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Investigating the formation and diagnostic value of ω-(o-alkylphenyl)alkanoic acids in ancient pottery

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ABSTRACT

Long chain ω-(o-alkylphenyl)alkanoic acids (APAAs) derived from the heating of unsaturated fatty acids have been widely used for the identification of aquatic products in archaeological ceramic vessels. To date, little attention has been paid to the diagnostic potential of shorter chain (<C$_{20}$) APAAs, despite their frequent occurrence. Here, a range of laboratory and field experiments and analyses of archaeological samples were undertaken to investigate whether APAAs could be used to further differentiate different commodities. The results of this study provide new insights regarding conditions for the formation of APAAs and enable us to propose novel criteria to distinguish different natural products.

KEYWORDS: organic residue analysis, lipid, archaeological pottery vessels, ω-(o-alkylphenyl)alkanoic acids, heating experiments, experimental archaeology.

INTRODUCTION

For the last three decades, lipid residue analysis has been used to study the techno-function of ancient ceramic vessels. Based on the archaeological biomarkers concept (Evershed 2008), it is possible to trace organic molecules, or suites of molecules, extracted from pots to organisms likely to have been exploited in the past. This approach has provided valuable insights into human activities, technology and economies (Heron and Evershed 1993; Evershed 2008; Regert 2017). The identification of specific lipid markers (biomarkers) using gas chromatography-mass spectrometry (GC-MS) has been used to track a range of commodities in ancient pottery, such as aquatic resources (Copley et al. 2004; Lucquin et al. 2016b; Gibbs et al. 2017; Shoda et al. 2017; Admiraal et al. 2019; Bondetti et al. 2020), beehive products (Roffet-Salque et al. 2015; Shoda et al. 2018), edible plants (Dunne et al. 2016; Heron et al. 2016; Bondetti et al. 2020) and various types of resins, wood tars and pitches (Heron et al. 1994; Mitkidou et al. 2008; Heron et al. 2015; Rageot 2015).

Lately, a great deal of attention has been paid to the detection of ω-(o-alkylphenyl)alkanoic acids (APAAs). These compounds do not occur naturally but are formed during protracted heating of mono- and polyunsaturated fatty acids (MUFAs, PUFAs) present in animal and plant tissues (Matikainen et al. 2003; Hansel et al. 2004; Evershed et al. 2008; Cramp and Evershed 2014). Due to their high stability over time, these compounds have been identified in vessels from a wide range of archaeological contexts (Copley et al. 2004; Lucquin et al. 2016b; Gibbs et al. 2017; Shoda et al. 2017; Bondetti et al. 2020). One particular application has been to overcome the challenge of identifying aquatic products in pottery. Aquatic products are rich in PUFAs that readily degrade in the burial environment and therefore

rarely encountered. As APAAs are produced from these liable precursor molecules, their presence along with other more robust compounds such as isoprenoid fatty acids (IFAs; e.g. 4,8,12-TMTD, phytanic and pristanic acids) (Ackman and Hooper 1968; Copley et al. 2004; Hansel et al. 2004; Cramp and Evershed 2014; Lucquin et al. 2016a) and long-chain dihydroxy fatty acids (Hansel and Evershed 2009; Cramp et al. 2019) have brought to light a range of examples of aquatic resource processing in the archaeological record.

More specifically, the presence of long chain APAAs ($\geq C_{20}$) provides the most convincing evidence for the cooking of aquatic commodities, since they are formed from their long-chain MUFA and PUFA precursors (especially n-3 fatty acids $C_{20:5}$ and $C_{22:6}$) which are only present in significant amount in aquatic organisms, such as freshwater and marine animals (Cramp and Evershed 2014). For example, the detection of APAAs has shown that Early Woodland hunter-gatherer pottery in North America was used for processing aquatic resources, hitherto contested (Taché et al. 2019). Similarly, APAAs have been identified in some of the earliest pottery in the world, revealing the motivations for pottery innovation (Craig et al. 2013).

While the use of APAAs to identify aquatic products in pottery represents a significant advance in organic residue analysis, APAAs with a shorter chain-length homologues (i.e. $<C_{20}$) are readily generated through heating non-aquatic products, especially tissues rich in unsaturated fatty acids (UFAs). These include a wide range of foodstuffs including vegetable fats and oils as well as terrestrial adipose fats (Heron and Evershed 1993; Evershed et al. 2008). Therefore, the detection of APAAs with 16 and 18 carbon atoms (i.e. ω-(o-alkylphenyl)hexadecanoic acid and ω-(o-alkylphenyl)octadecanoic acid) is currently of

limited diagnostic value, despite the fact that these compounds are frequently recovered from archaeological pots.

The synthesis of APAAs involves a number of different reactions encompassing mainly alkali isomerization and aromatization steps (Matikainen et al. 2003; Hansel et al. 2004; Evershed et al. 2008). Crucially, during this process, various double bond rearrangements occur, resulting in the formation of several isomers. Controlled heating experiments undertaken by Evershed and co-workers (Evershed et al. 2008), have shown that the distribution of APAA isomers with 18 carbon atoms (APAA-C_{18}) differed according to the number and the position of unsaturations in the fatty acid from which it was derived. Similarly, the difference in the APAA-C_{18} isomeric distribution in thermally degraded rapeseed oil, cod liver oil and horse adipose fat was interpreted as a direct consequence of the relative amounts of precursor $C_{18:1}$, $C_{18:2}$, $C_{18:3}$ fatty acids present in these products. Furthermore, Shoda et al. (2018) noted the dominance of two APAA-C_{18} isomers in pottery where starchy plants, such as nuts and cereals, were processed. Based on these observations, it appears that the isomeric distribution of APAA-C_{18} may provide an additional diagnostic tool for the identification of foodstuffs cooked in pottery. Considering that this has not yet been properly investigated, this research set out to explore the value of APAA-C_{18} isomeric distribution as a diagnostic tool to identify commodities processed in ancient pottery. This was done through a series of experiments involving the heating of different fats and oils, and through the comparison with the distribution of APAAs observed in archaeological samples.

Previous studies (Matikainen et al. 2003; Hansel et al. 2004; Evershed et al. 2008) involving different natural commodities (rapeseed oil, horse adipose fat and cod liver oil) have shown that APAAs were formed when UFAs are subjected to protracted heating (≥17 hours at temperatures above 270°C), although a shorter cooking time and lower temperatures have so far not been assessed. Yet, understanding the minimum time and temperature needed to form these compounds is often important for interpretative purposes. Secondly, these studies have suggested that APAAs are only formed in the presence of fired clay, containing the metal ions (Redmount and Morgenstein 1996; Mallory-Greenough et al. 1998) required for the prior alkali isomerization step. And thirdly, anaerobic conditions are regarded as necessary to produce APAAs, promoting the cyclization process. To that extent, our experiments gave us an additional opportunity to reassess the conditions for APAAs formation in order to better interpret results, particularly with respect to ancient culinary practices.

MATERIAL AND METHOD

Laboratory and field experiments

For all the experiments, wheel-thrown replica pottery vessels were used. Vessels were made with "Standard Red" clay, chosen for its relatively high content of metal ions (Al_2O_3- 22.78, Fe_2O_3- 7.37, CaO- 0.57, MgO- 0.86, K_2O- 1.6, Na_2O- 0.1) known to catalyse the isomerization reaction involved in APAA formation (Fig. 1) (Raven et al. 1997; Evershed et al. 2008). No temper was added to the matrix preventing any organic exogenous contamination and the pots were fired at 700°C by an experimental potter (Mr. Graham Taylor, Experimental Archaeologist and Ancient Pottery Technology Specialist, Rothbury, UK). The ceramic

powder used for the laboratory experiments was obtained by crushing one of these replica vessels with a mortar and pestle.

The first series of laboratory experiments was designed to examine the duration of heating on APAA formation. *Ca.* 65 mg of rapeseed oil (Commercial Organic, cold-pressed, extra virgin rapeseed oil, UK) was sealed under nitrogen in borosilicate glass tubes (Fisherbrand, UK; 12 mL) either with or without the addition of ceramic powder (100 mg). Each tube was heated at 270°C for a duration of 1, 5, 10 or 17 h (Table S1, A). The second series of experiments was designed to examine the effect of temperature on APAA formation. *Ca.* 65 mg of rapeseed oil was placed in open glass tubes and heated at 100, 150, 200, 250 or 270°C for 5 h with or without the addition of ceramic powder (Table S1, A). The third series examined the relationship between APAA formation and precursor fatty acids. *Ca.* 20 mg of pure fatty acids $C_{18:0}$, $C_{18:1}$ (*cis*-9-Octadecenoic acid), $C_{18:2}$ (*cis*-9,*cis*-12-Octadecadienoic acid) and α-$C_{18:3}$ (α-Lnn, *cis*,*cis*,*cis*-9,12,15-Octadecatrienoic acid) were heated in duplicate in open glass tubes with or without powdered ceramic (100 mg) for 5 hours at 270 °C (Table S1, A). Finally, in the last series of laboratory experiments, a selection of foodstuffs, including meat, fish and edible plants (leafy vegetables, fruits, nuts and cereals) were heated for 5 hours at 270°C with the presence of ceramic powder (Table S1, B). Prior to heating, a sub-sample of each raw foodstuffs was retained for analysis.

Experiments were also conducted in the field (YEAR centre, University of York), with the aim of simulating cooking conditions over an open fire. Portions of red deer meat, salmon flesh and chestnut flour were individually placed into replica pots, submerged in water and heated over an open fire. A thermocouple was used to measure the temperature on the

outside of the vessels for each pot. The pots were left to boil for 1 hour and regularly refilled with water. Subsequently, each pot was emptied and reused for another hour in the same manner. This action was repeated five times for the chestnut flour and 15 times for the meat and fish (Table S2). Each commodity was boiled in three separate replica vessels along with one blank, which was filled with water. Following the experiments, all pots were split into two parts, one was directly analysed and the other was buried for six months (from May to November 2018) at YEAR centre (Lat. 53.95; Long. -1.09; pH_{soil}=7.16) prior to analysis.

All animal products used in this study were acquired commercially or were killed or taken legally. The export of several fish samples from the Russia Federation was authorised by The Secretary of State for Environment and Rural Affairs office (Authorisation number: ITIMP18.0277).

Lipid analysis

For the cooking experiments, *ca*. 1 g of pottery was drilled following cleaning of the vessel surface with a modelling drill to remove any exogenous contamination. Any carbonized surface deposits (foodcrusts) that were formed during cooking, were detached from the surface of the pot using a sterile scalpel and were finely crushed. An aliquot of *ca.* 20 mg of foodcrusts was weighed out for the analysis. For the experiments undertaken in the laboratory, the residue, formed by heating the food products with the ceramic powder, were used. In addition, lipids were extracted from each of the raw foodstuffs in order to confirm the absence of APAAs.

Lipid extraction was performed following established acidified methanol protocols (Craig et al. 2013; Papakosta et al. 2015). Briefly, the samples were placed into glass vials to which methanol was added (4 mL for potsherds and raw foodstuffs, and 1 mL foodcrusts samples) along with an internal standard (*n*-tetratriacontane: 10 µg). The mixture was then ultrasonicated for 15 min before acidification with concentrated sulphuric acid (800 µl and 200 µl, respectively) and heated for 4 hours at 70 °C. After cooling, the lipids were extracted with *n*-hexane (3 x 2 mL). Finally, a second internal standard was added (*n*-hexatriacontane: 10 µg) and the samples were directly analysed by Gas Chromatography-Mass Spectrometry (GC-MS).

An Agilent 7890A Series Gas Chromatograph (Agilent Technologies, Cheadle, Cheshire, UK) coupled to either an Agilent 5977B Mass-selective detector or an Agilent 5975C Inert XL mass selective detector with a quadrupole mass analyser (Agilent technologies, Cheadle, Cheshire, UK) was used to analyse the samples. In both cases a splitless injector was employed and held at 300°C. The GC column was directly connected to the ion source of the mass spectrometer. The ionisation energy of the MS was 70 eV and spectra were obtained by scanning between m/z 50 and 800. All the samples were run on a DB23 (50%-Cyanopropyl)-methylpolysiloxane column (PN 122-2362; 60 m x 250 µm x 0.25 µm; J&W Scientific, Folsom, CA, USA) in selected ion monitoring mode (SIM) and using a temperature program setup to better detect and resolve the three isoprenoid fatty acids (phytanic and pristanic acids and 4,8,12-TMTD) and the ω-(o-alkylphenyl) alkanoic acids (Shoda et al. 2017). The temperature was set at 50°C for 2 min and increased at a rate of 10 °C/min until 100 °C. The temperature was then raised by 4 °C/min to 140 °C, then by 0.5 °C/min to 160 °C and finally by 20 °C /min to 250 °C, where the temperature was maintained for 10 min.

Helium was used as carrier gas at a flow rate of 1.5 mL/min. Raw foodstuffs were also analysed in Total Ion Chromatogram mode (TIC) in order to quantify their initial fatty acids content prior heating. The relative abundance of APAAs isomers C_{18} and C_{20}, were obtained by integration of the ions m/z 290, 318 respectively and carried out using MassHunter software (version B.07.01/Build 7.1.524.0).

Lipid extraction of archaeological samples followed the same protocol as the experimental samples and is described in Bondetti et al (2020) and Shoda et al (2018). These samples were all analysed by GC-MS using the same instrument and SIM method program. Statistical tests were conducted using PAST3 software (version 3.25 for Windows). Detailed explanations on the choice of statistical tests applied are given in the Supplementary Material.

RESULTS AND DISCUSSION

Under what conditions do APAAs form in archaeological ceramics?

Time and temperature This first set of experiments demonstrates that the production of the APAAs requires less intensive heating conditions than previously observed (Evershed et al. 2008; Table 1). Whilst experiments confirm their occurrence in the rapeseed oil heated for 17 hours, we found that APAAs are readily formed after just one hour of heating at 270°C. The experiments also indicate that heating at 200°C for 5 hours is sufficient to generate APAAs (Table 1). The experiments suggest that APAAs are more likely to form when the UFA precursors are in direct contact with the pottery wall, where temperatures in

excess of 200°C are easily achieved even when the vessels are used to heat (boil) liquid contents. This point is verified by experiments conducted on the open fire, where the external ceramic surface frequently reached temperatures greater than 300°C. Here, appreciable amounts of APAAs were formed in all the experiments (deer, salmon and chestnut flour) following five or 15 hours of simulated cooking (Table S1). Interestingly, the proportion of APAAs compared to other compounds was observed to increase following burial, especially for the salmon experiment where APAAs were only identifiable after burial. This is due to the relative loss of other more soluble and labile compounds during exposure to the burial environment, enriching the relative abundance of APAAs in the extracts.

Importantly, the APAA-C_{18} isomeric distribution is not significantly altered by the length of heating (Kruskal-Wallis; Chi^2 = 0.05; p = 1; Fig. S1a). Likewise the distribution of the isomers according to the heating temperature conditions remains similar overall (Kruskal-Wallis; Chi^2 = 0.49; p = 0.78), although a slight smoothing of the profile is observed with the increase in temperature (Fig. S1b). Overall, heating conditions appear to have little influence on the APAAs formation process allowing for further investigation of the diagnostic value of APAA-C_{18} isomeric distribution in archaeological context.

  Do APAAs form in the absence of ceramic? The experiment also shows that APAAs are produced in either the presence or absence of ceramic powder, for as short a duration as 1 hour with heating at 270°C or 5 hours at 200°C (Table S1, A; Fig. S2). This could suggest that, instead of the prior alkali isomerisation, the APAAs were formed here via the allylic radical intermediates mechanism, an alternative pathway described by Matikainen et al. (2003). However, it is worth noting that these experiments were undertaken in glass tubes, where

metal ions are also present, as part of the silicate glass composition (Norman et al. 1998), and therefore could have contributed to the isomerization process. However, due to the amorphous structure of silicate glasses, metal ions are likely to be less accessible than in low fired and powdered ceramic (Rice 1987). This may explain the lower conversion of UFAs to APAAs observed during our experiments carried out without pottery powder (Fig. S2). Overall the experiments support the observation that the pottery matrix assists the formation rate of such compounds (Evershed et al. 2008). Nevertheless, APAAs can also be produced by heating the UFA precursors in other kinds of containers, providing a minimal amount of metal ions, such as stone bowls or griddle stones (Admiraal et al. 2019). They have also been identified in charred food remains that have no clear association with a mineral artefact (Heron et al. 2016). Overall, this suggests that the steric properties, as previously proposed by Evershed et al. (2008), and/or the chemical composition of the cooking container influence, to a certain extent, the reaction but that other mechanisms could also be important requiring further inquiry.

Evacuated vs aerobic conditions Finally, these experiments also demonstrate that APAAs can be produced under fully aerobic conditions (Table 1 and Table S1, A), contrary to previous reports (Evershed et al. 2008), and therefore formation does not require the UFA precursors to be trapped in the ceramic matrix. Nevertheless, differences in the isomeric distribution of APAA-C_{18} are noted between the experiments in evacuated and fully aerobic conditions perhaps affecting the formation process. Whilst in both cases thermal degradation induced the formation of isomers A to I, the rapeseed oil heated in the open tubes produced greater relative amounts of E and F isomers (Fig. S1). In contrast, the rapeseed oil heated under anaerobic conditions exhibits a higher prevalence of the G

isomer. Interestingly, the distribution of the APAA-C_{18} isomers obtained by heating salmon, chestnut flour and red deer undertaken in the field experiments are not significantly different to those carried out in the laboratory in open tubes (Kruskal-Wallis test: chestnut flour, Chi^2 = 1.22; p = 1; salmon; Chi^2 = 0.93; p = 0.99; red deer; Chi^2 = 0.19; p = 0.91), either before or after burial. These findings suggest that the formation of APAAs during cooking is more likely to occur under aerobic conditions, and that the isomeric distribution remains stable over time even when subjected to natural degradation processes.

What degree of resolution can APAAs offer for product identification?

Distinguish different foodstuffs based on APAA-C_{18} distribution Different foodstuffs were heated in order to assess whether analysis of APAA-C_{18} could provide further diagnostic information. A wide range of foodstuffs was selected, including meat, fish and edible plants (leafy vegetables, fruits and cereals) either raw, or as purified oils (Table S1). These commodities were all subjected to the same experiments involving identical heating conditions (5h, 270°C, presence of ceramic powder and using open-air conditions; Table S1). For all the samples the whole set of APAA-C_{18} isomers (n=9, from A to I; Fig. 2) were produced. Analysis of the foodstuffs prior to heating found no evidence of APAAs. The percentage contribution of each isomer to the total was then computed by the integration of the m/z 290 ion (Table S1). Variability in the distribution of APAAs isomers resulting from the laboratory experiments were investigated using principal component analysis (PCA).

The PCA results show that the first two principal components (Fig. 3), represent 57.2% and 32.8% of the total variance in the dataset, respectively. Interestingly, PC1 effectively discriminates fruits, cereals and non-leafy vegetables (n = 20, Fig. 3 - orange markers) from leafy vegetables (n = 4, Fig. 3 - green markers). These groups correspond to the predominance of E and H isomers respectively, which have large positive and negative loadings on PC1 (E = 0.68 and H = -0.54). Therefore, we suggest that the relative abundance of E and H APAA-C_{18} isomers could offer a novel index to broadly differentiate these classes of edible plant products in ancient pottery.

Based on the PCA, we calculated the E/H ratio and were able to effectively separate three groups of food products (Fig. 4); i) cereals/fruits/non-leafy vegetables, ii) leafy vegetables, such as cabbage and spinach, and iii) animal products, including aquatic and terrestrial animals. The distribution of E/H ratios in the first group (n = 20; x̄ = 5.3 ± 1.6) is significantly different to the leafy vegetables (n = 4; x̄ = 1.2 ± 0.7; Mann-Whitney test: U = 0; z = 3.1; $p <$ 0.01) and/or animal products (n = 15; x̄ = 3.2 ± 1.2; Mann-Whitney test: U = 40; z = 3.7; $p <$ 0.01), as shown in Figure 4. Further experiments are needed to investigate how mixing of different foodstuffs may affect the E/H ratio or else theoretical values could be crudely predicted based on the proportion of unsaturated fatty acids in the original foodstuffs. A seemingly obvious limitation is that mixing for cereals/fruits/non-leafy vegetables and leafy vegetables is likely to produce intermediate E/H ratios matching animal fats.

While we have shown that the impact of temperature on relative distribution of APAA-C_{18} isomers is minimal, the E/H ratio is negatively correlated with heating temperature (Spearman; R = -1; p = 0.33), although we would need to increase the sample size to confirm

this first observation. The impact of the duration of heating is, however, negligible (T-test: t = 2.1; *p* = 0.08). Therefore, low E/H ratios, that are typically found in leafy vegetables could be theoretically produced through thermal alteration. Nevertheless, high APAA-C_{18} E/H ratios could still be used as a proxy to help distinguish cereals/fruits/non-leafy vegetables in ancient pottery, as these are unlikely to result from either mixing or extensive heat alteration. Overall, the approach would be particularly powerful when used in conjunction with other molecular and isotopic proxies.

To explore the application of this index in an archaeological context, the distribution of APAA-C_{18} isomers was determined in pottery from three sites; Zamostje 2 (Neolithic; *ca.* 6,600–4,000 cal BC), a riverine hunter-gatherer site located in Russia and two early agricultural sites, Joto (Yayoi period; *ca.* 20–230 cal AD) in Japan, and Tianluoshan (Neolithic; *ca.* 5,000–4,000 cal BC) located in China. These sites were chosen due to their strong association of pottery with the processing of either fish or plant products.

The samples (n=35) of Middle Neolithic pottery (*ca.* 5,000–4,000 cal BC) from Zamostje were found in close association with freshwater fish (Bondetti et al. 2020) and all met established molecular criteria for the identification of aquatic products (Hansel et al. 2004; Evershed 2008; Cramp and Evershed 2014; Lucquin et al. 2016a) and are associated with charred surface deposits with high bulk $\delta^{15}N$ values (x̄ = 10.7 ± 2.2 ‰), also characteristic of aquatic resources (Dufour et al. 1999; Craig et al. 2013; Choy et al. 2016). Two samples were obtained from the early agricultural site of Joto where SEM has previously identified the charred remnants of a layer of rice pericarp tissue in two surface deposits. Bulk isotope analysis from these samples exhibit values consistent with starchy plants ($\delta^{15}N_{mean}$ = 0.6 ±

1.8 ‰; $C:N_{mean}$ = 17.9 ± 4.6) (Shoda et al. 2011; Yoshida et al. 2013). Twenty samples were

obtained from Tianluoshan, the majority (n=12) of these were charred surface deposits

associated with starchy plants as indicated by the presence of levoglucosan, a range of

phytosterols and relatively low $\delta^{15}N$ bulk isotope and high C:N ratio values ($\delta^{15}N_{mean}$ = 4.8 ±

1.7 ‰; $C:N_{mean}$ = 16.0 ± 3.6; Shoda et al. 2018). Several other Tianluoshan pots were used

for processing terrestrial resources, including beeswax (n = 1) and aquatic products,

supported by overall higher $\delta^{15}N$ values (Shoda et al. 2018). Finally, further 4 samples had

both criteria, demonstrating some degree of mixing.

The E/H ratio of vessels (Fig. 4) from these three sites show good correspondence with the

presumed contents based on previous analysis (Shoda et al. 2011; Shoda et al. 2018;

Bondetti et al. 2020). The vessels used for animal fats from Zamostje 2 and Tianluoshan

have a mean E/H ratio of 2.5 (± 0.9) and 3.0 (± 1.1) respectively. While the E/H ratio for

vessels focused on plant processing from Joto (5.0 ± 0.48) and Tianluoshan (4.7 ± 0.6) is

relatively higher supporting their function for cooking starchy plants. Interestingly, samples

from Tianluoshan with molecular evidence for plant and aquatic products still have E/H

ratios that fall within the range of the cereals/fruits/non-leafy vegetables reference

samples, although they have on average a slightly lower ratios ($\bar{x}$ = 4.5 ± 0.8) compared to

samples with starchy plant biomarkers only.

The analysis of unsaturated fatty acids $C_{18:1}$, $C_{18:2}$, α-$C_{18:3}$ both undertaken here on pure

compounds (Table S1A, Fig. S3) and previously published Evershed et al. (2008) shows that

the APAA-C_{18} isomeric distribution is dependent on the relative abundance of UFAs-C_{18} in

the initial foodstuffs.

Overall, however, the isomeric distribution observed in the foodstuffs after heating showed no clear correlation with their initial fatty acid content (Table S1A and B, Table S3, Table S4), indicating that a more complex series of reactions is involved in their formation, most likely related to both the original proportion of UFA and the position of their unsaturations. Interestingly, spinach and cabbage, dominated by α-$C_{18:3}$ (Pereira et al. 2001), display a similar isomeric distribution to that obtained by heating α-$C_{18:3}$ (Mann-Whitney test: U = 36; z = 0.353, p = 0.72 for spinach and U = 37; z = 0.27, p = 0.79 for cabbage), leading to a dominant formation of APAA-C_{18} isomers F, G, H (Table S1A and B, Fig. S3). Moreover, previous thermal degradation of γ-$C_{18:3}$ and α-$C_{18:3}$ (Evershed et al. 2008), heated under the same conditions, resulted in a significant alteration of the isomeric distribution and supports this assumption. Therefore, it may not be possible to predict the APAA-C_{18} distribution based on a product's original UFAs content, necessitating empirical investigations as described above.

Distinguishing aquatic from terrestrial resources (APAA-C_{20} vs. APAA-C_{18}) As expected for aquatic products where $C_{20:x}$ UFAs are particularly abundant (Passi et al. 2002; Wirth et al. 2002; Cramp and Evershed 2014), APAAs containing 20 carbon atoms (i.e. ω-(o-alkylphenyl)ecosainoic acid, APAA-C_{20}) were readily formed. As stated previously, APAA-C_{20} are important criteria to highlight the processing of aquatic products in ancient pottery (Hansel et al. 2004; Cramp and Evershed 2014). However, these compounds are not exclusively produced by processing of aquatic products. The thermal degradation of other animal products, such as elk, beaver, pork and red deer fats also yielded APAA-C_{20} (Table S1). Similarly, trace amounts of APAA-C_{20} were detected in some of the heated plant

samples (e.g. broomcorn millet, quinoas, rice, sesame, and acorn; Table S1). In all cases, they are derived from trace amounts of $C_{20:x}$ UFA precursors present in these foodstuffs.

Consequently, the reliability of using APAA-C_{20} as biomarkers of aquatic resources may be questionable, especially when other aquatic derived compounds (e.g. isoprenoid fatty acids, APAA-C_{22}) are absent. This would appear to be a major limitation of the approach considering that APAA-C_{22} are observed much less frequently than the C_{20} homologous. Nevertheless, our results also show that the relative abundance of APAA-C_{20} (obtained by the integration of the m/z 318 ion) in aquatic products is much greater than those observed in other foodstuffs. For example, the ratio of APAA-C_{20} to APAA-C_{18} (APAA C_{20}/C_{18}) of aquatic animals (n=9; $\bar{x}$ = 0.21 ± 0.03) is significantly higher than both terrestrial plants (n= 5; $\bar{x}$ = 0.02 ± 0.00; Mann-Whitney test: U = 0; z = 2.93, p < 0.01) and terrestrial animals (n= 5; $\bar{x}$ = 0.04 ± 0.00; T-test: t = 2.41; z = 2.93; p = 0.03). This ratio therefore provides a useful criterion to separate aquatic commodities from the other foodstuffs (Fig. 5). The APAA C_{20}/C_{18} ratio observed in the different foodstuffs is strongly correlated with the relative abundances of precursor UFAs, $C_{18:x}$ and $C_{20:x}$ (Spearman; R = 0 .84; p < 0.01).

We suggest that a value of 0.06 for the APAA C_{20}/C_{18} ratio could be used as an interim threshold to distinguish aquatic sources from terrestrial products, since this is the lowest value observed for aquatic products and remains higher than any other type of resources (e.g. terrestrial animals and plants; Fig. 5). Preferential degradation processes differentially acting on the two homologous potentially could compromise the utility of this approach, for example due to differences in solubility. However, in the burial experiments conducted here on pots used to cook salmon, the APAA C_{20}/C_{18} ratio was still greater than 0.06 (n = 3; $\bar{x}$ =

0.10 ± 0.00) following 6 months burial (Fig. 5). Nevertheless, differential preservation of APAAs C_{18} and C_{20} in different burial contexts should be a focus of future investigations. Interestingly, the APAA C_{20}/C_{18} ratio obtained from Middle Neolithic pottery at Zamostje 2 (n = 32; x̄ = 0.11 ± 0.04) and Tianluoshan (n=7; x̄ = 0.08 ± 0.04) mostly fall within the range of modern aquatic data (Fig. 5) confirming their use for processing aquatic resources (Bondetti et al. 2020; Shoda et al. 2018).

CONCLUSIONS

The thermal degradation of a wide range of commodities brought new insights with regard to the interpretation of APAAs in ancient ceramic vessels. Indeed, the distribution of APAA-C_{18} isomers could offer novel diagnostic biomarkers to identify the processing of certain plants in archaeological pottery, such as leafy vegetables and cereals. Finally, these experiments have shown that APAA-C_{20} isomers are not exclusively formed from heating aquatic products. However, the APAA C_{20}/C_{18} ratio can potentially be used to determine whether the APAA-C_{20} arose from the processing of aquatic or terrestrial products and provides a useful complementary molecular tool to identify aquatic processing in ancient pottery. The stability of APAA C_{20}/C_{18} ratio should be assessed under a range of different environmental scenarios. Investigations should also examine the correspondence of this ratio with other molecular and isotopic data in archaeological samples.

Furthermore, our experiments show that:

- APAAs form relatively rapidly *ca.* 1 hour of heating.

- Heating at 200°C is sufficient for APAA formation.

- APAAs form under aerobic conditions and are readily formed by simulated cooking on an open fire.

- The presence of pottery is not a prerequisite for their formation, even though it greatly enhances their synthesis due to the accessibility of the metal ions present in the matrix promoting alkali isomerization.

This study shows that the production of APAAs requires much less intensive cooking conditions than previously thought, which probably explains why these compounds are frequently encountered in archaeological pottery. This has important implications for the interpretation of the mode of cooking as it implies that they could theoretically form during a single cooking event rather than from many hours of protracted heating and extensive re-use of a vessel as previously thought. While APAAs are frequently identified in archaeological pottery, they are also notably absent in many archaeological contexts despite large systematic investigation (Whelton et al. 2018; Cubas et al. 2020). This is surprising given that APAAs are so easily formed from a wide range of products and, even more so, considering that other fatty acid thermal degradation products are frequently encountered in vessels from these contexts (e.g. long-chain ketones C_{33} and C_{35}; Raven et al. 1997; Cubas et al. 2020). Further investigations are therefore needed to examine the formation of APAAs in relation to the physical and chemical properties of the ceramic matrices and to examine whether all burial conditions are conducive to their preservation.

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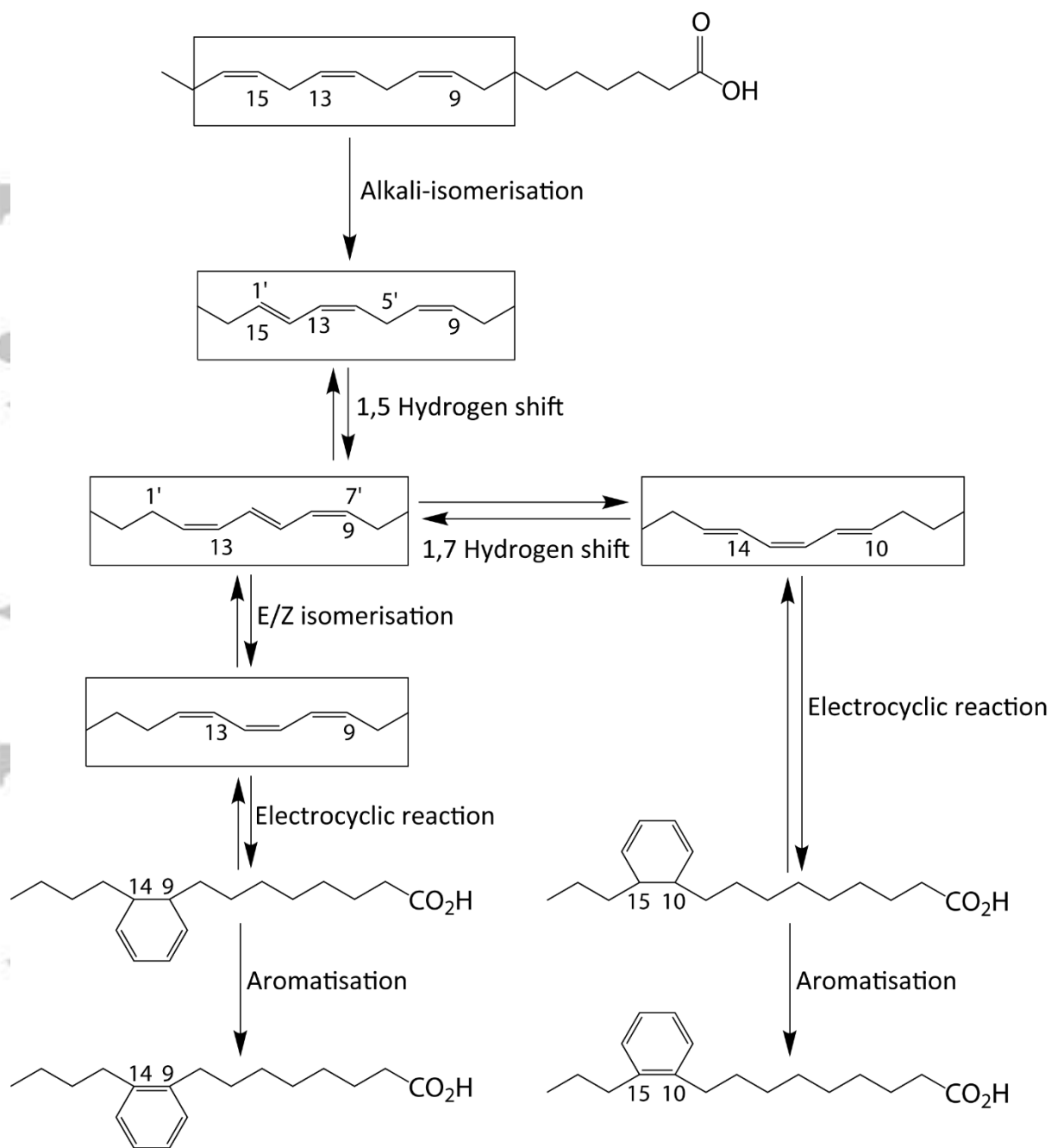
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Figure 1. Scheme of the reaction pathway for the formation of ω-(o-alkylphenyl)octadecanoic acid (APAA) through heating of cis, cis, cis-9, 12, 15-octadecatrienoic acid (after Hansel et al. 2004)
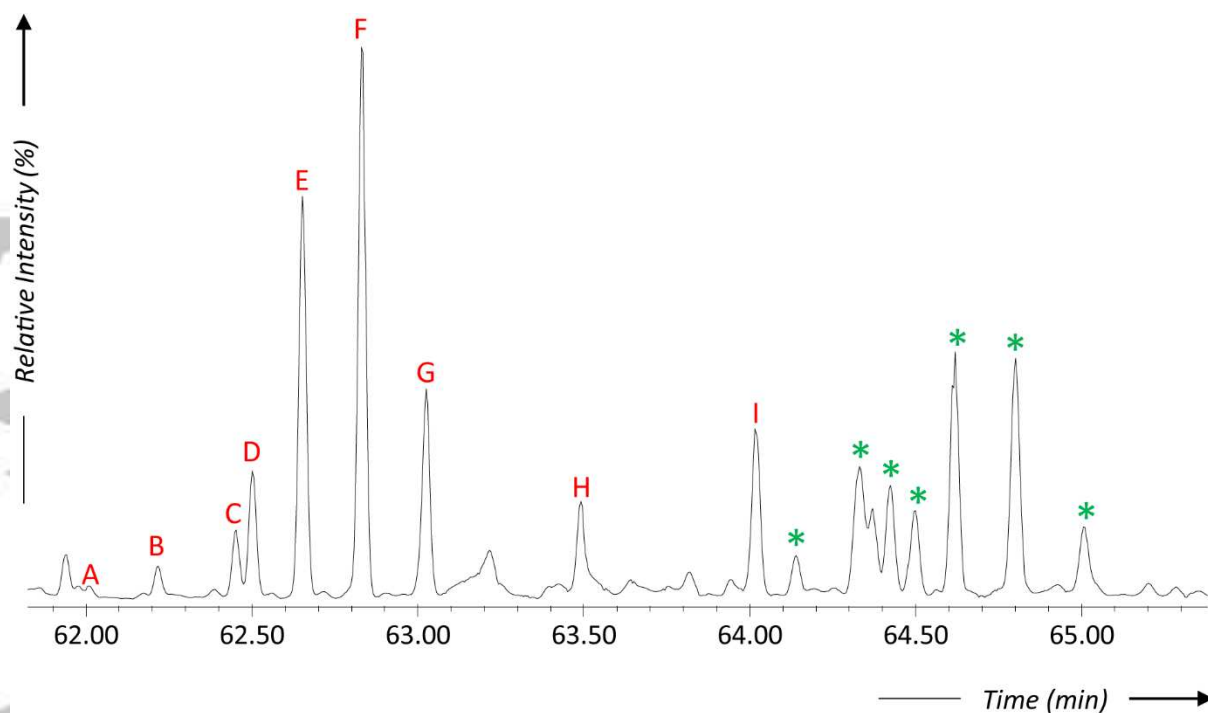
Figure 2. Partial SIM chromatogram (*m/z* 105 ion) of cooked Viviparus shellfish showing the distribution of the ω-(o-alkylphenyl)alkanoic acids with 18 (letters from A to I corresponding to the isomers) and 20 (*) carbon atoms.
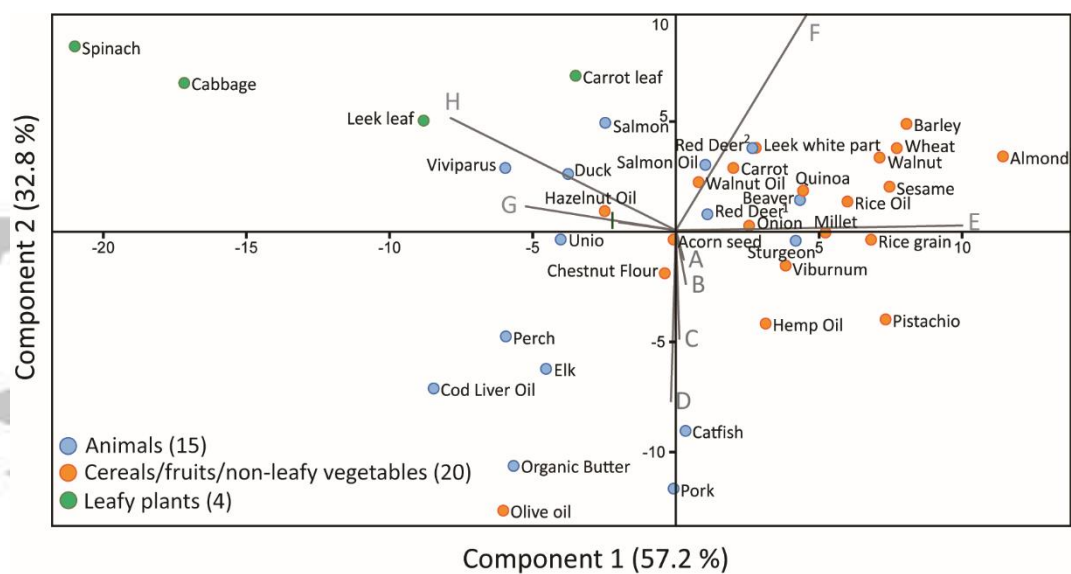
Figure 3. Principal component analysis (PCA) scatter plot of the first two principal components (PCs) based on the APAA-C_{18} isomeric distribution derived from different foodstuffs subjected to heating in the laboratory with the ceramic powder at 270°C for 5 hours.
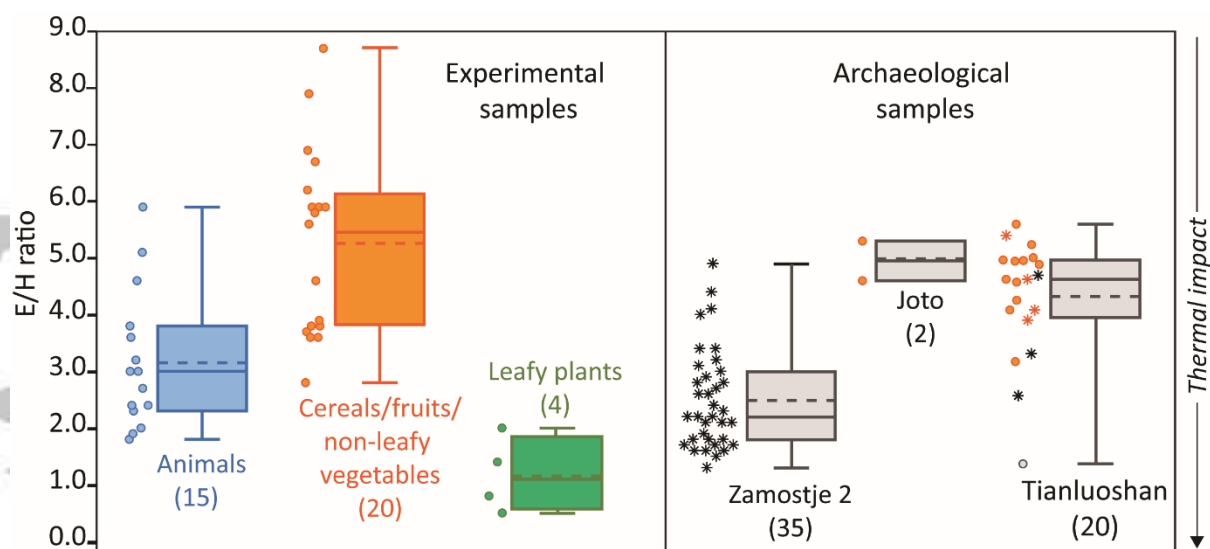
Figure 4. Boxplots of E/H ratio of modern references thermally degraded in the laboratory and archaeological samples. Samples with aquatic biomarkers are indicated by an asterisk, samples with plant and beeswax biomarkers are in orange and grey respectively. Plots represent median (solid line), mean (dashed line), ranges and quartiles. The arrow (thermal impact) shows the effect of increasing temperature on the E/H ratio.
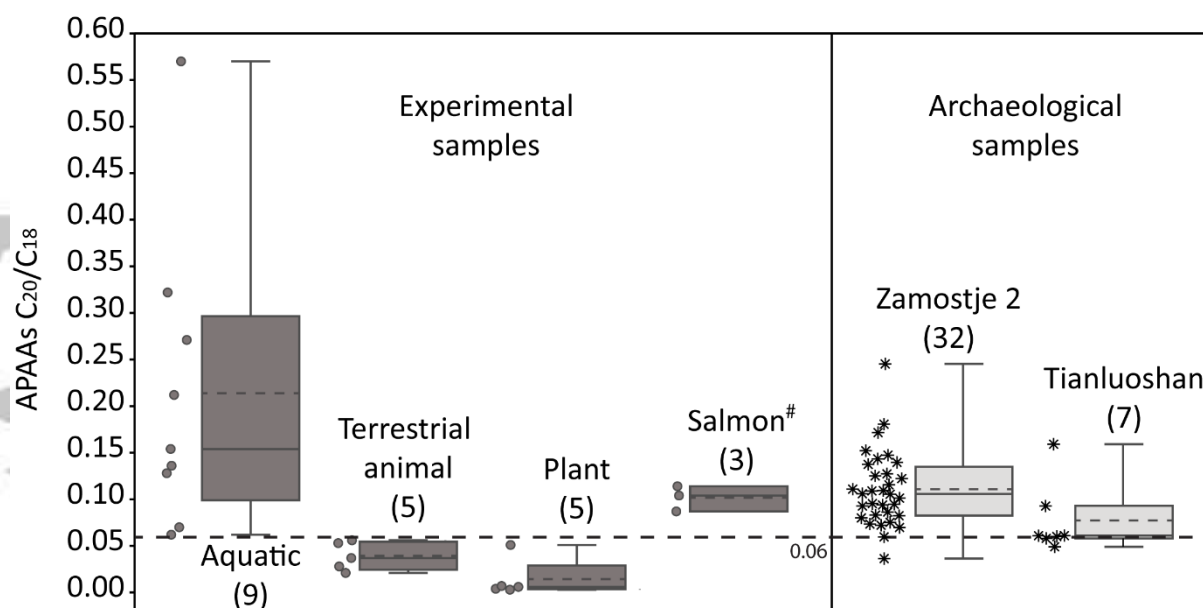
Figure 5. Boxplots of APAA C_{20}/C_{18} ratio of modern references, heated either in the laboratory or during field experiments after 6 months burial (#), and archaeological samples containing aquatic sources. Plots represent median (solid line), mean (dashed line), ranges and quartiles.

Table 1. Table summarizing the experimental parameters carried out in laboratory and thermal conditions required to form APAAs from rapeseed oil and various pure unsaturated fatty acids $C_{18:x}$.

Product	Cooking time (h)	Cooking temperature (°C)	Approximate product amount (mg)	Sealed	APAAs-C_{18} formed with pottery powder	APAAs-C_{18} formed without pottery powder
Rapeseed oil	1	270	65	✓	✓	✓
Rapeseed oil	5	270	65	✓	✓	✓
Rapeseed oil	10	270	65	✓	✓	✓
Rapeseed oil	17	270	65	✓	✓	✓
Rapeseed oil	1	270	65	×	✓	✓
Rapeseed oil	5	270	65	×	✓	✓
Rapeseed oil	5	250	65	×	✓	✓
Rapeseed oil	5	200	65	×	✓	✓
Rapeseed oil	5	150	65	×	×	×
Rapeseed oil	5	100	65	×	×	×
$C_{18:0}$	5	270	20	×	×	×
$C_{18:0}$	5	270	20	×	×	×
$C_{18:1}$	5	270	20	×	✓	✓
$C_{18:1}$	5	270	20	×	✓	✓
$C_{18:2}$	5	270	20	×	✓	✓
$C_{18:2}$	5	270	20	×	✓	✓
α-$C_{18:3}$	5	270	20	×	✓	✓
α-$C_{18:3}$	5	270	20	×	✓	✓