Integrated Approach for the Chemical Characterization of the Whole Wall Cell Components: Application to Archaeological Wood

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ABSTRACT

Alum-treated and untreated archaeological wood samples from the Oseberg collection were investigated by means of an integrated approach based on innovative NMR spectroscopy procedures, gel permeation chromatography and analytical pyrolysis coupled with gas chromatography/mass spectrometry (Py-GC/MS). The three-dimensional network that links the lignocellulosic components in the wood matrix (cellulose,

hemicelluloses and lignin) makes it impossible to dissolve wood in its native form in conventional molecular solvents, thus limiting the applicability of analytical techniques based on wet chemical pretreatments. We adopted ionic liquids (ILs) as non-derivatizing solvents that provided an efficient dissolution of the wood. Highly substituted lignocellulosic esters were obtained under mild conditions by the reaction of solubilized wood dissolved in ionic liquid with either acetyl chloride or benzoyl chloride. Using an alternative, we also phosphytilated the wood by reaction with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholan. As a result, the functionalized wood developed an enhanced solubility in molecular solvents, thus enabling a complete characterization by means of spectroscopic (2D-HSQC-NMR and ^{31}P-NMR) and chromatographic (Gel Permeation Chromatography) techniques.

In addition, Py-GC-MS was used for the analysis of the archaeological samples, without any sample pretreatment, in order to investigate the degradation undergone by the lignocellulosic components on the basis of their pyrolysis products. The application of these combined techniques to the Oseberg wood samples enabled the results to be compared and integrated. This also led to the chemical characterization of the whole wall cell and the evaluation of its state of preservation. The high depletion of carbohydrates and high extent of lignin oxidation were highlighted in the alum-treated objects, whereas a good preservation state was found for the untreated wood of the Oseberg ship.

Keywords: Waterlogged archaeological wood; Ionic liquid; NMR; GPC, Py(HMDS)-GC/MS

1. INTRODUCTION

The Oseberg find (834 AD) was uncovered in 1904 near Oslo in Norway. It was a burial mound made for two women of high standing. In addition to the Oseberg ship and a number of metal artefacts and textiles, a collection of wooden objects was recovered, comprising a ceremonial wagon, three sleds and animal head posts, cooking kits, weaving tools and looms, agricultural implements, and ship gear [1]. This collection represents one of the richest, most complete collections of Viking wooden objects in the world, most of which is exhibited at the Viking Ship Museum in Oslo, Norway. At the beginning of the 20th century, most of these wooden objects were treated with alum ($KAl(SO_4)_2 \cdot 12H_2O$), aimed at strengthening the wooden structure [2]. After a hundred of years, most of the treated objects have undergone dramatic conservation problems: the wood is now highly acidic ($pH \leq 2$) and the structural integrity has been seriously compromised, turning the wood into powder in the worst cases [3].

The *Saving Oseberg* project started in 2014 in order to safeguard the Oseberg artefacts and develop preservation strategies. The research includes a detailed chemical characterisation of the degraded wood and of the inorganic salts, in order to reveal the extent and the causes of the deterioration processes. To date, the degradation of the wood has been found to be directly related to the alum conservation treatment. The release of sulphuric acid and the concentration of Fe and Ca are believed to play a role in the hydrolysis of carbohydrates and oxidation of lignin [3]. Nevertheless, the mechanisms behind the observed deterioration are complex and difficult to fully clarify. The Oseberg case represents a unique example of deteriorated archaeological wood that requires further investigation.

The evaluation of the degradation state of archaeological wood can be carried out using various approaches and analytical instrumental techniques. Of these, methods have been developed using FTIR, NMR and Py-GC/MS, which have proven to provide detailed molecular information on the organic components of archaeological wood [4-6].

The best results to elucidate the structure of archaeological woods were summarised in an exhaustive overview regarding the most promising ^{13}C high-resolution solid-state NMR techniques published by Bardet [7]. Unfortunately, solid state ^{13}C-NMR is not sensitive enough to detect some important intermonomeric bonds present in the lignin structure together with important functional groups i.e. carboxylic and alcoholic moieties.

A complete picture of the chemical features of lignin was achieved after the development of liquid NMR analytical tools, such as qualitative and quantitative heteronuclear single quantum coherence (2D-HSQC), quantitative ^{13}C-NMR and ^{31}P-NMR experiments [5, 6, 8, 9].

However, this approach is based on the isolation of lignin from the wood matrix, which may result in some chemical and structural modifications, even when mild conditions are applied during the extraction procedure. Another significant limitation is that as a consequence of the extraction process, this approach does not evaluate the presence of the remaining lignin-carbohydrate complexes (LCCs) [10, 11], which is an important feature in the study of wood degradation [12].

Given that the three-dimensional lignin network that binds lignocellulosic components together makes it practically impossible to dissolve wood in its native form in conventional molecular solvents, a non-derivatizing solvent needs to be found that provides efficient dissolution to various reagents. This would then result in a homogeneous reaction environment suitable for preserve the native structure of the wood for sensitive analyses.

Ionic liquids (ILs) have been used as such solvents [13, 14]. The wood in a homogeneous solution can be easily derivatized without introducing any artefact. Highly substituted lignocellulosic esters can be obtained under mild conditions by reacting wood dissolved in ionic liquid with either acetyl chloride or benzoyl chloride in the presence of pyridine**.** Alternatively, the lignocellulosic material can be phosphitylated by reaction with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane in the presence of pyridine. As a result, the

functionalized wood develops an enhanced solubility in molecular solvents, thus enabling a complete characterization by means of spectroscopic (2D-HSQC-NMR, ^{31}P NMR) [15] and chromatographic techniques (GPC) [16].

2D-HSQC-NMR facilitates a detailed investigation of the chemical structure of archaeological wood samples, by identifying the cross-peak related to polysaccharides (cellulose and hemicelluloses) and to the aliphatic and aromatic domains of lignin. ^{31}P-NMR analyses enable an absolute quantification of several chemical groups, namely aliphatic alcohols, syringyl and condensed phenols (S-OH + cond.), guaiacyl phenols (G-OH), *p*-hydroxycumaryl units (H-OH) and carboxylic acids (COOH).

In addition, highly substituted lignocellulosic esters have been found to exhibit an enhanced solubility in tetrahydrofuran (THF), and a different instrumental response when submitted to GPC-UV analysis. Specifically, benzoylated specimens led to the UV-detection of the whole substrate components, which are cellulose, hemicelluloses, and lignin, regardless of their possible chemical connections. On the other hand, acetylated specimens have been found to account for the sole contribution of LLCs (and possibly free lignin), due to the lack of chromophores in the unbound polysaccharide portion [16, 17]. The GPC-UV analyses of each cellulosic and hemicellulosic fraction have also provided a valuable method for the assessment of LCC-bound polysaccharide nature.

These GPC, 2D-NMR and ^{31}P-NMR analytical techniques were applied here for the first time on archaeological wood from the Oseberg collection. Two samples were alum-treated and one sample was not treated with anything. The whole wood material was extensively characterized, in order to obtain results on the state of preservation of the wood components, in particular modifications of lignin, depolymerisation of cellulose and the structure of lignin-carbohydrate complexes.

These results were integrated and compared with those obtained by Py(HMDS)-GC/MS on non-fractionated samples. In fact analytical pyrolysis, and in particular Py-GC/MS,

provides detailed molecular information on archaeological wood degradation [18, 19]. This technique does not require sample pre-treatment, thereby providing results without altering the sample during preparation. In addition, it requires a very limited amount of sample (50-100 µg), which is always important when sampling precious archaeological artefacts. The *in situ* derivatisation of pyrolysis products using HMDS as a silylating agent is crucial in order to obtain pyrograms with a high resolution and to avoid retention of polar compounds at the Py-GC interface [20, 21].

Py(HMDS)-GC/MS was previously applied to some samples from the Oseberg collection [22] and the alum-treated samples were analyzed without any washing pre-treatment. The samples were ground, homogenized and dried before analysis and the results were interpreted, revealing a high depletion of carbohydrates in the samples and a high level of lignin oxidation. Nevertheless, pyrolysis reactions of organic materials can be influenced by the presence of significant amounts of inorganic compounds in the sample [23, 24], and the possible occurrence of “pyrolytic artefacts” is key when interpreting the results obtained from archaeological wood samples.

In the present work, the samples were washed before Py(HMDS)-GC/MS analysis, in order to remove as much alum as possible. We then compared the results obtained by Py(HMDS)-GC/MS on washed samples from the Oseberg collection with those previously obtained without washing the samples [22]. In addition, a comparison with the results obtained by GPC and NMR methods led to a discussion of the accuracy of information obtained by both these methods.

2. MATERIALS AND METHODS

2.1. Reagents

All reagents were purchased from Sigma-Aldrich and used as received. Xylan, from birchwood, was purchased from Sigma.

2.2. Samples

Fragments of archaeological waterlogged woods were obtained from the Museum of Cultural History, University of Oslo, Norway. The samples named Frag5 and Frag9 were alum-treated in 1904 and were taken from two fragments of the collection with a very poor structural integrity. The wood structure was severely compromised, thus identifying the wood species was challenging. However, analyses at low magnification revealed that the wood was a diffuse porous hardwood, probably birch (*Betula* sp.). The sample named Oseberg was not alum-treated and was taken from the Oseberg ship. The wood species was oak (*Quercus sp.*). Sound oak wood was used as a reference wood material to compare the results obtained by Py(HMDS)-GC/MS.

For the analyses, all the samples were washed with deionized water until neutrality was reached (in order to remove any alum). For Py(HMDS)-GC/MS analysis, the samples were oven-dried for 24 h at 40-50 °C. They were then homogenised and powdered using a ball mill made of zirconium oxide (Pulverisette 23, Fritsch GmbH, Germany) before analysis.

For NMR and GPC analyses, the samples were freeze-dried and ground in a mortar. The wood powder was Soxhlet extracted with *ca.* 250 mL of hexane for 48 h. The oak reference wood powder was Soxhlet extracted with *ca*. 250 mL of an acetone-water solution (9:1) for 48 h. It was then submitted to an alkaline extraction with NaOH (0.0075 mol L^{-1}) for 1 h (liquid-to-wood ratio 50:1) to remove tannins and then dried in an oven at 50 °C until a constant weight was reached. The dry extractive-free wood powders were ground in a planetary ball mill for 24 h at 300 rpm, using a 100 mL zirconium grinding bowl (zirconium dioxide 95%) in the presence of eight zirconium balls (10 mm in diameter each).

2.3. Methods

Our characterization method is shown in Figure 1.

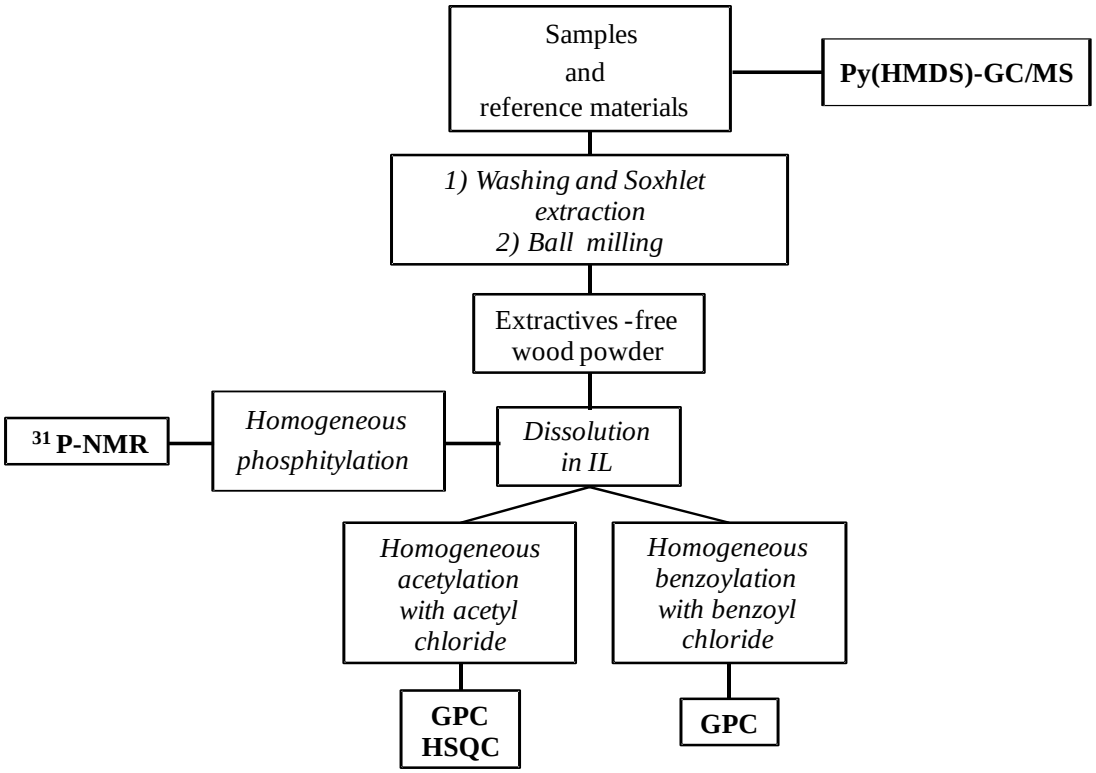

Figure 1. The chemical characterization approach applied in this work.

2.3.1. Lignin isolation

Lignin extraction was performed according to a modification of the milled wood method developed by Holmbom and Stenius [25]. The extractive-free wood powder (400 mg) was refluxed under a nitrogen atmosphere for 2 h in a 0.1 M HCl dioxane-water solution (50 mL, 85:15%), and then allowed to cool to room temperature. The insoluble material left after lignin solubilization was collected by centrifugation (3000 rpm, 15 min). The supernatant was added dropwise into a 0.01 M HCl aqueous solution (250 mL), which was then kept at +4°C overnight to enable complete lignin precipitation. The precipitate was collected by centrifugation (3000 rpm, 15 min), washed with acidified distilled water (pH 2), and freeze dried.

2.3.2. Lignin acetylation

The extracted lignin (50 mg) was acetylated in a pyridine-acetic anhydride solution (1:1 v/v, 4 mL), and kept overnight at 40°C. After stripping with ethanol, toluene, and chloroform (25 mL × 3, each solvent), the sample was dried under vacuum. The acetylated lignin was solubilized in THF for GPC and in DMSO-d_6 for 2D-HSQC-NMR analyses.

2.3.3. Wood benzoylation in ionic liquid

Benzoylation reaction was performed in 1-allyl-3-methylimidazolium chloride ([amim]Cl, 950 mg), on the extractive-free wood powders (50 mg) with benzoyl chloride, as reported by Salanti et al. [16]. The procedure was slightly modified and at the end of the reaction, 200 µL of iodomethane were added and left to react for 15 minutes extra in order to convert the carboxylic acids into methyl esters. The benzoylated wood samples were solubilized in THF (1 mg mL^{-1}) for GPC analysis.

2.3.4. Wood Acetylation in Ionic Liquid

Acetylation reaction was performed in 1-allyl-3-methylimidazolium chloride ([amim]Cl, 950 mg), on the extractive-free wood powders (50 mg) and birch xylans with acetyl chloride, as reported by Salanti et al. [16]. The procedure was slightly modified and at the end of the reaction, 200 µL of iodomethane were added and left to react for 15 minutes extra in order to convert the carboxylic acids into methyl esters. The acetylated wood samples were solubilized in THF (1 mg mL^{-1}) for GPC analysis and in d_6-DMSO for NMR analyses (50 mg mL^{-1}).

2.3.5. ^{31}P-NMR Analyses

1-allyl-3-methylimidazolium chloride ([amim]Cl, 950 mg) was added to ~ 50 mg of pulverized wood material in a 8 mL dried sample bottle equipped with a magnetic stirrer. It was then vortexed and heated at 80°C until the solution was clear (overnight). Pyridine (200 µL, 2.50 mmol) was added in one portion and the sample vortexed until visibly homogeneous (20 s). The sample was allowed to cool to room temperature. Then 2-

chloro-4,4,5,5-tetramethyl-1,3,2- dioxaphospholane (300 µL, 1.89 mmol) was added in one portion and vortexed until a cream-paste was formed (30 s). A pre-prepared deuterated chloroform stock solution of $Cr(acac)_3$ (1 mg/mL, 500 µL) was added in four portions (125 µL each), vortexing between each addition. Finally, an e-HNDI solution (121.5 mM, $CDCl_3$/pyridine 4.5:0.5, 200 µL) was added in one portion and the solution vortexed (30 s). Further dilutions with the $Cr(acac)_3/CDCl_3$ solution were necessary to achieve the complete solubility of the samples (required optimum dilution: 2000 µL). ^{31}P NMR spectra were recorded on 800 µL solutions on a Bruker Avance 500 MHz instrument [15]. The ^{31}P NMR data reported are the average of three experiments. The maximum standard deviation was ±0.02 mmol g^{-1} (P=0.05, n=3).

2.3.6. GPC Analyses

Acetylated lignin, acetylated and benzoylated wood samples were dissolved in THF (1 mg mL^{-1}) and analyzed at a flow rate of 1 mL min^{-1}.

The analyses were performed on a Waters 600 E liquid chromatography system connected to an HP1040 ultraviolet UV detector set at 240 or 280 nm. The injection port was a Rheodyne loop valve equipped with a 20 µL loop. The GP-column system was composed of a sequence of an Agilent PL gel 5 µm, 500 Å, and an Agilent PL gel 5 µm, 10^4 Å. The solvent used was tetrahydrofuran (Fluka 99.8%). PL Polymer Standards of Polystyrene from Polymer Laboratories were used for calibration. The peak molecular weight M_p is defined as the molecular weight of the species with maximum absorbance. M_p values reported are the average of three replicate analyses (M_p: ±100 g mol^{-1}, P = 0.05, n=3).

2.3.7. 2D-HSQC-NMR Analyses

Two-dimensional Heteronuclear Single Quantum Coherence spectra were run in DMSO-d_6 on acetylated wood samples. The inverse detected ^{1}H-^{13}C correlation spectra were measured on a Varian Mercury 400 MHz NMR instrument at 308 K. The spectral width

was set at 5 kHz in F2 and 25 kHz in F1. Altogether 128 transients in 256 time increments were collected. The polarization transfer delay was set at the assumed coupling of 140 Hz and a relaxation delay of 2 s was used. The spectra were processed using Π/2 shifted squared sinebell functions in both dimensions before Fourier transformation.

2.3.8 Py(HMDS)-GC-MS

Analytical pyrolysis was performed using 1,1,1,3,3,3-hexamethyldisilazane (HMDS, chemical purity 99.9%, Sigma Aldrich Inc., USA) as a silylation agent for the *in situ* thermally-assisted derivatisation of pyrolysis products. The instrumentation consisted of a micro-furnace Multi-Shot Pyrolyzer EGA/Py-3030D (Frontier Lab) coupled to a gas chromatograph 6890 (Agilent Technologies, USA) equipped with an HP-5MS fused silica capillary column (stationary phase 5 % diphenyl - 95 % dimethyl-polysiloxane, 30 m × 0.25 mm i.d., Hewlett Packard, USA) and with a deactivated silica pre-column (2 m × 0.32 mm i.d., Agilent J&W, USA). The GC was coupled with an Agilent 5973 Mass Selective Detector operating in electron impact mode (EI) at 70 eV. The pyrolysis temperature was 550 °C and interface temperature was 250 °C. Similar amounts (*ca.* 100 µg) of sample and HMDS (5 µL) were inserted into the platinum cup. Chromatographic conditions were as follows: initial temperature 50 °C, 1 min isothermal, 10 °C min^{-1} to 100 °C, 2 min isothermal, 4 °C min^{-1} to 190 °C, 1 min isothermal, 30 °C min^{-1} to 280 °C, 30 min isothermal. Carrier gas: He (purity 99.995%), constant flow 1.0 mL min^{-1}. After instrumental analysis, the compounds were identified by comparing their mass spectra with spectra reported in the Wiley and NIST libraries or in the literature [27]. Samples were analysed in triplicate. Semi-quantitative calculations were performed as follows: peak areas were normalised with respect to the sum of the peak areas of all identified pyrolysis products for each sample, and the data were averaged and expressed as percentages.

The percentage abundances were used to calculate the H/L ratio (ratio between the sum of holocellulose and lignin pyrolysis products) and the S/G ratio (ratio between the sums of syringyl- and guaiacyl-lignin pyrolysis products). The standard deviations associated with the calculated parameters were also calculated.

3. RESULTS

3.1. ^{31}P-NMR

The ^{31}P-NMR spectra of reference oak lignin, Frag5, Frag9 and Oseberg archaeological wood samples are reported in Figure 2. After specific regions of the spectra (corresponding to aliphatic alcohols, syringyl, guaiacyl, *p*-hydroxycoumaryl phenols and carboxylic acid) had been integrated the various chemical groups were quantified. The quantitation outputs are reported in Table 1. These also include the ^{31}P-NMR data of oak reference wood, as reported in the literature [9].

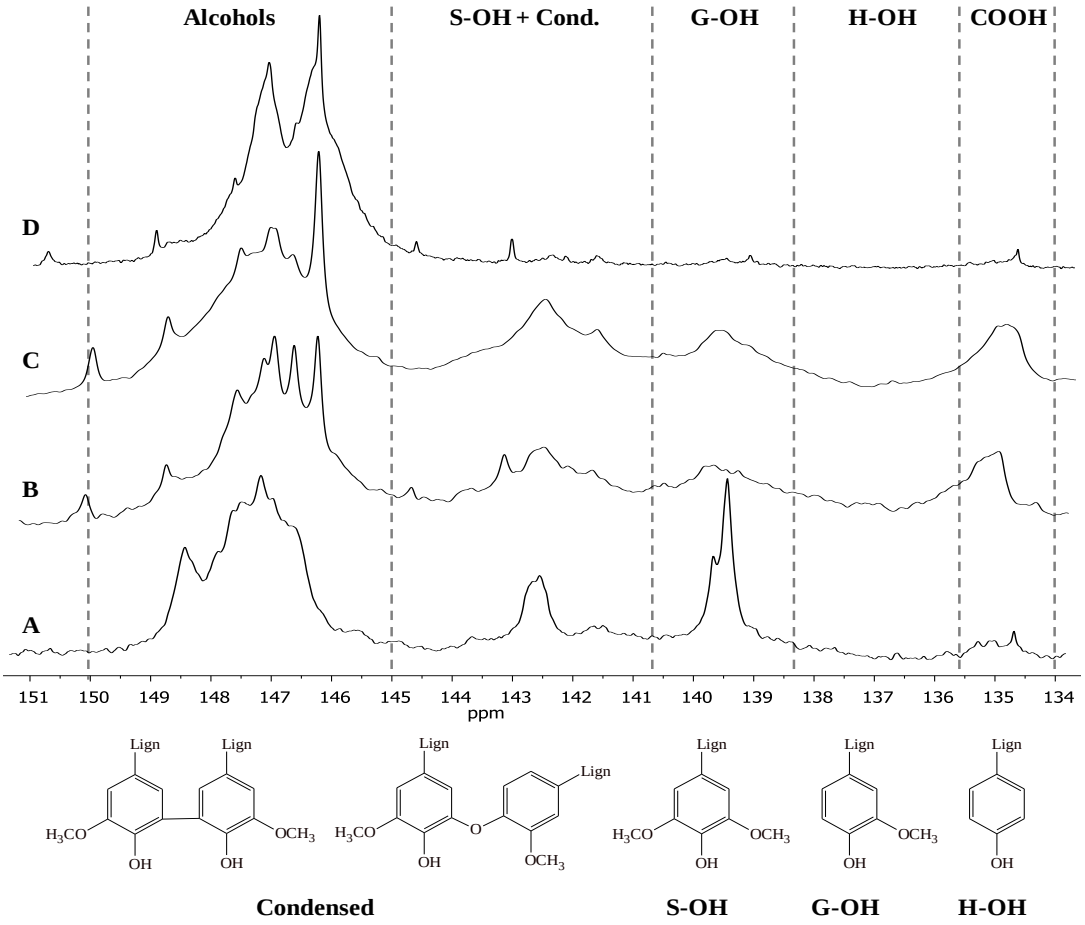

Figure 2. ^{31}P-NMR spectra of reference oak lignin (A), Frag5 (B), Frag9 (C) and Oseberg (D); the structures of condensed (Cond.), syringyl (S-OH), guaiacyl (G-OH) and *p*-hydroxycoumaryl phenol units (H-OH) are reported below.

(mmol g^{-1})	Oak wood Reference	Oseberg (D)	Frag9 (C)	Frag5 (B)	Oak Lignin Reference (A)
Alcohol	-	11.50	1.90	2.42	3.42
S-OH + Cond.	0.18	0.22	0.85	0.95	0.85
G-OH	0.16	0.21	0.44	0.64	0.51
H-OH	nd	nd	0.11	0.15	0.08
COOH	0.09	0.26	0.40	0.49	0.27

Table 1. ^{31}P-NMR data obtained for the oak reference wood [9] Oseberg, Frag9, Frag5 and oak reference lignin samples. Data are expressed in mmol g^{-1}, ± 0.02. The maximum standard deviation was ±0.02 mmol g^{-1} (P=0.05, n=3).

The ^{31}P NMR spectrum of the Oseberg sample (**D**) was dominated by a broad bimodal peak related to the derivatization of primary and secondary hydroxyl groups of cellulose and hemicelluloses. Other peaks pertaining to phenols and carboxylic acids were present in a low abundance. The ^{31}P-NMR spectra of alum-treated archaeological wood samples Frag9 (**B**) and Frag5 (**C**) were similar to that of the reference lignin (**A**), with a distribution of hydroxyl moieties (alcohols, phenols and carboxylic acids) indicating a huge loss in cellulose and hemicelluloses. This was highlighted by the increase in derivatized phenols signal.

Both the spectra of Frag9 and Frag5, especially, were characterized by sharp peaks related to monomeric sugars overlapping the broad peak of the aliphatic hydroxyls group. In addition, the shoulder corresponding to secondary alcohols (148.5 ppm) was less intense than the derivatized reference lignin spectrum, indicating a possible oxidation of hydroxyl groups at the α-position of the β-O-4 structure. Furthermore, the peak related to carboxylic acids (136 ppm) was relatively high for the all archaeological samples (especially for Frag5 and Frag9).

The quantitative results (Table 1) highlighted that the amount of detected phenols in Frag5 and Frag9 was comparable to the reference lignin, whereas the amount of phenols in the Oseberg sample was similar to the sample of reference sound wood. Taken together, the analyses indicated that the application of the alum treatment (Frag5 and Frag9) had resulted in a high loss of polysaccharides and in a considerable increase in carboxylic functionalities.

3.2. GPC: Gel Permeation Chromatography

Benzoylated and acetylated samples were analyzed by GPC-UV at 240 and 280 nm, respectively, to maximize their analytical response. The chromatograms obtained are reported in Figure 3. As previously discussed [16], after benzoylation, polysaccharides and lignin have a similar instrumental response. Therefore, the chromatograms of benzoylated samples are assumed to report the molecular weight distribution of the whole wall cell components (cellulose, hemicelluloses, lignin, and LCCs). On the other hand, acetylated sample chromatograms account exclusively for the molecular weight distribution of those lignocellulosic fractions that naturally contain aromatic moieties (LCCs and free lignin), due to the higher instrumental response observed for lignin compared to free acetylated polysaccharides. Concerning Frag5 and Frag9, a comparison between the benzoylated archaeological wood samples and the corresponding benzoylated sound oak reference specimen showed a significant decrease in the molecular weight of the archaeological samples, above all related to cellulose degradation (i.e., by hydrolysis). Instead, a comparison between the same samples after acetylation suggested the probable degradation of LCCs.

With regard to the Oseberg sample, both the benzoylated (BZ) and the acetylated (AC) samples had a comparable molecular weight distribution compared to the reference oak wood. The numerical outputs (M_p of the molecular weight distribution curve for BZ and AC derivatization) are summarized in Table 2. It should be underlined that the absolute value of the wood molecular weight could not be precisely estimated as a consequence of the use of polystyrenes as the calibration standard. In any case, our main focus is a comparison between archaeological and reference wood samples, which provides reliable indications despite possible errors in the absolute values.

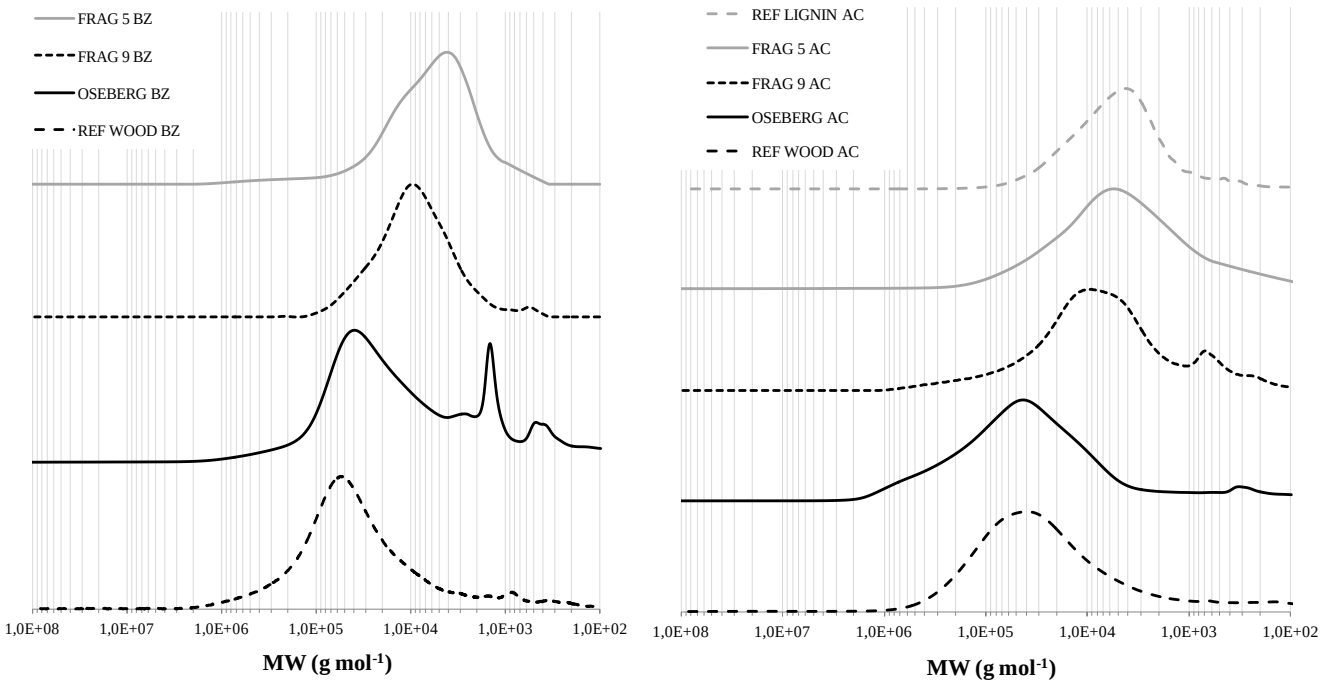

Figure 3. GPC-UV profiles of Frag5, Frag9, Oseberg and reference oak wood samples after benzoylation, (left), and GPC-UV profiles of reference lignin, Frag5, Frag9, Oseberg, and reference oak wood samples after acetylation (right).

(g mol^{-1})	**Mp BZ**	**Mp AC**
Reference oak wood	55800	43700
Oseberg	39700	43200
Frag9	9700	10430
Frag5	3300	6670
Oak reference lignin	-	4200

Table 2. M_p values (g mol^{-1}) of reference oak wood, Oseberg, Frag9, Frag5 and reference lignin samples after benzoylation (BZ) and acetylation (AC). Maximum standard deviation ±100 g mol^{-1} (P = 0.05, n=3).

3.3. 2D-NMR-HSQC

The high solubility achieved after wood acetylation enables the analysis of derivatized wood by means of 2D NMR techniques after dissolution in DMSO-d_6 (Figure 4). As a reference, the HSQC spectra of acetylated oak lignin and acetylated xylans from birch are reported. The interpretation was accomplished by consulting the literature data [26-30]. It was observed that the HSQC spectrum acquired for Frag5 almost matched the spectrum of lignin. All the main intermonomeric bonds were clearly detected: cross-peaks of the β-O-4, β-5 and β-β structure in the aliphatic region (50-90/3.0-6.0 ppm), and cross-peaks of S and G units in the aromatic domain (100-130/6.0-8.0 ppm). In addition, a cross-peak related to methyl esters (-$COOCH_3$) was visible at 60-3.5 ppm, indicating a significant amount of acidic functionalities, also according to the results obtained by ^{31}P-NMR.

The relative intensity of the S' units increased for the archaeological samples. The S' units are related to the oxidation of the C at the α position in β-O-4 type structures. The benzylic position is usually prone to oxidation by H-abstraction through a radical mechanism, due to the aromatic stabilization of the ketyl radical formed [31]. The result of this reaction is a ketone. A high level of degradation was thus observed for Frag5, where hydrolysis (both on cellulose and hemicelluloses) and oxidation (lignin) had changed the wood structure extensively. The situation was quite similar for Frag9, although the peaks related to the sugar moiety (coloured grey in Figure 4) were more visible compared to the Frag5 sample. In particular, X2, X3, X4 and X5 cross-peaks were evident, corresponding to the carbon atoms C2, C3, C4, and C5 in the 4-O-methyl-glucuronoxylan units of hemicelluloses. The X1 cross-peak was not detected. The Oseberg spectrum was dominated by signals arising from cellulose (C1, C2, C3, C4, C5 and C6); but also hemicelluloses (in particular X4 and X5 signals) and lignin (intermonomeric bonds and aromatic units) were clearly detected, indicating a good state of preservation.

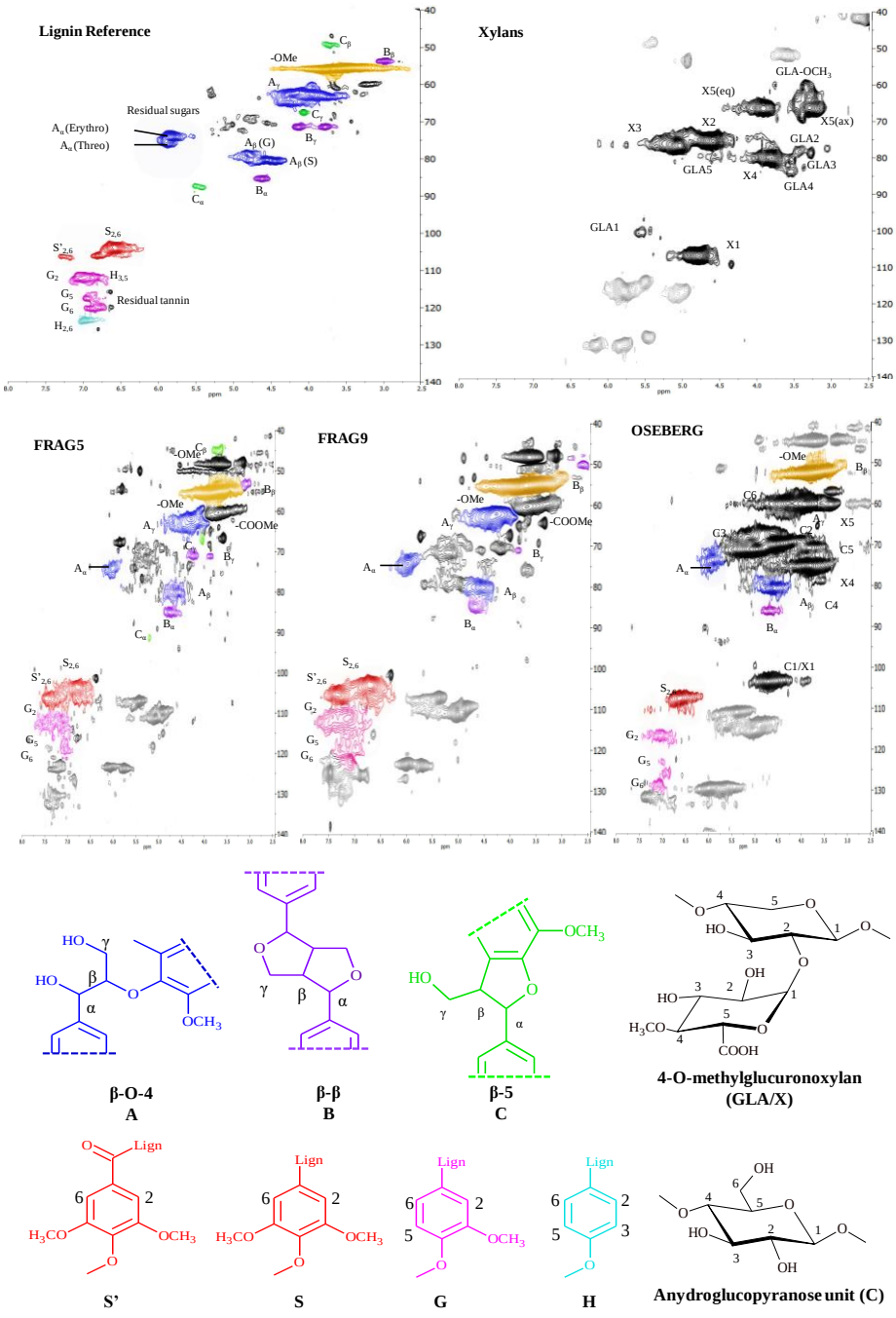

Figure 4. 2D-HSQC-NMR spectra of acetylated oak lignin reference, acetylated xylan from birch, Frag5, Frag9 and Oseberg samples. At the bottom, aliphatic and aromatic lignin structures are reported using different colours; polysaccharide structures are reported in black. A) β-O-4 alkyl-aryl ethers (blue); B) β-β resinols (purple); C) β-5 phenylcoumarans (green); S) syringyl units (red); S') oxidized syringyl units bearing a carbonyl at Cα (red);

G) guaiacyl units (pink); H) p-hydroxycumaryl units (light blue); C_n) anydroglucopyranose units of cellulose; GLA/X) 4-O-methtylglucuronixylan units of hemicelluloses.

3.4. Py(HMDS)-GC-MS

Analytical pyrolysis coupled with GC/MS was applied to the archaeological samples and the oak reference sample without any sample pre-treatment, except for washing, grinding and drying the samples. The pyrograms for the oak reference and the Oseberg sample were similar, showing both holocellulose (cellulose and hemicelluloses) and lignin pyrolysis products with high abundances. The pyrograms for Frag5 and Frag9 samples were dominated by lignin pyrolysis products especially for Frag5, which showed only a few peaks, ascribed to holocellulose pyrolysis products with very low abundances. Figure 5 reports the pyrolytic profiles of the reference oak sample and the Oseberg, Frag9 and Frag5 archaeological samples.

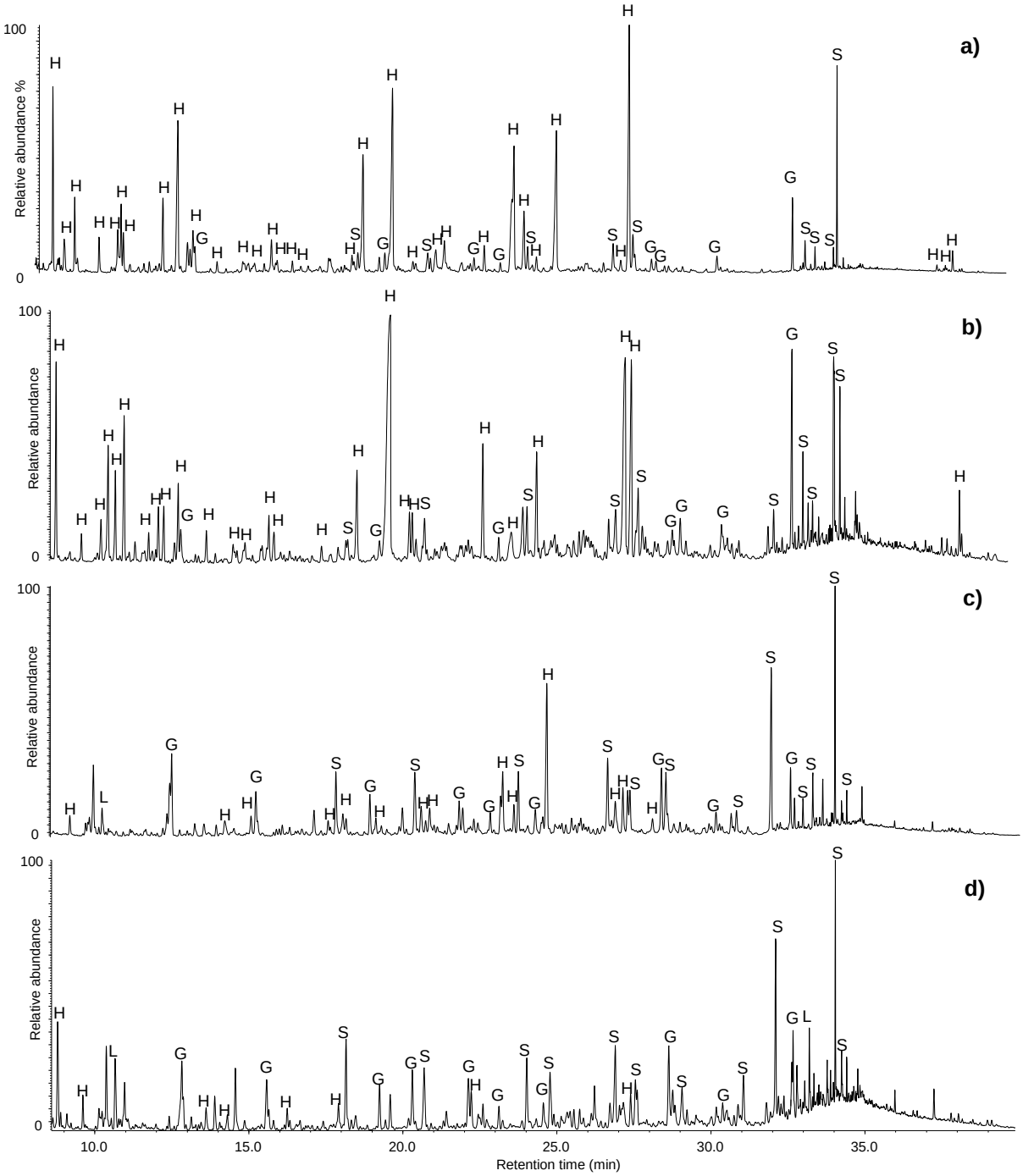

Figure 5. Py(HMDS)-GC/MS chromatographic profiles of **a)** reference oak wood **b)** Oseberg, **c)** Frag9 and **d)** Frag5. Letters refer to the attribution of the pyrolysis products to the corresponding wood component: H = holocellulose; L = lignin; G = guaiacyl-lignin; S = syringyl-lignin.

The pyrolytic H/L (holocellulose/lignin) ratio was calculated for the four samples analyzed and the results obtained for the archaeological samples were compared with those obtained for the sound oak reference sample (Table 3). The pyrolytic H/L ratio is a commonly used parameter to estimate the preferential loss of polysaccharides or lignin in an archaeological wood sample by comparing the value with that obtained for sound wood [4, 32]. This semi-quantitative calculation highlighted that the Oseberg sample had undergone a loss of holocellulose of around 10% (H/L 2.2 ± 0.2), confirming its very good state of preservation, whereas the loss of holocellulose for Frag9 and Frag5 was much higher, leading to very low H/L ratios (0.4 ± 0.1 and 0.1 ± 0.0, respectively). These results were in agreement with those obtained by other techniques, especially 2D-HSQC, which enabled us to measure a slightly better preservation of carbohydrates in Frag9 than in Frag5.

The pyrolytic S/G (syringyl/guaiacyl lignin) ratio was also calculated. This parameter is known to provide information on the preferential degradation of syringyl or guaiacyl units in the lignin network involving the methoxy groups [19, 33, 34]. The results showed that the Oseberg sample had a lower S/G ratio (1.4 ± 0.1) compared to the reference oak wood sample (1.9 ± 0.1), indicating that, despite the good preservation of carbohydrates, some chemical changes in the lignin had occurred. This is a common situation when wood degradation is at a low/medium extent, since syringyl moieties are less stable than guaiacyl moieties [35]. For Frag5 and Frag9, the S/G ratios (2.3 ± 0.4 and 2.2 ± 0.3, respectively) were comparable to those obtained for the reference wood. This result should be interpreted as the comparable degradation of both guaiacyl and syringyl components of lignin in the alum-treated archaeological samples.

Table 3. Semi-quantitative results obtained by Py(HMDS)-GC/MS. H/L ratios, S/G ratios and percentage abundance of categories of lignin pyrolysis products for the oak reference, Oseberg, Frag9, and Frag5 samples.

	Oak Reference wood	Oseberg	Frag9	Frag5
H/L	3.7 ± 0.3	2.2 ± 0.2	0.4 ± 0.1	0.1 ± 0.0
S/G	1.9 ± 0.1	1.4 ± 0.1	2.2 ± 0.3	2.3 ± 0.4
Categories of lignin pyrolysis products (%)				
Monomers	29.1 ± 1.6	31.7 ± 1.5	21.5 ± 1.7	14.6 ± 0.6
Short chain	9.2 ± 0.2	10.3 ± 0.3	21.5 ± 3.2	26.3 ± 1.2
Long chain	16.0 ± 0.8	11.4 ± 0.6	6.1 ± 0.8	8.4 ± 0.3
Carbonyl	13.1 ± 0.4	11.1 ± 0.3	19.8 ± 1.0	18.4 ± 0.2
Carboxyl	3.7 ± 0.3	4.1 ± 0.3	15.6 ± 1.4	20.3 ± 1.2
Demethylated/demethoxylated	15.4 ± 1.1	22.3 ± 1.2	7.7 ± 1.0	7.9 ± 0.7

Lignin pyrolysis products were divided into six categories according to their pyrolytic formation and molecular structure: monomers, short-chain, long-chain, carbonyl, carboxyl, and demethylated/demethoxylated compounds. The percentage abundance of each category was calculated and referred to the total abundance of lignin pyrolysis products (Table 3). The changes in the percentage distribution of the categories of lignin pyrolysis products were used to highlight lignin degradation pathways. This interpretative approach provides detailed information on the chemical changes undergone by the residual lignin present in the samples [18, 19, 22]. The percentage abundances of lignin pyrolysis products divided into categories are reported in Figure 6.

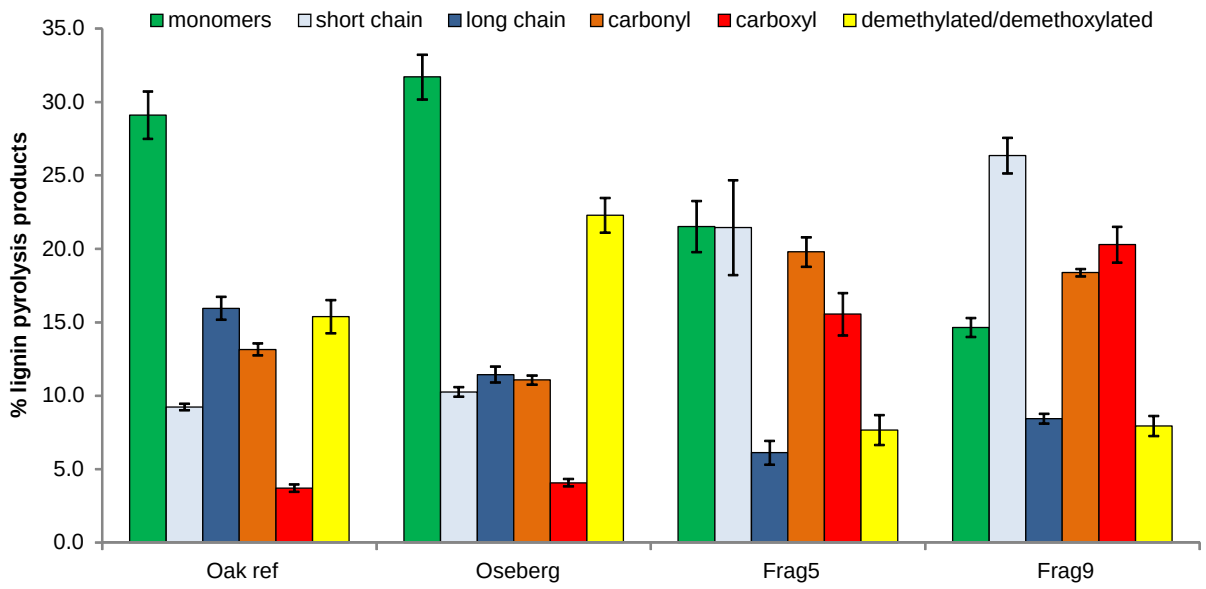

Figure 6. Percentage abundances of categories of lignin pyrolysis products in the samples analyzed.

The results show a very similar distribution for the oak reference wood and the Oseberg sample. Except for a slight relative increase in demethylated/demethoxylated compounds in the Oseberg sample, the rest of the distribution was almost identical. This indicated that the lignin in this sample had undergone degradation to a very low extent, above all related to the demethylation of methoxy groups, in agreement with the observation regarding the S/G ratio. As regards the Frag5 and Frag9 samples, there was a relative increase in lignin pyrolysis products with carbonyl and carboxyl functionalities, compared to the oak reference wood. This indicates a notable oxidation of lignin, as previously observed in alum-treated samples from the Oseberg collection [22], and in agreement with the results obtained by NMR techniques. In addition, the relative abundances of monomers and short chain pyrolysis products were different in Frag5 and Frag9, compared to the reference sample. A relative decrease in monomers and an increase in short-chain pyrolysis products indicate an alteration in the phenylpropanoid side chains originally present in the lignin units, likely due to cleavage of the bonds and oxidation of some positions.

4. DISCUSSION

The comparative interpretation of the data obtained in this work by GPC, NMR and Py-GC/MS analysis highlighted that the approaches applied all provide reliable and detailed information on the degradation processes occurring in archaeological wood. There was a very good agreement between the results obtained by the different techniques, although the information provided by this multi-analytical approach is rather complementary. NMR characterizes and semi-quantitatively evaluates different types of intermonomeric bonds that underwent degradation through HSQC experiments, whereas ^{31}P-NMR quantifies the amount of the different types of phenolic, alcoholic, and acidic groups in wood samples. GPC provides information on the depolymerisation of the polysaccharide fraction and on the presence of LCC complexes. On the other hand, investigation of the profiles of pyrolysis products by Py-GC/MS does not provide information on the intermonomeric bonds, but it can be exploited to estimate the relative content of holocellulose and lignin in degraded wood. In addition, categorisation of the lignin pyrolysis products enables oxidation, depolymerisation and demethylation phenomena to be highlighted.

The qualitative and quantitative results obtained were compared and interpreted by an integrated approach. The wood constituting the sample named Oseberg (not alum-treated) underwent processes resulting in a limited loss of polysaccharides. The ^{31}P-NMR analysis evidenced a slight increase in the relative amount of phenols, the GPC profile indicated a small decrease in the molecular weight (M_p) of the benzoylated sample, while the H/L ratio measured by Py-GC-MS was low compared to the sound reference wood (2.2 *vs* 3.7). A limited extent of demethylation of the lignin units was also detected. Overall, the Oseberg sample was comparable to the reference sound wood, demonstrating a good state of preservation.

On the other hand, the archaeological samples Frag5 and Frag9 (alum treated) were severely altered and were more similar to lignin in terms of chemical parameters. ^{31}P-NMR analyses revealed a higher phenol concentration, while GPC outputs indicated a strong decrease in the molecular weight (in order of depolymerisation: Frag5 > Frag9 >> Oseberg > reference). Also the H/L ratios calculated from Py-GC-MS analyses were extremely low, approaching almost zero for Frag5. 2D-HSQC spectra resembled the reference lignin spectra and the signals related to cellulose were completely lost. Residual xylans were detected in Frag9 (H/L ratio 0.4), while Frag5 showed the presence of monomeric sugars as also highlighted by the sharp peaks in the ^{31}P-NMR spectrum. This could be indicative of the presence of monomeric sugars chemically bonded to lignin as residue of the degraded cellulose and LCCs (Lignin Carbohydrate Complexes).

The results highlighted that the alum treatment led to a strong depolymerisation of the polysaccharide fraction (involving both cellulose and hemicelluloses), leaving a lignin-rich material. This chemical modification had a huge impact on the mechanical properties of the artifacts. The alum treatment also had an important impact on the lignin structure.

Py-GC-MS analyses provided evidence of side chain shortening and oxidation in both Frag5 and Frag9. ^{31}P-NMR highlighted a decrease in the amount of secondary alcohol connected to the increase in oxidized S' unit in the HSQC spectra resulting from the oxidation of a certain amount of β-O-4 structures. These observations suggest a relationship between the lignin oxidation and the chain shortening. Considering that the benzylic position of lignin moieties is the most sensitive to radical oxidation by H abstraction, the side chain shortening could arise from the beta-scission of the ketone formed. Finally, the strong increase in carboxylic acids in Frag5 and Frag9 is likely to be the last step in the lignin oxidation.

The results were also compared with the Py-GC/MS results obtained for other samples from the Oseberg collection (named "185 series") and presented in a previous publication

[22]. In the work presented here the samples were washed before Py-GC/MS analysis and most of the alum was removed, whereas in the other work the samples were analysed without washing. The relative amount of acid lignin pyrolysis products in the 185 series was found to be higher than in the samples presented here, and the shortening of the side chain occurred to such an extent that the monomers were almost undetected in the pyrolysis profiles.

There are several possible reasons for this: i) the high inorganic content in the non-washed samples may lead to some “pyrolytic artefacts” by catalysing radical oxidation reactions; ii) the washing process may remove wood degradation products such as vanillic and syringic acids weakly bound to the lignin network; iii) there is an intrinsic variability in the degradation state of the Oseberg samples. Finding the exact reason is difficult, as is recommending whether or not samples should be washed, which is beyond the scope of this work. However, more systematic investigations regarding the advantages and drawbacks of pre-washing archaeological wood samples that have been highly contaminated by inorganic salts before performing Py(HMDS)-GC/MS might help in better understanding the problem.

5. CONCLUSIONS

The use of ILs and the combined techniques applied in this work facilitated an extensive chemical characterization, without the fractionation of the whole wall cell components, thus providing a good picture of the state of preservation of the archaeological wood samples. The approach has several advantages: i) time- and material-consuming steps for fraction isolation were avoided; ii) the amount of material needed for the analyses was reduced to a minimum (150 mg); iii) the possible generation of analytical artefacts was minimized.

We believe these results should help in making decisions regarding how to re-treat the Oseberg collection, as the characterization of the residual wooden material is the first step

in the evaluation of innovative conservation methods and consolidation treatments. Recent results of the research into efficient consolidation strategies have identified those materials that have the potential to chemically interact with the residual wood structure as the most promising ones. It is therefore extremely important to investigate and quantify the functional groups present in the degraded wood polymeric network, in order to foresee and model their interaction with the consolidating agent.

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