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A direct temperature-resolved tandem mass spectrometry study of cholesterol oxidation products in light-aged egg tempera paints with examples from works of art

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ABSTRACT

Cholesterol (**1**) constitutes approximately 5% of the lipid fraction of eggs. The compound is therefore abundant in fresh egg tempera paints. The fate of cholesterol upon light ageing of egg tempera paint binding medium was investigated by direct temperature resolved mass spectrometry (DTMS) and tandem mass spectrometry (DTMSMS). Cholesterol oxidation products (COPs) such as 5,6-epoxycholestan-3-ol (**2**) and 3-hydroxycholest-5-en-7-one (**3**) were positively identified in light-aged egg binding medium. Given the fast rate of oxidation of cholesterol, the corresponding oxidation products are better markers for egg tempera than the cholesterol molecule itself. Cholesterol and COPs were discovered in paints on German baroque altar pieces from the 16th and 18th C and in a 20th C glaze on a Mark Rothko Seagram Mural painting at Tate by DTMS fingerprinting analysis of paint microsamples.

1. Introduction

The oxidation of cholesterol (**1**) has been studied extensively for instance in relation to atherosclerosis and cancer and dozens of cholesterol oxidation products have been identified [1,2]. These include relatively stable oxidation products as well as compounds that are formed in the very early stages of oxidation (e.g., cholesterol hydroperoxides).

The research presented here was carried out to investigate the oxidation of cholesterol in egg tempera paints. This study is part of the ERA project [3,4], which introduced and tested the idea of monitoring the quality of the museum environment by evaluating changes in the physical properties and chemical composition of paint systems. Mock egg tempera paintings were prepared and exposed in different museum environments and the physical and chemical changes thus produced, were assessed. These were then compared with the changes observed upon exposure of similar samples in the laboratory to standardized accelerated ageing conditions, such as elevated temperature and light intensity. Physical changes were determined using thermal and dynamic mechanical analytical methods [5,6–8] by the project partners at Birkbeck College (London, UK) and using spectroscopic methods such as infrared, ultraviolet and visible spectroscopy by another partner group at IROE, now IFAC (Florence, Italy).

Previously published work shows that changes on the molecular level can be successfully determined qualitatively and semi-quantitatively by direct temperature-resolved mass spectrometry (DTMS) combined with discriminant analysis (DA). DTMS highlights mainly chemical changes in the lipidic part of the tempera-binding medium [9–11]. Changes in the glycerolipids have been studied in detail using matrix-assisted laser desorption/ionisation Fourier transform ion cyclotron resonance mass spectrometry (MALDI-FTICRMS) [12] and electrospray ionisation FTICRMS [13]. These investigations pointed at oxidation of the glycerolipids as a main process that occurs on light exposure of the egg tempera paint systems. As cholesterol is a relatively abundant compound in the lipid fraction of eggs (5%), oxidation of this lipid is also thought to play an important role in the exposure-induced chemistry of the egg tempera paint systems.

The traditional methodology for the determination of cholesterol oxidation products (COPs) in complex matrices involves extraction, followed by thin-layer chromatography (TLC) or derivatization and gas chromatography (GC). Although exposure to alkaline conditions of the samples before analysis leads to breakdown and hence to a lower recovery of some of the COPs [14–16], saponification has also been performed by some researchers to study the oxidation of cholesteryl esters. Luby et al. [17], for instance, used TLC for the separation of cholesterol and COPs in

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saponified butter extracts, while Nam et al. [18] determined a variety of COPs in raw meat using trimethylsilyl derivatization and GC. Efforts are also being made to devise a methodology for a rapid and economical analysis of COPs in blood using TLC with or without silylation [19].

A strongly preferred position for autoxidation of cholesterol (**1**) is the 7-position (see Scheme 1) for the numbering of the carbon atoms in cholesterol. The initiation of the autoxidation reaction involves the formation of a carbon-centred allylic C-7 radical, which reacts with molecular oxygen and subsequently abstracts a hydrogen radical, leading to the formation of 7-hydroperoxycholesterol [1]. This compound is then able to react to cholest-5-ene-3,7-diol (**4**) and 3-hydroxycholest-5-en-7-one (**3**), which are known as the major autoxidation products of cholesterol. Other positions that are sensitive to oxidation include C-20 and C-25 [1].

The presence of COPs in eggs and egg products has been investigated by a number of groups since the 1960s. A review article on the analysis and the biological effects of sterol oxides in foodstuffs was published in 1983 [20]. The COPs identified in spray-dried or air-exposed egg yolk include the aforementioned major products as well as cholestane-3,5,6-triol, and 5,6-epoxycholestan-3-ol (**2**) [14,21]. The latter is reported to derive from cholesterol by reaction with 7-hydroperoxycholesterol or the hydroxyl radical ($OH^{\bullet}$) [1]. Some researchers have investigated the prerequisites for the formation of epoxy cholestanols during spray-drying [22]. Lai et al. [23] identified the presence of NO_x as an important factor in the rapid formation of COPs in gas-fired spray driers. Tsai and Hudson [15] used high-performance liquid chromatography (HPLC) for the isolation of epoxy-cholestanols from egg powders. In their research MS, NMR and IR spectroscopy were used for identification of the compounds. Employing extraction, liquid chromatography pre-separation and derivatization GC, van de Bovenkamp et al. [16] determined the presence of 7-hydroxycholesterol (**4**), 7-ketocholesterol (**3**) and 5,6-epoxy-cholesterol (**2**) in fresh egg yolk at the sub-μg/g level. Yang and Chen [24] used TLC and derivatization-GC for the determination of COPs in a selection of Chinese egg products. They found that between 1 and 2% of the cholesterol was transformed to 7-β-hydroxycholesterol (**4**) and 20-hydroxycholesterol by various methods of egg processing.

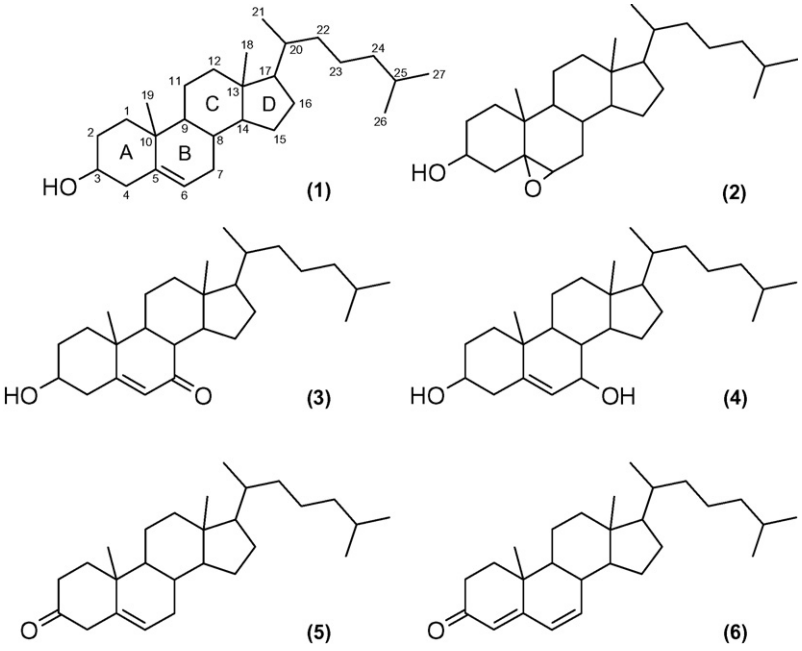
Kim and Nawar [25] have shown that the rates of cholesterol oxidation in model systems that contain different triacylglycerols vary strongly and that interpretation of the observations is very difficult. It is not the purpose of this paper to resolve reaction mechanisms or the kinetics of cholesterol oxidation in the egg tempera-binding medium. Rather, it is our intention to identify marker peaks indicative for cholesterol oxidation products traced by the DTMS-DA methodology applied for the evaluation of exposed tempera paint-based dosimeters [9,10,11,13,26]. The assignment of the marker peaks will be discussed first, followed by a presentation of experimental data from DTMSMS of standards and egg tempera paint. The spectra will be compared and discussed in the light of mass spectral data on sterols reported in the literature. Examples of fingerprinting information discovered in paint samples from works of art will be given.

2. Experimental

2.1. Egg tempera samples

The experiments described in this paper were carried out on egg binding medium samples that derived from a larger series of samples that was prepared for laboratory and museum exposure and included a great variety of pigmented paints [10]. The preparation of the egg tempera samples was based on a traditional recipe [27]. The egg white was separated from the yolk and beaten, so that a layer of foam formed on the liquid. The beaten egg white was left to stand overnight. Then, the foam was separated from the liquid part of the egg white and discarded. The yolk was mixed with the remaining part of the egg white and three drops of apple cider vinegar were added. The binding medium was coated on a sheet of Melinex® using a Byk Gardner (Geretsried, Germany) film applicator at 100 μm wet layer thickness.

The dry films were exposed to high levels of visible light in the MOLART light-ageing facility at the Limburg Conservation Institute (SRAL) in Maastricht, The Netherlands. The light-ageing facility uses 12 Philips TLD-36W/96 fluorescent daylight tubes to illuminate a surface of 1.2 m^2. The resulting light intensity during the 21 days' exposure of the egg-only tempera sample was 10,200 lx. Perspex



Scheme 1. Cholesterol (**1**) and main cholesterol oxidation products: 5,6-epoxy-cholestan-3-ol (**2**), 3-hydroxycholest-5-en-7-one (**3**), cholest-5-en-3,7-diol (**4**), 5-cholesten-3-one (**5**) and cholesta-4,6-diene-3-one (**6**).

(PMMA) filters were used to absorb most of the already low intensities of UV radiation produced by the fluorescent lights. The light ageing facility was located in an air-conditioned room. Therefore, the temperature and relative humidity were kept constant at values of 22 °C and 40–44%, respectively.

64-Day light-aged samples were obtained on accelerated light ageing in the facility of the Tate Gallery in London (UK). This ageing facility used 6 Philips TLD94 58 W daylight rendering fluorescent tubes that were filtered with a Perspex VE ultraviolet filter which has a cut-off wavelength at about 400 nm. Tubes were changed regularly to maintain a constant sample illuminance of about 20 klx. Cooling fans prevented excessive heating of the environment, keeping the average temperature at 28–29 °C and the relative humidity at 27–28%. The light intensity during the exposure of the tempera test samples was 18 klx.

2.2. Paint samples from works of art

Sample WSK P8 is a red glaze on silver leaf from the inside of pulpit canopy in the Catholic Pilgrimage Church *Die Wies* dedicated to the Flagellated Christ. The *Die Wies* Church in Upper Bavaria's the so-called Pfaffenwinkel is the only Bavarian monument besides the palace of Würzburg (Würzburger Residenz) recorded in UNESCO's World Heritage List of cultural and natural properties. The lavishly embellished pulpit with canopy (also sounding board) was designed by Dominikus Zimmermann and completed in 1753–1756.

Sample R4A1 is an opaque green glaze on silver leaf on the bust of St. Marinus (c. 1760). The bust depicting the St. Marinus belongs to the furnishings of the former Benedictine monastery church in Rott/Inn (administrative district: Rosenheim). The architect of the abbey church was the great Johann Michael Fischer (1692–1766). The church was consecrated in 1763. The wooden bust of St. Marinus in silver leaf technique, one of the patron saints of the church is part of the side altarpiece to the east dedicated to Christ.

Sample BisP3 was taken from the Holy Bishop presently in the Mainfränkisches Museum Würzburg (Sculpture Collection, Inv.-Nr. 44737). The sample was taken from the back of the bishop's neck where remains of 16th century polychromy are visible. The wooden sculpture of the Holy Bishop is dated c. 1505–1510 and thought to be made by Tilman Riemenschneider (1460–1531).

Sample S3A was taken in a glazed area near the right edge of the painting *Red on Maroon* (Tate Modern London, inventory number T01165) made as part of the Seagram Mural Painting Series painted by Mark Rothko (1903) between 1958 and 1960 in New York.

2.3. Reference compounds

5-Cholesten-3β-ol (cholesterol) (**1**), 5β,6β-epoxycholestan-3β-ol (**2**), 5-cholesten-3β-ol-7-one (**3**), 5-cholestene-3β,7β-diol (**4**), 5-cholesten-3-one (**5**) and cholesta-4,6-diene-3-one (**6**) were obtained from Sigma–Aldrich Chemie (Steinheim, Germany).

2.4. Trimethylsilyl derivatization

A dichloromethane/ethanol (7:3, v/v) extract of 21-day light-aged egg material was evaporated to dryness in GC auto-injector vials. To the dried extract were added 50 μl per mg sample (original sample weight, before extraction) of bis(trimethylsilyl) trifluoroacetamide, containing 1% trimethylchlorosilane (Fluka, Zwijndrecht, The Netherlands) and 50 μl per mg sample (weight before extraction) of pyridine. The reaction vials were flushed with dry nitrogen and placed in an oven for 60 min at 65 °C. After a subsequent cooling period, the solvent was removed under a gentle flow of dry nitrogen and the residue was redissolved in dichloromethane.

2.5. DTMS(MS)

Although the sensitivity of the DTMS method allows analysis of smaller samples, approximately 1 mg was scraped off the Melinex support and homogenised into ethanol (~100 μl), in order to ensure statistically more representative sampling. The exact sample size and volume of the ethanol added varied with the composition of the tempera test system, i.e., the pigment-volume concentration. Aliquots of 1 μl of the sample suspension was deposited on the 0.1 mm diameter, platinum/rhodium (90:10) filament (Drijfhout, The Netherlands) of the DTMS probe. DTMS analysis of the egg tempera samples was performed on a JEOL JMS-SX 102A double focusing mass spectrometer with B/E geometry. In the ion source of this instrument, the wire was resistively heated by ramping the current at a rate of 0.5 A/min. Using this ramp the temperature was linearly increased from ambient to approximately 800 °C in 2 min. Desorbed and pyrolysed material was ionised by 16 eV electron impact ionisation. The mass spectrometer was scanned over an m/z range of 20–1000 using a 1-s cycle time.

DTMSMS experiments were performed on a JEOL JMS-SX/SX 102A four-sector instrument of B/E-B/E geometry (which was kindly made available by Prof. Dr. A.R.J. Heck of the Bijvoet-Institute of Utrecht University before it was acquired by the FOM-Institute for Atomic and Molecular Physics). Ions produced in the ion source with a kinetic energy of 8 kV were selected by MS1 (first two sectors of the instrument). They were thus passed through the collision cell, which was at ground potential. Helium was used as collision gas. The gas pressure was set to produce a precursor ion signal intensity of 90% of the initial signal intensity on MS^2 (no collision gas). The resulting pressure in the collision cell was 3.5×10^{-4} Pa.

DTMS analysis of the reference materials was performed on the same instrument, using μM ethanol solutions. Similarly to previously described, aliquots of 1–2 μl were applied to the platinum/rhodium (90:10) filament. The remaining experimental parameters are identical to those of the previously described DTMS analysis.

3. Results and discussion

3.1. DTMS analysis

As a first step in the investigations reference compounds of cholesterol oxidation products were analysed. The choice of references for COPs is based on the major, and relatively stable, oxidation products described in the literature [1,14–16,21]. Analysis of the ethanol solutions of reference materials by 16 eV EI DTMS allowed the attribution of the DTMS molecular and fragment peaks of cholesterol and COPs. Table 1 lists the cholesterol related m/z peaks (first column), possible assignment (second and third column) and proposed structure (fifth column). The analysis also provided for the relative intensities of the m/z peaks of the compounds under 16 eV EI DTMS conditions (fourth column). This information is important in the interpretation of the changes observed in the composition of the egg tempera dosimeters induced by accelerated light and thermal ageing and exposure to the museum environment.

The molecular and main fragment ions of the cholesterol (**1**) and corresponding oxidation products can be detected at scan range 25–40 and mass range m/z 200–450. This area of the DT mass spectrum was studied in more detail and the results are discussed below. Previous investigations using discriminant analysis of the direct temperature-resolved mass spectra of the light ageing series of the egg tempera samples indicated which mass spectrometric peaks increased and which decreased on light ageing [10]. Fig. 1 shows the total ion current thermogram (A) and the DT mass spectrum over the desorption window of cholesterol (scans 25–40)

Table 1
Assignment of *m*/*z* peaks in the DTMS spectra of cholesterol and selected cholesterol oxidation products.

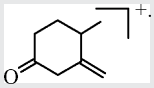
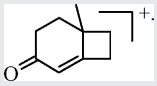
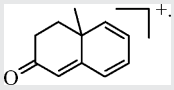
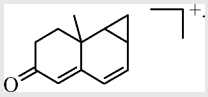
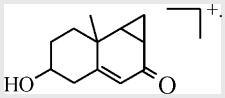
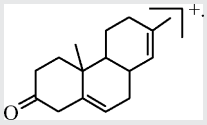
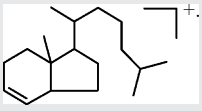
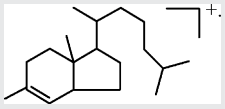
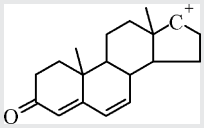
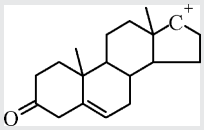
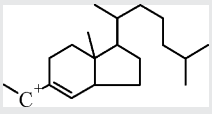
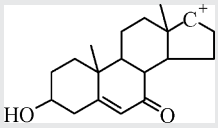
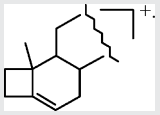
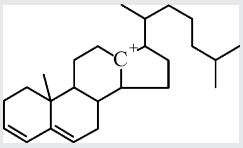
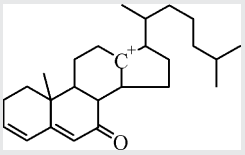
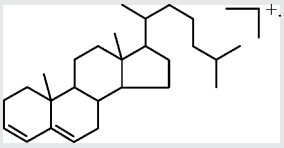
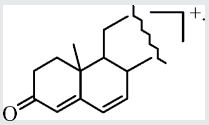
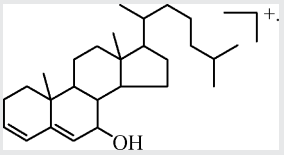
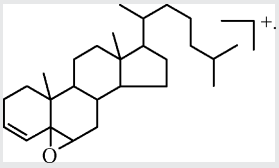
m/*z*	Compound/fragment			Proposed structure
	Loss	Origin	16 eV	
124	$M^{\bullet+}-260$	5-Cholesten-3-one (**5**)	9%	
136	$M^{\bullet+}-246$	Cholesta-4,6-diene-3-one (**6**)	26%	
160	$M^{\bullet+}-222$	Cholesta-4,6-diene-3-one (**6**)	18%	
174	$M^{\bullet+}-208$	Cholesta-4,6-diene-3-one (**6**)	14%	
192	$M^{\bullet+}-208$	5-Cholesten-3-ol-7-one (**3**)	10%	
229	$M^{\bullet+}-155$	5-Cholesten-3-one (**5**)	15%	
247	$M^{\bullet+}-135$ $M^{\bullet+}-139$	Cholesta-4,6-diene-3-one (**6**) 5-cholesten-3-ol (**1**)	32% 8%	
	$M^{\bullet+}-137$	5-cholesten-3-one (**5**)	4%	
261	$M^{\bullet+}-123$	5-Cholesten-3-one (**5**)	6%	
269	$M^{\bullet+}-113$	Cholesta-4,6-diene-3-one (**6**)	26%	
271	$M^{\bullet+}-113$	5-Cholesten-3-one (**5**)	12%	
275	$M^{\bullet+}-111$ $M^{\bullet+}-109$	5-Cholesten-3-ol (**1**) 5-Cholesten-3-one (**5**)	24% 11%	
287	$M^{\bullet+}-113$	5-Cholesten-3-ol-7-one (**3**)	5%	
299	$M^{\bullet+}-85$	5-Cholesten-3-one (**5**)	3%	
301	$M^{\bullet+}-85$	5-Cholesten-3-ol (**1**)	15%	$M^{\bullet+}$–C22–C27
342	$M^{\bullet+}-42$	5-Cholesten-3-one (**5**)	5%	

Table 1 (*Continued*)

m/z	Compound/fragment			Proposed structure
	Loss	Origin	16 eV	
353	$M^{\bullet+}-33$	5-Cholesten-5-en-3-ol (**1**)	11%	
367	$M^{\bullet+}-33$	5-Cholesten-3-ol-7-one (**3**)	5%	
368	$M^{\bullet+}-18$	5-Cholesten-3-ol (**1**)	21%	
369	$M^{\bullet+}-15$	5-cholesten-3-one (**5**)	8%	$M^{\bullet+}-CH_3$
371	$M^{\bullet+}-15$	5-Cholesten-3-ol (**1**)	18%	$M^{\bullet+}-CH_3$
382	$M^{\bullet+}$ $M^{\bullet+}-18$	Cholesta-4,6-diene-3-one (**6**) 5-cholesten-3-ol-7-one (**3**)	100% 10%	
384	$M^{\bullet+}$	5-Cholesten-3-one (**5**)	100%	For structure see Scheme 1
	$M^{\bullet+}-18$	5-Cholesten-3,7-diol (**4**)	100%	
	$M^{\bullet+}-18$	Cholest-5,6-epoxide-3-ol (**2**)	19%	
386	$M^{\bullet+}$	5-Cholesten-3-ol (**1**)	100%	For structure see Scheme 1
400	$M^{\bullet+}$	5-Cholesten-3-ol-7-one (**3**)	100%	For structure see Scheme 1
402	$M^{\bullet+}$ $M^{\bullet+}$	Cholest-5,6-epoxide-3-ol (**2**) 5-Cholesten-3,7-diol (**4**)	100% 15%	For structure see Scheme 1 For structure see Scheme 1

Data presented include relative intensities derived from the DTMS 16 eV run of the ethanol solutions of pure reference compounds.

(B) of the ethanol homogenate of an unexposed egg-only tempera paint-based dosimeter. The base peak (*m/z* 386) in Fig. 1B is also the base peak in the 16 eV EI spectrum of cholesterol. Indicated with (C) are the peaks relating to EI 16 eV fragment ions, which are also described in Table 1. Fig. 2 shows the summation mass spectrum of 64-day light-aged egg-only tempera (over the same scan range). This spectrum shows high intensities of the peaks *m/z* 384, 386 and 400. These peaks can be attributed to cholesterol (*m/z* 386) and corresponding oxidation products (*m/z* 384 and 400). The peaks labelled "G" derive from glycerolipids [10]. The "BG" label indicates a peak that originates from background noise (chemical noise).

Fig. 1B shows no evidence for the presence of COPs in unexposed egg-only tempera. Comparison with the spectrum of egg-only tempera light-aged for 64 days (Fig. 2) shows that the cholesterol peaks have decreased and new peaks characteristic of the oxidation products are detected in the light-aged tempera. These include *m/z* 402 [base peak of cholesterol epoxide (**2**), but also detected in other oxidised cholesterol products such as 7-hydroxycholesterol (**4**), details in Table 1], *m/z* 400 [5-cholesten-3-ol-7-one (**3**)], *m/z* 384 [base peak of 5-cholesten-3-one (**5**) and 7-hydroxycholesterol (**2**), but also present in the 16 eV spectra of other oxidation products such as cholesterol 5,6-epoxide (**2**) and 5-cholesten-3-ol-7-one (**3**)] and *m/z* 382 [dehydration product of 5-cholesten-3-ol-7-one (**3**)]. The mass thermograms (MTs) of *m/z* 384, 386, 400 and 402, derived from the DTMS data of 64-day light-aged egg-only tempera are compared in Fig. 3. The first peak in the MTs of *m/z* 384, 400 and 402 (scan number 34) occurs at a higher scan number than that for cholesterol (scan number 30). The increase in desorption temperature is attributed to the higher polarity of the COPs. This is supported by the results from the analysis of the ethanol solution of the reference COPs, in particular 5-cholesten-3-one (**5**) and 7-hydroxycholesterol (**4**). The former desorbs at a slightly higher than cholesterol and the latter one at a much higher scan number (and hence temperature). Both compounds show *m/z* 384 as the base peak in their 16 eV EI DTMS spectrum. The different

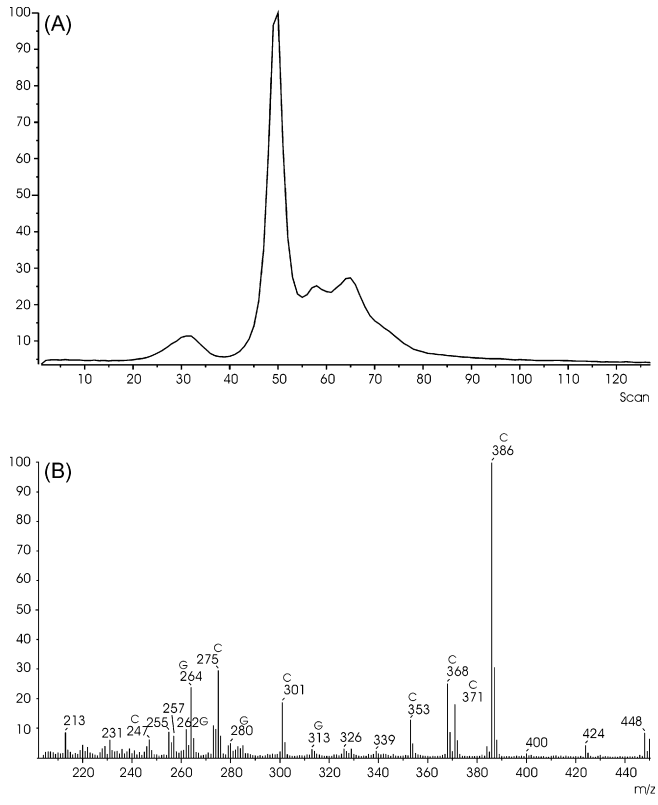


Fig. 1. TIC (A) and summation spectrum (scans 25–40) (B) of unexposed egg-only tempera paint-based dosimeter.

desorption temperatures of the two oxidation products therefore also explain the shape of the MT for *m*/*z* 384.

The most important peaks related to cholesterol and COPs, observed in Figs. 1 and 2, are listed in Table 1. This table only specifies assignments on the basis of the literature on cholesterol oxidation in egg yolk, or in exceptional cases, on the basis of pathways of progressed oxidation of egg yolk COPs. Hydroperoxides are not included in the table, given their presumed short life-time in their chemical matrix and the instability of their molecular ions under EI conditions. A specified example of the structure of the ion [28] or molecule mentioned in the second column of Table 1 is given in the fifth column of the same table. As an aid to the interpretation of the table, Scheme 1 shows the numbering of the cholesterol skeleton as well as the structures of selected COPs.

The peaks *m*/*z* 275 and *m*/*z* 301, can derive from cholesterol as well as its oxidation products (see Table 1) and are specially important 16 eV EI fragment peaks in the spectra of cholesterol (**1**) and 5-cholesten-3-one (**5**).

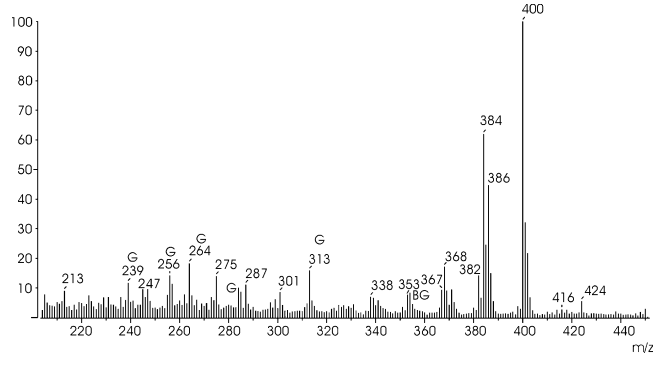


Fig. 2. Partial (*m*/*z* 250–420) summation mass spectrum of the cholesterol desorption window (scans 25–40) for 64-day light-aged egg-only tempera. Peaks labelled G derive from glycerolipids; BG from background (chemical noise).

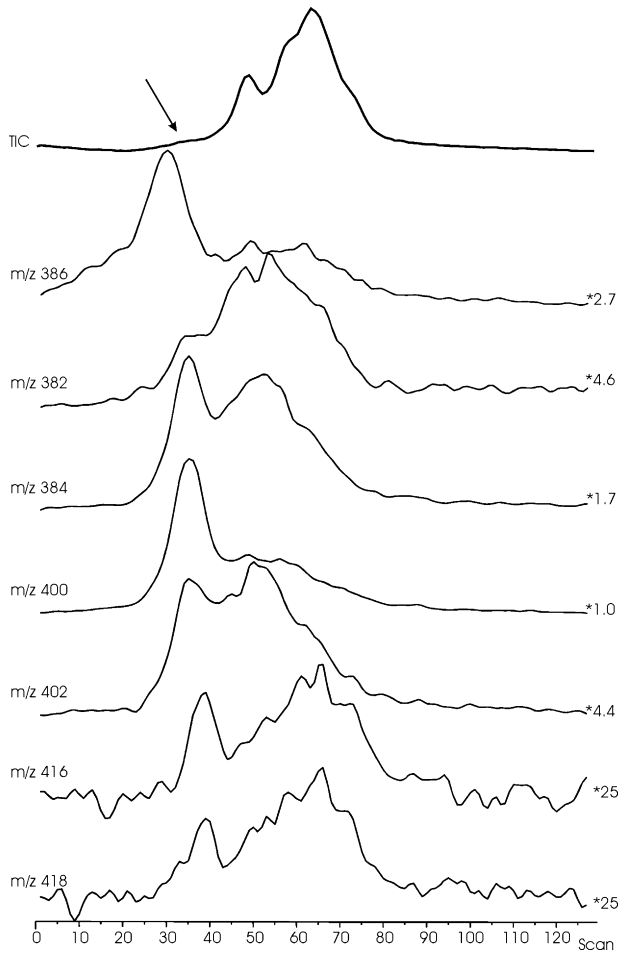


Fig. 3. TIC and MTs of *m*/*z* 386, 384, 400, 402, 416 and 418 derived from the DTMS data of 64-day light-aged egg-only tempera. The MTs are normalized to the maximum intensity of *m*/*z* 400; the normalization factors are given in parentheses.

The peak at *m*/*z* 368 is a known 16 eV EI fragment ion peak of cholesterol (approximately 21% of the height of the base peak *m*/*z* 386). The height of *m*/*z* 368 relative to *m*/*z* 386 in the partial spectrum of the artificially aged egg tempera sample (Fig. 2), suggests that it does not originate from the EI fragmentation alone. There is another possible source, *viz*. a fragment due to loss of a fatty acid neutral from a cholesterol ester molecular ion. Indications for the latter source were found in the DTMS analysis of a similar unpigmented egg paint system to which mastic had been added [13]. It should be noted that the formation of cholestadiene by catalytic dehydration of cholesterol [1] cannot be excluded in egg tempera samples where large amounts of inorganic paint components are present.

Cholesta-4,6-diene-3-one (**6**) could partially account for the peak at *m*/*z* 382 (base peak in the spectrum of the reference compound). Further evidence comes from other fragments ions of the compound (e.g., *m*/*z* 247), which are also present and show the same MT. Smith [1] has suggested three pathways for the oxidative formation of cholesta-4,6-dien-3-one (**6**) from cholesterol (**1**). One of these involves successive oxidation, isomerisation and dehydration of cholest-5-en-3,7-diol (**4**). Another is based on the reaction of cholest-5-en-3-one (**5**) with singlet oxygen ($^1O_2$), and suggests 5-hydroperoxycholest-6-en-3-one as an intermediate.

Experiments by Chicoye et al. [14] indicated that cholest-5-en-3-ol-7-one (**3**) is transformed to cholesta-3,5-dien-7-one under

conditions of saponification (81 °C in KOH solution). Thus, in the case of the lead white tempera, the basicity of the lead hydroxy carbonate pigment ($Pb_2(OH)_2CO_3$) may account for the high intensity of the *m/z* 382 peak in the DTMS spectra of light-aged lead white pigmented tempera [13]. In this connection, it should be noted that *m/z* 400 is the highest peak in the cholesterol mass window of the DTMS spectra of lipid extracts of light-aged lead white tempera (data not shown). This indicates that transformation of ketocholesterol to cholestadienone can also take place during the desorption process on the DTMS analytical process.

The peak at *m/z* 384 may derive from the molecular ion of 5-cholesten-3-one (**5**), which can be formed from cholesterol (**1**) on reaction with $^3O_2$ [1]. It can also originate from 7-hydroxycholesterol (**4**) and although *m/z* 384 is not the base peak of cholesterol-5,6-epoxide (**2**) the presence of this compound may also contribute to the overall intensity of *m/z* 384. As mentioned before, 5-cholest-3-ol-7-one (**3**) is another well-known (and abundant) COP. The *m/z* 400 is the base peak of the 16 eV spectrum of the reference compound. Attribution of the peak at *m/z* 400 to this compound is therefore plausible. The possibililty of a minor contribution from 7-hydroxycholest-5-en-3-one is not excluded as this is one of the structures involved in the aforementioned pathway for the formation of cholesta-4,6-dien-3-one (**6**) from cholest-5-en-3,7-diol (**4**).

Two of the COPs mentioned in the introduction have a molecular weight of 402 Da, *viz.* 5,6-epoxy-cholestan-3-ol (**2**) and cholest-5-en-3,7-diol (**4**). Clearly, other cholestenediols may also be responsible for the peak at *m/z* 402. Cholest-5-ene-3,20-diol and cholest-5-ene-3,25-diol, for instance, have been detected in egg products [29].

If the possibility of multiple oxygenation of cholesterol is taken into account, COPs with molecular weights between 414 and 420 Da can be expected. Fig. 2 therefore focuses especially on some of these masses. Inspection of the figure shows that two of the peaks in this mass window might be tentatively assigned to COPs on the basis of their MTs, *viz.* *m/z* 416 and 418. The MTs of these peaks are also shown in Fig. 3. The shift of the local maximum of these MTs (marked by arrows) with respect to the maximum of the MTs of *m/z* 400 and 384 is again attributed to the higher polarity of these compounds as a result of their higher degree of oxygenation. The MTs of the other peaks between *m/z* 414 and 420 (data not shown) do not show a local maximum in the desorption window of cholesterol. According to the extensive literature on the oxidation of cholesterol, progressed oxidation can occur. The two most preferred sites on the sterol skeleton for progressed oxidation are the 7- and the 25-position. Thus, 5,6-epoxycholestan-3,25-diol and 5-cholesten-3,7,25-triol are suggested as the most probable COPs for *m/z* 418 and 3,25-dihydroxycholest-5-en-7-one for *m/z* 416. The formation of the latter two products by autoxidation of 5-cholesten-3,25-diol has been suggested by Smith et al. [30]. Similarly, autoxidation of the well-known COP 5-cholesten-3-ol-7-one at the 25-position was proposed as an explanation for the formation of 3,25-dihydroxycholest-5-en-7-one.

As indicated in Table 1, loss of water from molecular radical cations is also part of the attribution of the peaks in Fig. 2. Thus, water loss from the $M^{\bullet+}$ of ketocholesterols can contribute to the intensity of the peak at *m/z* 382. It should be noted however that the relative intensity of *m/z* 382 in the 16 eV EI DTMS spectrum of cholest-5-en-3-ol-7-one (**3**) is less than 20% of the base peak ($M^{\bullet+}$). In the 16 eV EI DTMS spectrum of cholest-5-en-3,7-diol, the $M^{\bullet+}-H_2O$ peak (*m/z* 384) is the base-peak and the molecular ion (*m/z* 402) shows a relative intensity of 15% (data not shown). It must be assumed that the spectra of other cholestenediols will also show a significant peak at *m/z* 384. 5,6-Epoxycholestan-3-ol produces $M^{\bullet+}-H_2O$ ions at a relative intensity of 20% of the molecular ion (base-peak) and will therefore also contribute to the signal intensity at *m/z* 384.

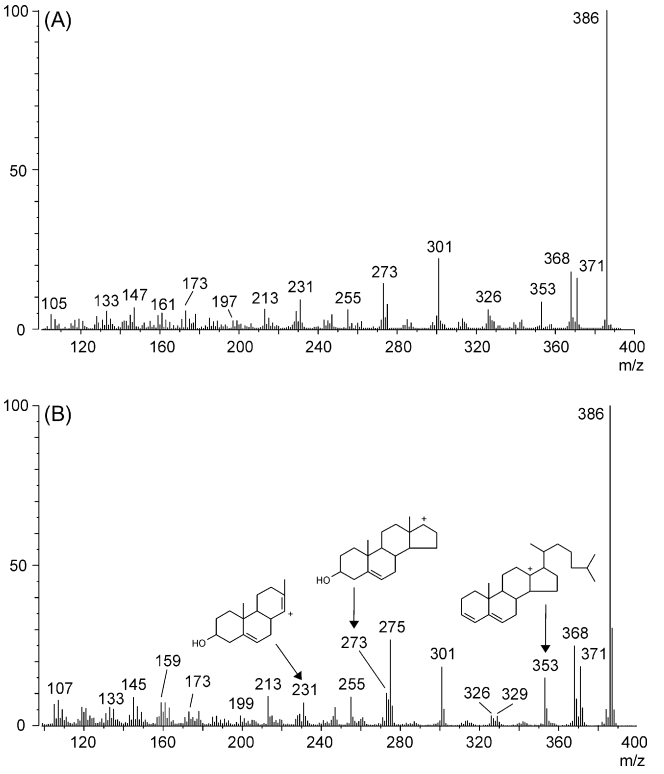


Fig. 4. DTMSMS spectrum of peak *m/z* 386 from 21-day light-aged egg-only tempera (A) and DTMS spectrum (EI, 70 eV) of cholesterol (B).

The ion at *m/z* 402, in turn, could partly originate from water loss of a COP with molecular weight 420. Cholestane-3,5,6-triol, which is known as a hydration product of 5,6-epoxy-cholestan-3-ol [1], has been detected in an 8-year old egg yolk sample [29]. Water loss from the molecular ion of this compound is highly probable, due to the presence of three hydroxyl groups and may even explain the absence of a peak at *m/z* 420 in Fig. 2. Hence, the presence of small quantities of the compound in the light-aged tempera sample cannot be excluded. Since Fig. 2 indicates the presence of COPs with a molecular weight of 418 Da, which probably contain more than one hydroxyl-group, fragmentation of the molecular ions of these compounds by loss of water may also partly account for the *m/z* 400 peak.

3.2. DTMSMS of COPs

Although it seems evident that *m/z* 386 originates from cholesterol (**1**), the peak was subjected to DTMSMS analysis for confirmation. Fig. 4A shows the DTMSMS spectrum of peak *m/z* 386 from sample of 21-day light-aged egg-only tempera. Comparison with the 70 eV electron impact ionisation spectrum of cholesterol (**1**) (Fig. 4B) indicates that peak *m/z* 386 in the DTMS spectra of egg-only tempera may, in fact, be attributed to cholesterol (**1**). Characteristic peaks for the fragmentation of cholesterol (**1**) are observed in both spectra. These include peaks at *m/z* 371 ($M^{\bullet+}-CH_3^{\bullet}$), *m/z* 368 ($M^{\bullet+}-H_2O$), *m/z* 353 ($M^{\bullet+}-CH_3^{\bullet}-H_2O$), *m/z* 301 (loss of C22–27), *m/z* 275 (loss of C1–7) [28,31], *m/z* 273 (loss of C20–27+H), *m/z* 231 (loss of C16–17+C20–27+H) and *m/z* 213 (loss of C16–17+C20–27+H+H_2O).

Fig. 5A shows the DTMSMS spectrum of the *m/z* 400 peak from light-aged (21 days at 10,000 lx) egg-only tempera. Comparison of this spectrum with the DTMSMS spectrum of a 5-cholesten-3-

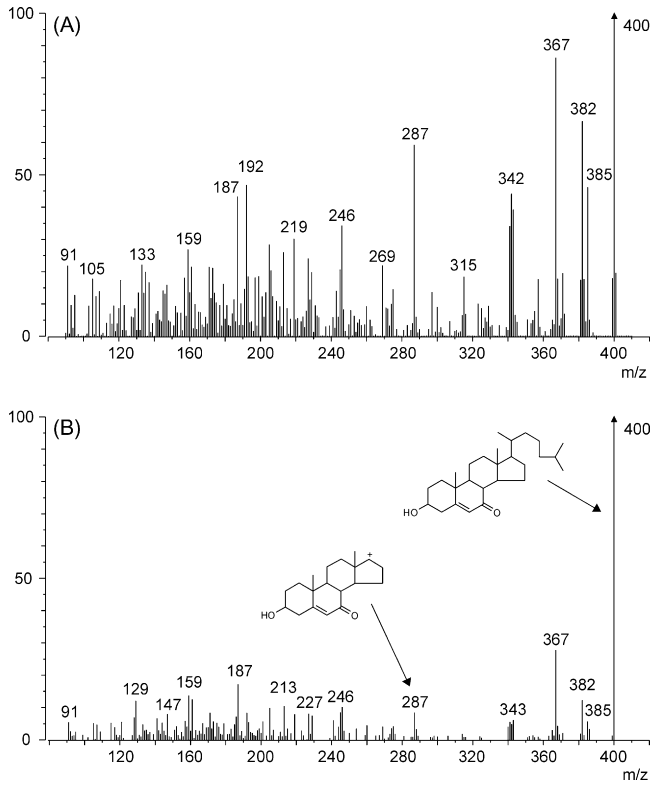


Fig. 5. DTMSMS spectra of peak *m*/*z* 400 from 21-day light-aged egg-only tempera (A) and 3-hydroxycholest-5-en-7-one (B).

ol-7-one (**3**) standard (Fig. 5B) shows great similarities. *m*/*z* 385 and *m*/*z* 382 are attributed to loss of a methyl radical and a water molecule, respectively. The peak at *m*/*z* 367 is the combined result of these two fragmentations. Fragmentation at the side chain (bond C17–20) leads to the formation of *m*/*z* 287 through loss of a $C_8H_{17}^{\bullet}$ radical. The peak at *m*/*z* 205 derives from a rearrangement fragmentation in the C-ring that breaks the bonds C8–14 and C12–13 and involves the transfer of one hydrogen. The same fragmentation, combined with the loss of water, results in *m*/*z* 187. Each of these peaks in the MSMS spectrum of the standard compound is also present in Fig. 5A. A few peaks in the DTMSMS spectrum of *m*/*z* 400 from the light-aged egg-only sample do not appear in the spectrum of the standard compound (e.g., 297 and 315). This suggests that other components must be present in the light-aged tempera, which form *m*/*z* 400 ions. Possibly fragment ions with *m*/*z* 400 resulting from water loss of COPs with molecular weight 418 Da contribute to the peak. Differences in internal energy of the ions may account for differences in relative intensities of important peaks in the mass spectra. The presence of a peak at *m*/*z* 192 in the 70 eV EI spectrum of 5-cholesten-3-ol-7-one (**3**) confirms this notion.

The DTMSMS spectrum of peak *m*/*z* 402 from the light-aged egg-only tempera sample (21 days) shown in Fig. 6A closely resembles that of the molecular ion of 5,6-epoxy-cholestan-3-ol (**2**) shown in Fig. 6B. Differences between the spectra may derive from a variety of factors. First of all there may be a minor difference in internal energy of the precursor ions. In addition, Tsai and Hudson [15] have shown that configurational differences in the structure of 5,6-epoxy-cholestan-3-ol (**2**) are expressed in the mass spectra. Although discrimination between the two epimers is not possible on the basis of the spectra presented, 5,6-epoxy-cholestan-3-ol (**2**) is positively identified in the light-aged egg-only tempera. It should be noted that the 5β,6β-epoxide is thermodynamically more stable than the 5α,6α-epoxide and that this is used to explain the higher relative abundance of the β-epoxide [23]. 5,6-Epoxycholesterols are formed on reaction of cholesterol with active oxygen species such as singlet oxygen ($^1O_2$) and hydroperoxide [30]. The high abundance of 5,6-epoxycholesterols may thus be explained as interaction between cholesterol and hydroperoxide functionalities of (oxidised) egg glycerolipids. As mentioned earlier, 5-cholesten-3,7-diol (**4**) is another likely candidate to (partly) account for the peak *m*/*z* 402. The DTMSMS spectrum of the molecular ion of this compound is shown in Fig. 6C. The lack of specificity of the DTMSMS spectrum and the strong contribution of 5,6-epoxy-cholestan-3-ol (**2**) to the spectrum in Fig. 6A do not allow a partial attribution of the peak at *m*/*z* 402 to 5-cholesten-3,7-diol (**4**). However, it may be recalled that cholest-5-en-3,7-diol (**4**) shows a relatively small molecular ion peak *m*/*z* 402 under 16 eV EI conditions. It is likely, therefore, that the cholest-5-en-3,7-diol (**4**) will be the main contributor to the intensity at *m*/*z* 384.

The MSMS peaks from *m*/*z* 384 in the spectra of 5-cholesten-3-one (**5**) ($M^{\bullet+}$), 5-cholesten-3,7-diol (**4**) and 5,6-epoxy-cholestan-3-ol (**2**) (both $M^{\bullet+}-H_2O$) did not allow unambiguous identification of the structure of the precursor ion.

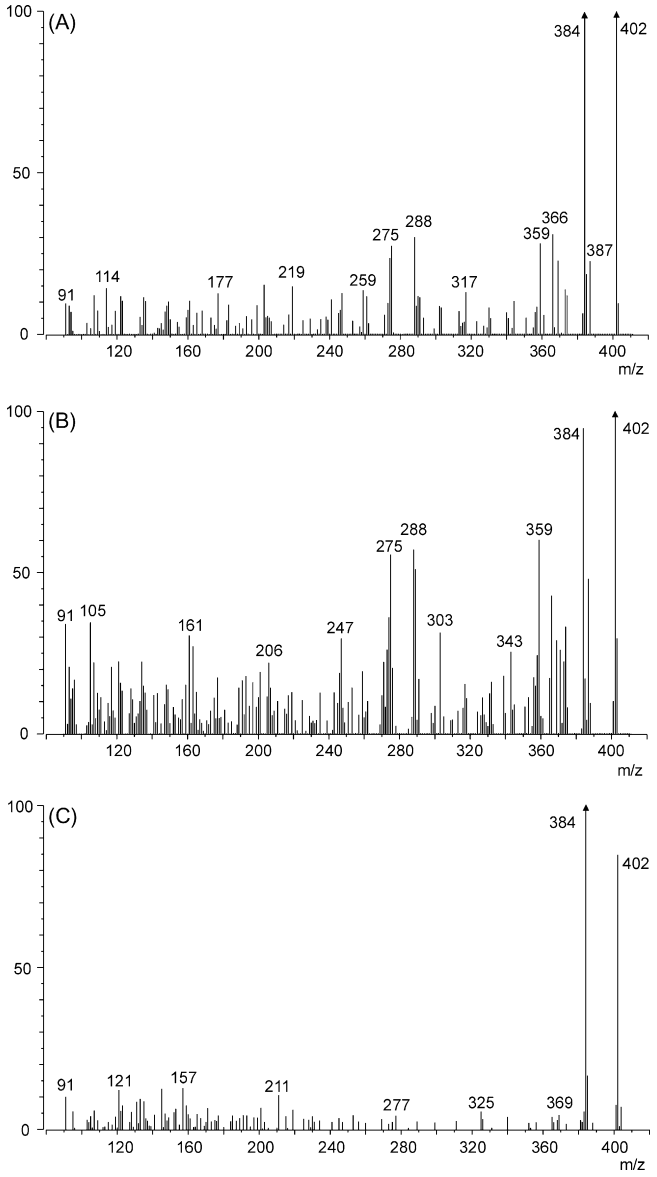


Fig. 6. DTMSMS spectra of peak *m*/*z* 402 from 21-day light-aged egg-only tempera (A), 5,6-epoxy-cholestan-3-ol (B) and cholest-5-en-3,7-diol (C).

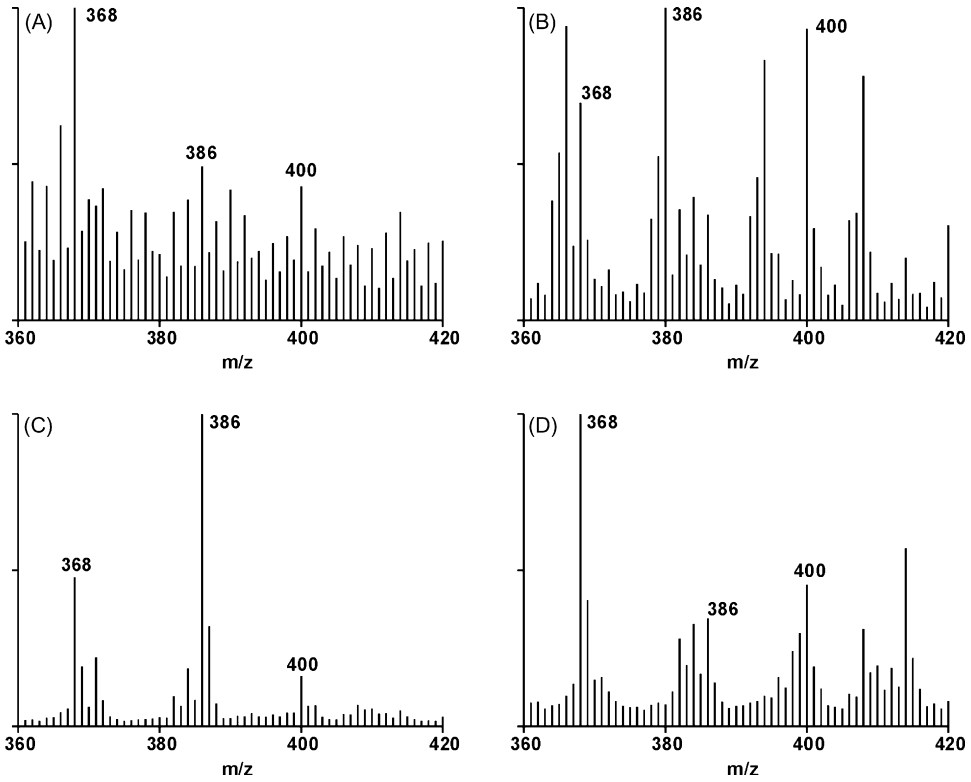


Fig. 7. Partial DTMS data highlighting the appearance range of cholesterol and its oxidation products in samples from (A) an opaque green glaze on silver leaf on the bust of St. Marinus (c. 1760) from the side altarpiece in the former Benedictine Monastery Church in Rott am Inn (Bavaria); (B) polychromy remnant from the Holy Bishop presently in the Mainfränkisches Museum Würzburg; (C) red glaze on silver leaf from the inside of pulpit canopy in the Catholic Pilgrimage Church *Die Wies* in Pfaffenwinkel (Bavaria); (D) a discolored glaze on Red on Maroon (Tate Nr. 01165) by Mark Rothko (1903–1970).

The question of the identity of the presence of cholestenediols was also investigated by means of a DTMS analysis of trimethylsilyl (TMS) derivatized standards and samples. DTMS analysis of silylated COPs, such as 5-cholesten-3-ol-7-one (**3**), 5-cholesten-3-one (**5**) and 5,6-epoxy-cholestan-3-ol (**2**), indicated that epoxy- and keto-functionalities are not silylated. The spectrum of TMS derivatized cholest-5-en-3,7-diol showed a molecular ion at *m*/*z* 546 and a base peak at *m*/*z* 456, indicating that the loss of trimethylsilanol ($HOSi(CH_3)_3$) is an important feature in the spectra of silylated cholestenediols. The observation of a peak at *m*/*z* 546 and an intense peak at *m*/*z* 456 in the partial (cholesterol desorption window) DTMS spectrum of the silylated extract of 21-day light-aged egg-only tempera (data not shown) indicate the presence of COPs with two hydroxyl functionalities on a cholestene skeleton in the light-aged sample. Furthermore, in a similar way, the observation of peaks at *m*/*z* 472 and 474 in the same spectrum confirms the presence of hydroxycholestenone [probably cholest-5-en-3-ol-7-one (**3**)] and 5,6-epoxy-cholestan-3-ol (**2**) in the 21-day light-aged egg-only tempera sample.

3.3. COP's in microsamples from works of art

DTMS is a commonly used microanalytical technique for the analysis of samples from paintings [32]. The data are usually complex mass spectral fingerprints as a result of the evaporation and pyrolysis of many different compounds present in multilayered paints. Cholesterol is occasionally encountered and often considered to be a contaminant from the handlers of the works of art. The presence of cholesterol and several of its oxidation products is more indicative of the original use of egg-based products by the artist. Fig. 7 shows four partial mass spectra summarized in the evaporation range of the DTMS data sets from three German Baroque surface finishes of altar pieces (Fig. 7A–C) and a colored glaze from a 20th century painting by Mark Rothko (1903) (Fig. 7D). The Rothko painting Tate Nr. 01165 (Red on Maroon) was extensively investigated establishing the presence of cholesterol compounds, egg proteins and dammar resin as a surface glaze [33]. Despite the young age of the film, the dammar was highly oxidised which is presumably due to the presence of synthetic ultramarine. One of the oxidation products of the dammar in the film characterised by its molecular ion at *m*/*z* 414 (3-oxo-25,26,27-trisnor-dammarano-24,20-lactone) in Fig. 7D is evidence for the highly oxidising conditions [34]. The 3,5-cholestadiene (*m*/*z* 368) as water elimination product of cholesterol (*m*/*z* 386) is a relative intense peak in the DTMS data. Evidence for COP's is *m*/*z* 400 (**3**), 384 (**5**) and 382 (**6**). This information suggests that Rothko is at least using egg yolk as one of the constituents of his colored glaze.

The partial DTMS from the red glaze on the pulpit canopy (1753–1756) in Die Wies in Upper Bavaria has an intense *m*/*z* 386 from preserved cholesterol in the glaze. The *m*/*z* 368 is proposed to be the water elimination product due to electron ionisation as well to the thermal process of desorption on the platinum/rhodium analytical filament. The smaller *m*/*z* 400, 384 and 382 can be interpreted as molecular ions of COP's. The *m*/*z* 400 from the 7-oxo-cholesterol (**3**) is a relatively intense peak in the DTMS of the polychrome on the back of the neck the Holy Bishop (dated c. 1505–1510) made by Tilman Riemenschneider (1460–1531). The presence of other cholesterol derived ions (*m*/*z* 368, 382, 384, 386) supports the proposal that egg yolk might have been one of the constituents of the surface coating. The DTMS of the green glaze on silver from the bust of St. Marinus (polychromy from the 1760s) in the former Benedictine Monastery church in Rott am Inn shows evidence for cholesterol and thus egg derived constituents by the ions with *m*/*z* 400, 386 and 368. The reason why egg yolk or whole egg is used by artists in their media is often unknown. We can

speculate that it was used to emulgate the materials used to make the glaze paint into a free flowing brushable substance.

4. Conclusion

The results presented show that DTMSMS and the combined use of DTMS with trimethylsilyl derivatization are useful tools for the characterization of molecular changes in light-aged egg tempera samples. Using DTMSMS, 5,6-epoxycholestan-3-ol (**4**) and 3-hydroxycholest-5-en-7-one (**3**) were positively identified in light-aged egg tempera binding medium. The former compound is likely to have been formed as a result of interaction between peroxidised glycerolipids and cholesterol. DTMS of trimethylsilyl derivatized light-aged egg-only tempera allowed the identification of cholestenediol. The assignment of cholesterol-related m/z peaks in previous publications [9,10] was thereby confirmed and refined. The m/z peaks in the DTMS spectra of (aged) egg tempera paint systems have now been identified. They can be used as an aid in the interpretation of differences observed in tempera paint-based dosimetry. As the oxidation of cholesterol develops early in the life-time of egg tempera paint and is accelerated by the presence of inorganic pigments, cholesterol itself is not a good marker for egg as a binding medium in paintings. Cholesterol oxidation products may be better markers, provided they survive the attrition of time. The DTMS data from the Baroque altar pieces indeed evidence the survival of some cholesterol and various cholesterol oxidisation products over the centuries. The cholesterol oxidation products in the Rothko painting are also evidence for rather oxidising conditions in the glaze that now is discolored.

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