PERGAMON

Organic Geochemistry 32 (2001) 45–55

Organic Geochemistry

www.elsevier.nl/locate/orggeochem

Evolution of the chemical composition of *Ginkgo biloba* external and internal leaf lipids through senescence and litter formation

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Received 7 June 1999; accepted 18 October 2000
(returned to author for revision 3 May 2000)

Abstract

External and internal lipids were analysed in fresh, senescent and litter leaves of *Ginkgo biloba* to derive qualitative and quantitative information on the changes that occur during the very early stages of diagenesis, and to identify potential biomarkers of the only living Ginkgoale. Analysis of fresh leaves led to the identification of additional compounds, including several series of phenolic constituents, and showed differences between the external and internal lipids (absence/presence of some components, relative abundances and distributions of some series). Pronounced differences concerned with the evolution of content (as wt.% of whole leaves) were observed between the internal lipids (regular decrease from fresh to senescent and to litter leaves) and the external lipids (no significant changes in content). Molecular studies showed variations in the relative abundances and distributions of most constituents of *G. biloba* leaf lipids which reflect different degrees of stability during the first stages of diagenesis. It also appeared that (i) the phenolic components and the co-occurrence of α-tocopherol and nonacosan-10-ol could be useful biomarkers of *G. biloba*, and (ii) most of the internal lipids continue to be exported to the leaf surface where they are further degraded during senescence and litter formation.

Keywords: *Ginkgo biloba*; Leaf lipids; Early degradation; Litter leaves; Senescent leaves

1. Introduction

Higher plant detritus constitutes a large and continually renewed pool of reactive organic matter in estuarine and coastal marine environments, and higher plants are a major contributor of organic matter to sediments (Tissot and Welte, 1984). The nature and distributions of lipid components is often used to differentiate higher plant organic matter from bacterial or algal organic matter, in marine environments (e.g. Goutx et al., 1990; Harada et al., 1995; Zegouagh et al., 1996), fluvial environments (e.g. Jaffé et al., 1995) or lacustrine environments (e.g. Cranwell, 1984; Rieley et al., 1991). Nevertheless, while the lipid composition of modern organisms has been extensively investigated, in order to identify molecular biomarkers, the very early stages of their diagenesis has only been studied recently (e.g. de Leeuw et al., 1995; Jaffé et al., 1996). The present study focuses on the examination of changes in leaf lipids of *Ginkgo biloba* through the early stages of degradation (fresh, senescent and litter leaves) in order

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0146-6380/01/$ - see front matter
PII: S0146-6380(00)00152-2

to identify biomarkers of the Ginkgoale and their preservation potential.

The Ginkgoale family is now restricted to a single species, *G. biloba,* naturally occurring in one specific area in China. However, this family was widespread, as numerous species during the Mesozoic (Steward and Rothwell, 1993). Accordingly, the abundance of this family should be recorded as biomarker assemblages in Mesozoic sediments. *G. biloba* is considered as a living fossil, its morphology has not changed for more than 100 million years. It exhibits dichotomous leaves, the leaf venation is an open forking system without vein fusion (Ozenda, 1991). Leaf lipids of *G. biloba* were first investigated by Ageta (1959), after extraction with ethanol: they contained about 10% fatty acids, 15% esters, 75% paraffins and wax alcohols. The main component, called ''ginnol'', was the secondary alcohol, nonacosan-10-ol. More recently, Gülz et al. (1992) analysed the epicuticular waxes of *G. biloba* leaves, extracted by dipping in chloroform, and found in addition to the usual constituents of higher plant leaf lipids (hydrocarbons, wax esters, aldehydes, primary alcohols, fatty acids) more specific compounds: nonacosan-10-ol, benzyl acyl esters, γ-tocopherol and acetates of fatty alcohols. Other authors have also examined the distribution of hydrocarbons and fatty acids (Casal and Moyna, 1979), and of secondary alcohols (Holloway et al., 1976; Casal and Moyna, 1979).

Leaf lipids in higher plants are located in several distinct pools. Numerous authors have reported the presence of abundant amounts of lipids inside leaves, including intracuticular waxes and other internal lipid pools (e.g. Holloway, 1980; Walton, 1990; Riederer and Schreiber, 1995). Nevertheless, the most frequently studied leaf lipids are the epicuticular waxes. In contrast to the internal lipids, the external lipids are easily extractable by brief contact with a low polarity solvent. Epicuticular waxes consist of a thin continuous lipid layer covering all the aerial organs of higher plants. These external lipids act as a natural interface between land plants and their aerial environment (e.g. Eglinton and Hamilton, 1967; Jeffree et al., 1976; Baker, 1980), and play an important protective role against drought, cold or pathogenic agents (e.g. Martin and Juniper, 1970; Weete et al., 1978; Riederer and Schreiber, 1995). This layer exhibits a dynamic chemical composition in order to adapt to variations in the environment (e.g. Baker, 1980; Bianchi, 1995; Riederer and Schreiber, 1995). It is often considered that migration of compounds, between the interior and the surface of the leaf, plays an important role in this adaptation of the chemical composition of external lipids (Cassagne and Lessire, 1975; Giese, 1975; Lessire et al., 1980). The external lipids are generally assumed to be produced within the epidermal cells and then excreted to the cuticle *via* extrusion through pores, diffusion in solvents or transport by proteins and carbohydrates (Kolattukudy et al., 1976; Baker, 1980; Hallam, 1980).

Previous studies on *G. biloba* leaf lipids focused on external lipids of fresh leaves. However, when diagenetic changes and geochemical implications are considered, information on the internal pool appears important as well, due to (i) the abundance of this pool and (ii) the role of some of its components as a source of the epicuticular waxes. Accordingly, both internal and external lipids of *G. biloba* leaves were analysed in fresh, senescent and litter leaves. The lipid extracts were studied by gas chromatography (GC) and gas chromatography–mass spectrometry (GC/MS). Since the morphology of epicuticular waxes is known to be related to chemical composition (e.g. Kolattukudy et al., 1976; Jeffree et al., 1976; Baker, 1980), the leaves studied were also examined by scanning electron microscopy (SEM).

2. Experimental

2.1. Samples

Leaves of *G. biloba* were collected from a rural area (Massif Central, France) where urban pollution is minimal. Due to the well-known changes in lipid composition during leaf growth (e.g. Avato et al, 1984; El-Otmani et al., 1987; Gülz et al., 1991) fresh mature leaves (green leaves) were collected in September 1998. Senescent leaves (yellow) and leaves from litter (brown) were collected in December and January, respectively. Each batch of samples consisted of approximately 30 leaves, collected on several representative branches of the same tree for the fresh and senescent samples, and at the base of this tree for the litter leaves. After collection, leaves were dusted free of any adhering particles with pre-extracted cotton wool, and dried overnight at 50°C.

2.2. SEM

Alteration of epicuticular wax morphology can occur when the usual procedure of sample preparation for SEM is used, especially dehydration by ethanol (Reed, 1980). Samples were therefore prepared for SEM by directly sputtering the dried leaves with gold, using an EDWARDS E306 sputter coater. A JEOL JSM-840A electron microscope was used for examination at 12–17 kV.

2.3. Extraction of leaf lipids

2.3.1. External lipids

Whole leaves (2–4 g) were extracted by dipping in solvent (20 ml; 15 s; ×2) at room temperature. In order to minimise the extraction of internal lipids *n*-heptane was chosen due to its weak ability to penetrate tissues

(e.g. Salasoo, 1983; Hamilton, 1995). The extracts were evaporated under vacuum, and weighed.

2.3.2. Internal lipids

Dichloromethane and methanol constitute an efficient solvent mixture, allowing the extraction of non-polar as well as some polar lipids (Kates, 1972). These solvents appear adequate to identify biomarkers of plant lipids in sedimentary organic matter. Moreover, methanol, as other alcohols, is able to inactivate the possible enzymatic degradation of lipids during extraction of plant tissues (Kates, 1972). As a result, after heptane extraction, the leaves were crushed and the internal lipids were extracted with 30 ml of CH_2Cl_2/CH_3OH (2/1, v/v) by strirring overnight at room temperature. The mixture was centrifugated (10 min at 4000 rpm) and the solvent was evaporated under vacuum.

2.4. Analyses

No chromatographic fractionation was performed in order to limit losses of lipids during the separation procedure. Each lipid sample was analysed by GC and GC/MS both as total extract and as the trimethylsilylated extract.

2.4.1. Trimethysilylation

As described in Perry and Hulyalkar (1965) an aliquot of lipid (approximately 1 mg) was stirred in 130 μl of pyridine/hexamethyldisilazane/trimethylchlorosilane (10/2/1, v/v/v) at room temperature. The derivatized extract was then directly recovered, after separation from the subsequently formed precipitate, by centrifugation for 3 min at 13,000 rpm.

2.4.2. GC

GC analyses were conducted using an INTERSMAT IGC 121 FL fitted with a fused silica capillary column, coated with Rtx-5MS (30 m×0.32 mm i.d., 0.23 μm film; RESTEK). The temperature program used was 100–300°C at 4°C/min followed by an isothermal period at 300°C for 20 min. Helium was used as carrier gas with the split injector and the FID detector temperatures held at 300°C. Due to the large diversity of chemical structures in the lipids, absolute quantification using internal standards could not be performed. Accordingly, variations in the abundances of two given series were gauged from variations in the ratio of the intensities of their maxima on the GC-trace.

2.4.3. GC/MS

GC/MS analyses were performed using a Hewlett-Packard 5890A chromatograph coupled with a Hewlett-Packard 5980 Série II mass spectrometer, scanning from 40 to 800 Da, electron energy 70 eV, scan time 1.3 s. The chromatographic conditions were the same as above. Compounds were identified by comparison of their retention times and mass spectra with those of reference compounds or from literature data.

3. Results and discussion

3.1. Fresh leaves

3.1.1. External lipids

The external lipids recovered by short heptane extractions account for 0.5 wt.% of the dried leaves. GC/MS analysis of the extracts allowed the identification of a number of constituents (Table 1). The major compound in the external lipids of fresh *G. biloba* leaves (Fig. 1a) is nonacosan-10-ol **1** (''ginnol'' Appendix, Ageta, 1959; Holloway et al., 1976; Gülz et al., 1992). This secondary alcohol is a common component of epicuticular waxes of conifer needles, where it forms small crystalline wax tubules on the leaf surface (Jeffree et al. 1976; Baker, 1980; Gülz et al., 1992). Such tubules are observed by SEM (Fig. 2a and b) on the fresh leaves of *G. biloba* in agreement with previous work (Baker, 1980; Gülz et al., 1992). This crystalline wax covers the epidermis of *G. biloba* fresh leaves (Fig. 2b). The tubules that constitute a dense network, are short (about 1 μm in length) and cylindrical (about 0.1 μm in diameter), and are distributed in all directions (Fig. 2b). Nonacosan-10-one 2, occurs in much lower relative abundance than the corresponding alcohol in the external lipids: it most probaby derives from oxidation of nonacosan-10-ol. The small shoulder (Fig. 1) observed on the large peak of nonacosan-10-ol corresponds to α-tocopherol **3**, identified here for the first time in *G. biloba* leaf lipids.

The main series of compounds on the GC trace corresponds to 3-alkylphenols **4**, also termed ''cardanols'' (Itokawa et al., 1987). Silylation reveals the presence of another series of phenolic compounds identified as 2-carboxy-3-alkylphenols **5** or ''anacardic'' or ''ginkgolic'' acids (Itokawa et al., 1987). The latter compounds are not detected without silylation, probably due to their spontaneous decarboxylation in the GC injector. This becomes evident from the $C_{17:1}$alkylphenol/C_{27}*n*-alkane ratio which significantly decreases from 0.96 to 0.57 after silylation, showing (i) that carboxyphenols contributed to series **4** in the chromatogram of the crude extract, and (ii) that series **4** and **5** were present in similar concentrations. Fragmentograms of *m/z* 180 (alkylphenols, Fig. 3b) and *m/z* 219 (carboxyphenols, Fig. 3c) in the trimethylsilylated extract allow differentiation of the distributions of each series from those observed on the crude extract at *m/z* 108 (Fig. 3a). The alkyl chains of both series are characterised by having the same lengths (13, 15 or 17 carbons) and number of unsaturations (0–2). However, while the alkylphenol series is markedly dominated by the $C_{17:1}$ homologue, the carboxyphenols series exhibits a smooth distribution

Table 1
Composition of external and internal lipids from *Ginkgo biloba* fresh leaves, senescent leaves and leaves from a litter

		Fresh[b]		Senescent[b]		Litter[b]	
Identified compounds	Diagnostic fragment ions	External	Internal	External	Internal	External	Internal
n-Alkanes	43, **57**, 71,...,M^+	21–35 (**27**)	–	21–35 (**27**)	–	25–35 (**27**)	–
Phenols[c]	**180**, M-15[a]	13, 15, 17 (**17:1**)	13, 15, 17 (**17:1**[d])	13, 15, 17 (**17:1**)	13, 15, 17 (**17:1**[d])	13, 15, 17 (**17:1**)	13, 15, 17 (**17:1**[d])
Carboxyphenols[c]	**219**, M-15[a]	13, 15, 17 (**15:1**)	13, 15, 17 (**15:1**)	13, 15, 17 (**17:1**)	13, 15, 17 (**13**)	13, 15, 17 (**15:1**)	13, 15, 17 (**15:1**)
Dimethoxycoumarins[c]	208, **221**, M^+	12, 14, 16 (**14:1**)	12, 14, 16 (**14:1**)	12, 14, 16 (**12**)	Traces	Traces	Traces
Nonacosan-10-ol	157, 297, M^+	29[d]	29	29[d]	29	29[d]	29
Nonacosan-10-one	155, 295, M^+	29	–	29	29	29	29
α-Tocopherol	**165**, M^+	29	29	29	29	29	29
γ-Tocopherol	**151**, M^+	–	Traces	–	Traces	–	–
n-Acids	**117**, M-15[a]	12–32 (**16**,24)	10–30 (**16**)	12–30 (**16**, 24)	12–30 (**16**)	7–20 (**16**)	11–24 (**16**)
n-Primary alcohols	**75**, M-15[a]	24–30 (**28**)	14–24	14–28 (**28**)	16–28	18	–
Phytol	**71**, 123, 196, M^+	–	20	20	20	20	20
Phytadienes	**68**, 123,...,M^+	–	20	20	20	20	20
Isoprenic ketone	M-58, 250, M^+	–	18	18	18	18	18

[a] Diagnostic fragment ions of silylated derivatives.
[b] Carbon number range (**max**, submax).
[c] Carbon number of the hydrocarbon chain.
[d] Main component of the extract. –: undetected.

with a slight maximum at $C_{15:1}$. Although, these series were not previously detected in *G. biloba* leaf extracts, the occurrence of the $C_{15:1}$ and $C_{17:1}$ homologues was reported by Itokawa et al. (1987) in methanolic extracts from *G. biloba* seeds.

A series of three compounds, tentatively identified as dimethoxyalkylcoumarins **6** with C_{12}, $C_{14:1}$ and $C_{16:1}$ alkyl chains, was detected in low amounts. As suggested by Barrero et al. (1994), from his analysis of the legume *Onosis pubescens*, these dimethoxyalkylcoumarins may be biosynthetically linked to the phenolic series **4** and **5**. Dimethoxyalkylcoumarins have not been reported previously in *G. biloba* leaf extracts, but a series of dihydroisocoumarins **7** was recently identified in *G. biloba* fruits, where they were considered to be derivatives of carboxyphenols **5** (Choukchou-Braham et al., 1994). Plant phenolics, including coumarins or carboxyphenols, are usually considered to play an important role in the resistance to attack by insects or fungi (Bennett and Wallsgrove, 1994). The presence of such compounds in *G. biloba* leaf lipids may have played a role for the persistence of this species through geological time. Moreover, these phenolic compounds may be diagnostic biomarkers of *G. biloba* since: (i) they are present in substantial amounts and (ii) they are present in multiple organs (leaves, seeds, fruits).

C_{21}–C_{35} predominantly odd-numbered *n*-alkanes were also detected on the GC trace, maximising at C_{27} (the large peak of nonacosan-10-ol includes a weak contribution from the coeluting C_{31} *n*-alkane). This distribution is consistent with those reported in literature for *G. biloba* leaf lipids, although minor variations were noted (C_{19}–C_{35}, max. C_{27}, Gülz et al., 1992; C_{22}–C_{31}, max. C_{27}, Casal and Moyna, 1979). The presence of a series of saturated fatty acids ranging from C_{12} to C_{32}, displaying a strong even/odd predominance, was confirmed by GC analysis of the trimethylsilylated extract. Their distribution is bimodal with a maximum at C_{16} and a submaximum at C_{24}. Trimethylsilylation also reveals the presence, in low amounts, of predominantly even-numbered, primary fatty alcohols. They range from C_{24} to C_{30}, with a maximum at C_{28}. The distribution of the two latter series is slightly different from those previously reported in the literature (Holloway et al., 1976; Casal and Moyna, 1979; Gülz et al., 1992). The difference between the observed distributions may reflect changes in the chemical composition of external lipids in response to environmental variations (Kolattukudy et al., 1976; Gülz et al., 1991; Lockheart et al., 1997). These differences also confirm that ubiquitous compounds such as *n*-alcohols or *n*-acids cannot easily constitute dependable biomarkers.

3.1.2. Internal lipids

Internal lipids are much more abundant than external lipids (17.1 wt.% of the dried leaves). Most of the

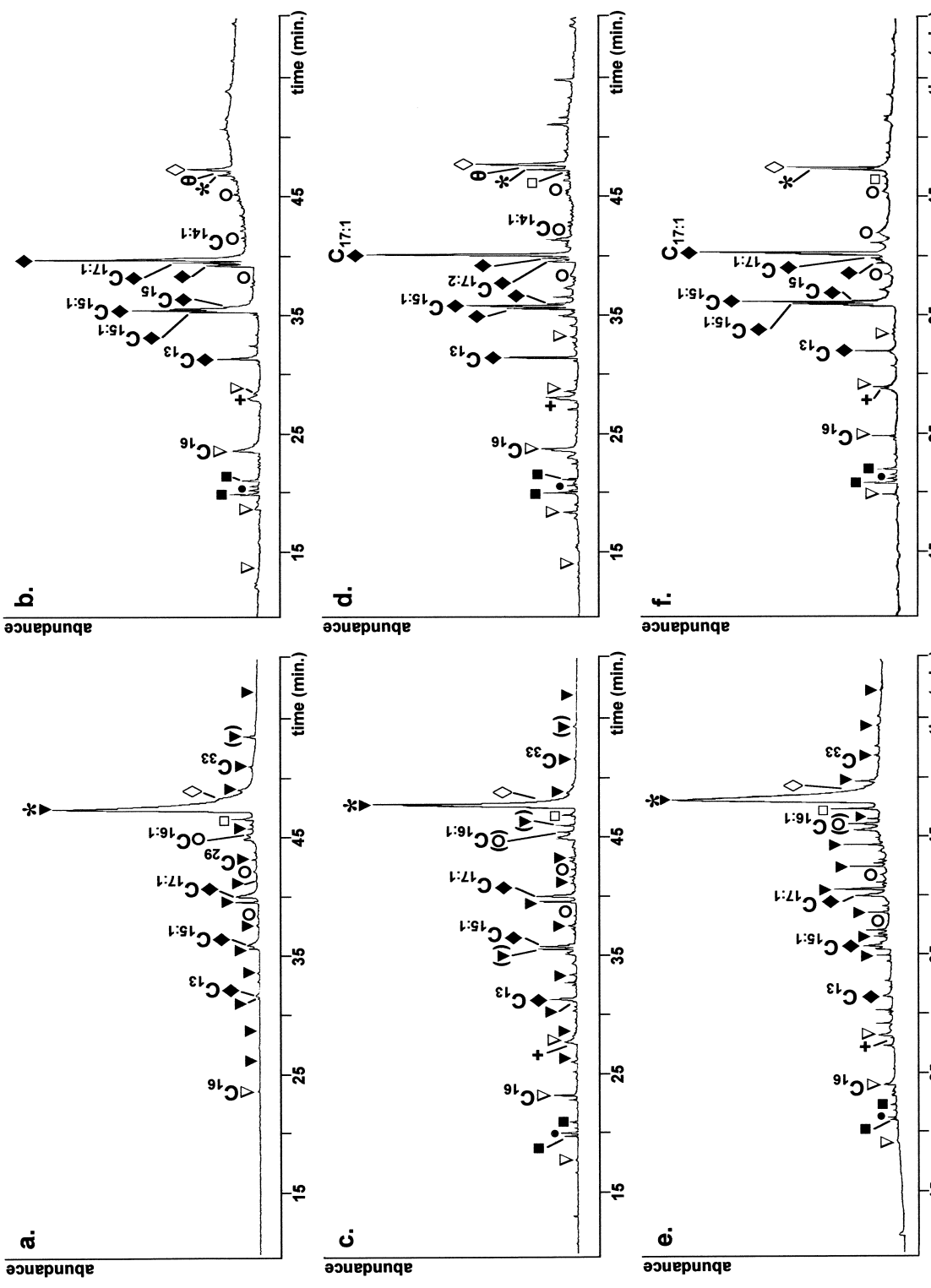

Fig. 1. Chromatograms of *G. biloba* crude extracts. Fresh leaves: (a) external lipids, (b) internal lipids. Senescent leaves: (c) external lipids, (d) internal lipids. Leaves from a litter: (e) external lipids, (f) internal lipids. ▼: *n*-Alkanes; ▽: *n*-acids; ◇: α-tocopherol; θ: γ-tocopherol; *: nonacosan-10-ol; □: nonacosan-10-one; ○: dimethoxyalkylcoumarins; ◆: native alkylphenols, and alkylphenols resulting from the spontaneous decarboxylation of the carboxyphenols; ●: isoprenic ketone; +: phytol; ■: phytadienes.

constituents of the external lipids are also detected in the internal lipids (Table 1 and Fig. 1b). This likely reflects the well-documented migration processes (Cassagne and Lessire, 1975; Giese, 1975; Lessire et al., 1980) between the internal and external pools, used to adapt the chemical composition of epicuticular waxes to environmental variations (Herbin and Robins, 1969; Weete et al., 1978; Lockheart et al., 1997). However, *n*-alkanes are not present in the internal lipids, they thus appear as specific compounds to *G. biloba* external leaf lipids. Nonacosan-10-one **2** is also lacking in the internal extract, which indicated that the oxidation of nonacosan-10-ol is a surficial process in the fresh leaves. The abundance of this secondary alcohol, when compared with the phenolics, was much lower than in the external lipids (Fig. 1b), which is in agreement with its role in the formation of wax crystalloids. Internal lipids consist dominantly of phenolic compounds. Alkylphenols **4** constitute the most abundant series of the internal lipids, followed by carboxyphenols **5**; the distribution of these series is the same as in the external lipids. The dimethoxyalkylcoumarin **6** distribution is also similar in internal and external lipids.

In addition to α-tocopherol **3**, trace amounts of γ-tocopherol **8** were detected (cf. Gülz et al., 1992). Acyclic isoprenoids were also identified including phytol, two phytadienes and 6, 10, 14 trimethylpentadecan-2-one **9**. Phytadienes have been shown to be artificially produced from phytol by numerous analytical procedures such as acidification, saponification and high temperatures during GC injection (Grossi et al., 1996). However, phytadienes may constitute original components of *G. biloba* since (i) they are detected in the trimethylsilylated extracts and (ii) Grossi et al. (1996) suggested that analyses of trimethylsilylated extracts produces no phytadienes as artifacts. Phytadienes, phytol and the C_{18} isoprenic ketone are generally considered to be degradation products of the phytyl chain of chlorophyll (de Leeuw et al., 1977; Rontani et al., 1996). Hence, their exclusive presence amongst the internal lipids is consistent with the location of chlorophyll inside leaf tissues, i.e. in the mesophylle.

The carbon range of fatty acids is similar in internal and external lipids (Table 1); however, the distribution is unimodal for internal acids with only one maximum (C_{16}) in the shortest homologues. The *n*-alcohol distribution is also different in internal lipids when compared to those present in the external extract: their chain length is shortened when compared with external alcohols and they exhibit no marked dominant homologue in the internal pool (Table 1). *n*-Acid and *n*-alcohol distributions thus suggest that the longest homologues of these series are relatively more abundant in the external pool than in the internal pool. This finding may be due (i) to a preferential degradation of the shortest compounds

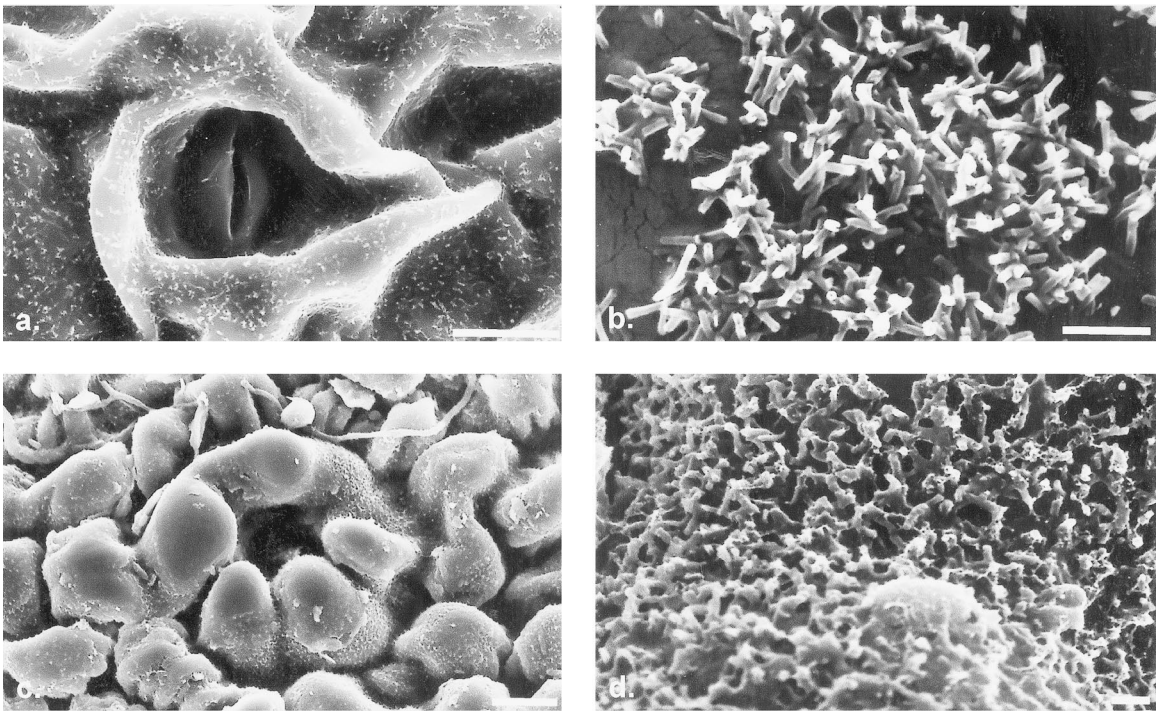

Fig. 2. *G. biloba* surface observed by SEM. Fresh leaves: (a) stomata, (b) arrangement of the wax crystalloids. Leaves from a litter: (c) stomata, (d) aspect of the degraded wax; scale bar = 100 μm in (a) and (c), 1 μm in (b) and (d).

on the leaf surface, or (ii) to a preferential transfer of the longest homologues to the external pool.

3.2. Senescent leaves and leaves from litter

While the content of the internal lipids, as wt.% of the whole leaves, regularly decreases from the fresh to senescent leaves and to the litter, the content of external lipids is quite constant in the three samples studied (Table 2). Since SEM examination provided no evidence for cellular disruption, this maintenance of the relative amount of external lipids cannot be due to contamination by internal lipids during external lipids extraction. Due to the much higher abundance of the internal lipid pool, the net result is a marked decrease in the total content of lipids in the leaves during senescence and litter formation. It appears, therefore, that (i) the external lipids generally exhibit a lower degree of degradation than the other lipid constituents and/or relative increase owing to some transfer from the internal lipids, and (ii) the internal lipids undergo substantial degradation, which accounts for the observed decrease in total lipids, and are generally more affected than the other leaf constituents. Leaf lipid degradation is also reflected by epidermis morphology: while wax crystalloids are still well preserved on the surface of the senescent leaves, they are no longer observed by SEM in the litter (Fig. 2c and d). Crystalline wax is extensively degraded at the top of the epidermis cells which thus appears smooth (Fig. 2c), and the wax remaining is disintegrated without any clearly visible tubule (Fig. 2d).

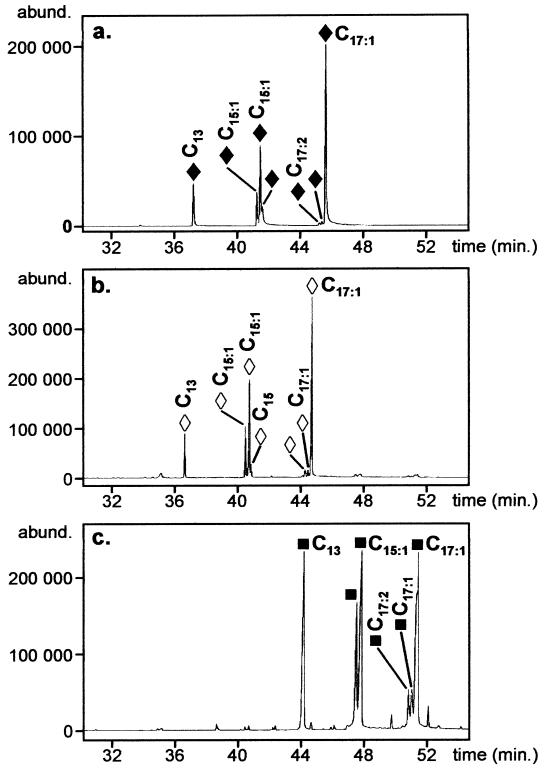

Fig. 3. Extract ion chromatograms (EIC) for the phenolic compounds in external lipids of *G. biloba* fresh leaves. (a) EIC at *m*/*z* 108 of the extract before silylation corresponding to the native alkylphenols and the alkylphenols resulting from the spontaneous decarboxylation of the carboxyphenols; (b) EIC at *m*/*z* 180 of the trimethylsilylated extract corresponding to the alkylphenols; (c) EIC at *m*/*z* 219 of the trimethylsilylated extract corresponding to the carboxyphenols.

In both internal and external pools, the lipid constituents of senescent leaves and of the litter are chiefly the same as those from fresh leaves. The chemical composition does not undergo major qualitative changes, however some variations in the distribution and relative abundance of almost all constituents are noted (Table 1, Fig. 1c, d, e and f). This should reflect different degrees of degradation and/or differential transfers between internal and external lipids.

n-Alkanes exhibit a preferential degradation of the shortest homologues during the early stages of degradation since the C_{21}–C_{24} compounds are not detected in the litter. While the maximum of the *n*-alkane series remains at C_{27} during leaf senescence and litter formation, the odd/even predominance tends to disappear (Fig. 1). That may reflect a bacterial contribution; however, while bacterial *n*-alkanes exhibit a strong even/odd predominance, they generally contain short, C_{16} to C_{20}, homologues (e.g. Ratledge and Wilkinson, 1988). Since the abundance of *n*-alkanes is rather small, the reduction of the odd/even predominance is more likely the result of degradation of these compounds. Indeed, Herbin and Robins (1969) showed that when *n*-alkanes represent a small percentage of the leaf lipids, the odd-over-even carbon number dominance tended to disappear.

Phenolics are the main compound classes of internal lipids from senescent and litter leaves. In the external lipid fraction, the ratio of the amount of phenolic compounds to *n*-alkanes remains constant in the senescent stage (1.10) but decrease to 0.50 in the litter. Phenolic compounds are sensitive to oxidation, which is consistent with the present observations. Carboxyphenols **5** become slightly more abundant than alkylphenols **4** in external lipids. The distribution of the latter series remains unchanged through the early stages of degradation, while that of carboxyphenols exhibits some variations (Table 1).

Table 2
Yield of *Ginkgo bioba* lipids (wt.% of the dried leaves)

Samples	External lipids	Internal lipids	Total lipids
Fresh leaves	0.5	17.1	17.6
Senescent leaves	0.5	12.9	13.4
Litter	0.5	11.4	11.9

The C_{12} homologue becomes dominant in the dimethoxyalkylcoumarins **6** series during the early stages of degradation. Although these compounds decreased to trace amounts in senescent leaves for internal lipids, dimethoxyalkylcoumarins decreased to trace amount only at the litter stage for external lipids.

γ-Tocopherol **9** is not detected in the internal lipids extracted from litter leaves. In contrast, the relative abundance of α-tocopherol **3** with respect to phenolics markedly increases during leaf senescence and litter formation in the internal lipids (0.21 for the fresh leaves when compared with 0.49 and 0.53 for the senescent and litter leaves, respectively). Several hypotheses can be considered to explain this apparent increase in the relative abundance of α-tocopherol. Firstly, this may reflect contributions from microbial tocopherol since most autotrophic microorganisms contain tocopherols (e.g. Hughes and Tove, 1982); however, no trace of microorganisms were detected by SEM examination. Secondly, this increase in the relative abundance of α-tocopherol may reflect a release, upon degradation, of tocopheryl units contained in leaf macromolecules since van Bergen et al. (1994) showed, on fossil seeds, that the insoluble fractions of plants could include tocopheryl units. Finally, such an apparent increase in the relative abundance of α-tocopherol may be due to better preservation of α-tocopherol when compared with phenolics; α-tocopherol is considered to be one of the potential precursors of pristane in sedimentary organic matter (Goossens et al., 1984).

While nonacosan-10-one **2** is not detected internally in fresh leaves, it is detected in small amounts in senescent and litter leaves probably indicating that, during these stages of degradation, nonacosan-10-ol **1** can also be oxidised inside *G. biloba* leaves. Moreover the relative abundance of the secondary ketone with respect to nonacosan-10-ol **1** regularly increases in the external lipids during leaf senescence and litter formation (0.01 for the fresh leaves, when compared with 0.07 and 0.22 for the senescent and litter leaves, respectively). That reflects the progressive oxidation of the secondary alcohol, which probably explains the disintegrated aspect of the wax tubules (Fig. 2c and d). Nevertheless, the relative abundance of nonacosan-10-ol with respect to phenolics (0.07 for the fresh leaves, when compared with 0.28 and 0.40 for the senescent and litter leaves, respectively) or to α-tocopherol (0.30 for the fresh leaves, when compared with 0.57 and 0.76 for the senescent and litter leaves respectively) increases in the internal lipids pointing to slower degradation for nonacosan-10-ol than for phenolics and α-tocopherol. In contrast, in the external lipids, the ratio of the secondary alcohol to the phenolics remains relatively constant through the early stages of degradation, possibly illustrating a continuous exportation of nonacosan-10-ol to the leaf surface where it was also further degraded.

Chlorophyll-derived compounds (phytol, phytadienes and the C_{18} isoprenoid ketone **9**) appear in the external lipids at the senescent stage; they exhibit increasing relative abundances in internal lipids through senescence and litter formation, reflecting their secondary nature. Their absence in the external lipids of fresh leaves suggests that chlorophyll, which is located inside the leaf, was degraded internally and that these degradation products were then exported to the leaf surface.

n-Alcohols exhibit a wider distribution, shifted to shorter homologues, in the external lipids from senescent leaves (C_{14}–C_{28}) when compared with fresh leaves (C_{24}–C_{30}). This may reflect export of the internal alcohols during leaf senescence since the distribution of the latter is characterised by the presence of shorter homologues. The *n*-alcohol series is reduced to a single homologue (C_{18}) in the external lipids of the litter. The distribution of the series in the internal lipids is similar in fresh and senescent leaves, but they are not detected in litter leaves (Table 1). Analyses of external and internal lipids of the litter thus indicate that alcohols, which are known to be labile compounds, are probably extensively degraded by this stage. In spite of the more oxidising conditions at the leaf surface, degradation appears more advanced in the internal lipids than in external ones where the C_{18} homologue is still observed for litter samples. Taken together, these observations suggest that internal alcohols are first exported to the leaf surface, where they are further degraded, during the early stages of degradation.

Although fatty acid distributions remain constant in senescent leaves, they are considerably reduced in litter leaves; the carbon range is shortened in both internal and external lipids, and the distribution becomes unimodal in the external lipids (Table 1). The preferential degradation of the longest homologues thus observed is unusual (e.g. Lajat et al., 1990; Meyers and Eadie, 1993). This may be due to (i) a partial migration of acids from the internal pool (which does not exhibit a submaximum for long chain fatty acids) to the leaf surface, and/or (ii) the shortening of long chain fatty acids under the degradative effects of bacteria, as suggested by Hankin and Kolattukudy (1968).

4. Conclusions

Chemical characterisation of the lipids of the fresh leaves of *G. biloba* led to the identification of additional compounds to those previously found (e.g. Casal and Moyna, 1979; Gülz et al., 1992), including α-tocopherol and phenolic components (alkylphenols, carboxyphenols and dimethoxyalkylcoumarins). The latter could constitute distinctive biomarkers for *G. biloba* since (i) they are present in substantial amounts in the leaves and (ii) they have been detected in other organs such as seeds and fruits.

Separate analyses of the internal and external lipids showed the predominance of the internal pool (>90% of the total lipids). A number of common compounds occur in these two pools, in agreement with migration processes. However, several qualitative and quantitative differences are observed: (i) absence of nonacosan-10-one and *n*-alkanes in the internal pool, (ii) dominance of phenolics in this pool while nonacosan-10-ol is much more abundant in the external lipids, (iii) presence of γ-tocopherol and chlorophyll-derived products (phytol, phytadienes and the C_{18} isoprenoid ketone **9**) in the internal pool, and (iv) differences in fatty acid and primary alcohol distributions (shorter homologues are relatively more abundant in the internal pool).

Comparison of the fresh, senescent and litter leaves showed that the content of internal lipids (as wt.% of whole leaves) decreased, whereas the lipids of the external pool remained relatively constant. It therefore appears that the internal lipids as a whole, are more degraded than other leaf constituents. The external pool exhibits a lower degree of alteration and/or benefits from transfers of the internal pool. Molecular studies indicate that all the lipid constituents of *G. biloba* leaves were affected to a certain degree by degradation, but the chemical composition of the external and internal pools do not undergo major qualitative changes during senescence and litter formation. However, variations in the distributions and relative abundances of most leaf lipid constituents are noted. Thus, the differences are in agreement with the stability scale established by Cranwell (1981) for lipid preservation in sediments. *n*-Alkanes, nonacosan-10-ol, α-tocopherol and, to a lesser extent, fatty acids are relatively less degraded whereas phenolic components and primary alcohols are preferentially altered. Nevertheless, phenolics still dominate the internal pool in the litter leaves, and might be useful as biomarkers of *G. biloba*. The co-occurrence of α-tocopherol and nonacosan-10-ol has also a potential as a marker of *G. biloba* in palaeoenvironments.

Some of the degradation patterns observed (decrease of the dimethoxyalkylcoumarins to trace amounts, degradation of *n*-alcohols) appear earlier in the internal pool than in the external one in spite of the more oxidising conditions at the leaf surface. Such a feature, in conjunction with the observations of degradation products of chlorophyll, nonocosan-10-ol, changes in *n*-alcohol distribution, and lipid contents strongly suggests that most of the internal lipid constituents continue to be exported to the leaf surface where they are further degraded during senescence and litter formation.

Acknowledgements

We are grateful to M. Grably, C. Girardin, G. Bardoux and especially Y. Pouet for technical assistance and GC/MS facilities. We would like to thank S. Bertholon for providing the *G. biloba* samples. We are also indebted to N. Poirier, B. Allard, A. Derieux, P. Metzger and Y. Zegouah for helpful discussions. This paper was significantly improved thanks to suggestions by P.F. van Bergen. Thanks to J.W. Collister and one anonymous reviewer who provided constructive reviews of the manuscript. The study was supported by grants from *Fédération de Recherche en Ecologie Fondamentale et Appliquée* (F.R.3), *C.N.R.S.* and especially *Naturalia et Biologia*.

Associate Editor—**J.W. Collister**

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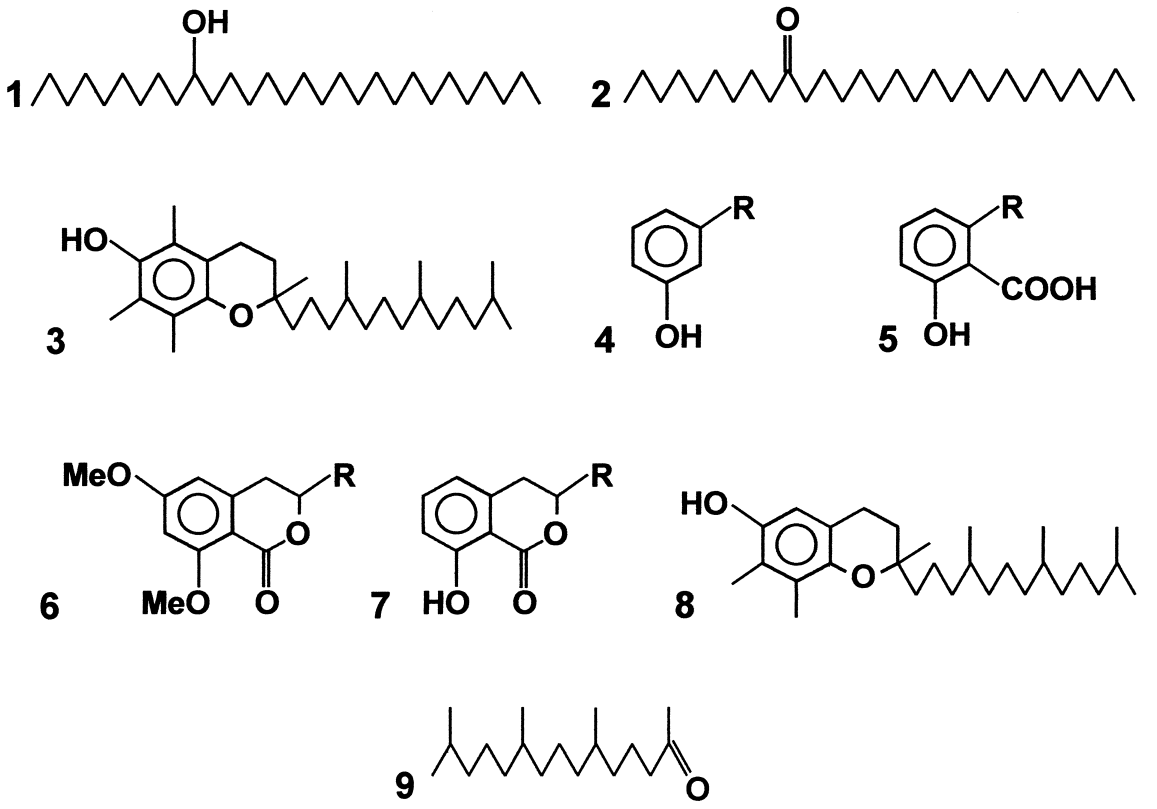
Appendix



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