



# Characterisation of archaeological wood: A case study on the deterioration of a coffin

Claudia Crestini <sup>a,\*</sup>, Nesrin M.N. El Hadidi <sup>b</sup>, Giuseppe Palleschi <sup>a</sup>

<sup>a</sup> Dipartimento di Scienze e Tecnologie Chimiche, Tor Vergata University, Via della Ricerca Scientifica 00133, Rome, Italy

<sup>b</sup> Conservation Department, Faculty of Archaeology, Cairo University, Giza 12613, Egypt

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## ABSTRACT

An advanced strategy for the improvement of conservation techniques applied to archaeological wood requires a deep insight in its structure. The authors report here a new approach to the structural characterisation of ancient wood. A sample of an ancient Egyptian artefact was studied by means of scanning electron microscopy, quantitation of the residual amount of lignin and carbohydrates. Its residual lignin was isolated and characterised by <sup>31</sup>P-, <sup>1</sup>H-NMR and 2D-Homonuclear NMR HOHAHA techniques. These analyses allowed the description of structural details of the ancient wood.

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## 1. Introduction

The study of archaeological wood is to date focusing an increasing interest due to a novel awareness of the relevance of the valorization of cultural heritage. The chemical characterisation of archaeological wood is of paramount importance in order to elucidate both the nature of degradation processes in archaeological wood samples and to design and develop new consolidation and conservation techniques for ancient wooden artifacts. Little is known about the chemical transformations of the different wood components during ageing. Under favorable conditions archaeological wood samples survived in good conditions, however bacteria can slowly degrade wood for centuries and heavily affect the cellulose content [1] yielding a residual wood characterised by a high degree of fragility and softness. This is due to the loss of cellulose and hemicellulose, the degradation of lignin and the alteration of low molecular weight extractable compounds. The studies dealing with the analysis of ancient wood have been mainly focused on the use of scanning electron microscopy (SEM) [2], FT-IR spectroscopy [3] solid state <sup>13</sup>C-NMR CP-MAS spectroscopy [4,5] pyrolysis GC-MS spectrometry of volatile wood extractives [6–8] and X-ray diffraction [9]. SEM of wood allows the identification of the nature of microbiological attack that occurred by the characterisation of the different morphological characteristics of the residual cell walls [2]. FT-IR spectroscopy with the aid of deuterium exchange method allows to clarify the ageing process of archaeological wood as a change in the state of order on a macromolecular structural level. X-ray diffraction is a technique that

can identify the presence of heteroatoms. It was applied at the determination of sulfates and sulphides and sulfur accumulated in waterlogged wood. GC-MS pyrolysis allows the identification of volatile species in ancient wood, while solid state CP-MAS spectrometry was used for the quick estimation of the degree of decay of waterlogged archaeological wood. The cellulose content of archaeological wood samples is usually low as compared to lignin. Despite extensive efforts aimed at elucidating the wood conservation state, little is known about the structure of the residual lignin in archaeological wood [1]. An investigation of this kind is of pivotal relevance in order to point out two main topics. Firstly the elucidation of degradation processes of lignin in archaeological samples may indicate alternative routes to the selection of conservative strategies. Moreover, the structural characterisation of the modification induced by ageing and different environmental conditions may shed light on the pathways of lignin oxidation.

To the best of our knowledge there are only a few reports in literature dealing with the isolation and characterisation of archaeological lignins [7,10]. In this study the isolation of MWL from fossil wood and its analysis by GC-MS pyrolysis was reported.

Available lignin characterisation techniques currently used in pulp and paper processes studies constitute an invaluable tool to elucidate lignin modification processes occurring upon ageing in archaeological wood samples [11]. More specifically, high resolution solution nuclear magnetic resonance techniques have proven to be excellent analytical tools for the structural analysis of lignins. <sup>31</sup>P-NMR, when applied to soluble lignin samples, is a powerful technique which allows the identification and quantitative evaluation of the different phenolic, aliphatic and carboxylic OH groups after a simple process of phosphitylation with a suitable reagent [12–14]. Through <sup>31</sup>P-NMR it is possible to identify the amount of guaiacyl, syringyl, *p*-hydroxyphenyl, aliphatic OH groups belonging to

\* Corresponding author. Tel.: +39 067259 4734; fax: +39 067259 4734.  
E-mail address: [crestini@stc.uniroma2.it](mailto:crestini@stc.uniroma2.it) (C. Crestini).

residual carbohydrates and to lignin itself, and to evaluate the amount of condensed lignin subunits such as diphenyl methane and 5-5' biphenyl groups [12–14]. Recently, the structural characterisation of lignin from archaeological waterlogged wood has been accomplished by means of  $^{31}\text{P}$ -NMR spectroscopy [15]. Invaluable information about the backbone of lignin may come from 2D homocorrelated NMR spectroscopy (HOHAHA). Such technique may provide qualitative evaluation of the kind of lignin interunit bonding of soluble samples [16,17]. Lignin from wheat straw decayed by white-rot fungi was previously studied by means of  $^{31}\text{P}$ -NMR and 2D homo- and heteronuclear-NMR techniques. It was possible to identify the main degradative patterns and elucidate the structural details of the degraded lignin [18]. These protocols could constitute powerful tools to analyse archaeological samples since they can provide direct information on the conservation state of ancient lignins.

We report here the study of the structure of wood and MWL extracted from an ancient coffin present at the Egyptian Museum of the Faculty of Archaeology (registration no. 852) – Cairo University (Egypt). The archaeological sample was taken from a wooden coffin, which dates back to the Graeco–Roman period in Egypt. This coffin was found during the first Egyptian University excavations, which were carried out in Touna El Gebel – Egypt, in 1931. The funerary monument of “Padykam”, scribe of the town Khemenou (*Hermoupolis Magna*) was discovered. Inside his tomb three wooden coffins were found, one of which belonged to his father Dhouty – Iou (Gabra 1932).

## 2. Experimental

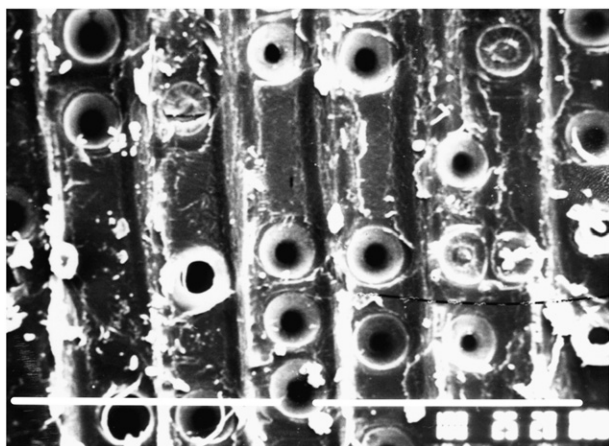
**General:**  $^1\text{H}$ -,  $^{31}\text{P}$ - and 2D-NMR spectra were recorded with a Bruker AM 400 spectrometer. All solvents were of ACS reagent grade and were redistilled and dried according to standard procedures.

### 2.1. Scanning electron microscopy

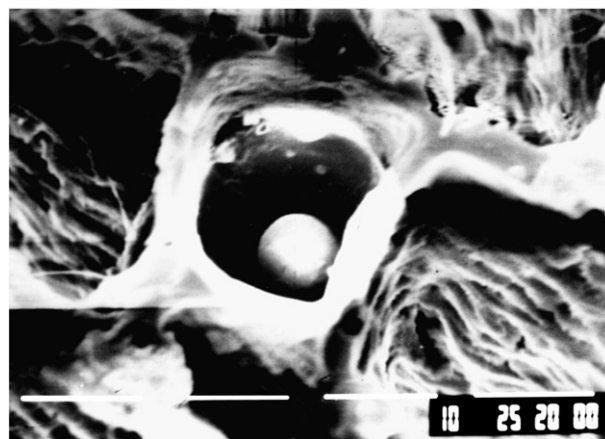
Through sputter coating the samples were covered with gold and studied with a Joel T200 SEM. The coffin conservation state appeared homogeneous from a macroscopic examination. Therefore three wood samples were taken from three distinct regions of the coffin and analysed by SEM analysis. They were found analogous.

### 2.2. Extractives, lignin and carbohydrate contents

Extractives content was determined gravimetrically by standard methods after continuous extraction of lignin with organic solvents. The organic layers were collected and the solvents evaporated under reduced pressure. The dried samples were accurately weighed. Lignin



**Fig. 1.** Longitudinal section in Cypress wood taken from the part of the coffin box “foot area”, which shows tracheids arranged in longitudinal rows, each with a number of bordered pits which are penetrated by fungal mycelium. SEM micrograph bar 100  $\mu\text{m}$ .



**Fig. 2.** Cross section in Cypress wood taken from the inner surface of the coffin “head area”, the inner cavity of the quadrangular tracheids is occupied with dense fungal mycelium. A fungal spore is shown in the inner cavity of the tracheid. SEM micrograph bar.

content in wood samples was determined by the Klason method [19]. Carbohydrate content was determined according to the Nelson method by acidic hydrolysis in sulfuric acid followed by spectrophotometric titration at 500 nm, using a glucose solution as standard [19].

### 2.3. Lignin isolation

Milled lignin was prepared from ultraground extractive free powder according to Bjorkman's procedure. Extractive free powders were ultraground for three weeks in a rotatory ball mill. In the case of lignin extraction from the archaeological wood sample the milling time was reduced to 48 h as explained below. The ML fraction was then extracted with dioxane/water (96:4, v/v). The residue was concentrated under reduced pressure and freeze-dried. Purification was performed by dissolving the lignin in 90% acetic acid. The solution was then added dropwise to stirred water. The precipitated lignin was centrifuged and freeze-dried. It was then dissolved again in a mixture of 1,2-dichloroethane/ethanol (2:1, v/v) and precipitated by the addition of diethyl ether. The purity was confirmed by UV and Klason lignin content measurements.

### 2.4. $^{31}\text{P}$ -NMR analysis

Quantitative  $^{31}\text{P}$  NMR spectra were obtained using methods identical to those described by Argyropoulos et al. [12,13]. The chemical shifts were referenced to phosphoric acid. Derivatization of the lignin samples with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane was performed as described previously. The  $^{31}\text{P}$  NMR spectroscopic data reported in this effort are averages of three phosphitylation experiments followed by quantitative  $^{31}\text{P}$  NMR acquisitions. The maximum standard deviation of the reported data was  $2 \times 10^{-2}$  mmol/g, while the maximum standard error was  $1 \times 10^{-2}$  mmol/g.

### 2.5. Lignin acetylation

Acetylation was carried out suspending 200 mg of lignin in 10 mL of pyridine/acetic anhydride (1:1) at 25 °C for 48 h. HCl (4 M) was then added to obtain pH 3, and the mixture was stirred 12 h. The residue was centrifuged washed with water, centrifuged again, and freeze-dried [19].

### 2.6. Two dimensional NMR spectroscopy (HOHAHA)

Such NMR spectra were acquired at 25 °C using 30 mg of lignin dissolved in 0.6 mL  $\text{CDCl}_3$  on a Bruker 400 MHz spectrometer using a

**Table 1**

Carbohydrates, lignin and extractive contents of ancient coffin wood (AC) and recent *Cupressus sempervirens* (RC).

	AC	RC
Extractives (%)	45	20
Klason lignin (%)	43	35
Sugar content (%)	11	45

10 mm inverse detection probe (DHP). The chemical shifts were reference to TMS. HOHAHA spectra were acquired over a 9.5 ppm window in both  $F_2$  and  $F_1$ , with MLEV-17 spin lock length 80 ms 256 increments were acquired. After  $F_2$  zero-filling, Fourier transformation and squared cosine-bell apodization, the transformed data matrix was  $4096 (F_2) \times 256 (F_1)$  real points.

### 3. Results and discussion

#### 3.1. Scanning electron microscopy

The wood samples that were taken from the coffin box were desiccated and brittle, therefore it was not possible to carry out standard techniques of sampling and optical microscopy, or to make thin-sections in order to identify wood. The only solution was to prepare the samples for scanning electron microscopy by fixing small pieces of appropriate size on stubs with double-sided cellophane tape [20].

The sample was identified as Cypress wood, *Cupressus sempervirens*. Fig. 1 (SEM  $\times 500$ ) shows tracheids arranged in longitudinal rows. The bordered pits are penetrated by fungal mycelium. A higher resolution as in Fig. 2 (SEM  $\times 2000$ ) showed the inner cavity of the quadrangular tracheids, that were covered with dense fungal mycelium. A fungal spore is shown in the inner cavity of this tracheid. These data clearly show that the sample underwent a heavy fungal decay.

Previous microbiological studies of the coffin revealed that the cellulolytic fungi that affected the wood during its years of burial in the soil produce exo- $\beta$ -1,4-glucanase and endo- $\beta$ -1,4-glucanase, which decompose cellulose. The first enzyme is responsible for the initial attack of more highly ordered forms of native cellulose. The latter completes the degradation to the short-chains, cellobiose [21]. The effect of such decay on the wood structure was next studied.

#### 3.2. Extractives, lignin and carbohydrates contents

The study of the residual wood structure proceed with the determination of the extractives, lignin and carbohydrate contents [11,22]. The commonly applied protocols were modified according to the lower strength of the ancient wood. The sample was ball milled. However, in view of the low mechanical strength of the residual wood, the milling time was reduced to 48 h. The powdered wood was then

submitted to continuous extraction with organic solvents. By this means it was possible to isolate the low molecular weight constituents coming from the wood decomposition (Table 1) [23].

On the residual wood the quantitative analysis of carbohydrate and lignin content was performed by acid hydrolysis. The carbohydrate content was estimated by a spectrophotometric technique, while the lignin amount was calculated as Klason insoluble and soluble lignin [11].

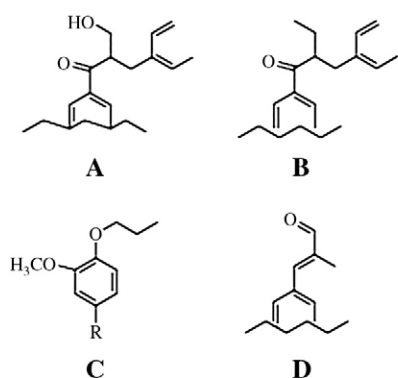
In order to clarify the nature and extent of modifications induced by ageing and fungal attack on the coffin wood, a recent sample of *C. sempervirens* was studied as reference. Data from the ancient coffin wood (AC) were compared to those obtained from the recent sample (RC). Thus standard procedures were employed for the isolation of lignin, extractives, carbohydrate and lignin contents. Scheme 1 shows the results obtained. As expected from ageing processes, the extractives content was found substantially increased in the archaeological sample AC from 20 to 45%. This indicates an extensive degradation of the original biopolymers. The carbohydrate content was found to be decreased from 45 to 17%, while the total lignin content increased from 35 to 43%. These data are in accord with a microbiological attack by a cellulolytic microorganism, and confirm the substantial stability of lignin to biodegradation.

#### 3.3. Characterisation of ancient lignin

The attention was next turned to the structural characterisation of the residual ancient lignin. The lignin was isolated according to standard procedures [19]. The presence of fungal body did not interfere with the NMR results. In fact it cannot be dissolved in dioxane and was not extracted during lignin isolation. This is also shown by the absence of NMR signals of nitrogen containing species that at  $^{31}\text{P}$ -NMR show typical chemical shift.

Fig. 3 shows the  $^1\text{H}$ -NMR spectra of the ancient (Fig. 3A) and recent (Fig. 3B) lignin samples. Typical lignin signals such as the methoxy groups, acetylated aliphatic and phenolic OH groups, carboxylic acids and aldehyde end units were detected in the  $^1\text{H}$ -NMR spectrum of the ancient lignin. Between 3.5 and 6 ppm overlapped peaks from lignin side chains occurred. Noteworthy, intense aliphatic signals were found between 1.2 and 1.7 ppm. These signals were more intense than other common lignin signals.

Aliphatic substructures are found commonly in lignins in low amounts. Their origin is still unclear. They have been attributed to the presence of fatty acid contaminants [24] or to the occurrence of reduction steps during overall lignin oxidations, i.e. in disproportionation processes. Such process may in principle occur on the aliphatic side chain, or after cleavage of the aromatic ring by decarboxylation processes of muconate derivatives. This hypothesis is supported by the common detection of these signals in lignins submitted to oxygen delignification [25], hydrogen peroxide oxidation catalyzed by metal-porphyrins [26] and laccase oxidations [18]. In this study, the lignin



Structure	Chemical shift (ppm)	Chemical shift (ppm)
A	4.27	5.24
	4.14	5.24
	4.27	4.12
B	5.31	1.99
	5.33	1.28
	5.33	1.99
C	4.06	0.95
	4.04	1.30
	4.04	1.58
D	6.94	2.26
	7.09	2.29

**Scheme 1.** Lignin substructures and chemical shifts identified by 2D-NMR in lignin isolated from the Graeco-Roman coffin box of "Dhouty-lou".

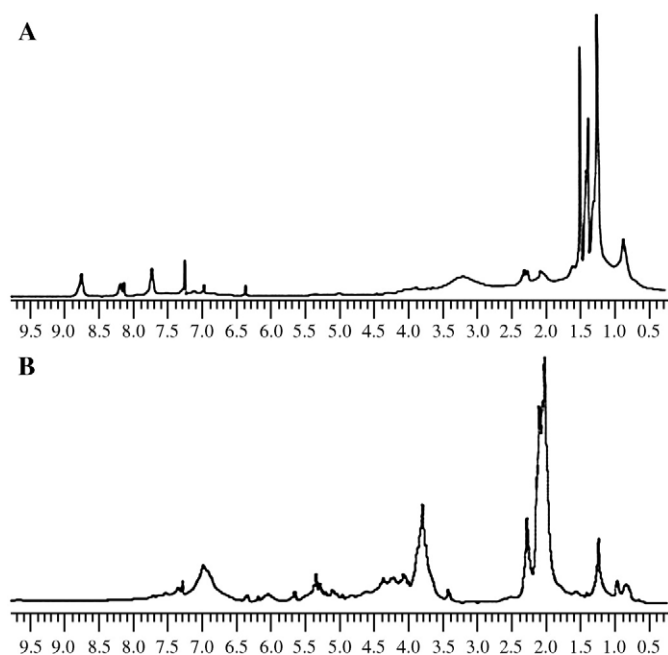


Fig. 3.  $^1\text{H}$ -NMR of acetylated lignin from the Graeco-Roman Coffin box of "Dhouty-lou" (A), and from a modern *Cupressus* wood (B).

was subject to exhaustive solvent extraction before the acetylation and NMR analysis. It is interesting to note that strong aliphatic resonances were also revealed in lignins from wheat straw after extensive fungal decay. In that cases the resonances were assigned by means of  $^{31}\text{P}$ -NMR and 2D-NMR to reduced phenylpropanoic chains [18]. Thus it seems not likely that such peaks are due to low molecular weight contaminants. Another interesting feature of this spectrum is the occurrence of sharp resonances in the aromatic proton region. This leads to hypothesize a structure composed by short oligomeric chains rather than a true polymeric lignin backbone. However the overall aspect of the spectrum still reminds the lignin one.

With the aim to characterise in more detail the structure of wheat lignin and to identify the nature of the observed signals by establishing the complete spin systems responsible for those signals, a 2D homocorrelated NMR experiment (HOHAHA), providing enhanced spin lock lengths, enough to detect the full spin system, was performed on samples of acetylated lignins.

Scheme 1 represents the different substructures identified from the HOHAHA spectrum of the aliphatic region of the lignin isolated from the ancient coffin. As expected from the  $^1\text{H}$ -NMR the aliphatic region shows several strong correlations. More specifically, four different spin systems, A–D containing aliphatic signals were identified and tentatively assigned. Surprisingly the lignin backbone was found still intact with the presence of signals representative of partially oxidised  $\beta$ -O-4 interunit bondings as the main binding pattern. In fact the spin system A belongs to  $\alpha$ -carbonyl- $\beta$ -O-4 arylether units A (Scheme 1). Traces of phenyl coumarane units (not shown) were also found although at a very low extent. Correlation peaks B correspond to  $\alpha$ -carbonyl- $\beta$ -O-4 arylether units with a methyl  $\gamma$ -carbon B. The presence of aliphatic side chain is also found in correlation peaks C. Furthermore, cinnamyl units were identified in

correlations D. The correlations C and D were previously identified in white-rotted lignin [18]. One could hypothesize that fungal degradation of lignin may occur by common reaction pathways. The correlation peaks referred to the aliphatic side chain confirmed the extensive occurrence of aliphatic reduced lignin side chains as previously shown by the  $^1\text{H}$ -NMR.

A  $^{31}\text{P}$ -NMR analysis of the ancient lignin sample and of a recent reference wood after suitable phosphorylation, allowed to characterise in detail the modifications induced by ageing on the distribution of the aliphatic and phenolic OH groups present in the biopolymer.

Table 2 shows the distribution of OH groups in the ancient and recent lignin samples. The absence of peaks in the syringyl region (142–143 ppm) confirms that the sample belongs to a softwood species. The aliphatic OH groups were found substantially decreased in the decayed wood, indicating the occurrence of side-chain oxidation reactions, oxidative coupling processes, and/or disproportionation processes. These data confirm the occurrence of oxidised  $\beta$ -O-4 subunits shown by the 2D-NMR spectrum and the high amount of aliphatic protons found in the proton spectra. The phenolic guaiacyl and *p*-OH groups that are present in significant amounts in the recent sample, were found to be highly decreased (Table 2) in the ancient sample. This distribution suggests the occurrence of oxidative coupling reactions, and/or aromatic ring cleavage processes. Thus a heavy degradation of the original lignin structure, leading to a residual lignin with a substantial lower content of phenolic and aliphatic OH groups occurred.

Diphenylmethane and biphenyl lignin subunits are usually generated upon lignin oxidation processes. Information about condensed subunits that cannot be detected by 2D-NMR is conveniently evidenced from absorbances in  $^{31}\text{P}$ -NMR spectra. In accord with the described behaviour, the content in condensed OH groups was found to be increased (Table 2). This is expected from oxidative coupling reactions. Interestingly, among the condensed phenolic units, the diphenylmethane units were increased, while 5-5' biphenyl units were decreased. Table 2 shows also the carboxylic acid content. They were increased by more than 250%, as a result of oxidative processes.

#### 4. Conclusions

The wood of the ancient coffin was heavily affected by microbiological ageing. A fungal cellulolytic attack in the past during the burial years was the major reason for the degradation of the coffin, with the consequent decrease in carbohydrate content. However the lignin structure was subjected to an extensive degradation as shown by the high amount of low molecular weight extractive compounds detected. The structural analogies of this lignin with fungal decayed lignin suggest the occurrence of analogous degradation pathways. The lignin was found to be constituted by a lower content of aliphatic and phenolic OH groups. Side-chain oxidation processes occurred, yielding to the formation of diphenylmethane and aliphatic subunits. The lignin side chains were extensively oxidised and an increase of carboxylic acids is evident. Aromatic ring cleavage reactions might also have occurred. Finally, the residual side chains showed a largely reduced content in hydroxy and alkoxy groups with respect to native lignin i.e. they are mainly non-oxygenated hydrocarbon chains. The residual lignin has a structure substantially different from the recent sample: it is more condensed and more recalcitrant to further

Table 2

Distribution of OH groups in the ancient (AC) and recent (RC) lignin samples as revealed by quantitative  $^{31}\text{P}$ -NMR spectroscopy.

	Aliphatic OH <sup>a</sup>	Guaiacyl OH <sup>a</sup>	<i>p</i> -hydroxyphenyl OH <sup>a</sup>	Condensed OH <sup>a</sup>	Diphenylmethane OH <sup>a</sup>	5-5' diphenyl OH <sup>a</sup>	COOH <sup>a</sup>
AC	2.62	0.444	0.078	0.309	0.231	0.004	2.207
RC	4.59	0.733	0.581	0.108	0.099	0.006	0.523

<sup>a</sup> mmol/g of lignin.



degradation. In view of these findings one could plan strategic conservative treatments aimed at mimicking the cellulose missing from the sample.

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