Molecular changes in egg tempera paint dosimeters as tools to monitor the museum environment
van den Brink, O.F.

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6. MALDI-FTMS analysis of oxygenated triglycerides and phosphatidylcholines in egg tempera paint dosimeters

Previous chapters discussed results that were obtained by direct temperature resolved mass spectrometric analysis of egg tempera test systems. This technique highlights the chemical changes in the lipidic part of the tempera binding medium. Changes in the mastic triterpenoids, egg cholesterol and triacylglycerols were observed. Changes observed in the triacylglycerol mass window pointed at addition of oxygen to unsaturated triacylglycerols. Phospholipids, although abundantly present in egg were not detected by DTMS. The present chapter reports analyses that were carried out to further investigate the changes in triacylglycerols and diacylphosphatidylcholines in light-aged and pollutant-exposed egg-only tempera. Furthermore, the effect of the addition of inorganic pigments to the fresh binding medium is assessed.

6.1 Introduction

Since the glycerolipids mainly contain unsaturated fatty acids they are prone to oxidation. The oxidation of phosphatidylcholines has been studied extensively by Barclay [1]. Chan broadly reviewed the autoxidation of unsaturated lipids [2]. In a more recent book Frankel gives a systematic overview of the processes in lipid oxidation [3]. His book also discusses methods for the determination of the extent of oxidation. Several essays have been developed to determine the state of oxidation of unsaturated lipids. The thiobarbituric acid (TBA) assay for instance focuses on TBA reactive substances, such as malonic dialdehyde [4], whereas the HPLC/iron thiocyanate assay quantifies the abundance of phospholipid hydroperoxides [5]. Murphy and Harrison [6] have reviewed the analysis of phospholipids by FAB-MS. In more recent work FAB-MS was used for the analysis of intermediate products of lipid oxidation in phosphatidylcholine liposomes [7]. Research by Harvey et al. [8] and by Marto et al. [9] showed that phospholipids can also be easily analysed by matrix-assisted laser
desorption/ionisation Fourier transform mass spectrometry (MALDI-FTMS). Triglycerides have been studied widely using a great variety of ionization techniques. In the most recent research ESI [10], laser desorption [11] and MALDI [12, 13] have successfully been applied to generate pseudomolecular ions with great efficiency.

In principle ESI is a softer ionization technique than MALDI, and hence forms molecular ions more efficiently without loss due to fragmentation. However, as was pointed out by Duffin et al. [14], the ionization efficiency is greatly influenced by the analyte polarity. Therefore, in this comparative study we use MALDI-FTMS for rapid and simultaneous analysis of diacylphosphatidylcholines and triacylglycerols.

6.2 Experimental

6.2.1 Materials and sample preparation

The preparation of the egg-only tempera samples subjected to MALDI-FTMS analysis is described in Chapter 1. The light ageing series (0, 4, 8, 16, 32 and 64 days), the NOx/SO2 exposed sample and the 21-day thermally aged sample of the egg-only tempera test system were analysed. In addition the unexposed azurite (basic copper carbonate) and lead white (basic lead carbonate) were analysed.

Direct temperature-resolved mass spectrometry (DTMS) was performed as described in the experimental section of Chapter 2.

6.2.2 Matrix-assisted laser desorption/ionisation Fourier transform ion cyclotron resonance mass spectrometry (MALDI-FTMS)

Samples were scraped off the Melinex support, extracted with dichloromethane/ethanol (2:1), and centrifuged. A drop of the supernatant was applied on the MALDI probe, which had been coated with a layer of 2,5-dihydroxybenzoic acid (DHB, Aldrich, Steinheim, Germany). FTMS analysis was performed on a modified Bruker Spectrospin (Fällanden, Switzerland) APEX 7.0e FT-ICR-MS instrument with an Infinity™ Cell and a home-built external ion source for CI, EI, FAB, ESI and MALDI [15]. For MALDI a 600 ps laser pulse
(337nm, 0.48 mJ) from a Photon Technology PL2300 (London, Ontario, Canada) nitrogen laser was used to desorb and ionise the analytes from the probe inside the ion source of the FTMS instrument. The laser light impinging on the probe under a 45° angle produced a 4.5 mm² spot. Ions were trapped in the ICR-cell using a 900 µs trapping delay (p2). Spectra were acquired in broadband mode (128k datapoints). Data of 100 shots were summed to obtain the spectra presented.

6.2.3 High performance size exclusion chromatography (HPSEC)

Samples were homogenised in tetrahydrofuran (HPLC grade, Fluka, Buchs, Switzerland) and centrifuged. 100 µl of the supernatant was injected into an Applied Biosystems 480 injector module. The sample was separated on a Polymer Laboratories 10³ Å (300 x 7.5 mm i.d., 5 µm particle size) HPSEC column, using a 1 ml/min flow of THF from an Applied Biosystems 400 solvent delivery system. Detection of eluted components was performed by a LDC/Milton Roy SM 4000 programmable wavelength UV detector, using 240nm UV-light. Data acquisition was accomplished using a PC with Chrompack Maestro HPLC software (version 2.3 for Windows). Polystyrene standards (Polymer Laboratories, Zeist, The Netherlands) were used to calibrate the HPSEC separation.

6.3 Results and discussion

6.3.1 Direct temperature-resolved mass spectrometry

The results obtained on unexposed and light-aged egg-only tempera by direct temperature-resolved mass spectrometry (DTMS) on a sector instrument (see Chapter 3) indicated changes in the triacylglycerols (TAGs) [16-18]. Figure 1 shows the DTMS spectra from m/z 500 to m/z 950 of unaged egg-only tempera stored in the dark (A) and exposed to light for 16 days (B). The clusters of peaks at m/z 820-910 originate from TAGs. Peaks at m/z 540-620 are diglycerides and fragments from TAGs and phospholipids. In the spectrum of the light-aged sample new clusters of peaks appear at masses roughly 16 mass units higher than the TAG peaks. Although oxygenation of TAGs seems evident, the exact nature of the changes cannot be identified on the basis of these DTMS results.
Moreover, phospholipids (PLs) although abundantly present in egg [19] cannot be identified in the DTMS spectra, due to the extensive fragmentation of PLs under EI conditions resulting in mass spectrometric overlap with fragments of TAGs in the diglyceride region of the mass spectrum. Phospholipids can be detected by chemical ionisation DTMS on a sector instrument, but in that case still high-resolution experiments would be required for the identification of the products of the ageing processes. Hence, MALDI-FTMS was selected for the simultaneous investigation of the degree of oxygenation of triglycerides and phospholipids.

Figure 1 Comparison of DTMS spectra of control (A) and 16-day light-aged (B) egg-only tempera samples.

6.3.2 MALDI-FTMS of the unaged egg-only tempera

Figure 2 shows the MALDI-FTMS spectrum of the DCM:EtOH extract of the control egg-only tempera sample. Here sodium cationised molecular ions of both triacylglycerols and phosphatidylcholines (DAPCs) are observed. The triacylglycerols are observed between m/z 850 and 910, the DAPCs between m/z
720 and 820. Diglyceride peaks, observed between \( m/z \) 560 and 660 (data not shown), originate from hydrolysed TAGs and PLs, which are present as such in the sample, and from fragments due to loss of fatty acids from TAG pseudomolecular ions and loss of phosphate groups from PLs.

In the triacylglycerol mass window, primarily three clusters of peaks are observed: 1) C53-TAGs, 2) C55-TAGs, and 3) C57-TAGs. The peak at \( m/z \) 881, for example, corresponds to a sodium cationised C55-TAG with three unsaturations (TAG55:3). Table 1 compares the molecular distribution of triacylglycerols as determined from the mass spectra with values found in the literature [19]. The table shows that TAGs reported to be present in egg in low relative abundance (< 1%) are not detected in our experiments. Furthermore, compared with the literature, the more unsaturated TAGs are observed in the MALDI-FTMS spectra with higher relative abundance. Thus far, it is not clear whether this is due to the original composition of the egg which is known to be affected by the diet of the chicken, or due to mass spectrometric effects such as enhanced ion formation from unsaturated TAGs in the MALDI process. Another, less likely, explanation would be that the sample preparation in the method used by Kuksis, which is rather lengthy compared to the sample preparation used here, had caused degradation of unsaturated TAGs before their measurement.

**Figure 2** MALDI-FTMS spectrum of unaged egg-only tempera sample. Numbers above peaks indicate the degree of unsaturation of the diacylphosphatidylcholine (DAPC) or triacylglycerol (TAG).
Table 1  Experimental abundance of egg triacylglycerols determined by MALDI-FTMS compared with literature values.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Principal composition [19]</th>
<th>Rel. abundance in literature (%) [19]</th>
<th>Rel. ab. (%) By MALDI-FTMS</th>
<th>+O</th>
<th>+2O</th>
<th>+3O</th>
<th>+4O</th>
</tr>
</thead>
<tbody>
<tr>
<td>C51:0</td>
<td>16:0-16:0-16:0</td>
<td>0.1</td>
<td>n.d.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C51:1</td>
<td>16:0-18:1-14:0</td>
<td>0.6</td>
<td>n.d.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C51:2</td>
<td>14:0-18:1-16:1</td>
<td>0.4</td>
<td>n.d.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C53:0</td>
<td>16:0-18:0-16:0</td>
<td>0.3</td>
<td>n.d.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C53:1</td>
<td>16:0-18:1-16:0</td>
<td>5.7</td>
<td>2.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C53:2</td>
<td>18:0-16:1-16:1</td>
<td>3.5</td>
<td>3.4</td>
<td>32.64</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C53:3</td>
<td>16:0-18:2-16:1</td>
<td>1.1</td>
<td>2.2</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C53:4</td>
<td>14:0-18:2-18:2 (0.15)</td>
<td>0.3</td>
<td>n.d.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16:1-18:2-16:1 (0.15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C55:0</td>
<td>16:0-18:0-18:0</td>
<td>0.2</td>
<td>n.d.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C55:1</td>
<td>16:0-18:1-18:0</td>
<td>9.5</td>
<td>4.9</td>
<td>32.64</td>
<td>16-64</td>
<td>16-64</td>
<td>16-64</td>
</tr>
<tr>
<td>C55:2</td>
<td>16:0-18:1-18:1</td>
<td>33.0</td>
<td>24.3</td>
<td>4-64</td>
<td>16-64</td>
<td>16-64</td>
<td>16-64</td>
</tr>
<tr>
<td>C55:3</td>
<td>16:0-18:2-18:1</td>
<td>21.3</td>
<td>33.9</td>
<td>4-64</td>
<td>16-64</td>
<td>16-64</td>
<td>16-64</td>
</tr>
<tr>
<td>C55:4</td>
<td>16:1-18:2-18:1 (3.8)</td>
<td>6.7</td>
<td>9.1</td>
<td>4-64</td>
<td>16-64</td>
<td>16-64</td>
<td>16-64</td>
</tr>
<tr>
<td></td>
<td>16:0-18:2-18:2 (2.9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C55:5</td>
<td>???</td>
<td></td>
<td>n.d.</td>
<td>0.9</td>
<td>16-32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57:0</td>
<td>18:0-18:0-18:0</td>
<td>&lt;0.1</td>
<td>n.d.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57:1</td>
<td>18:0-18:1-18:0</td>
<td>0.3</td>
<td>n.d.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57:2</td>
<td>18:0-18:1-18:0</td>
<td>1.9</td>
<td>1.5</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57:3</td>
<td>18:1-18:1-18:1</td>
<td>5.9</td>
<td>5.6</td>
<td>8-64</td>
<td>16-64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57:4</td>
<td>18:1-18:2-18:1</td>
<td>7.0</td>
<td>6.7</td>
<td>8-32</td>
<td>16-64</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>C57:5</td>
<td>18:1-18:2-18:2</td>
<td>1.0</td>
<td>3.6</td>
<td>8</td>
<td>64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57:6</td>
<td>18:2-18:2-18:2</td>
<td>1.0</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The MALDI-FTMS spectrum of the unaged egg-only tempera sample also shows C42-DAPCs and C44-DAPCs. For comparison and identification the MALDI-FTMS spectrum of egg yolk DAPCs (Sigma, St Louis MO, USA) is shown in Figure 3. Both the reference spectrum and the spectrum of the unaged egg-only tempera sample show sodiated and protonated molecular ions of DAPCs, albeit in different ratios. Furthermore, the FTMS spectra of the reference material and the unaged egg-only tempera sample indicate loss of trimethylamine from sodium cationised DAPCs leading to [M-59+Na]+ ions. This observation and interpretation agree with a publication by Marto [9] and corrections thereof [20]. Potassium cationised DAPCs were observed at low relative abundances in the unaged egg-only tempera sample only. These peaks were identified by their exact masses.
Diacylphosphatidylethanolamines (DAPEs), although found to contribute up to 24% of the PL fraction in egg yolk [19], are not observed in our data. Analysis of commercially available phosphatidylethanolamine extracted from egg (data not shown) showed that this compound class can be detected by MALDI-FTMS as [M+Na]$^+$ and [M-H+2Na]$^+$ pseudomolecular ions. Egg yolk DAPEs mainly consist of arachidonic acid residues (C20:4) which oxidise quickly upon exposure to air. In the first stage of oxidation of arachidonoyl phospholipids one to three oxygen atoms are introduced, resulting in hydroxy, 1,3-cyclic diol and thromboxane structures [21]. More progressed oxidation leads to formation of a variety of chain-shortened products [22]. The arachidonic acid containing DAPEs were observed unaltered in the MALDI-FTMS spectrum of commercially available DAPEs isolated from chicken egg yolk; oxidation products were not observed (data not shown). This proves that our sample preparation method for MALDI-FTMS does not lead to oxidation of the polyunsaturated fatty acyl chains. We could rule out charge competition processes between DAPE and DAPC in the MALDI ion production as explanation of the absence of DAPEs in the spectra, using MALDI-ToF-MS experiments with a mixture of palmitoyl-oleoyl-glycerophosphocholine and of palmitoyl-oleoyl-glycerophosphoethanolamine (both species were observed; data not shown). Hence the absence of DAPEs in the spectra must be caused by the chemical behaviour of these substances in the paint samples rather than by experimental factors, and further study is necessary to resolve this issue.
Table 2: Experimental abundance of egg diacylphosphatidylcholines determined by MALDI-FTMS compared with literature values.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Experimental relative abundance (%)</th>
<th>Relative abundance (%) in the literature [19]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-C42:1</td>
<td>40.3</td>
<td>41.2</td>
</tr>
<tr>
<td>PC-C42:2</td>
<td>33.6</td>
<td>24.5</td>
</tr>
<tr>
<td>PC-C44:1</td>
<td>9.8</td>
<td>10.6</td>
</tr>
<tr>
<td>PC-C44:2</td>
<td>14.8</td>
<td>15.3</td>
</tr>
<tr>
<td>PC-C44:3</td>
<td>1.5</td>
<td>2.1</td>
</tr>
<tr>
<td>PC-C44:4</td>
<td>n.d.</td>
<td>2.7</td>
</tr>
<tr>
<td>PC-C46:4</td>
<td>n.d.</td>
<td>3.6</td>
</tr>
</tbody>
</table>

n.d. = not detected

Table 2 compares the relative abundance of DAPCs observed by FTMS of the unexposed egg-only tempera sample with those determined by Kuksi [19]. This table only includes species that have been observed by Kuksi with a relative abundance higher than 2%. These compounds account for 93% of all egg DAPCs. The table shows that the relative abundances determined by MALDI-FTMS are very similar to those observed by other workers, although some difference is observed for the doubly unsaturated C42-DAPCs. Another difference with respect to our data is that fourfold unsaturated C44-DAPCs and C46-DAPCs were not detected in our MALDI-FTMS, but have been observed by Kuksi with 2.7% and 3.6% abundance respectively.

6.3.3 Changes in the TAGs upon light ageing

Figure 4 compares the FTMS spectra in the triacylglycerol mass window (m/z 870 – 950) for all light-aged samples. The figure shows that two major changes occur upon light ageing. First, within each cluster the intensities of the more unsaturated TAGs decrease relative to the less unsaturated TAGs. The average number of unsaturations per TAG, based on an assumed equal response factors of all TAGs for MALDI-FTMS, decreases from 2.8 in the unaged egg-only tempera sample to 1.7 in the 64-day light-aged sample. Second, clusters of peaks are introduced at approximately 16 a.m.u. higher than the depleted peaks. The average exact mass difference between the peaks formed and the peaks depleted, 15.994 +/- 0.006 a.m.u., agrees exactly with the exact mass of oxygen (15.9949). This unambiguously proves that light ageing leads to oxygenation of the TAGs. SORI-CAD [23] and resonant excitation CAD experiments [24, 25] on singly oxygenated TAGs formed upon light ageing showed which one of the three fatty acid had undergone oxygenation (see also Chapter 7).
Figure 4 Comparison of triacylglycerol mass ranges in MALDI-FTMS spectra egg-only tempera samples light-aged for 0, 4, 8, 16, 32, and 64 days.
Another significant difference between the MALDI-FTMS spectra of light-exposed and unexposed egg-only tempera is the emergence of peaks in the ranges m/z 910-915 and m/z 935-940, which indicates multiple oxygenation of C55-TAGs and C57-TAGs. Introduction of up to four oxygen atoms is observed in the light-aged samples. The average degree of unsaturation of all intact TAGs in the unaged material is 2.81. The degree of unsaturation of the sum of the oxidised and unoxidised material does not change upon light ageing, and its average over all light ageing samples with ageing times between 0 and 64 days is 2.83 ± 0.03. This implies that the net result of light ageing is the introduction of oxygen without addition or abstraction of hydrogen and suggests that structures like hydroxides, epoxides, hydroperoxides, and epidioxides are forming predominantly upon ageing. The results in the last three columns of Table 1, in particular the observation of triply and fourfold oxygenated TAG55:2 and fourfold oxygenated TAG55:3, indicate that at least in some cases more than one oxygen is introduced per double bond. This suggests that hydroperoxide, epidioxide, hydroxy epoxide or diol functionalities form upon oxidation of the unsaturated fatty acid moieties. Such structures have been observed in autoxidation studies of unsaturated fatty acid methyl esters [3] and trilinoleoylglycerol [26]. Theoretically it can also indicate that oxygenation may lead to a decrease in the degree of unsaturation. As the degree of unsaturation is conserved such a process should be compensated by the formation of unsaturated oxygen containing functional groups, such as a keto functionality by loss of water from a hydroperoxy group.

Based on its exact mass, the peak at m/z 907.697 in the spectrum of the 16-day light-aged egg-only tempera sample is attributed to doubly oxygenated TAG55 with elemental composition C_{55}H_{96}O_{8} (exact mass 907.700), rather than to TAG57:3 (exact mass 907.773). This example shows that the resolution (mass accuracy) of the FTMS data allows a precise determination of the degree of oxygenation of TAGs.

The total intensity (based on peak height) of the unreacted TAGs was determined and the same was done for the singly, doubly, three and fourfold oxygenated TAGs. Normalisation of each mass spectrum was carried out by summation of the intensity (height) of all oxidised and unreacted TAGs. Intensities of the nominal mass peak (all ¹²C) and the first isotope peak (¹³C₁) were summed; contributions of second isotope peaks (¹³C₂) of compounds 2 amu lighter than the peak of interest were neglected because of their small contribution. The relative intensities of the unreacted, singly and multiply oxygenated compounds were used to calculate the index for the degree of oxygenation of the TAGs using the following formula:
MALDI-FTMS of oxidised egg glycerolipids

\[ \text{IDOX}_{\text{TAG}} = \sum_{n=1}^{4} n \times I_n \]

in which \( \text{IDOX}_{\text{TAG}} \) is the degree of oxygenation; \( n \) is the number of oxygen atoms introduced to the TAG; and \( I_n \) is the total relative intensity of the all \( n \)-fold oxygenated TAGs after normalisation.

**Figure 5** plots \( \text{IDOX}_{\text{TAG}} \) as a function of the exposure time. The figure suggests that the degree of oxygenation slowly develops in the first 8 days of light ageing, and plateaus after 16 days. **Figure 6** plots the relative abundances of the unaffected TAGs and the oxygenated TAGs as a function of the exposure time, and gives a more detailed view of the oxygenation processes. The curve of the unreacted TAGs in this figure indicates that the relative abundance of the unreacted TAGs decreases rapidly as the ageing time increases. Furthermore, the intensity of singly oxygenated species appears to plateau after 16 days of light ageing, indicating that the rate of formation and depletion of this species have equilibrated. The curves of the multiply oxygenated species indicate that multiple oxygenation mainly happens after single oxygenation. After 16 (or 32) days of light ageing the relative concentrations of triply and quadruply oxygenated species also appear to plateau, indicating that these species are not only formed, but also depleted during light ageing.

![Degree of oxygenation of TAGs and DAPCs](image)

**Figure 5**  Degree of oxygenation of triacylglycerols (solid line) and diacylphosphatidylcholines (dotted line) as function of light ageing time.
Figure 6 Relative abundance of unreacted and oxygenated triacylglycerols as function of light ageing time.

Table 3 Effect of light ageing time on the relative abundance of unreacted, oxygenated and chain shortened triacylglycerols.

<table>
<thead>
<tr>
<th>Light exposure time</th>
<th>Unreacted TAGs</th>
<th>Oxygenated TAGs</th>
<th>Oxidative cleavage products</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>4</td>
<td>0.90</td>
<td>0.10</td>
<td>0.00</td>
</tr>
<tr>
<td>8</td>
<td>0.82</td>
<td>0.14</td>
<td>0.04</td>
</tr>
<tr>
<td>16</td>
<td>0.10</td>
<td>0.78</td>
<td>0.12</td>
</tr>
<tr>
<td>32</td>
<td>0.08</td>
<td>0.70</td>
<td>0.22</td>
</tr>
<tr>
<td>64</td>
<td>0.05</td>
<td>0.76</td>
<td>0.19</td>
</tr>
</tbody>
</table>

The literature suggests oxidative cleavage of the fatty acid chain as a result of progressed oxidation [27]. In the FTMS spectra of 16-, 32- and 64-day light-aged egg-only tempera (Figure 4) peaks are observed at m/z 789.615, 787.602, and 785.588. The exact m/z of these peaks agree with the elemental compositions C₄₆H₈₆O₈Na (789.621), C₄₆H₈₄O₈Na (787.606), and C₄₆H₈₂O₈Na (785.590) respectively, and suggest a net addition of two oxygen atoms and chain shortening by a C9 unit of unsaturated C55-TAGs. The presence of oxidatively cleaved TAGs is further evidenced by fragment peaks at m/z 505 and m/z 533 (not shown) due to loss of one of the C₁₆ or C₁₈ fatty acid chains from the pseudomolecular ions of the oxidatively cleaved species. In the 16-, 32- and 64-day light-aged samples (Figure 4) peaks have also been observed at m/z 799.570, 801.587 and 803.606. Their exact masses agree with the elemental compositions C₄₆H₈₆O₉Na (799.569), C₄₆H₈₂O₉Na (801.585) and C₄₆H₈₄O₉Na (803.601), suggesting either progressed oxidation of oxidatively cleaved TAGs or oxidative
cleavage of TAGs with two oxidised fatty acid chains. **Table 3** shows the relative abundances of unreacted TAGs, oxygenated TAGs and oxidative cleavage products in the light-aged samples. These data clearly show that oxidative cleavage follows a similar trend as the oxygenation of TAGs.

Another process that consumes oxygenated TAGs is cross-linking [28-30]. To verify whether cross-linking plays a role in the light ageing of egg, control and aged samples were extracted with THF and analysed by SEC. **Figure 7** compares the SEC chromatograms of the control, 4-, 16-, and 64-day light-aged egg-only tempera samples. The figure clearly shows that cross-linking plays an important role in the light ageing of egg, and hence may be seen as one of the possible causes of depletion of oxygenated TAGs. Furthermore, TAGs are affected by hydrolysis leading to the formation of diglycerides. In fact, this is thought to be a major cause of the decrease in the signal to noise ratio of the TAGs upon light ageing. Hydrolysis reactions have been further confirmed by data on light-aged egg yolk tempera paints studies by extractive silylation and GCMS [31] and by ESI-FTMS (see **Chapter 7**).

![Figure 7](image)

**Figure 7**  HPSEC chromatograms of unaged and light-aged egg samples (0, 4, 16, and 64 days). Calibration against polystyrene.

**Figure 8** compares the effects of thermal ageing (A) and exposure to air pollutants (B). Thermal ageing (21 days at 60°C) appears to have no effect on the TAGs, as no significant change of the FTMS spectrum in the TAG mass range is observed (see also **Figure 4** for comparison). The calculated degree of oxygenation is zero. Exposure to NOX and SO2 (10 ppm, 17 ppm) for four days,
Figure 8  TAG range of the MALDI-FTMS spectra of thermally aged (A) and air pollutant [NO\textsubscript{x}, SO\textsubscript{2}] exposed (B) egg, and azurite (C) and lead white (D) pigmented tempera.

also in the dark, appears to greatly enhance oxygenation. The FTMS spectrum shows extensive oxygenation and depletion of unsaturated TAGs. The degree of oxygenation, calculated by the aforementioned method is 0.87. The spectrum also shows the oxidative cleavage product at m/z 787.601 (inset). More products of oxidative cleavage are observed at m/z 771.611 and 783.565 corresponding to
the masses of doubly and fourfold unsaturated oxygenated C46-TAGs with elemental compositions $\text{C}_{46}\text{H}_{84}\text{O}_{7}\text{Na}$ (771.611) and $\text{C}_{46}\text{H}_{80}\text{O}_{5}\text{Na}$ (783.575) respectively. These peaks were not observed in the FTMS spectra of light-aged egg-only tempera samples. Further research is required to determine whether these products are unique and can be used as markers for exposure to NO$_x$ and SO$_2$. Gallon and Pryor [32] have determined that NO$_2$ addition products are formed when methyl linoleate is exposed to NO$_2$ in the absence of oxygen. In the presence of oxygen (air), as was the case with the exposure of the tempera test systems to NO$_x$/SO$_2$, hydroperoxides are observed as primary oxidation products.

The presence of metal salts as inorganic pigments in tempera paint may lead to metal catalysed oxidation (MCO) of unsaturated lipids. MCO of unsaturated lipids has been studied extensively [33]. The effects of a variety of pigments on the chemistry of linseed oil has been studied by Rasti and Scott [34]. These workers also observed a catalytic effect of the copper containing pigment verdigris on oxidative reactions in the drying of linseed oil [35]. It has been stated by others that different pigments variously affect oxidative degradation in egg tempera paint [36, 37]. Extractive silylation GCMS experiments carried out in our group [31] indicate that the presence of inorganic pigments strongly influences the chemical composition of the egg tempera binding medium. Additions of inorganic pigments to the egg and mastic binding medium of our tempera paint systems appear to greatly enhance oxidation. Figures 8C and 8D show this phenomenon in unexposed azurite and lead white temperas respectively. As a control experiment, a mixture of egg and mastic that had been coated on Melinex and stored in the dark along with the pigmented paints was analysed by MALDI-FTMS. No significant difference between the resulting spectrum and the spectrum of the unaged egg-only tempera sample was observed. This indicates that the oxidation can only be explained by the presence of the pigments. The degree of oxygenation of the lead white pigmented sample was 0.84. The azurite pigmented sample shows an even higher degree of oxygenation (1.42) and complete depletion of polyunsaturated TAGs. These results are confirmed by DTMS analysis of the same systems [31, 38, 39] (see also Chapter 3). The observation that copper catalyses the autoxidation of unsaturated TAGs to a greater extent than lead is in agreement with the literature [33].

6.3.4 Changes in the DAPCs upon light ageing

Figure 9 compares the phospholipid m/z ranges (m/z 700 – 830) of the mass spectra of unexposed and 8-, 32- and 64-day light-aged egg-only tempera samples. Three changes are clearly observed in this comparison. Firstly as in the light ageing of the TAGs, the more unsaturated DAPCs are preferentially
depleted. This can be seen by comparing the peak ratio of doubly unsaturated DAPCs to singly unsaturated DAPCs during light ageing. The average degree of unsaturation of the DAPCs decreases from 1.5 for the unaged sample to 1 for the 64-day light-aged sample. Secondly, the signal to noise ratio decreases greatly upon light ageing due to hydrolysis [31]. Thirdly, new peaks appear at \( m/z \) 737 and 739. The mass difference between these peaks and the depleted peaks agrees with the exact mass of an oxygen atom, thus again indicating oxygenation of the DAPCs. Given their exact masses, the peaks at \( m/z \) 737.443 and 739.461 in the spectrum of the 8-day light-aged sample were identified as the potassium [\( \text{M+K-N(CH}_3)_3 \text{]}^+ \) ions. Unlike the observations in the oxidation of TAGs, products of multiple oxidation of phospholipids are not observed in the samples that were exposed for longer times (16, 32, 64 days). This may be explained by the lower degree of unsaturation of the phospholipid fraction as compared with the TAGs, resulting in a lower reactivity in oxidative environments. However, Porter and coworkers [40] observed introduction of hydroperoxide groups into palmitoyl-linoleoyl- glycerophosphocholines (24.5% of egg DAPC) after 16 hours of exposure to air at room temperature. Hydroperoxides were also found upon reaction with HO• radicals in solution [7]. This suggests that the presence of other materials in the egg causes transformation of hydroperoxides. Indeed, cysteine residues in proteinaceous materials are known to reduce lipid hydroperoxides to hydroxides [33]. The appearance of singly oxygenated DAPCs could also point to intermolecular oxygen transfer from hydroperoxides to form epoxides and hydroxides observed in lipid monolayers [41]. This would imply that phospholipids could interact with each other in the dried tempera paint film, for instance, in intact low-density lipoprotein (LDL). In related experiments [42] we have found evidence that LDLs are still intact in the unaged unpigmented tempera material.

The development of the relative abundance of oxygenated DAPCs as a function of the ageing time is shown in Figure 5. The oxygenation only starts after 8 days of light ageing and develops more gradually than the oxygenation of TAGs. Again this is interpreted as due to the lower degree of unsaturation of DAPCs as compared to TAGs in egg. Fukuzawa et al. [43] observed that the rate of peroxidation of egg DAPC liposomes, measured as oxygen consumption, increased drastically after a lag-period. The length of this lag-period was found to depend on the concentration of DAPC hydroperoxides endogenously present in the liposomes. Longer lag-times were observed when less endogenous hydroperoxide was present. Such autocatalytic processes may also explain the observed delay in oxygenation of the DAPC fraction in our systems.
MALDI-FTMS of oxidised egg glycerolipids

Figure 9  DAPC range of the MALDI-FTMS spectra of unaged, and 8-, 32- and 64-day light-aged egg. Peaks marked with * originate from oxidative cleavage products of triacylglycerols.
Figure 10 shows the DAPC range of FTMS spectra of the thermally aged egg-only tempera sample (A), the lead white pigmented sample (B), and the azurite pigmented tempera (C). The spectrum of the thermally aged sample closely resembles that of the unaged egg-only tempera sample. This is in agreement with the observations in the TAG range. The spectrum of the sample that had been exposed to SO$_2$ and NO$_x$ is not included here because it shows almost no contribution from peaks of DAPC origin. The high acidity in the paint film as a consequence of the exposure may have caused hydrolysis of the DAPCs.

The DAPCs in the lead white pigmented sample show oxygenation as indicated by the peaks at m/z 737, 739, 796 and 798. Furthermore, the peaks observed at m/z 735.452 and 794.527 have exact masses corresponding to the oxygenated $[(\text{DAPC}42;3+\text{O})+\text{Na}-\text{N}(\text{CH}_3)_3]^+$ (735.457) and $[(\text{DAPC}42;3+\text{O})+\text{Na}^+]$ (794.531) respectively. This suggests that specific oxidation products are formed in the presence of a pigment. The DAPC-range of the FTMS spectrum of the azurite pigmented tempera is more complicated. In addition to protonated, sodium cationised DAPCs and fragments thereof also the copper cationised singly unsaturated DAPC42:1 was identified at m/z 822-825. The singly unsaturated DAPC44:1 was observed at low intensity at 850 and 852 (data not shown). The absence of peaks from doubly unsaturated DAPCs indicates that the DAPC fraction has undergone vast chemical changes due to the presence of the azurite pigment. In addition to the singly oxygenated DAPCs that were observed in the spectrum of the lead white tempera, the azurite tempera spectrum shows high intensities of protonated singly oxygenated C42-DAPCs (m/z 772-775) and peaks from doubly oxygenated DAPCs (m/z 810-813). These latter peaks were assigned on the basis of their exact mass and presence of peaks due to the characteristic loss of trimethylamine from the sodium cationised molecules. The inset shows that the peaks at m/z 810 and 811 consist of doublets. The heavier and less intense peaks of the doublets originate from the sodium cationised singly unsaturated DAPC44:1. Although based on the exact mass the peaks at m/z 814.557 and m/z 816.572 could be interpreted as $[(\text{DAPC}42;1+2\text{O})+\text{Na}]^+$ (814.557) and $[(\text{DAPC}42;0+2\text{O})+\text{Na}]^+$ (816.573), these peaks were attributed to $[(\text{DAPC}44;4+2\text{O})+\text{H}]^+$ (814.559) and $[(\text{DAPC}44;3+2\text{O})+\text{H}]^+$ (816.575) because fragment peaks due to loss of N(CH$_3$)$_3$ were not observed. In a similar way it was determined that the high relative intensity of the peak at m/z 798.560 is caused by the combination of $[(\text{DAPC}42;1+\text{O})+\text{Na}]^+$ (exact mass 798.563) and $[(\text{DAPC}44;4+\text{O})+\text{H}]^+$ (exact mass 798.565).
Figure 10 DAPC range of the MALDI-FTMS spectra of thermally aged egg (A), lead white pigmented tempera (B), and azurite pigmented tempera (C). Peaks marked with * originate from or have contributions from oxidative cleavage products of triacylglycerols.
6.3.5 **Implications for paintings and paint-based dosimetry**

The results presented here unequivocally show that oxidative changes occur in the egg tempera binding medium when exposed to visible light. The rates with which a glycerolipid in the egg-only tempera is oxygenated depends on its structure. The DAPC fraction is found to be oxidised at a slower initial rate than the TAGs. The number of unsaturations in the compound determines the rate of oxygenation. Progressed oxidation leads to multiple insertions of oxygen and is accompanied by oxidative cleavage reactions and the formation of THF extractable cross-linked material. Lipid oxidation products, unlike the native glycerolipids, are very reactive towards amine moieties in the proteinaceous fraction of the egg. This type of lipid protein interaction plays an important role in the anhydrous conditions in the dry paint film [31]. The increased reactivity of the lipid fraction towards the proteinaceous components in egg tempera paint leads to changes in the size and nature of the polymeric network. Hence, exposure to light as a consequence of the very function of a painting leads to important changes in the overall composition of the egg tempera binding medium. The high $IDOX_{TAG}$ of the sample exposed to $NO_x$ and $SO_2$ in the dark implies that air pollution can be an important factor in the quality of the museum environment and the effects that it has on the art objects.

The observation that the inorganic pigments lead white and azurite, when present in egg tempera paint, catalyse the oxidation of their binding medium in the early stage of the drying of the film strongly suggests that the organic chemical composition of a paint is influenced by its pigments. Based on the observation that pigments can, by catalytic action, change the chemistry of the binding medium, it must be expected that temperas with different pigments respond differently to environmental conditions. Thus the MALDI-FTMS results obtained on the pigmented temperas suggest that a variety of paint systems should be exposed for successful environmental monitoring using paint-based dosimeters. In fact dosimetry of the museum environment in six different sites using pigmented temperas has shown that there is a pigment dependent difference in chemical response to the environmental conditions [17, 18] (see Chapter 3).

In view of the potential for detailed analysis, the question can be posed whether MALDI-FTMS should be used instead of DTMS (EI) for the evaluation of chemical changes in tempera paint based dosimeters. The main advantage of DTMS is its multi-component analytical potential. It allows, in one analytical run, the evaluation of changes in a greater variety of compound classes than glycerolipids alone. Besides changes in the glycerolipids, DTMS also detects changes in the cholesterol, and the formation of cross-linked material is also reflected in the DTMS spectra [16-18] (see Chapters 2 and 3). MALDI-FTMS
as applied here, on the other hand, allows the investigation of changes in the TAGs and DAPCs. The latter are not observed as molecular ions in the DTMS runs. Changes in the glycerolipids can be studied in much more detail, due to this specificity and the high resolution of the FTMS data. Hence, MALDI-FTMS is a good technique for supportive, ancillary identification of chemical changes observed by fingerprinting DTMS.

Although the degree of unsaturation of TAGs is found not to change upon oxygenation and the number of possible functional groups introduced into the fatty acyl chains can be limited to relatively few, detailed kinetic information on the rates of oxygenation of fatty acyl chains with different degrees of unsaturation cannot yet be derived from the present data. Investigation of the structure of oxidation products should contribute to the understanding of the reactions that take place during photo-oxidation of the egg tempera binding medium. ESI-FTMSMS work reported in Chapter 7 is aimed at determination of the fatty acyl side chain on which the oxidation has taken place and work in progress is aimed at determination of the functional groups that are formed upon oxygenation.

6.3.6 Other applications of the methodology

The oxidation of lipids also plays a role in a variety of diseases, including atherosclerosis. Oxidative cleavage products of phospholipids can even act as inflammatory mediators [44]. Progressed oxidation of unsaturated lipids in food leads to the formation of off-flavour volatile compounds [3]. The present MALDI-FTMS method also can be used as for rapid direct screening of the oxygenation of TAGs and DAPCs in biochemical and food samples. The simple sample preparation for MALDI-FTMS makes this analytical method very suitable for determination of the degree of oxygenation of lipids in complex matrices. The high resolution of the mass spectrometric data allows determination of the number of oxygen atoms taken up by a TAG or DAPC molecule and hence expression of the oxygen uptake by these glycerolipids as the degree of oxygenation irrespective of the functional groups introduced upon oxygenation.

6.4 Conclusions

MALDI-FTMS is a rapid and sensitive method for the simultaneous analysis of TAGs and DAPCs and their oxidation products in a complex mixture such as egg. DHB is a suitable matrix for these compounds, mainly producing sodiated molecular ions and characteristic fragment ions.
Chapter 6

The high mass accuracy (mass difference accuracy) of the FTMS data is used to determine the elemental composition of TAG and DAPC oxidation products. Indications were found for products of oxidative cleavage of triacylglycerols.

Triglycerides and diacylphosphatidylcholines are sensitive to light-induced oxidation as an increasing function of the degree of unsaturation. The results obtained on the light ageing series confirm the notion that oxygenation of glycerolipids plays an important role in the light ageing of tempera test systems. Moreover, the trend observed by MALDI-FTMS of the egg glycerolipids compares very well with that observed by DTMS and DA of the whole test system. The results provide a scientific basis for the use of test paintings as environmental monitors.

Exposure of egg glycerolipids to high concentrations of SO₂ and NOₓ in the dark results in extensive oxidation. These observations suggest that the combination of exposure to light and sulphur oxides and nitrogen oxides imposes considerable oxidative stress on unsaturated glycerolipids.

The presence of basic lead carbonate and basic copper carbonate, which are common pigments in traditional egg tempera paint, leads to extensive oxidation of egg glycerolipids in the curing stage of an egg tempera paint.

References


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