

Chapter 2

Distribution of Cutan in Modern Leaves

Abstract Cutan, a resistant non hydrolysable aliphatic biopolymer, was first reported in the cuticle of *Agave americana* and has generally been considered ubiquitous in leaf cuticles along with the structural biopolyester cutin. Because leaves and cuticles in the fossil record almost always have an aliphatic composition, it was argued that selective preservation of cutan played an important role in leaf preservation. However, the analysis of leaves using chemical degradation techniques involving hydrolysis to test for the presence of cutan reveals that it is absent in 16 of 19 taxa (angiosperm and gymnosperm), including many previously reported to contain cutan on the basis of pyrolysis data. Cutan is clearly much less widespread in leaves than previously thought and its presence or absence does not exert any major bias on the preservation of leaves in the fossil record. In the absence of cutan, other constituents—cutin, plant waxes and internal plant lipids—are incorporated into the geomacromolecule and contribute to the formation of a resistant fossil geopolymer.

Keywords Aliphatic biopolymers • Algaenan • Aliphatic • Cuticle

Introduction

Leaf fossils (including both complete leaves and their isolated cuticles) are widely used in applied paleobotany. Jones and Rowe (1999) provide a useful summary of many examples. Applications include paleobiodiversity analysis (Burnham 1993), paleoclimate analysis to determine both temperature and precipitation (Wolfe 1995; Wilf 1997; Wiemann et al. 1998; Jacobs 2002; Kowalski 2002; Kowalski and Dilcher 2003; Sun et al. 2003; Wilf et al. 2003; Liang et al. 2003; Glasspool et al. 2004), understanding plant paleoecology (Burnham et al. 1999; Royer et al. 2005) and vegetational history (Mai 1995; Collinson and Hooker 2003; Hill 2004), documenting insect/plant interaction (Glasspool et al. 2003), determining vegetation response to key global change events (Wing et al. 2005) and reconstructing

paleoatmospheric carbon dioxide levels (Royer et al. 2001). Underpinning all of this research is the assumption that either (i) the fossil record of leaves is representative of ancient plants and vegetation or (ii) the biases in the leaf fossil record are understood. Some aspects of taphonomic bias that result from transport processes or growth environment are partly understood and can be taken into account through a detailed understanding of facies associations. For example, high proportions of stenophylls and non-entire margined leaves at streamsides may yield an anomalously cool climate signal compared to the surrounding vegetation (Liang et al. 2003).

The outer cuticle of leaves is made up of the structural biopolymer cutin (primarily C₁₆ and C₁₈ hydroxy fatty acids; Kolattukudy 1980), which is hydrolysable under basic conditions, surface waxes (soluble in organic solvents) and, in some cases, the resistant non hydrolysable aliphatic biopolymer cutan (Nip et al. 1986a, b). The outer cuticle protects the internal tissues composed of more labile biopolymers—polysaccharides, lignin, and proteins. Chemical analyses of fossil leaves and cuticles have shown that although relatively ‘younger’ fossil material may preserve carbohydrates and lignin (Briggs et al. 2000, and references therein) fossil leaves and cuticles older than the Tertiary very often yield a dominant aliphatic signal (Nip et al. 1986a, b; Tegelaar et al. 1991; Logan et al. 1993; van Bergen et al. 1994; Möhle et al. 1997, 1998; Collinson et al. 1998; Stankiewicz et al. 1998b; Gupta et al. 2007a, b).

Tegelaar et al. (1991) argued that the leaf fossil record is biased in favor of leaves containing the highly resistant, highly aliphatic, non-hydrolysable macromolecule cutan in their cuticles. Any bias due to the presence or absence of cutan must be understood if data from fossil leaves are to be applied reliably in multidisciplinary research.

Cutan is a long chain aliphatic biopolymer resistant to hydrolysis (i.e., an aliphatic residue is recovered after base and acid hydrolysis: Nip et al. 1986a, b; Tegelaar et al. 1989c; McKinney et al. 1996; Mosle et al. 1997, 1998; Schouten et al. 1998) and was therefore interpreted as a diagenetically stable polymer that can survive in the fossil record with little chemical change (Nip et al. 1986a, b; Tegelaar et al. 1989a). Its diagenetic stability is due to largely non-hydrolysable ether linkages and sterically protected ester functional groups (for structural details refer to McKinney et al. 1996; Schouten et al. 1998). Cutan was first described and documented by Nip et al. (1986a, b) from the leaf cuticle of modern *Agave americana*, a monocotyledon flowering plant. Nip et al. (1986a) reported that the leaf cuticle of another monocotyledon, *Clivia miniata*, gave similar results to *Agave* and also contained cutan. Tegelaar et al. (1991) reported a characteristic cutan signature (an aliphatic signal of an extended homologous series of *n*-alkanes, *n*-alk-1-enes and *n*-alkadienes) in chromatograms generated by Curie-point pyrolysis of isolated complete cuticles of 11 of 13 modern plants analysed. However, this result was based on analysis using solely pyrolysis GC-MS, and did not involve isolating the cutan biopolymer from the modern plants using hydrolytic techniques. Tegelaar et al. (1991) equated the aliphatic signal with the material defined as non-ester cutin in maturing *Clivia miniata* cuticles by Schmidt and Schönherr (1982) and interpreted it as cutan. The apparently widespread occurrence of an aliphatic signal in

modern plants, combined with the dominantly aliphatic composition of fossil leaves and cuticles, led Tegelaar et al. (1991) to infer that selective preservation of cutan accounts for the preservation of fossil leaves, thus biasing the fossil record in favor of leaves containing the biopolymer cutan.

Mösle et al. (1997, 1998) and Collinson et al. (1998) sought to demonstrate the presence or absence of cutan on the basis that it should be recoverable as a residue after acid and base hydrolysis as initially documented by Nip et al. (1986b). Collinson et al. (1998) analysed leaf cuticles of modern *Ginkgo biloba* (previously thought to contain cutan: Nip et al. 1986b; Tegelaar et al. 1991), as well as a wide variety of modern conifers (Collinson et al. 1998), but were unable to detect cutan in the living plant cuticles despite the presence of aliphatic components in their fossil equivalents. However, Boom et al. (2005) suggested that the use of oxidative conditions (hydrogen peroxide and acetic acid) to isolate the *Ginkgo* and conifer cuticles from the leaves could have broken down the cutan.

In order to investigate the importance of cutan in the taphonomy of plants, we tested for its presence (as a highly aliphatic polymer resistant to base and acid hydrolysis) in a variety of modern leaves (Table 2.1). Whole leaves were used as the starting point for the analyses to avoid any possibility of altering the cuticles by chemical isolation. The analyses included genera with an extensive leaf fossil record (e.g. *Acer*, *Quercus*), and with minimal or no known fossil record (e.g. *Gossypium*). Other taxa were included in the analyses to enable comparisons with previous work (e.g., Tegelaar et al. 1991): the monocotyledonous flowering plants *Agave americana* and *Clivia miniata* previously reported to contain cutan (including the ‘type’ example of cutan in *Agave*, the taxon in which it was originally defined), *Ginkgo* and the conifers *Metasequoia*, *Sciadopitys*, *Abies* and *Pinus*, previously reported to lack cutan.

Samples and Preparation

Samples of modern leaves (indicated on Table 2.1) were collected fresh from the University of Bristol and University of Nancy botanic gardens during November 2002 and April 2003, respectively. The selected leaves were well developed and mature. These gardens are situated several miles from the town center and hence the chemistry of the leaves is unlikely to be affected by urban pollution. The experimental protocol used for detecting the presence of cutan is outlined in Fig. 2.1. The modern leaves were crushed in liquid nitrogen using a mortar and pestle. They were transferred to glass vials and subjected to rigorous solvent extraction at room temperature for 8 h by adding 2:1-CH₂Cl₂ (dichloromethane): CH₃OH (methanol) in an ultrasonic bath, in order to remove the soluble lipids. The lipid extract was retained for later analysis. The insoluble residue (Residue 1) was dried in a flow of N₂ and subjected to base hydrolysis (saponification) to remove hydrolysable constituents, e.g. the biopolyester cutin. This involved preparing a solution of 1 M methanolic NaOH solution (in 95:5 v/v methanol: water) by dissolving two pellets (0.2 g each)

Table 2.1 Modern leaves and cuticles studied using chemical degradation techniques which reveal the presence (+) or absence (–) of cutan

Family	Genus	Species	Cutan
Gymnosperms			
Araucariaceae ^c	<i>Araucaria</i>		–
Cupressaceae ^c	<i>Cupressus</i>		–
Ginkgoaceae ^{a,d}	<i>Ginkgo</i>	<i>biloba</i>	–
Pinaceae ^a	<i>Abies</i>	<i>grandis</i>	–
Pinaceae ^c	<i>Abies</i>		–
Pinaceae ^c	<i>Cedrus</i>		–
Pinaceae ^c	<i>Picea</i>		–
Pinaceae ^a	<i>Pinus</i>	<i>sylvestris</i>	–
Pinaceae ^c	<i>Pinus</i>		–
Podocarpaceae ^b	<i>Podocarpus</i>		+
Podocarpaceae ^c	<i>Podocarpus</i>		–
Sciadopityaceae ^b	<i>Sciadopitys</i>	<i>verticillata</i>	–
Sciadopityaceae ^c	<i>Sciadopitys</i>		–
Taxodiaceae ^c	<i>Athrotaxis</i>		–
Taxodiaceae ^c	<i>Cunninghamia</i>		–
Taxodiaceae ^c	<i>Glyptostrobus</i>		–
Taxodiaceae ^c	<i>Metasequoia</i>		–
Taxodiaceae ^c	<i>Sequoia</i>		–
Taxodiaceae ^c	<i>Sequoiadendron</i>		–
Taxodiaceae ^c	<i>Taxodium</i>		–
Flowering plants			
Aceraceae ^a	<i>Acer</i>	<i>campestre</i>	–
Agavaceae ^{a,b,d,e}	<i>Agave</i>	<i>americana</i>	+
Betulaceae ^a	<i>Betula</i>	<i>alba</i>	–
Cactaceae ^b	<i>Cereus</i>		+
Clethraceae ^b	<i>Clethra</i>		–
Clusiaceae ^b	<i>Clusia</i>	<i>multiflora</i>	+
Clusiaceae ^b	<i>Clusia</i>	<i>rosea</i>	+
Ericaceae ^a	<i>Erica</i>	<i>herbaceae</i>	–
Fagaceae ^a	<i>Castanea</i>	<i>sativa</i>	–
Fagaceae ^a	<i>Quercus</i>	<i>robur</i>	–
Liliaceae ^{a,e}	<i>Clivia</i>	<i>miniata</i>	+
Malvaceae ^a	<i>Gossypium</i>	<i>hirsutum</i>	–
Myrsinaceae ^b	<i>Myrsine</i>	<i>guyanense</i>	–
Orchidaceae ^b	Unidentified epiphyte		+
Poaceae ^b	Unidentified		–
Rosaceae ^a	<i>Prunus</i>	<i>laurocerasus</i>	+ ^g
Rutaceae ^a	<i>Citrus</i>	<i>limon</i>	–
Salicaceae ^a	<i>Populus</i>	<i>hybrida</i>	–
Solanaceae ^{a,f}	<i>Lycopersicon</i>	<i>esculentum</i>	–
Vitaceae ^a	<i>Vitis</i>	<i>vinifera</i>	–

^aAnalysis of whole leaves, this investigation

^bEnzymatically isolated cuticles (Boom et al. 2005)

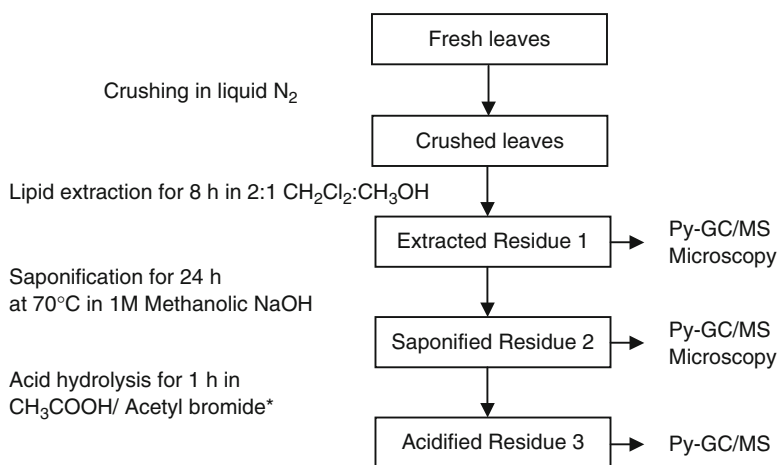
^cChemically isolated cuticle (Collinson et al. 1998)

^dChemically isolated cuticle (Mösle et al. 1998)

^eOther references: see text

^fFruit cuticle

^gResistance to acid hydrolysis not tested



*Applied to *Agave*, *Pinus*, and *Acer* to test the acid resistance of constituent biopolymers

Fig. 2.1 Analytical protocol used to detect the presence of cutan in leaves

of NaOH in 0.5 ml double distilled water and 9.5 ml methanol. This solution was added to Residue 1, which was then refluxed at 70 °C for 24 h in a reactitherm (Mösle et al. 1997). Thus, the resultant residue (Residue 2) is devoid of constituents that can be hydrolysed under basic conditions (e.g. cutin). Residue 2 from *Agave americana*, *Pinus sylvestris*, and *Acer campestre* was further subjected to acid hydrolysis (Mösle et al. 1997) as a control to test the acid resistance of *Agave*, one gymnosperm and one angiosperm, as cutan by definition should be resistant to acid treatment as well. The resultant residue (Residue 3) is devoid of constituents hydrolysable under basic and acidic conditions (e.g. lignin, cutan) and hence is resistant to both base and acid hydrolysis.

Untreated leaf, Residue 1 and Residue 2 of modern *Quercus robur* and *Pinus sylvestris* were studied using transmission electron microscopy (as described in Collinson et al. 1998) to determine the effects of base hydrolysis (saponification) on the leaf cuticle.

Flash pyrolysis-GC-MS was conducted on the residues of the extant leaves after lipid extraction (Residue 1), and after base hydrolysis (Residue 2) and, where present, after acid hydrolysis (Residue 3). Flash pyrolysis involves the thermal fragmentation of the chemical constituents of the sample at high temperatures in an inert gas stream. These fragments are then separated and identified by gas chromatography-mass spectrometry. Flash pyrolysis reveals bulk macromolecular information and it has been used extensively in the molecular characterisation of both modern and fossil plant tissues (see van Bergen 1999 for review). Samples were analysed with a Perkin Elmer GC/MS. A CDS (Chemical Data System) AS-2500 Pyroprobe pyrolysis unit was used with both the injector and interface temperature at 290 °C. 100–150 µg of tissue sample was introduced into quartz tubes and pyrolysed at 610 °C.

Pyrolysis products were separated using a DB-1 fused silica capillary column (30 m, 0.25 mm i.d., 0.1 μm film thickness) to evaluate the distribution of pyrolysis products of leaf tissue (Gupta and Pancost 2004), especially the *n*-alkyl component (for greater insight into the polar (non-aliphatic) compounds see Ralph and Hatfield 1991). The GC oven was programmed from 40 (held for 4 min) to 320 $^{\circ}\text{C}$ at 5 $^{\circ}\text{C min}^{-1}$ and held at that temperature for 15 min. Helium was the carrier gas. The MS was operated at 70 eV scanning over the range m/z 45–600 at 1 scan s^{-1} with an emission current of 300 μA (full scan mode). Two to three replicate samples were analysed to check the consistency of the runs. Compounds were identified using the NIST mass spectral library and from published spectra (Ralph and Hatfield 1991; Bland et al. 1998).

Analytical Results

The pyrolysis-GC/MS profile of *Agave americana* after solvent extraction (Residue 1) revealed a dominance of *n*-alkane and *n*-alk-1-ene homologues ranging in carbon number from C_8 to C_{35} and maximising at C_{28} (Fig. 2.2a), indicating the presence of a dominant *n*-alkyl component, which is characteristic of *Agave americana* (Tegelaar et al. 1991). Phenols and polysaccharide pyrolysis products are also present. Fatty acyl components were detected in relatively low abundance, as the *Agave* cuticle tissue was sampled from a fully developed, mature part of the plant where the ratio between cutin and cutan is relatively low; the proportion of cutin is greater in immature parts of the plant (Tegelaar et al. 1991). The pyrolysis-GC/MS profiles of Residue 2 (after saponification/base hydrolysis, Fig. 2.2b) and Residue 3 (after acid and base hydrolysis, Fig. 2.2c) show the persistence of *n*-alkane/alk-1-ene homologues including those with carbon chain length $>\text{C}_{20}$. These *n*-alkene/alkanes indicate the presence of cutan, consistent with results from a number of other studies (Nip et al. 1986a, b; Tegelaar et al. 1989c, 1991; McKinney et al. 1996; Möhle et al. 1997; Villena et al. 1999).

The pyrolysate of the angiosperm *Prunus laurocerasus* after extraction contains components related to polysaccharides, lignin and cutin, i.e., saturated and unsaturated C_{16} and C_{18} fatty acyl moieties (Fig. 2.3a; for details on the molecular structure and chemical formula of these compounds see Ralph and Hatfield 1991; van Bergen 1999). Also present is an *n*-alkyl component represented by *n*-alkane/alk-1-ene homologues ranging up to *n*- C_{32} ; *n*-alkane/alk-1-ene homologues from *n*- C_{26} to $_{31}$ are the most abundant. Inset Fig. 2.3a shows the distribution of the *n*-alkanes and *n*-alkenes separately to show more clearly the *n*-alkyl building components. As with *Agave*, the pyrolysate of Residue 2 from *Prunus laurocerasus* contains *n*-alkane/alk-1-ene homologues, including long chain homologues (Fig. 2.3b). The pyrolysate of post saponification Residue 2 of *Clivia miniata* also contains *n*-alkane/alk-1-ene homologues (data not shown) and a similar residue remains following acid hydrolysis (see also Villena et al. 1999).

In striking contrast, in all other leaves analysed, although *n*-alkane/alk-1-ene homologues are present in the pyrolysate of the extracted leaves (Residue 1), they

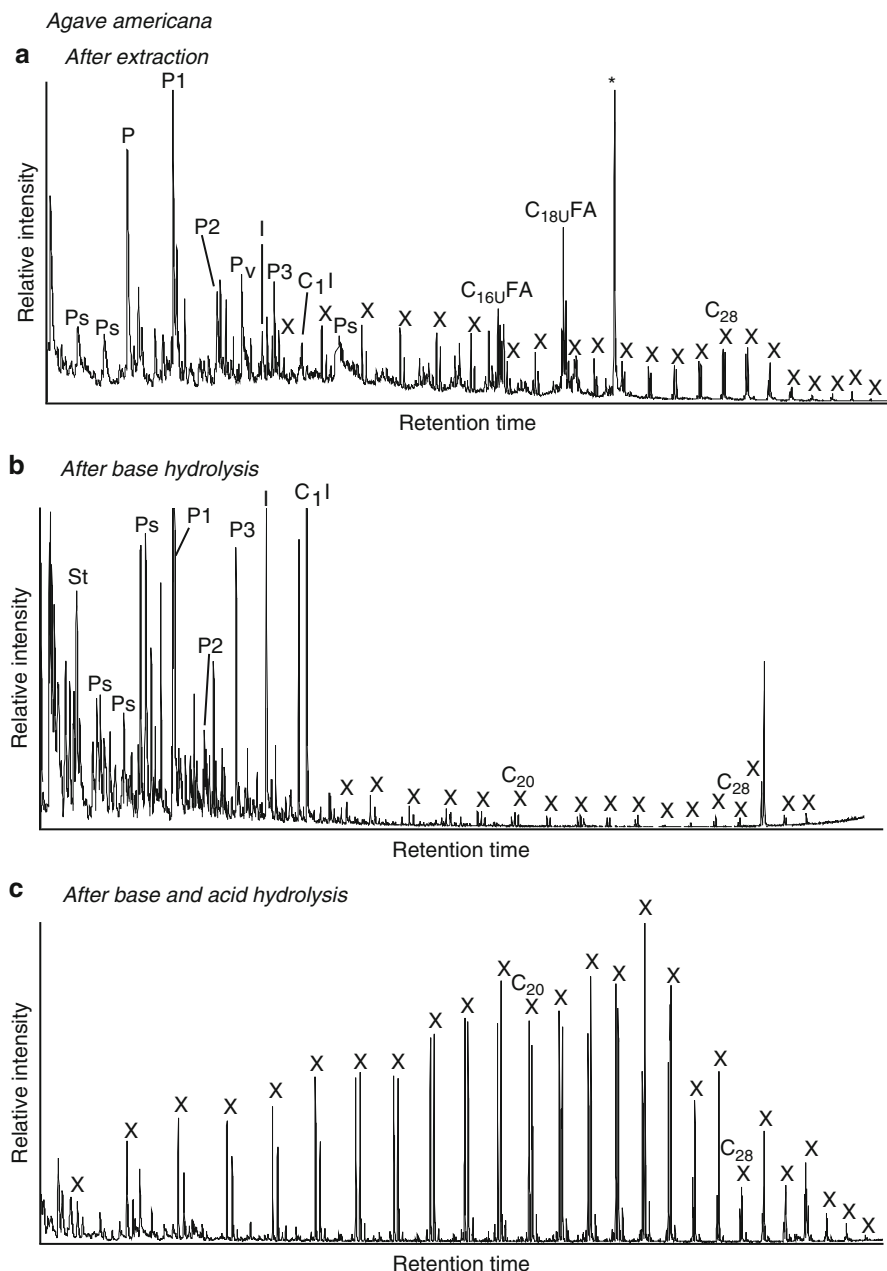


Fig. 2.2 Partial ion chromatogram showing py-GC/MS analyses of modern *Agave americana* cuticle and associated tissue **(a)** after lipid extraction (Residue 1); **(b)** after saponification (Residue 2); and **(c)** after saponification and acid hydrolysis (Residue 3). Note the presence of *n*-alkane/alk-1-ene homologues in all three fractions. Ps: polysaccharide pyrolysis products; P: phenol; Pn: alkyl phenols, where n denotes the number of carbon atoms in the alkyl component, Pv: vinyl phenol (derived from cutin), I: indole; C₁I: methyl indole; St: styrene; C₁₆UFA: C₁₆ unsaturated fatty acid, and C₁₈UFA: C₁₈ unsaturated fatty acid, X: *n*-alkane/alk-1-ene homologues (C_n refers to the carbon chain length). *contaminant. Peak at C₂₉*n*-alkane is exaggerated due to co-elution with a contaminant

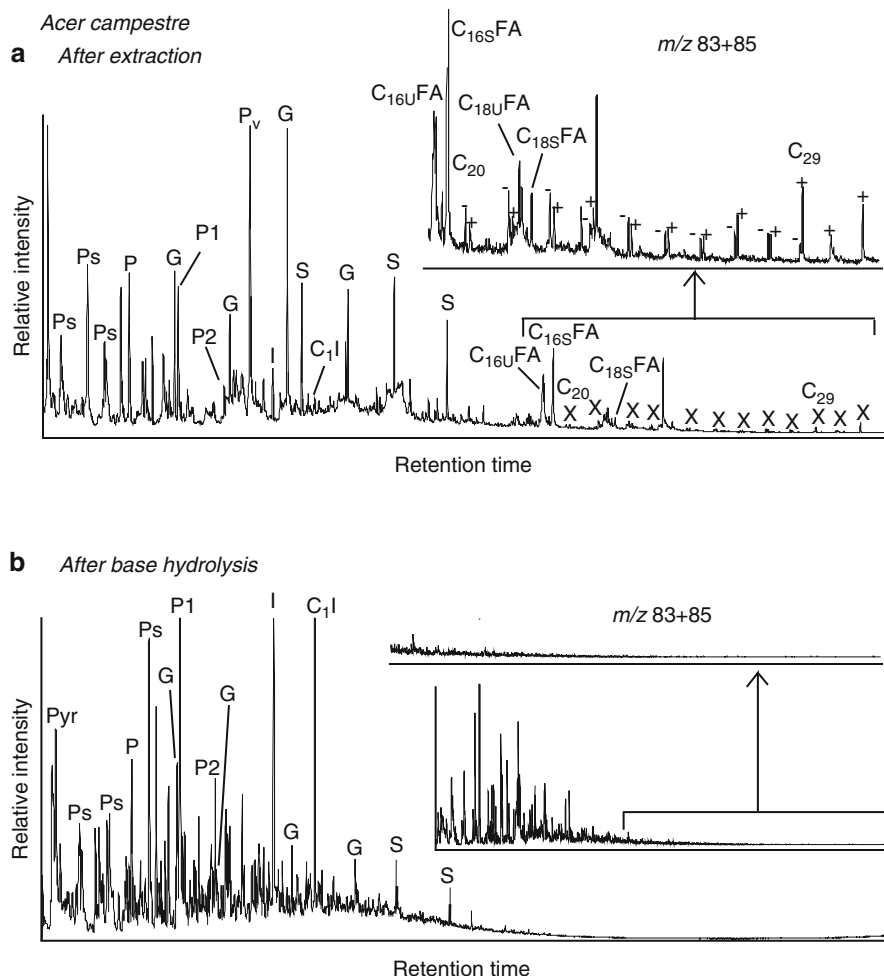


Fig. 2.4 Partial ion chromatogram showing the pyrolysis-GC/MS analysis of modern *Acer campestre* leaf (**a**) after lipid extraction (Residue 1); and (**b**) after lipid extraction followed by saponification (Residue 2). Note the presence of long-chain *n*-alkane/alk-1-ene homologues in trace amounts in the extracted plant tissue and its absence post saponification (as revealed by inset m/z 83+85 mass chromatograms). *Pyr* pyrrole derivative. Other legends same as in Figs. 2.2 and 2.3

in *Agave* or *Prunus*. The m/z 83+85 mass chromatogram (Fig. 2.4a inset) focuses on this homologous series of *n*-alkanes and *n*-alk-1-enes, which range in carbon number to C_{31} . The pyrolysate of Residue 2, following saponification, contain moieties related mainly to polysaccharides and lignin. Fatty acyl moieties and homologous series of *n*-alkanes and *n*-alk-1-enes are absent (Fig. 2.4b) in the pyrolysate. Saponification is expected to remove cutin, and the absence of fatty acyl moieties and vinyl phenol (Tegelaar et al. 1989b) in the pyrolysates of Residue 2 indicates that this has occurred.

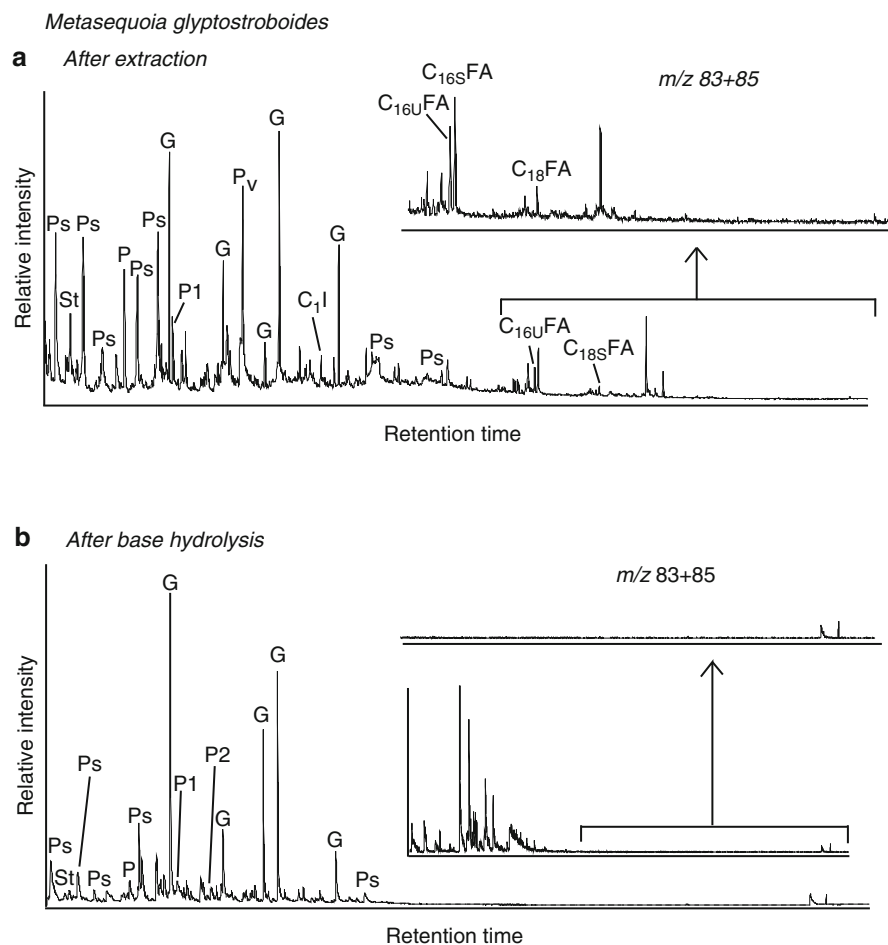


Fig. 2.5 Partial ion chromatogram showing the pyrolysis-GC/MS analysis of modern *Metasequoia glyptostroboides* leaf (**a**) after lipid extraction (Residue 1); and (**b**) after lipid extraction followed by saponification (Residue 2). Note the presence of long-chain *n*-alkane/alk-1-ene homologues in trace amounts in the extracted plant tissue and its absence post saponification (as revealed by inset m/z 83+85 mass chromatograms). Other legends same as in Figs. 2.2 and 2.3

Figure 2.5a shows the pyrolysis trace of the gymnosperm *Metasequoia glyptostroboides*. Lignin, polysaccharides and cutin moieties are abundant, whereas the *n*-alkanes and *n*-alk-1-enes are detected in extremely subordinate relative abundance (also see inset m/z 83+85 mass chromatogram; Yang et al. 2005, Fig. 3). The pyrolysate of Residue 2 post saponification (Fig. 2.5b) similarly contains polysaccharide and lignin moieties but no aliphatic component.

Data on all species investigated as part of this study are presented in Table 2.1. In all of these apart from *Agave*, *Prunus* and *Clivia* (see above), *n*-alkane/alk-1-ene homologues were present in the pyrolysate of Residue 1, albeit in low (but variable)

relative abundances that may be produced, for example, from secondary reactions in the pyroprobe or non extractable physically entrained waxes (e.g. wax esters; Sylvie Derenne, personal communication). However, none contained *n*-alkane/alk-1-ene homologues after saponification. In addition to *Agave*, two other examples (*Pinus* and *Acer*) of Residue 2 were subjected to acid hydrolysis. A small amount of Residue 3 was obtained and in both cases this yielded only lignin moieties upon pyrolysis and no aliphatic polymer, confirming the absence of cutan. This indicates that a single acid hydrolysis procedure does not remove lignin from a crushed leaf preparation but confirms the absence of any highly aliphatic resistant residue.

The Occurrence of Cutan in Modern Leaves

In this study, following solvent extraction, pyrolysis of all of the modern leaves (Residue 1) yielded predominantly carbohydrate, lignin, and protein moieties together with C₁₆ and C₁₈ fatty acids, reflecting the bulk composition of the leaf (Ralph and Hatfield 1991; Gupta and Pancost 2004). Residue 1 pyrolysates are also characterised by a series of *n*-alkane/*n*-alk-1-ene homologues. Because, the source of such an aliphatic signal from biological components other than cutan is unclear, this aliphatic signature was previously interpreted to result from the pyrolysis of cutan. The ubiquity of the signal prompted the hypothesis that cutan accounts for the aliphatic signal typically found in leaf fossils (Table 2.2; Tegelaar et al. 1991). However, in our study, pyrolysis of the residue after saponification (Residue 2) of 16 out of 19 leaves released products related solely to lignin and carbohydrates; no aliphatic components (neither fatty acids nor *n*-alkane/*n*-alk-1-ene homologues) were detected. Transmission electron microscopy of Residue 2 confirmed the absence of cuticle after hydrolysis (e.g., Möhle et al. 1997, 1998). However, cell walls were retained after the treatment, consistent with the presence of lignin and polysaccharide moieties in the pyrolysates post saponification. The absence of *n*-alkane/alk-1-ene homologues after saponification was also noted in modern *Quercus* leaf litter (van Bergen et al. 1998).

The absence of the aliphatic signal in the pyrolysates of Residue 2 reveals that the aliphatic components in the majority of leaves analysed are hydrolysable and thus, by definition, are not cutan. This means that cutan is absent in most of the flowering plant leaves previously interpreted as containing it (Tegelaar et al. 1991). The exceptions are *Agave americana*, *Clivia miniata* and *Prunus laurocerasus*, all of which yielded a residue diagnostic of cutan after saponification, a residue that was retained in *Agave* and *Clivia* (*Prunus* not tested in our study) in Residue 3 after acid hydrolysis. The presence of cutan in *Agave* and *Clivia* is concordant with many results from other laboratories (Nip et al. 1986a, b; Tegelaar et al. 1989c; McKinney et al. 1996; Möhle et al. 1997, 1998; Schouten et al. 1998; Villena et al. 1999) and proves that our protocol, using the whole leaf as a starting material, is able to detect cutan.

Cuticles isolated enzymatically in previous studies Boom et al. (2005) recorded cutan in the eudicots *Clusia rosea*, *C. multiflora* and *Cereus* sp., a cactus—presumably

Table 2.2 Molecular composition of leaves and cuticles in the fossil record revealing the presence of an ubiquitous *n*-alkyl component

Age	Fossil	Locality	Lithology	Pro	PS	P+	B+	Lig	Pr	F.A.	Aliphatic geopolymer	References
2-20 Ka	<i>Hymenaea</i>	Kenya	Amber	-	+	+	+	+	+	14, 16, 18:1	7-26 (even distrib.)	Stankiewicz et al. (1998a)
Miocene (Upper)	<i>Sciadopitys teritaria</i>	Eschweiler, Germany		N.A.	N.A.	N.A.	+	N.A.	N.A.	16, 16:1, 16:2	6-31 (24-29)	Tegelaar et al. (1991)
Miocene (Upper)	<i>Pinus leitzii</i>	Gozdnica, Poland	Clay-silt	-	+	+	+	+	-	N.A.	N.A.	Stankiewicz et al. (1997)
Miocene (Upper)	<i>Sequoia langsdorfii</i>	Gozdnica, Poland	Clay-silt	-	+	+	+	+	-	N.A.	N.A.	Stankiewicz et al. (1997)
Miocene	<i>Glyptostrobus europaeus</i>	Orawa, Poland		-	-	+	+	+	-	14, 16, 18	13-27 (19-25)	Almendros et al. (1999a)
Miocene (Upper)	<i>Quercus</i>	Kreutzau, Germany		-	-	+	+	-	+	-	10-35 (even distrib.)	Nip et al. (1986a,b)
Miocene (Upper)	<i>Quercus palaecerris</i>	Ardèche, France	Diatomite	-	-	+	+	+	+	16, 18	10-31 (26-31)	Gupta et al. (2007a)
Miocene (Upper)	<i>Quercus suber</i>	Ardèche, France	Diatomite	-	-	+	+	+	+	16, 18	10-31 (26-31)	Gupta et al. (2007a)
Miocene (Upper)	<i>Quercus</i> sp.	Ardèche, France	Diatomite	-	-	+	+	+	+	-	10-31 (27-31)	Gupta et al. (2007a)
Miocene (Upper)	<i>Quercus hispanica</i>	Ardèche, France	Diatomite	-	-	+	+	+	+	16, 18	8-29 (26-29)	Gupta et al. (2007a)
Miocene (Upper)	<i>Pinus</i>	Ardèche, France	Diatomite	-	-	+	+	+	+	14, 16, 18	10-32 (26-31)	Gupta et al. (2007a)
Miocene (Upper)	<i>Populus alba</i>	Ardèche, France	Diatomite	-	-	-	+	-	+	-	9-30 (26-30)	Gupta et al. (2007a)
Miocene (Upper)	<i>Acer pseudocampestre</i>	Ardèche, France	Diatomite	-	-	+	+	+	+	16, 18	10-32 (26-31)	Gupta et al. (2007a)

Miocene (Upper)	<i>Vitis teutonica</i>	Ardèche, France	Diatomite	-	-	+	+	+	+	16	9-31 (26-31)	Gupta et al. (2007a)
Miocene (Upper)	<i>Castanea vesca</i>	Ardèche, France	Diatomite	-	-	+	+	+	+	16, 18	10-31 (26-31)	Gupta et al. (2007a)
Miocene (Upper)	<i>Tilia mastajana</i>	Ardèche, France	Diatomite	-	-	+	+	+	+	16	10-33 (26-31)	Gupta et al. (2007a)
Miocene (Upper)	<i>Robinia</i> sp.	Ardèche, France	Diatomite	-	-	+	+	+	+	16	10-31 (26-31)	Gupta et al. (2007a)
Miocene (Mid)	<i>Amentotaxus gladiifolia</i>	Salzhausen, Germany		N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	+	Tegelaar et al. (1991)
Miocene (Mid)	<i>Magnolia</i>	Clarkia, USA	Clastic	-	-	+	+	+	+	-	+	Logan et al. (1993)
Miocene (Mid)	<i>Quercus</i>	Clarkia, USA	Clastic	-	-	+	+	+	+	-	+	Logan et al. (1993)
Miocene (Mid)	<i>Metasequoia</i>	Clarkia, USA	Clastic	-	+	+	+	+	+	-	+	Yang et al. (2005)
Oligocene	Conifer	Enspel, Germany	Clastic	-	-	+	+	+	+	6-30*	8-32 (11-15)	Gupta et al. (2007b)
Oligocene	Unidentified angiosperm	Enspel, Germany	Clastic	+	+	+	+	+	+	-	8-27 (12-20)	Gupta et al. (2007b)
Oligocene	<i>Hymenaea</i>	Dominica	Amber	-	-	+	+	-	+	16, 18:1, 18	7-26 (even distrib.)	Stankiewicz et al. (1998a)
Paleocene	<i>Ginkgo adiantoides</i>	Wyoming, USA	Mudrock	-	-	+	-	+	-	-	7-24 (even distrib.)	Collinson et al. (1998)
Paleocene/Eocene	<i>Metasequoia</i>	Ellesmere Island	Lignite	-	+	+	+	+	-	16	-	Yang et al. (2005)
Eocene	<i>Ocotea obtusifolia</i>	Tennessee, USA		N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	+	Tegelaar et al. (1991)
Eocene	<i>Berryophyllum saffordii</i>	Kentucky, USA		N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	+	Tegelaar et al. (1991)

(continued)

Table 2.2 (continued)

Age	Fossil	Locality	Lithology	Pro	PS	P+	B+	Lig	Pr	F.A.	Aliphatic geopolymer	References
Eocene (Mid)	<i>Rhodomyrtophyllum</i>	Geiseltal, Germany		N.A.	N.A.	N.A.	+	-	+		6-34 (27-32)	Tegelaar et al. (1991)
Eocene (Mid)	<i>Polyspora hallensis</i> ¹	Geiseltal, Germany		-	+	N.A.	+	+	+	16,18	6-34 (10-15 and 30-34)	Tegelaar et al. (1993)
Eocene (Mid)	<i>Metasequoia</i>	Axel Heiberg Island	Lignite	-	+	+	+	+	-	-	+	Yang et al. (2005)
Eocene (Lower)	cf. <i>Sapindus fructiferus</i>	Tennessee, USA		N.A.	N.A.	N.A.	+	N.A.	N.A.	+	+	Tegelaar et al. (1991)
Eocene	Unidentified	Messel, Germany		-	-	+	+	-	+	-	10-35 (even distrib.)	Nip et al. (1986a,b)
Cretaceous	<i>Squamastrabus tigrensis</i>	Patagonia, Argentina		-	-	+	+	-	-	6-18	9-29 (21 to 26)	Almendros et al. (1999b)
Cretaceous	<i>Ginkgo adiantoides</i>	North Dakota, USA	Mudrock	-	-	+	+	-	+	-	7-30 (8-13)	Mösle et al. (1998)
Cretaceous (Upper)	<i>Ginkgo coviacea</i>	China	Marl	-	-	+	+	-	+	-	7-28 (11-15)	Mösle et al. (1998)
Cretaceous (Upper)	<i>Protodleycaria ilicoides</i>	S.-Quedlinburg, Germany		N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	+	+	Tegelaar et al. (1991)
Cretaceous (Lower)	<i>Frenelopsis</i>	Central Spain	Carbonate	-	-	+	+	-	-	-	7-27 (7-15)	Mösle et al. (1998)
Cretaceous (Lower)	<i>Abietites linkii</i>	NW Germany	Coal	-	-	+	+	-	-	-	6-30 (7-13)	Mösle et al. (1998)
Cretaceous	<i>Abietites</i>	Osterode, Germany		-	-	+	+	-	-	-	6-30 (6-15)	Nip et al. (1986a,b)
Cretaceous (Lower)	<i>Frenelopsis oligostomata</i>	Cerro de la Mesa (Spain)	Limestone	-	-	+	N.A.	-	-	14, 16, 18*	N.A.	Nip et al. (1986a,b)
Jurassic	<i>Ginkgo huttonii</i>	Scalby Ness, U.K.		-	-	+	+	-	-	-	7-23 (7-15)	Nip et al. (1986a,b)

Jurassic	<i>Ginkgo huttonii</i>	Yorkshire, UK	-	-	+	+	-	-	7-17 (9-14)	Mösle et al. (1997)
Jurassic (Mid)	<i>Pachypteris</i>	Scarborough, U.K.	Mudstone	-	+	+	-	-	10-28 (10-15)	Ewbank et al. (1996)
Permian	<i>Callipteris conferta</i>	Nahe, Germany	-	-	+	+	-	-	6-29 (7-14)	Nip et al. (1986a,b)
Carboniferous (Upper)	<i>Reticulopteris</i>	Indiana, USA	Coal ball	-	-	+	-	-	7-32 (10-16)	van Bergen et al. (1994)
Carboniferous (Upper)	<i>Neuropteris</i>	Indiana, USA	Coal ball	-	-	+	-	-	6-32 (10-20)	van Bergen et al. (1994)
Carboniferous (Upper)	<i>Karinopteris</i>	Indiana, USA	Paper coal	-	-	+	-	-	6-30 (6-13)	van Bergen et al. (1994)
Carboniferous (Upper)	<i>Alathopteris</i>	Kansas, USA	Coal ball	-	-	+	-	-	6-33 (9-17)	van Bergen et al. (1994)
Carboniferous (Step B)	Cordaite	Lone Star Lake, USA	Coal	-	+	+	-	+	6-30 (9-16)	Stankiewicz et al. (1998b)
Carboniferous (Step B)	<i>Walchia</i>	Garnett, USA	Limestone	-	+	+	-	-	7-30 (10-15)	Mösle et al. (2002)
Carboniferous (Step B)	<i>Walchia</i>	Hamilton, USA	Limestone	-	+	+	-	-	7-30 (10-15)	Mösle et al. (2002)
Carboniferous (Step B)	<i>Cordaites</i>	Lone Star Lake, USA	Coal	-	+	+	-	-	6-30 (10-15)	Mösle et al. (2002)
Carboniferous (Wes A)	Cordaite	Joggins, Canada	Clastic	-	+	+	-	-	6-30 (9-16)	Stankiewicz et al. (1998b)
Carboniferous (Wes A)	Pteridosperm	Joggins, Canada	Clastic	-	+	+	-	+	6-30 (9-14)	Stankiewicz et al. (1998b)
Carboniferous (Wes A)	<i>Cordaites</i>	Joggins, Canada	Clastic	-	+	+	-	-	6-30 (10-15)	Mösle et al. (2002)

(continued)

Table 2.2 (continued)

a stem not a leaf, one monocot (an epiphytic orchid) and one species of *Podocarpus* (a conifer). The cuticles were enzymatically isolated; the residue following acid and base hydrolysis yielded a highly aliphatic signal upon pyrolysis. There are two other recent reports of cutan: 1. in fruit cuticles of green pepper *Capsicum annuum* (Chefet [2003](#)), based on the presence of a residue from enzymatically isolated cuticles subjected to acid and base hydrolysis; 2. in stems of *Arabidopsis* (Xiao et al. [2004](#)), based on a nonsaponifiable residue from enzymatically isolated cuticles. The materials were not analysed chemically. The *Capsicum* example meets the definition of cutan as yielding a non-saponifiable and non hydrolysable residue (although confirmation of the aliphatic signal would be preferable). It is not known, however, whether the *Arabidopsis* nonsaponifiable residue contained a highly aliphatic component or was composed entirely of lignin and polysaccharide moieties derived from the cell wall, which is clearly evident in the published TEM images of the cuticles (Xiao et al. [2004](#): Fig. 7).

Cutan and the Leaf Fossil Record

Cutan occurs in leaves of the living plants *Agave americana*, *Clivia miniata*, *Clusia rosea* and *C. multiflora*, an unnamed epiphytic orchid, *Cereus* (presumably stems), *Prunus laurocerasus* (resistance to acid hydrolysis not tested), and one *Podocarpus* species. There is no known fossil record of *Agave*, *Clivia*, epiphytic orchids, or cacti (e.g. Collinson et al. [1993](#); Herendeen and Crane [1995](#)). Fossil Clusiaceae are represented in the late Cretaceous (Crepet and Nixon [1998](#)) but by fossil flowers not leaves. There is one record of Clusiaceae leaves, preserved as impressions (i.e. organic material is absent) from the Tertiary of India (Ambwani [1991](#)), but their identity is equivocal. *Prunus laurocerasus* has been reported from the Pliocene and Miocene of continental Europe (as *Laurocerasus*: Palamarev and Petkova [1987](#)). However, *Prunus* leaf fossils are generally rare and the rosaceous subfamily Amygdaloideae (to which *P. laurocerasus* belongs: Lee and Wen [2001](#)) is typically represented by fruit stones in Paleogene and younger strata (Mai [1984](#)). *Podocarpus* leaves and pollen have been recorded from the Cretaceous, Paleogene, and Neogene of Australia (Hill [1994](#), [2004](#)), although the leaves or phylloclades of many other Podocarpaceae are usually far more abundant and more diverse than *Podocarpus* leaves (e.g. Hill [1994](#), Table 12.1, p. 284). Although cutan has been reported in *Podocarpus* (Boom et al. [2005](#)) it may not be present in all species (Collinson et al. [1998](#)).

This study has shown that cutan is absent in the leaves of the living flowering plants *Acer campestre*, *Quercus robur*, *Castanea sativa*, *Citrus limon*, *Betula alba*, *Populus hybrida*, and *Gossypium hirsutum*, and the conifers *Pinus sylvestris*, *Metasequoia glyptostroboides* and *Abies grandis* (see Table 2.1 for complete list). We are not aware of a fossil record for *Citrus* or *Gossypium* leaves (e.g. Collinson et al. [1993](#)). Fossil *Castanea* leaves are infrequent but are recorded in the late Neogene of Europe (Kvaček and Walther [1989](#)) and the Oligocene and Neogene of Japan and China (Tanai [1995](#)). Fossil *Betula* (Crane [1989](#); Walther [1999](#); Hably

et al. 2000) and *Populus* (Collinson 1992; Mai and Walther 1991) leaves are also infrequent but are known from the Paleogene onwards. Studies of fossil *Pinus* tend to focus on cones (Mai 1995) but leaves are also recorded in the Paleogene and Neogene (e.g. Kvaček and Rember 2000; Walther 1999). Leaves of *Acer*, *Quercus* and *Metasequoia* are abundant, diverse and widespread in the fossil record. *Acer* and *Quercus* are particularly well represented in North America, Asia and Europe from the Oligocene onwards (Prochazka and Buzek 1975; Daghljan and Crepet 1983; Wolfe and Tanai 1987; Kvaček and Walther 1989, 1998; Mai and Walther 1991; Mai 1995; Tanai 1995; Liu et al. 1996; Fotyanova 1997; Walther 1999; Kvaček and Rember 2000; Hably et al. 2000). *Metasequoia* is absent in the Cainozoic of Europe (Kvaček and Rember 2000; LePage et al. 2005) but has an extensive record from the Cretaceous onwards elsewhere in the Northern Hemisphere (LePage et al. 2005; Yang et al. 2005). It is important to note that these leaf fossils include both compressions and impressions. Impression fossils lack original organic material. If the presence of cutan in the cuticle were an important factor in leaf preservation leaves lacking cutan might be represented by a predominance of impression fossils over compression fossils but this is not the case.

The above data indicate that the presence of cutan in the leaf is not a strong predictor for a particularly abundant, widespread or diverse leaf fossil record of that taxon, nor is the presence of cutan a prerequisite for such a leaf fossil record. This does not mean that the presence of cutan in some leaves plays *no* role in the formation of fossils; cutan is resistant to a variety of diagenetic reactions and may influence leaf preservation. However, the lack of a correlation between the presence of cutan and a fossil record indicates that a variety of other factors, such as proximity to a depositional setting, redox conditions in the depositional setting and rates of burial, are far more important.

Explanations for the Aliphatic Component in Fossils

Most fossil leaves and cuticles are characterised by a strong aliphatic signal irrespective of plant type, enclosing lithology, depositional environment, locality and age (Table 2.2). The similarity between this aliphatic composition and that of cutan was one of the main reasons to invoke cutan as a primary control on fossil preservation (Tegelaar et al. 1991). It is now unclear if it was appropriate to place such an emphasis on the aliphatic composition of fossil organic matter, because the aliphatic content is generally overestimated when analysed by conventional pyrolysis and solid state ^{13}C NMR (e.g. forest soils investigated by Poirier et al. 2000). Nonetheless, it remains critical to identify the source of fossil aliphatic macromolecules in order to understand the chemical reactions that underpin fossil leaf taphonomy.

Selective preservation—The aliphatic composition of leaf fossils (Table 2.2) was interpreted previously as a direct consequence of decay resistance and selective preservation of the diagenetically stable aliphatic biopolymer cutan (Nip et al. 1986a, b; Tegelaar et al. 1991; de Leeuw and Largeau 1993). Cutan occurs in modern *Podocarpus*

and a similar aliphatic signal has been reported in a Neogene cuticle (Wijninga 1996) suggesting that cutan survived into the fossil record (Boom et al. 2005). However, the fossil sample was not identified as *Podocarpus*, but was one of five unidentified isolated dispersed cuticles (Wijninga 1996, Fig. 2.4), some of which were found in association with *Podocarpus* wood. There is no direct evidence for cutan preservation in fossils. Combined with the lack of cutan in many leaves with diverse fossil records, the above indicates that selective preservation of cutan is no longer tenable as an explanation for the highly aliphatic signal found in most leaf fossils.

Migration from sediment—Given the widespread occurrence of aliphatic components in sediments, insect, and plant fossils, the occurrence of aliphatic components in fossil leaves might be attributed to migration (Baas et al. 1995; van Bergen et al. 1995). This possibility, however, has been countered by several lines of evidence: (1) Aliphatic polymers are characteristically insoluble, and therefore relatively immobile (see Briggs 1999 for discussion); (2) An aliphatic signal was detected in Tertiary *Hymenaea* leaves trapped in amber (Table 2.2), where they are protected from external contamination (Stankiewicz et al. 1998a); (3) The aliphatic signatures in co-occurring plant and insect fossils from the Upper Carboniferous of North America are different, indicating that they could not have been introduced solely from the matrix (Stankiewicz et al. 1998b) and the internal morphology of the cuticle is altered indicating diagenesis; (4) The composition of artificially matured insect tissue is aliphatic (Stankiewicz et al. 2000) showing that endogenous organic matter can generate an aliphatic composition, as observed in fossils; (5) Thermochemolysis (TMAH assisted pyrolysis: Challinor 1989, 1991a, b; de Leeuw and Baas 1993; Martin et al. 1994; Almendros et al. 1998, 1999a; McKinney et al. 1996) of co-occurring insect and plant fossils and the associated organic rich matrix revealed differences in the distribution of the constituent fatty acyl components indicating that the aliphatic component of the fossil is endogenously derived (Gupta et al. 2007b); (6) Logan et al. (1995) showed that leaf lipids in the Miocene *Clarkia* sediments were concentrated on the leaf surfaces without migrating into the surrounding sediment. Introduction from other sources such as sediment is not tenable as an explanation for the highly aliphatic composition of leaf fossils.

In-situ polymerisation of labile aliphatics—In the absence of a diagenetically-stable aliphatic biopolymer in the living relatives, the preservation and aliphatic character of the fossil leaves cannot be explained by selective preservation. Migration from an external source can also be excluded. Thus, the aliphatic composition of the fossil leaves and cuticles (Table 2.2) must have been derived endogenously from other compounds present in the leaf tissues.

Pyrolysates of fossil leaves from the Tertiary of the Ardèche showed the dominance of C₁₆ and C₁₈ fatty acyl moieties (Gupta et al. 2007a). Thermochemolysis released the fatty acyl moieties, ranging in carbon number from C₈ to C₃₂ with a predominance of C₁₆ and C₁₈ homologues, that form part of the geopolymer. C₁₆ and C₁₈ fatty acyl homologues in equivalent modern leaves occur in cutin, phospholipid fatty acids (PLFA) and as triacylglycerides, sterol esters, other complex lipids and free fatty acids (FA). Thus, polymerisation of labile aliphatic components present in the cuticle and internal leaf tissue (cutin, PLFA, FA) during diagenesis is a

potential source of the aliphatic component of the fossil leaf macropolymer. Cutin can become crosslinked (Deshmukh et al. 2003) or intermolecularly ether linked (Schmidt and Schönherr 1982) making it diagenetically stable. As cutin contains C_{16} and C_{18} units these could be the source of the corresponding short chain *n*-alkyl component observed in the fossils. As previously suggested by Collinson et al. (2000) and Finch and Freeman (2001), long chain waxes (Eglinton and Hamilton 1967; Walton 1990) can be incorporated into the fossil geomacromolecule to account for the higher molecular weight long chain *n*-alkanes and *n*-alkenes generated during pyrolysis. Thus, while selective preservation of the biopolymer cutan cannot explain the preservation of fossil leaves, their aliphatic composition may be attributed to *in situ* polymerisation (Briggs 1999; Stankiewicz et al. 2000) of extractable and non-hydrolysable lipid components resulting in an aliphatic geopolymer (not inherited from the biopolymer cutan), a process that may be of widespread importance in the fossilization of organic materials.

The Ecology and Physiology of Plants with Cutan-Containing Leaves

There is no one-to-one correlation between the occurrence of cutan and leaf succulence or thick evergreen leaves with thick cuticles. The leaves of the cutan-containing *Agave* and the stems of the cactus *Cereus* are succulent, but the leaves of *Kalanchoe*, which are also succulent, lack cutan (Finch and Freeman 2001; not based on oxidative isolation, *contra* Boom et al. 2005). The cutan-containing leaves of *Clivia*, *Clusia* and epiphytic orchids are relatively fleshy but much less succulent. Leaves of *Podocarpus* and *Prunus laurocerasus* are evergreen and relatively thick with thick cutan-containing cuticles but leaves that lack cutan, such as *Citrus limon*, are similar in texture. The leaves of *Pinus* are evergreen with thick cuticle but lack cutan; nonetheless they are needle-like with a very low surface area to volume ratio, an adaptation to drought.

Cutan occurs in some CAM plants but it is absent in others, and it is present in plants using the C_3 photosynthetic pathway. *Clusia rosea* and *C. multiflora* both contain cutan (Boom et al. 2005); the former exhibits C_3 or CAM plasticity, and the latter is an obligate C_3 plant (Herzog et al. 1999; Lüttge 1999). Thus, some CAM plants, some succulent plants and some plants with thick cuticles do not contain cutan. A clear correlation cannot be made between any of these attributes and the presence of cutan and, on the basis of the small sample currently available, it is not yet clear that the presence of cutan in cuticles is an adaptation for drought resistance (*contra* Boom et al. 2005).

Most importantly, this study shows that the highly aliphatic signal in fossils is not due to the selective preservation of cutan and we suggest that it derives from *in situ* polymerisation of more labile aliphatic components such as waxes, internal lipids, and cutin. Thus neither the ecology and physiology of plants with cutan-containing leaves nor the presence or absence of cutan in leaves exert any major bias on the preservation of leaves in the fossil record.

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