

Leaf cuticle analyses: implications for the existence of cutan/non-ester cutin and its biosynthetic origin

Jana Leide¹, Klaas G. J. Nierop^{2,†}, Ann-Christin Deininger¹, Simona Staiger¹, Markus Riederer^{1,*} and Jan W. de Leeuw^{2,3}

¹University of Würzburg, Julius-von-Sachs-Institute for Biosciences, D-97082 Würzburg, Germany, ²Department of Earth Sciences, Faculty of Geosciences, Utrecht University, 3584 CB Utrecht, The Netherlands and ³NIOZ Royal Netherlands Institute for Sea Research, P.O. Box 59, 1790 AB, Den Burg, The Netherlands [†]Present address: Geolab, Faculty of Geosciences, Utrecht University, 3584 CB Utrecht, The Netherlands

*For correspondence. E-mail riederer@uni-wuerzburg.de 202028March2020126/141162

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- **Background and Aims** The cuticle of a limited number of plant species contains cutan, a chemically highly resistant biopolymer. As yet, the biosynthesis of cutan is not fully understood. Attempting to further unravel the origin of cutan, we analysed the chemical composition of enzymatically isolated cuticular membranes of *Agave americana* leaves.
- **Methods** Cuticular waxes were extracted with organic solvents. Subsequently, the dewaxed cuticular membrane was depolymerized by acid-catalysed transesterification yielding cutin monomers and cutan, a non-hydrolysable, cuticular membrane residue. The cutan matrix was analysed by thermal extraction, flash pyrolysis and thermally assisted hydrolysis and methylation to elucidate the monomeric composition and deduce a putative biosynthetic origin.
- **Key Results** According to gas chromatography–mass spectrometry analyses, the cuticular waxes of *A. americana* contained primarily very-long-chain alkanolic acids and primary alkanols dominated by C₃₂, whereas the cutin biopolymer of *A. americana* mainly consisted of 9,10-epoxy ω -hydroxy and 9,10, ω -trihydroxy C₁₈ alkanolic acids. The main aliphatic cutan monomers were alkanolic acids, primary alkanols, ω -hydroxy alkanolic acids and alkane- α,ω -diols ranging predominantly from C₂₈ to C₃₄ and maximizing at C₃₂. Minor contributions of benzene-1,3,5-triol and derivatives suggested that these aromatic moieties form the polymeric core of cutan, to which the aliphatic moieties are linked via ester and possibly ether bonds.
- **Conclusions** High similarity of aliphatic moieties in the cutan and the cuticular wax component indicated a common biosynthetic origin. In order to exclude species-specific peculiarities of *A. americana* and to place our results in a broader context, cuticular waxes, cutin and cutan of *Clivia miniata*, *Ficus elastica* and *Prunus laurocerasus* leaves were also investigated. A detailed comparison showed compositional and structural differences, indicated that cutan was only found in leaves of perennial evergreen *A. americana* and *C. miniata*, and made clear that the phenomenon of cutan is possibly less present in plant species than suggested in the literature.

Key words: *Agave americana*, *Clivia miniata*, *Ficus elastica*, *Prunus laurocerasus*, cuticular waxes, cutin, cutan, non-ester cutin, Fourier transform infrared spectroscopy (FTIR), flash pyrolysis, thermally assisted hydrolysis and methylation (THM).

INTRODUCTION

The cuticle covers all primary aerial organs of higher plants. Based on the extracellular position, the cuticle represents the boundary layer between the atmosphere and the plant interior (Yeats and Rose, 2013; Fich *et al.*, 2016). The main functions of the cuticle are minimization of uncontrolled water loss and apoplastic solutes, protection from mechanical damage, inhibition of post-genital organ fusions and reflection and attenuation of solar radiation. The heterogeneous cuticle shows a species-, organ- and tissue-specific composition and varies in thickness and structure. The barrier properties are influenced by the physico-chemical characteristics of the cuticular components, of which the variability also depends on the plant developmental stage and is affected by environmental stress (Lendzian, 1984; Kerstiens, 1996; Ribeiro da Luz, 2006; Martin and Rose, 2014).

Generally, the cuticle can be divided into two distinct components: the cuticular waxes and the cutin matrix. The cuticular waxes are solvent-extractable mixtures containing up to 150 different compounds in varying quantities. These complex mixtures are mainly composed of homologous series of very-long-chain alkanolic acids, primary and secondary alkanols, *n*-alkanals, *n*-alkanes, *n*-alkanones and alkyl esters. In addition to these aliphatic compounds with chain lengths of 20–60 carbon atoms, pentacyclic triterpenoids and sterols occur in cuticular waxes. These cuticular waxes provide the main barrier properties by impregnating the cutin matrix as well as covering the cutin matrix as cuticular wax film and/or crystalloids.

The biopolymer cutin is non-extractable but hydrolysable. In contrast to the cuticular waxes, the cutin matrix mainly consists of alkanolic acids with chain lengths of 16 and 18 carbon atoms. Characteristic cutin monomers are for the most part ω -hydroxy

alkanoic acids with mid-chain functionalities, mostly hydroxyl, carbonyl and epoxy groups. These long-chain alkanolic acids are esterified into a complex polymeric network with linear and branched domains by primary and secondary ester bonds (Sachleben *et al.*, 2004; Deshmukh *et al.*, 2005; Stark and Tian, 2006). Additionally, minor proportions of aromatic acids, such as cinnamic acid derivatives, are linked within the complex biopolyester. The viscoelastic cutin matrix substantially contributes to mechanical integrity. Fibrous polysaccharides from the underlying cell wall intermingle with the cutin biopolymer and are responsible for the elastic capacity of the cuticle (Graça *et al.*, 2002; Johnson *et al.*, 2007; Domínguez *et al.*, 2011; Khanal and Knoche, 2017).

Despite the compositional complexity of the cuticle, considerable progress in understanding its biosynthesis has been made over the past 20 years. The first pathways underlying cuticle biosynthesis are identical for the aliphatic compounds of cuticular waxes and cutin and involve the *de novo* biosynthesis of C₁₆ and C₁₈ alkanolic acid precursors in the plastids of epidermal cells. These plastid-derived precursors are elongated to very-long-chain alkanolic acid precursors (≥20 carbon atoms) and are converted by reduction, decarboxylation, hydroxylation and oxidation reactions into characteristic cuticular wax compounds in the endoplasmic reticulum of the epidermal cells (Kunst *et al.*, 2006; Yeats and Rose, 2013; Joubès and Domergue, 2018). To generate cutin monomers, such as 9,ω- or 10,ω-dihydroxy C₁₆ alkanolic acid, 9,10-epoxy ω-hydroxy C₁₈ alkanolic acid and 9,10,ω-trihydroxy C₁₈ alkanolic acid, C₁₆ and C₁₈ alkanolic acids are modified in the endoplasmic reticulum by the oxidation of terminal and/or mid-chain carbon atoms. Finally, the cutin monomers and the cuticular wax compounds are exported to the apoplastic space, where cutin polymerization and cuticular wax deposition occurs (Fich *et al.*, 2016).

Infrequently, a highly resistant biopolymer remains after cuticle extraction and hydrolysis, which has been designated non-ester cutin or cutan (Schmidt and Schönherr, 1982; Nip *et al.*, 1986b). Spectroscopic, chemical and thermal degradation approaches have been applied to this non-hydrolysable component, revealing the highly aliphatic character with a small proportion of aromatic moieties (McKinney *et al.*, 1996; Schouten *et al.*, 1998; Boom *et al.*, 2005). Both cutin and cutan are predominantly aliphatic biopolymers but cutan is often considered as an ether-linked network of very-long-chain aliphatic moieties (Villena *et al.*, 1999; Boom *et al.*, 2005). Cutan increases stiffness and strength but restricts extensibility of the cuticle (Schmidt and Schönherr, 1982; Fernández *et al.*, 2016; Khanal and Knoche, 2017).

Commonly, the cutan matrix is considered a minor cuticular component but the leaf cuticle of a few plant species possesses significant amounts of cutan. It was documented in the cuticle of gymnosperms, for example *Podocarpus* sp. (Podocarpaceae) and angiosperms, such as the monocotyledonous *Agave americana* (Asparagaceae), *Clivia miniata* (Amaryllidaceae) and eudicotyledonous *Clusia multiflora*, *Clusia rosea* (Clusiaceae), *Ficus elastica* (Moraceae), *Prunus laurocerasus* (Rosaceae), *Sonneratia alba* (Lythraceae) and *Ilex latifolia* (Aquifoliaceae; Villena *et al.*, 1999; Boom *et al.*, 2005; Gupta *et al.*, 2006; Azuma *et al.*, 2010; Takahashi *et al.*, 2012; Guzmán-Delgado *et al.*, 2016). These perennial evergreen plant species exhibit

some morphological characteristics, such as succulent or thick leaves with a thick cuticle (Boom *et al.*, 2005; Deshmukh *et al.*, 2005).

There is controversy about the monomeric composition and biosynthetic origin of cutan – whether it is structurally related to cutin or possibly derived from it. Understanding the composition and the structure of cutan might be crucial for comprehending the plant cuticle. The nature of linkages between cutan monomers remains speculative, too. Mostly based on analyses of *A. americana*, different models have been proposed for the monomeric and polymeric cutan structure, assuming that cutan monomers are linked together via non-hydrolysable, ester or ether bonds (Nip *et al.*, 1986b; McKinney *et al.*, 1996; Schouten *et al.*, 1998; Villena *et al.*, 1999; Deshmukh *et al.*, 2005).

Our study focuses on the extraction of cutan, and whether its previously reported quantitative correlation with the amount of 9,10-epoxy ω-hydroxy C₁₈ alkanolic acid relates to a cutin-based origin (Schmidt and Schönherr, 1982), or, alternatively, that cutan is biosynthetically related to the very-long-chain cuticular wax compounds. Using the leaf cuticle of *A. americana*, a study was conducted with the aim of investigating the cuticular components by applying a modification of the extraction protocol of Schmidt and Schönherr (1982) in order to remove cuticular waxes and cutin, and purifying a proper cutan residue (Fig. 1). This enabled us to test whether this protocol, originally applied to *C. miniata*, yielded a cutan component similar to that derived using the approach used by Nip *et al.* (1986b). Additionally, the compositional heterogeneity of cuticular waxes and cutin was analysed along a gradient from the base to the apex of the adaxial leaf surface to verify the hypothesized conversion of cutin to cutan (non-ester cutin) along this leaf gradient, as previously suggested (Schmidt and Schönherr, 1982). Different chemical degradation techniques and analytical methods were applied to the enzymatically isolated cuticular membrane. Furthermore, the cuticular composition of *A. americana* was compared with that of *C. miniata*, *F. elastica* and *P. laurocerasus* to investigate whether or not the presence of cutan is related to the chemical composition of cuticular waxes or the cutin matrix.

MATERIALS AND METHODS

Plant material

Agave americana, *Clivia miniata* and *Ficus elastica* plants were cultivated in a growth chamber with 75 % relative humidity, a 14-h photoperiod at 450 μmol photons m⁻² s⁻¹, and a temperature regime of 22/18 °C (day/night). *Prunus laurocerasus* plants grew in the Botanical Garden of Würzburg. Fully expanded leaves were investigated.

Leaf trait measurements

The one-sided surface area of the leaf lamina without petiole was determined according to the pixel values of the planar surface scanned at high resolution (600 dpi) in comparison with a reference area (Adobe Photoshop 7.0.1). After leaf size measurement and determination of fresh weight and water-saturated fresh weight, the leaf lamina was oven-dried

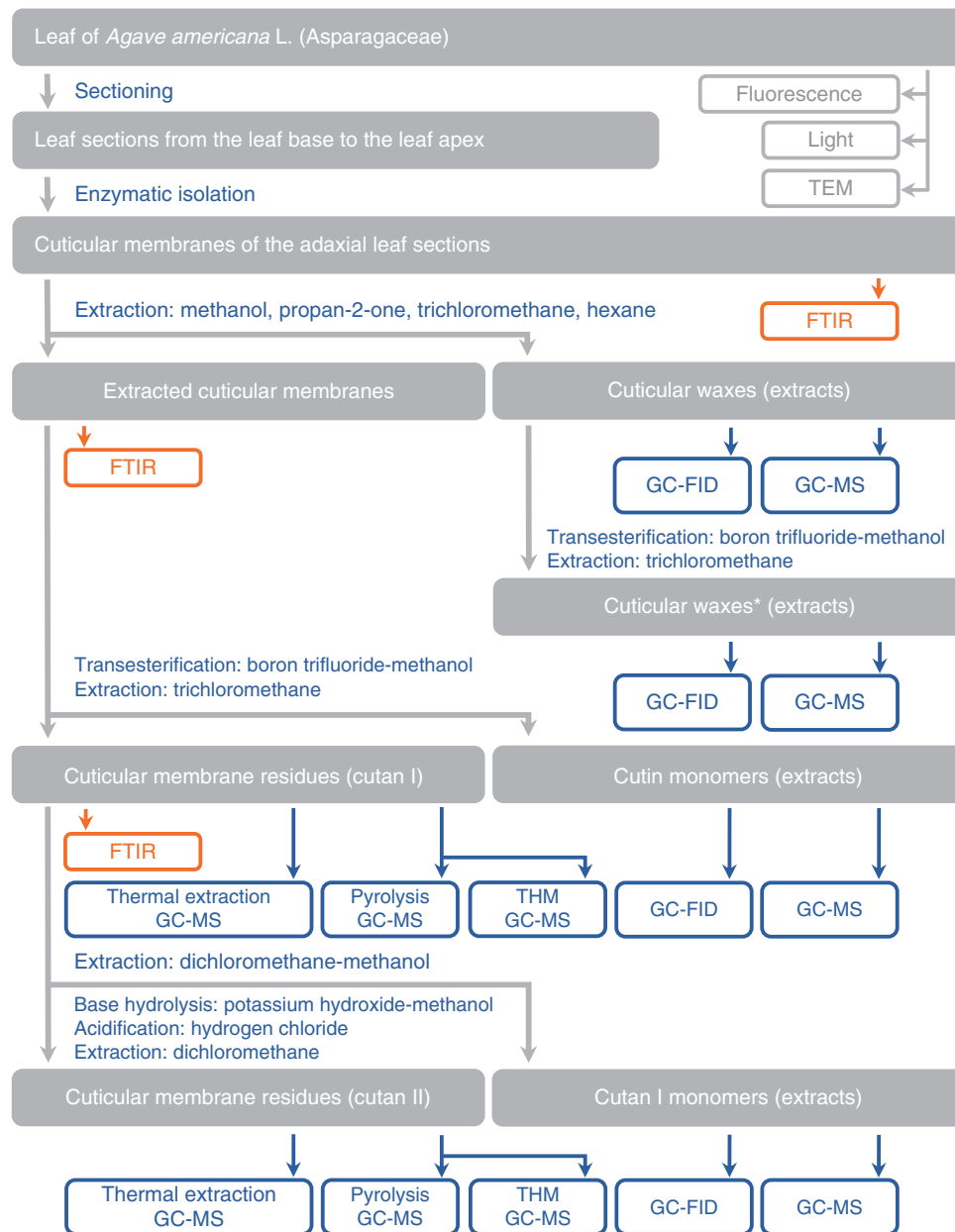


FIG. 1. Scheme for the microscopic (autofluorescence; light; transmission electron microscopy, TEM), spectroscopic (Fourier transform infrared spectroscopy, FTIR) and gas chromatographic examination of the *Agave americana* leaf cuticle. Cuticular waxes before and after acid-catalysed transesterification (*) as well as transesterified cutin monomers were analysed by gas chromatography with flame ionization (GC-FID) and mass spectrometry (GC-MS). Cutan monomers were obtained directly from the cuticular membrane residue after removal of the cutin monomers (cutan I) or from the cuticular membrane residue after further extraction, base hydrolysis and extraction (cutan II) and detected by thermal extraction-GC-MS, flash pyrolysis-GC-MS and THM-GC-MS.

at 60 °C for 7 d (Mettler UF55) and the dry weight was determined (Mettler PM480 Delta Range). The specific leaf surface area was calculated as the quotient of the one-sided surface area of the leaf lamina and the dry weight. For leaf water content measurement, the fresh weight of the leaf lamina was related to the dry weight. The leaf dry matter content was calculated by dividing the dry weight of the leaf lamina by the water-saturated fresh weight. Leaf mass per surface area was calculated from the ratio of the dry weight and the one-sided surface area of the leaf lamina. Degree of succulence was calculated as the difference between water-saturated fresh

weight and dry weight divided by the double-projected surface area of the leaf lamina.

Microscopic examination

For fluorescence microscopy, sections were cut out of the leaf middle and embedded in a tissue-freezing medium (Tissue-Tek O.C.T., Sakura) and frozen to −20 °C. Slices with a thickness of ~30 µm were cut with a microtome (CM1900 Cryostat, Leica Microsystems) and examined by fluorescence microscopy

(Leica DMR HC; excitation filter BP 355–425, suppression filter LP 470).

For transmission electron microscopy, leaf sections ~2 mm × 1 mm × 1 mm in size were placed in a fixative consisting of 5 % glutaraldehyde (Merck) in 0.1 M sodium cacodylate buffer (Roth), pH 7.2. After overnight incubation at 4 °C, samples were washed three times in 0.1 M sodium cacodylate buffer on ice for 10 min (twice) and overnight. Subsequently, samples were post-fixed in 2 % osmium tetroxide (Electron Microscopy Sciences) in 0.1 M sodium cacodylate buffer on ice for 2 h. These three washing steps were repeated at room temperature in 0.05 M sodium cacodylate buffer. Samples were dehydrated at room temperature with a series of ethanol dilutions (AppliChem): 30 and 40 % for 15 min, 50 % for 90 min, 60 and 75 % for 30 min, 90 and 100 % for 60 min, 100 % overnight. Samples were infiltrated at room temperature by immersion in propylene oxide (Sigma) for 1 h (twice), followed by immersions in Spurr's resin (Sigma) with propylene oxide (25:75) for 6 h, Spurr's resin with propylene oxide (50:50) overnight and Spurr's resin with propylene oxide (75:25) for 8 h. Finally, samples were infiltrated twice with Spurr's resin overnight and for 4 h (Spurr, 1969). Polymerization of Spurr's resin was performed at 60 °C for 48 h. Slices with a thickness of ~0.5 µm were cut, stained with methylene blue–azure II and examined by light microscopy (Leica DMR HC microscope). The cuticle thickness was determined. Slices with a thickness of ~70 nm were cut with a diamond knife (Diatome), stained with 2.5 % uranyl acetate (Merck) for 15 min and Reynolds' lead citrate for 10 min (Reynolds, 1963), washed in water three times and dried. Slices were examined by transmission electron microscopy (JEM-2100, Jeol) at 200 kV. The cuticle ultrastructure was determined.

Leaf sectioning and isolation of cuticular membranes

Leaves of *A. americana* were bisected and divided into 11 sections. All spines were removed. Leaves of *C. miniata* were bisected and divided into eight sections. Leaf discs of *F. elastica* and *P. laurocerasus* were punched out. The cuticular membranes were enzymatically isolated by incubating these leaf sections or discs at room temperature with pectinase (Trenolin Super DF, Erbslöh), and cellulase (Celluclast, Novo Nordisk AIS) in 20 mM citric acid (Merck), pH 3.0, containing 1 mM sodium azide (Sigma–Aldrich). The enzyme solution was exchanged weekly. Isolated cuticular membranes were extensively washed in deionized water and air-dried. The cuticle weight was gravimetrically determined using a balance with a precision of 0.1 g (Sartorius MC1 Analytic AC 210S).

Solvent extraction and derivatization of cuticular waxes

For cuticular wax extraction, enzymatically isolated cuticular membranes were extracted by successively immersion in methanol (Roth) for 5 min combined with an ultrasonic treatment at room temperature (twice), in propan-2-one (Roth) at 50 °C overnight, in trichloromethane (Roth) for 5 min combined with an ultrasonic treatment at room temperature

(twice) and in hexane (Roth) at 50 °C overnight. As an internal standard, *n*-tetracosane (Sigma–Aldrich) was added. The extracts were combined and the solvents were evaporated under a continuous flow of nitrogen. Before gas chromatographic analysis, cuticular wax extracts were derivatized with *N,O*-bis-trimethylsilyl-trifluoroacetamide (Macherey–Nagel) in pyridine (Merck) at 60 °C for 60 min to transform functional groups containing cuticular wax compounds into their corresponding trimethylsilyl derivatives.

To release building blocks of esterified cuticular waxes, the extracted cuticular waxes of *A. americana* leaves were transesterified with boron trifluoride in methanol (methanolysis; Fluka) at 70 °C overnight. Sodium chloride-saturated aqueous solution (AppliChem), trichloromethane, and *n*-tetracosane as an internal standard were added to all reaction mixtures. From this two-phase system, cuticular wax compounds were extracted three times with trichloromethane. The combined organic phases were dried over anhydrous sodium sulphate (AppliChem). All extracts were filtered and the organic solvent was evaporated under a continuous flow of nitrogen. Subsequently, a derivatization was performed using *N,O*-bis-trimethylsilyl-trifluoroacetamide in pyridine to convert functional groups containing cuticular wax compounds to their trimethylsilyl derivatives.

Cuticular wax analysis by gas chromatography–mass spectrometry

The qualitative composition of cuticular waxes was determined by temperature-controlled capillary gas chromatography (6890N GC, Agilent Technologies) and on-column injection (DB-1, length 30 m, inner diameter 320 µm, film thickness 0.1 µm; Agilent J&W GC column) with helium carrier gas inlet pressure programmed at 50 kPa for 5 min, 3 kPa min^{−1} to 150 kPa, and at 150 kPa for 40 min using a mass spectrometric detector (ionization energy 70 eV, mass range *m/z* 10–800; 5975 MSD, Agilent Technologies). Separation of the cuticular waxes was achieved using an initial temperature of 50 °C for 2 min, raised by 40 °C min^{−1} to 200 °C, held at 200 °C for 2 min, and subsequently raised by 3 °C min^{−1} to 320 °C and maintained at 320 °C for 30 min. Quantitative composition of cuticular waxes was studied using capillary gas chromatography (7890A GC, Agilent Technologies) and flame ionization detection under the same gas chromatographic conditions but with hydrogen as carrier gas.

Acid-catalysed transesterification, extraction and derivatization of cutin monomers

To depolymerize the non-extractable cutin matrix, de-waxed and enzymatically isolated cuticular membranes were transesterified with boron trifluoride in methanol (methanolysis) at 70 °C overnight (twice) to release methyl esters of cutin acids. Sodium chloride-saturated aqueous solution, trichloromethane, and *n*-dotriacontane (Sigma–Aldrich) as an internal standard were added to all reaction mixtures. From this two-phase system, the transesterified cutin monomers were

extracted three times with trichloromethane. The combined extracts were dried over anhydrous sodium sulphate. All extracts were filtered and the organic solvent was evaporated under a continuous flow of nitrogen. Derivatization with *N,O*-bis-trimethylsilyl-trifluoroacetamide in pyridine was performed at 60 °C for 60 min.

Cutin analysis by gas chromatography–mass spectrometry

Analysis of cutin monomers was performed similarly to the gas chromatographic analysis of cuticular waxes. Separation of cutin monomers was carried out at 50 kPa for 60 min, 10 kPa min^{−1} to 150 kPa, and at 150 kPa for 30 min using a temperature program of 50 °C for 1 min, raised by 10 °C min^{−1} to 150 °C, held at 150 °C for 2 min, and subsequently raised by 3 °C min^{−1} to 320 °C and maintained at 320 °C for 30 min. Qualitative and quantitative composition was studied using capillary gas chromatography with mass spectrometric and flame ionization detection under the same chromatographic conditions. Single compounds were identified based on the electron ionization mass spectra using authentic standards, the Wiley 10th/NIST 2014 mass spectral library (W10N14, John Wiley & Sons) or by interpretation of the spectra, by the retention times and/or by comparison with literature data, and quantitated using the internal standard.

Preparation of cutan

After cutin transesterification, the cuticular membrane residue, designated cutan I, was air-dried and then extracted with dichloromethane and methanol (9:1; VWR) using a Soxhlet apparatus for 24 h. The cuticular membrane residue obtained was air-dried followed by base hydrolysis with 1 M potassium hydroxide in methanol (Merck) at 70 °C for 24 h. After this, the solution including the solid residue was allowed to cool down, and acidified to pH <3 with 2 N hydrogen chloride. Subsequently, dichloromethane was added and the mixtures were vigorously stirred followed by removal of the dichloromethane fraction. This procedure was performed three times. Then, the aqueous phase was removed from the solid residue, which was washed with water (three times), with methanol (three times) and with dichloromethane (three times). The cuticular membrane residue was air-dried and designated cutan II.

Cutan analysis by thermal extraction and flash pyrolysis gas chromatography–mass spectrometry

The solvent extracts of cutan I were derivatized using *N,O*-bis-trimethylsilyl-trifluoroacetamide in pyridine, after which they were further diluted in ethyl acetate prior to analysis. They were on-column injected using a Trace GC Ultra gas chromatograph (Thermo Fisher Scientific) onto a CP-Sil 5 CB fused silica column (length 30 m, inner diameter 320 µm, film thickness 0.1 µm). The gas chromatograph was operated at a constant flow of 2 mL min^{−1}. The oven was programmed starting at 70 °C to rise to 130 °C at a rate of 20 °C min^{−1} and then to

320 °C at a rate of 4 °C min^{−1}, followed by an isothermal hold for 20 min. The Trace DSQ mass spectrometer operated within a scanning range of *m/z* 50–800.

Cutan I and cutan II were examined by thermal extraction. Samples were pressed onto nickel/iron Curie point wires and inserted into the pyrolysis unit (Curie-Point pyrolyser, Horizon Instruments), which had a set-point temperature of 250 °C. Subsequently, all compounds that evaporated at this temperature were injected on-line onto the gas chromatography column. The pyrolysis unit was directly connected to a gas chromatograph (Carlo Erba GC8060) through a splitless injector set at 280 °C and the products were separated by a CP-Sil 5 fused silica column (length 25 m, inner diameter 320 µm, film thickness 0.4 µm; Varian). Helium was used as carrier gas. The oven was initially kept at 40 °C for 1 min, heated at a rate of 7 °C min^{−1} to 320 °C and maintained at this temperature for 15 min. The column was coupled to a mass spectrometer (mass range *m/z* 45–650, ionization energy 70 eV, cycle time 0.7 s; Fisons MD800). Pyrolysis was carried out via flash heating at 600 °C for 5 s. Similar to thermal extraction, the compounds released by, in this case flash pyrolysis, were on-column injected, separated and detected identically to those released by thermal extraction.

Cutan analysis by thermally assisted hydrolysis and methylation

Thermally assisted hydrolysis and methylation (THM), performed by flash heating using the Curie-point pyrolyser in the presence of tetramethylammonium hydroxide, was employed at 350 °C, and in the case of *C. miniata* also at 510 and 600 °C to provide more insight into the composition of polar cutan monomers. Both ester and ether bonds were cleaved and the resulting hydroxyl and carboxyl groups were transformed *in situ* to the corresponding methyl ethers and methyl esters, respectively. Prior to THM, a droplet of 25 % tetramethylammonium hydroxide in water (Sigma–Aldrich) was added to the samples, which were dried under a 100-W halogen lamp (Philips). Analysis of the THM products by gas chromatography–mass spectrometry was performed under conditions similar to those applied to conventional pyrolysis. Identification of the compounds was based on their mass spectra using an NIST library or by interpretation of the spectra, by the retention times and/or by comparison with literature data.

Cuticle analysis by Fourier transform infrared spectroscopy

The enzymatically isolated cuticular membrane, the solvent-extracted cuticular membrane and the cuticular membrane residue after transesterification with boron trifluoride in methanol were scanned with a Fourier transform infrared spectrometer (Bruker Tensor 27) in transmission mode at 20 °C. The temperature was adjusted using a custom-made sample holder connected to the water circuit of a thermostat (DC30-K20, Thermo Scientific Haake). Infrared spectra were recorded in the wavenumber range from 4000 to 400 cm^{−1}. The resolution was set to 2 cm^{−1} with an acquisition time of 120 scans. The OPUS 7 (Bruker) software was used to analyse the infrared

spectra. Transmission was transformed into absorbance. The baseline of the spectra was adjusted and smoothed.

RESULTS

Comparative study of *Agave americana* adaxial leaf sections

Leaves of *A. americana* were bisected and divided into 11 sections and the adaxial surface area was determined (Supplementary Data Fig. S1A). The leaf part at the apex enlarged gradually from 0.9 to 6.7 cm² (sections 1–4). In the middle part, the leaf broadened with a surface area between 7.5 and 7.2 cm² (sections 5–7). The leaf base had a lower surface area, ranging from 6.1 to 4.5 cm² (sections 8–11; Supplementary Data Fig. S1B). Analysing the leaf sections from the leaf apex to the leaf base, a cuticle weight from 827.7 µg cm⁻² (section 1) to 1502.0 µg cm⁻² (section 11) was documented (Fig. 2A). Extraction with different organic solvents reduced the cuticle weight by ~17 %. The cuticle weight without solvent-extractable compounds ranged from 676.6 µg cm⁻² at the leaf apex (section 1) to 1192.6 µg cm⁻² at the leaf base (section 11). Accordingly, the difference was higher at the leaf base: 309.4 and 324.8 µg cm⁻² (sections 10 and 11) in comparison with ~150.6 µg cm⁻² in the leaf middle and at the leaf apex (sections 1–9).

Cuticular waxes of *Agave americana* adaxial leaf surface

Analysis of *A. americana* cuticular waxes of the adaxial leaf sections from the leaf apex to the leaf base by gas chromatography revealed an increasing cuticular wax load, ranging from 32.3 to 77.7 µg cm⁻² (sections 1–11; Fig. 2B). These cuticular waxes obtained by solvent extraction consisted mainly of alkanolic acids (18 %) and primary alkanols (60 %), most prominently octacosanol (C₂₈), triacontanol (C₃₀), dotriacontanol (C₃₂) and tetratriacontanol (C₃₄) and their corresponding alkanolic acid derivatives (Fig. 3A). Moderate quantities of alkane- α,ω -diols (8 %), *n*-alkanals (7 %), *n*-alkanes (2 %), secondary alkanols (2 %) and alkyl esters (2 %) were identified in the cuticular waxes. The very-long-chain aliphatic wax compounds had a chain length distribution between 20 and 34 carbon atoms, and the chain length of alkyl esters was between C₄₀ and C₅₂.

The detailed analysis of the cuticular wax accumulation of the leaf sections showed no qualitative differences but a quantitative gradient from the leaf apex to the leaf base. Mainly, the amount of alkanolic acids (4.5–14.7 µg cm⁻²), primary alkanols (19.0–50.9 µg cm⁻²), *n*-alkanals (1.5–4.4 µg cm⁻²) and secondary alkanols (0.5–1.1 µg cm⁻²) was distinctly higher at the leaf base.

After acid-catalysed transesterification, the cuticular wax quantity and quality differed. Without transesterification, the cuticular wax amount averaged 45.5 µg cm⁻² at the adaxial leaf surface, whereas the cuticular wax amount after transesterification was distinctly higher, ~159.0 µg cm⁻² (Supplementary Data Fig. S2). Nevertheless, this transesterification approach confirmed the presence of alkanolic acids (35 %) and primary alkanols (44 %), most prominently octacosanol (C₂₈), triacontanol (C₃₀), dotriacontanol (C₃₂) and tetratriacontanol (C₃₄), alkane- α,ω -diols (7 %) and *n*-alkanes (1 %), though in different amounts. Additionally, α,ω -dicarboxylic acids (6 %), ω -hydroxy alkanolic

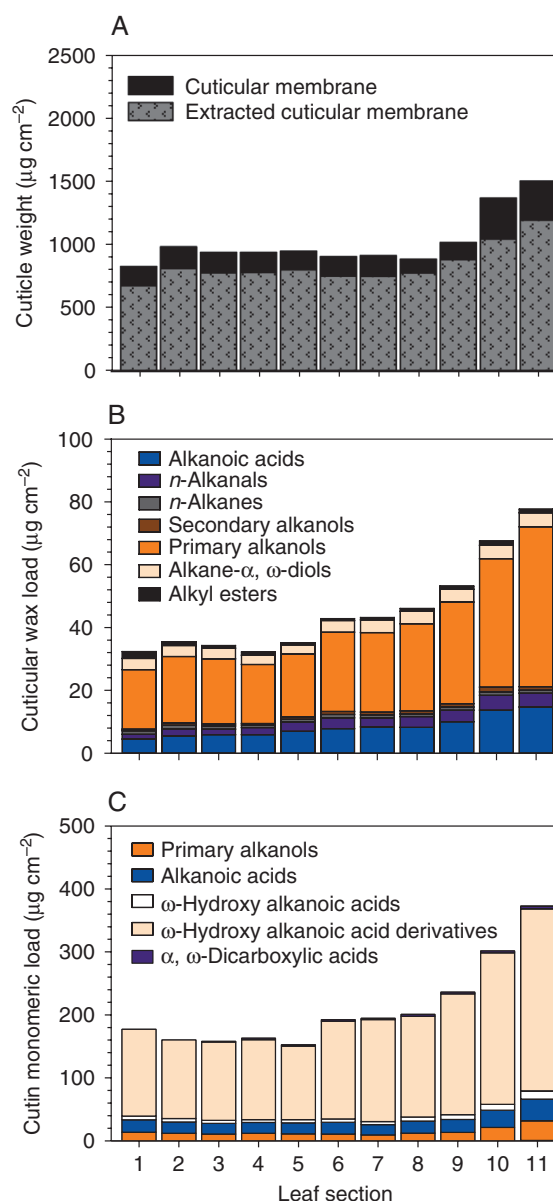


FIG. 2. Investigation of the *Agave americana* adaxial leaf surface along a gradient from the leaf apex (section 1) to the leaf base (section 11). (A) Gravimetric analysis of the enzymatically isolated cuticular membrane. (B, C) Determination of the amount of cuticular waxes (B) and cutin monomers (C) of the adaxial leaf surface by gas chromatography with flame ionization and mass spectrometry. Data are shown as mean values ($n = 4$).

acids (6 %) and ω -hydroxy alkanolic acid derivatives (<1 %) were identified only in the cuticular waxes after transesterification. The very-long-chain aliphatic wax compounds had a chain length distribution between 20 and 36 carbon atoms; only 9,10-epoxy ω -hydroxy and 9,10, ω -trihydroxy alkanolic acids, present in trace amounts, had a carbon chain length of 18.

Cutin deposition on the *Agave americana* adaxial leaf surface

After acid-catalysed transesterification of the extracted cuticular membrane, the released cutin monomers from the

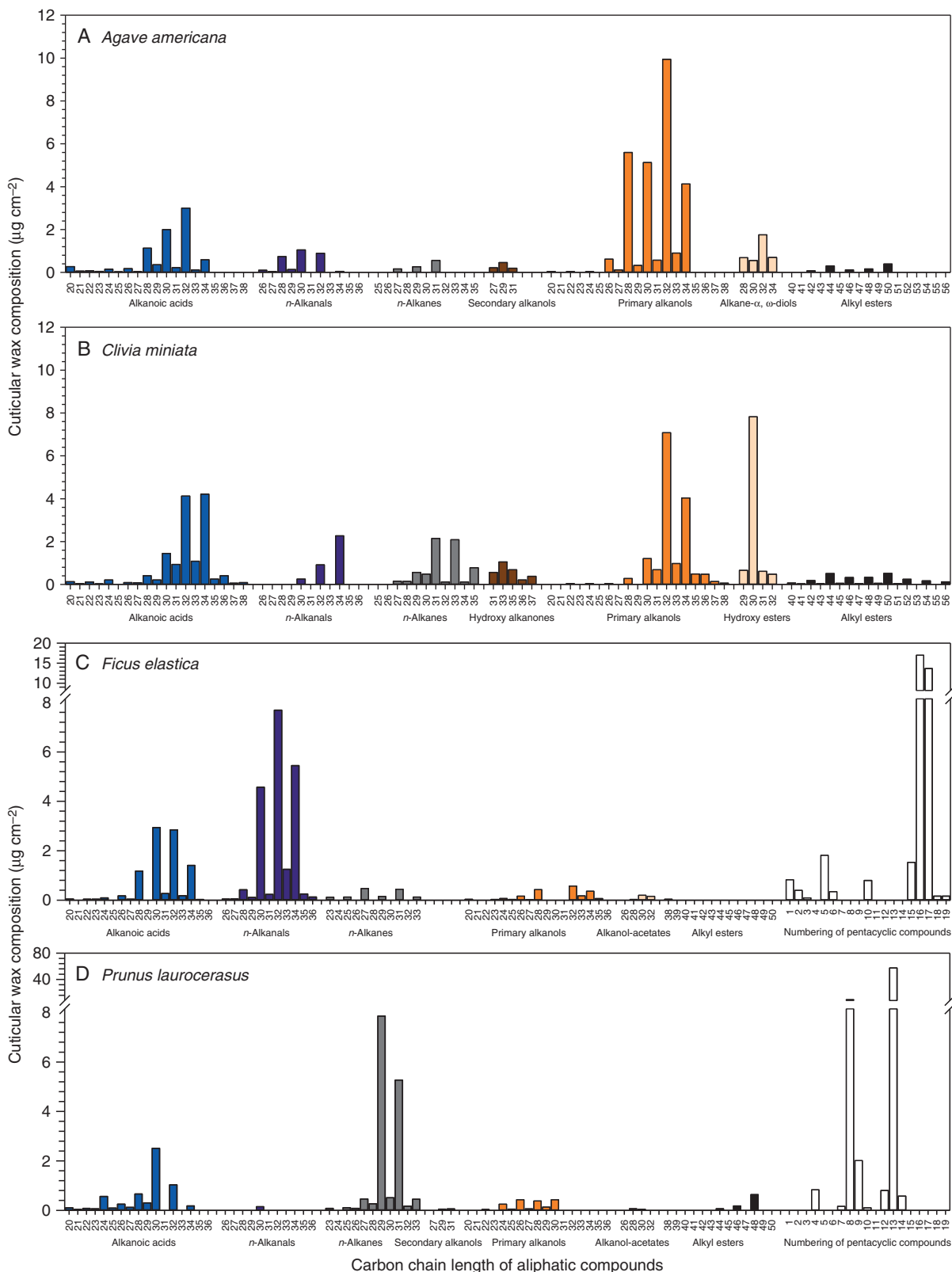


FIG. 3. Detailed cuticular wax composition of the adaxial leaf surface of *Agave americana* (A), *Clivia miniata* (B), *Ficus elastica* (C) and *Prunus laurocerasus* (D) detected by gas chromatography with flame ionization and mass spectrometry. Carbon chain length is shown for the aliphatic wax compound classes. Pentacyclic triterpenoids are numbered: lupeol (1), lupenone (2), betulone (3), betulonic acid (4), β -amyrin (5), β -amyrin derivative (6), erythrodiol (7), oleanolic acid (8), hederagenin (9), α -amyrin (10), α -amyrenone (11), uvaol (12) and ursolic acid (13), taraxerol (14), friedelanol (15), friedelin (16), fernenol (17), hopanol (18) and hopenone (19). Data are shown as mean values ($n = 4$). Data for *A. americana* and *C. miniata* represent an average over the total adaxial leaf surface.

A. americana adaxial leaf sections were analysed by gas chromatography, showing the presence of primary alkanols and cutin acids. Quantitative but not qualitative differences were determined by comparing the monomeric cutin level of the adaxial leaf sections along a gradient from the leaf apex to the leaf base (Fig. 2C). The leaf apex (section 1) had a lower cutin load ($177.1 \mu\text{g cm}^{-2}$) compared with the leaf base (section 11; $368.8 \mu\text{g cm}^{-2}$).

In detail, the aliphatic compounds had a chain length distribution between 16 and 34 carbon atoms and were dominated by the C_{16} and C_{18} cutin acids. The most predominant aliphatic cutin monomers were 9,10-epoxy ω -hydroxy C_{18} alkanolic acid and 9,10, ω -trihydroxy C_{18} alkanolic acid (Fig. 4A). Smaller amounts of very-long-chain compounds in the cutin fraction included primary alkanols, alkanolic acids, ω -hydroxy alkanolic acids and α,ω -dicarboxylic acids, mainly with carbon chain lengths of 28, 30, 32 and 34, also present in the cuticular waxes after acid-catalysed transesterification.

Cutan deposition on the *Agave americana* adaxial leaf surface

The THM analysis of cutan I, which was yielded after solvent extraction and acid-catalysed transesterification, showed homologous series of methylated alkanolic acids, primary alkanols and, in lower abundance, ω -hydroxy alkanolic acids and alkane- α,ω -diols, all of which ranged from C_{26} to C_{34} . Additionally, substantial amounts of 9,10, ω -dihydroxy C_{16} alkanolic acid and 9,10, ω -trihydroxy C_{18} alkanolic acid were detected (Fig. 5A). Cutan I, the cuticular membrane residue obtained from *A. americana* leaves after solvent extraction and acid-catalysed transesterification, was subjected to thermal extraction, which released methyl esters of C_{14} , C_{16} and C_{18} as well as methyl esters of C_{28} , C_{30} , C_{32} and C_{34} alkanolic acids. In addition, C_{30} and C_{32} primary alkanols and C_{29} and C_{31} *n*-alkanes were identified (Fig. 5B). The solvent extracts consisted of the same compounds, albeit the C_{34} monomers were more abundant, which was due to the more efficient solvent extraction compared with the thermal extraction (Fig. 5C). The maximum temperature available for the latter is 250°C , resulting in less efficient extraction of higher boiling compounds. Hence, compounds with longer carbon chains were negatively discriminated.

Exhaustive solvent extraction, base hydrolysis, acidification and extraction of cutan I yielded cutan II. Upon thermal extraction of cutan II, only trace amounts of *n*-alk-1-enes and *n*-alkanes as well as methylated C_{30} and C_{32} alkanolic acids were detected (Fig. 5D).

The products of the cutan II after flash pyrolysis were dominated by *n*-alka- α,ω -dienes, *n*-alk-1-enes and *n*-alkanes ranging from C_7/C_8 to C_{35} , maximizing at C_{29} and with a sharp decrease after C_{31} (Fig. 6A, B). The second most abundant compound class comprised *n*-alkan-2-ones ranging from C_{21} to C_{35} , with a clear odd-over-even predominance, and dominated by C_{31} and C_{33} (Fig. 6C). In addition, traces of *n*-alken-2-ones were identified. Methylated C_5 to C_{34} alkanolic acids, dominated by C_{30} and C_{32} , were also present, though in trace amounts (Fig. 6D).

Upon THM of cutan II, methylated alkanolic acids predominated; their distribution was relatively similar to those released after THM of cutan I, ranging from C_5 to C_{34} , with a strong

even-over-odd predominance, and was dominated by C_{16} , C_{30} , C_{32} and C_{34} (Fig. 7A, D). A second compound class consisted of C_{26} to C_{34} primary alkanols with a strong even-over-odd predominance, dominated by C_{32} , C_{30} , C_{34} and C_{28} in decreasing abundance (Fig. 7B). A third compound class comprised alkane- α,ω -diols with decreasing abundance: C_{32} , C_{30} , C_{34} and C_{28} . A fourth class of compounds included ω -hydroxy alkanolic acids with chain lengths C_{32} , C_{30} , C_{28} and C_{26} in decreasing order. Traces of 9,10, ω -dihydroxy C_{16} alkanolic acid and smaller amounts of 9,10, ω -trihydroxy C_{18} alkanolic acid were also present in cutan II, though in much lower abundance released from cutan II as compared with cutan I upon THM. Apart from these aliphatic compounds, a small number of aromatic compounds were detected. Benzene-1,3,5-triol and its derivatives with a methyl or an ethyl group were found in decreasing abundance (Figs 7C and 8).

Comparison of the relative abundance of very-long-chain alkanolic acids, primary alkanols, ω -hydroxy alkanolic acids and alkane- α,ω -diols in the cuticular wax, the cutin, the cutan I and the cutan II component of *A. americana* clearly indicates their similar if not identical distribution patterns and the predominance of the carbon chain length 32 in these compound classes (Fig. 9A–D).

Cuticular waxes and cutin deposition on the *Clivia miniata*, *Ficus elastica* and *Prunus laurocerasus* adaxial leaf surface

Based on gas chromatographic analysis, the cuticular wax load ranged from 55.3 to $95.5 \mu\text{g cm}^{-2}$ in *C. miniata*, *F. elastica* and *P. laurocerasus* (Table 1). The cuticular waxes of the *A. americana* and *C. miniata* adaxial leaf surface were rather similar and consisted mainly of alkanolic acids (25 %) and primary alkanols (29 %), most prominently triacontanol (C_{30}), dotriacontanol (C_{32}) and tetratriacontanol (C_{34}) and corresponding alkanolic acid derivatives (Fig. 3B). In smaller amounts, hydroxy esters (17 %), *n*-alkanes (13 %), *n*-alkanals (6 %), hydroxy alkanones (5 %) and alkyl esters (5 %) were identified in the cuticular waxes of *C. miniata*. The very-long-chain aliphatic wax compounds had a chain length distribution between 20 and 38 carbon atoms, and the chain length of alkyl esters was between C_{40} and C_{56} .

The adaxial leaf surface of *F. elastica* and *P. laurocerasus* revealed cuticular waxes of very-long-chain aliphatic compounds (33.4 and $24.3 \mu\text{g cm}^{-2}$, respectively) and pentacyclic compounds (36.8 and $71.2 \mu\text{g cm}^{-2}$, respectively). The pentacyclic wax compounds were dominated by triterpene alkanols and triterpene alkanones, mainly friedelin (46 %) and fernenol (37 %), in *F. elastica*, and by triterpene acids, mainly ursolic acid (81 %) and oleanolic acid (13 %), in *P. laurocerasus*. In *F. elastica*, the aliphatic wax fraction predominantly contained alkanolic acids (28 %) and *n*-alkanals (60 %), most prominently triacontanol (C_{30}), dotriacontanol (C_{32}) and tetratriacontanol (C_{34}) and their corresponding alkanolic acids (Fig. 3C). Lower amounts of primary alkanols (6 %), *n*-alkanes (4 %), alkanol-acetates (1 %) and alkyl esters (<1 %) were identified. The very-long-chain aliphatic wax compounds had a chain length distribution between 20 and 40 carbon atoms. In *P. laurocerasus*, the aliphatic wax fraction contained mainly alkanolic acids (25 %) and *n*-alkanes

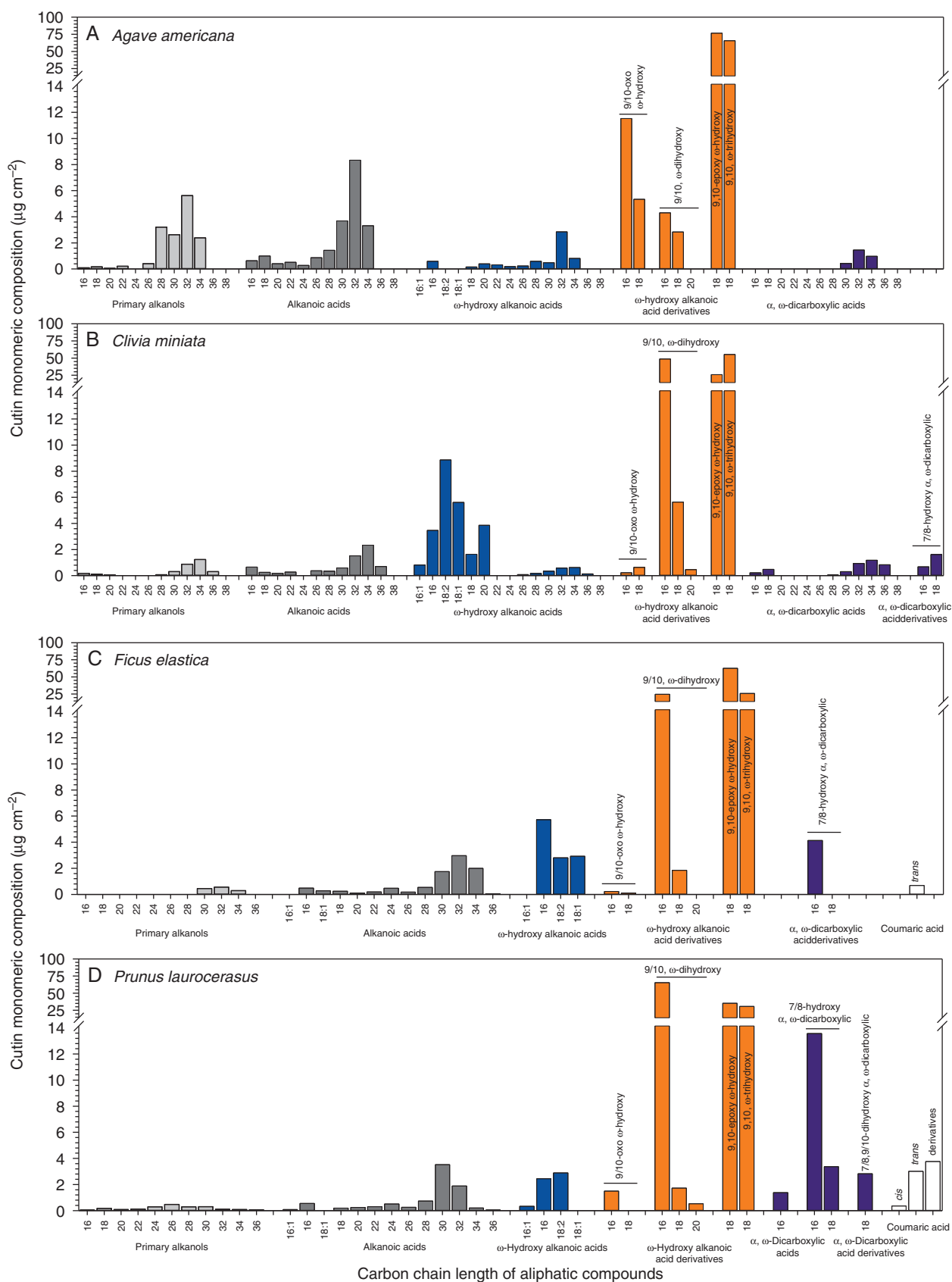


FIG. 4. Detailed cutin monomeric composition of the adaxial leaf surface of *Agave americana* (A), *Clivia miniata* (B), *Ficus elastica* (C) and *Prunus laurocerasus* (D) after acid-catalysed transesterification detected by gas chromatography with flame ionization and mass spectrometry. Data are shown as mean values ($n = 4$). Data for *A. americana* and *C. miniata* represent an average over the total adaxial leaf surface.

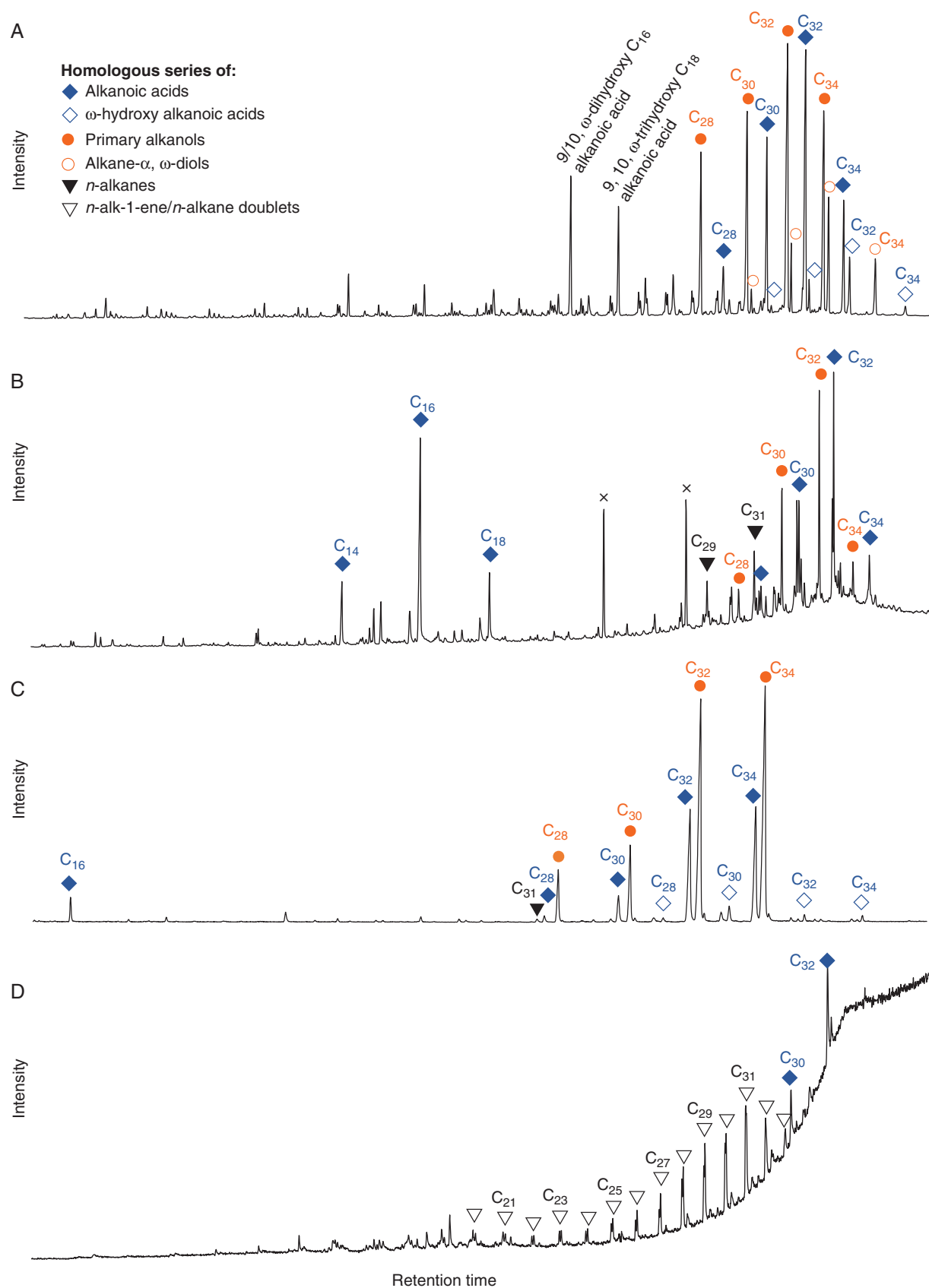


FIG. 5. (Partial) gas chromatograms of cutan I upon THM (A), thermal extraction (B) and solvent extraction (C) as well as thermally extracted cutan II (D) obtained from the adaxial leaf surface of *Agave americana*. Alkanoic acids were identified as methyl esters. Contaminants are marked (x).

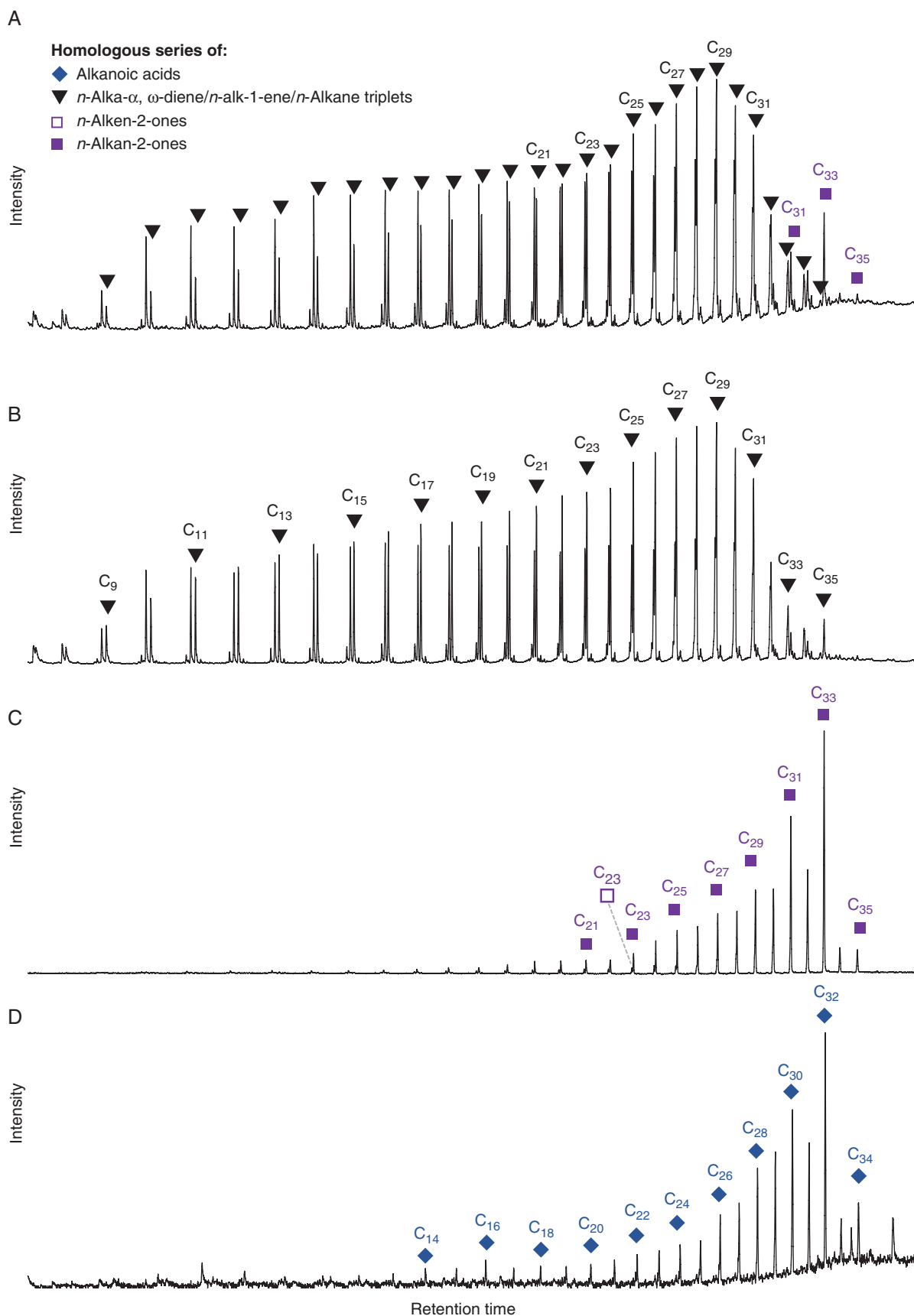


FIG. 6. (Partial) gas chromatograms of cutan II on the adaxial leaf surface of *Agave americana* upon flash pyrolysis. Total ion current represents all reaction products (A), m/z 55 and 57 highlight triplets of *n*-alka- α , ω -dienes, *n*-alk-1-enes, *n*-alkanes (B), m/z 59 indicates doublets of *n*-alken-2-ones, *n*-alkan-2-ones (C), and m/z 74 and 87 represent methylated alkanoic acids (D).

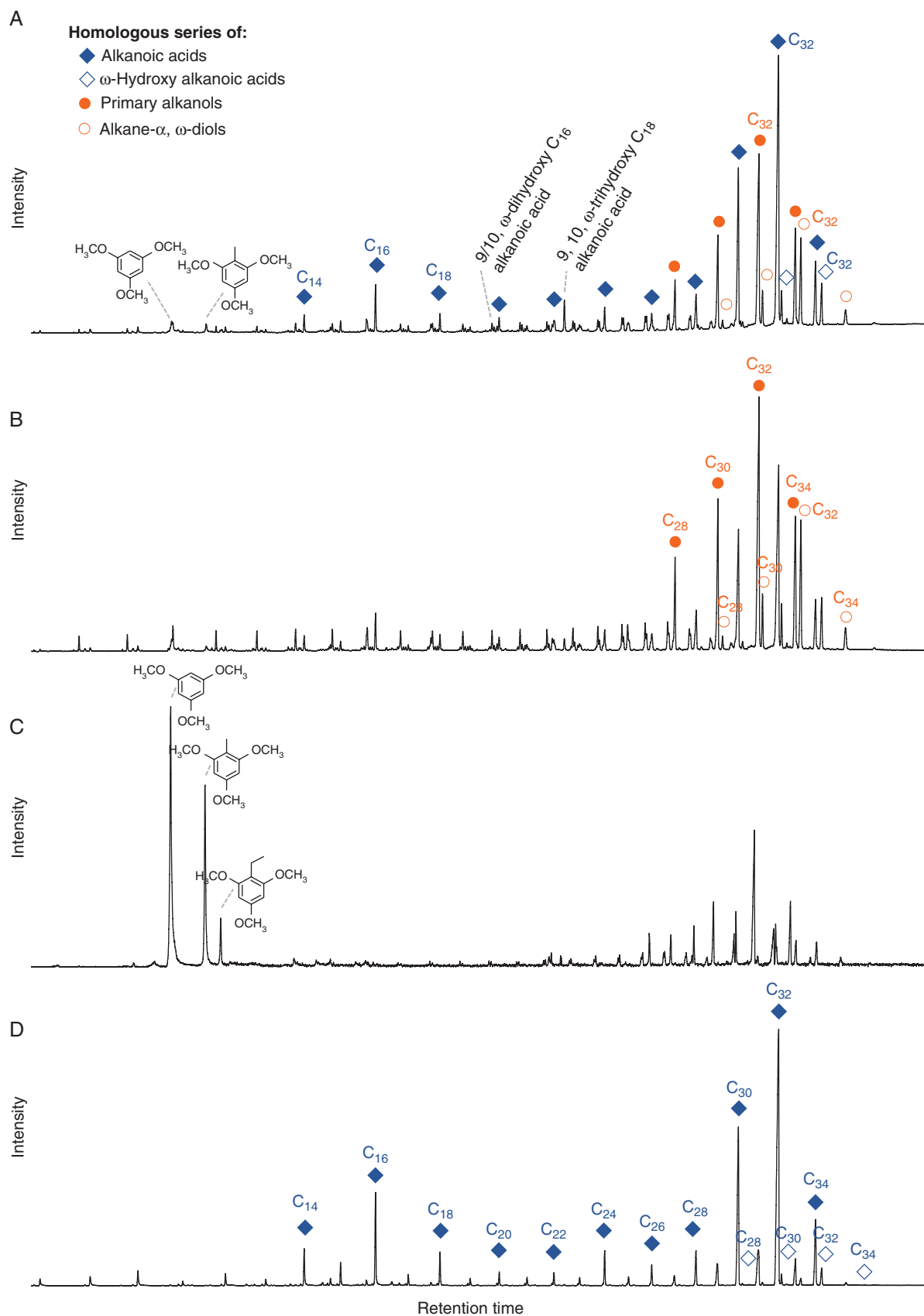


FIG. 7. (Partial) gas chromatograms of cutan II of the adaxial leaf surface of *Agave americana* upon THM at 350 °C. Total ion current represents all reaction products (A), m/z 69 highlights methylated primary alkanols (B), m/z 168, 182 and 196 indicate benzene-1,3,5-triol and derivatives (C), and m/z 74 and 87 represent methylated alkanolic acids (D). Methylated ω -hydroxy alkanolic acids, ω -hydroxy alkanolic acid derivatives and alkane- α , ω -diols are indicated.

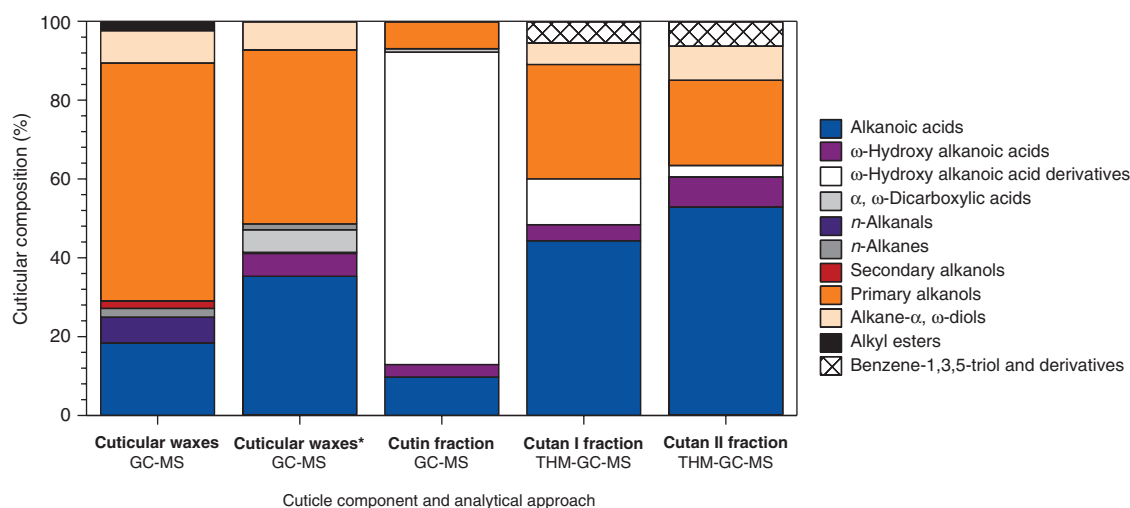


FIG. 8. Compositional analysis of the adaxial leaf surface of *Agave americana*. Cuticular waxes before and after acid-catalysed transesterification (*) as well as cutin monomers were analysed by gas chromatography with flame ionization (GC-FID) and mass spectrometry (GC-MS). Cutan I and cutan II monomers were detected by THM-GC-MS.

(63 %), most prominently nonacosane (C_{29}), hentriacontane (C_{31}), triacontanoic acid (C_{30}) and dotriacontanoic acid (C_{32} ; Fig. 3D). Also detected, in smaller amounts, were primary alkanols (7 %), alkyl esters (4 %), *n*-alkanals (1 %) alkanol-acetates (<1 %) and secondary alkanols (<1 %). The very-long-chain aliphatic wax compounds had a chain length distribution between 20 and 34 carbon atoms and the chain length was between C_{42} and C_{50} for alkyl esters.

The amount of cutin monomers of *C. miniata*, *F. elastica* and *P. laurocerasus* was similar to that of *A. americana*, ranging from 142.1 to 179.7 $\mu\text{g cm}^{-2}$ (Table 1). In contrast to *A. americana*, the adaxial leaf surface of *C. miniata*, *F. elastica* and *P. laurocerasus* showed a high abundance of cutin monomers with a carbon chain length of both 16 and 18 carbon atoms. C_{16} cutin acids (25–47 %) and C_{18} cutin acids (43–68 %) were present in substantial amounts. The predominant cutin monomers were 9/10, ω -dihydroxy C_{16} alkanolic acid, 9,10-epoxy ω -hydroxy C_{18} alkanolic acid and 9,10, ω -trihydroxy C_{18} alkanolic acid. In addition, unsaturated C_{16} and C_{18} cutin acids were detected in *C. miniata* (8 %), *F. elastica* (4 %) and *P. laurocerasus* (2 %). As detected for *A. americana*, very-long-chain primary alkanols, alkanolic acids, ω -hydroxy alkanolic acids and α,ω -dicarboxylic acids up to a carbon chain length of 38 were identified in the cutin fraction of *C. miniata* (Fig. 4B). Very-long-chain primary alkanols and alkanolic acids were present in the cutin fraction of *F. elastica* and *P. laurocerasus*. In addition, aromatic coumaric acid and derivatives were detected (≤ 4 %; Fig. 4C, D).

Similar to *A. americana*, a cuticle analysis of the adaxial leaf surface of *C. miniata* along a gradient from the leaf apex to the leaf base was performed and showed quantitative variations for cuticular wax and cutin accumulation, though qualitative differences were not detected (Supplementary Data Figs S1C, D and S3A–C).

Cutan deposition on the *Clivia miniata*, *Ficus elastica* and *Prunus laurocerasus* adaxial leaf surface

The cuticular membrane residue after cuticular wax extraction and acid-catalysed cutin transesterification (cutan I) was subjected

to exhaustive solvent extraction, base hydrolysis, acidification and extraction. In the case of *C. miniata* a residual cutan II was obtained, whereas for both *F. elastica* and *P. laurocerasus* almost no cuticular membrane residue was found (Table 1).

Upon THM at 350 °C, cutan I of *C. miniata* released mainly methylated C_{14} , C_{16} and C_{18} alkanolic acids as well as ω -hydroxy $C_{18:1}$ alkanolic acid, 9/10, ω -dihydroxy C_{16} alkanolic acid and 9,10, ω -trihydroxy C_{18} alkanolic acid (Supplementary Data Fig. S5A). The flash pyrolysis products of cutan II of *A. americana* and *C. miniata* were *n*-alka- α,ω -dienes, *n*-alk-1-enes and *n*-alkanes in the range between C_7/C_8 and C_{34}/C_{35} , maximizing at C_{29} and showing a sharp decrease after C_{31} (Supplementary Data Fig. S4). Upon THM at 350 °C, cutan II of *C. miniata* released almost no monomers (Supplementary Data Fig. S5B). By contrast, THM at temperatures of 510 and 600 °C released small amounts of methylated alkanolic acids ranging from C_8 to C_{36} with a strong even-over-odd predominance dominated by C_{30} , C_{32} and C_{34} in addition to C_{14} , C_{16} and C_{18} (Supplementary Data Fig. S5C, D). Moreover, ω -hydroxy alkanolic acids ranging from C_{28} to C_{34} were identified, whereas primary alkanols were almost absent. Furthermore, a homologous series of *n*-alka- α,ω -dienes, *n*-alk-1-enes and *n*-alkanes ranging from C_8 to C_{35} was detected.

Spectroscopic study of *Agave americana*, *Clivia miniata*, *Ficus elastica* and *Prunus laurocerasus* adaxial leaf surface

Fourier transform infrared analysis was performed on the cuticular membrane of *A. americana*, *C. miniata*, *F. elastica* and *P. laurocerasus* leaves (Supplementary Data Fig. S6A). Absorbance maxima were obtained by stretching and bending vibrations of aliphatic, aromatic and oxygen-bearing moieties in the spectral range from 3400 to 700 cm^{-1} (Fig. 10A–D). The infrared spectra were predominated by asymmetrical and symmetrical carbon–hydrogen stretching vibrations of methylene groups located at 2919 and 2850 cm^{-1} corresponding to the presence of aliphatic moieties. Moreover, aliphatic bending vibration of methylene groups led to an intermediate absorbance at 1464, 1319 and 720 cm^{-1} . Absorbance intensities in the spectral range from

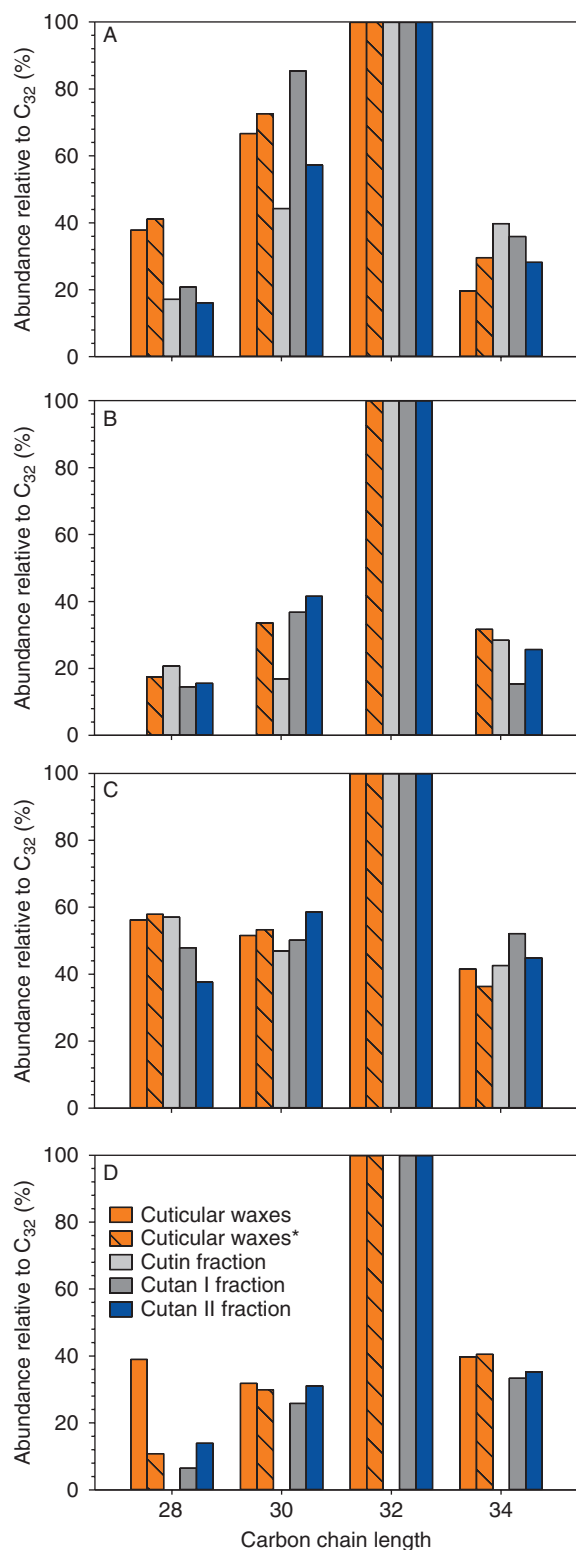


FIG. 9. Comparative analysis of extractable cuticular waxes before and after acid-catalysed transesterification (*), and cutin, cutan I and cutan II fractions of the adaxial leaf surface of *Agave americana*. Cutan I and cutan II monomers were detected by THM-gas chromatography-mass spectroscopy. Very-long-chain aliphatic compounds were identified for alkanolic acids (A), ω -hydroxy alkanolic acids (B), primary alkanols (C) and alkane- α,ω -diols (D).

1650 to 1500 cm^{-1} were assigned to the stretching vibration of aromatic moieties: carbon-carbon single bonds conjugated with carbon-carbon double bonds. High absorbance at $\sim 3380 \text{ cm}^{-1}$ indicated a stretching vibration of hydroxyl groups with an intermolecular linkage. Strong stretching vibration of carbonyl groups of ester bonds centred at 1734 cm^{-1} with a shoulder at 1717 cm^{-1} indicating a hydrogen bonding. Absorbance intensities at 1167 cm^{-1} showed an asymmetrical carbon-oxygen-carbon stretching vibration of ester bonds and at 1062 or 1037 cm^{-1} carbon-oxygen stretching vibration of free hydroxyl groups.

Infrared spectra of the cuticular membrane of *A. americana*, *C. miniata*, *F. elastica* and *P. laurocerasus* leaves showed mainly absorbance similarities. Differences were primarily found for the absorbance intensity ratio of the carbonyl group stretching vibration and the carbon-oxygen-carbon stretching vibration of ester bonds, respectively, versus the stretching vibration of methylene groups, which were lower for *F. elastica* and *P. laurocerasus* leaves. Additionally, the intermolecular hydroxyl group stretching vibration was relatively high in *P. laurocerasus* compared with the stretching vibration of methylene groups.

After solvent extraction, the cuticular membrane was gravimetrically reduced by 12 % for *C. miniata* to 22 % for *P. laurocerasus* (Supplementary Data Fig. S6B). At particular wavenumbers, the absorbance intensity ratio of the cuticular membrane of *A. americana*, *C. miniata*, *F. elastica* and *P. laurocerasus* leaves changed when compared with the stretching vibration of methylene groups. The absorbance intensities of the carbonyl group stretching vibration of ester bonds boosted for *A. americana*, *F. elastica*, *P. laurocerasus* leaves, and the intermolecular hydroxyl group stretching vibration was intensified in *C. miniata* and *P. laurocerasus* leaves. In addition, the stretching vibration of aromatic moieties increased for *F. elastica* and *P. laurocerasus* leaves.

After solvent extraction and acid-catalysed transesterification, the cuticular membrane residue amounted to <20 % of the initial cuticle weight for *F. elastica* and *P. laurocerasus* leaves, respectively, and was fibrous (Supplementary Data Fig. S6C). In this mode, a further analysis was technically not feasible. In contrast, the membranous cuticular residue was gravimetrically reduced to 34 % for *A. americana* and 43 % for *C. miniata* leaves. Scanning of the cuticular membrane residue of *A. americana* and *C. miniata* leaves showed a decline of absorbance intensities of the carbonyl group stretching vibration of ester bonds and the intermolecular hydroxyl group stretching vibration. The stretching vibration of aromatic moieties and the carbon-oxygen stretching vibration of free hydroxyl groups increased.

Leaf attributes of Agave americana, Clivia miniata, Ficus elastica and Prunus laurocerasus

Transverse sections of the adaxial leaf surface showed the relatively thick cuticle of these four plant species. The micrographs highlighted the cuticularization of the outer periclinal, epidermal cell wall, and the cuticular pegs pointed to a thickening of the cuticle between anticlinal, epidermal cell walls (Fig. 11A–C). The leaf cuticle ultrastructure was found to be different. The leaf cuticle of *A. americana* showed a lamellate

TABLE 1. Characterization of *Agave americana*, *Clivia miniata*, *Ficus elastica* and *Prunus laurocerasus* leaves: specific leaf surface area, leaf dry matter content, leaf mass per surface area, leaf water content and degree of succulence. Cuticular properties of the adaxial leaf surface are summarized. Data for *A. americana* and *C. miniata* represent an average over the total adaxial leaf surface. Data are shown as mean values ($n \geq 4$)

Clade	Angiosperms/monocotyledons		Angiosperms/eudicotyledons	
Order	Asparagales		Rosales	
Family	Asparagaceae	Amaryllidaceae	Moraceae	Rosaceae
Species	<i>Agave americana</i>	<i>Clivia miniata</i>	<i>Ficus elastica</i>	<i>Prunus laurocerasus</i>
Life form	Perennial evergreen herb	Perennial evergreen herb	Perennial evergreen woody shrub/tree	Perennial evergreen woody shrub
Climate	Permanent hot, arid	Permanent hot, humid	Permanent hot, humid	Seasonal cold, moderate humid
Photosynthesis	Crassulacean acid metabolism	C ₃ pathway	C ₃ pathway	C ₃ pathway
Leaf traits	Simple, spiny, linear	Simple, entire, linear	Simple, entire, ovate	Simple, serrate, lanceolate
Specific surface area (m ² kg ⁻¹)	2	7	9	7
Dry matter content (mg g ⁻¹)	102	112	206	376
Mass per surface area (g m ⁻²)	543	137	117	145
Water content (g g ⁻¹)	0.9	0.9	0.8	0.6
Succulence (g m ⁻²)	2321	522	227	124
Cuticular waxes (μg cm ⁻²)	45	55	70	96
Aliphatic compounds (%)	100	100	63	25
Principal compound class	primary alkanols	primary alkanols	<i>n</i> -alkanals	<i>n</i> -alkanes
Pentacyclic compounds	–	–	37 %	75 %
Principal compound	–	–	Friedelin	Ursolic acid
Cutin (μg cm ⁻²)	211	176	142	180
Aliphatic compounds (%)	100	100	99.6	96
Principal compound	9,10-epoxy ω-hydroxy C ₁₈ alkanolic acid	9,10,ω-trihydroxy C ₁₈ alkanolic acid	9,10-epoxy ω-hydroxy C ₁₈ alkanolic acid	9/10,ω-dihydroxy C ₁₆ alkanolic acid
Aromatic compounds	–	–	0.4 %	4 %
Principal compound	–	–	Coumaric acid	Coumaric acid
Cutan I				
Aliphatic compounds (%)	94	99	100	100
Principal compound class	Alkanolic acids	Alkanolic acids	Alkanolic acids	Alkanolic acids
Aromatic compounds (%)	6	1	–	–
Principal compound	Benzene-1,3,5-triol	Benzene-1,3,5-triol	–	–
Cutan II				
Aliphatic compounds (%)	94	98	–	–
Principal compound class	Alkanolic acids	Alkanolic acids	–	–
Aromatic compounds (%)	6	2	–	–
Principal compound	Benzene-1,3,5-triol	Benzene-1,3,5-triol	–	–
Cuticle weight (μg cm ⁻²)	1021	644	761	721
Cuticle thickness (μm)	6	5	6	5
Cuticle ultrastructure	Lamellate	Lamellate/reticulate	Lamellate/reticulate	Reticulate

layering, whereas *C. miniata* and *F. elastica* possessed a lamellate outer layering merging into a reticulate inner zone. The leaf cuticle of *P. laurocerasus* showed a reticulate ultrastructure. The leaf cuticle thickness was between 4.9 μm for *C. miniata* and 6.5 μm for *A. americana* (Table 1). Moreover, the cuticular membrane of the adaxial *A. americana* leaf surface was comparatively heavy and weighed 26–37 % more in comparison with those of *C. miniata*, *F. elastica* and *P. laurocerasus*.

Several leaf traits of *A. americana*, *C. miniata*, *F. elastica* and *P. laurocerasus* were measured. Comparing these plant species, *A. americana* had the lowest values of specific leaf surface area (1.9 m² kg⁻¹) and leaf dry matter content (102.5 mg g⁻¹). In contrast, leaves of *A. americana* exhibited the highest leaf water content (0.9 g g⁻¹) and leaf mass per surface area (542.7 g m⁻²). In comparison with *A. americana*, the leaf mass per surface area for *C. miniata*, *F. elastica* and *P. laurocerasus* was ~75 %

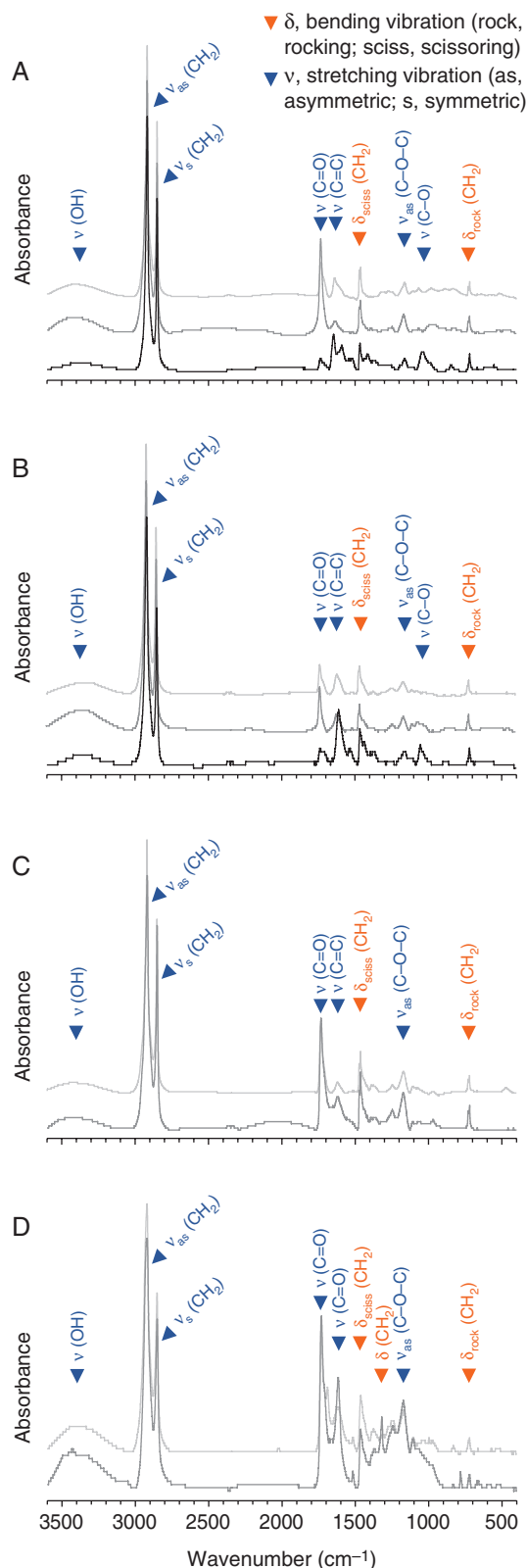


FIG. 10. Fourier transform infrared spectroscopy of the adaxial leaf surface of *Agave americana* (A), *Clivia miniata* (B), *Ficus elastica* (C) and *Prunus laurocerasus* (D). The infrared spectra of the enzymatically isolated cuticular membrane (light grey), the solvent-extracted cuticular membrane (dark grey) and the transesterified cuticular membrane residue (black) were scanned. Absorbance maxima of stretching (v) and bending (δ) vibration of aliphatic, aromatic and oxygen-bearing moieties are marked.

lower. Furthermore, *A. americana* had a >90 % higher degree of succulence compared with *F. elastica* and *P. laurocerasus*.

DISCUSSION

Dissimilarities of leaf cutan-depositing plant species as reported in the literature

Components of the plant cuticle are cuticular waxes, cutin, in some cases cutan, and polysaccharide moieties originating from the cell wall. Up to the present, only a few plant species have been described to accumulate the biopolymer cutan as a cuticular component of leaves. Besides *A. americana*, these cutan-depositing leaves have been reported to occur in, amongst others, *C. miniata*, *F. elastica* and *P. laurocerasus* (Schmidt and Schönherr, 1982; Villena *et al.*, 1999; Boom *et al.*, 2005; Guzmán-Delgado *et al.*, 2016).

Agave americana, *C. miniata*, *F. elastica* and *P. laurocerasus* are natively non-co-occurring evergreen perennials with different thermal tolerances and requirements for water and light and represent different photosynthetic pathways. Similarly, as determined in this study, leaf traits of these four plant species were divergent. The rigid, succulent and thick leaves of the rosette-forming *A. americana* had a relatively high leaf water content, from 0.8 to 0.9 g g⁻¹, and a high leaf mass per surface area. The leaf dry matter content of *A. americana* leaves was low and also the specific leaf surface area, at 2–4 m² kg⁻¹ (Raveh *et al.*, 1998; Vendramini *et al.*, 2002). Leaves of clump-forming *C. miniata* were described as long and strappy and had a leaf dry matter content comparable to *A. americana*, of 112 mg g⁻¹. The glossy, non-succulent and thick leaves of *F. elastica* had a higher leaf dry matter content of 206 mg g⁻¹ and a high specific leaf surface area between 6 and 9 m² kg⁻¹ (Choong *et al.*, 1992). The sclerophyllous leaves of *P. laurocerasus* were oblong, coriaceous and shiny. The leaf water content of *P. laurocerasus* averaged only 0.6–0.7 g g⁻¹ and the leaf mass per surface area was between 120 and 193 g m⁻². The leaf dry matter content of *P. laurocerasus* was relatively high, from 267 to 376 mg g⁻¹ (Castro-Díez *et al.*, 2000; Ceccato *et al.*, 2001; Sancho-Knapik *et al.*, 2010).

In general, the cuticle thickness of leaves ranges from <1 to 10 μ m (Schreiber and Schönherr, 2009), inclusive of *A. americana*, *C. miniata*, *F. elastica* and *P. laurocerasus* (Wattendorff and Holloway, 1980, 1982, 1984; Mérida *et al.*, 1981; Becker *et al.*, 1986; Choong *et al.*, 1992; Gouret *et al.*, 1993; Guzmán-Delgado *et al.*, 2016). However, investigations of the cuticle weight of leaves showed wide differences between plant species. Holloway and Baker (1970) as well as Schreiber and Riederer (1996) specified a range between <20 and 1373 μ g cm⁻². These previous studies list the cuticle weight for *A. americana*, *C. miniata*, *F. elastica* and *P. laurocerasus* between 333 and 730 μ g cm⁻². However, our analysis showed a range between 644 and 1021 μ g cm⁻², though confirming that the leaf cuticle of *A. americana* had the highest cuticle weight. Likewise, the cuticle ultrastructure of leaves can vary. According to Holloway (1982b), the leaf cuticle of *A. americana*, *C. miniata* and *F. elastica* has a polylamellate or lamellate outer layering and an inner, mainly reticulate zone, whereas *P. laurocerasus* leaves have a reticulate cuticle ultrastructure (Wattendorff and Holloway, 1980, 1982; Holloway, 1994).

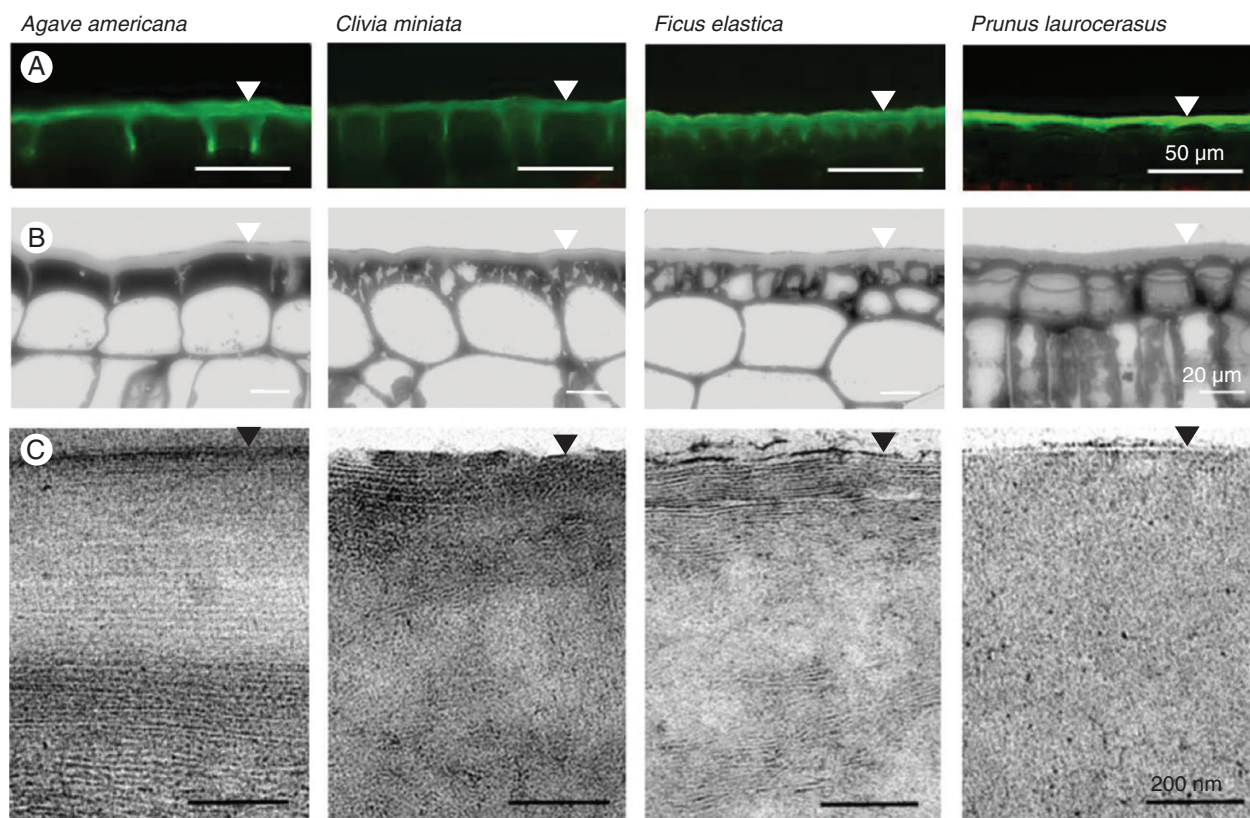


FIG. 11. Transverse sections of the adaxial leaf surface of *Agave americana*, *Clivia miniata*, *Ficus elastica* and *Prunus laurocerasus*: micrographs using autofluorescence (A), light micrographs stained with methylene blue and azure II (B) and transmission electron micrographs stained with uranyl acetate and Reynolds' lead citrate (C). The outside margin of the cuticle is marked with arrowheads.

Spectroscopic analysis of the cuticular membrane of *Agave americana*, *Clivia miniata*, *Ficus elastica* and *Prunus laurocerasus*

To illustrate similarities and differences between the cuticular membrane of cutan-depositing leaves, a Fourier transform infrared analysis was performed. Structural information related to aliphatic, aromatic and oxygen-bearing moieties were obtained for the cuticular membrane of *A. americana*, *C. miniata*, *F. elastica* and *P. laurocerasus* leaves.

As expected, the overall picture of the cuticular membrane was characterized by similar spectroscopic properties; only small differences occurred (Chamel and Maréchal, 1992; Villena *et al.*, 1999, 2000; Heredia-Guerrero *et al.*, 2014; Guzmán-Delgado *et al.*, 2016). Due to the fact that the most intense infrared absorbance was found for methylene groups, the cuticular membrane was indicated as predominantly aliphatic in composition. Strong absorbance of carbonyl groups, intermediate absorbance of carbon–oxygen–carbon bonds and of intermolecular hydroxyl groups give rise to a polymeric association via ester bonds. Both findings confirmed that the structural basis of the cuticular membrane is formed by an aliphatic biopolyester, the cutin matrix (Holloway, 1982a; Ramirez *et al.*, 1992). For this reason, the extracted cuticular membrane without cuticular waxes exhibited intensified absorbance of functional groups that were related to ester bonds. Additionally, the absorbance of aromatic moieties increased in comparison with the aliphatic moieties for the extracted cuticular membrane

of *F. elastica* and *P. laurocerasus* leaves, suggesting the presence of possibly cinnamic acid derivatives cross-linked within the cutin matrix.

After extraction and transesterification, a membranous cuticular residue was only obtained for *A. americana* and *C. miniata* leaves. A weakened absorbance of functional groups associated with ester bonds was detected for this cuticular membrane residue. Besides aliphatic moieties, which were predominantly present, aromatic moieties were cross-linked within this biopolymer via ester and possibly ether bonds (McKinney *et al.*, 1996; Schouten *et al.*, 1998; Villena *et al.*, 1999; Boom *et al.*, 2005).

Compositional analysis of cuticular waxes and cutin matrix of *Agave americana*

Previous studies using different extraction procedures and methods for detection and sampling of various leaf sections showed that the chemical composition of cuticular waxes and the cutin matrix of *A. americana* was purely aliphatic. The cuticular wax load was quantified, ranging from 10 to 115 $\mu\text{g cm}^{-2}$, and the cutin deposition ranged from 85 to 210 $\mu\text{g cm}^{-2}$ (Holloway and Baker, 1970; Guerrero *et al.*, 2006). A study by Holloway and Baker (1970) identifying alkanolic acids, primary alkanols, *n*-alkanals, *n*-alkanes and secondary alkanols as cuticular wax compound classes of *A. americana* leaves corroborates our

results, though alkane- α,ω -diols and alkyl esters were not identified by these authors. Our detailed analysis showed that homologous series of very-long-chain alkanolic acids and primary alkanols were the major wax compound classes of *A. americana* leaves, dominated by compounds with a carbon chain length of 32.

Gravimetrically, the cuticular wax amount was estimated at $181 \mu\text{g cm}^{-2}$ by subtracting the cuticle weight after solvent extraction from the cuticle weight without extraction. In comparison, a gas chromatographic analysis of the cuticular waxes yielded a cuticular wax coverage of only $45 \mu\text{g cm}^{-2}$. However, supplementing the solvent extraction with an acid-catalysed transesterification prior to gas chromatographic analysis, the cuticular waxes accounted for $159 \mu\text{g cm}^{-2}$, indicating a major contribution of complex, high-molecular-weight esterified cuticular wax compounds. These compounds escape the gas chromatographic window and are not reported in the literature comparing predominantly cuticular wax loads only after solvent extraction.

The cutin matrix of *A. americana* leaves represents 9,10-epoxy ω -hydroxy and 9,10, ω -trihydroxy C_{18} alkanolic acids as principal monomeric compounds. Similar to previous studies, alkanolic acids, ω -hydroxy alkanolic acids, 9/10-oxo ω -hydroxy alkanolic acids and 9/10, ω -dihydroxy alkanolic acids were also shown to be present in the cutin matrix as C_{16} and C_{18} cutin monomers (Matic, 1956; Holloway and Baker, 1970; Espelie et al., 1982; Holloway, 1982a). The presence of homologous series of very-long-chain alkanolic acids, ω -hydroxy alkanolic acids, α,ω -dicarboxylic acids and primary alkanols in the cutin fraction of *A. americana* leaves potentially indicates that a small but significant amount of cuticular waxes is embedded in the cuticular matrix, which was not removed by the procedure followed to extract the cuticular waxes.

An examination of fully expanded leaves along a gradient from the leaf apex to the leaf base showed that the cuticle weight was twice as much at the leaf base in comparison with the leaf apex. Strengthening these findings, the cuticular wax and cutin deposition was detected to be 2-fold higher at the leaf base of *A. americana* compared with the leaf apex. Differences in the cuticular lipid composition were not detected between these leaf sections. Analysing selected sections of young leaves of *A. americana* at the apex, the middle and the base, Guerrero et al. (2006) found a decline of >80 %, from 115 to $20 \mu\text{g cm}^{-2}$, for cuticular waxes and of ~60 %, from 205 to $85 \mu\text{g cm}^{-2}$, for the cutin matrix. This contrasting distribution of cuticular components probably indicates tissue-specificities or developmental differences in *A. americana* leaves or consequences of different extraction procedures.

Comparative analysis of the cuticular waxes and the cutin matrix of *Agave americana*, *Clivia miniata*, *Ficus elastica* and *Prunus laurocerasus*

Quantitative analyses of cuticular waxes and cutin sometimes reveal high divergences, mostly due to different extraction, gravimetric and chromatographic methods. Based on gravimetric analyses, previous studies documented that the cuticular wax proportion of the leaf cuticle of *A. americana*,

C. miniata, *F. elastica* and *P. laurocerasus* was between 5 and 25 % (Mérida et al., 1981; Gouret et al., 1993; Schreiber and Riederer, 1996; Khanal et al., 2013; Guzmán-Delgado et al., 2016). Previous studies also specified the cuticular leaf wax coverage in *A. americana* as between 10 and $115 \mu\text{g cm}^{-2}$, in *C. miniata* as $113 \mu\text{g cm}^{-2}$, in *F. elastica* as $87 \mu\text{g cm}^{-2}$ and in *P. laurocerasus* from 28 to $83 \mu\text{g cm}^{-2}$ (Holloway and Baker, 1970; Schreiber and Riederer, 1996; Jetter et al., 2000; Guerrero et al., 2006; Zeisler and Schreiber, 2016).

The cuticular wax coverage of *A. americana*, *C. miniata*, *F. elastica* and *P. laurocerasus*, determined in our study by gas chromatographic analysis, ranged from 45 to $96 \mu\text{g cm}^{-2}$, not taking into account the cuticular waxes potentially present in the cutin fraction. It is noteworthy that the very-long-chain aliphatic wax fraction was from 24 to $55 \mu\text{g cm}^{-2}$ in these four plant species, with a different compound class preference and/or carbon chain length distribution. Considering the aliphatic wax compounds, two biosynthetic pathways can be distinguished: an alkanol-forming and an alkane/alkanal-forming pathway (Joubès and Domergue, 2018). The cuticular waxes of *A. americana* and *C. miniata* possessed aliphatic wax compounds, which preferentially originated from the alkanol-forming pathway of cuticular wax biosynthesis (Fagerström et al., 2013). On the other hand, the cuticular waxes of *F. elastica* and *P. laurocerasus* contained aliphatic compounds mainly biosynthesized via the alkane/alkanal-forming pathway and, additionally, pentacyclic triterpenoids in substantial quantities (Jetter et al., 2000; Guzmán-Delgado et al., 2016; Zeisler and Schreiber, 2016). Pentacyclic triterpenoids are generated via cyclization of 2,3-oxidosqualene, ring expansions and skeletal rearrangements of carbocation species (Thimmappa et al., 2014).

The cutin biopolyester of leaves varies in amount and composition. Gravimetrically, the cutin proportion accounted for 43–76 % of the leaf cuticle of *A. americana*, *C. miniata*, *F. elastica* and *P. laurocerasus* as reported by Holloway and Baker (1970), Mérida et al. (1981), Gouret et al. (1993) and Guzmán-Delgado et al. (2016). Previous analyses substantiated the cutin deposition on leaves as between 85 and $205 \mu\text{g cm}^{-2}$ for *A. americana*, up to $200 \mu\text{g cm}^{-2}$ for *C. miniata* and $199 \mu\text{g cm}^{-2}$ for *P. laurocerasus* (Schmidt and Schönherr, 1982; Graça et al., 2002; Guerrero et al., 2006). The cutin quantity of *A. americana*, *C. miniata*, *F. elastica* and *P. laurocerasus*, obtained in this study by gas chromatographic analysis, ranged between 142 and $211 \mu\text{g cm}^{-2}$, but is potentially overestimated because of the non-extracted cuticular waxes embedded in the cuticular matrix.

The polymeric cutin matrix can be broadly characterized according to the carbon chain length of the major aliphatic monomers. The leaf cutin of *C. miniata*, *F. elastica* and *P. laurocerasus* possessed both C_{16} and C_{18} hydroxy alkanolic acids in large amounts. In leaves of *C. miniata*, *F. elastica* and *P. laurocerasus*, 9/10, ω -dihydroxy C_{16} alkanolic acid, 9,10-epoxy ω -hydroxy C_{18} alkanolic acid and 9,10, ω -trihydroxy C_{18} alkanolic acid clearly dominated the cutin composition. In contrast, *A. americana* leaves showed high amounts of 9,10-epoxy ω -hydroxy C_{18} alkanolic acid and 9,10, ω -trihydroxy C_{18} alkanolic acid (Holloway, 1982a).

Furthermore, very-long-chain aliphatic compounds up to a carbon chain length of 38 were identified in the cutin fraction

but at a considerably lower level than the C_{16} and C_{18} cutin monomers. These very-long-chain alkanolic acids, ω -hydroxy alkanolic acids, α,ω -dicarboxylic acids or primary alkanols exhibited a species-specific carbon chain length distribution similar to the cuticular waxes of *A. americana*, *C. miniata*, *F. elastica* and *P. laurocerasus*, respectively. Likewise, the very-long-chain aliphatic compounds had a predominant carbon chain length of 30, 32 or 34. Small amounts of aromatic coumaric acid and derivatives produced via the phenylpropanoid biosynthetic pathway were detected only in the leaf cutin of *F. elastica* and *P. laurocerasus*, probably interacting in the cutin monomer cross-linking.

Compositional analysis of cutan: differentiation between cutan I and cutan II

A number of different methodological approaches have been applied to the non-extractable, non-hydrolysable cutan to analyse its monomeric composition, and different cutan polymeric structures were tentatively suggested based on this composition (McKinney *et al.*, 1996; Schouten *et al.*, 1998; Sachleben *et al.*, 2004; Boom *et al.*, 2005; Deshmukh *et al.*, 2005). Based on gravimetric analyses, the proportion of this cuticular component was determined to be between 13 and 56 % of the leaf cuticle of *A. americana*, *C. miniata*, *F. elastica* and *P. laurocerasus* (Gouret *et al.*, 1993; Villena *et al.*, 1999; Guerrero *et al.*, 2006; Guzmán-Delgado *et al.*, 2016).

In our study, solvent extraction and acid-catalysed transesterification of the leaf cuticle of *A. americana*, *C. miniata*, *F. elastica* and *P. laurocerasus* resulted in a cuticular membrane residue, cutan I. After additional solvent extraction, base hydrolysis, acidification and extraction of cutan I, no cuticular membrane residue was obtained for *F. elastica* and *P. laurocerasus*, whereas on the contrary *A. americana* and *C. miniata* did yield the operationally defined cutan II. The fact that Gouret *et al.* (1993) and Guzmán-Delgado *et al.* (2016) obtained a non-extractable, non-hydrolysable residue of the cuticular membrane of *F. elastica* and *P. laurocerasus* leaves might be due to different approaches. Acid hydrolysis with 6 N hydrogen chloride at 180 °C for 18 h and base-catalysed transmethylation with 0.1 M sodium methoxide in methanol at 80 °C for 3 h, respectively, may result in the production of insoluble artefacts under these harsh conditions (Poirier *et al.*, 2003). Alternatively, it cannot be excluded that the successive sequence of solvent extraction, acid-catalysed transesterification, solvent extraction, base hydrolysis, acidification and extraction is required to obtain a proper cutan residue.

Even cutan II of *A. americana* and *C. miniata* was not completely devoid of cuticular waxes and/or cutin monomers. Most likely, extraction procedures following acid-catalysed cutin transesterification were hampered to some extent due to the hydrophobic nature of the cutan matrix, which protects the cuticular waxes from being completely extracted on the one hand and limits the accessibility of the polar boron trifluoride in methanol to fully depolymerize the cutin matrix by acid-catalysed transesterification on the other hand. Although the modified approach used by Schmidt and Schönherr (1982) did not remove all cuticular waxes and cutin monomers from the

cuticular membrane, their approach resembles that of the more drastic procedure used by Nip *et al.* (1986a, b), suggesting that the operationally defined non-ester cutin is probably compositionally very similar to cutan.

Both solvent and thermal extraction of *A. americana* cutan I yielded methylated C_{14} , C_{16} , C_{18} , C_{28} , C_{30} , C_{32} and C_{34} alkanolic acids and, additionally, C_{30} and C_{32} primary alkanols and C_{29} and C_{31} *n*-alkanes. In particular, the presence of the latter compounds in cutan I potentially indicates that a certain portion of the cuticular waxes was still present. Upon THM of *A. americana* cutan I, methylated alkanolic acids and ω -hydroxy alkanolic acids as well as primary alkanols and alkane- α,ω -diols were identified. Their carbon chain length distribution, dominated by 28, 30, 32 and 34, was relatively similar to those in the cuticular waxes. Moderate quantities of ω -hydroxy C_{16} and C_{18} alkanolic acid derivatives in cutan I indicated also that acid-catalysed transesterification was insufficient to release all cutin monomers. These results further confirm the incompleteness of the cuticular wax extraction and acid-catalysed cutin transesterification.

The thermal extraction of *A. americana* cutan II showed only trace amounts of compounds, such as methylated alkanolic acids, that were significantly lower when compared with those of cutan I. The thermal extracts of *A. americana* cutan II comprised predominantly *n*-alk-1-enes and *n*-alkanes, implying some low-temperature pyrolysis. The presence of homologous series of *n*-alka- α,ω -dienes, *n*-alk-1-enes and *n*-alkanes in the range between C_7/C_8 and C_{35} , as found in *A. americana* cutan II, was typical of the flash pyrolysis products of cutan (Nip *et al.*, 1986a; Boom *et al.*, 2005). In addition, Tegelaar *et al.* (1989) and Boom *et al.* (2005) also encountered the homologous series of *n*-alkan-2-ones in pyrolysis products of *A. americana* cutan in a similar distribution. However, Tegelaar *et al.* (1989) did not detect these *n*-alkan-2-ones after acid hydrolysis and therefore suggested that these compounds are possibly derived from ether-linked aliphatic moieties by flash pyrolysis.

Even the cutan II of *A. americana* was not completely devoid of cutin monomers. The methylated alkanolic acids identified by thermal extraction and flash pyrolysis were not documented earlier, and likely reflect residual alkanolic acids produced during acid-catalysed transesterification. Similar to McKinney *et al.* (1996), the predominating compounds detected by THM included even-over-odd dominated alkanolic acids for *A. americana* cutan II. Traces of 9/10, ω -dihydroxy C_{16} alkanolic acid and 9/10, ω -trihydroxy C_{18} alkanolic acid represent characteristic cutin leftovers in *A. americana*. The 9/10, ω -trihydroxy C_{18} alkanolic acid identified in its methylated version represented also 9/10-epoxy ω -hydroxy C_{18} alkanolic acid as these epoxy groups were hydrolysed and methylated by THM (Del Río and Hatcher, 1998). In contrast to McKinney *et al.* (1996), homologous series of ω -hydroxy alkanolic acids, primary alkanols and alkane- α,ω -diols were identified in the cutan II of *A. americana*. These compounds might have been overlooked by McKinney *et al.* (1996) due to co-elution of the primary alkanols with the alkanolic acids, whereas the ω -hydroxy alkanolic acids and alkane- α,ω -diols were only identified here. The methylated primary alkanols of cutan II were very similarly distributed to those released by THM of cutan I, suggesting that these compounds were non-hydrolysable and possibly cross-linked via ether bonds.

In summary, the cutan II of *A. americana*, as cutan proper, is a biopolymer, which mainly consists of very-long-chain aliphatic compounds in which C₃₂ monomers predominate. The very-long-chain aliphatic moieties bearing one or two functional groups are likely ester- and possibly also ether-linked to the aromatic benzene-1,3,5-triol derivatives and to each other, as proposed in previous studies (McKinney *et al.*, 1996; Schouten *et al.*, 1998; Villena *et al.*, 1999; Deshmukh *et al.*, 2005). Furthermore, these very-long-chain aliphatic moieties apparently prevent to some extent ester bonds from being cleaved by acid or base hydrolysis, which also explains why both solvent extraction and acid-catalysed transesterification limited the complete removal of cuticular wax and cutin compounds. The close resemblance of the very-long-chain lipid distribution of the cuticular wax compounds and the ones released by THM strongly suggests a biosynthetic wax origin of the non-extractable, non-hydrolysable cutan. Hence, cutan precursors of *A. americana* are initially subjected to an elongation process before further functionalization and cross-linking by ester and possibly ether bonds to produce the cutan biopolymer.

For the cutan of *C. miniata*, the picture was less clear. Upon THM at 350 °C, *C. miniata* cutan I released mainly C₁₄, C₁₆ and C₁₈ alkanolic acids as well as methylated ω-hydroxy C_{18:1} alkanolic acid, 9/10,ω-dihydroxy C₁₆ alkanolic acid and 9/10,ω-trihydroxy C₁₈ alkanolic acid, of which the latter in particular are characteristic cutin monomers of *C. miniata*. These findings are in line with results obtained for *A. americana* cutan I indicating that the removal of cutin monomers from the cuticular matrix was not complete.

Due to pyrolysis, concurrent with THM, a mixture of pyrolysis and THM products was generated at temperatures of 510 and 600 °C, whereas at 350 °C almost no products were generated. *n*-Alka-α,ω-dienes, *n*-alk-1-enes and *n*-alkanes ranging between C₈ and C₃₅ were THM products at temperatures of 510 and 600 °C, implying that the cutan II of *C. miniata* was probably less accessible to tetramethylammonium hydroxide. Together, these results suggest that *C. miniata* cutan II has a structure that remains stable at a higher temperature than that of *A. americana*. Apart from this, and looking at the THM products only, a picture similar to that found for *A. americana* is seen for the alkanolic acids and ω-hydroxy alkanolic acids. Primary alkanols were less abundantly present, which might be due to the weaker reactivity of tetramethylammonium hydroxide in the presence of *C. miniata* cutan II, the pyrolysis of which takes more energy, leaving less energy for THM. Consequently, an underestimation of methylated hydroxyl groups as well as aromatic moieties relative to easier-to-methylate carboxyl groups resulted in a picture dominated by alkanolic acid methyl esters.

Conclusions

Different analytical approaches were applied to investigate cuticular waxes, cutin monomers and cutan monomers of evergreen perennials: *A. americana*, *C. miniata*, *F. elastica* and *P. laurocerasus*. Contrary to what was previously assumed, cutan II as cutan proper was only encountered as a cuticular component in leaves of *A. americana* and *C. miniata*. Hence, non-extractable, non-hydrolysable cutan is possibly present to a lesser extent in plant species than suggested in the literature

due to less appropriate extraction procedures, which mainly result in cutan I, which besides cutan proper contains leftovers of cuticular waxes and cutin, in contrast to cutan II, when present. Very-long-chain cuticular wax compounds and cutan II monomers showed a similar carbon chain length distribution. Therefore, the origin of the aliphatic building blocks of cutan II is probably related to the biosynthesis of the aliphatic wax compounds and involves the elongation and conversion of C₁₆ and C₁₈ precursors to very-long-chain cutan monomers with different functional groups. Minor proportions of benzene-1,3,5-triol and derivatives suggested that these aromatic moieties form the polymeric core of the cutan matrix, to which the aliphatic moieties are linked. The cuticular composition of *A. americana* and *C. miniata* leaves might be responsible for a specific cuticle ultrastructure, possibly representing adaptations of the cuticular barrier properties to environmental conditions.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. **Figure S1**: photographs of the adaxial leaf side of *Agave americana* and *Clivia miniata*. **Figure S2**: cuticular wax composition of the adaxial leaf surface of *Agave americana* after solvent extraction and acid-catalysed transesterification. **Figure S3**: investigation of the *Clivia miniata* adaxial leaf surface along a gradient from the leaf apex to the leaf base. **Figure S4**: gas chromatogram of cutan I of the adaxial leaf surface of *Clivia miniata*. **Figure S5**: (partial) gas chromatograms of cutan I and cutan II of the adaxial leaf surface of *Clivia miniata*. **Figure S6**: photographs of the cuticular membrane of *Agave americana*, *Clivia miniata*, *Ficus elastica* and *Prunus laurocerasus* leaves.

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