Study of the copper distribution in contaminated soils of hop fields by single and sequential extraction procedures. *Sci. Total Environ.* **2000**, *263*, 11–22.
- (14) Lapierre, C.; Monties, B.; Rolando, C. Preparative thioacidolysis of spruce lignin: isolation and identification of main monomeric products. *Holzforschung* **1986**, *40*, 47–50.
- (15) Santimone, M. Titration studies of guaiacol oxidation by horseradish peroxidase. *Journal canadien de biochimie* **1975**, *53*, 649–657.
- (16) Lewis, N. G.; Razal, N. G.; Yamamoto, E. Lignin degradation by peroxidases in organic media. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7925–7927.
- (17) Durot, N.; Gaudard, F.; Kurek, B. The unmasking of lignin structures in wheat straw by alkali. *Phytochemistry* **2003**, *63*, 617–623.
- (18) Effland, M. J. Modified procedure to determine acid-insoluble lignin in wood and pulp. *Tappi J.* **1977**, *60*, 143–144.
- (19) Monties, B. Dosage de la lignine insoluble en milieu acide: influence du prétraitement par hydrolyse acide sur la lignine Klason. *Agronomie* **1984**, *4*, 387–392.
- (20) Rolando, C.; Monties, B.; Lapierre, C. Thioacidolysis. In *Methods in Lignin Chemistry*; Lin, S. Y., Dence, C. W., Eds.; Springer-Verlag: Berlin Heidelberg, 1992; pp 334–349.
- (21) Lapierre, C.; Monties, B.; Rolando, C. Thioacidolysis of diazomethane-methylated pine compression wood and wheat straw in situ lignins. *Holzforschung* **1988**, *42*, 409–411.
- (22) Lequart, C.; Kurek, B.; Debeire, P.; Monties, B. MnO₂ and oxalate: an abiotic route for the oxidation of aromatic components in wheat straw. *J. Agric. Food Chem.* **1998**, *46*, 3868–3874.
- (23) Lapierre, C.; Pollet, B.; Monties, B. Thioacidolysis of spruce lignin: GC-MS analysis of the main dimers recovered after Raney Nickel desulfuration. *Holzforschung* **1991**, *45*, 61–68.
- (24) Lapierre, C.; Pollet, B.; Rolando, C. New insights into the molecular architecture of hardwood lignins by chemical degradative methods. *Res. Chem. Intern.* **1995**, *21*, 397–412.
- (25) Blakeney, A. B.; Harris, P. J.; Henry, R. J.; Stone, B. A. A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohyd. Res.* **1983**, *113*, 291–299.
- (26) de Ruiter, G. A.; Schols, H. A.; Voragen, A. G. J.; Rombouts, F. M. Carbohydrate analysis of water soluble uronic-acid-containing polysaccharides with high performance anion exchange chromatography using methanolysis combined with TFA hydrolysis is superior to four other methods. *Anal. Biochem.* **1992**, *207*, 176–185.
- (27) Cathala, B.; Chabbert, B.; Joly, C.; Dole, P.; Monties, B. Synthesis, characterisation, and water sorption properties of pectin-dehydrogenation polymer (lignin model compound) complex. *Phytochemistry* **2001**, *56*, 195–202.
- (28) Scalbert, A.; Monties, B.; Lallemand, J. Y.; Guitet, E.; Rolando, C. Ether linkage between phenolic acids and lignin fractions from wheat straw. *Phytochemistry* **1985**, *24*, 1359–1362.
- (29) Iiyama, K.; Lam, T. B. T.; Stone, B. Phenolic acid bridges between polysaccharides and lignin in wheat internodes. *Phytochemistry* **1990**, *29*, 733–737.
- (30) Durot, N. Déstructuration des parois végétales par le carbonate et les métaux de transition: une étude modèle sur la biodégradation abiotique de la paille en sols de craie et sur l'incorporation de pesticides aux lignines. Thèse de Doctorat, Université de Reims Champagne Ardenne, # 2002REIMS003, <http://www.sudoc.abes.fr/>, 2002.
- (31) Kurek, B.; Monties, B. Influence of spruce colloidal lignins on the veratryl alcohol oxidation by the lignin peroxidase of *Phanerochaete chrysosporium*. In *Biotechnology in the pulp and paper industry*; Kuwahara, M., Shimada, M., Eds.; UNI publishers CO: Kyoto, 1992; pp 309–314.
- (32) Lapierre, C.; Jouin, D.; Monties, B. On the origin of the alkali solubility of gramineae lignins. *Phytochemistry* **1989**, *28*, 1401–1403.

Received for review February 25, 2003. Revised manuscript received November 6, 2003. Accepted December 1, 2003. This work was supported by a grant from "EuropolAgro" #98A02.

JF030132M