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Stereochemistry of C_{18} Monounsaturated Cork Suberin Acids Determined by Spectroscopic Techniques Including ^{1}H-NMR Multiplet Analysis of Olefinic Protons

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ABSTRACT:
Introduction – Suberin is a biopolyester responsible for the protection of secondary plant tissues, and yet its molecular structure remains unknown. The $C_{18:1}$ ω-hydroxyacid and the $C_{18:1}$ α,ω-diacid are major monomers in the suberin structure, but the configuration of the double bond remains to be elucidated.
Objective – To unequivocally define the configuration of the $C_{18:1}$ suberin acids.
Methods – Pure $C_{18:1}$ ω-hydroxyacid and $C_{18:1}$ α,ω-diacid, isolated from cork suberin, and two structurally very close $C_{18:1}$ model compounds of known stereochemistry, methyl oleate and methyl elaidate, were analysed by NMR spectroscopy, Fourier transform infrared (FTIR) and Raman spectroscopy, and GC–MS.
Results – The GC–MS analysis showed that both acids were present in cork suberin as only one geometric isomer. The analysis of dimethyloxazoline (DMOX) and picolinyl derivatives proved the double bond position to be at C–9. The FTIR spectra were concordant with a *cis*-configuration for both suberin acids, but their unambiguous stereochemical assignment came from the NMR analysis: (i) the chemical shifts of the allylic ^{13}C carbons were shielded comparatively to the *trans* model compound, and (ii) the complex multiplets of the olefinic protons could be simulated only with $^3J_{HH}$ and long-range $^4J_{HH}$ coupling constants typical of a *cis* geometry.
Conclusion – The two $C_{18:1}$ suberin acids in cork are (*Z*)-18-hydroxyoctadec-9-enoic acid and (*Z*)-octadec-9-enedoic acid.

Supporting information can be found in the online version of this article.

Keywords: *Cis/trans* configuration; ^{1}H-NMR multiplet analysis; cork suberin; (*Z*)-18-hydroxyoctadec-9-enoic acid; (*Z*)-octadec-9-enedoic acid

Introduction

Cork is a tissue found in the outer bark of plants that exhibit secondary growth (as trees typically are), ensuring protection against physical and biotic aggressions, preventing water loss and affording insulation to internal tissues. Cork cell walls have a specific polymeric component, suberin, which accounts for up to 50% of its dry sweight, and is thus believed to be the main component responsible for the vital barrier properties. Commercial cork is extracted from the cork-oak (*Quercus suber* L.), the only known tree able to produce thick layers of continuous cork tissue in a few years growth. Cork, as a material, has a wide array of properties of technical interest and, because of that, is used in an enormous number of applications, some of which are without viable substitutes. Despite its fundamental role in trees and plants, and its relevance for cork properties, suberin is still very poorly understood.

Suberin is a polyester that, after ester-breaking depolymerisation, releases a mixture of monomers, including long-chain (C_{16} to C_{24}) α,ω-diacids and ω-hydroxyacids and glycerol. The composition of suberin from different plant sources is variable, but is typically dominated by C_{18} α,ω-diacids and ω-hydroxyacids with mid-chain substituents, which can be an unsaturated, an epoxide or a vicinal-diol group (Kolattukudy, 2002). Although the composition of suberins from various sources differs in the relative proportion of C_{18} mid-chain substituted acids, the monounsaturated $C_{18:1}$ are always present in significant quantities. For instance, in cork suberin they amount up to 25% of all C_{18} mid-chain substituted acids, but there are cases, such as in potato skin suberin, where they reach close to 100% (Graça and Pereira, 2000). These high levels of $C_{18:1}$ α,ω-diacid and $C_{18:1}$ ω-hydroxyacid monomers indicate a major role in the macromolecular structure of all suberins. The stereochemistry of their mid-chain double bonds will affect the spatial macromolecular arrangements and therefore the physical behaviour of the suberins.

Stereospecific *syn*-hydroxylation provided indirect evidence that unsaturated cork suberin acids, that is, the ω-hydroxyacid, 18-hydroxyoctadecenoic acid (Hyd18:1) and the α,ω-diacid, octadecene-1,18-dioic acid (Di18:1) were present both as *cis* and *trans* isomers (Ribas and Seoane, 1954a; Seoane, 1966).

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However, these authors pointed out that the results would be reliable only if the naturally present suberin acids with mid-chain diol groups were completely removed prior to the *syn*-hydroxylation. In a later work, these same $C_{18:1}$ acids, but from potato skin suberin, were tentatively assigned as *cis*, based solely in the fact that they were liquid at room temperature (Rodríguez and Ribas, 1972).

The *cis* or *trans* configuration also can be determined using physical methods (Eliel and Wilen, 1994). The objective of the present work was the determination of the double bond configuration of Hyd18:1 and Di18:1 using two NMR approaches: the analysis of ^{1}H and ^{13}C chemical shifts of the olefinic protons and carbons; and the analysis of the splitting pattern of the complex multiplets that arise from the olefinic protons in the ^{1}H-NMR spectra, extracting the coupling constants by computer simulation. Two $C_{18:1}$ model compounds, structurally similar to the $C_{18:1}$ suberin acid methyl esters, with known double bond *cis* and *trans* stereochemistry were co-analysed, namely methyl oleate and methyl elaidate. In addition to the one-dimensional NMR analyses, both $C_{18:1}$ suberin acids were fully characterised by two-dimensional NMR, Fourier transform infrared (FTIR) and Raman spectroscopy, electron-induced mass spectrometry (EIMS), and the double bond position confirmed using picolinyl and dimethyloxazoline (DMOX) derivatives.

Experimental

Chemicals and reagents

Methyl oleate (*cis*-octadec-9-enoic acid methyl ester) and methyl elaidate (*trans*-octadec-9-enoic acid methyl ester) with purity above 99% were obtained from Sigma-Aldrich (Sintra, Portugal) and used as received. All solvents used were of HPLC grade (Merck, Germany).

Cork suberin C_{18} monounsaturated acids

Methyl 18-hydroxyoctadecenoate (Hyd18:1_Me) and dimethyl 1,18-octadecenodioate (Di18:1_Me) were obtained from cork suberin after methanolysis depolymerisation, followed by a multi-step isolation and purification process, which is now part of a patent submission (data not shown). The purity of these suberin acids was checked by GC–MS (> 99.9%) and their structure confirmed by EIMS, one-dimensional NMR (^{1}H, ^{13}C) and two-dimensional correlation NMR (correlation spectroscopy (COSY), heteronuclear single-quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC)) (online Supporting information, Fig. SM1).

For the preparation of picolinyl esters and DMOX derivatives (see following sections), the two $C_{18:1}$ suberin acid methyl esters (Hyd18:1_Me and Di18:1_Me) were hydrolysed to free carboxylic acids in a 0.5 M KOH ethanol:water 9:1 solution and recovered after acidification to an organic phase. After drying and trimethylsilyl (TMS) derivatisation, the completeness of the methyl esters hydrolysis to free acids was checked by GC–MS.

Cis/*trans* separation by GC–MS analysis

Analytical samples were prepared in comparable pyridine and N, O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) solutions, although only Hyd18:1_Me needed to be derivatised, to keep the chromatographic conditions identical between model compounds and suberin acids.

Methyl oleate and methyl elaidate mixtures. Methyl oleate (3 μL, ca. 2.6 mg, 0.77 mmol) and methyl elaidate (3 μL, ca. 2.6 mg, 0.77 mmol) were each separately diluted in pyridine (340 μL) and BSTFA (340 μL). An aliquot (100 μL, ca. 0.4 mg) of each of these solutions was taken and mixed in a vial to which pyridine (800 μL) was added.

Methyl 18-hydroxyoctadecenoate (Hyd18:1_Me) and dimethyl 1,18-octadecenodioate (Di18:1_Me). Di18:1_Me (1.6 mg, 0.54 mmol) and Hyd18:1_Me (1 mg, 0.31 mmol) were dissolved in pyridine (202 μL and 130 μL respectively) and derivatised with BSTFA (202 μL and 130 μL respectively), for 30 min, in an oven at 60 °C.

GC–MS analysis. The analytical samples were injected on a 7890A gas chromatograph coupled to a 5975C mass spectrometer detector (Agilent Technologies, Santa Clara, CA, USA) using the following conditions: column DB5-MS (60 m, internal diameter 0.25 mm, film thickness 0.25 μm); oven temperature was from 200 °C to 300 °C with a heating rate of 0.3 °C/min, and a helium flow rate of 1 mL/min. Injections were made in splitless mode, with an injector temperature of 300 °C. Mass spectrometer conditions: electron ionisation 70 eV; source temperature 230 °C and quadrupole temperature 150 °C; transfer line temperature was kept at 310 °C.

Double bond position

Picolinyl esters. Picolinyl esters were prepared using a modified method of Gunstone (1999). Thus, each of the suberin acids (2 mg) was reacted with oxalyl chloride (1 mL) overnight at room temperature. The excess of oxalyl chloride was then removed in a warm water bath under a nitrogen flow.

To the cooled (0 °C, ice bath) acid chlorides was added a cooled (0 °C, ice bath) solution (1 mL) of 3-hydroxymethylpyridine (HMP, 34.8 mg) in dichloromethane (1.7 mL). After 30 min at 0 °C, the reaction mixtures were allowed to warm to room temperature over 2.5 h. The dichloromethane and excess of HMP were removed in a warm water bath under a nitrogen flow and the resultant picolinyl esters dried in a vacuum oven, at 45 °C, over phosphorus pentoxide, overnight. Each picolinyl ester was then derivatised with pyridine and BSTFA (120 μL of each per mg of ester), prior to GC–MS analysis.

DMOX derivatives. The procedure to synthesise the DMOX derivatives was adapted from Harvey (1992). Thus, each suberin acid (3 mg) was reacted with 2-amino-2-methylpropanol (15.5 mg) for 6 h in an oil bath at 160 °C. Both reaction mixtures were allowed to cool to room temperature and subsequently derivatised with BSTFA and pyridine as described for the picolinyl esters prior to GC–MS analysis.

GC–MS analysis. The TMS-derivatised solutions of the picolinyl esters and DMOX derivatives were injected into the GC–MS system previously described. The oven temperature programme was: 5 min at 100 °C, followed by a temperature increase from 100 °C to 250 °C at a rate of 8 °C/min, and then from 250 °C to 300 °C at 3 °C/min; the final temperature was kept for 20 min. Injection and mass spectrometer conditions were as described above.

FTIR analysis

The FTIR absorption spectra (32 scans per spectrum) were acquired on an Alpha-P spectrometer (Bruker Optik, Karlsruhe, Germany) with a spectral resolution of 4 cm^{-1} and a wavenumber range from 4000 cm^{-1} to 400 cm^{-1}. The spectra were obtained by attenuated total reflectance (ATR), with a diamond cell and the pressure clamp applied directly over the samples, which were liquid at room temperature.

Raman analysis

Vibrational Raman spectra were obtained in an apparatus consisting of a double monochromator Spex 1403 (Horiba, Kyoto, Japan), with an argon ion laser line at 514.5 nm, model 2016 (Spectra-Physics, *Santa Clara, CA*, USA) and a R928 photomultiplier detector (Hamamatsu Photonics, Shizuoka, Japan). The spectra were acquired at room temperature, with 90° geometry, a resolution of 4 cm^{-1}, a time of integration of 1 s and an exit power of 1 W. The liquid suberin acid methyl ester samples were placed in glass tubes and analysed at room temperature.

NMR analysis

The NMR spectra were recorded on an Avance II + 600 spectrometer (Bruker Biospin, Rheinstetten, Germany), operating at 600.13 MHz for protons and 150.96 MHz for carbons, equipped with a cryoprobe and pulse gradient units, capable of producing magnetic field pulsed gradients in the *z* direction of 56.0 G/cm. All NMR spectra were acquired in deuterated chloroform with 0.03% of TMS at a temperature of 300 K. The ^{1}H spectra chemical shifts were referenced to TMS (0.00 ppm) and the ^{13}C spectra to chloroform (77.00 ppm). The sample concentration used was 5 mg/500 μL placed in 3 mm or 5 mm diameter NMR tubes suitable for 600 MHz. The NMR spectra were further processed, and spin simulations made using MestReNova, Version 7.1.2 (Mestrelab Research, Santiago de Compostela, Spain).

Results and discussion

In this analysis of the unsaturated suberin acids from cork, without commercial standards available for reference, it was not known if one or both geometric isomers were present and, if both, in what relative proportions. Furthermore, assignment of *cis* or *trans* configuration by NMR is facilitated by the presence of different substituents close to the double bond. However, both Hyd18:1 and Di18:1 are highly symmetrical molecules in relation to the double bond, with identical alkyl substituents up to seven carbons away.

Determination of *cis*/*trans* character by GC–MS

The $C_{18:1}$ *cis* and *trans* model compounds were separated in this present study with methyl oleate eluting 30 s earlier than methyl elaidate (Fig. 1A). Using the same chromatographic conditions, each of the two $C_{18:1}$ suberin acid methyl esters showed a single chromatographic peak (Fig. 1B and C). Due to the structural similarity between the model compounds and the $C_{18:1}$ suberin acids, these results indicate that the $C_{18:1}$ cork suberin acids, Hyd18:1 and Di18:1 exist only as one of the isomers, either *cis* or *trans*.

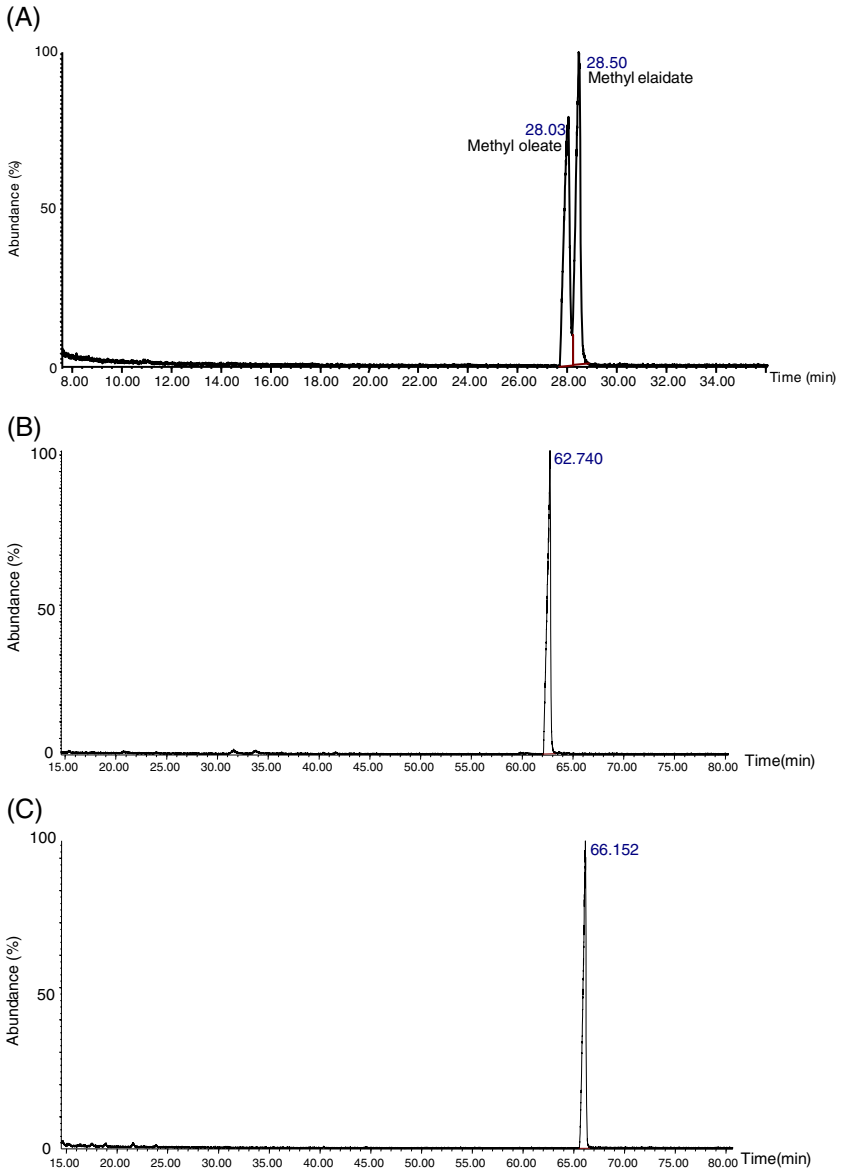


Figure 1. GC–MS ion chromatograms of: (A) 1:1 mixture of methyl oleate (t_R = 28.03 min) and methyl elaidate (t_R = 28.50 min); (B) purity control of dimethyl (*Z*)-octadec-9-enedioate (Di18:1); and (C) methyl (*Z*)-18-hydroxyoctadec-9-enoate (Hyd18:1) (analysed as TMS derivative), isolated from cork suberin.

Double bond position

Chemical degradation studies indicated that the double bond was at C–9, for both Hyd18:1 (Ribas and Seoane, 1954b) and Di18:1 (Ribas and Seoane, 1954a). To confirm this assignment, the double bond position was determined here by mass spectrometry, after derivatisation of the $C_{18:1}$ cork suberin acids to the corresponding picolinyl esters and 4,4-dimethyloxazoline derivatives (Harvey, 1992; Graça and Pereira, 2000). In these derivatives, the presence of the nitrogen atom close to the carboxyl group of the monounsaturated long-chain acids, under typical GC–MS electron ionisation conditions, gives a characteristic pattern of molecule fragmentation that allows the recognition of the double bond position, particularly when it is located mid-chain (Harvey, 1992).

The GC–EIMS spectra of Hyd18:1 DMOX–TMS derivative and Di18:1 picolinyl–TMS derivative are presented in Fig. 2A and B respectively. The mass spectra of Hyd18:1 and Di18:1 TMS derivatives, together with the mass spectra of Hyd18:1 picolinyl–TMS derivative, Di18:1 DMOX–TMS and bis-DMOX derivatives, are shown as online Supporting information (Figs SM2 to SM6, respectively). The Hyd18:1 DMOX–TMS spectrum (Fig. 2A) showed the ions at *m/z* 113 and *m/z* 126 typical of the DMOX moiety, and the successive losses of 14 amu (CH_2 group) starting at *m/z* 320 ($M^{+\bullet} - 103$) until *m/z* 222. The 14 amu sequence restarts at *m/z* 196 until the end of the alkyl chain, at *m/z* 98. The ions of *m/z* 222 and *m/z* 196 had a difference of 26 amu, thus indicating the double bond position at C – 9.

Similarly, the spectrum of Di18:1 picolinyl ester, TMS derivative (Fig. 2B) showed ions at *m/z* 92, *m/z* 108, *m/z* 151 and *m/z* 164 indicative of the pyridine ring being present. A series of 14 amu losses was found from *m/z* 358 ($M^{+\bullet} - CO_2TMS$) until *m/z* 260 and restarted at *m/z* 234, until the end of the alkyl chain. As with the case of the DMOX derivatives, this gap of 26 amu between *m/z* 260 and *m/z* 234, indicated the double bond position at C–9. Previous work with Di18:1 *bis*-DMOX derivative, but extracted from the suberin of potato periderm, also led to the same assignment of the double bond position at C–9 (Graça and Pereira, 2000).

FTIR and Raman analysis

The FTIR spectra of the two $C_{18:1}$ suberin acids, Hyd18:1_Me and Di18:1_Me and of the two $C_{18:1}$ model compounds are compared in Fig. 3, with the most diagnostic bands for *cis* and *trans* assignment annotated. The Raman spectra of the Hyd18:1_Me and Di18:1_Me suberin acids, together with the complete list

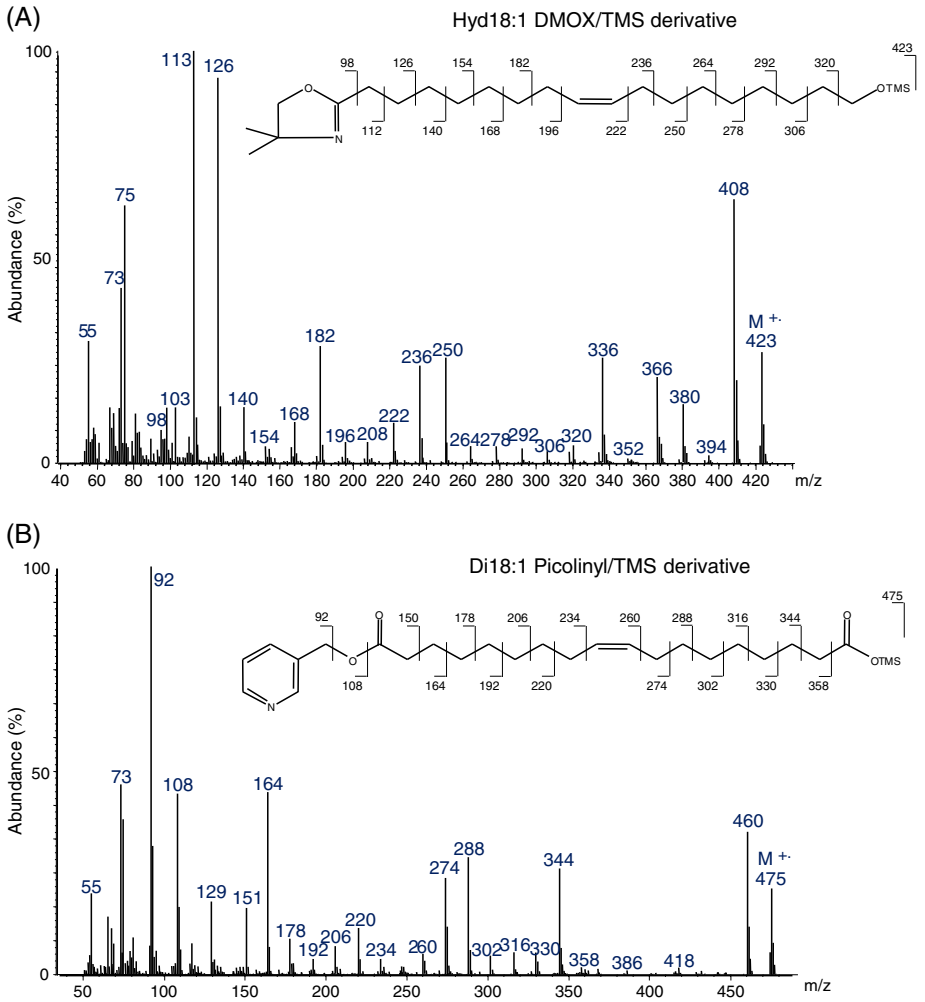


Figure 2. Assignment of the double bond position. Electron ionisation mass spectrum and fragmentation pattern of: (A) (*Z*)-18-hydroxyoctadec-9-enoic acid (Hyd18:1) DMOX–TMS derivative; (B) (*Z*)-octadec-9-enedioic acid (Di18:1) picolinyl–TMS derivative.

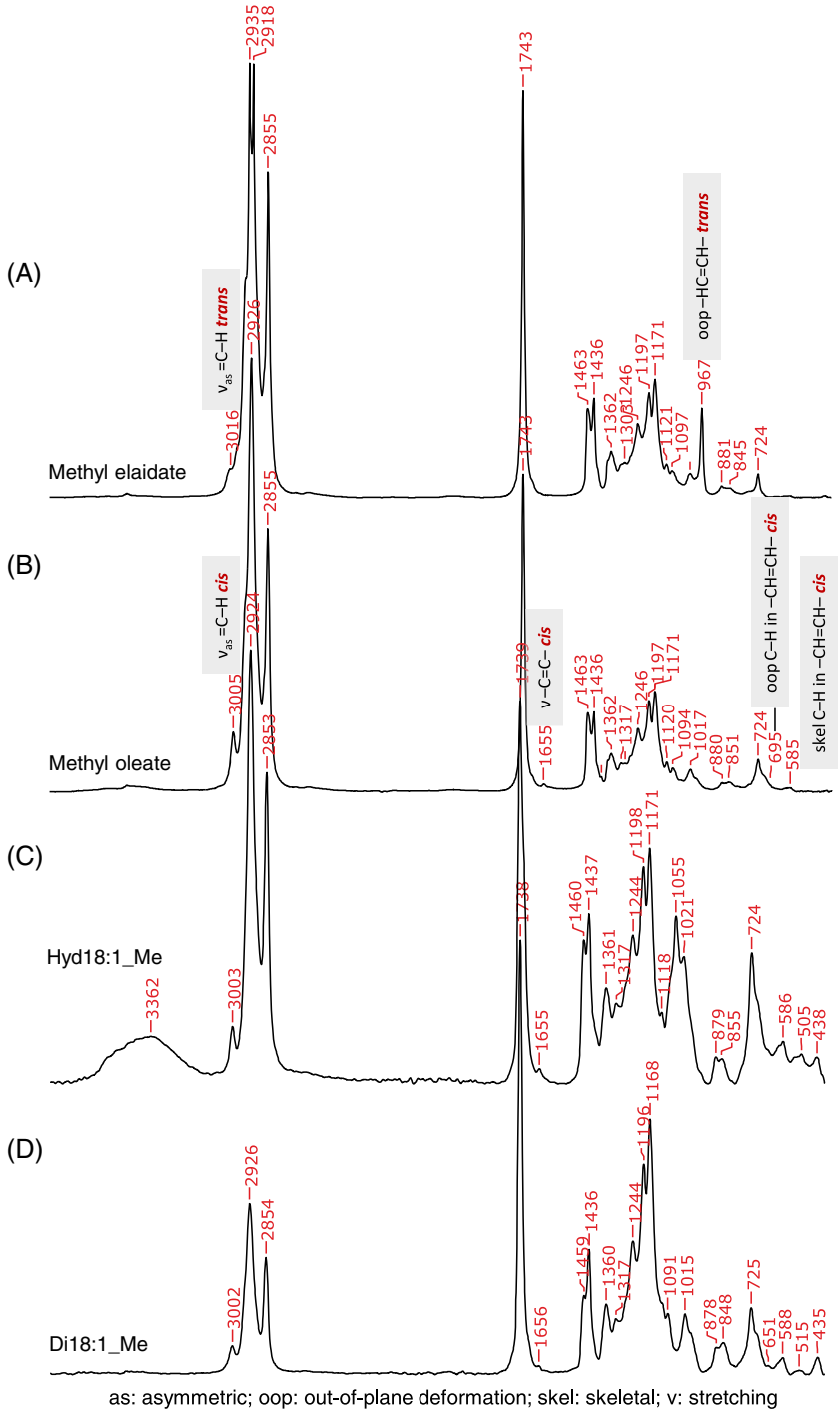


Figure 3. The FTIR spectra of: (A) methyl (*E*)-octadec-9-enoate (methyl elaidate); (B) methyl (*Z*)-octadec-9-enoate (methyl oleate); and cork suberin acids (C) methyl (*Z*)-18-hydroxyoctadecenoate (Hyd18:1_Me) and (D) dimethyl (*Z*)-1,18-octadecenodioate (Di18:1_Me). The main absorption bands assignable to *cis* or *trans* configurations are annotated.

of tentative band assignments for all FTIR and Raman spectra are presented as online Supporting information (Fig. SM7 and Tables SM1 and SM2, respectively). Up to five modes of vibration can be used to differentiate *cis* and *trans* isomers, that is, the =C–H stretching vibration, the C=C–H in-plane and out-of-plane deformation vibrations, and the C=C stretching and skeletal vibrations (Günzler and Gremlich, 2002).

A weak to medium intensity absorption band at 3090–3010 cm^{-1} is typical of the =C–H stretching vibration, with *trans* isomers absorbing at a higher wavenumber than their equivalent *cis* isomers (Socrates, 2001). This was observed in the $C_{18:1}$ model compounds with absorptions at 3005 cm^{-1} and 3016 cm^{-1} for methyl oleate and methyl elaidate respectively (Fig. 3). In the case of Hyd18:1_Me and Di18:1_Me this same band was at 3002 cm^{-1} and 3003 cm^{-1} respectively, values close to the one observed in the *cis* model compound.

Absorptions for C=C stretching in *cis* isomers typically are below 1665 cm^{-1}, while the *trans* isomers absorb above this

frequency (Socrates, 2001). A weak band at ca. 1655 cm^{-1} was observed for methyl oleate, Hyd18:1_Me and Di18:1_Me supporting the *cis* configuration. No such absorption band was observed for methyl elaidate (Fig. 3).

The Raman analysis of methyl oleate and methyl elaidate showed a C = C stretching band at ca. 1656 cm^{-1} for the former *cis* isomer and ca. 1670 cm^{-1} for the latter *trans* (Bailey and Horvat, 1972). The Raman spectra of Hyd18:1_Me and Di18:1_Me had strong bands at 1662 and 1660 cm^{-1} respectively (Fig. SM7), supporting the *cis* configuration.

Another possible diagnostic vibration band for *cis* and *trans* discrimination comes from the C = C–H in-plane deformation, which is not intense in the FTIR spectra, but is relatively strong in Raman spectra of *cis* isomers. This band is present at ca. 1267 cm^{-1} for methyl oleate but not observed in methyl elaidate (Sadeghi-Jorabchi *et al.*, 1991). In the Hyd18:1_Me and Di18:1_Me Raman spectra, a medium to strong band assignable to this vibration is found at 1266 and 1270 cm^{-1} respectively (Fig. SM7), therefore supporting a *cis* configuration for these suberin acids.

In the FTIR spectra, the relatively strong absorption band at 980–955 cm^{-1}, typical of the C = C–H out-of-plane deformation, is known to be unique to *trans* isomers (Chapman, 1965). This band is prominent in the FTIR spectrum of methyl elaidate at 967 cm^{-1} (Fig. 3) but is not observed for methyl oleate or the $C_{18:1}$ suberin acids spectra, thereby indicating a *cis* configuration. In Raman spectra, however, the most intense band for the C = C–H out-of-plane deformation is found for *cis*-isomers. This band was observed at 882 cm^{-1} for methyl oleate, but was not well defined in methyl elaidate (Beattie *et al.*, 2004). The observation of a band at 882 cm^{-1} in the Raman spectra of Hyd18:1_Me and Di18:1_Me supports a *cis* configuration.

Further support for the *cis* configuration was the observation of a weak band (shoulder) around 695 cm^{-1} and another weak band at ca. 965 cm^{-1} assigned to the wagging (out-of-plane) deformation vibrations and the skeletal vibrations respectively of the C–H bond in *cis*–CH = CH– groups (Socrates, 2001) (Fig. 3).

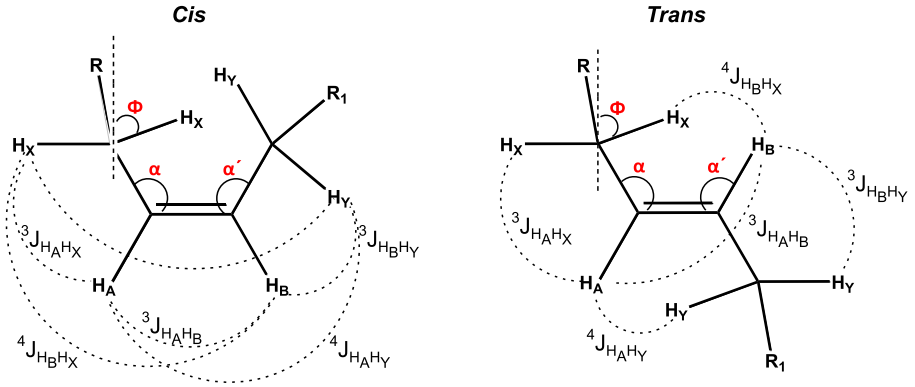
The analysis of the FTIR and Raman therefore indicate that the $C_{18:1}$ acids from cork suberin have, at least, a dominant *cis* configuration.

NMR analysis

The relative symmetry of these unsaturated acids results in an overlapping complexity for the olefinic proton resonances that precludes a straightforward analysis of coupling constants to assign configuration. Therefore, an examination of the chemical shifts of the allylic ^{13}C carbons (Fig. 4) and the estimation of the J_{HH} olefinic coupling constants through spectra simulation were used to determine configuration about the double bonds.

Chemical shifts and *cis* and *trans* configuration. The assignment of ^{1}H and ^{13}C chemical shifts of the suberin acid methyl esters, Hyd18:1_Me, and Di18:1_Me, and of the model compounds methyl oleate and methyl elaidate, are presented as online Supporting information (Tables SM3 and SM4), with the two latter compared with published results. The molecular schemes of the *cis* and *trans* double bonds are presented in Fig. 4, with the olefinic (A and B) and allylic (X and Y) positions identified. The ^{1}H and ^{13}C chemical shifts of the olefinic and allylic positions are shown in Table 1. In this table, the differences between the chemical shifts of the *cis* and *trans* model compounds, $\Delta(\text{ppm}) = \delta^{cis} - \delta^{trans}$, are also given.

In the model compounds, a small difference of $\delta^{cis} - \delta^{trans} = -0.03$ ppm was observed in the olefinic protons, and of +0.05 ppm in the allylic protons (Table 1) showing that the *cis* olefinic protons are slightly more shielded than the *trans* olefinic protons, and that the *cis* allylic protons are more deshielded than the *trans* allylic protons. Similar differences were observed



Φ = torsional angle; α and α´= bond (valence) angle; A and B: olefinic protons; X and Y: allylic protons
$^3J_{H_AH_B}$ = olefinic coupling; $^3J_{H_AH_X}$ = olefinic/allylic coupling; $^3J_{H_BH_Y}$ = olefinic/allylic coupling;
$^4J_{H_BH_X}$ = allylic coupling; $^4J_{H_AH_Y}$ = allylic coupling

Figure 4. Molecular schemes of double bonds of *cis* and *trans* configuration with reference to the coupling constants.
$R = [CH_2]_6 - CO_2CH_3$
Suberin acids
$R_1 = [CH_2]_7 - OH$: methyl (*Z*)-18-hydroxyoctadecenoate (Hyd18:1_Me)
$R_1 = [CH_2]_6 - CO_2CH_3$: dimethyl (*Z*)-1,18-octadecenodioate (Di18:1_Me)
Model compounds
$R_1 = [CH_2]_6 - CH_3$: methyl (*Z*)-octadec-9-enoate (methyl oleate)
$R_1 = [CH_2] - CH_3$: methyl (*E*)-octadec-9-enoate (methyl elaidate)

Table 1. Chemical shifts (ppm) of the olefinic and allylic ^{1}H and ^{13}C in cork suberin acids methyl (*Z*)-18-hydroxyoctadecenoate (Hyd18:1_Me) and dimethyl (*Z*)-1,18-octadecenodioate (Di18:1_Me), and model compounds methyl (*Z*)-octadec-9-enoate (methyl oleate) and methyl (*E*)-octadec-9-enoate (methyl elaidate)

NMR	Compound type	Proton	Hyd18:1_Me	Di18:1_Me	Methyl oleate	Methyl elaidate	$\Delta = \delta^{cis} - \delta^{trans}$
^{1}H	Olefinic	H_9 and H_{10}	5.34	5.34	5.35	5.38	−0.03
	Allylic	H_8 and H_{11}	2.01	2.01	2.01	1.96	+0.05
^{13}C	Olefinic	C_9	129.74	129.79	129.74	130.20	−0.46
		C_{10}	129.85	129.79	129.99	130.47	−0.48
	Allylic	C_8	27.11	27.11	27.20	32.59	−5.39
		C_{11}	27.09	27.11	27.14	32.54	−5.40

in other alkyl disubstituted olefins, as in *cis*- and *trans*-2-pentene and *cis*- and *trans*-3-hexene, which were simulated through theoretical calculations taking into account the double bond magnetic anisotropy and steric effects (Abraham *et al*., 2001). In these respects, for the suberin acids, Hyd18:1_Me and Di18:1_Me, the chemical shifts of their olefinic and allylic protons were almost coincident with those observed in methyl oleate (Table 1) and, therefore, compatible with a *cis* configuration.

Also diagnostic for the *cis* or *trans* configuration assignment in these olefins are the ^{13}C chemical shifts. Comparing methyl oleate and methyl elaidate, the difference between the olefinic carbons was relatively minor, ca. −0.47 ppm, but a much larger difference was observed in the allylic carbons, ca. −5.40 ppm, showing a significant upfield shift for the *cis* isomer (Table 1). This comparative shielding of the *cis*-allylic carbons is probably due to the so-called γ-effect, attributed to the van der Waals interactions between spatially close groups located at three-bond distance (Günther, 2001). In the *cis* double bond configuration, this effect is much more important due to the forced spatial proximity of the opposite methylene allylic groups (Fig. 4). This same shielding of −5.40 ppm in the *cis* allylic carbon was also observed in *cis*- and *trans*-2-butene (Allman, 2010).

The absolute value of the ^{13}C chemical shifts of allylic carbons can be by itself diagnostic of the *cis* or *trans* configuration. In several $C_{18:1}$ fatty acids, when the double bond is close to mid-chain, the chemical shifts of the allylic carbons were found to be very close to 27 ppm for the *cis* isomer, and 32 ppm for the *trans* isomer (Gunstone, 1993). This reasoning can be applied to both 9-unsaturated $C_{18:1}$ suberin acids, Hyd18:1_Me and Di18:1_Me analysed here, which showed allylic carbon chemical shifts of ca. 27 ppm (Table 1), and therefore is concordant with a *cis* configuration.

Coupling constants and cis/trans configuration. The $^{3}J_{HH}$ between the two olefinic protons is typically the main diagnostic coupling constant used for *cis* or *trans* configuration assignment. The magnitude of this coupling constant depends on the overlapping extent of the orbitals of the two =C–H bonds, which is highest when they are parallel (Pavia *et al*., 2009). The orbital parallelism depends on the dihedral angle of =C–H bonds and on the H–C=C bond (valence) angles (α and α′, Fig. 4). In the *trans* configuration, the dihedral angle is 180°, due to the antiparallel arrangement of the =C–H orbitals (Pavia *et al*., 2009), whereas in the *cis* configuration, the parallelism is smaller due to the bond angles (Fig. 4), thus explaining the comparatively higher coupling constants observed in *trans* isomers.

The $^{3}J_{HH}$ between the olefinic and allylic vicinal protons (Fig. 4) is not affected by the double-bond configuration, because the conformation of the allylic protons can change by rotation around the C–C bond. More relevant can be the $^{4}J_{HH}$ long-range allylic coupling, due to the transmission of spin information through the π-electrons, which adds a $J(\pi)$ contribution to the $J(\sigma)$ component (Pavia *et al*., 2009). These long-range couplings are dependent on the configuration and conformation, namely on the torsional angle (Φ) of the allylic C–H bond (as defined in Fig. 4). The sign of the $J(\pi)$ component of the $^{4}J_{HH}$ allylic coupling is negative, whereas the sign of the $J(\sigma)$ component is positive (Günther, 2001), and therefore the sign and magnitude of the $^{4}J_{HH}$ allylic coupling constant reflects the stereochemistry. Typically, in alkyl disubstituted olefins, the $^{4}J_{HH}$ allylic coupling constant is higher in magnitude in *trans* isomers (Rummens and Haan, 1970).

To determine the coupling constants discussed above, the olefinic proton multiplets from the suberin acids and $C_{18:1}$ model compounds were simulated (Fig. 5, Table 2). In Fig. 5, the acquired signals are compared with the simulated multiplets, with their corresponding splitting diagrams. The spin systems, the chemical shifts and the coupling constants used in the simulation are shown in Table 2.

Two spin systems were used in the simulation. One, ABX_2Y_2, for the two model $C_{18:1}$ fatty acids and Hyd18:1_Me, which have non-identical alkyl substituents in relation to the double bond. The other, an $AA'X_2X_2'$ system was used for Di18:1_Me, although one could expect it to have an A_2X_4 spin system, due to the complete symmetry of the molecule in relation to the double bond. The simulation showed that relatively minor variations in the coupling constants had a major impact in the multiplet patterns (Table 2). A simulation of the olefinic protons signals of the model compounds has already been published (Schaumburg and Bernstein, 1968), but the acquired spectra did not match ours, due to differences in the NMR frequencies used (60 MHz).

In our simulation, the $^{3}J_{HH}$ olefinic coupling constants used were unequivocally within the *cis* range for methyl oleate (11.0 Hz), Di18:1_Me (10.5 Hz) and Hyd18:1_Me (10.5 Hz), and within the *trans* range for methyl elaidate (16.0 Hz). The $^{4}J_{HH}$ allylic coupling constants, including their magnitude and signal, had a significant impact on the simulated spectra. In fact, only negative coupling constants could approach the acquired splitting patterns of the olefinic protons. As discussed above, *trans* isomers have higher $^{4}J_{HH}$ allylic coupling constants in absolute value, which was confirmed by the values used to simulate the methyl elaidate olefinic multiplet (−2.6 and −2.3 Hz), compared with the ones used in methyl oleate (−1.5 Hz) and in both suberin acids Di18:1_Me (−1.5 Hz) and Hyd18:1_Me (−2.0 Hz) (Table 2).

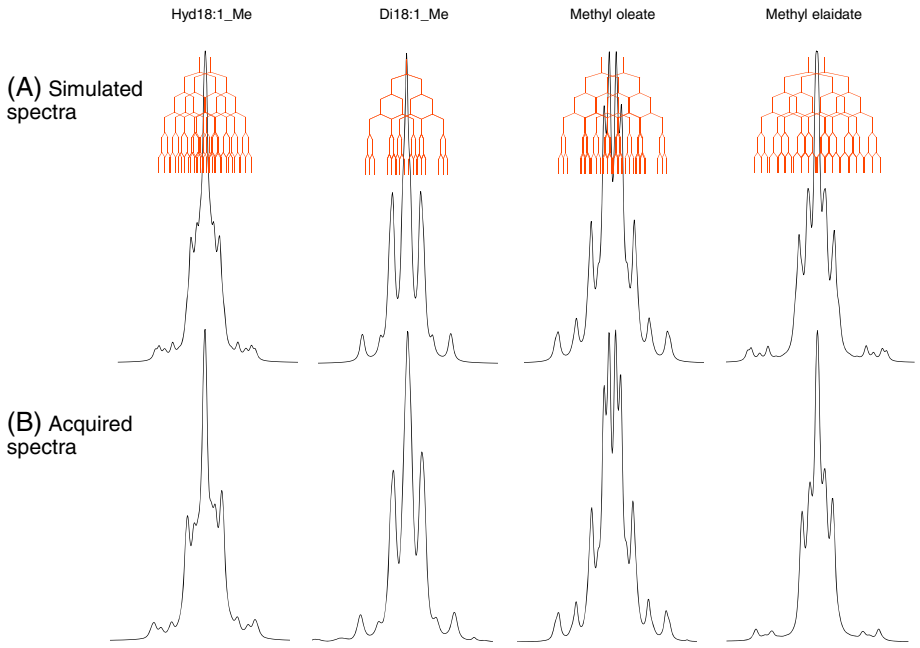


Figure 5. NMR olefinic proton multiplets of: (A) simulated and (B) acquired from methyl (*Z*)-18-hydroxyoctadecenoate (Hyd18:1_Me) and dimethyl (Z)-1,18-octadecenodioate (Di18:1_Me) from cork suberin; and the model compounds methyl (*Z*)-octadec-9-enoate (methyl oleate) and methyl (*E*)-octadec-9-enoate (methyl elaidate).

Table 2. Simulation of olefinic proton NMR multiplets: spin systems, chemical shifts (ppm) and coupling constants (Hz) used in the simulation. Cork suberin acids methyl (*Z*)-18-hydroxyoctadecenoate (Hyd18:1_Me) and dimethyl (*Z*)-1,18-octadecenodioate (Di18:1_Me), and model compounds methyl (*Z*)-octadec-9-enoate (methyl oleate) and methyl (*E*)-octadec-9-enoate (methyl elaidate)

Variable		Hyd18:1_Me	Di18:1_Me	Methyl oleate	Methyl elaidate
Spin system		ABX_2Y_2	$AA'X_2X_2'$	ABX_2Y_2	ABX_2Y_2
Chemical shifts	A	5.3525	5.35	5.356	5.388
	B	5.3460	5.35	5.344	5.379
	X	2.0150	2.01	2.015	2.015
	Y	2.0050	2.01	2.005	2.005
Coupling constants	$^3J_{HH}$ olefinic	10.5	10.5	11.0	16.0
	$^3J_{HH}$ olefinic/allylic	$H_AH_X = 6.5$	$H_AH_X = 7.3$	7.5	$H_AH_X = 8.0$
		$H_BH_Y = 6.5$	$H_BH_Y = 7.0$		$H_BH_Y = 7.9$
	$^4J_{HH}$ allylic	−2.0	−1.5	−1.5	$H_AH_Y = -2.3$
					$H_BH_X = -2.6$

The configuration of $C_{18:1}$ suberin acids and the molecular structure of suberin

The above results demonstrated that both $C_{18:1}$ suberin acids are found, at least in cork, only in the *cis* configuration. These two $C_{18:1}$ suberin acids play a major role in the suberin structure: they have been found in significant quantities in all suberins studied so far, sometimes in overwhelming proportions, such as in potato periderm, where they represent more than 70% of all long-chain monomers. The *cis* stereochemistry of these monomers can be determinant for the macromolecular structure of suberin. An important point of discussion is whether the suberin acids are organised in a totally amorphous manner, or if they have some order at molecular or macromolecular level, as in semi-crystalline polymers. A disordered amorphous molecular arrangement in suberin would be in line with cork properties, such as low rigidity or a glass transition temperature estimated at 18 °C (Dionisio *et al*., 1995). However, a glass transition phenomenon in cork might apply to only a part of the material, because it also includes in its composition, besides suberin, other biopolymers such as lignin and polysaccharides (Mano, 2002). Also, an ordered molecular arrangement in suberin could justify the regular lamellar structure known to exist in suberised cell walls (Sitte, 1962).

Taking into account that the $C_{18:1}$ acids also exist in suberin as glycerol esters (Graça and Santos, 2007) a fairly comparable situation of *cis*-$C_{18:1}$ fatty acids esterified to glycerol can be found in plant fats and oils, namely in the triacylglycerol of oleic acid. The molecular packing of triolein in the solid phase has been thoroughly studied and is known to have up to three different

crystalline forms, each associated with different conformations around the *cis* double bond of the oleic acid units (Larsson *et al*., 2006). Altogether, this shows that the $C_{18:1}$ acids in suberin can also be part of an ordered molecular arrangement.

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References

Abraham RJ, Canton M, Griffiths L. 2001. Proton chemical shifts in NMR: Part 17. Chemical shifts in alkenes and anisotropic and steric effects of the double bond. *Magn Reson Chem* **39**: 421–431.

Allman R. 2010. *Nuclear magnetic resonance*. CMR: Peak splitting. http://www.wavesignal.com/forensics/NMR.html (accessed October 2012).

Bailey GF, Horvat RJ. 1972. Raman spectroscopic analysis of the *cis/trans* isomer composition of edible vegetable oils. *J Am Oil Chem Soc* **49**: 494–498.

Beattie JR, Bell SEJ, Moss BW. 2004. A critical evaluation of Raman spectroscopy for the analysis of lipids: Fatty acid methyl esters. *Lipids* **39**: 407–419.

Chapman D. 1965. *The Structure of Lipids*. Methuen: London; 52–93.

Dionisio MSC, Correia NT, Mano JF, Ramos JJM, Fernandes AC, Saramago B. 1995. Absorbed water in the cork structure – a study by thermally stimulated currents, dielectric-relaxation spectroscopy, isothermal depolarization experiments and differential scanning calorimetry. *J Mater Sci* **30**: 4394–4400.

Eliel EL, Wilen SH. 1994. *Stereochemistry of Organic Compounds*. John Wiley & Sons, Inc: New York; 555–573.

Graça J, Pereira H. 2000. Suberin structure in potato periderm: Glycerol, long-chain monomers, and glyceryl and feruloyl dimers. *J Agric Food Chem* **48**: 5476–5483.

Graça J, Santos S. 2007. Suberin: a biopolyester of plants' skin. *Macromol Biosci* **7**: 128–135.

Gunstone FD. 1993. The composition of hydrogenated fats by high-resolution C-13 nuclear-magnetic-resonance spectroscopy. *J Am Oil Chem Soc* **70**: 965–970.

Gunstone FD. 1999. The preparation of derivatives for lipid analysis. In *Lipid Synthesis and Manufacture*, Gunstone FD (ed.). Sheffield Academic Press: Sheffield; 321–346.

Günther H. 2001. *NMR Spectroscopy*. John Wiley & Sons, Inc: New York; 108–128.

Günzler H, Gremlich H-U. 2002. *IR Spectroscopy*. Wiley-VCH: Weinheim; 185–190.

Harvey D. 1992. Mass spectrometry of picolinyl and other nitrogen-containing derivatives of lipids. In *Advances in Lipid Methodology – One*, Christie WW (ed.). The Oily Press: Dundee; 19–80.

Kolattukudy P. 2002. Suberin from plants. In *Biopolymers – Polyesters I*. Doi Y, Steinbüchel A (ed.). Wiley-VCH: Weinheim; 41–73.

Larsson K, Quinn P, Sato K, Tiberg F. 2006. *Lipids: Structure, Physical Properties and Functionality*. The Oily Press: Brigwater; 18–25.

Mano JF. 2002. The viscoelastic properties of cork. *J Mater Sci* **37**: 257–263.

Pavia D, Lampman G, Kriz G, Vyvyan J. 2009. *Introduction to Spectroscopy*. Brooks/Cole: Belmont; 233–247.

Ribas I, Seoane E. 1954a. Química del corcho. XI. Ácidos no saturados. *Anal Real Soc Fís Quím* **50B**: 963–970.

Ribas I, Seoane E. 1954b. Química del corcho. XII. Ácido 18-hidroxi-9-octadecenoico y derivados. *Anal Real Soc Fís Quím* **50B**: 971–976.

Rodríguez B, Ribas I. 1972. Investigaciones químicas sobre el corcho de solanium tuberosum L. (patata). *Anal Quím* **68**: 303–308.

Rummens FHA, Haan JW. 1970. Spectroscopic studies on olefins – III: NMR of *cis*- and *trans*- disubstituted olefins. *Org Magn Resonance* **2**: 351–355.

Sadeghi-Jorabchi H, Wilson RH, Belton PS, Edwardswebb JD, Coxon DT. 1991. Quantitative analysis of oils and fats by Fourier transform Raman spectroscopy. *Spectrochim Acta A* **47**: 1449–1458.

Schaumburg K, Bernstein HJ. 1968. Calculation of the NMR spectrum of double-bond protons in aliphatic systems. *Lipids* **3**: 193–198.

Seoane E. 1966. Ácido 18-hidroxi-trans-9-octadecenoico, aislado del corcho en forma de ácido treo-9,10,18-trihidroxioctadecanoico. *Anal Real Soc Fís Quím* **62B**: 563–568.

Sitte P. 1962. Zum feinbau der suberinschichten im flaschenkork. *Protoplasma* **54**: 555–559.

Socrates G. 2001. *Infrared and Raman Characteristic Group Frequencies – Tables and Charts*, 3rd edn. John Wiley & Sons, Ltd: Chichester; 68–81.

Supporting information

Supporting information can be found in the online version of this article.