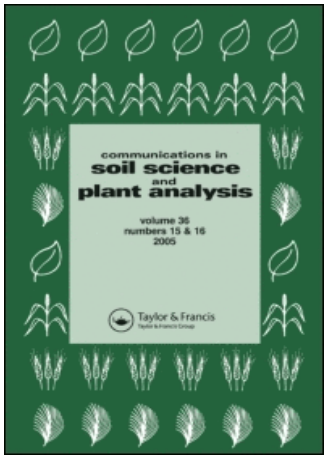

Communications in Soil Science and Plant Analysis
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597241

Spectroscopic Characterization of Aliphatic Moieties in Four Plant Cuticles

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Online Publication Date: 01 October 2007
To cite this Article: Johnson, Elizabeth J., Dorot, Orna, Liu, Judong, Chefetz, Benny and Xing, Baoshan (2007) 'Spectroscopic Characterization of Aliphatic Moieties in Four Plant Cuticles', Communications in Soil Science and Plant Analysis, 38:17, 2461 - 2478
To link to this article: DOI: 10.1080/00103620701588841
URL: http://dx.doi.org/10.1080/00103620701588841

Communications in Soil Science and Plant Analysis, 38: 2461–2478, 2007

ISSN 0010-3624 print/1532-2416 online
DOI: 10.1080/00103620701588841

Spectroscopic Characterization of Aliphatic Moieties in Four Plant Cuticles

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Abstract: Aliphatic components of tomato, pepper, and apple fruit cuticles, and the leaf cuticles of mature olive trees, were characterized using elemental analysis, ^{13}carbon (C) nuclear magnetic resonance (NMR), and Fourier transform infrared spectroscopy (FTIR). Cuticular fractions isolated for analyses included bulk, dewaxed, nonsaponifiable, and nonhydrolyzable cuticles. Results from ^{13}C NMR and FTIR spectra indicate that the cuticles of all the plant materials studied are comprised of extractable lipids, polysaccharides, and cutin, whereas the cuticles extracted from the olive leaf, pepper fruit, and apple fruit also contained nonsaponifiable, nonhydrolyzable

Received 1 June 2006, Accepted 8 September 2006
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residues, likely to be cutan. Hydrogen (H)/C and [oxygen (O) + nitrogen(N)]/C atomic ratios for the olive leaf, pepper fruit, and apple fruit cuticle fractions indicate that their bulk cuticle, dewaxed cuticle, and lipid fractions are more aliphatic than but have a similar polarity to their respective cutan-like fraction. These results provide evidence that pepper fruit, apple fruit, and olive leaf cuticles each contain a cutan-like fraction, but in the olive leaf and apple fruit, this fraction has a slightly different chemical structure from that of the pepper fruit and makes up a smaller percent of the total cuticle.

Keywords: ^{13}C NMR, cutan, cutin, FTIR, plant cuticle

INTRODUCTION

The plant cuticle is a membrane of predominantly lipid composition that encompasses the fruit and leaves of terrestrial vegetation. The main function of the cuticular membrane is to serve as a waterproof, protective barrier to the environment (Kolattukudy 2002). The structure and chemical composition of cuticular material varies by plant but can usually be characterized by two classes of hydrophobic hydrocarbons, soluble wax and insoluble aliphatic cutin (Jeffree 1996). Cutin is the structural component of the plant cuticle, which is attached to the outside of the epidermal wall in the aerial parts of angiosperms and gymnosperms. Cuticle wax is a complex mixture of long-chain hydrocarbons that function to prevent desiccation of the plant and act as a barrier to water diffusion. The primary role of cutin is to serve as the polymer matrix binding the wax in the cuticle. Cutin's high-molecular-weight polymer structure consists of cross-linked, 16- or 18-C chain, hydoxyl-fatty acids and hydroxyepoxy-fatty acids that can be depolymerized and solubilized upon saponification (Jeffree 1996). Many species of plants also have a recently identified third class of hydrophobic hydrocarbon, cutan (Tegelaar et al. 1989). Cutan is operationally defined as the residual cuticular material that is nonsaponifiable and nonhydrolyzable. It is comprised of an amorphous three-dimensional network linked by ether bonds containing double bonds and free carboxylic groups (Villena, Dominguez, and Heredia 1999; Sachleben et al. 2004). Some plant cuticles contain cutin but do not appear to contain cutan (such as *Lycopersicon esculentum*), and others have cutan but not cutin (such as *Beta vulgaris*), whereas many species are composed of varying ratios of cutin and cutan that differ in abundance based on plant maturity and location in the plant (such as *Agave americana*) (Tegelaar et al. 1991; Villena, Dominguez, and Heredia 1999).

The plant cuticle components cutin and cutan have demonstrated selective preservation during decomposition with little or no alteration (Almendros et al. 1996; Lichtfouse et al. 1998; Nierop 1998), especially cutan, which is often identified in fossilized cuticles (Tegelaar et al. 1991). Cuticular membranes from decomposing plants are one of the primary

sources of aliphatic moieties present in soil organic materials (Zech et al. 1997; Chefetz et al. 2002; Kögel-Knabner et al. 1992). As plant cuticular materials decompose, the resistant nature of the aliphatic biopolymers in the cuticle causes a relative increase in the aliphatic moieties in humified soil organic material (Nierop 1998; Tegelaar et al. 1989). Recently, several studies have emphasized the importance of the aliphatic-rich components of the soil organic matter, specifically cutin and cutan, in the sorption and sequestering of hydrophobic organic contaminants (HOC) (Gunesekara and Xing 2003; Kang and Xing 2005; Chefetz, Deshmukh, and Hatcher 2000; Mao et al. 2002; Salloum, Chefetz, and Hatcher, 2002). As cuticular materials are the precursor to the aliphatic organic materials in the soil, a study of the chemical composition of plant cuticles may facilitate explanations of this soil behavior.

The chemical composition of plant cuticular materials may hold economic implications for agriculture and industry, as well as a better understanding of the sorption potential of HOCs to the organic matter in soil and sediments. The objective of this study was to comprehensively characterize the aliphatic moieties of cutin and cutan isolated from four plant cuticles (tomato fruit, pepper fruit, apple fruit, and olive leaf) using elemental analysis, attenuated total reflectance (ATR)–Fourier transform infrared spectroscopy (FTIR), and solid-state ^{13}carbon (C) nuclear magnetic resonance (NMR). This article represents the baseline characterization in an ongoing experiment monitoring the variation in fruit cuticle composition and structure during decomposition.

MATERIALS AND METHODS

Plant Cuticles

Cuticular materials were isolated and purified from the fruits of tomato (*Lycopersicon esculentum*), pepper (*Capsicum anuum*), and apple (*Malus pumila* "macintosh"). These fruits were chosen because they represent typical New England crops, and they have large thick cuticles that are isolated easily from the remaining fruit material. The fruits were purchased at a grocery store located in Hadley, Massachusetts, USA. Plant cuticular materials were also isolated from the leaves of mature olive trees (*Olea europaea*), growing in Rehovot, Israel. Olive leaves were chosen due to their thick cuticle, which is easily isolated from the leaf.

Isolation of Plant Cuticular Fractions

To isolate the skin of the tomato, the fruit was blanched in boiling water for 20 s and submerged in ice water. The loosened skins were removed by hand

(Luque et al. 1994). The pepper and apple fruit skins were manually peeled off and boiled in water for 1 h, after which any remaining pulp was manually scraped off. Immediately after picking, olive leaves were soaked in water and washed several times in distilled water to remove dust. Isolation of the olive leaf cuticle began with the chemical extraction procedure described next.

Aliphatic cuticular materials were isolated from the fruit skins and untreated leaves using a modified extraction–depolymerization procedure as described by Kögel-Knabner et al. (1994). In brief, once the skins were removed from the fruit, they were incubated in a solution of ammonium oxalate (16 g/L) and oxalic acid (4 g/L) at 90°C for 24 h and extensively washed with distilled water to remove any residual pectinaceous material. The resulting material is the bulk cuticle fraction (C1). Cuticular waxes (CW) were removed from the bulk cuticle by soxhlet extraction with chloroform–methanol (1:1 v/v) at 60°C for 24 h to yield dewaxed cuticle (C2). Because of the large quantity of wax (~50%) in apple bulk cuticle, it was possible to isolate the cuticle wax from the chloroform–methanol solvent using a Buchi R-200 Rotovaper (Flawil, Switzerland). To depolymerize the cutin polymer, the dewaxed cuticle fraction was saponified in 1% potassium hydroxide (KOH) in methanol for 3 h at 70°C, under refluxing conditions, producing the nonsaponifiable cuticle fraction (C3). Carbohydrates were removed from this fraction by acid hydrolysis using 6M hydrochloric acid (HCl) and refluxed for 6 h at 90°C, resulting in the nonhydrolyzable cuticle fraction (C4). All samples were freeze-dried, ground, and sieved (<0.18 mm) before analysis.

As indicated, the cuticle fractions isolated were referred to as C1 (bulk cuticle), C2 (dewaxed cuticle), C3 (nonsaponifiable cuticle), C4 (nonhydrolyzable cuticle), and CW (cuticle wax). Each of these labels was preceded with letters T, P, A, or O when referencing a fraction isolated specifically from tomato fruit, pepper fruit, apple fruit, or olive leaf cuticle, respectively.

Characterization of the Cuticular Fractions

Elemental analysis for C, hydrogen (H), and nitrogen (N) content for the three fruit cuticle fractions were determined using a Vario ELIII elemental analyzer (Elementar, Germany) (Xing et al. 2005), and the olive leaf content was determined using an EA 1108 automated elemental analyzer (Fison Instruments, Milan, Italy). Randomly chosen fruit cuticle samples were run in triplicate. Olive leaf cuticular materials were run in duplicate. Ash content was measured by igniting samples at 800°C for 4 h. Oxygen content was calculated by the mass difference. Hydrogen/C and (O + N)/C atomic ratios were calculated and presented in Table 1.

FTIR spectroscopy of the fruit cuticles was performed using a Perkin-Elmer Spectrum One spectrometer with a Perkin-Elmer universal attenuated total reflectance (ATR) sampling accessory (Wellesley, MA) (Kang and

Table 1. Percent yield, elemental analysis,[a] and atomic ratios of isolated cuticles

Sample	Isolation		Yield[b,c] (% wt)	C (%)	H (%)	N (%)	H/C	$(O^d + N)/C$
Tomato fruit	Bulk cuticle	TC1	100.0	63.67	9.31	1.19	1.76	0.26
	Dewaxed cuticle	TC2	89.1 ± 1.9	62.90	9.21	1.29	1.76	0.28
Pepper fruit	Bulk cuticle	PC1	100.0	62.98	9.34	1.26	1.78	0.32
	Dewaxed cuticle	PC2	93.6 ± 1.2	62.27	9.31	1.44	1.79	0.36
	Saponified cuticle	PC3	24.7 ± 2.5	41.16	6.16	3.28	1.80	0.77
	Hydrolyzed cuticle	PC4	6.4 ± 0.2	64.67	1.37	1.37	1.33	0.24
Apple fruit	Bulk cuticle	AC1	100.0	67.24	9.79	0.73	1.75	0.25
	Dewaxed cuticle	AC2	55.0 ± 0.2	61.54	9.03	1.03	1.76	0.35
	Saponified cuticle	AC3	12.2 ± 1.3	39.55	5.49	2.24	1.67	0.87
	Hydrolyzed cuticle	AC4	2.9 ± 0.1	57.72	4.93	1.57	1.02	0.46
	Cuticle wax	ACW	41.9 ± 3.2	77.19	11.36	0.02	1.77	0.11
Olive leaf	Bulk cuticle	OC1	100.0	67.40	9.62	0.34	1.71	0.26
	Dewaxed cuticle	OC2	73.8	60.73	8.80	0.42	1.74	0.38
	Saponified cuticle	OC3	21.7	40.08	5.42	1.18	1.62	1.02
	Hydrolyzed cuticle	OC4	2.46	59.34	4.66	0.69	0.94	0.46

[a]The ash content for all cuticular isolations was negligible (<0.5%).
[b]The yields of the isolations of each sample were calculated to the percentage content of their respective bulk cuticles.
[c]Standard deviation for yield values of the olive leaf cuticular fractions were <5%.
[d]Oxygen content was calculated by the mass difference.

Xing 2005). Samples were analyzed between 4000 and 650 cm^{-1}, and 200 scans were collected per sample. No sample preparation or baseline correction was required. Olive leaf cuticular fraction FTIR spectroscopy was performed using a Nicolet 550 Magna-IRTM Spectrometer (Nicolet Instruments, Madison, Wisc., USA). Two-mg samples were finely ground, mixed with 98 mg KBr, and compressed into pellets. Samples were analyzed between 4000 and 400 cm^{-1}, and 40 scans were collected per sample. A baseline correction was performed using 4000, 2000, and 860 cm^{-1} as zero absorbance points.

Solid-state cross-polarization magic angle spinning ^{13}C nuclear magnetic resonance (CP-MAS ^{13}C NMR) spectra were obtained for the three fruits using a Bruker DSX-300 spectrometer (Karlsruhe, Germany) operated at the ^{13}C frequency of 75 MHz. The acquisition parameters were contact time, 1 ms; acquisition delay, 5 s; spinning speed, 5 kHz; and number of scans, 3000 (Chen et al. 2005). CP-MAS ^{13}C NMR spectra were obtained for the olive leaf cuticle using the following acquisition parameters: contact time, 2 ms; recycle delay, 1 s; spinning speed, 13 kHz; and number of scans, 10,000 (Chefetz 2003). The spectra for all plant cuticles were divided into the following chemical shift regions: paraffinic C (0–50 ppm), alcohols, amines, carbohydrates, ethers, methoxyl, and acetal C (50–109 ppm), aromatic and phenolic C (109–163 ppm), carboxyl and carbonyl C (163–190 ppm), and ketone C (190–220 ppm) (Kang et al. 2003). The 0- to 109-ppm region of the spectra represents the aliphatic C content, and the 109–163 ppm region represents the aromatic C content. Total aliphaticity was determined by expressing aliphatic C content as a percentage of the aliphatic plus aromatic C content, and total aromaticity was calculated by expressing aromatic C content as a percentage of the aromatic plus aliphatic C content.

RESULTS AND DISCUSSION

Elemental Analysis and Yield

Yields and elemental composition of isolated plant cuticular fractions are presented in Table 1. Isolation yields for each cuticular fraction were calculated to the percentage content of their respective bulk cuticles. The percent of external lipids removed from the tomato and pepper bulk cuticles (C1) by soxhlet extraction was relatively small, 11 and 7%, respectively, when compared to the 26 and 45% extracted from the olive leaf cuticle and apple bulk cuticle, respectively. The very high wax content of the apple cuticle permitted the successful isolation of a large portion (~93%) of the extracted wax (ACW) for further characterization. The dewaxed cuticle (C2) represented 89, 93, and 74% of the tomato fruit, pepper fruit, and olive leaf bulk cuticle, respectively, whereas the apple dewaxed cuticle

comprised a little over half of its bulk cuticle (55%). Once the soluble external waxes were removed from the fruit cuticle, the predominant remaining chemical components were cutin, sugars, and cutan, in varying abundances, depending on the plant.

Because the tomato cuticle is a cutan-free cuticle (Tegelaar et al. 1991), it was unnecessary to analyze the C3 and C4 cuticular fractions. The tomato C3 fraction was isolated, but only to calculate the percent saponified cutin monomers in the cuticle. The cutin monomers removed by saponification represented a large portion of the bulk cuticle in all four plant samples, corresponding to 65, 61, 42, and 52% by weight for the tomato fruit, pepper fruit, apple fruit, and olive tree leaf, respectively. The final isolation, acid hydrolysis, eliminated the remaining cuticular polysaccharides and resulted in a nonsaponifiable, nonhydrolyzable, cutan-like residue (C4), which comprised 6.8, 2.9, and 2.5% of the pepper fruit, apple fruit, and olive leaf bulk cuticles, respectively. When percentage yields were recalculated to the percentage content of their respective dewaxed cuticles, instead of their bulk cuticles (i.e., normalizing for the large wax content in the apple cuticle), the percent of cutin monomers and cutan-like residue (C4) appeared more comparable among the plant cuticles. The recalculated percentages of cutin monomers removed by saponification were 70, 65, 76, and 71% for the tomato fruit, pepper fruit, apple fruit, and olive leaf cuticles, respectively, and the percentages of cutan-like residue were 7.3, 5.3, and 3.3% for PC4, AC4, and OC4, respectively. The yields for the isolated tomato and pepper fruit cuticles are consistent with literature values (Chefetz 2003; Chen et al. 2005; Round et al. 2000).

The general elemental analysis trends were similar among the isolated fractions of the four plant cuticle samples. The bulk, dewaxed, and nonhydrolyzable cuticles of all plant cuticle samples were each composed of approximately 65% organic C. The lowest organic C content was in the nonsaponifiable sample, approximately 40% in all samples. The highest organic C content was found in the apple cuticle wax (77%). The aliphaticity, as represented by the H/C atomic ratio, ranged between 1.66 and 1.80 for the C1, C2, C3, and CW fractions (as available) for each of the plant cuticles isolated. The C4 fractions had the lowest aliphaticity, 1.33, 1.02, and 0.94 for the pepper fruit, apple fruit, and olive leaf, respectively. The polarity, as determined by the $(O + N)/C$ atomic ratio, was highest in the C3 fraction ($\sim$1.0 for all three isolated samples), which is indicative of the higher N and polysaccharide content of this fraction. The polarity of the remaining fractions was much lower, ranging greatest in the apple fruit sample, from 0.10 (ACW) to 0.42 (AC4). In general, elemental analysis results indicate that the cuticular fractions isolated were primarily aliphatic in composition and encompassed a wide range in polarity. The ^{13}C NMR and ATR-FTIR spectra for each fraction, as described next, confirmed the elemental analysis results.

Spectroscopic Characterization

Figures 1A–4A illustrate the ATR-FTIR and FTIR spectra for the isolated cuticular fractions from the plant cuticles studied. Infrared spectra obtained for the tomato fruit and pepper fruit (TC1 and PC1) and the dewaxed cuticle (C2) fractions isolated from all plant cuticles illustrate many similarities. The AC1 and OC1 spectra were overshadowed by bands associated with their larger wax content and are discussed separately. The broad spectral band at $\sim$3300 cm^{-1} was assigned to the stretching vibration of the hydrogen-bonded, hydroxyl functional groups. Most of the remaining pronounced bands on the spectra were attributed to the fatty acids and esters comprising cutin polymer. These include two strong absorption bands located at 2931 and 2850 cm^{-1}, associated with the asymmetric and symmetric stretching vibrations of the methylene groups, respectively, as well as smaller bands at 1473, 1463, 1313, and 723 cm^{-1}, which corresponded to methyl group bending vibrations (Chamel and Marechal 1992). A relatively strong band at 1731 cm^{-1} and its shoulder at 1700 cm^{-1} were assigned to the potentially hydrogen-bonded, C-O stretching vibration of the carbonyl bond in the ester group within the cutin polymer (Chen et al. 2005). Narrow bands at 1163 and 1100 cm^{-1} were assigned to the asymmetric and symmetric C-O-C stretching vibrations of the ester bonds, respectively. These bands are associated with the interesterification of the cutin monomers in the polymer form. Peaks associated with C-O stretching in polysaccharides in the bulk and dewaxed cuticle were identified at 1247, 1063, and 1030 cm^{-1}. The aromatic domain of the cuticle was associated with a weak adsorption band located at 1650–1500 cm^{-1}, which corresponded to the presence of phenolic compounds at the cuticular level.

After saponification, the characteristics of the FTIR spectra changed significantly. The saponification with KOH in methanol caused the ester bonds in

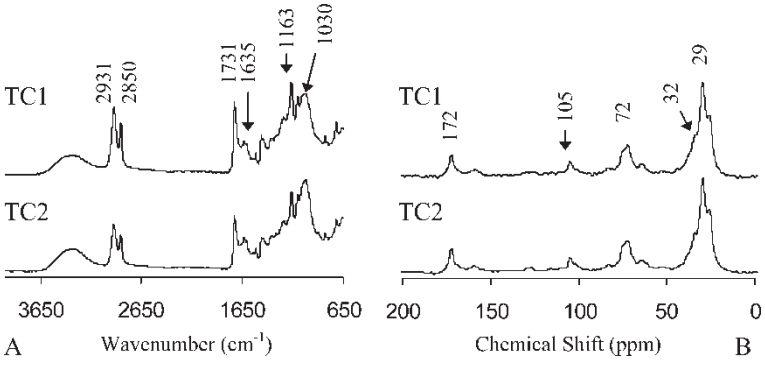

Figure 1. ATR-FTIR (A, left side) and solid-state CPMAS ^{13}C NMR (B, right side) spectra of the tomato fruit bulk cuticle (TC1) and dewaxed cuticle (TC2).

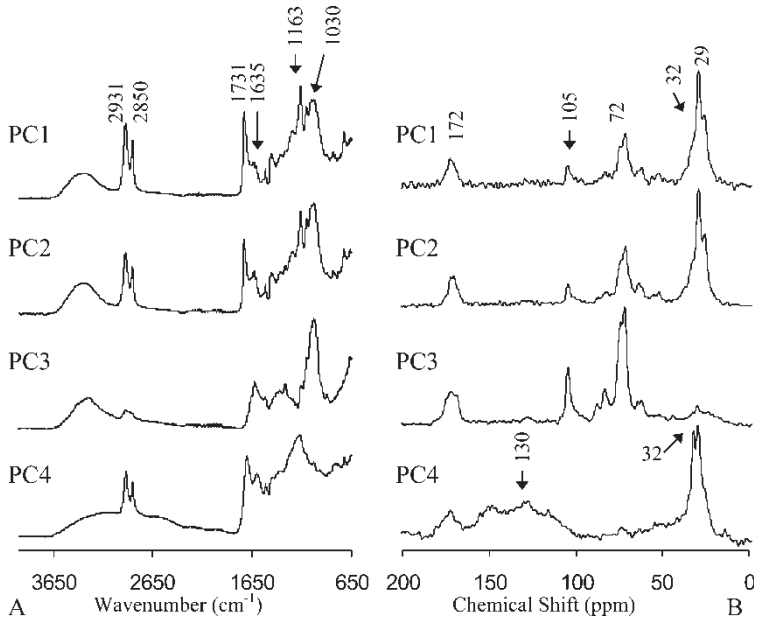

Figure 2. ATR-FTIR (A, left side) and solid-state CPMAS ^{13}C NMR (B, right side) spectra of the pepper bulk cuticle (PC1), dewaxed cuticle (PC2), nonsaponified cuticle (PC3), and nonhydrolyzable cuticle (PC4).

the cutin polymer to break, corresponding to removal of hydroxyl fatty acids, the esters bonding the cutin monomers, and some phenolics that had been linked by ester bonds. As such, the spectra of the C3 fraction was characterized by a broad peak around 1030 cm^{-1} and a peak at 1635 cm^{-1}, associated with cuticular polysaccharides and ionized carboxyl groups, respectively. The increase in polar functional groups in the C3 fraction, suggested by the elemental analysis, was confirmed by the predominance of polysaccharide bands in these spectra. Other adsorption bands presented include those associated with the aromatic structures of the cuticle (1650–1500 cm^{-1}).

To isolate the nonsaponifiable, nonhydrolyzable, cutan-like fraction (C4) of the cuticle, the polysaccharides must be removed. This was accomplished by hydrolyzing the C3 fraction of the pepper fruit, apple fruit, and olive leaf cuticles. The C4 fraction of the pepper fruit exhibited slightly different spectra from the apple fruit and olive leaf cuticles. Peaks at 2931 and 2850 cm^{-1} (stretching vibrations of methylene groups), 1700 cm^{-1} (carbonyl stretch of the carboxyl group), and 1163 cm^{-1} (C-O-C stretching vibrations in the ester groups) dominated the PC4 spectrum. The bands around 1700 cm^{-1} are particularly strong in OC4. Villena, Dominguez, and Heredia (2000) and Chefetz (2003) indicated that the presence of these bands (representing the cutin fatty acids linked by ester bonds) after saponification may be due

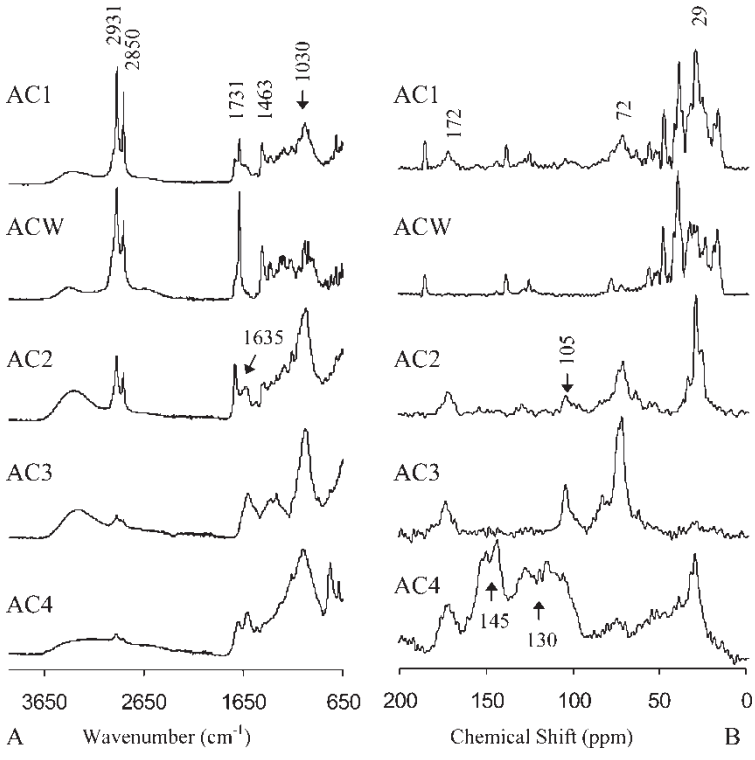

Figure 3. ATR-FTIR (A, left side) and solid-state CPMAS ^{13}C NMR (B, right side) spectra of the apple fruit bulk cuticle (AC1), dewaxed cuticle (AC2), nonsaponified cuticle (AC3), nonhydrolyzable cuticle (AC4), and cuticular wax (ACW).

to a partial molecular shielding by polysaccharides, present in a high degree of crystallinity, thus protecting the cutin structure from the saponification treatment. Because of this shielding, the elemental analysis, and FTIR data for the PC4 fraction were similar to the results obtained by Chefetz (2003) and not those obtained by Chen et al. (2005), in which the pepper cuticle may have been more purely saponified. The AC4 and OC4 spectra contained the same bands as the PC4, except the bands at 2931 and 2850 cm^{-1} were of a much lower intensity. Other dominant bands in all the C4 samples included 1515 cm^{-1}, associated with the double bonded C stretching vibration in the aromatic ring, a band around 1600 cm^{-1}, indicating the presence of phenolics, and a band at 830 cm^{-1}, corresponding to C-H and C-C out-of-plane bending vibration in the aromatic ring. The presence of these bands indicates that the cutan-like C4 fraction has a greater aromatic component than the other fractions (Chen et al. 2005).

The cuticular wax (ACW) removed from the bulk apple cuticle (AC1) was isolated from the organic solvent. The dominant peaks in the ACW

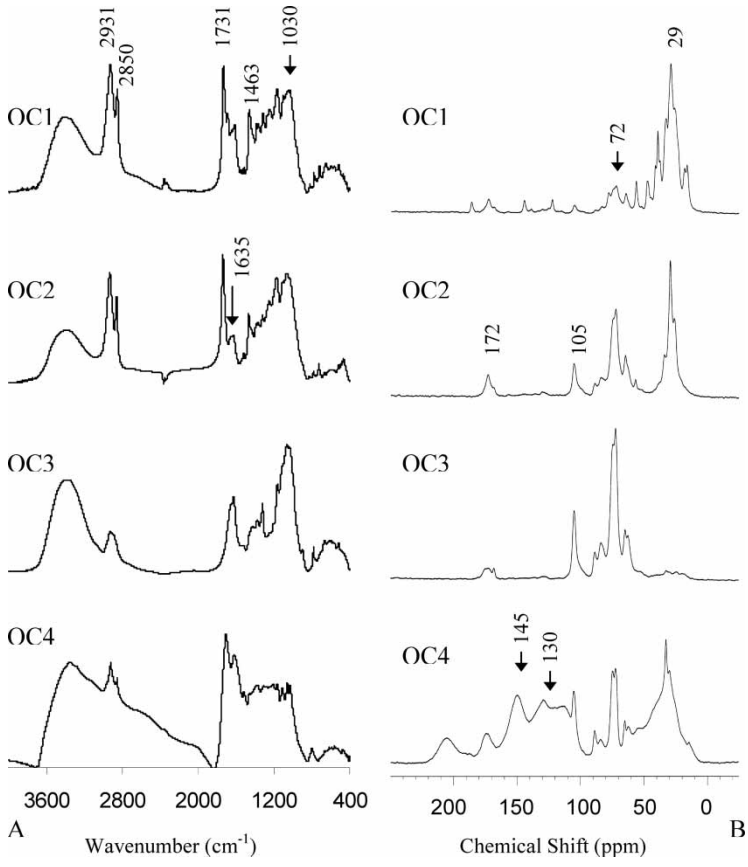

Figure 4. FTIR (A, left side) and solid-state CPMAS ^{13}C NMR (B, right side) spectra of the olive leaf bulk cuticle (OC1), dewaxed cuticle (OC2), nonsaponified cuticle (OC3), and nonhydrolyzable cuticle (OC4).

spectrum were indicative of the cuticular wax's aliphatic nature: 2931 and 2850 cm^{-1}, representing the vibrations of the methylene group; a shoulder at 3012 cm^{-1}, associated with the double-bonded C-H stretch in the fatty acids of the olefinic groups; and 1731 cm^{-1}, indicating the C-O double bond stretching in the carbonyl group of the aliphatic wax ester (Veraverbeke et al. 2005). This confirmed the elemental analysis data, which indicated a high aliphaticity and low polarity for ACW. Other bands included 1463 cm^{-1}, associated with methyl group scissoring in the wax hydrocarbon chains, and 1030 and 997 cm^{-1}, representing C-O and H-O deformations in polysaccharide primary and secondary alcohols, respectively (Dubis, Dubis, and Morycki 1999; Ramirez et al. 1992). According to Dubis, Dubis, and Poplawski (2001), the small band at 699 cm^{-1} in our data was representative of the small aromatic component in cuticular wax. Because of the high

percentage of wax in the apple fruit and the relatively high percentage in the olive leaf bulk cuticle (Table 1), the AC1 and OC1 spectra also contained these spectral bands. It should be noted that the wax analyzed on the apple cuticle is most likely a combination of both natural epicuticular waxes and those added during fruit processing.

The results for the solid-state ^{13}C NMR spectra for the isolated cuticular fractions are presented in Figures 1B–4B. The NMR spectra for the bulk and dewaxed cuticle (C1 and C2) of all plant cuticles studied exhibited a dominant peak at 29 ppm (methylene C), which suggests a highly aliphatic nature. Once the free lipids were removed, these peaks were assigned to the paraffinic C in the fatty acids of the biopolymer cutin (Kögel-Knabner et al. 1994). The integration data for these isolations provided further confirmation by indicating aliphatic C content ranging between 87% and 96% and paraffinic C content between 38% and 73% (Table 2). Other peaks included 64 ppm, 72 ppm (O-alkyl carbon in carbohydrates), 105 ppm (anomeric C of polysaccharides), 130 ppm (C-substituted aromatic C), and 172 ppm (carboxyl/amide C). Similar to the FTIR analysis, additional peaks were identified on the AC1 and OC1 spectra due to influence of large wax content and are discussed separately.

The saponified cuticle NMR spectra for the pepper fruit, apple fruits, and olive leaf were also very similar and were dominated by carbohydrate-type C. Peaks in the polysaccharide C region were identified at 64, 72, 74, 82, and 105 ppm, confirming the FTIR results, which indicated high polysaccharide content. According to Chefetz (2003), the significant reduction in the methylene C peaks (29 and 31 ppm) suggests that these cuticles are composed primarily of cutin, with a relatively small quantity of cutan. Other research has indicated that the loss of methylene C peaks after saponification confirms the presence of cutin and the complete absence of cutan (Kögel-Knabner et al. 1994; Tegelaar et al. 1989). Although the aliphatic C content was not greatly reduced, the percent paraffinic C decreased to 14, 12, and 8% for PC3, AC3, and OC3, respectively, further indicating the removal of the aliphatic cutin monomers. A small broad peak around 130 ppm was present after the saponification, indicating an aromatic component to the C3 fraction. The resonances in the carboxyl region (172 ppm) that remained after saponification most likely correspond to ester type linkages that may have become encapsulated in the hydrophobic network of the macromolecule and were thus protected from the saponification (McKinney et al. 1996).

To isolate the cutan-like fraction (C4) of the pepper fruit, apple fruit, and olive leaf cuticle, the C3 fraction was acid hydrolyzed. Based on the original definition of cutan by Tegelaar et al. (1989), this residual fraction is cutan. In this article, it is referred to as a "cutan-like" fraction because of the apparent incomplete saponification of the C2 fraction, as illustrated in the ATR-FTIR and NMR spectra for the C3 fractions. The NMR spectra of the C4 fraction confirmed the desired removal of polysaccharides from the sample, as indicated by the loss of the peaks around 72 ppm and 105 ppm.

Table 2. Integration of CPMAS ^{13}C NMR spectra[a] for fruit cuticular isolations

Sample[b]		Distribution percentages of C chemical shift (ppm)										
		0–50	50–61	61–96	96–109	109–145	145–163	163–190	190–220	Aliphatic C (%)	Aromatic C (%)	Paraffinic C (%)
Tomato fruit	TC1	49.2	4.4	20.1	5.5	7.5	4.0	7.2	2.2	87	13	49
	TC2	49.4	4.9	20.4	5.5	6.9	4.0	7.4	1.7	88	12	49
Pepper fruit	PC1	45.6	4.3	25.2	5.5	6.8	2.8	10.4	2.4	89	11	46
	PC2	42.2	5.0	27.7	5.3	6.0	2.3	9.9	1.6	91	9	42
	PC3	14.2	4.5	45.4	10.9	6.6	6.6	2.8	2.1	89	11	14
	PC4	31.2	3.9	7.7	3.7	5.5	10.9	10.4	6.6	56	44	31
Apple fruit	AC1	55.5	5.2	15.3	3.2	8.9	2.4	7.0	2.6	88	12	56
	AC2	37.5	4.7	28.6	6.5	7.7	3.7	8.6	2.7	87	13	38
	AC3	12.1	4.5	43.3	11.2	8.9	4.2	12.5	3.4	85	16	12
	AC4	18.8	4.5	10.9	8.7	30.0	13.8	8.0	5.3	50	52	19
	ACW	80.0	8.0	4.8	0.0	4.8	0.0	1.6	0.8	95	5	80
Olive leaf	OC1	73.0	3.8	13.0	1.4	3.0	1.4	3.8	0.30	95	5	73
	OC2	41.5	3.9	38.0	7.5	2.6	1.0	5.0	0.0	96	4	42
	OC3	8.3	4.3	65.4	14.7	2.0	0.7	4.6	0.0	97	3	8.3
	OC4	26.8	4.3	14.5	7.4	21.5	13.3	6.5	5.7	60	40	27

[a]Aliphatic C: aliphatic C region (0–109 ppm) divided by aliphatic and aromatic regions (0–163 ppm); aromatic C: aromatic C region (109–163 ppm) divided by aliphatic and aromatic regions (0–163 ppm); paraffinic C: paraffinic C region (0–50 ppm) divided by aliphatic and aromatic regions (0–163 ppm).

[b]Isolation symbol C1 represents the bulk cuticle, C2 represents the dewaxed cuticle, C3 represents the saponified cuticle, C4 represents the acid-hydrolyzed cuticle. The letters preceding these symbols (T, P, A, and O) represent tomato fruit, pepper fruit, apple fruit, and olive leaf, respectively.

As with the FTIR spectra, the spectra for the apple fruit and olive leaf C4 fractions varied slightly from the pepper fruit. The PC4 fraction yielded spectra similar to those described in the literature (Chefetz 2003); it was composed of 31% paraffinic C, and the NMR spectra exhibited dominant peaks at 32 and 29 ppm. Whereas the AC4 and OC4 fractions were similar to the PC4 in their aliphaticity (56, 50, and 60% aliphatic C for PC4, AC4, and OC4, respectively), the apple fruit and olive leaf were composed of only 19 and 27% paraffinic C, respectively, and demonstrated weaker peaks at 32 and 29 ppm. Chefetz (2003) determined that dominant methylene C peaks in the nonsaponifiable, nonhydrolyzable fraction confirmed the presence of cutan in the cuticle. The aromatic C contents in the C4 fractions each increased approximately 35% over the C3 fractions, indicating that the aromatic core of the cuticle was exposed after the removal of the soluble lipids, cutin monomers, and polysaccharide components (Chen et al. 2005) and confirming that cutan is composed of both aromatic and aliphatic components. The aromatic regions of the C4 cuticular fractions, especially AC4 and OC4, exhibited peaks centered in the vicinity of 147, 130, and 110 ppm, characteristic of O-aryl, C-substituted, and protonated C in the aromatic rings of lignin structures (Kögel-Knabner et al. 1994), although the characteristic lignin peak at 55 ppm (methoxyl groups) did not appear to be present in the isolated C4 fractions' spectra.

Because cutan has an operational definition, it is difficult to assess whether the isolated C4 fractions are cutan-like in nature or merely cuticle residues and products remaining from an incomplete extraction procedure. Most of the literature on cutan has centered on cuticles that are composed of a high percentage of cutan, such as the leaves of *Clivia miniata* and *Agave americana* (Villena, Dominguez, and Heredia 1999; McKinney et al. 1996; Deshmukh et al. 2005). The ^{13}C NMR spectra provided by these researchers illustrate cutan with dominant methylene C peaks and weak peaks in the aromatic region. The only spectral data available from a cuticle with high cutin and low cutan content is pepper cutan, presented by Chefetz (2003). The isolated pepper cuticular fractions correlated very well to those described in Chefetz's 2003 study, and as such, we contend that the PC4 fraction is cutan-like in nature. The weaker methylene C peaks in the AC4 and OC4 fractions make its assessment more ambiguous.

A classical work studying the isolation of spruce needle cuticles demonstrated that the cuticle did not contain cutan, despite the presence of weak NMR methylene C peaks in the acid-hydrolyzed fraction (Kögel-Knabner et al. 1994). Similar to the C3 spectra in this study, methylene C peaks were not present after the saponification of the spruce needle cuticles. The authors of that study determined that the reappearance of these peaks after acid hydrolysis indicated that they were most likely representative of residual cutin acids, not cutan, but this may not be true in the apple fruit and olive leaf cuticles. Compared to the spectral changes through isolation of cuticles with high cutan content, the loss of the methylene C peak after

saponification may appear to indicate that cutan is not present in the cuticles. However, Chefetz (2003) demonstrated that in cuticles with high cutin and low cutan content, a loss of methylene C peaks after saponification followed by a relative increase in aliphatic peaks after hydrolysis indicates the presence of a low percentage of cutan in the cuticle. Kögel-Knabner et al. (1994) determined that the spruce needle cuticle's nonsaponifiable, non-hydrolyzable residue was composed of lignin and cutin residues. The apple fruit and olive leaf cutan-like residue in the current study does not appear to have a strong signature lignin NMR peak at 55 ppm (Figures 3B and 4B); therefore, this explanation of the acid-hydrolyzed spectra may not accurately describe our spectral data.

The dominant peaks in the apple cuticular wax spectrum ACW were almost all in the paraffinic region and include 16 ppm (terminal methyl), 29 and 32 ppm (amorphous and condensed methylene, respectively), 39 ppm (methylenes in free fatty acids), 48 ppm (methines in carboxylic acids/esters), and 56 ppm (methines in epoxide groups). The paraffinic C in the ACW was 80%, whereas the aromatic C comprised 5% of the fraction, confirming the FTIR analysis. Other strong peaks were located at 78 (methines attached to ester-linked midchain hydroxyls) and 125 and 138 ppm (both olefinic and/or aromatic carbon) (Deshmukh et al. 2005; Lichtfouse et al. 1998). The wax content was high enough in both the AC1 and OC1 to exhibit some of these peaks.

CONCLUSION

In general, all the cuticular fractions isolated from the tomato fruit, pepper fruit, apple fruit, and olive leaf were highly aliphatic in composition. More than 60% of the tomato and pepper fruit bulk cuticles and more than 50% of the olive leaf bulk cuticle were composed of the aliphatic biopolymer cutin. The apple cutin content is slightly lower than the other two fruits because of its high wax content. The presence of cutan in pepper cuticles, which was known from previous studies, was confirmed and determined to be $\sim$6% of the bulk cuticle. This is much lower than previously studied cuticles containing cutan, which were closer to 50–60% cutan. The low content may explain why the methylene C peaks were diminutive in the PC3 cuticular fraction NMR spectrum, yet demonstrated a relative increase after the saponification and removal of the polysaccharides.

The presence or absence of cutan in apple fruit and olive leaf cuticles was more ambiguous. The FTIR and NMR spectra for AC3 and OC3 appeared much like the PC3, but the AC4 and OC4 spectra were slightly different from the PC4. The apple fruit and olive leaf nonhydrolyzable cuticles produced spectra with weaker methylene C peaks and stronger peaks in the aromatic region than the pepper fruit. One possible explanation is that similar to tomato, the apple fruit and olive leaf cuticles contain only cutin,

and the residual peaks, including aliphatic signature in the AC4 and OC4, are from the incomplete removal of cutin monomers during saponification. However, based on both the operational definition of cutan and the presence of polymethylene C peaks in the NMR C4 spectra, we have concluded that the apple fruit and olive leaf cuticles may contain a cutan-like residue.

The deviation of the AC4 and OC4 fractions from the PC4 fraction may be due to low cutan content in the cuticle, variation in spectral characteristics from the highly aliphatic cutan studied in the literature, and the presence of residue from incomplete saponification. The cutan of apple fruit and olive leaf cuticles comprises a very small portion of the total cuticle, which may have prevented it from exhibiting the characteristic spectra exhibited by published samples composed of a high percentage of cutan. Unlike the cutan characterizations that have been published in the literature thus far, the apple fruit and olive leaf cutan exhibits strong peaks in the aromatic regions and have weaker methylene C signatures. The presence of a stronger aromatic component in the apple fruit and olive leaf cutan may simply be a structural characteristic of the cutan from these species of plant. Because of their low cutan yield, any incomplete removal of cuticular materials during the saponification would have a more dominant presence in the apple fruit and olive leaf cutan spectra than if there was a large cutan yield. In this experiment, both the FTIR and NMR have illustrated peaks (previously discussed) that indicate the presence of residual saponifiable materials that were not properly extracted. These peaks may act to overrun the AC4 and OC4 spectra, preventing a clear picture of the cutan structures. Therefore, using the operational definition of cutan, and the arguments posed previously, it is our opinion that apple fruit and olive leaf cuticles may contain cutan-like materials.

ACKNOWLEDGMENTS

The authors thank Baoliang Chen and Talya Dayton for their assistance in the laboratory. This project was supported by Research Grant Number IS-3385-03R from BARD, the United States–Israel Binational Agricultural Research and Development Fund, and the Federal Hatch Program (MAS 00960).

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