Molecular studies of fresh and aged triterpenoid varnishes

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5. Artificial ageing of varnish triterpenoids in solution

Abstract

A new method is presented for the artificial ageing of triterpenoid samples. Varnish samples in solution are exposed to the radiation of a fluorescent tube device. The solvents dichloromethane and acetone, and the photosensitisers Merocyanine 540 and FotoFenton 2 dissolved in acetonitrile or dichloromethane were found to generate reactive species, which induce similar oxidation and cross-linking processes in the triterpenoid samples as found in varnish films on paintings. Information was obtained on the oxidation mechanisms of the model compounds hydroxydammarenone and oleanolic acid. Hydroxydammarenone is first oxidised to an ocotillone type molecule, which is subsequently oxidised to a lactonised molecule. In addition, a compound with a shortened side chain is formed, probably from the ocotillone type molecule. Oxidation of the A-ring was not observed. The solvent ageing of oleanolic acid first results in the formation of small amounts of compounds with additional keto groups at C3 and C11. In addition, the double bond at C12 of oleanolic acid becomes oxidised and a keto group is formed at this position. The compound formed by this latter process is the main compound after prolonged solvent ageing. Decarboxylation of the carboxylic acid at C17 of oleanolic acid was not observed. This method of solvent ageing can be successfully used for the preparation of cross-linked fractions of aged dammar and mastic varnishes, in addition to the investigation of the oxidation mechanism of triterpenoids.

5.1. Introduction

Dammar and mastic are complex mixtures of a large number of triterpenoids with different carbon skeletons. As discussed in Chapter 2, both fresh dammar and mastic resin consist of more than 10 triterpenoid compounds. Films of varnishes subjected to either ‘natural’ or artificial ageing, see Chapter 3 and 4,
result in even more complex mixtures due to oxidation and cross-linking processes. Especially side chain oxidation of dammarane type molecules and oxidation of C11, C17 and C28 of oleanane/ursane type molecules was found to take place. However, it is difficult to assess the exact oxidation mechanisms of the triterpenoid constituents by investigation of the chemical composition of ‘naturally’ or artificially aged varnishes. It is more straightforward to model the oxidation processes, which are found to take place in paintings, by subjecting a single pure triterpenoid compound to these ‘ageing’ processes. Naturally, one would like to age the model compound as a film, because a varnish ages on a painting as a film as well. However, unlike a fresh resin solution, the solution of one pure compound in a solvent does not produce a homogeneous solid film. The addition of compounds with good film forming properties, such as an acrylate, may improve this, but this addition may complicate the investigation of the ageing processes. Therefore, a new approach was developed to investigate the ageing of triterpenoids in a dissolved state.

Specific types of reactive species can be generated in solution, which initiate and generate ageing reactions in triterpenoid solutions. Therefore, this approach allows for more control over the modelling experiments. The reagents can move freely in the solution, unlimited by low diffusion rates encountered in solid films. Therefore, the rate of the ageing reactions in the solution is no doubt higher than that in a solid film. By ageing in solution, other disadvantages related to the ageing of films are avoided. For example, the comparison of films is complicated when their surfaces are not constant. Subsequently the availability of oxygen and light energy is not constant. Another disadvantage is the observation that photochemical damage is largely a surface phenomenon [1], which implies that an aged resin film is not uniform in chemical composition.

Ageing processes are thought to occur via radical reactions. Photochemical methods, which generate a large number of radicals or other reactive species in the reagent solution, were investigated for their capability to ‘artificially age’ triterpenoids. Photosensitisers can be used for this purpose, which are compounds that create these reactive species in the presence of light. The photosensitiser absorbs at the irradiating wavelength. It may subsequently transfer its energy to either a substrate or to molecular oxygen. Furthermore, it may exchange an electron or hydrogen atom with the substrate or exchange an electron with oxygen. Depending on the type of photosensitiser used, singlet oxygen, superoxide anion, OH radicals and alkyl radicals can be produced [2]. The advantage of these photochemical methods is that specific radicals may be produced in high yield. Induction is strictly dependent on the dose of radiation and stops immediately once irradiation is ended [2]. Two types of photosensitisation reactions exist: radical
photosensitised oxidation (type I) and the oxidation by singlet oxygen (type II). Polyunsaturated fatty acids and cholesterol give rise to characteristic peroxidation products depending on the type of photosensitisation reaction [3, 4]. This dependence on type of photosensitisation reaction may also apply to the peroxidation of triterpenoids.

Photosensitisers are mainly used in the biomedical research in order to study peroxidation processes. Systems such as membranes or whole cells contain numerous substances that may act as a photosensitiser. A number of photosensitisers are ketones. A solvent such as acetone can therefore act as a photosensitiser. We investigated a number of solvents for their capability of generating ageing processes in triterpenoids. In addition, two commercially available photosensitisers with different actions were tested: Merocyanine 540 (MC540) and FotoFenton 2 (FF2) (2-hydroxyacetophenone oxime) (figure 1). These two specific photosensitisers were chosen, because of their solubility in organic solvents. MC540 is a photosensitizing dye of great therapeutic interest [5, 6]. MC540 lacks specificity, because it is reported to produce singlet oxygen in addition to other reactive oxygen species, including oxygen radicals, thus giving rise to both type I and type II reactions [6-8]. FF2 resembles the hydroxyl radical formation of Fenton’s reagent [2], but produces fewer side products. FF2 generates hydroxyl radicals by photochemical cleavage of the photosensitiser [9]. This method of solvent ageing was used to gain insight into the oxidation and cross-linking mechanisms of triterpenoids. By using relatively large amounts of these photosensitisers and applying relatively long exposure times, we investigated whether this photochemical approach could simulate the natural ageing process of triterpenoid samples as seen on paintings.

Figure 1 Molecular structure of Merocyanine 540 (MC540) and FotoFenton 2 (FF2).
Two pure triterpenoid compounds, hydroxydammarenone and oleanolic acid, were solvent aged. Hydroxydammarenone is abundant in fresh dammar and mastic resin. Oleanolic acid itself is not abundantly present in the fresh resins, but its molecular structure is very similar to that of oleanonic acid. Sample solutions were light exposed in a closed system, a jar containing a lid. The lid was necessary, because of the rapid evaporation of the organic solvents suitable for dissolving triterpenoids. Therefore, the amount of oxygen decreases during the reaction period, which complicates the investigation of the reaction kinetics. In order to reduce this problem, we used small volumes of reaction products in relatively large vials, thereby creating a large oxygen to reagent ratio. For future experiments, an ageing device should be constructed, which allows the irradiation of solutions under constant oxygen pressure.

For the current experiments, the solutions were light exposed in a fluorescent tube device (13,000-13,500 lux), which was constructed by R. Hoppenbrouwers of the Stichting Restauratie Atelier Limburg (SRAL). UV/VIS radiation is emitted with a wavelength range from approximately 850 nm down to about 305 nm. The chemical composition of the aged solutions was determined with direct temperature-resolved mass spectrometry (DTMS), gas chromatography mass spectrometry (GCMS) and size exclusion chromatography (SEC) coupled with a diode array UV/VIS detector. This chapter first describes the effect of a number of different solvents on the light ageing of hydroxydammarenone. Second, the effect of the two photosensitisers on the solvent ageing of hydroxydammarenone and oleanolic acid is investigated. Third, it is investigated whether the solvent ageing of fresh dammar and mastic simulates the ageing processes as found on paintings. Finally, some observations concerning cross-linking processes are described.

5.2. Effect of solvents

In order to test whether certain solvents are capable of inducing changes in a reagent during light exposure, hydroxydammarenone was dissolved in six different solvents and light exposed for two weeks. The solvents methanol, toluene, acetonitrile (ACN), ethanol, dichloromethane (DCM) and acetone were tested. Figure 2 shows the DTMS summation spectra of pure hydroxydammarenone (a) and of hydroxydammarenone after two weeks of exposure in ethanol (b), acetone (c) and DCM (d). As will be described in Chapter 6, hydroxydammarenone is represented by peaks at m/z 109, m/z 205, m/z 355 and m/z 424. When hydroxydammarenone was dissolved in methanol, ethanol, toluene and ACN, no molecular changes were induced during the two weeks of light exposure.
exposure, as shown for ethanol in Figure 2(b). Rao et al. [10] demonstrated that the dammarane type molecule, cabraleone, is A-ring oxidised when a solution of this triterpenoid compound in methanol is irradiated by UV light. This A-ring oxidation
is not observed in our own experiments, in which a methanol or ethanol solution of hydroxydammarenone was light exposed for two weeks. This implies that in our experimental setup the A-ring oxidation pathway is not accessible, probably due to very low UV light levels. Exposure in acetone and DCM resulted in major chemical changes as seen in Figure 2(c) and (d). Figure 2(c) shows that the characteristic mass peaks of hydroxydammarenone have almost disappeared and a number of new peaks at m/z 143, m/z 399 and m/z 443 are present. As described in Chapter 6, these peaks are characteristic of an ocotillone type molecule. This oxidised triterpenoid is usually found as the main triterpenoid constituent of aged dammar varnishes from paintings, as described in Chapter 3. A similar kind of side chain oxidation could also be achieved by Bielmann [11] after treatment with p-nitroperbenzoic acid in ether. Figure 2(d) shows that some hydroxydammarenone is still present in addition to a certain amount of ocotillone type molecules after exposure in DCM. Compared to the irradiated hydroxydammarenone in acetone, exposure in DCM results in a lower degree of oxidation.

How can we rationalise the oxidising abilities of acetone and dichloromethane? Acetone is known to act as a photosensitiser due to its keto group. Indig et al. [12] demonstrated that acetone can be optically excited by a mercury lamp, forming triplet acetone. Carbonyl compounds after excitation can remove a hydrogen atom from a substrate giving rise to an alkyl radical [2]. Another ketone, benzophenone, is often used for the sensitised oxidation of lipids [13, 14]. Acetone absorbs UV light with a wavelength up to 350 nm [15]. The fluorescent tube device used for light exposure emits radiation with a wavelength down to about 305 nm. The borosilicate glass sample vials absorb UV radiation with wavelengths below 315 nm, but transmit UV radiation with longer wavelengths [1]. Therefore, acetone directly absorbs the UV radiation, acts as a photosensitiser and probably oxidises the triterpenoid sample.

The action of DCM is not entirely clear. DCM absorbs UV light below 240 nm [15]. As mentioned above, the sample solution is not irradiated with light of this wavelength. However, triterpenoids usually contain keto groups and in some cases aldehyde groups. These functional groups absorb UV radiation at relatively high wavelengths (270-300 nm), which results in an \( n \rightarrow \pi^* \) transition [16]. Hydroxydammarenone was found to have an absorption band between 260 and 330 nm. The triterpenoid itself can therefore absorb UV light during light exposure and subsequently abstract a hydrogen atom from a substrate. It is inferred that DCM is a good substrate for this hydrogen abstraction, which gives rise to radical formation. The resulting CHCl\(_2\) radical may subsequently expel a chlorine radical forming a carbene (\( \cdot \)CHCl). A large number of radicals can be formed via this
mechanism, which may be responsible for the oxidation of hydroxydammarenone in DCM during irradiation. This hypothesis needs further investigation.

5.3. **Effect of the addition of photosensitisers**

Photosensitisers added to the reagent solution can generate specific types of radicals or reactive oxygen species when irradiated by light. This could give more control on the reaction process, because certain photosensitisers only generate one specific type of reactive oxygen species or radical species [2]. It was investigated whether two specific photosensitisers, Merocyanine 540 (MC540) and FotoFenton 2 (FF2), could induce changes in hydroxydammarenone when irradiated by light.

Hydroxydammarenone and a photosensitiser were dissolved in ACN, because ACN was found to be a ‘non-active’ solvent during irradiation. Figure 3 shows the DTMS summation spectra of hydroxydammarenone in ACN (a), in ACN with FF2 (b), and in ACN with MC540 (c) after an exposure period of two weeks. Both photosensitisers are observed to induce changes in hydroxydammarenone. Figure 3(b) shows that a certain amount of hydroxydammarenone is still present, deduced from the peak at m/z 424. In addition, peaks at m/z 143 and m/z 399 point to the formation of an ocotillone type molecule. A peak at m/z 414 is known to represent the oxidised triterpenoid with a lactonised side chain, as described in Chapter 3. This compound could also be produced by Mills et al. by oxidation of hydroxydammarenone with chromic acid [17]. A number of peaks are present, which could not be assigned yet, such as m/z 82, 125, 315 and 359. GCMS analysis showed that these peaks represent trace compounds, which could not be identified by their 70 eV mass spectra. It is possible that the DTMS peak at m/z 125 represents a compound with a dehydrated ocotillone type side chain. The molecular ion region of the DTMS spectrum depicted in Figure 3(c) mainly shows peaks that represent the lactonised molecule and ocotillone type molecules. In addition, a peak at m/z 424 representing hydroxydammarenone is not present at all in Figure 3(c), which indicates that MC540 induced relatively more changes than FF2. The DTMS spectra of Figure 3(b) and 3(c) show similar peaks, which indicates that MC540 and FF2 probably induce similar oxidation reactions, independent of the type of reactive species generated.

In order to get information about the oxidation mechanism of hydroxydammarenone, it was irradiated in ACN with FF2 for one week and two weeks. GCMS (figure 4 and Table I) demonstrates that after one week of irradiation hydroxydammarenone (8) is partly oxidised to an ocotillone type molecule (7). Small amounts of other compounds are present of which only a molecule with a shortened side chain (25) and the molecule with the lactonised side chain (26)
could be identified. After two weeks of irradiation, almost all of the hydroxydammarenone has been oxidised to mainly the same three compounds as just mentioned. The relative distribution of these compounds has changed. The molecule with the lactonised side chain (26) seems to have been formed mainly during the second week of irradiation. Probably it is formed by oxidation of the
ocotillone type molecule (7) shown in Figure 5. The compound with the shortened side chain (25) is probably formed directly from hydroxydammarenone (8).

Figure 4 Gas chromatograms of hydroxydammarenone and FF2 in ACN after one week (a), or two weeks (b) of solvent ageing.

Figure 5 Proposed oxidation mechanism of hydroxydammarenone.
### Table I  List of compounds, with their corresponding molecular weights (MW), identified in triterpenoid samples. Peak labels correspond to those used in other figures of this thesis.

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<th>Label</th>
<th>Compound name</th>
<th>MW</th>
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<tbody>
<tr>
<td>1</td>
<td>Dammaradienone (3-oxo-dammar-20(21),24-diene)</td>
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</tr>
<tr>
<td>2</td>
<td>Nor-α-amyrone (3-oxo-28-nor-urs-12-ene)</td>
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<td>3</td>
<td>Dammaradienol (3β-hydroxy-dammar-20,24-diene)</td>
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<tr>
<td>4</td>
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<tr>
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<td>6</td>
<td>Oleanonic acid (3-oxo-olean-12-en-28-oic acid)</td>
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<td>20,24-Epoxy-25-hydroxy-dammaran-3-one&lt;sup&gt;1&lt;/sup&gt;</td>
<td>458</td>
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<td>Oleanonic aldehyde (3-oxo-olean-12-en-28-al)</td>
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<td>Moronic acid (3-oxo-olean-18-en-28-oic acid)</td>
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<td>18</td>
<td>(Iso)masticadienonic acid (3-oxo-13α,14β,17βH,20αH-lanosta-8,24-dien-26-oic acid or 3-oxo-13α,14β,17βH,20αH-lanosta-7,24-dien-26-oic acid)</td>
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<td>26</td>
<td>3-Ox-25,26,27-trisnor-dammarano-24,20-lactone&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>40</td>
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<td>3,12-dioxo-olean-28-oic acid</td>
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<td>45</td>
<td>3-hydroxy-12-oxo-olean-28-oic acid</td>
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<sup>1</sup>The configuration at C-20 and C-24 was not determined.

<sup>2</sup>The configuration at C-20 was not determined.
Fresh dammar resin and aged dammar varnishes both contain compounds with an oxidised A-ring, which contains a carboxylic acid group at C2 and an isopropenyl group at C5 (4 and 5) (Chapter 2 and 3). These compounds are probably formed by oxidation of triterpenoid compounds with a keto group at C3. However, it is not known whether this type of A-ring oxidation takes place during biosynthesis by enzymatic action, after excretion, or afterwards as a result of ageing on a painting. The model compound hydroxydammarenone, used for the solvent ageing experiments, contains a keto group on C3 and is therefore a good precursor for the A-ring oxidation. GCMS demonstrates that this A-ring oxidation does not take place during solvent ageing (Figure 4). In addition, the majority of the oxidised triterpenoids found in aged varnishes from paintings contains a keto group at C3 (Chapter 3), which implies that this A-ring oxidation is not likely to take place during ageing on paintings. The oxidised A-ring is probably formed during biosynthesis of the resin in the tree and therefore tree dependent, or formed during exposure to the relatively harsh outdoor conditions after exudation of the resin from the tree.

After establishment of the effect of photosensitisers in the ‘nonactive’ solvent ACN, it was investigated whether an additional photosensitiser effects the rate and the type of oxidation reactions that occur during light exposure in the ‘active’ solvent DCM. Figure 6 shows the gas chromatograms of hydroxydammarenone in DCM (a), in DCM with FF2 (b), and in DCM with MC540 (c) after an exposure period of two weeks. Table I lists the compounds identified. Figure 6(a) shows that hydroxydammarenone (8) is still the main compound, in addition to a small amount of an ocotillone type molecule (7), after irradiation in DCM, which indicates a small degree of oxidation. The presence of FF2 and MC540 clearly has an effect on the rate of the oxidation reactions. Figure 6(b) shows that hydroxydammarenone has been oxidised to an ocotillone type molecule (7), a lactonised molecule (26) and a molecule with a shortened side chain (25) in the presence of FF2. After exposure in the presence of MC540 (Figure 6(c), only the lactonised molecule (26) and the molecule with a shortened side chain (25) are present. As described above, the molecule with the lactonised side chain (26) is probably formed via oxidation of the ocotillone type molecule (7). Therefore, it can be concluded that MC540 has accelerated the oxidation reactions to a higher degree than FF2. It can also be concluded that the different combinations of solvents and photosensitisers used for the oxidation of hydroxydammarenone result in the same oxidation processes, as depicted in Figure 5. Another important observation was the fact that light is necessary for induction of the molecular changes. Storage in the dark at room temperature of a triterpenoid
solution in DCM or acetone with or without an additional photosensitiser did not result in triterpenoid oxidation.

Fresh dammar and mastic resin also contain molecules with other carbon skeleton types, such as the isomeric oleanane/ursane skeletons. Oleanolic acid was used as the second model compound. Figure 7 shows the gas chromatograms of oleanolic acid after exposure in DCM with FF2 for one week (a) and two weeks (b). Table I lists the compounds identified. After one week of solvent ageing, oleanolic acid (43) is still the main compound with some traces of oxidised compounds. Small amounts of compounds with additional keto groups at C3 (6) and C11 (44) are formed (figure 8). In addition, a compound is formed which is represented by peaks at m/z 235, 263 and 470 (compound is not labeled). This unidentified compound is probably related to the unidentified oleanane type

Figure 6  Gas chromatograms of hydroxydammmarenone in DCM after two weeks of solvent ageing without a photosensitiser (a), with FF2 (b), or with MC540 (c).
Artificial ageing of varnish triterpenoids in solution

molecule (28), which is often found in aged varnishes from paintings and represented by peaks at m/z 233, 263 and 468. The difference between these two unidentified compounds is probably the presence of the keto or hydroxyl group on C3. Another compound was formed after one week of ageing (45), which became the main compound after two weeks of ageing. The double bond at C12 of oleanolic acid (43) becomes oxidised and a keto group is formed at this position (figure 8). This compound has only been found in traces in aged varnishes from paintings. Therefore, two weeks of ageing of oleanolic acid in DCM with FF2 results in the formation of compounds that are oxidised to a higher degree than those found in aged varnishes from paintings.

Fresh dammar resin and aged dammar varnishes both contain compounds with a noroleanane or norursane skeleton (2 and 14) (Chapter 2 and 3). These compounds are oxidised during ageing on the painting to compounds 30 and 31 (Chapter 3). The noroleanane/norursane type molecules are reported to be formed by decarboxylation of compounds with a carboxylic acid at C17 [18]. However, it is not known whether this decarboxylation process takes place during biosynthesis.
by the action of enzymes, after excretion, or afterwards as a result of ageing on a painting. The model compound oleanolic acid, used for the solvent ageing experiments, contains a carboxylic acid group on C17 and is therefore a good precursor compound to study whether decarboxylation can be induced. GCMS demonstrates that the 28-nor-derivative of the oleanane compound was not formed during solvent ageing (Figure 7), which implies that the carboxylic acid group at C17 is strongly bound in oleanolic acid. In addition, the majority of the oxidised oleanane/ursane type triterpenoids found in aged varnishes from paintings contains a carboxylic acid group at C17 (Chapter 3), which implies that decarboxylation is not likely to take place during ageing on paintings. The noroleanane/norursane type molecules are probably formed during biosynthesis of the resin in the tree and are therefore tree dependent, or formed during exposure to the relatively harsh outdoor conditions after exudation of the resin from the tree.

5.4. **Solvent ageing of dammar and mastic resin**

The application of solvent ageing to hydroxydammarenone and oleanolic acid by combinations of solvents and photosensitisers was described above. Similar dammarane and oleanane type oxidation products were formed as were found in aged varnishes from paintings. The conclusive test, for investigating whether this solvent ageing method simulates the ageing processes as found on paintings, is to subject dammar and mastic resin to this ageing method. Of all six solvents tested, only DCM was able to dissolve the complete resins due to the polymeric fraction present in both resins. Therefore, dammar and mastic were irradiated in DCM with FF2 for one and two weeks. Figure 9 (dammar) and 10

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**Figure 8** *Proposed oxidation mechanism of oleanolic acid.*

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Figure 9  Gas chromatograms of fresh dammar (a), dammar with FF2 in DCM after one week (b) or two weeks (c) of solvent ageing, and the gas chromatogram of a dammar varnish aged on a painting (d).

(mastic) depict the gas chromatograms of the fresh resins (a), after solvent ageing for one week (b) and two weeks (c). For comparison the gas chromatograms of an aged dammar and mastic varnish from a painting are shown (Figure 9(d) and 10(d)). Table I lists the compounds identified. The dammar and mastic solutions had turned yellow after only one week of solvent ageing. This colour aspect will be dealt with below. A number of changes are apparent when comparing the
chromatograms of fresh dammar (Figure 9(a)) and of the one week solvent aged dammar (Figure 9(b)). Mainly side chain oxidation of the dammarane type molecules has taken place, resulting in the oxidation of compounds 1, 5 and 8 to compounds 4, 7, 25, 26, 29 and 41. Compound 41 has been found only in trace amounts in aged dammar varnishes from paintings. Part of the oleanane/ursane type compounds with the aldehyde side chain (9 and 11) is oxidised to compounds with an acid group (6 and 10) as deduced from the peak ratios of these compounds. These modifications were also found to take place during ageing of varnishes on paintings (Figure 9(d)), as described in Chapter 3. As seen from the signal to noise ratio, the dammar sample that was solvent aged for two weeks (Figure 9(c)) contains only traces of triterpenoid compounds, as will be described below. Only some highly oxidised dammarane type molecules (7, 25 and 26) are detected by GCMS, because dammarane type compounds are the major constituents of fresh dammar resin (Figure 9(a)).

The dammar solution that was exposed for 1 week resembles the painting varnish most, when the gas chromatograms of the solvent aged and naturally aged varnish are compared. The dammar solution that was exposed for two weeks (Figure 9(c)) contains a relatively higher amount of the dammarane type compound with the lactonised side chain (26) than of the dammarane type compound with the ocutillone side chain (7). More than 10 aged dammar varnishes from paintings have been analysed by GCMS (Chapter 3). In all cases, the lactonised compound was less abundant than the ocutillone type compound. Consequently, two weeks of solvent ageing results in a dammar sample that is relatively more oxidised than the aged dammar varnishes that are usually found on paintings. A number of oxidised oleanane/ursane type molecules, such as 27, 28, 30, 31 and 32, are usually present in small amounts in aged varnishes from paintings (Figure 9(d)). These compounds are not observed in the gas chromatograms of the solvent aged samples (Figure 9(b) and 9(c)). It is possible that these oxidised compounds are formed some time after one week, but formed in amounts below the detection limit and therefore not present in Figure 9(c). Only dammarane type compounds are present in Figure 9(c), because oleanane/ursane type molecules are much less abundant than dammarane type molecules in dammar resin (Chapter 2). Another explanation, for the absence of the oxidised oleanane/ursane type compounds in the solvent aged samples, is the possibility that the oxidation of dammarane type molecules is relatively more accelerated than the oleanane/ursane type molecules during solvent ageing. Possibly the activation energy of the oxidation reactions of the oleanane/ursane compounds is relatively high.

After one week of solvent ageing of mastic resin (Figure 10(b)), the relative amount of a large number of compounds has decreased (8, 9, 13, 18, 19, 20 and 21). Strikingly, all of these compounds, except 9, have a tetracyclic euphane or
Artificial ageing of varnish triterpenoids in solution

dammarane structure (or a bicyclic structure (13)). Two compounds, 6 and 17, were found to be relatively stable after one week of ageing and traces of oxidised oleanane type molecules are formed (27 and 28). Oxidised compounds with a tetracyclic structure are not formed. The fate of these tetracyclic structures is not known. Processes such as degradation, cross-linking or isomerisation may give rise to compounds with other cyclic structures. After two weeks of solvent ageing

Figure 10  Gas chromatograms of fresh mastic (a), mastic with FF2 in DCM after one week (b) or two weeks (c) of solvent ageing, and the gas chromatogram of a dammar varnish aged on a painting (d).
(Figure 10(c)) the only compound left of fresh mastic resin is moronic acid (17), which is confirmed to be a very stable compound and a useful marker for aged mastic varnish [19]. Oxidised compounds with the dammarane (7 and 26) or oleanane skeleton (27 and 28) are present. A main constituent becomes the oxidised oleanane type molecule (42), which is found only in trace amounts in aged varnishes from paintings (Figure 10(d), not labeled). One week of solvent ageing of mastic resembles the aged mastic varnish from a painting best, as is the case with the solvent ageing of dammar resin. Two weeks of solvent ageing results in a chemical composition of the resin that is relatively more oxidised than the aged varnishes that are usually found on paintings.

5.5. **Cross-linked fractions in solvent aged resins**

Earlier it was noted that the signal to noise ratio of the gas chromatograms of dammar resin in particular decreased during solvent ageing. This phenomenon has also been observed in aged varnishes from paintings (Chapter 3) and is probably caused by cross-linking processes. DTMS was used to obtain additional information on the occurrence of these cross-linking processes. Figure 11 and 12

![Figure 11 DTMS total ion currents of fresh dammar resin (a) and of dammar after one week (b) or two weeks (c) of irradiation in DCM with FF2.](image)
show the DTMS TICs of the dammar (Figure 11) and mastic (Figure 12) samples that had been solvent aged in DCM with FF2 for one and two weeks. Two main peaks are present in the TICs. As described in the previous chapters, the first peak represents volatile (triterpenoid) material. At higher scan numbers and thus higher temperature the cross-linked fraction pyrolyses, which is represented by the second peak. Particularly in the case of dammar, the TICs clearly show that the cross-linked fraction increases during solvent ageing. The same process also occurs in mastic resin, but to a lower extent. The DTMS TICs of the pure compounds, hydroxydammarenone and oleanolic acid, contain a peak at relatively high temperature as well (not shown), which indicates that these pure compounds also cross-link during solvent exposure.

The dammar and mastic resin solutions were found to become yellow after solvent exposure in DCM with as well as without an additional photosensitiser. A

Figure 12  DTMS total ion currents of fresh mastic resin (a) and of mastic after one week (b) or two weeks (c) of irradiation in DCM with FF2.
yellow appearance of the sample is caused by light absorption around 400-430 nm. Size Exclusion Chromatography (SEC) in combination with a UV/VIS diode array detector was used to investigate the spectroscopic characteristics of some solvent aged samples. Figure 13(a) shows the SEC traces of the dammar sample that had been solvent aged in DCM with FF2 for one week at 240 nm (solid line) and at 400 nm (dashed line). For comparison, the SEC trace at 240 nm of fresh dammar resin (dotted line) is shown. The trace at 240 nm of the solvent aged dammar shows that two peaks are present, which are not well resolved. The peak at 400 dalton represents the triterpenoid fraction. The (shoulder) peak at 900/1000 dalton can be attributed either to cross-linked dimerised triterpenoids or to highly oxidised triterpenoids, as already discussed in Chapter 3. The SEC trace at 400 nm clearly shows that the SEC fraction at 900/1000 dalton absorbs more light at 400 nm than the triterpenoid fraction. These results are similar to the results obtained with scrapings of aged varnishes from paintings (Chapter 3).

Figure 13  SEC traces at 240 nm (solid line) and at 400 nm (dashed line) of dammar after one week of solvent ageing in DCM with FF2 (a). In addition, the SEC trace at 240 nm (dotted line) of fresh dammar resin is shown in Figure (a). Figure (b) shows the SEC traces at 240 nm (solid line) and at 400 nm (dashed line) of dammar after three and a half weeks of solvent ageing in DCM (b).
One dammar sample was exposed in DCM for a relatively long period of time (three and a half weeks). After exposure the dammar solution had turned dark yellow to brown. Figure 13(b) shows the SEC traces of this dammar sample at 240 nm (solid line) and at 400 nm (dashed line). It is clear that a high degree of cross-linking has taken place during this relatively long period of light exposure in DCM. Higher molecular weight material up to 10 KDa has been formed. The SEC trace at 400 nm clearly demonstrates that the higher molecular weight fractions absorb light in the blue light region. A much higher degree of cross-linking has taken place after three and a half weeks of solvent ageing as compared to ageing on paintings (Chapter 3). This dammar sample, which was aged for a relatively long period of time, is not comparable to that of a varnish aged on a painting. In general, the chance of cross-linking is relatively high in the solvent ageing method. Molecules have much more freedom of movement in solution. Secondly, the solvent ageing method leads to the generation of a relatively large amount of radicals or other reactive species in the resin solution, which increases the probability of radical recombination (quenching) resulting in cross-linking. Thirdly, the concentration of oxygen decreases in the vials during light exposure, which may cause a shift to occur from oxidation to cross-linking reactions.

It was observed that a yellowish precipitate was deposited on the glass jar after some period of solvent ageing of dammar in DCM with or without a photosensitiser. This precipitate was found to be soluble in tetrahydrofuran (THF). The solvent ageing conditions were exactly the same as used above for the dammar ageing in DCM with FF2, except for a higher temperature of the fluorescent tube device during exposure. The temperature was about 10-15 degrees higher than in the experiments described above, which resulted in an increased reaction rate. The type of oxidation reactions was found to be the same. In general, the temperature was found to be a very important factor for the rate of the ageing reactions. Under these experimental conditions the precipitate was observed to be formed already after one week of solvent ageing. DTMS analysis of the nine days exposed dammar in DCM solution (a) and the precipitate formed (b) shows that the precipitate consists of cross-linked material, because it appears later in the temperature profile (figure 14). The total summation spectrum of the precipitate (Figure 14(c)) shows a large number of peaks, which is indicative of complex material. Figure 14(d) depicts the DTMS spectrum of the cross-linked fraction of an aged dammar varnish from a painting. The spectra of the solvent aged (Figure 14(c)) and “naturally” aged cross-linked fraction (Figure 14(d)) are very similar. An important difference between the two spectra of Figure 14(c) and (d) is the presence of the peaks at m/z 36 and m/z 38 in the spectrum of the solvent aged material. These peaks point to the elimination of hydrogen chloride, which
indicates that some incorporation of chlorine occurs during solvent ageing. This observation is consistent with the hypothesis that DCM itself is involved in the
formation of radicals.

The chemical structure of the higher molecular weight fraction will be investigated further with Nuclear Magnetic Resonance Spectroscopy (NMR). The method of solvent ageing is very well suited for the preparation of a large batch of this cross-linked material needed for NMR.

5.6. Conclusions

Dissolution in the solvents DCM and acetone and subsequent light exposure induces changes in triterpenoid substances, like oxidation and cross-linking, which are similar to the molecular changes seen during “natural” ageing of triterpenoid varnishes on paintings. Other solvents, such as methanol, ethanol, acetonitrile and toluene, were found not to induce oxidation reactions during light exposure of triterpenoid samples. The photosensitisers MC540 and FF2 were also found to be useful for the simulation of natural oxidation processes either in ‘active’ or ‘non-active’ solvents. Light is the driving force of these ageing processes. Information was obtained on the oxidation mechanisms of the model compounds hydroxydammarenone and oleanolic acid. Hydroxydammarenone is first oxidised to an ocotillone type molecule, which is subsequently oxidised to a lactonised molecule. In addition, a compound with a shortened side chain is formed, probably from the ocotillone type molecule. Only side chain oxidation of this dammarane type molecule occurs during solvent ageing. Oxidation of the A-ring was not observed. The solvent ageing of oleanolic acid first results in the formation of small amounts of compounds with additional keto groups at C3 and C11. In addition, the double bond at C12 of oleanolic acid becomes oxidised and a keto group is formed at this position. The compound formed by this latter process is the main compound after prolonged solvent ageing. Decarboxylation of the carboxylic acid at C17 of oleanolic acid was not observed. SEC in combination with UV/VIS diode array detection demonstrated that the yellow colour of a dammar sample, which was light exposed in DCM for a relatively long period of time, was caused by the formation of relatively high molecular weight material. This chapter leaves a number of questions unresolved, such as the role of specific reactive species generated by the ‘active’ solvents and the photosensitisers and the effect of different wavelengths of the radiation.

Nevertheless, the photochemical technique of solvent ageing can be regarded as a useful technique for a number of reasons. Information can be gained on the type of oxidation products that are formed during ageing. It can be determined which compounds can be used as stable markers for aged triterpenoid
varnishes. Furthermore, it can be used as a preparation technique for the cross-linked fraction of aged dammar and mastic varnishes. Finally, this technique is very simple from an experimental point of view, very rapid, without film forming problems and controllable, because there is no reaction without light. It should be investigated whether it can also be successfully used for the artificial ageing of other painting materials. A disadvantage of the current investigations is that kinetic measurements were not possible due to the decreasing oxygen concentration. A device that controls the oxygen concentration is presently being developed in our laboratory.

5.7. Experimental

5.7.1. Sample preparation

Hydroxydammarenone (also called dipterocarpol) (Aldrich), oleanolic acid (Aldrich), dammar resin (A.J. van der Linde, Amsterdam, The Netherlands), mastic (H. Schmincke & Co., Erkrath, Germany), Merocyanine 540 (Molecular Probes, Leiden, the Netherlands) and FotoFenton 2 (Molecular Probes, Leiden, the Netherlands) were used for solvent ageing. Methanol (Merck, p.a.), ethanol (Merck, p.a.), dichloromethane (Fluka, >99.8%, HPLC grade), acetone (Merck, p.a.), toluene (Aldrich, 99.5+%, A.C.S. reagent) and acetonitrile (Sigma-Aldrich, 99.93%, HPLC grade) were used as solvents. Hydroxydammarenone and oleanolic acid solutions were made in the concentration of 1 mg/ml. Photosensitisers were added in a concentration of 0.2 mg/ml. Dammar and mastic solutions were made in a concentration of 2 mg/ml. Photosensitisers were added in a concentration of 0.4 mg/ml. Solutions of 300 µl were irradiated in borosilicate glass vials (Chromacol, type 1, class A, 4 ml content). MC540 was not completely soluble in DCM solutions. The aged varnishes of which the gas chromatograms are depicted in Figures 9(d) and 10(d) are described in Chapter 3 (varnish A and C).

5.7.2. Ageing conditions

The glass vials containing triterpenoid solutions were irradiated in a fluorescent tube device, which was constructed by R. Hoppenbrouwers of the Stichting Restauratie Atelier Limburg (SRAL). The device was equipped with fluorescent tubes (TLD, 36 Watt, 96.5 (Philips), 13,000-13,500 lux). The
temperature was kept between 18°C and 30°C with an average temperature of 25°C. The average temperature of the solvent ageing of the sample, of which the results are shown in Figure 14, was about 37°C. After exposure, samples were stored in the freezer.

5.7.3. DTMS

After solvent ageing, the solutions were diluted (10 times). Approximately 2 µl was applied directly to the DTMS probe. In the case of fresh dammar and mastic resin, about 50-100 µg was homogenised in approximately 100-200 µl ethanol. An aliquot (about 2 µl) of the resulting suspensions was applied to the DTMS probe by using a syringe (SGE, 5 µl. All samples on the probe were dried in vacuo prior to introduction in the ion source. DTMS analysis was performed in a JEOL SX-102 double focusing mass spectrometer (B/E) using a direct insertion probe equipped with a Pt/Rh (9/1) filament (100 micron diameter). Ions were generated by electron impact (16 eV) in an ionisation chamber kept at 180 °C and were accelerated to 8 kV. The mass spectrometer was scanned from m/z 20-1000 with a 1-second cycle time. The probe filament was temperature programmed at a rate of 0.5 A/min to an end temperature of 800 °C. Data were acquired using a JEOL MP-7000 data system.

5.7.4. GCMS

The GCMS analysis of fresh dammar and mastic resin is described in Chapter 2. An aliquot of 50 µl of the solvent aged samples was evaporated to dryness. For methylation, according to the method of Hashimoto et al. [20], aliquots of 250 µl of methanol, 25 µl of benzene and 10 µl of TMSdiazomethane were added to the samples. The mixtures were left at room temperature for 30 minutes. After evaporation to dryness, the samples were dissolved in 25 µl dichloromethane (1 µl injection). GCMS experiments were performed as described in Chapter 4.

5.7.5. SEC

SEC experiments were carried out as described in Chapter 3.
Chapter 5

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