Composition of Suberin Extracted upon Gradual Alkaline Methanolysis of *Quercus suber* L. Cork

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The monomeric composition of suberin extracts obtained by gradual alkaline methanolysis of *Quercus suber* cork was determined by gas chromatography−mass spectrometry (GC-MS). Results show that 1-alkanols and alkanoic and α,ω-alkanedioic acids are preferentially removed upon mild alkaline conditions, whereas mid-chain-modified ω-hydroxyalkanoic acids are preferentially removed under stronger alkaline conditions. Saturated ω-hydroxyalkanoic acids are found to be abundant in all suberin extracts. These results are consistent with two distinct suberin fractions with different locations in cork cell walls and/or esterification degrees. It is proposed that these fractions correlate with the two main suberin peaks in the solid state ^{13}C NMR spectra of cork and suberin extracts. Quantitative GC-MS analysis showed that suberin monomers comprise ∼30% (w/w) of the suberin extracts, the remaining comprising nonvolatile structures with high M_n values, as measured by vapor pressure osmometry. The presence of a large fraction of high molecular weight aliphatic structures in suberin extracts is supported by the corresponding NMR spectra.

Keywords: *Suberin; cork; Quercus suber L.; GC-MS; NMR; alkaline methanolysis*

INTRODUCTION

Suberin is a biopolyester that is a component of the cell walls in the periderm of higher plants, arranged in a characteristic multilamellar structure (Sitte, 1975; Kolattukudy, 1980). It is known that suberin composition varies among plants of different species, but some variability also occurs within plants of the same species (Jensen and Östman 1954; Holloway 1972, 1983).

The outer bark of *Quercus suber* L., commonly known as cork, presents highly suberized cell walls that confer special properties to this material such as low permeability to liquids, elasticity, and resilience. Cork is a material with great economic importance, widely used in the manufacture of stoppers and insulating and decorative panels. The average chemical composition of reproduction cork is approximately 40% suberin, 22% lignin, 20% polysaccharides, 15% extractives, and 1% ash (Pereira, 1988).

The most abundant monomers of cork suberin were isolated and identified early on by Seoane and Ribas (1951), Jensen and Östman (1954), Guillemonat and Traynard (1962), and Duhamel (1964). The polymeric structure of cork suberin was studied by selective chemical treatments (Rodríguez and Ribas, 1972; Agullo and Seoane, 1981, 1982). The molecular dynamics and interactions of suberin with the other components in cork cell walls were studied more recently by solid state NMR spectroscopy (Gil et al., 1997; Lopes et al., 1999a,b). It was found that a distinct family of suberin methylene groups, identified by the peak at 33 ppm in the ^{13}C NMR spectrum of cork, are closer to $-CH_2O-$ groups and carbohydrate moieties in cork cell walls (within a few nanometers). The remaining methylenes, identified by the peak at 30 ppm, comprise most of the suberin methylenes and are characterized together with a small fraction of lignin-like moieties by high motional freedom in cork cell walls (Gil et al., 1997; Lopes et al., 1999b). The detailed monomeric composition of cork suberin was assessed with high-performance gas chromatography combined with mass spectrometry (GC-MS). These studies were reported by Holloway (1972, 1983), Arno et al. (1981), and, more recently, Vallejo et al. (1997), Graça and Pereira (1997), and Bento et al. (1998), using different depolymerization techniques. It was found that the constituent monomers of suberin are predominantly alkanoic acids, α,ω-alkanedioic acids, ω-hydroxyalkanoic acids, dihydroxyacids, trihydroxyacids, epoxyacids, and 1-alkanols with even-carbon-numbered chain lengths of $C_{16}-C_{26}$. Small amounts of ferulic acid (Holloway, 1983; Vallejo et al., 1997; Graça and Pereira, 1998; Bento et al., 1998) and glycerol (Graça and Pereira, 1997; Bento et al., 1998) were also found in cork suberin extracts.

Despite the extensive studies by GC-MS on the monomeric composition of cork suberin, most of the published papers present semiquantitative results, seldom attempting calibration of the GC-MS system with internal standards and pure reference compounds. Moreover, the identification of suberin monomers by mass spectrometry, usually after derivatization to the corresponding methyl ester trimethylsilyl ethers, has been limited in most papers to a particular fraction of suberin that is volatile enough to be eluted in GC. In this work, we use GC-MS, vapor pressure osmometry (VPO), solution state ^1H NMR spectroscopy, and solid state ^{13}C NMR spectroscopy to characterize qualitatively and quantitatively the composition of suberin extracted upon gradual alkaline methanolysis of cork and obtain additional structural information on the suberin polymer.

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EXPERIMENTAL PROCEDURES

Isolation and Derivatization of Suberin. Good quality *Q. suber* L. cork from the Portuguese region of Alentejo was ground and sieved, and the granulometric fraction <0.425 mm was used. Extractive free cork was obtained by exhaustive extraction of cork powder sequentially with dichloromethane, ethanol, and water. Suberin extracts were obtained by 3 h of reflux of individual 1.5 g portions of extractive free cork in 250 mL of dry methanol with different concentrations of sodium methoxide in the 0.01−3.0% range. After filtration, each of the resulting individual residues was again refluxed in 100 mL of methanol for 15 min and filtered. The two methanolic extracts were combined and acidified to pH 6 with 0.25 M H_2SO_4 and concentrated in a rotary evaporator. The resulting concentrate was suspended in 100 mL of water and extracted three times with 200 mL of chloroform. The combined chloroform solutions were dried over anhydrous sodium sulfate, and the solvent was evaporated to dryness (Pereira, 1988). Suberin extracts were quantified gravimetrically, and results were expressed in percent of cork dry weight. This depolymerization method yields methyl esters from carboxylic groups and methoxyhydrins from epoxide groups; however, a fraction of free acids is also produced. Polar suberin monomers such as glycerol, in principle, are not extracted with chloroform, remaining in the aqueous phase. The suberin samples obtained will be designated hereafter as follows: suberin A, extracted with 0.01% $NaOCH_3$; suberin B, extracted with 0.02% $NaOCH_3$; suberin C, extracted with 0.03% $NaOCH_3$; and suberin D, extracted with 3.0% $NaOCH_3$.

For quantification, a known amount (~0.26 mg) of HPLC grade cholesterol in acetone was added to each suberin extract (~18 mg). The mixture was dissolved in diethyl ether, chloroform, and methanol (4:5:1), and freshly prepared diazomethane was added to convert the suberin free acids into their methyl esters. After standing at room temperature for 30 min, the solution was evaporated to dryness with a rotary evaporator. The residue was dissolved in 250 μL of pyridine, and components containing hydroxyl groups were converted into their trimethylsilyl (TMS) ethers by adding 250 μL of bis-(trimethylsilyl)trifluoroacetamide and 50 μL of trimethylchlorosilane. After the mixture had stood at 70 °C for 30 min, the methyl ester TMS ethers were immediately analyzed by GC-MS to avoid degradation of the sample (Ekman, 1983).

GC-MS Analysis. Four GC-MS runs were made for the extracts of suberin A, C, and D and six GC-MS runs for the extract of suberin B. The samples were analyzed using an HP system equipped with a DB-1 fused silica capillary column (J&W Scientific, 30 m × 0.32 mm and 0.25 μm film thickness). The chromatograph was operated at 150−285 °C with a heating rate of 4 °C/min and then for 10 min at 285 °C. Injector and detector temperatures were held at 220 and 300 °C, respectively. The carrier gas was helium (35 cm/s). The GC system was calibrated with pure reference compounds (representative of the major suberin monomers) relative to cholesterol, the internal standard, and the multiplication factors needed to obtain correct quantification from peak areas were calculated as an average of three GC-MS runs. Mass spectra were obtained in the EI mode (70 eV), and the identification of compounds was based on comparison with previously published data, reference compounds, and ion fragmentation patterns.

VPO Analysis. VPO analyses were made on a Knauer instrument operating at 45 °C and calibrated with pure benzil ($C_{14}H_{10}O_2$) solutions. A second calibration was performed with pure 1-eicosanol. The differential (millivolts) was measured relative to pure solvent for three or four solutions of benzil in chloroform, with known concentrations in the 0.001−0.004 mol dm^{-3} range. After calibration, three solutions of each suberin extract were prepared in chloroform, with known concentrations in the 2.0−10.0 g dm^{-3} range, and the respective differential (millivolts) was measured relative to pure solvent. The ratio of the calibration curve slope to the sample curve slope gave the number-average molecular weight (M_n) of the respective suberin extract in g mol^{-1} units. Several measurements were made for each sample to evaluate the reproducibility of the M_n measurements.

Table 1. Gravimetric Quantification of Suberin Extracts (Expressed in Percent of Dry Cork Weight) and Corresponding M_n Values Determined by VPO

sample	% (w/v) $NaOCH_3$ in methanol	% (w/w) extracted suberin	M_n (g mol^{-1}) range	av	SD[a]
suberin A	0.01	3.0−6.0	528−627	580	46
suberin B	0.02	7.0−18.0	722−868	793	60
suberin C	0.03	50.0−55.0	597−697	636	37
suberin D	3.0	40.0−45.0	755−968	861	71

[a] SD, standard deviation.

NMR Analysis. ^{1}H NMR spectra of $CDCl_3$ suberin solutions were acquired at 300.13 MHz on a Bruker AMX 300 spectrometer. Chemical shifts are given in parts per million relative to TMS. Solid state ^{13}C NMR spectra were obtained on a Bruker 400P spectrometer, operating at a proton frequency of 400 MHz. Suberin samples were packed in 4 mm rotors and spun at the magic angle with spinning rates of 4−5 kHz. The single-pulse excitation ^{13}C spectra were recorded with a carbon 90° pulse of 4.7 μs, a recycle delay of 6 s, and high-power decoupling during acquisition. The cross-polarization magic angle spinning (CP/MAS) ^{13}C spectrum was recorded with a proton 90° pulse of 4.3 μs, a contact time of 1 ms, and a recycle delay of 4 s.

RESULTS AND DISCUSSION

Suberin Depolymerization and Isolation. Suberin was extracted from cork after depolymerization by sodium methoxide-catalyzed methanolysis. Previous studies (Holloway and Deas, 1973; Ekman, 1983) established that alkaline methanolysis ($NaOCH_3/CH_3OH$) is the least harsh depolymerization method to determine suberin monomeric composition, because epoxy acids can be detected indirectly in the form of methoxyhydrins. The high temperatures and the aqueous alcoholic alkaline medium used in alcoholic saponification (NaOH/ROH, with water associated to the alcohol) promote the degradation of the unstable epoxy groups to *vic*-diol groups, whereas aqueous alkaline hydrolysis (NaOH/H_2O) liberates large amounts of polyphenols that interfere with the subsequent extraction of the acids with ether or chloroform.

Results from the gravimetric quantification of extracted suberin, expressed in percent of dry cork weight, are shown in Table 1. Suberins A and B correspond to extracts resulting from partial depolymerization of native suberin with very mild alkaline methanolysis (0.01 and 0.02% $NaOCH_3$ in methanol, respectively). Suberin C results from depolymerization of native suberin with mild alkaline methanolysis (0.03% $NaOCH_3$ in methanol). Suberin D is extracted upon strong alkaline methanolysis conditions (3.0% $NaOCH_3$ in methanol), following a standard method proposed by Pereira (1988) for gravimetric quantification of cork suberin. The yield of suberin extraction increases with $NaOCH_3$ concentration up to 0.03% and then decreases slightly for higher concentration (Table 1). Taking into account the particularly pasty nature of the suberin fraction extracted with 0.03% $NaOCH_3$ and considering that the same drying conditions were used for all samples, it is proposed that the higher yield of this extraction may be due to the presence of significant amounts of occluded solvent (methanol). This possibility will be discussed further in a later section.

Identification of Suberin Monomers. Figure 1 shows the gas chromatogram of the methyl ester TMS

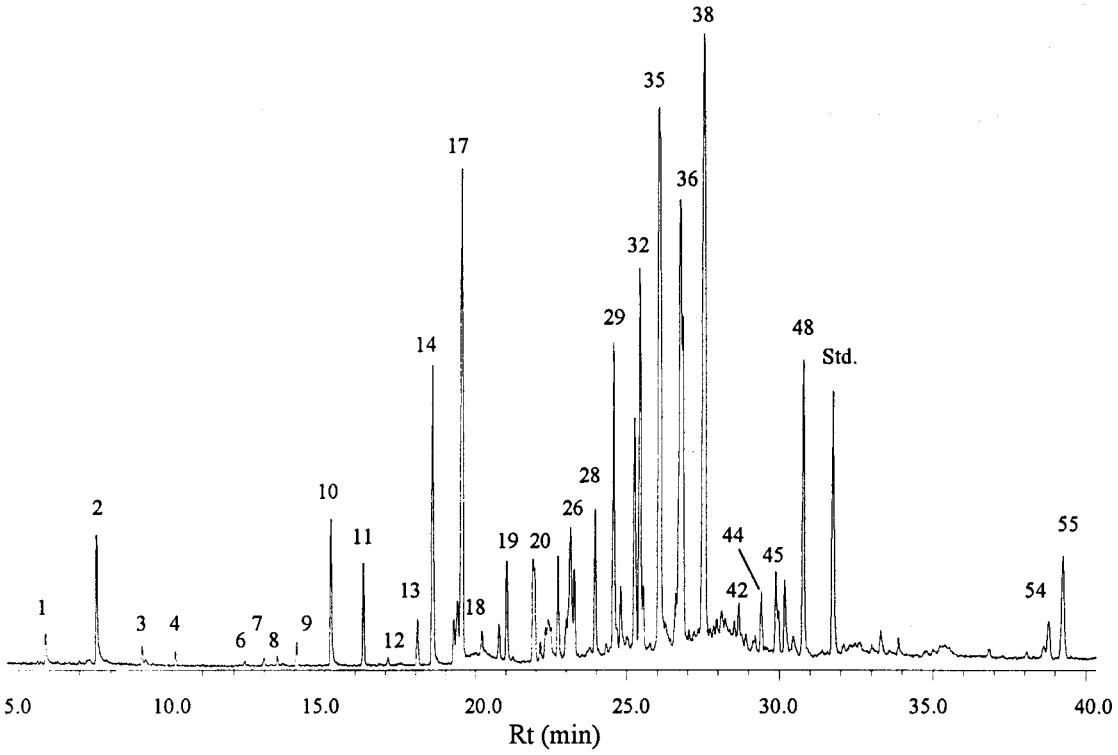

Figure 1. Total ion chromatogram of the methyl ester TMS ether derivatives of suberin D monomers. Peak numbers refer to the compounds listed in Table 2.

Table 2. Identification of Methyl Ester TMS Ether Derivatives of Suberin Monomers Detected by GC-MS

peak	M_r	suberin monomer	peak	M_r	suberin monomer
1[a]	222	*cis*-ferulic acid	29[d,h]	460	9-hydroxy-10-methoxyoctadecane-1,18-dioic acid + 10-hydroxy-9-methoxyoctadecane-1,18-dioic acid
2[a]	222	*trans*-ferulic acid			
3[a]	270	hexadecanoic acid	30[a]	382	tetracosanoic acid
4[b]	314	1-hexadecanol	31[d,h]	504	9-methoxy-10,18-dihydroxyoctadecanoic + 10-methoxy-9,18-dihydroxyoctadecanoic acid
5[a,c]	294	*cis*-octadeca-9,12-dienoic acid			
6[a]	296	octadec-9-enoic acid	32[d,e,h]	518	*threo*-9,10-dihydroxyoctadecane-1,18-dioic acid
7[a]	298	octadecanoic acid	33[b]	426	1-tetracosanol
8[a]	340	1-octadec-9-enol	34[b]	428	21-hydroxyheneicosanoic acid
9[a]	342	1-octadecanol	35[d,e,h]	562	*threo*-9,10,18-trihydroxyoctadecanoic acid
10[a,d]	314	hexadecane-1,16-dioic acid	36		unidentified hydroxyacid(s)
11[a,c,e]	358	16-hydroxyhexadecanoic acid	37[a]	398	docosane-1,22-dioic acid
12[a]	326	eicosanoic acid	38[a,e]	442	22-hydroxydocosanoic acid
13[c]	370	1-eicosanol	39	488	C_{20} homologue of **29**
14[a,d,e]	340	octadec-9-ene-1,18-dioic acid	40		derivative of **39**
15[a,e]	342	octadecane-1,18-dioic acid	41		derivative of **39**
16	446	dihydroxyhexadecanoic acid	42[b]	454	1-hexacosanol
17[a,d,e]	384	18-hydroxyoctadec-9-enoic acid	43[b]	456	23-hydroxytricosanoic acid
18[b]	386	18-hydroxyoctadecanoic acid	44		derivative of **39**
19[a]	354	docosanoic acid	45		unidentified hydroxyacid
20[b]	398	1-docosanol	46[b]	426	tetracosane-1,24-dioic acid
21	368	eicosene-1,20-dioic acid	47		unidentified pentacyclic triterpenol
22[a]	356	9-oxo-octadecane-1,18-dioic acid	48[b]	470	24-hydroxytetracosanoic acid
23[f]	356	9,10-epoxy-octadecane-1,18-dioic acid + unidentified	std[a]	458	cholesterol
			49	542	unidentified pentacyclic triterpenol
24[g]	472	dihydroxyoctadecenoic acid	52[i]	514	cerin
25[a,e]	400	9,10-epoxy-18-hydroxyoctadecanoic acid	53[i]	426	friedelin
26[a]	370	eicosane-1,20-dioic acid	54[i]	542	betulinic acid
27[b]	412	20-hydroxyeicosenoic acid	55[i]	586	betulin
28[b]	414	20-hydroxyeicosanoic acid			

[a] Matches mass spectrum of Wiley 275 library. [b] Same fragmentation pattern as other homologous compounds. [c] Matches mass spectrum of pure standard compound. [d] Matches mass spectrum of Kolattukudy and Agrawal (1974). [e] Matches mass spectrum of Eglinton and Hunneman (1968) and Eglington et al. (1968). [f] Matches mass spectrum data of Arno et al. (1981). [g] Matches mass spectrum data of Holloway (1983). [h] Matches mass spectrum data of Vallejo et al. (1997). [i] Matches mass spectrum data of Seoane et al. (1971).

ether derivatives of suberin D monomers. The comparison of this chromatogram with those obtained for suberins A, B, and C (not shown) indicates similar qualitative composition in all extracts but different quantitative proportions of suberin monomers. Table 2 shows the identified suberin monomers and the molecular weight of the corresponding methyl ester TMS ether derivatives. Small amounts of 3,4-dimethoxybenzoic acid, 2-(4-hydroxy-3-methoxyphenyl)ethanol, 3-vanillylpropanol, and nonadioic acid were detected at very short retention times. Ferulic acid was identified in all suberin extracts (peaks 1 and 2) in both cis and trans

configurations. This is the most common aromatic component of cork suberin extracts, and it is known to be esterified with aliphatic ω-hydroxyacids (Guillemonat and Traynard, 1962; Graça and Pereira, 1998).

Some pentacyclic triterpenoids such as cerin, friedelin, betulinic acid, and betulin, along with smaller amounts of other unidentified triterpenoids (peaks 47 and 49), were detected at long retention times in suberin extracts A and B. Suberin C contained preferentially friedelin and betulin, whereas suberin D contained betulinic acid and betulin (peaks 54 and 55). The presence of triterpenoids in suberin extracts of solvent-pre-extracted cork has been also reported by Seoane et al. (1971), Agullo and Seoane (1982), and Vallejo et al. (1997).

The main suberin components eluted by GC and identified by EI-MS (Table 2) include ω-hydroxyalkanoic, α,ω-alkanedioic, alkanoic, dihydroxyalkanedioic, trihydroxyalkanoic, hydroxyepoxyalkanoic, epoxyalkanedioic acids, and 1-alkanols with chain lengths of C_{16}−C_{26}. The mass spectra of the TMS ether methyl ester derivatives of most of these suberin monomers (Table 2) are found in the literature (Eglinton and Hunneman, 1968; Eglinton et al., 1968; Seoane et al., 1971; Kolattukudy and Agrawal, 1974; Arno et al., 1981; Holloway, 1983).

Some hydroxylated compounds in Table 2 (peaks 16, 36, 39−41, 44, and 45) are unidentified probably due to the occurrence of positional isomer and/or conformational isomer mixtures in the same chromatographic peak. The mass spectrum of compound **16** has some ion peaks common to dihydroxyhexadecanoic acid (*m*/*z* 387 $[M - 59]^+$, 273, 231, and 73), but it was not possible to make a clear identification. However, small amounts of 10,16- and 9,16-dihydroxyhexadecanoic acids (major isomers) have been reported previously in cork suberin by Holloway (1972, 1983). The mass spectrum of compound **21** shows characteristic peaks of eicosene-1,20-dioic acid (*m*/*z* 309 $[M - 59]^+$, 276 $[M - 92]^+$, 74, and the base peak 55) but with contamination of oxo functionality.

The monomers identified in all suberin extracts covered >90% of the components eluted by GC analysis (Figure 1). Most of these components had been previously identified by other authors, but monomers **4**, **5**, **8**, **22**, **24**, **34**, and **43** (Table 2) are reported here for the first time.

Monomeric Composition of Suberin Extracts. The average monomeric composition of the suberin extracts obtained with different $NaOCH_3$ concentrations is summarized in Table 3. The qualitative compositions are similar for all suberin extracts, but quantitative differences are detected.

Suberin D results from complete extraction of cork suberin; therefore, its composition was chosen as the one reflecting the composition of the whole native polymer. Moreover, suberin D results from methanolysis conditions also used in previous studies of cork suberin (Holloway, 1983; Vallejo et al., 1997, 1999), which enables a straightforward comparison with those published results to be made. In fact, the monomeric composition of suberin D is quite similar to that obtained by Vallejo et al. (1997) by methanolysis of Spanish *Q. suber* cork with 0.5 M (2.7%) $NaOCH_3$ in methanol. In the present study, the content of ferulic acid (1.3%) is smaller than that reported (5.3-9.1%) by Vallejo et al. (1997), and the α,ω-alkanedioic acids are more abundant (17.3%) in the present work than in the previous study (6.1−10.2%). However, it should be noted that Vallejo et al. (1997) analyzed cork samples immediately after removal from the tree, whereas we have analyzed cork samples after a prolonged storage (∼12 months). Such differences in suberin composition are expected between those stages (Conde et al., 1999). In addition, the natural variability of cork suberin may also contribute to such differences.

Alkanoic acids and 1-alkanols (C_{16}−C_{26}) are minor components in cork suberin D (Table 3). The abundances of the homologues chain length are similar for both functional classes and confirms the previous observations by Holloway (1972, 1983): docosanoic acid is the major alkanoic acid followed by tetracosanoic acid, whereas 1-docosanol is more abundant than 1-tetracosanol and this more abundant than 1-eicosanol. The content of alkanoic acids in suberin D is also in agreement with results reported by Arno et al. (1981), Vallejo et al. (1997), and Graça and Pereira (1998).

The content of α,ω-alkanedioic acids (C_{16}−C_{24}) is 17.3% of the total suberin D monomers. Graça and Pereira (1998) and Bento et al. (1998) reported similar contents for this homologous series of suberin monomers. The abundance of the homologues has the following sequence: docosane-1,22-dioic acid > octadec-9-ene-1,18-dioic acid > hexadecane-1,16-dioic acid > tetracosane-1,24-dioic acid. The ω-hydroxyalkanoic acids (C_{16}−C_{24}) are commonly the major components of all suberins (Holloway, 1983), comprising 34.2% of the total monomers in suberin D. This value is in agreement with the content of ω-hydroxyalkanoic acids reported by Vallejo et al. (1997) and Bento et al. (1998) for suberin extracts isolated in similar conditions. The most abundant monomer in this homologous series, as well as in the total suberin composition, is 22-hydroxydocosanoic acid. The 18-hydroxyoctadec-9-enoic acid and 24-hydroxytetracosanoic acids are the next most abundant homologues of this monomer series.

The total amount of 9,10-epoxyoctadecane-1,18-dioic acid is 5.3% of the suberin D monomers, taking into account that part of this monomer is converted to the corresponding methoxyhydrin compound after alkaline methanolysis. The 9,10-epoxy-18-hydroxyoctadecanoic acid amounts to 4.6% in suberin D, by the same reasoning. The corresponding 9,10-dihydroxyacids amount to 5.0 and 12.7%, respectively. The use of aqueous alkaline hydrolysis or concentrated mineral acids promotes degradation of epoxy groups and yields a larger amount of the corresponding 9,10-dihydroxylated monomers. Therefore, previous studies of cork suberin by Holloway (1972) and Rodriguez and Ribas (1972) do not mention the existence of epoxyacids resulting from alkaline hydrolysis, and recent studies employing alkaline methanolysis report 9,10-epoxyacid contents similar to ours (Vallejo et al., 1997) or even larger, with the corresponding decrease of 9,10-dihydroxyacid contents (Holloway, 1983; Graça and Pereira, 1998).

The differences detected among suberin extracts A−D can give some insight on the macromolecular structure of native suberin. Figure 2 shows schematically the chain-length abundance of the suberin aliphatic monomers extracted with gradual alkaline methanolysis conditions. The major components of all suberin extracts are aliphatic monomers with chain lengths of C_{18} and C_{22}, the C_{18} monomers occurring predominantly as 9,10-substituted α,ω-alkanedioic and ω-hydroxyalkanoic

Table 3. Composition of *Q. suber* Cork Suberin Extracts Obtained with Different Alkaline Methanolysis Conditions and Determined by GC-MS as Percent Area of the Methyl Ester TMS Ether Derivative Peaks

	suberin A		suberin B		suberin C		suberin D	
monomer	av	SD[b]	av	SD	av	SD	av	SD
alkanoic acids	**10.1**	0.2	**8.5**	0.2	**5.4**	1.7	**2.6**	0.01
C16:0	0.4	0.1	0.4		0.3		0.2	
C18:2(9,12)	0.1	0.1		0.1				
C18:1(9)	0.3	0.2	0.2	0.1			trace	
C18:0	0.2	0.2	0.2	0.03			trace	
C20:0	0.3	0.9	0.3	0.2	trace	0.1	trace	0.1
C22:0	5.2	1.2	4.3	0.07	2.9	0.6	1.3	0.2
C24:0	3.6		3.1		2.2		1.1	
α,ω-alkanedioic acids	**29.4**		**18.8**		**24.1**		**17.3**	
C16:0	2.9	0.4	1.5	0.4	1.9	0.2	1.6	0.1
C18:1(9)	4.7	0.03	3.6	0.3	5.0	2.3	4.1	0.2
C18:0	1.0	0.4			0.4	0.2	0.5	0.1
C20:1	5.0	3.0	1.2	0.7	2.2	1.2	0.3	0.01
C20:0	3.6	0.5	2.6	0.2	3.0	1.0	2.6	0.2
C22:0	9.7	0.7	8.0	0.5	10.4	4.9	7.1	5.1
C24:0	2.5	0.7	1.9	0.2	1.2	0.1	1.1	0.3
ω-hydroxyalkanoic acids	**27.1**		**27.6**		**24.8**		**34.2**	
C16:0 16-OH	0.4	0.03	0.6	0.03	0.4	0.1	1.1	0.1
C18:1(9) 18-OH	2.6	0.5	3.5	0.1	3.8	1.5	8.8	0.6
C18:0 18-OH	0.3	0.2	trace				trace	
C20:1(10) 20-OH	0.2	0.1	0.5	0.06	0.6	0.1	1.2	0.3
C20:0 20-OH	1.5	0.1	2.5	0.1	1.1	0.1	2.2	0.4
C21:0 21-OH	0.3	0.03					trace	
C22:0 22-OH	15.7	2.6	14.5	0.8	14.7	8.5	16.3	1.3
C23:0 23-OH	0.2	0.01	trace				trace	
C24:0 24-OH	5.9	0.4	6.0	0.04	4.2	1.8	4.6	0.3
substituted alkanoic acids	**9.6**		**11.6**		**14.6**		**29.6**	
C16:0 di-OH	1.6	0.08	2.6	0.1	0.9	0.4	0.9	0.2
C18:1 di-OH	0.3	0.03	0.6	0.1	0.9	0.4	1.3	0.3
C18:0 9,10-epoxy, 18-OH	1.1	0.06	1.4	0.2	0.7	0.04	0.8	0.6
C18:0 9-OH, 10-OCH_3, 18-OH	0.8	0.05	2.5	0.09	2.2	0.6	3.8	0.3
C18:0 9,10,18-OH	4.6	1.8	3.0	0.05	9.0	2.1	12.7	2.8
C20:0 di-OH	1.2	0.3	1.5	0.07	0.9	0.3	10.1	0.3
substituted alkanedioic acids	**14.1**		**23.9**		**24.2**		**10.3**	
C18:0 9,10-epoxy + 9-oxo	0.5	0.03	0.8	0.06	0.8	0.2	0.9	0.2
C18:0 9-OH, 10-OCH_3	5.1	1.0	12.8	1.1	10.4	5.9	4.4	0.7
C18:0 9,10-OH	8.5	2.2	9.3	0.7	13.0	7.3	5.0	1.5
1-alkanols	**8.3**		**8.0**		**5.1**		**4.7**	
C16:0	0.1	0.05	0.2	0.03	0.6	0.2	0.2	0.09
C18:1(9)	0.3	0.1	0.2	0.03	0.5	0.3	0.2	0.09
C18:0	0.4	0.1	0.4	0.03	0.7	0.1	0.3	0.09
C20:0	1.1	0.5	1.3	0.03	0.4	0.3	0.5	0.07
C22:0	5.1	1.4	3.9	0.1	1.8	0.6	2.0	0.1
C24:0	1.0	0.4	1.6	0.09	1.1	0.1	1.0	0.1
C26:0	0.3	0.1	0.4	0.03	trace		0.5	0.01
ferulic acid	0.4	0.2			0.5	0.1	1.3	0.4
unidentified	1.0	0.5	1.6	0.3	1.3	0.1		
yield of GC-MS analysis[a] (%)	27		28		9		29	

[a] Total yields were determined by calibration of the GC-MS system with pure reference samples relative to cholesterol, the internal standard. [b] SD, standard deviation.

acids and the C_{22} monomers as saturated α,ω-alkanedioic and ω-hydroxyalkanoic acids. Suberin A is particularly enriched in saturated 1-alkanols and alkanoic, ω-hydroxyalkanoic, and α,ω-alkanedioic acids; therefore, the chain length of C_{22} is the most abundant (36%) in these extracts. Suberin extracts B−D contain 39−46% of C_{18} monomers due to higher quantities of 9,10-dihydroxy- and 9,10-epoxyoctadecane-1,18-dioic acids and 18-hydroxyoctadecanoic acids.

The relative amounts of alkanoic acids and 1-alkanols in suberin extracts increase for milder alkaline methanolysis (Table 3). In other words, during the first steps of alkaline treatment, linkages involving these monomers are readily broken and alkanoic acids and 1-alkanols are preferentially removed. These suberin monomers contain a single functional group being, most likely, located at the endpoint of suberin chains or directly attached to the cell wall matrix as part of short suberin chains. The α,ω-alkanedioic acids are also preferentially removed with very mild alkaline treatment (0.01% $NaOCH_3$), as shown in Table 3. The preferential removal of these monomer families can be explained as follows: (i) they may be preferentially located in sites more accessible to methanolysis in cork cell walls and/or (ii) they may constitute esterified suberin chains with a high $COOR/CH_2$ ratio, therefore easily depolymerized, dissolved, and removed from cork.

The relative amount of mid-chain-modified ω-hydroxyalkanoic acids in suberin extracts increases for stronger alkaline methanolysis (Table 3). This monomer family requires stronger alkaline treatment to be dissolved and removed from cork. This suggests that these monomers may be located in sites which are less accessible to methanolysis, such as the core of the suberin polymer. In addition, they may also constitute

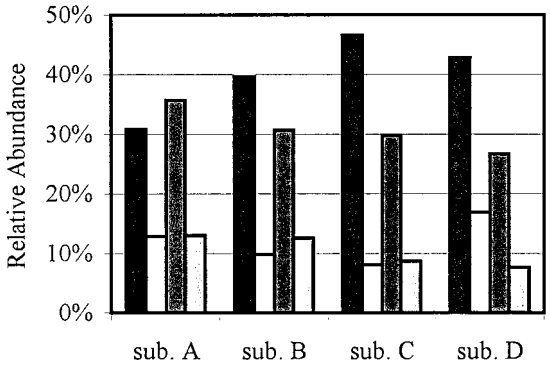

Figure 2. Chain-length abundance of the aliphatic monomers of suberin extracts obtained upon gradual alkaline methanolysis of *Q. suber* cork. The bars represent, from left to right in each grouping, C18, C20, C22, and C24.

less esterified suberin chains, that is, with a lower $COOR/CH_2$ ratio. Because the chain length of all monomer families is similar, with C_{18} and C_{22} being the most abundant, it would be expected that all suberin chains have similar degrees of esterification, resulting from the polymerization of the monomer α,ω-functionalities. Therefore, the existence of less esterified suberin chains may imply the presence of other aliphatic components with very long carbon chains. These aliphatic components may dissolve in the methanolic solution along with the suberin monomers but would not be detected by GC-MS analysis due to their higher molecular weight. This possibility will be discussed further.

The saturated ω-hydroxyalkanoic acids are the most abundant monomer family of suberin, evenly dispersed in the suberin domain of cork cell walls, because the relative amounts of this monomer family are similar in all suberin extracts (Table 3).

Quantitative Analysis of Suberin Monomers. It is noteworthy that calibration of the GC-MS system with pure reference compounds, relative to an internal standard, was not attempted in previous studies on the monomeric composition of cork suberin. Therefore, all previously published results are semiquantitative. In this study the quantification of suberin monomers was performed after calibration of the GC-MS system with commercially available pure standards. Considering the large number of monomers in suberin extracts and the difficulty in finding such commercial pure compounds, the determination of the calibration factors relative to cholesterol, the internal standard, was limited to five suberin monomers representative of the major homologous families. Each calibration factor was assumed to be similar for homologous compounds and therefore was used to correct peak areas of other suberin monomers.

All peak areas of saturated and monounsaturated alkanoic acids were multiplied by a factor of 3.34 obtained for octadec-9-enoic acid, the peak area of octadeca-9,12-dienoic acid was multiplied by a factor of 2.23, the peak areas of α,ω-alkanedioic acids were multiplied by a factor of 1.35 obtained for docosane-1,22-dioic acid, the peak areas of ω-hydroxyalkanoic acids were multiplied by a factor of 1.24 obtained for 16-hydroxyhexadecanoic acid, and peak areas of 1-alkanols were multiplied by a factor of 1.11 obtained for 1-eicosanol. The resulting total yields of the GC-MS analysis of suberin extracts are shown in Table 3. Together, the suberin monomers comprised on average 27−29% (270−290 mg/g) of suberin extracts A, B, and D. The remaining suberin extract (~70%) may contain nonvolatile and/or high molecular weight structures, which cannot be eluted by GC or detected by EI-MS (limited to m/z ~700). To our knowledge, the presence in suberin extracts of a very significant fraction of nonvolatile and/or high molecular weight structures was not previously reported by other research teams. Suberin C shows a much lower yield in suberin monomers detectable by GC-MS (9% or 90 mg/g). Such yield can result from (i) a greater fraction of high molecular weight components and/or (ii) an overstated weight of this suberin extract due to the presence of occluded solvent (methanol). Further evidence will be presented and discussed in the following sections.

Evidence of High Molecular Weight Structures in Suberin Extracts. All suberin extracts were submitted to VPO analysis to determine their M_n and to confirm the existence of structures with molecular weight higher than those compounds detected by GC-MS analysis. The results are shown in Table 1. Some dispersion in M_n values for each suberin sample was observed, as expected due to its heterogeneous nature. The M_n values of suberin extracts range from 528 to 968 g mol^{-1}, increasing from suberin A to suberin D, except for suberin C. The reduced M_n value of suberin C can be explained if occluded solvent (methanol) exists in this extract, because low molecular weight molecules can cause a pronounced decrease in the M_n value. The estimation of the "average molecular weight" for the suberin monomers shown in Table 3 (considering methylated ester groups and free hydroxyl groups) resulted in values of 353−362 g mol^{-1}, meaning that the higher values determined by VPO reveal the presence of high molecular weight structures in the same suberin extracts. In fact, a high molecular weight fraction with m/z ranging from 1000 to 1500 was previously detected by our research team using DCI-MS analysis in cork suberin extracts (Cordeiro et al., 1998). Our previous study on condensed aromatic moieties in suberin extracts (Lopes et al., 1998) provides a partial answer relating to the nature of these high molecular weight structures; it showed that the proportion of condensed aromatic structures increases with increasing alkaline concentration of methanolic solutions (due to alkaline-catalyzed condensation reactions). This is consistent with the variation of M_n values from suberin A to suberins B and D. High molecular weight aliphatic structures are also likely to occur in suberin extracts. These structures may consist of very long carbon chains, as suggested above.

Spectroscopic Characterization of Nonderivatized Suberin Extracts. The nonderivatized suberin extracts were characterized by solution state ^1H NMR and solid state ^{13}C NMR spectroscopy. Figure 3 shows the solution state ^1H NMR spectra of suberin C and suberin D extracts dissolved in $CDCl_3$. These spectra contain structural information consistent with the monomeric composition of suberin extracts C and D (Table 3), because both extracts contain aliphatic methyl groups (δ 0.6−1.0, 1.9−2.3%), aliphatic methylenes (δ 1.0−1.7, 67.1−71.1%), allylic protons (δ 2.0, 2.0−2.1%), CH_2COO groups (δ 2.28 t, 6.7−7.3%), $COOCH_3$ groups (δ 3.9 s, 1.4−1.7%), $COOCH_2$ groups (δ 4.03 and 4.15 t, 2.2−2.7%), vinylic protons (δ 5.32 t, 0.8−1.2%), primary alcohols (δ 3.63 t, 1.0−3.0%), secondary alcohols (δ 4.5−5.2, 1.0−2.1%), aromatic methoxyl groups (δ 3.76 s, 2.1−3.0%), phenylpropanoid $HC_{\alpha,\beta}OR$ groups (δ 5.4−6.4,

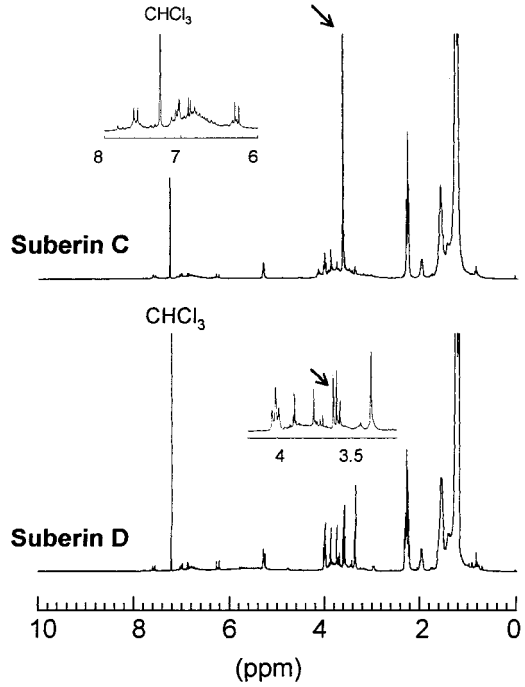

Figure 3. Solution state ^{1}H NMR spectra of suberin extracts C and D dissolved in $CDCl_3$. The arrows show the peaks assigned to (occluded) methanol (3.6 ppm).

1.1−3.6%), and aromatic protons (δ 6.4−8.0, 3.3−3.4%). The low content of aromatic and methoxyl protons, expressed by the low-intensity signals at 6.4−8.0 and 3.76 ppm, is consistent with the low content of ferulic acid and other possible condensed aromatic structures in suberin extracts. Comparison of the two spectra shows that suberin D is richer in primary and secondary hydroxyl groups (δ 3.63 t and 4.5−5.2). These observations are consistent with the increased content of mid-chain-hydroxylated ω-hydroxyalkanoic acids in suberin D (Table 3). The triplet at 4.03 ppm in both spectra, assigned to $COOCH_2$ protons, shows that a fraction of the existing carboxylic groups are still esterified in suberin extracts. The remaining carboxylic groups were converted into methyl esters ($COOCH_3$, δ 3.9 s) or free carboxylic acids.

The sharp singlet at ~3.64 ppm is assigned to methanol (indicated by the arrows in Figure 3). From the intensity of the methanol peak compared to that of the suberin peaks, it becomes clear that methanol contamination is much more pronounced in suberin C than in suberin D (Figure 3). Such comparison may be made because the drying conditions were identical in the preparation of both samples. The methanol peak accounts for 7.8% of the total proton signal in the suberin C spectrum, whereas in the spectrum of suberin D it overlaps with the primary alcohol triplet, which accounts for 2.0% of the spectrum signal. Considering all results obtained for suberin C, it may be concluded that mild alkaline methanolysis conditions (0.03% $NaOCH_3/CH_3OH$) can lead to the extraction of most of the suberin polymer with significant occlusion of methanol, which explains the corresponding decreased M_n value (Table 1).

Additional information about the nature of native suberin can be obtained from their solid state ^{13}C NMR spectra (Figures 4 and 5). The spectra are dominated by the methylene peaks at 25−33 ppm and contain smaller signals assigned to free carboxylic acids (177−

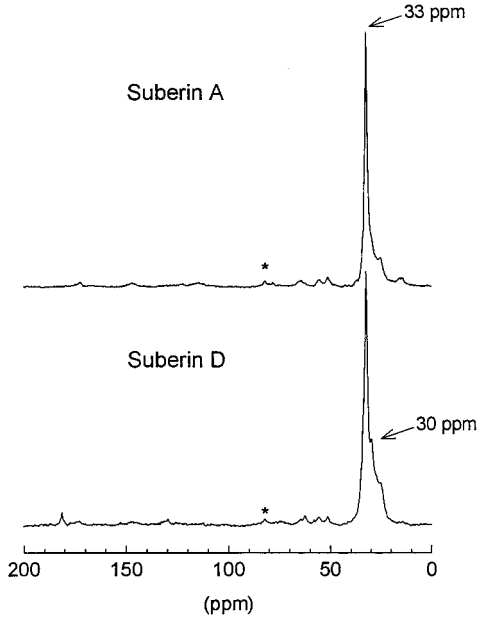

Figure 4. Solid state ^{13}C CP/MAS NMR spectra of suberin extracts A and D, acquired at 5 kHz with a contact time of 1 ms. The asterisks denote spinning sidebands.

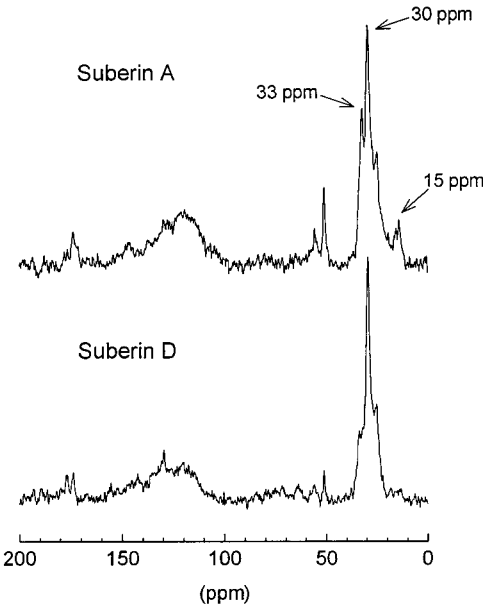

Figure 5. Solid state single-pulse ^{13}C NMR spectra of suberin extracts A and D, acquired at 5 kHz with a recycle delay of 6 s.

181 ppm), carboxylic esters (172−174 ppm), unsaturated carbons (130 ppm), secondary alcohols (75 ppm), primary alcohols (62−64 ppm), and methoxyl groups (56 ppm). The aromatic signals (115 and 147 ppm) are very weak in all spectra. Figure 4 shows the CP/MAS spectra of suberin extracts A and D. In these spectra, the signals observed arise from the carbons situated in rigid environment. Figure 5 shows the single-pulse excitation spectra of the same samples, selecting the peaks corresponding to the most mobile carbon sites. Due to the complementarity of these experiments, discussion of the overall composition of the extract must take into account both experiments.

In both the CP/MAS and the single-pulse spectra of extracts A and D, it is clear that the peak at 15 ppm is relatively stronger in extract A, which indicates an

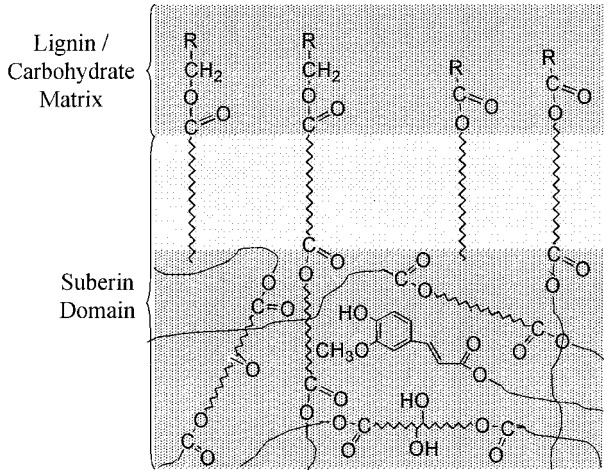

Figure 6. Proposed macromolecular organization of suberin polymer in cork cell walls. R, carbohydrate or lignin residues.

enrichment of methyl groups in that sample. This is consistent with the monomeric composition of suberin A (Table 3) enriched in 1-alkanols and alkanoic acids. In the aliphatic suberin region, the 33 ppm peak dominates the CP/MAS spectra, but it may be seen that the very weak shoulder at 30 ppm for sample A (Figure 4) increases in intensity compared to the 33 ppm peak for suberin extract D (Figure 4). Similarly, the 30 ppm peak is relatively stronger in the single-pulse spectrum of D (Figure 5) than in the spectrum of A. Furthermore, suberin D is also enriched in free carboxylic acids (181 ppm). The changes in the aliphatic regions of the spectra of extracts A and D support the previous observations on the corresponding cork residues that the 33 ppm methylenes, which are closer to $-CH_2O-$ groups and carbohydrate moieties, are preferentially extracted from cork upon very mild alkaline methanolysis (Gil et al., 1997). The free carboxylic acids in extract D may result from ester hydrolysis caused by water contamination during methanolysis or by mineral acid used to neutralize this particular extract. Considering all results obtained for suberin A, we suggest that 1-alkanols, alkanoic acids, and α,ω-alkanedioic acids, which compose suberin chains with higher $COOR/CH_2$ ratios, may be the monomers contributing preferentially to the methylene peak at 33 ppm and therefore involved in linkages to the carbohydrate moieties of the cell wall matrix (Gil et al., 1997; Lopes et al., 1999b). The very long carbon chains, with lower $COOR/CH_2$ ratios, which are not easily removed by alkaline methanolysis and are not detected by GC-MS analysis, may be the components contributing preferentially to the methylene peak at 30 ppm. This would be consistent with the increasing M_n values from suberin A to suberin D and the corresponding increase in the methylene peak at 30 ppm in the solid state ^{13}C NMR spectra.

Figure 6 shows a schematic representation of a possible macromolecular organization of suberin in cork cell walls. This model is a simple qualitative representation of the major suberin monomers and possible linkages within the suberin domain and between suberin and the cell wall lignin/carbohydrate matrix. The very long and quite mobile carbon chains are represented by lines because their structures are not fully established yet. The suberin that composes the secondary wall of cork cell walls is spatially segregated from the lignin/carbohydrate matrix of the middle lamella and primary wall, but it is attached to the cell wall matrix at discrete sites. These anchoring points involve particular families of suberin monomers, such as 1-alkanols, alkanoic acids, and α,ω-alkanedioic acids. This model is an improvement of the scheme suggested by Gil et al. (1997), resulting from the present results and recent advances in the characterization of cork by solid state NMR spectroscopy (Lopes et al., 1999b).

CONCLUSIONS

The present results represent some new advances in determining the macromolecular structure of cork suberin. The monomeric composition of suberin extracts obtained upon gradual alkaline methanolysis of cork was shown to comprise two types of aliphatic suberin chains in cork cell walls:

(i) Suberin chains that are promptly removed from cork upon very mild alkaline methanolysis (0.01% $NaOCH_3$) produce an extract particularly enriched in saturated 1-alkanols, alkanoic acids, and α,ω-alkanedioic acids. The saturated ω-hydroxyalkanoic acids are also an abundant monomer family in all suberin extracts. These chains may be preferentially located in sites more accessible to methanolysis in cork cell walls, such as endpoints of suberin chains or the linkage points of suberin chains to the cell wall matrix. In addition, they may constitute esterified suberin chains with a high $COOR/CH_2$ ratio; therefore, they would be easily depolymerized, dissolved, and removed from cork.

(ii) Suberin chains that are removed from cork upon stronger alkaline methanolysis (3.0% $NaOCH_3$) produce an extract particularly enriched in mid-chain-modified ω-hydroxyalkanoic acids. The saturated ω-hydroxyalkanoic acids are also an abundant monomer family in all suberin extracts. These chains may be located in sites less accessible to methanolysis in cork cell walls, such as the core of the suberin polymer, and/or may constitute less esterified suberin chains with a lower $COOR/CH_2$ ratio.

The quantitative GC-MS analysis showed that 70% of all suberin extracts was not detected. VPO analysis confirmed the existence of high molecular weight structures in suberin extracts, which exhibited M_n values ranging from 528 to 968 g mol^{-1}. The characterization of suberin extracts by solid state ^{13}C NMR spectroscopy has shown that one type of suberin methylene at 33 ppm, closer to $-CH_2O-$ groups and carbohydrate moieties of the cell wall matrix (Gil et al., 1997; Lopes et al., 1999b), is preferentially removed from cork upon very mild alkaline methanolysis. The present results suggest that 1-alkanols, alkanoic acids, and α,ω-alkanedioic acids may be the monomers contributing preferentially to the methylene peak at 33 ppm and therefore involved in linkages to the cell wall lignin/carbohydrate matrix. The remaining methylenes at 30 ppm, characterized by high motional freedom and preferentially removed upon stronger alkaline methanolysis, may have preferential contributions from very long carbon chains with high molecular weight, not easily removed by alkaline methanolysis and not detected by GC-MS. A recent study on the characterization of a polymeric suberin fraction isolated enzymatically from cork, which exhibits different chemical features from the suberin monomers detected by GC-MS, supports the existence of such aliphatic chains (Rocha et al., unpublished results).

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