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# Visible light-induced oxidation of unsaturated components of cutins: a significant process during the senescence of higher plants

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**Abstract**

9-Hydroperoxy-18-hydroxyoctadec-10(*trans*)-enoic and 10-hydroperoxy-18-hydroxyoctadec-8(*trans*)-enoic acids deriving from type II (i.e. involving $^1O_2$) photooxidation of 18-hydroxyoleic acid were detected after visible light-induced senescence experiments carried out with *Petroselinum sativum* and subsequent cutin depolymerisation. These results showed that in senescent plants, where the $^1O_2$ formation rate exceeds the quenching capacity of the photoprotective system, $^1O_2$ can migrate outside the chloroplasts and affect the unsaturated components of cutins. Significant amounts of 9,18-dihydroxyoctadec-10(*trans*)-enoic and 10,18-dihydroxyoctadec-8(*trans*)-enoic acids resulting from the reduction of these photoproducts of 18-hydroxyoleic acid were also detected in different natural samples. These results well support the significance of the photooxidation of the unsaturated components of higher plant cutins in the natural environment.


*Keywords:* *Petroselinum sativum*; Cutins; Higher plants; Senescence; 18-Hydroxyoleic acid; Photooxidation; Singlet oxygen

## 1. Introduction

Photochemical damage in cells of phototrophic organisms is not exclusively caused by UV radiation (Nelson, 1993). In fact, owing to the presence of chlorophylls, which are efficient photosensitizers (Foote, 1976), numerous lipid components of these organisms are being photodegraded during senescence by photosynthetically active radiation (Rontani, 2001).

When a chlorophyll molecule absorbs a quantum of light energy, an excited singlet state ($^1$Chl) is formed. In healthy cells of phototrophic organisms the primary route for energy from the excited chlorophyll singlet state ($^1$Chl) is the fast photochemical reactions of photosynthesis (Foote et al., 1970). In senescent cells this pathway is not functional; thus accelerated production of the longer-lived triplet state ($^3$Chl; by intersystem crossing) and toxic oxygen species (singlet oxygen ($^1O_2$), hydrogen peroxide, superoxide ion and hydroxyl radical; by reaction of $^3$Chl with ground state oxygen) are expected (Rontani, 2001). The rate of formation of these potentially damaging species can then exceed the quenching capacity of the photoprotective system of the cells and photodegradation can occur (Merzlyak and Hendry, 1994). Probably due to its long lifetime in hydrophobic micro-environments and thus in

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phytodetritus (Suwa et al., 1977), $^1O_2$ plays a key role in the photodegradation of most of the unsaturated lipid components of phototrophic organisms (i.e. chlorophyll phytyl side-chain, sterols and unsaturated fatty acids) (Rontani, 2001, 2005).

In healthy cell environment where physical and chemical quenchers abound, the average lifetime of $^1O_2$ has been estimated to be 10–40 ns, which is <1% of its lifetime in pure water (Rodgers and Snowden, 1982). This corresponds to an $^1O_2$ diffusion distance of less than 10 nm, which is less than the thickness of a cell membrane (Girotti, 2001). This suggests that $^1O_2$ produced within a cell would have little chance of escaping. However, this is not the case in senescent phytoplanktonic cells. Here, where the $^1O_2$ formation rate exceeds the quenching capacity of the photoprotective system, $^1O_2$ can migrate outside the phytodetritus and affect the attached bacteria (Rontani et al., 2003).

In the present work, we intend to check in higher plants whether $^1O_2$ produced during the senescence can migrate outside the chloroplasts and affect some unsaturated components of cutin. Cutin is present in the cuticle (the outer layer of the epidermal cells of primary plant tissues, such as leaves) (Graça et al., 2002), it is known to be an insoluble polyester polymer whose depolymerisation mainly affords $C_{16}$ and $C_{18}$ ω-hydroxycarboxylic acids (Deas and Holloway, 1977; Kolattukudy, 1977, 1980). Some of the unsaturated $C_{18}$ ω-hydroxycarboxylic acids such as 18-hydroxyoctadec-9(*cis*)-enoic (18-hydroxyoleic) and 18-hydroxyoctadeca-9(*cis*),12(*cis*)-dienoic (18-hydroxylinoleic) acids (Kolattukudy, 1980) constitute potential targets for $^1O_2$. This study focuses on the 18-hydroxyoleic acid since it is widely distributed in higher plants (Kolattukudy, 1980) and its type II (i.e. involving $^1O_2$) photooxidation products are relatively stable and easy to characterize.

## 2. Results and discussion

### 2.1. Photosensitized oxidation of 18-hydroxyoleic acid in solution

As in the case of monounsaturated fatty acids (Frankel, 1998), photosensitized oxidation of 18-hydroxyoleic acid involves a direct reaction of $^1O_2$ with the carbon–carbon double bond by a concerted ''ene'' addition (Frimer, 1979) and leads to the formation of a hydroperoxide at each unsaturated carbon. This results to the production of a mixture of 9- and 10-hydroperoxides with an allylic *trans* double bond (Fig. 1).

After irradiation of 18-hydroxyoleic acid in pyridine in the presence of haematoporphyrin as sensitizer and subsequent $NaBH_4$ reduction and silylation, a group of two unresolved peaks corresponding to 9,18-dihydroxyoctadec-10(*trans*)-enoic and 10,18-dihydroxyoctadec-8(*trans*)-enoic acids was detected (Fig. 2(b)). These compounds were characterized by GC/EIMS on the basis of allylic cleavage at the carbon bearing –$OSiMe_3$ groups (Fig. 2(a)). The fragment ion at *m*/*z* 288 corresponds to a known rearrangement of a trimethylsilyl group towards the carboxylic group of 9,18-dihydroxyoctadec-10(*trans*)-enoic acid (Capella and Zorzut, 1968; de Leeuw et al., 1980).

Fairly polar solvents such as chloroform favour allylic rearrangement of allyl hydroperoxides (Lythgoe and Trippett, 1959). These processes, which can act on *cis* and *trans* allyl hydroperoxides, are highly stereospecific and produce only *trans* configurations (Porter et al., 1995). In chloroform, 9-hydroperoxy-18-hydroxyoctadec-10-enoic and 10-hydroperoxy-18-hydroxy-octadec-8-enoic acids rearrange to 11-hydroperoxy-18-hydroxyoctadec-9(*trans*)-enoic and 8-hydroperoxy-18-hydroxyoctadec-9(*trans*)-enoic acids, respectively, (Fig. 1). The coelution of the corresponding silylated 9,18-, 10,18-, 11,18- and 8,18-dihydroxyacids allowed to confirm the *trans* configuration of the double bond of the initial 9- and 10-hydroperoxides.

### 2.2. Irradiation of Petroselinum sativum leaves

In order to study the photochemical fate of 18-hydroxyoleic acid in cutins, we irradiated *P. sativum* leaves. Parsley was selected for these experiments on the basis of the relatively high 18-hydroxyoleic acid content of its cutins (Fig. 3(a)). The fraction resulting from cutin depolymerisation was in fact mainly comprised of even-carbon number dominated $C_{22}$–$C_{30}$ *n*-alkan-1-ols and 16-hydroxyhexadecanoic, 18-hydroxyoleic, 18-hydroxylinoleic and isomeric 10(8-10),16-dihydroxyhexadecanoic acids (Fig. 3(a)). Detached leaves were allowed to senesce in darkness or in light for different periods. During visible light-induced incubation parsley leaves underwent yellowing and then browning without notable fungal infection, while under darkness leaves remained green. Estimation of photodegradation progress involved quantification of 3-methylidene-7,11,15-trimethylhexadecan-1,2-diol, 9-hydroxy-octadec-10-enoic and 10-hydroxyoctadec-8-enoic acids in the extracts obtained before depolymerisation. The presence of these well known specific oxidation products of chlorophyll phytyl side-chain (Rontani, 2001) and oleic acid (Frankel, 1998), respectively, attests to the involvement of $^1O_2$-mediated oxidation processes in parsley leaves. After incubation, high amounts of methoxyhydrins and chlorohydrins resulting from the degradation of 9,10-epoxy-18-hydroxyoctadecanoic and 9,10-epoxy-18-hydroxyoctadec-12-enoic acids during the treatment (Holloway and Deas, 1973) appeared in the fraction resulting from cutin

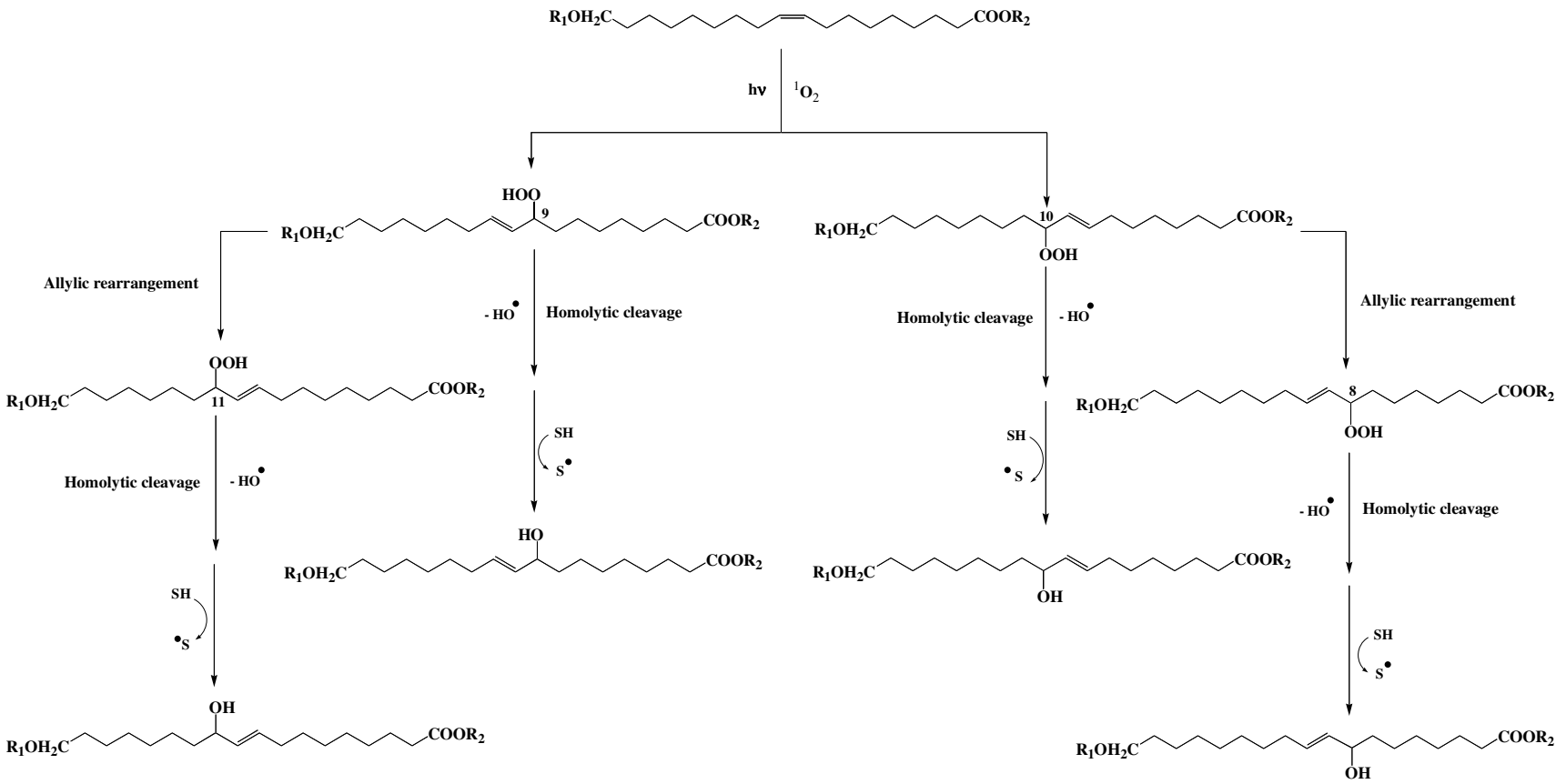


Fig. 1. Photosensitized oxidation of 18-hydroxyoleic acid in cutin polymers.

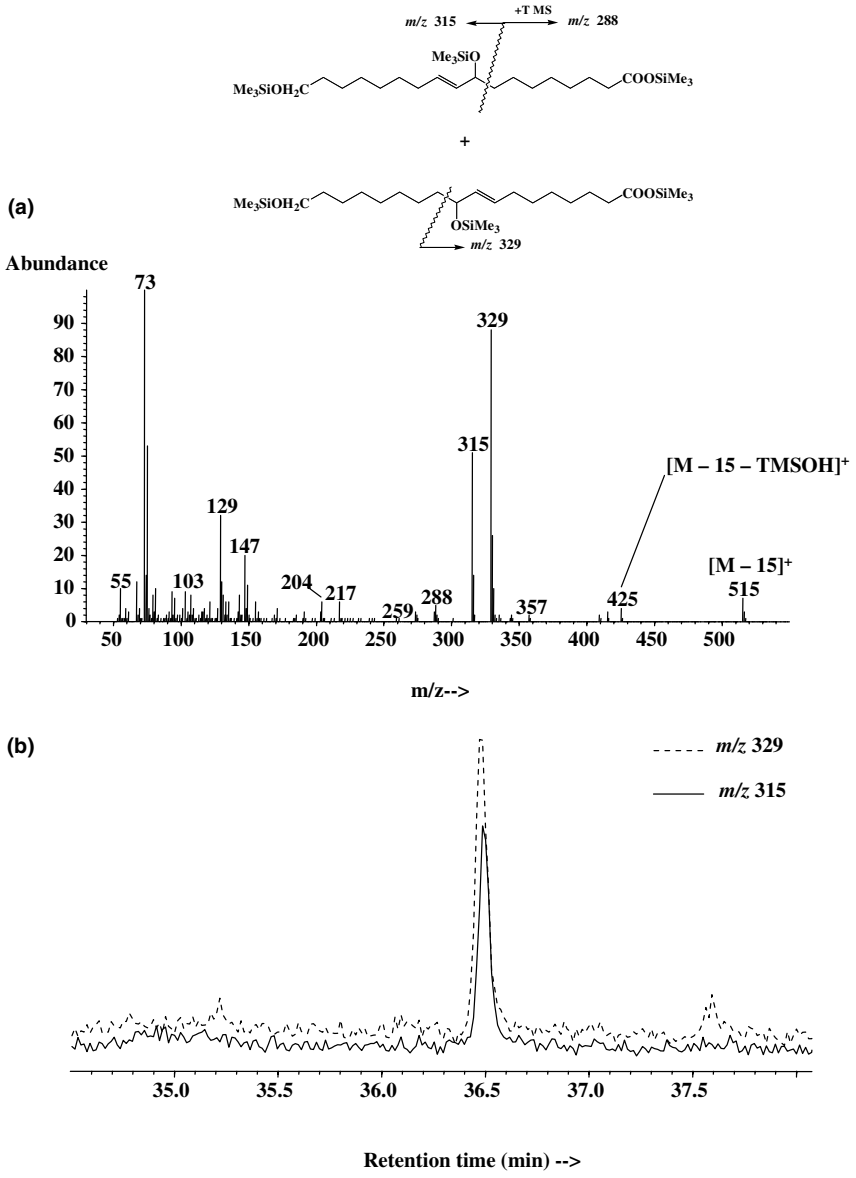


Fig. 2. EI mass spectrum (a) and mass chromatograms of *m/z* 329 and 315 (b) showing the production of 9,18-dihydroxyoctadec-10(*trans*)-enoic and 10,18-dihydroxyoctadec-8(*trans*)-enoic acids after photosensitized oxidation of 18-hydroxyoleic acid in the presence of haematoporphyrin (as sensitizer) and subsequent $NaBH_4$-reduction and silylation.

depolymerisation (peaks 6–9 in Fig. 3(b)). These $C_{18}$ epoxyacids, which were also present but in small amounts in fresh leaves, are well known components of cutins (Holloway, 1973; Holloway and Deas, 1973; Kolattukudy, 1980; Blée, 1998, 2002). The formation of these epoxides was attributed to the involvement of a peroxygenase (Lequeu et al., 2003). In parsley, this enzyme seems to mainly intervene during the senescence. The reactive properties attributed to hydroperoxides have led to speculation that peroxygenases participate in the senescence process in plants (Thompson et al., 1998), but there are conflicting reports in the literature (Grossman and Leshem, 1978; Peterman and Siedow, 1985; Prakash et al., 1990). Thus, the involvement of such enzymes during senescence remains today a completely open question (Berger et al., 2001).

In addition to the degradation products of epoxides, we observed the production of significant amounts of 9,18-dihydroxyoctadec-10(*trans*)-enoic and 10,18-dihydroxyoctadec-8(*trans*)-enoic acids after irradiation (peak 5 in Fig. 3(b)). These compounds were only present in trace amounts before irradiation (peak 5 in Fig. 3(a)) or after incubation under darkness. The production of these compounds, which result from the photosensitized oxidation of 18-hydroxyoleic acid (see above) may be attributed to: (i) the migration of $^1O_2$ from the chloroplasts to the cuticle during the senescence or (ii) the $^1O_2$-mediated oxidation of 18-hydroxyo-

* *n*-Alkanols
**1**- 16-Hydroxyhexadecanoic acid
**2**- 18-Hydroxylinoleic acid
**3**- 18-Hydroxyoleic acid + docosan-1-ol
**4**- 9(8-10),16-Dihydroxyhexadecanoic acids
**5**- 9(8-11),18-Dihydroxyoctadec-10(8-10)(*trans*)-enoic acids
**6**- 9,18-Dihydroxy-10-methoxyoctadec-12-enoic + 10,18-dihydroxy-9-methoxyoctadec-12-enoic acids
**7**- 9,18-Dihydroxy-10-methoxyoctadecanoic + 10,18-dihydroxy-9-methoxyoctadecanoic acids
**8**- 9,18-Dihydroxy-10-chlorooctadec-12-enoic + 10,18-dihydroxy-9-chlorooctadec-12-enoic acids
**9**- 9,18-Dihydroxy-10-chlorooctadecanoic + 10,18-dihydroxy-9-chlorooctadecanoic acids
**10**- 3-Methylidene-7,11,15-trimethylhexadecan-1,2-diol (phytyldiol)

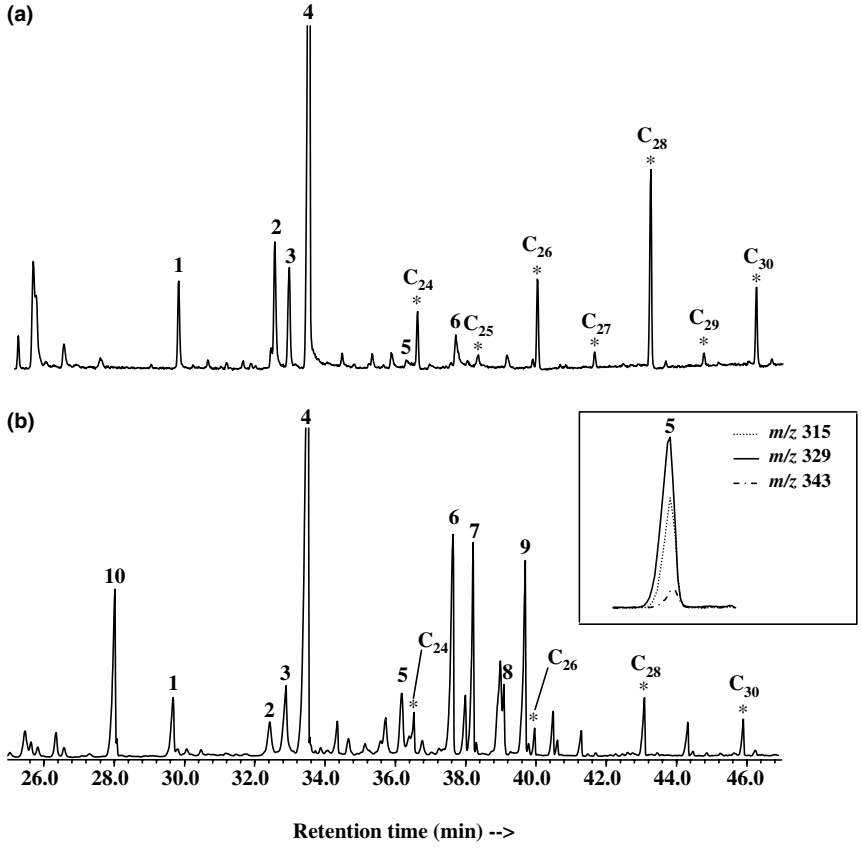


Fig. 3. Partial total ion current chromatogram of the silylated $NaBH_4$-reduced acidic fraction resulting from the cutin depolymerisation of *P. sativum* before (a) and after (b) visible light irradiation for 12 weeks (800 mol of photons $m^{-2}$).

leic acid within chloroplasts followed by its subsequent release. The last hypothesis was discarded since extracts obtained before depolymerisation contained only trace amounts of 18-hydroxyoleic acid.

Photochemically produced hydroperoxides may undergo three main degradative processes: (i) highly stereoselective free radical allylic rearrangement (Porter et al., 1995); (ii) homolysis of the O–O bond leading to carbonyl (dehydration), alcoholic (reduction), fragmentation (β-scission) or oxirane (radical cyclization) products (Frimer, 1979); (iii) heterolysis of the O–O bond leading to the formation of two carbonyl fragments (Hock cleavage), this proton-catalysed cleavage being initiated by migration of groups to positive oxygen (Frimer, 1979). After irradiation, the presence of 11,18-dihydroxyoctadec-9(*trans*)-enoic acid (exhibiting an informative mass spectrometric fragmentation at *m/z* 343 after silylation) (Fig. 3(b)) demonstrates the involvement of allylic rearrangement in senescent parsley leaves. These processes, which weakly intervene in phytodetritus (Rontani, 2001), seem to act more intensively in higher plants. Some tests were carried out in order to determine whether hydroperoxyacids, hydroxyacids or oxoacids were present before the treatment. The residues obtained after extraction were divided into two. One half was reduced with $NaBD_4$ and saponified, and the other acetylated (to dehydrate hydroperoxides to ketones) before saponification (Marchand and Rontani, 2001). Comparison of the amounts of unlabelled hydroxyacids present after acetylation

(naturally occurring hydroxyacids) and after reduction (naturally occurring + hydroperoxide reduction-derived hydroxyacids) allowed estimation of the proportion of hydroperoxyacids and hydroxyacids present in the samples, while quantification of labelled hydroxyacids after reduction afforded the amount of oxoacids. The allylic oxoacids could not be directly characterised since these compounds do not survive alkaline hydrolysis and are cleaved after hydration and retro-aldol reactions (Marchand and Rontani, 2001). The results obtained demonstrated that before the treatment 18-hydroxyoleic acid photoproducts comprised 90% of hydroperoxides, 10% of hydroxyacids and traces of oxoacids. Heterolytic cleavage of 9(8-11)-hydroperoxy-18-hydroxyoctadec-10(8-10)-enoic acids must also probably intervene; however, it was very difficult to differentiate the compounds resulting from this cleavage from these resulting from the cleavage of fatty acids oxidation products (Marchand and Rontani, 2001).

Interestingly, 18-hydroxylinoleic acid appeared to be photodegraded more quickly than 18-hydroxyoleic acid. This is in good agreement with the well known increasing rates of type II (i.e. involving singlet oxygen) photooxidation of fatty acids with their degree of unsaturations (Frankel, 1998; Rontani et al., 1998). Despite the strong degradation of 18-hydroxylinoleic acid, we failed to detect significant amounts of photoproducts deriving from this hydroxyacid. This is possibly due to: (i) the instability or the volatility of these compounds or (ii) the involvement of cross-linking reactions leading to the formation of macromolecular structures (Neff et al., 1988) non-amenable to gas chromatography.

### 2.3. Presence of photoproducts of 18-hydroxyoleic acid in environmental samples

The detection of substantial amounts of photoproducts of 18-hydroxyoleic acid in particulate matter and recent sediment samples collected in Mediterranean sea and in Camargue microbial mats (Table 1, Fig. 4) allowed to show that under natural conditions photochemical processes act extensively on the unsaturated components of higher plant cutins.

The involvement of allylic rearrangement is well supported by the presence of significant amounts of 11,18-dihydroxyoctadec-9(*trans*)-enoic acid in all the samples analyzed (Fig. 4). Parallel $NaBD_4$ reduction and acetylation assays (as previously described; Marchand and Rontani, 2001) allowed us to demonstrate that before the treatment 9(8-11)-hydroperoxy-18-hydroxyoctadec-10(8-10)-enoic acids were fully reduced to the corresponding hydroxyacids in the different samples analyzed. This result is in good agreement with the strong reduction of hydroperoxides deriving from the oxidation of fatty acids previously observed in Camargue microbial mats (Marchand and Rontani, 2003). This reduction was attributed to the high amounts of sulfides produced by sulfate-reducing bacteria in the mats especially during the night (Wieland and Kühl, 2001), which are known to easily reduce hydroperoxides (Mihara and Tateba, 1986). In contrast, hydroperoxides deriving from the oxidation of fatty acids or sterols are present in relatively high proportions in the particulate matter and recent sediments analyzed (Rontani and Marchand, 2000; Marchand and Rontani, 2001). The surprising strong reduction of 9(8-11)-hydroperoxy-18-hydroxyoctadec-10(8-10)-enoic acids observed in such samples could be attributed to the presence of highly detritic terrestrial material (probably originating from aeolian or Rhône inputs) in these samples. Indeed, the reduction of hydroperoxides deriving from the photooxidation of unsaturated components of higher plant cutins, which appeared to be relatively slow in senescent plants (see above), could intervene significantly in very old terrestrial material.

## 3. Conclusions

Visible light-dependent degradation of 18-hydroxyoleic acid has been studied in pyridine in the presence of haematoporphyrin as photosensitizer. These processes resulted to the production of 9-hydroperoxy-18-hydroxyoctadec-10(*trans*)-enoic and 10-hydroperoxy-18-hydroxyoctadec-8(*trans*)-enoic acids. These compounds were then detected after irradiation of senescent leaves of *P. sativum* in the acidic fraction resulting from cutin

Table 1
Concentrations (μg $g^{-1}$ dry weight) of 18-hydroxyoctadec-9-enoic acid and its photoproducts in particulate matter, sediment and microbial mat samples collected in Mediterranean sea

| | SOFI station particulate matter (m) | | SOFI station sediments (cm) | | | | | | Camargue microbial mats (mm) |
|---|---|---|---|---|---|---|---|---|---|
| | 56.5 | 142 | 0–2 | 2–4 | 4–6 | 6–8 | 8–10 | 10–12 | 0–5 |
| 18-Hydroxyoctadec-9-enoic acid | 2.0 | 1.7 | 1.6 | 0.8 | 0.8 | 1.3 | 0.7 | 1.5 | 4.6 |
| 9(8-11),18-Dihydroxyoctadec-10(8-10)-enoic acids | 2.3 | 1.6 | 0.3 | 0.3 | 0.4 | 0.4 | 0.2 | 0.4 | 7.0 |

**1**- 16-Hydroxyhexadecanoic acid
**2**- Hexadecane-1,16-dioic acid
**3**- 18-Hydroxyoleic acid
**4**- 9(8-10),16-Dihydroxyhexadecanoic acids
**5**- Octadec-9-ene-1,18-dioic acid
**6**- 9(8-10)-Hydroxyhexadecane-1,16-dioic acids
**7**- Octadecane-1,18-dioic acid
**8**- 9(8-11),18-Dihydroxyoctadec-10(8-10)(*trans*)-enoic acids
**9**- 20-Hydroxyeicosanoic acid
**10**- 9-Hydroxyoctadec-10-ene-1,18-dioic acid
**11**- Eicosane-1,20-dioic acid
**12**- 9,18-Dihydroxy-10-methoxyoctadecanoic + 10,18-dihydroxy-9-methoxyoctadecanoic acids
**13**- 9,10,18-Trihydroxyoctadecanoic acid (mixture of diastereoisomers)
**14**- 9,18-Dihydroxy-10-chlorooctadecanoic + 10,18-dihydroxy-9-chlorooctadecanoic acids
**15**- 22-Hydroxydocosanoic acid
**16**- Docosane-1,22-dioic acid

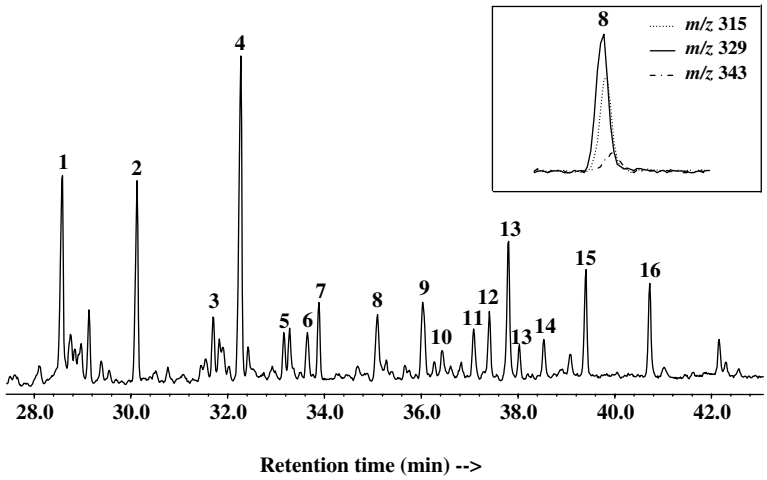


Fig. 4. Partial total ion current chromatogram of the silylated $NaBH_4$-reduced saponified fraction of Camargue microbial mats (core section 0–5 mm).

depolymerisation. This demonstrates that during the senescence of higher plants type II (i.e. involving $^1O_2$) photooxidation processes may act not only in chloroplasts (Rontani et al., 1996) but also in cuticles. In senescent plants, where the $^1O_2$ formation rate exceeds the quenching capacity of the photoprotective system, $^1O_2$ can migrate outside the chloroplasts and affect the unsaturated components of cutins.

Significant amounts of dihydroxyoctadecenoic acids resulting from the reduction of the photoproducts of 18-hydroxyoleic acid were also detected in particulate matter and recent sediment samples collected in Mediterranean sea and in Camargue microbial mats. These results well support the significance of the photooxidation of the unsaturated components of higher plant cutins in the natural environment.

## 4. Experimental

### 4.1. Chemicals

Heterologous expression of CYP94A1 in yeast and production of 18-hydroxyoleic acid: CYP94A1 was expressed in *Saccharomyces cerevisiae* as previously described (Tijet et al., 1998). Yeast cells were harvested, broken with glass beads (0.45 mm diameter) and microsomes were prepared by differential centrifugation. Microsomal membranes were resuspended in 100 mM sodium phosphate (pH 7.4), 30% (v/v) glycerol and 1.5 mM mercaptoethanol. The 18-hydroxyoleic acid was produced by incubation of $^{14}C$ radiolabelled oleic acid (50 Ci $mol^{-1}$ from NEN-Dupont, Stevenage, UK) with microsomes of transformed yeast. The standard assay (0.2 ml) contained 20 mM sodium phosphate (pH 7.4), 1 mM NADPH, plus a regenerating system (consisting of a final concentration of 6.7 mM Glc-6-P and 0.4 U of Glc-6-P dehydrogenase) and radiolabelled oleic acid (100 μM). The reaction was initiated by the addition of NADPH and was stopped by the addition of 0.1 ml of acetonitrile (0.2% acetic acid). The reaction products were resolved by TLC as described below. Incubation media were directly spotted on TLC plates, which were developed with a mixture of diethyl ether-light petroleum (boiling point, 40–60 °C) formic acid (50:50:1, v/v/v). The plates were scanned with a thin-layer scanner (Berthold LB 2723). The area corresponding to the 18-hydroxyoleic acid was scrapped and the metabolite

was eluted from the silica with 10 ml of diethyl ether–hexane (50:50, v/v) which was subsequently removed by evaporation.

9-Hydroperoxy-18-hydroxyoctadec-10(*trans*)-enoic and 10-hydroperoxy-18-hydroxy-octadec-8(*trans*)-enoic acids were produced after irradiation of 18-hydroxyoleic acid in dry pyridine in the presence of haematoporphyrin as sensitizer (Nickon and Bagli, 1961). 8-Hydro-peroxy-18-hydroxyoctadec-9(*trans*)-enoic and 11-hydroperoxy-18-hydroxy-octadec-9(*trans*)-enoic acids were obtained after highly stereospecific allylic rearrangement of these hydroperoxides in chloroform (Lythgoe and Trippett, 1959). Subsequent reduction of the different hydroperoxides in methanol with excess $NaBH_4$ afforded the corresponding dihydroxyacids.

### 4.2. Plant material

Parsley (*P. sativum*) leaves were purchased fresh in the market.

### 4.3. Photodegradation experiments

Leaf segments (1 g) were allowed to senesce at 20 °C either in light or in darkness in non-sterilized Petri dishes containing dry filter paper. Irradiations were carried out with two 30 W fluorescent lamp (Osram, Fluora). These lamps provided a spectral distribution of light well suited for photobiological processes involving chlorophyll-*a*. Irradiance (as PAR) was measured using a Licor LI 1000 data logger equipped with a LI 1935A spherical quantum sensor.

### 4.4. Leaf treatment

Leaf segments were placed in a mortar and intensively ground in the presence of methanol (30 ml per g). The extraction was repeated five times and the methanol extracts discarded. The residue was subsequently reduced in methanol (25 ml) by excess $NaBH_4$ or $NaBD_4$ using magnetic stirring for 30 min. After reduction, 25 ml of water and 2.8 g of potassium hydroxide were added for depolymerisation and the mixture was directly saponified by refluxing for 2 h. After cooling, the content of the flask was acidified to pH 1 and extracted three times with dichloromethane. The combined extracts were dried over anhydrous $Na_2SO_4$, filtered and concentrated by rotary evaporation.

### 4.5. Particulate matter, sediment and microbial mat samples

The SOFI station is located in the Gulf of Lion (Mediterranean sea) approximately 30 km from Marseilles at 43°04′ N, 5°08′ E. Two sediment traps (PPS5) with a 1 $m^2$ opening were deployed on 10/12/1997 along a fixed mooring under the euphotic layer (56.5 m) and 20 m from the bottom (142 m) and recovered on 20/02/1998. In order to avoid bacterial decomposition, the sample cups were filled before deployment with filtered (0.2 μm) seawater containing 5% of formaldehyde and 1.1 g $l^{-1}$ of sodium tetraborate. The top layer of the bottom sediment (12 cm) was collected at 162 m depth with a multitube corer (Multicoror type MarK IV, Bowers and Connelly, UK) (core diameter, 15 cm).

The microbial mats were collected with manual corers in Camargue (France) at sampling station no. 1 of the MATBIOPOL European project (43°30′ N, 4°30′ E). This station is a pristine site located in a large pond in the south of the Rhône delta, close to the sand barrier on the sea coast. The pond is used for the storage of water by the salterns of Salins de Giraud.

The treatments of these different samples were described previously (Marchand and Rontani, 2001, 2003).

### 4.6. Derivatization

After solvent evaporation, the residues were taken up in 300 μl of a mixture of pyridine and BSTFA (Supelco; 2:1, v/v) and silylated for 1 h at 50 °C. After evaporation to dryness under nitrogen, the residues were taken up in a mixture of ethyl acetate and BSTFA and analysed by gas chromatography/electron impact mass spectrometry (GC/EIMS).

### 4.7. Identification and quantification of oxidation products

Components were identified by comparison of retention times and mass spectra with those of standards and quantified (calibration with external standards) by GC/EIMS. For low concentrations or in the case of coelution, quantification was assessed by selected ion monitoring (SIM). Derivatives of regioisomeric hydroxy compounds, which often coelute, can be distinguished by their EI mass spectra to a great extent, since the main fragments are caused by α-cleavage relative to the functional group. The isomers could thus be detected and quantified from the ion currents of the specific fragment ions.

GC/EIMS analyses were carried out with a HP 5890 series II plus gas chromatograph connected to a HP 5972 mass spectrometer. The following conditions were used: 30 m × 0.25 mm (i.d.) fused silica column coated with HP5 (Hewlett–Packard; film thickness, 0.25 μm); oven temperature programmed from 60 to 130 °C at 30 °C $min^{-1}$ and then from 130 to 300 °C at 4 °C $min^{-1}$; carrier gas (He) maintained at 1.04 bar until the end of the temperature program and then programmed from 1.04 to 1.5 bar at 0.04 bar $min^{-1}$; injector (on column) temperature, 50 °C; electron energy, 70 eV; source temperature, 170 °C; cycle time, 1.5 s.

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