

Isolation and Characterization of a Lignin-Like Polymer of the Cork of *Quercus suber* L.

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Keywords

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Summary

A lignin-like polymer was successfully extracted from the cork of *Quercus suber* L. using an organosolv-based technique. This material was characterized by elemental analysis, functional group analysis, nitrobenzene oxidation followed by HPLC analysis of the oxidation products, FTIR and liquid ¹³C NMR. The evidence thus obtained indicated that the extracted material was composed mainly of a lignin-like polymer covalently bound to residual aliphatic structures which are not present in common lignins and which have been assigned to suberin. The latter is likely to be attached to the oxygenated side chains of the phenolic polymer but bonding through the aromatic ring can also be envisaged. No residual carbohydrates were detected. The phenolic polymer, composed mainly of guaiacyl-type units and small amounts of syringyl-type units, had a low methoxy content and a high degree of condensation. This polymer showed the presence of a fraction containing C₆C_n units with n < 3 or even n < 2.

Introduction

Cork from *Quercus suber* L. is assumed to be composed of suberin (ca. 40%), lignin (ca. 22%), polysaccharides (ca. 20%) and extractibles (waxes, tannins, ...) (ca. 15%) (Pereira 1988).

It has been proposed that suberin, a biopolyester also present in the underground parts of plants such as tubers and roots, in wound periderms or other plant organs, is composed of aliphatic and aromatic moieties (Kolattukudy 1980). In cork, the aliphatic fraction of suberin has been studied in detail (Arno *et al.* 1981; Holloway 1983) using depolymerisation methods followed by GC and GC/MS analysis of depolymerisation products. These products are constituted mainly by aliphatic hydroxyacids ranging from C₁₆ to C₃₀. The so-called aromatic fraction of suberin has not been studied in detail.

The nature of cork lignin is not completely understood and the ambiguity between its possible features and the aromatic part of suberin has not been resolved. Not only the chemical structures of these two polymeric components have not been clearly elucidated, but even the overall composition of cork is still a matter of debate. A key issue in this context is to find out whether these components form physical mixtures or are covalently bound.

Several attempts have been made to extract and characterize the lignin of cork (Zimmermann *et al.* 1985; Marques *et al.* 1994). Up to now only Björk-

man-based techniques (Björkman 1956) have been used. Zimmerman *et al.* (1985) in their attempts to isolate the lignin from cork did not find guaiacyl, syringyl or typical dilignol units which suggests that lignin was not present in the examined cork extracts. Marques *et al.* (1994) isolated a material called Milled Cork Lignin (MCL), composed not only of aromatic structures, but also of significative amounts of sugars and aliphatic moieties assigned to suberin. This extract was characterized using chemical methods, Py/GC/FID and FTIR. The structural differences between the isolated phenolic polymer and a classical lignin led to the proposal of the term "lignin-like polymer" for the former. These authors concluded that the polyphenolic part accounts for about 40% of cork, a figure which differs from the 22% obtained by traditional methods (Pereira 1988). Perra *et al.* (1993, 1995), using a slightly modified extraction scheme, isolated lignin and suberin fractions from beech bark and mentioned no suberin structures within the so-called lignin fraction. In all these studies, no conclusion has been drawn about possible covalent bonding between the "lignin-like polymer" and suberin.

In conclusion, the chemical structure of cork components and their organization in the cell walls is far from being thoroughly understood. Modern spectroscopic techniques such as liquid and solid state NMR have been recently applied to the characterization of cork components (Pascoal Neto *et al.* 1995). These techniques, used in tandem with more selective iso-

lation techniques for the cork components, can hopefully open new perspectives to the understanding of this material with unique properties and elucidate some of the controversies found in the literature.

In this paper, we aim at contributing to the understanding of the chemistry of the aromatic fraction of cork and its possible association to the aliphatic fraction of suberin, using another approach for the isolation of the so called "lignin-like polymer" of cork. For this we used an organosolv-based technique (Pascoal Neto *et al.* 1994), with an ethanol/water mixture as solvent, at high temperature, in the presence of an acidic catalyst. Acidic organosolv methods are claimed to produce lignins with little structural degradation and with low sugar content (Pascoal Neto *et al.* 1994). The material obtained by this technique was characterized by elemental analysis, functional analysis, nitrobenzene oxidation followed by HPLC analysis of the oxidation products, FTIR and liquid ^{13}C NMR.

Materials and Methods

High quality reproduction cork was ground to 20 mesh. The cork powder was extracted in a Soxhlet apparatus using sequentially dichloromethane, ethanol and water (8 hours for each solvent). The total amount of material extracted was 13.4% (o.d. cork basis).

The organosolv experiments were carried out in a 4842 PARR stirred reactor. The conditions were as follows: solvent mixture: ethanol/water, 50 : 50 (v/v); solvent/cork ratio: 20/1 (l/kg); catalyst: 0.10M acetic acid; temperature: 160°C; time-to-temperature: 0.5h; time-at-temperature: 4h. After the reaction time, the reactor was cooled and the solid fraction was separated from the liquid fraction (black liquor) by filtration. Then, the organic solvent was evaporated from the liquid fraction and a precipitate appeared in the remaining aqueous solution. This mixture (aqueous solution + precipitate) was extracted sequentially with petroleum ether and diethyl ether. Then, the precipitate in the aqueous solution was recovered by centrifugation and washed with water. This precipitate will be referred to in the following as Organosolv Cork Lignin-Like Polymer or simply OCL. In order to eliminate adsorbed aliphatic compounds (which could remain after the sequential extraction of OCL with petroleum ether and diethyl ether), a portion of OCL was extensively extracted with chloroform at room temperature for 3 days with constant stirring. This fraction was designated as Treated Organosolv Cork Lignin-Like Polymer or simply OCL-T.

The elemental analysis was performed at the laboratories of the Service Central d'Analyse of CNRS (Vernaison, France). The alkoxy groups were determined using the Viebock/Schwappach method (Chen 1992a).

Alkaline nitrobenzene oxidations of lignin and cork were performed in a 20ml steel reactor for 2.5h at 170°C. The solutions obtained were extracted with diethyl ether and the organic phase was discarded. The aqueous phase was acidified to pH 1–2 with aqueous HCl and the aromatic compounds, arising from the oxidation of lignin, were recovered by extraction with diethyl ether. After the elimination of the solvent by vacuum evaporation, the residual solid was dissolved in an acetonitrile/water mixture 1 : 2 (v/v) and the solution filtered through a 45µ Millipore filter and analyzed by HPLC using a C18 reverse phase column and a UV detector set at 280nm. The eluant was composed of a mixture of (A) water with 1% acetic acid and (B) methanol/ace-

tonitrile 7/3 in variable proportions, as follows: 0–22 min, 80% A + 20% B (0.7 ml/min); 22–37 min, 80–65% A + 20–35% B (0.7–1.2 ml/min); 37–41 min, 65–80% A + 35–20% B (1–2–0.7 ml/min). FTIR spectra were taken with a Mattson 7000 spectrophotometer using the standard KBr pellet technique.

^{13}C NMR spectra were recorded at 62.9MHz on a Bruker WM 250 spectrometer at 323K with TMS as reference. For the inverse gate sequence the parameters were as follows: 90° pulses, 10s delay, and 19230Hz sweep width (Robert 1992). The lignin-like polymers were dissolved in DMSO-d₆, while suberin was dissolved in CDCl₃.

Results and Discussion

Isolation and general characterization of the lignin-like polymer

Organosolv treatment of cork yielded 2.6% (o.d. extracted cork) of OCL. This relatively low yield can be explained by the resistance offered by the cork structure to the attack of the acidic medium. Cork is composed of closed cells (Pereira *et al.* 1987), with suberized cell walls which limit the diffusion of liquids and reactants into the cork structure. The ester bonds in suberin are quite resistant to acid hydrolysis (as compared to alkaline hydrolysis). In these conditions, cork maintains its cellular structure and reactions are limited to the external surface of cork particles: only about 23% of the initial cork material (including the 2.6% of OCL) was solubilised during the 4-hour treatment.

The FTIR and ^{13}C NMR spectra of OCL (Figs. 2 and 3) clearly show that this material is composed mainly of aromatic structures, with some residual aliphatic moieties which are not present in typical wood lignins (peaks at 2854 and 2930 cm⁻¹ in the FTIR spectra and at ca. 30ppm in the ^{13}C NMR spectra). After 3 days of extraction with chloroform, the resulting OCL-T lignin-like polymer still presented these aliphatic structures (Fig. 3) suggesting that they are (at least in part) covalently bonded to the aromatic polymer. There is no evidence of the presence of sugars in these samples, at least not in significant amounts.

The elemental composition of OCL was: C: 59.4%, O: 30.8%, H: 5.7%, which is not very different from common softwood and hardwood lignins (Fengel and Wegener 1984). However, the content of alkoxy groups of OCL, 11.4%, is low compared to common softwood and hardwood lignins (Fengel and Wegener 1984). In addition to methoxy groups, some ethoxy groups arising from ethoxylation reactions between ethanol and the aromatic rings, could have contributed to the total alkoxy groups content (Pascoal Neto *et al.* 1994), which would make the methoxy content even lower than 11.4%.

Nitrobenzene oxidation and HPLC analysis of products

The chromatograms of the nitrobenzene oxidation products of both cork and OCL are quite simple

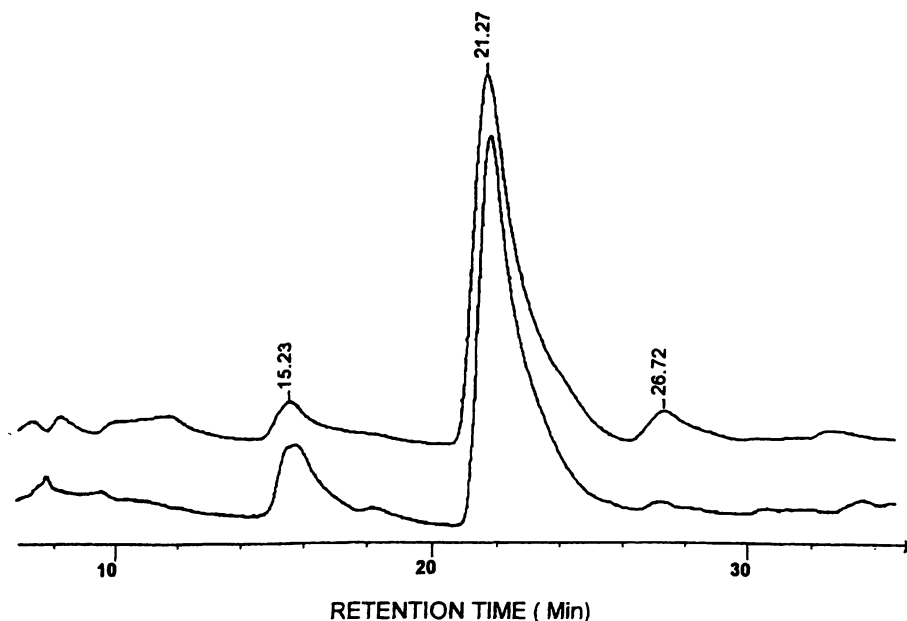


Fig. 1. Chromatograms from the HPLC analysis of the nitrobenzene oxidation products of cork (A) and organosolv cork lignin-like polymer (OCL) (B) (vanillin: RT = 21.4 min; vanillic acid: RT = 15.5 min; syringaldehyde: RT = 26.8 min).

(Fig. 1). The major oxidation products which were identified are vanillin, vanillic acid and syringaldehyde (Table 1). According to these results, the lignin-like polymer of cork is composed mainly of guaiacylpropane (G) units, confirming previous results for milled cork lignin (Marques *et al.* 1994); however, syringylpropane (S) units, are also present in cork, albeit in small amounts. No traces of *p*-hydroxybenzaldehyde were detected in our study. Interestingly, the G/S value of 8 obtained for OCL is significantly lower than that of cork (G/S 56) which indicates that the method used for the extraction of OCL is quite selective towards the syringylpropane-rich fraction of cork and would suggest a heterogeneous distribution of these two types of structural units in the cork cell wall.

The total amount of aromatic aldehydes and acids recovered after nitrobenzene oxidation of cork and OCL (Table 1) is quite low compared to results reported in literature for the nitrobenzene oxidation of wood lignins (Chen 1992b). This difference can be attributed to a high degree of condensation of the aromatic units in cork, namely through inter-aromatic C–C bonds (Chen 1992b).

Table 1. Analysis of the products of the alkaline nitrobenzene oxidation of cork and organosolv cork lignin-like polymer (OCL)

	Vanillin (%)	Vanillic acid (%)	Syringaldehyde (%)	Molar ratio (G/S)*
Cork	2.3	0.7	0.1	56
OCL	7.5	1.4	1.3	8

* G/S = number of guaiacylpropane units/number of syringylpropane units = (vanillin + vanillic acid)/(syringaldehyde)

FTIR characterization

We have used the band assignments and the criteria proposed by Faix (1991) for the interpretation of lignin FTIR spectra. The OCL spectrum (Fig. 2) is quite similar to the spectra of typical G-type Milled Wood Lignins (Faix 1991). The peak at 1269 cm^{-1} , corresponding to G units with C=O groups is the strongest. The relative intensities of the peaks at 1603 , 1512 and 1463 cm^{-1} are typical of G-type lignins ($1604 < 1512 > 1462$). In addition, the aromatic skeletal vibrations appear at 1512 cm^{-1} , instead of 1505 – 1507 cm^{-1} , which is characteristic of lignins rich in S units. However, a more detailed interpretation of the spectrum of OCL also showed the presence of syringyl units, confirming the results of the alkaline nitrobenzene oxidation. The band at 1330 cm^{-1} , which is typical of syringyl units in GS type lignins, is clearly visible; also, in the region of 1175 – 1065 cm^{-1} the spectrum shows a strong peak at 1126 cm^{-1} with a shoulder typical of GS lignins, instead of a strong band at 1140 cm^{-1} which is typical of G-type lignins. According to Faix (1991), this is a very sensitive criterion for the recognition of GS lignins. No clear evidence exists for the presence of H units (*p*-hydroxyphenyl propane) in this lignin-like polymer of cork.

^{13}C NMR characterization

The ^{13}C NMR spectra of OCL and OCL-T are given in Figure 3. They were recorded with the inverse gate sequence which allows quantitative analysis and signal intensity comparisons. The two spectra present the main and detailed characteristics of a G-type lignin structure with a very small syringyl contribution (Table 2), which confirm the results discussed

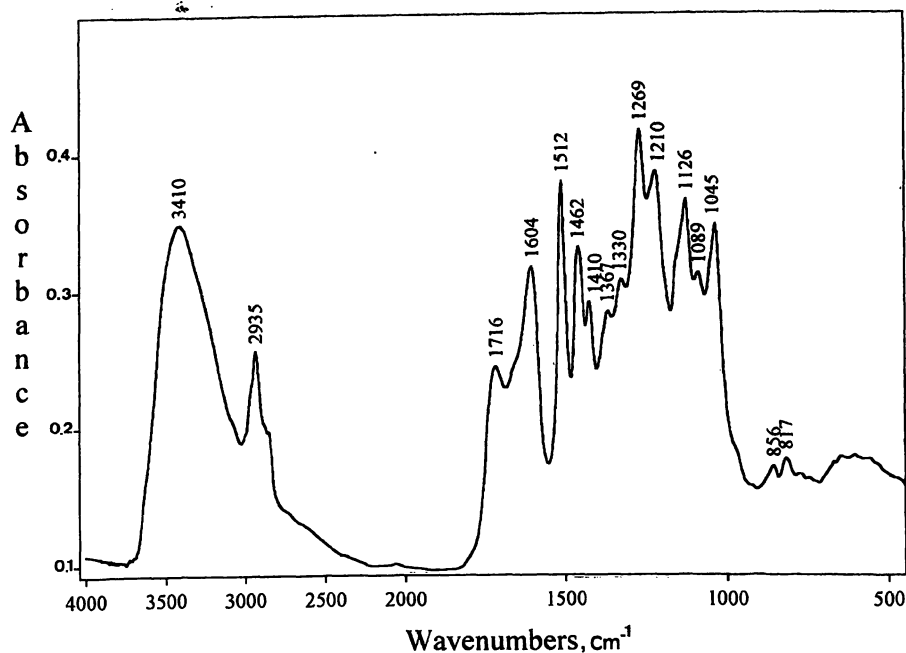


Fig. 2. FTIR spectrum of organosolv cork lignin-like polymer (OCL).

above. The absence of contaminating carbohydrates in these samples is evident. Nevertheless, the spectra reveal some qualitative and quantitative structural differences with respect to those of conventional lignins, which are important enough to suggest that this phenolic polymer is not a lignin, but rather a lignin-like macromolecule.

One of the main differences concerns the presence of signals S1, S2, S3 and S4 (Fig. 3) respectively at 174.3, 33.5, 29.0 and 24.2 ppm, on OCL and OCL-T spectra, which are not present in conventional lignin spectra. By comparison with the spectrum of suberin extracted by traditional alkaline methanolysis (results not shown), they can be assigned to the carboxy (S1) and $-\text{CH}_2-$ groups (S2, S3, S4) in the aliphatic chains of suberin. After extraction of OCL with CHCl_3 , the four S signals are still present in the OCL-T spectrum (Fig. 3, B) in approximately the same proportions as in OCL. This indicates that the phenolic polymer and the suberin moieties are likely to be covalently bound and not just physically mixed. This point is particularly relevant to the elucidation of the structure of both phenolic and suberin components in cork.

Another difference with respect to classical lignins can be observed by comparing the relative intensity of some signals like signal 26, 22 and 11. The signal centred at 63.2 ppm (made up of 25 and 26) identified on the DEPT spectra (not shown) as $-\text{CH}_2-$ is classically assigned to $\text{C}\gamma\text{H}_2\text{OH}$ with an α $\text{C}=\text{O}$, but its intensity is very high compared to that of signal 27 ($\text{C}\gamma\text{H}_2\text{OH}$ in general). A tentative explanation could be that of the two signals 25 and 26, 26 would arise from a terminal $\text{C}_x\text{H}_2\text{OR}$ (with $x = \beta$ or γ) in the side chain of the aromatic ring of OCL (as in the lignin

propane side chain), that is the same type of signal as 27 at 60.2 ppm, normally assigned to $\text{C}\gamma\text{H}_2\text{OH}$ in conventional lignin structures. A downfield shift is

Table 2. ^{13}C chemical shifts and signal assignments for organosolv cork lignin-like polymer (OCL) and treated organosolv cork lignin-like polymer (OCL-T)

Signal number	δ , ppm/TMS	Assignments*
S1	174.3	carbonyl groups in suberin
1	172.4	$\text{C}=\text{O}$ in ArCOOH and ArCOOR
2	170.4	$\text{C}=\text{O}$ in ArCOOH and ArCOOR
3	167.0	$\text{C}=\text{O}$ in ArCOOH and ArCOOR
4	152.4–152.7	$\text{C3/C3}'$ in non-phenolic 5-5'
5	149.6–149.7	C3 in G_e
6	147.7	C4 in G_e , C3/C5 in S_{ne}
7	145.1	$\text{C4/C4}'$ in non-phenolic 5-5' and S
9	135.2–135.5	C4 in G_e
10	132.4	$\text{C5/C5}'$ in non-phenolic 5-5'
11	129.5	vinyllic C in $\text{Ar}-\text{CH}=\text{CH}-\text{CH}_2\text{OR}$
12	125.4–125.5	$\text{C5/C5}'$ in phenolic 5-5'
14	119–119.2	C6 in G
15	115.4–115.6	C5 in G
17	112–112.1	C2 in G
18	104.5–104.6	C2/C6 in S
20	87.0	$\text{C}\beta$ in $\text{C}\beta\text{-O-4}$
21	83.9	$\text{C}\beta$ in $\text{C}\beta\text{-O-4}$
22	80–83	$\text{C}\alpha$ and $\text{C}\beta$ in $\text{C}\alpha\text{-O-R/C}\beta\text{-O-4}$
23	71.2–72.2	$\text{C}\alpha$ in $\text{G}\beta\text{-O-4}$
25, 26	63.1–63.2	$\text{C}\gamma\text{H}_2\text{OR}$, R = suberin $\text{C}\gamma\text{H}_2\text{OH}$ with a $\text{C}=\text{O}$
27	60.2	$\text{C}\gamma\text{H}_2\text{OH}$ in G
28	55.8–55.9	$\text{Ar}-\text{OCH}_3$
29	53.3	$\text{C}\beta$ in $\beta\text{-}\beta$ and $\beta\text{-5}$
S2	33.5	$-\text{CH}_2-$ in suberin
S3	28.9–29	$-\text{CH}_2-$ in suberin
S4	24.4	$-\text{CH}_2-$ in suberin

* S: syringyl unit; G: guaiacyl; e : etherified unit in C4; ne : non-etherified unit in C4.

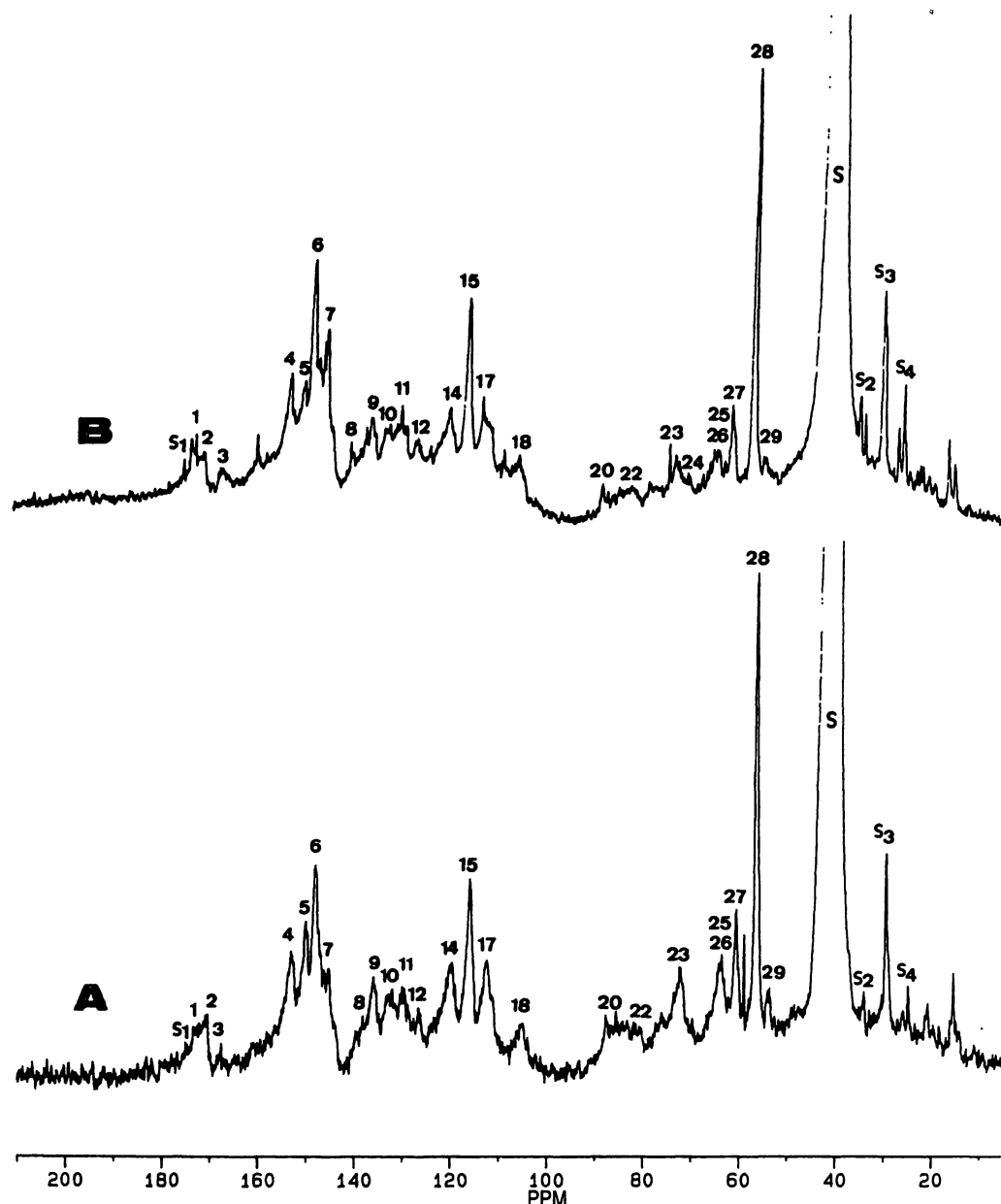


Fig. 3. ^{13}C NMR spectra of organosolv cork lignin-like polymer (OCL) (A) and treated organosolv cork lignin-like polymer (OCL-T) (B).

indeed expected when going from an alcohol to an ether or ester function, the 3 ppm shift found here being more in tune with an ester group. In our case, R could correspond to the carboxy functions between 167 and 175 ppm, in particular those belonging to the ester moieties encountered in suberin chains (signal S1 at 174.3 ppm). These results need further investigation with appropriate model compounds. The intensity of signal 22 is quite high compared to that of signal 20, the opposite occurring in classical lignins. This could be explained by the presence of C α carbons in such structures as β -O-4/ α -O-R. In extracted lignins the α -O-4 linkages are in negligible amounts because they are fragile and easily split off. In OCL and OCL-T, R could be suberin moieties in the C α -O-R linkages. Signal 11 was assigned to vinylic carbons in cinnamyl structures. Its

relative intensity is higher than in spectra of typical lignins.

The quantitative analysis of the OCL spectrum (Fig. 3, A) gives 0.8 methoxyl groups (signal 28), 3.8 quaternary aromatic carbons (signals 4–12 between 161 and 125 ppm) and 2.2 aliphatic C–O carbons (signals 20–27 and 29) per aromatic ring. One can explain (i) the low methoxy content, compared to softwood lignin, by the presence of *p*-hydroxyphenylpropane structures and (ii) the higher number of quaternary aromatic carbons by a high extent of substitution, from so-called condensation, on the aromatic ring. No *p*-hydroxyphenylpropane units could be clearly shown on the spectra, but condensed units are identified by signals 4, 7 and to a lesser extent by 12 which could arise from the classical condensed 5-5, β -5 and 4-O-5 structures. Linkages between

aromatic rings and suberin moieties could also exist and introduce more quaternary aromatic carbons. The presence of condensed 5-5 *p*-hydroxyphenylpropane units could explain the low methoxy content.

The number of aliphatic C–O carbons per aromatic unit in OCL, 2.2, is close to the figure usually obtained by the NMR analysis of spruce MWL, viz. 2.3 (Robert and Chen 1989). However, the quantitative analysis of the OCL-T spectrum (Fig. 3, B) gives 0.6 methoxyl groups, 3.8 quaternary aromatic carbons and only 1.3 aliphatic C–O carbons per aromatic ring. This means that the lignin-like polymer in cork contains aromatic units with an aliphatic side chain with less than 3 or even 2 carbon atoms per aromatic ring (C_6C_n , $n < 3$). This suggests that the $CHCl_3$ extraction eliminated some low DP phenylpropane units and that the aliphatic suberin chains might be bound preferentially to a phenolic polymer with an oxygenated aliphatic side chain shorter than that of typical lignins.

Conclusions

In an attempt to better characterize the phenolic polymer present in cork of *Quercus suber* L., we isolated with an organosolv-based technique, a lignin-like polymer which we assume to be covalently bound to a suberin fraction. Up to now, little attention was paid to such structures. This extraction method has the advantage of giving a sample free of carbohydrate contaminants, thus allowing a clearer inspection of the two other major components. Results obtained indicate that the phenolic polymer is composed mainly of guaiacyl-type units with a small amount of syringyl-type units and has a low methoxyl content and a high degree of condensation. Some structural information was acquired concerning the aliphatic oxygenated side chain of the aromatic polymer, viz. in C_6C_n units, n can be lower than 3 or even 2. The aliphatic fraction of suberin is covalently attached to the phenolic polymer, probably to the side chain of the C_6C_n units. Bonding of suberin to the aromatic ring should also be considered. In a next step, with the help of adequate model compounds and using other fractionation techniques, we will try to elucidate the nature of the association between the two polymeric systems and the influence of the extraction method on the structure of the outcoming macromolecules.

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