Free and bound fatty acid oxidation products in archaeological ceramic vessels

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While oxidation products of unsaturated fatty acids, for example dicarboxylic acids (hereafter diacids), must form during the use of unglazed ceramic vessels for the processing of animal and plant products, such components have never been observed during studies of absorbed lipids. Their absence from the extractable lipid fraction is presumed to be the result of their loss from potsherds through groundwater leaching. Lipid oxidation products, including short-chain dicarboxylic acids, ω -hydroxy acids and longer-chain hydroxy and dihydroxy acids, have now been observed as components probably covalently bound into solvent insoluble residues of potsherds recovered from waterlogged deposits. These components were only revealed following alkaline treatment of the insoluble residues. A similar mixture of diacids was observed in high abundance in the free lipid fraction of vessels recovered from an exceptionally arid deposit where groundwater leaching would never have occurred. These results confirm the formation of oxidation and probable polymerization products of unsaturated fatty acids during vessel use and burial.

Keywords: dicarboxylic acids; hydroxyacids; ceramics; alkaline treatment; organic residues; lipids

1. INTRODUCTION

Analytical studies of organic residues associated with archaeological vessels have focused primarily on the solvent extractable lipid components of visible surface deposits, and residues absorbed within the ceramic fabric. It has been shown that these lipid residues directly reflect the original contents of ceramic vessels (Evershed *et al.* 1992). A whole range of commodities, including olive oil, animal fats, beeswax and leaf waxes, have been identified by such analyses (Condamin *et al.* 1976; Evershed *et al.* 1991; Charters *et al.* 1995, 1997; Evershed *et al.* 1997a,b). Analysis of solvent-extractable lipids absorbed within the pottery matrix has also revealed information on the usage of ceramic vessels, by using quantitative analysis of the lipid profile down a pot to differentiate between cooking (with and without water) and storage vessels (Charters *et al.* 1993, 1997).

Degraded animal fats are the most common types of organic residue detected in domestic pottery vessels, particularly in cooking jars and certain specialist types of bowls (Rottländer 1990; Charters *et al.* 1993). The extracts typically comprise a mixture of triacylglycerols, diacylglycerols, monoacylglycerols and free fatty acids. The origin of these residues are attributed to degraded animal fats, due to the presence of a high concentration of

saturated fatty acids, particularly the C_{16:0} and C_{18:0} components (Evershed *et al.* 1991, 1992, 1997a). Fresh animal fats contain mainly triacylglycerols and distributions of the type seen in archaeological ceramics must arise through their partial hydrolysis to di- and monoacylglycerols, with the release of free fatty acids, seen eluting in the early part of gas chromatograms. This has been demonstrated in laboratory experiments designed to simulate the decay of lipids (Evershed & Charters 1995; Dudd *et al.* 1998). Animal fats introduced into the wall of replica ceramics and incubated under aerobic and anaerobic conditions reveal patterns of decay of the triacylglycerols analogous to those seen in extracts of archaeological potsherds, thus supporting the hypothesis that these archaeological sherds contain degraded animal fats. In addition to degradation during burial, alterations in lipid distributions occurring through long-term use have to be considered since vessels may have been used for many years. Studies of ethnographic vessels with a known history of use bridge the time-gap between the laboratory experiments and archaeological materials, and show that extensive hydrolysis occurs when vessels are used for many years (Charters 1996; Evershed *et al.* 1997b).

The oxidizing conditions and high temperatures that pottery vessels may be subjected to during their use will result in further chemical changes being induced in lipids associated with surface or absorbed residues. The chemical changes known to occur during the processing of modern foods (Davidek *et al.* 1990), highlight the major degradative processes that should also be seen in archaeological pottery vessels. One recognized novel degradation pathway involves the condensation of fatty acids with the

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elimination of carbon dioxide and water to give a series of long-chain secondary ketones (Evershed *et al.* 1995; Raven *et al.* 1997). These compounds have exactly analogous structures to the long-chain ketones found in the leaf waxes of higher plants, although the range of components seen is much more complex than would be expected from a single plant species. Other degradation products that are likely, include low molecular weight components arising through oxidation reactions at double bonds in unsaturated fatty acids and polymerized lipids formed through free-radical reactions. While evidence for the latter occurring in pottery has come from studies of insoluble residues in archaeological pottery involving pyrolysis-gas chromatography/mass spectrometry (Oudemans & Boon 1991; Regert *et al.* 1998), surprisingly, little evidence has been provided for the presence of degraded components that might form through oxidation of lipids, e.g. diacids (Seher *et al.* 1980) and hydroxy acids (Passi *et al.* 1981), even though their formation during food preparation is anticipated (Evershed *et al.* 1992; Simic *et al.* 1992; Hamilton *et al.* 1997). The reason for this paucity of information is thought to be a consequence of the rapid removal of these components from archaeological ceramics, due to their relatively enhanced solubility in water and reactivity. Hence, we have examined samples from extremes of depositional context, and also used the residue left after lipid extraction to provide evidence for incorporation of these components into insoluble residues. Here we report what we believe is the first identification of diacids in domestic pottery and pottery residues from Chalain in France and Qasr Ibrîm in Egypt.

2. MATERIALS AND METHODS

(a) *Samples*

Two sites have been studied. The first, a neolithic settlement at Chalain in France, is a waterlogged lacustrine deposit from which a variety of organic materials have been recovered, including wood, textiles, cereal cakes and charred surface residues in ceramic vessels. The second, an arid settlement at Qasr Ibrîm in Egyptian Nubia (approximately sixth century AD) (Plumley *et al.* 1977; Rowley-Conwy 1991), has revealed a plethora of organics such as seeds, plant remains, textiles, wood, coprolites and, again, surface residues in pottery, all of which exhibit exceptional morphological preservation and good preservation of the constituent biomolecules (O'Donoghue *et al.* 1996; van Bergen *et al.* 1997). The samples studied were taken from cooking vessels (Chalain) and lamps (Qasr Ibrîm).

(b) *Lipid extraction*

Charred surface residues were removed from the pottery using a sterile scalpel. Potsherds were surface cleaned with an electric hand-drill and then crushed to a fine powder using a mortar and pestle. A weighed amount (2 g) was transferred to a vial and *n*-tetratriacontane was added as internal standard (20 µl of a 1 mg ml⁻¹ solution in hexane). Either chloroform/methanol (Chalain samples) or dichloromethane/methanol (Qasr Ibrîm samples) were used for the extraction (10 ml, 2:1 v/v, 2 × 15 min ultrasonication). An aliquot of the total lipid extract was derived using *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (containing 1% v/v trimethylchlorosilane, 20–30 µl, 25 min (Chalain) or 60 min (Qasr Ibrîm), 70 °C).

(c) *Alkaline treatment of the insoluble fraction*

After solvent extraction, a portion (1 g of sherd) of the insoluble residue was transferred into a 50-ml round bottom flask; 10 ml of 0.5 M NaOH in MeOH/H₂O (9:1 v/v) were added and the mixture was stirred continuously whilst heating to 70 °C for 90 min. After cooling, the saponified mixture was centrifuged (2000 r.p.m., 20 min). An aliquot (3 ml) was acidified with a few drops of 1 M HCl and extracted with chloroform (3 × 3 ml). The solvent was evaporated under a stream of nitrogen gas and the treated residue left overnight in a vacuum desiccator to dry. The extracts were first prepared as their methyl ester derivatives by heating at 70 °C for 1 h with 14% w/v boron trifluoride-methanol complex (100 µl), then by adding double-distilled water and extracting into cyclohexane (200 µl). Trimethylsilylation was then performed as described above, and the sample dissolved in hexane prior to gas chromatographic analysis.

(d) *Gas chromatography and gas chromatography/mass spectrometry*

The gas chromatography (GC) analyses were performed on a Hewlett Packard 5890 gas chromatograph with on-column injection. Total lipid extracts were analysed on a 15 m × 0.32 mm internal diameter (i.d.) fused silica capillary column, coated with DB1 stationary phase (0.1 µm film thickness). The column temperature was held isothermally at 50 °C for 1 min immediately after injection, then was increased from 50 °C to 350 °C at 10 °C min⁻¹ and held at the final temperature for 10 min. After screening samples, the total lipid extracts from the Qasr Ibrîm lamps were then further analysed on a 50 m × 0.32 mm i.d. CP Sil 5 CB apolar capillary column (0.12 µm film thickness), with a temperature programme consisting of heating from 50 °C to 300 °C (held for 10 min) at 5 °C min⁻¹.

Saponified samples were separated on the same 50 m column as described for the lamp extracts. The temperature programme consisted of a 1-min isothermal hold at 40 °C followed by two ramps from 40 to 230 °C at 20 °C min⁻¹ and 230 to 300 °C at 4 °C min⁻¹. Hydrogen was used as carrier gas at a head pressure of 10 psi. Flame ionization detection was used to monitor the column effluent.

Gas chromatography/mass spectrometry (GC/MS) analyses were performed using a Carlo Erba 5160 Mega series chromatograph with on-column injection coupled to a Finnigan 4500 quadrupole mass spectrometer. The GC was operated using the same column and parameters as those described above. The mass spectrometer was operated in the electron ionization mode (70 eV); the interface and source block temperatures were 350 °C and 170 °C, respectively. The mass spectrometer was scanned over the *m/z* ranges 50–800 for the total lipid extracts and from 50 to 650 for the alkaline treated mixture in 0.95 s with an interscan delay of 0.05 s.

3. RESULTS AND DISCUSSION

A typical distribution of lipids seen in solvent extracts of cooking vessels recovered from the neolithic lake-dwelling settlements of Chalain in France is shown in figure 1. None of the total lipid extracts, from visible deposits and organic residues absorbed in the clay matrix, contained lipid oxidation products, such as diacids. Instead, the residues were dominated by mono-, di- and triacylglycerols and free fatty acids. Significantly, however, alkaline treatment of the insoluble residues

Figure 1. Partial gas chromatogram of a total lipid extract (trimethylsilylated) showing a characteristic pattern for the charred surface residues from vessels of Chalain. Body of vessel 4050 characterized by a broad distribution of triacylglycerols. The fatty acids are indicated by the common abbreviation (the number following the C indicates the number of carbon atoms in the fatty acid and the number after the colon indicates the degree of unsaturation). TAG, triacylglycerols; DAG, diacylglycerols; MAG, monoacylglycerols (the numbers on the peaks indicate the total number of acyl carbon atoms); IS, internal standard (*n*-tetratriacontane).

(recovered after solvent extraction) yielded appreciable amounts of oxygenated fatty acid derivatives. The gas chromatogram obtained from the insoluble residue by base treatment of a sherd from vessel 4175 revealed the presence of two homologous series of compounds (figure 2; for relevant chemical structures see figure 3).

A series of α,ω -dicarboxylic acids ranging from C_7 to C_{12} were identified by GC/MS and showed azelaic (C_9) acid as the dominant component. The mass spectra of the identified diacids are characterized by fragment ions $[M-15]^+$ and $[M-131]^+$. The latter ion occurs only in diacids with carbon chain lengths greater than C_5 , and arises through scission of the C–C bond between the α - and β -carbons. The other homologous series comprises C_8 to C_{12} α -hydroxy carboxylic acids (cf. Eglinton *et al.* 1968). These components were identified as their methyl ester trimethylsilyl ether derivatives according to their mass spectra, characterized by very intense ions at $[M-15]^+$ and $[M-47]^+$ with less abundant ions at $[M-31]^+$. Other oxygenated saturated carboxylic acids found included 9- and 10-hydroxyoctadecanoic acid (cf. Evershed *et al.* 1990) and two isomers of 9,10-dihydroxyoctadecanoic acid (cf. Gülaçar *et al.* 1990). The presence of unsaturated hydroxyoctadecenoic acids are also suggested on the basis of characteristic fragment ions seen in their mass spectra (figure 4). It is especially significant that they were absent from the solvent-soluble fraction, only being liberated upon base treatment. In addition to these oxidized compounds, saturated ($C_{16:0}$, $C_{17:0}$, $C_{18:0}$) and unsaturated ($C_{18:1}$) fatty acids were also liberated by base treatment. These data indicate a new mechanism for the preservation of degraded lipids in archaeological pottery through chemical bonding, possibly via ester linkages, into an insoluble polymeric matrix.

Significantly, these results support our contention that the majority of the low molecular weight lipid oxidation products that form in vessels during use and burial are lost by groundwater leaching. In order to test this hypothesis further, we examined lipid-containing vessels from an exceptionally arid archaeological site where percolation of groundwater would not have occurred during the entire period of burial. The vessels studied were unglazed lamps recovered from Qasr Ibrîm in Egyptian Nubia. Conventional lipid analyses revealed the presence of abundant diacids together with saturated free fatty acids and monoacylglycerols (for a typical extract, see figure 5). GC/MS analysis confirmed the diacids to comprise a homologous series, with carbon chain lengths ranging from C_4 to C_{14} , with azelaic acid (C_9) being the dominant component. The presence of abundant free diacids in the lamps is presumed to relate to their mode of use, with the heat from the flame during the burning of the illuminant oil or tallow being sufficient to bring about extensive oxidation, especially in unsaturated fatty acids. As no such compounds have been observed in solvent extracts of vessels recovered from more waterlogged deposits, the preservation of the unbound polyfunctional oxidation products at Qasr Ibrîm must relate to the unusually arid nature of the burial environment, limiting their loss from the vessels during burial.

All the diacids and hydroxy acids, identified either in the total lipid extracts of the samples from the dry site (Qasr Ibrîm), or in the saponified fraction of sherds from the waterlogged site (Chalain), are consistent with mechanisms known to be associated with oxidation of unsaturated fatty acids (Simic *et al.* 1992). The production of short-chain α,ω -dicarboxylic acids occurs by oxidative degradation of a carbon–carbon double bond. For each

Figure 2. Partial gas chromatogram of the base treated residue (as methyl ester-trimethylsilyl ether derivatives) of a potsherd from a neolithic cooking vessel. Peak identities: $C_{16:0}$, $C_{18:0}$, etc., indicate saturated fatty acids; $C_{18:1}$ indicates monounsaturated fatty acid; $\bigcirc C_x$ indicates α,ω -dicarboxylic acids with carbon chain length x ; $\bullet C_x$ indicates ω -hydroxy acids with carbon chain length x .

Figure 3. Structures of various fatty acid oxidation products found in the saponified residue of a neolithic potsherd (see figure 2). Structural identifications: (a) C_9 α,ω -dicarboxylic acid or nonanedioic acid (azelaic acid); (b) 9-hydroxyoctadecenoic acid; (c) 9,10-dihydroxyoctadecanoic acid; (d) ω -hydroxydodecanoic acid.

unsaturated fatty acid precursor, various diacids are known to be formed, with distributions reflecting (i) direct cleavage of the double bond; (ii) hydration followed by oxidative cleavage; (iii) ω -oxidation with subsequent bond cleavage; or (iv) a more complex pathway by ω -oxidation, hydration and then bond cleavage. These mechanisms are known to involve the production of hydroperoxide intermediates formed via radical reactions. The last stage of such a reaction sequence is the termination step that results in the formation of diacids. Clearly, such mechanisms are involved in the formation of the bound and free lipid oxidation products in the samples studied. The nature of the oxidation products of unsaturated lipids is dependent

on the precursors present in the processed materials, the duration and intensity of vessel use, and the burial conditions. Assumptions can be made from the distribution pattern of the homologues, for example, the prominence of the C_9 diacid implies the presence of moieties with unsaturation mainly at position 9 (most probably a Δ^9 fatty acid). However, the significant amounts of other diacid homologues indicate that unsaturated fatty acids with double bonds at other positions may also have been present.

The long-chain monohydroxyacids, identified in the vessel 4175 from Chalain, are assumed to be formed by the hydration of double bonds; for example, 10-hydroxyoctadecanoic acid is known to be produced by the degradation of oleic acid, whereas dihydroxylation of the double bond is responsible for the formation of 9,10-dihydroxyoctadecanoic acids. The unsaturated hydroxy acids result through oxidation of the double bond by dihydroxylation, either during the use of the vessel or during burial in the anaerobic waterlogged environment (figure 4). The results obtained from alkaline treatment of the insoluble material emphasize the possibilities that exist to obtain more complete insight into the molecular information preserved in archaeological materials.

4. CONCLUSIONS

The results presented in this paper confirm our expectations that extensive lipid oxidation occurs during vessel use and probably subsequently during burial. At the majority of sites the low molecular weight polyfunctionalized components appear to be lost from potsherds during burial by groundwater leaching. However, base treatment of insoluble carbonized residues strongly suggests that such compounds survive in pottery from these sites in a covalently bound (esterified) form. The presence of low molecular weight free diacids in pottery from the exceptionally arid site of Qasr Ibrim confirms that such components can persist in the absence of percolating groundwater.

Figure 4. One proposed degradation pathway for the formation of unsaturated hydroxy acids. The m/z values correspond to the fragment ions of the *bis*-TMS derivatives.

Figure 5. Partial gas chromatogram of the total lipid extract from the residue of lamp 6 from Qasr Ibrîm. C_x are α,ω -dicarboxylic acids where x refers to the carbon chain length. IS is the internal standard (*n*-tetratriacontane).

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