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# Cholesterol degradation in archaeological pottery mediated by fired clay and fatty acid pro-oxidants

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**Abstract:**

Cholesterol is generally absent in animal fat residues preserved in archaeological ceramic vessels. It is known from edible oil refining that during bleaching with activated clay sterols are degraded, largely *via* oxidation. Laboratory heating experiments using fired clay from replica pottery vessels promoted rapid degradation of cholesterol *via* oxidation. Furthermore, heating cholesterol with fatty acids (saturated and unsaturated) revealed additional degradation to occur independently of the ceramic matrix. As both conditions are met in archaeological pottery during animal (and plant) product processing involving heating, the very rare detection of sterols in organic residues can be explained.

**Keywords:** cholesterol; fatty acids; archaeology; degradation; clay; organic residue

Unglazed ceramic pots found commonly at archaeological sites are carriers of valuable information about past human diet and food procurement. This information resides in the chemical residues of food constituents, mainly lipids, that become absorbed in the porous ceramic matrix during food processing.[1, 2] Once absorbed, these lipids are relatively protected against ground water leaching and microbial degradation, and owing to their hydrophobicity, can survive over many millennia.[2]

The lipids recovered from archaeological pottery rarely retain the composition originally found in the commodities from which they derive. The changes observed can be separated into pre-burial, use-related modifications and post-burial (mainly microbial) changes. In particular, high heat (>270 ºC) during vessel use has been shown to lead to formation of a range of diagnostic degradation products, e.g. long-chain ketones from the condensation of fatty acids, or vicinal dihydroxy fatty acids and ω-(*o*-alkylphenyl)-alkanoic acids formed from monounsaturated and polyunsaturated fatty acids, respectively.[2-6] Pre- and post-burial changes are apparent from the hydrolysis of acyl lipids (such as triacylglycerols) to free fatty acids and di- and monoacylglycerols, as well as the preferential loss of polar compounds due to water leaching.[7, 8]

Sterols, such as cholesterol in animals and ß-sitosterol in plants, are essential membrane compounds and therefore present in any living eukaryotic organism and consequentially virtually in every food commodity. Yet, gas chromatography-mass spectrometry (GC-MS) analysis of several thousand lipid extracts has shown sterols are almost entirely absent in archaeology pottery. Indeed, so rare are sterols that where they are detected, modern contamination is suspected to be the most likely source, e.g. cholesterol from fingerprints.[9, 10] Since we have recently shown that sterols are reasonably resistant to post-burial degradation,[11] this makes degradation pre-burial, for example during cooking, the most likely explanation for the absence of sterols from organic residues.

Commercial edible oil refining, particularly the bleaching process, is known to lead to the degradation of sterols.[12-14] Interestingly, acid-activated clay suspensions are the most common solid phases used in oil bleaching.[12, 15] Furthermore, the heating of sterols with fatty acids and triacylglycerols has been found to promote lipid degradation.[16] Active clay surfaces and abundant acyl lipids would have been ubiquitous in archaeological ceramic cooking pots, and cooking processes would have involved applying substantial heat. Therefore, we rationalized that this might cause the degradation of sterols and explain their general absence in archaeological food residues in pottery. To test this hypothesis, laboratory heating experiments were performed using known lipids spiked on powdered clay ceramics. The progress of the experiments was monitored by GC analysis of trimethylsilylated solvent extracts of the ceramics, with quantification using internal standards.

In initial experiments cholesterol (212 µg) and oleic acid (58 µg) were applied to 0.5 g of powdered clay ceramic and heated (100 ºC, 14 h) in open glass vials. GC analysis showed that 73(±4)% of the cholesterol was degraded by this treatment (Experiment 1 in Table 1, Fig. 1a).

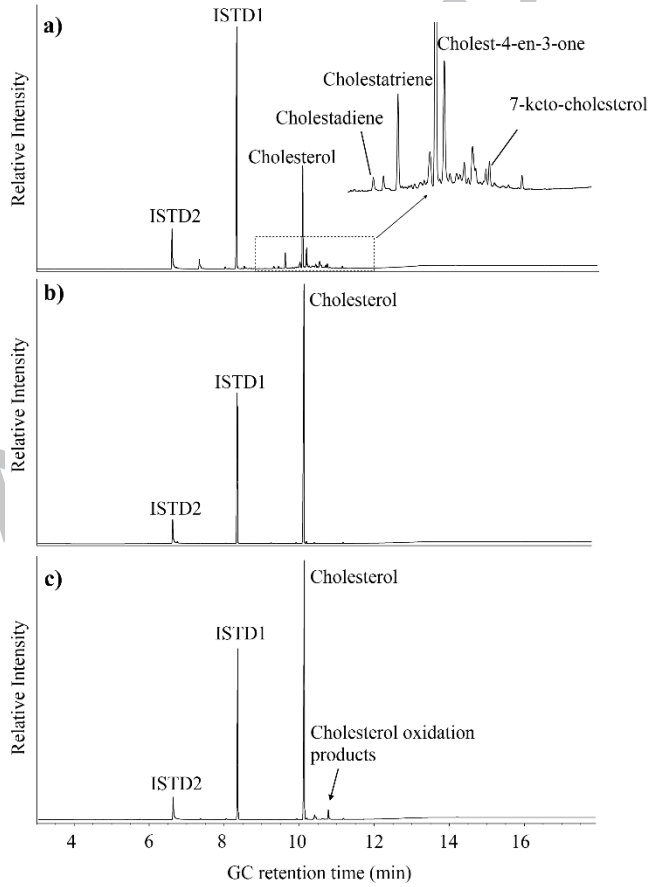

**Figure 1.** GC-FID chromatograms of cholesterol and cholesterol oxidation products extracted from experiments 1 (a), 4 (b) and 3 (c). The internal standards (ISTD1 and ISTD2) are at the same concentrations in all three chromatograms. The magnified section of chromatogram in panel a) shows the cholesterol oxidation products eluting between 9 and 12 min (identified by GC-MS).

The same pattern of degradation products and loss was observed when only cholesterol was spiked on clay. However, heating cholesterol without clay resulted in almost the complete recovery of cholesterol (Experiment 4 in Table 1, Fig. 1b). The addition of oleic acid and heating without clay led to minimal degradation of cholesterol and the formation of only low abundances of degradation products (Experiment 3 in Table 1, Fig. 1c). Previous studies suggested that cholesterol is stable at temperatures of 100 ºC, but the addition of clay clearly led to cholesterol degradation even at this relatively low temperature.[17, 18] Cholesterol degradation followed approximately first-order kinetics for the first 24 h ($R(Chol)=184.6e^{-0.071t}$, Fig. 2).

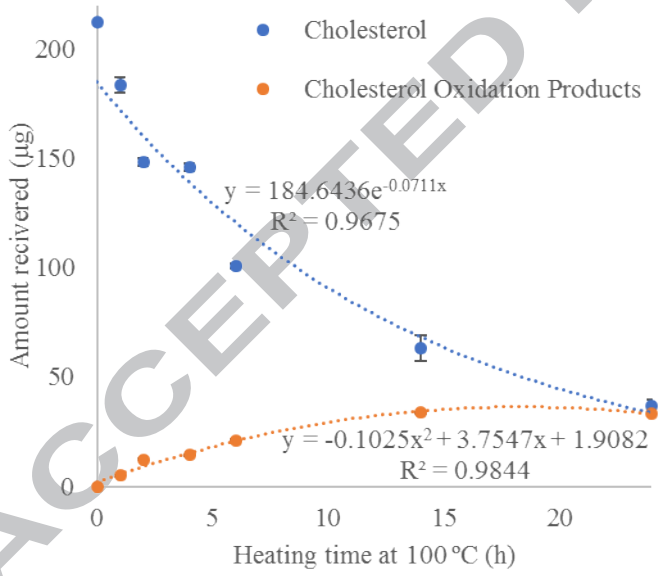


**Figure 2.** Cholesterol (blue) and cholesterol oxidation products (orange) recovered from clay after heating 212 µg cholesterol and 58 µg oleic acid at 100 ºC for 1, 2, 4, 6, 14, and 24 h (experiments 5-10 in Table 1).

Interestingly, GC-amenable oxidation products did not accumulate to account for cholesterol loss, such that after 24 h almost 180 µg of cholesterol was degraded, but

only *ca*. 30 µg of cholesterol degradation products could be accounted for. Most likely, the oxidation products underwent further reactions and were subsequently lost as volatile products from the reaction vessel or were polymers that had too high molecular weight to be analysed by the GC method used in this study.[19]

The main degradation products (identified by GC/Q-TOF MS) were cholesterol-dehydration products (cholestadiene and cholestatriene isomers), as well as the oxidation products cholest-4-en-3-one and 7-keto-cholesterol. Although almost 30 degradation products of cholesterol were detected (see ESI Table S1), the distribution did not correspond to the usually observed thermooxidation products or degradation products of cholesterol expected and reported in bleaching earth, i.e. steradienes, 7-α/β-hydroxycholesterol, 5-α/β-epoxycholesterol and cholestanetriol alongside 7-keto-cholesterol.[14, 17, 20, 21] While 7ß-hydroxycholesterol and 7-keto-cholesterol were detected they were not the main degradation products, which indicates different degradation pathways compared to those observed in edible oil refining. These differences could be due to different treatments of the clay in both processes, i.e. acid-activation for refining of oil and high-temperature firing for the production of pottery, or different availability of oxygen.

**Table 1.** Cholesterol recovery, heating time and initial composition of the experimental mixtures. The amount of cholesterol added was 212 µg.

| Experiment | Clay | Fatty acid, (µg spiked) | Ratio of cholesterol: fatty acid (w/w) | Time heated at 100 ºC (h) | Cholesterol recovered (%) (uncertainty from duplicates experiments unless stated otherwise) |
|---|---|---|---|---|---|
| 1 | Yes | Oleic acid (58 µg) | 4:1 | 14 | 27 (±4, n=4) |
| 2 | Yes | - | - | 14 | 30 (±5, n=4) |
| 3 | No | Oleic acid (58 µg) | 4:1 | 14 | 89 (±3) |
| 4 | No | - | - | 14 | 98 (±1) |
| | | | | | |
| 5 | Yes | Oleic acid (58 µg) | 4:1 | 1 | 86 (±2) |
| 6 | Yes | Oleic acid (58 µg) | 4:1 | 2 | 70 (±1) |
| 7 | Yes | Oleic acid (58 µg) | 4:1 | 4 | 69 (±1) |

| | | | | | |
|---|---|---|---|---|---|
| 8 | Yes | Oleic acid (58 µg) | 4:1 | 6 | 48 (±1) |
| 9 | Yes | Oleic acid (58 µg) | 4:1 | 14 | 30 (±3) |
| 10 | Yes | Oleic acid (58 µg) | 4:1 | 24 | 17 (±1) |
| | | | | | |
| 11 | Yes | Stearic acid (56 µg) | 4:1 | 14 | 27 (±2) |
| 12 | Yes | Oleic acid (58 µg) | 4:1 | 14 | 30 (±4, n=4) |
| 13 | Yes | Linoleic acid (58 µg) | 4:1 | 14 | 27 (±2) |
| | | | | | |
| 14 | Yes | Oleic acid (104 µg) | 2:1 | 14 | 28 (±1) |
| 15 | Yes | Oleic acid (214 µg) | 1:1 | 14 | 28 (±1) |
| 16 | Yes | Oleic acid 416 µg) | 1:2 | 14 | 25 (±3) |
| 17 | Yes | Oleic acid (2.1 mg) | 1:10 | 14 | 1 (±1) |
| 18 | Yes | Oleic acid (21 mg) | 1:100 | 14 | 1 (±1) |
| | | | | | |
| 19 | Yes | Stearic acid (412 µg) | 1:2 | 14 | 20 (±1) |
| 20 | Yes | Stearic acid (2.1 mg | 1:10 | 14 | 1 (±1) |
| 21 | Yes | Stearic acid (21 mg) | 1:100 | 14 | 2 (±1) |
| 22 | No | Stearic acid (21 mg) | 1:100 | 14 | 49 (±9) |
| 23 | No | Oleic Acid (2.1 mg) | 1:10 | 14 | 1 (±0) |
| 24 | No | Oleic Acid (21 mg) | 1:100 | 14 | 1 (±0) |
| 25 | No | Trioleate (2.1 mg) | 1:10 | 14 | 86 (±2) |
| 26 | No | Trioleate (21 mg) | 1:100 | 14 | 88 (±2) |

To test the effect of fatty acids as potential pro-oxidative agents, further heating experiments with fatty acids of different degrees of unsaturation, i.e. stearic acid (fully saturated), oleic acid and linoleic acid (mono-, and diunsaturated, respectively) were conducted using a ratio of 1:4 *w/w* fatty acid to cholesterol (Experiments 11-13 in Table 1). No significant differences were seen in the recovery of cholesterol between the fatty acids or a control without fatty acids. This showed that at these low ratios fatty acids had only a minimal effect on cholesterol degradation and the degree of unsaturation did not markedly affect reactivity. Accordingly, the clay was deemed to be the major influence factor on the observed degradation.

Cholesterol was then heated with different ratios of oleic acid, from the initially used ratio of 1:4 (fatty acid to cholesterol) to 100:1 *w/w* (experiments 14-18 in Table 1). It was observed that when the ratio reached 10:1 *w/w* cholesterol became almost

completely degraded, and the GC chromatograms showed a wide range of new peaks (Fig. 3a+b). The degradation products differed from those observed during experiments involving heating cholesterol with only clay or lower concentrations of fatty acids. Some of the most prominent new products formed were $C_6$-$C_9$ ω-diacids (identified by GC/Q-ToF MS) together with unidentified degradation products eluting from the GC later than cholesterol. The diacids likely formed from hydroperoxides produced through autoxidation at the double-bond in oleic acid (ESI, Fig. 1).[22] None of the potentially formed unsaturated diacids (i.e. deca-2-en-1,10-dioic acid and undeca-2-en-1,11-dioic acid from C-10 and C-11 peroxides) were observed, most likely due to further reactions leading to their degradation.

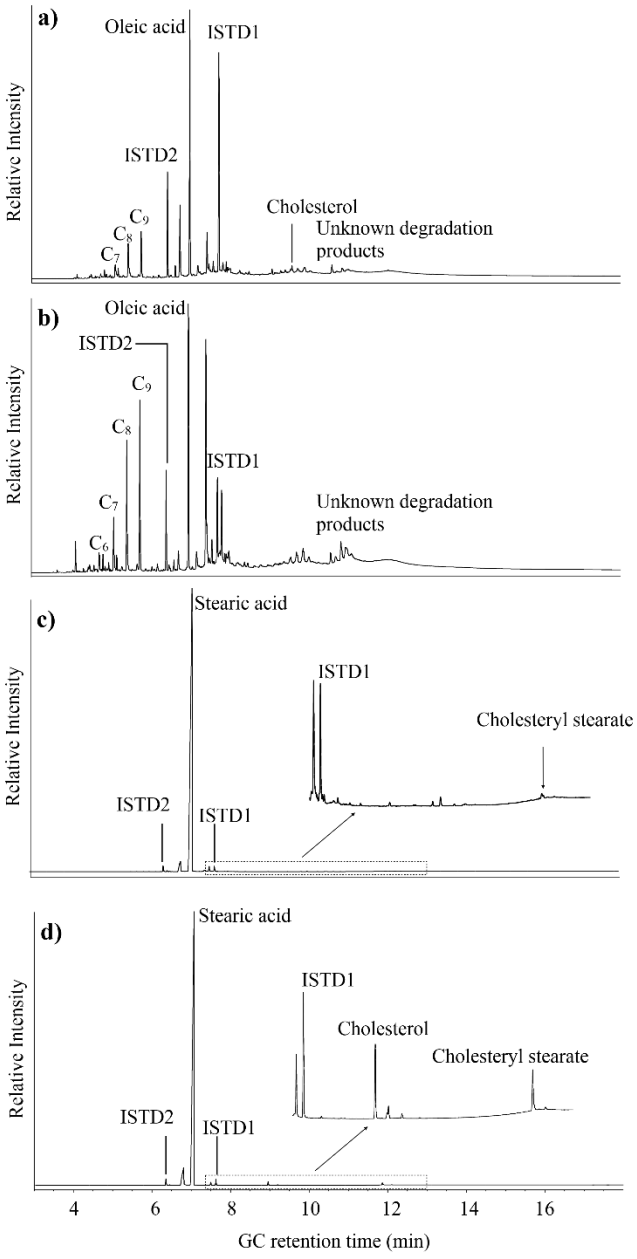

**Figure 3**. GC-FID chromatograms of the products of experiments 17 and 18 (Table 1) corresponding to oleic acid and cholesterol in ratios of a) 10:1 and b) 100:1 *w/w* showing the almost complete degradation of cholesterol, as well as c) and d) the chromatograms of the products of experiments 21 and 22 (stearic acid: cholesterol 100:1 *w/w*) with and without clay, respectively. Peaks labelled with $C_6$-$C_9$ are α,ω-diacids with 6-9 carbons.

Critically, the degradation seen in these experiments was observed even when oleic acid was replaced with the saturated *n*-alkanoic acid, stearic acid (Experiments 19-21 in Table 1). The conclusion is, therefore, that we are observing a fatty acid-dependent reaction independent from the oxidative reactivity of the clay surface observed before. The pattern of degradation products was very different when stearic acid was used, rather than oleic acid, with none of the diacids being observed and higher residual concentration of stearic acid (Fig. 3c). This was expected due to the absence of double bonds in stearic acid and the consequential lower susceptibility to autoxidation. When cholesterol alone was heated with an excess of stearic acid (100:1 *w/w*) in the absence of clay powder a moderate decrease of cholesterol was observed, but also the formation of a sizable peak which matched the retention time of cholesterol stearate (Fig. 3d). This suggests that significant esterification takes place under these conditions and the observed decrease of cholesterol is not only through oxidation in this case. While the respective oleic acid esters in the other experiments were possibly formed as intermediates, they were apparently not stable under the experimental conditions and underwent further degradation reactions. Noteworthy, heating of cholesterol with trioleate instead of oleic acid lead only to a moderate decrease in cholesterol (12-14%, experiments 25-26 in Table 1). Considering the evidence that sterol esters are more prone to oxidation than free sterols this could indicate that esterification and further degradation of the esters could also be an important reaction pathway here.[18, 23]

In summary, we have shown that both clay and fatty acids (present in high relative abundances, compared to cholesterol, consistent with those seen in animal fats) independently promote the degradation of cholesterol when heated at only 100 ºC. Free fatty acids promoted cholesterol degradation more strongly than triacylglycerols, but free fatty acids can be readily liberated from the TAGs during cooking steps and after absorption into the ceramic matrix. Since both fatty acids

and clay would have been commonplace during cooking in archaeological pottery (through the presence of the pot matrix and co-occurring lipids in foodstuffs), this could explain the general absence of cholesterol in preserved lipid residues. The latter makes the general survival and occurrence of cholesterol in archaeological cooking residues unlikely, and its detection should always be interpreted with caution.

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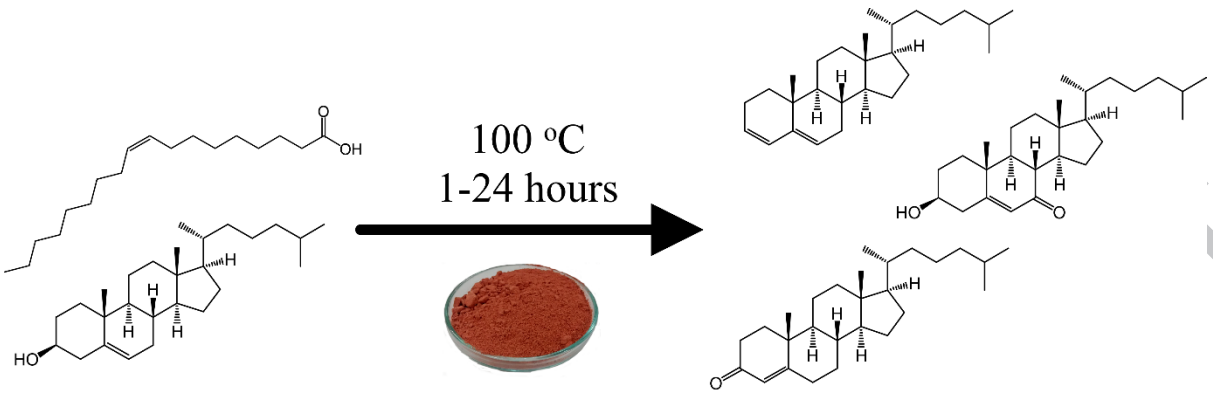
Highlights:

- Cholesterol was degraded when dosed on powdered clay and heated at 100 ºC
- Dehydration products and cholesterol oxidation products could be detected
- Fatty acids in tenfold or higher excess promoted drastic cholesterol degradation
- This explains the absence of dietary sterols in archaeological lipid residues




Cholesterol and fatty acids

Powdered clay ceramics

Cholesterol degradation