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INVESTIGATING THE FORMATION AND DIAGNOSTIC VALUE OF ω-(*O*-ALKYLPHENYL)ALKANOIC ACIDS IN ANCIENT POTTERY*

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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 provide new insights about the conditions for the formation of APAAs and enable the proposition of novel criteria to distinguish different natural products.

KEYWORDS: ARCHAEOLOGICAL POTTERY VESSELS, EXPERIMENTAL ARCHAEOLOGY, HEATING EXPERIMENTS, LIPID, ORGANIC RESIDUE ANALYSIS, ω-(O-ALKYLPHENYL) ALKANOIC ACIDS

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, 2015; Mitkidou *et al*. 2008; 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

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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-Trimethyltridecanoic (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 amounts 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 to process 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 and *ω*-[*o*-alkylphenyl]octadecanoic acids) is currently of limited diagnostic value, despite the fact that these compounds are frequently recovered from archaeological pots.

The synthesis of APAAs involves several different reactions encompassing mainly alkali isomerization and aromatization steps (Fig. 1) (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 *et al*. (2008) have shown that the distribution of APAA isomers with 18 carbon atoms (APAA-C_{18}) differed according to the number and 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 $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ 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 by a 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 ($\geq$ 17 h at $>$ 270°C), although a shorter cooking time and lower temperatures have so far not been assessed. Yet,

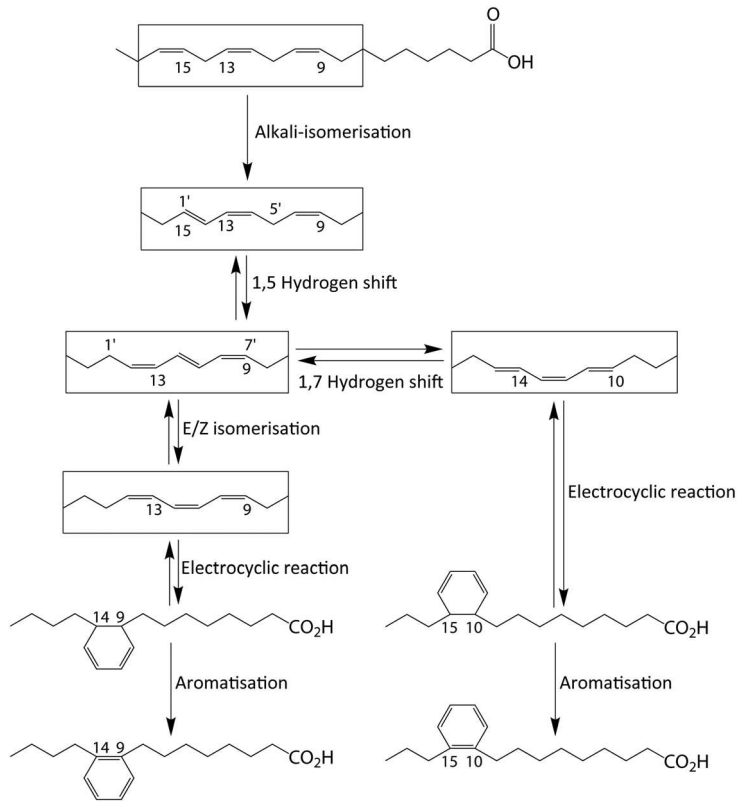

Figure 1 *Reaction pathway for the formation of ω-(o-alkylphenyl)octadecanoic acid (APAA) through the heating of cis, cis, cis-9, 12, 15-octadecatrienoic acid.Source: After Hansel et al. (2004).*

understanding the minimum time and temperature needed to form these compounds is often important for interpretative purposes. Second, 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. Third, anaerobic conditions are regarded as necessary to produce APAAs, promoting the cyclization process. To that extent, the present experiments gave an additional opportunity to reassess the conditions for the formation of APAAs in order to interpret the results better, 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; and 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. About 65 mg of rapeseed oil (Commercial Organic, cold pressed, extra-virgin rapeseed oil, UK) were 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 1, 5, 10 or 17 h (see Table S1, A, in the additional supporting information). The second series of experiments was designed to examine the effect of temperature on APAA formation. About 65 mg of rapeseed oil were placed in open glass tubes and heated to 100, 150, 200, 250 or 270°C for 5 h with or without the addition of ceramic powder (see Table S1, A, online). The third series examined the relationship between APAA formation and precursor fatty acids. About 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 h at 270°C (see Table S1, A, online). 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 h at 270°C with the presence of ceramic powder (see Table S1, B, online). Before heating, a subsample of each raw foodstuffs was retained for analysis.

Experiments were also conducted in the field (YEAR Centre, University of York, UK), 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 h and regularly refilled with water. Subsequently, each pot was emptied and reused for another 1 h in the same manner. This action was repeated five times for the chestnut flour and 15 times for the meat and fish (see Table S2 in the additional supporting information). 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, the other was buried for six months (May–November 2018) at the YEAR Centre (latitude 53.95; longitude –1.09; pH_{soil} 7.16) before analysis.

All animal products used were acquired commercially or were killed or taken legally. The export of several fish samples from the Russian Federation was authorized by The Secretary of State for Environment and Rural Affairs office (Authorisation Number ITIMP18.0277).

Lipid analysis

For the cooking experiments, about 1 g of pottery was drilled following cleaning of the vessel's surface with a modelling drill to remove any exogenous contamination. Any carbonized surface deposits (foodcrusts) formed during cooking were detached from the surface of the pot using a sterile scalpel and were finely crushed. An aliquot of about 20 mg of foodcrusts were weighed out for analysis. For the experiments undertaken in the laboratory, the residue formed by heating the food products with the ceramic powder was used. In addition, lipids were extracted from each raw foodstuff 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, 1 mL for 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 and 200 µl, respectively) and

heated for 4 h at 70°C. After cooling, the lipids were extracted with *n*-hexane (3 × 2 mL). Finally, a second internal standard was added (*n*-hexatriacontane, 10 µg) and the samples were directly analysed by GC-MS.

An Agilent 7890A Series Gas Chromatograph (Agilent Technologies, Cheadle, 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, 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 MS. The ionization 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 × 250 µm × 0.25 µm; J&W Scientific, Folsom, CA, USA) in selected ion monitoring (SIM) mode and using a temperature programme setup to better detect and resolve the three IFAs (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 10°C min^{-1} until 100°C. The temperature was then raised by 4°C min^{-1} to 140°C, then by 0.5°C min^{-1} to 160°C, and finally by 20°C min^{-1} to 250°C, where it was maintained for 10 min. Helium was used as carrier gas at a flow rate of 1.5 mL min^{-1}. Raw foodstuffs were also analysed in total-ion chromatogram (TIC) mode in order to quantify their initial fatty acids content before heating. The relative abundance of APAAs isomers C_{18} and C_{20} was obtained by integration of the ions *m/z* 290 and 318, respectively, and carried out using MassHunter software (v. B.07.01/Build 7.1.524.0).

Lipid extraction of archaeological samples followed the same protocol as the experimental samples and is described by 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 (v. 3.25 for Windows). For detailed explanations of the choice of statistical tests applied, see the additional supporting information.

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 h, we found that APAAs are readily formed after just 1 h of heating at 270°C. The experiments also indicate that heating at 200°C for 5 h 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 > 200°C are easily achieved even when the vessels are used to heat (boil) liquid contents. This point is verified by experiments conducted over an open fire, where the external ceramic surface frequently reached > 300°C. Here, appreciable amounts of APAAs were formed in all the experiments (deer, salmon and chestnut flour) following 5 or 15 h of simulated cooking (see Table S1 in the additional supporting information). Interestingly, the proportion of APAAs compared with 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.

Table 1 *Summary of the experimental parameters carried out in the laboratory and the thermal conditions required to form ω-(o-alkylphenyl)alkanoic acids (APAAs) from rapeseed oil and various pure unsaturated fatty acids (UFAs) $C_{18:x}$*

Product	*Cooking time (h)*	*Cooking temperature (°C)*	*Approximate product weight (mg)*	*Sealed*	*APAAs-C_{18} formed with pottery powder*	*APAAs-C_{18} formed without pottery powder*
Rapeseed oil	1	270	65	Yes	Yes	Yes
Rapeseed oil	5	270	65	Yes	Yes	Yes
Rapeseed oil	10	270	65	Yes	Yes	Yes
Rapeseed oil	17	270	65	Yes	Yes	Yes
Rapeseed oil	1	270	65	No	Yes	Yes
Rapeseed oil	5	270	65	No	Yes	Yes
Rapeseed oil	5	250	65	No	Yes	Yes
Rapeseed oil	5	200	65	No	Yes	Yes
Rapeseed oil	5	150	65	No	No	No
Rapeseed oil	5	100	65	No	No	No
$C_{18:0}$	5	270	20	No	No	No
$C_{18:0}$	5	270	20	No	No	No
$C_{18:1}$	5	270	20	No	Yes	Yes
$C_{18:1}$	5	270	20	No	Yes	Yes
$C_{18:2}$	5	270	20	No	Yes	Yes
$C_{18:2}$	5	270	20	No	Yes	Yes
α-$C_{18:3}$	5	270	20	No	Yes	Yes
α-$C_{18:3}$	5	270	20	No	Yes	Yes

Importantly, the APAA-C_{18} isomeric distribution is not significantly altered by the length of heating (Kruskal–Wallis; $Chi^2 = 0.05$; $p = 1$) (see Fig. S1a, in the additional supporting information). 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 (see Fig. S1b online). 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 h with heating at 270°C or 5 h at 200°C (see Table S1, A, and Fig. S2 in the additional supporting information). This could suggest that instead of the prior alkali isomerization, the APAAs were formed 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 (see Fig. S2 in the additional supporting information). 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 enquiry.

Evacuated versus aerobic conditions Finally, these experiments also demonstrate that APAAs can be produced under fully aerobic conditions (Table 1 and see Table S1, A, in the additional supporting information), 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–I, the rapeseed oil heated in the open tubes produced greater relative amounts of E and F isomers (see Fig. S1 in the additional supporting information). In contrast, the rapeseed oil heated under anaerobic conditions exhibits a higher prevalence of the G isomer. Interestingly, the distributions 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; $Chi^2 = 0.93$; $p = 0.99$; and 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?

Distinguishing different foodstuffs based on the APAA-C_{18} distribution Different foodstuffs were heated in order to assess whether the 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 (see Table S1 in the additional supporting information). These commodities were all subjected to the same experiments involving identical heating conditions (5 h, 270°C, presence of ceramic powder and using open-air conditions) (see Table S1 online). For all samples, the whole set of APAA-C_{18} isomers ($n = 9$, A–I) (Fig. 2) was produced. Analysis of the foodstuffs before 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 (see Table S1 online). 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 data set, 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.

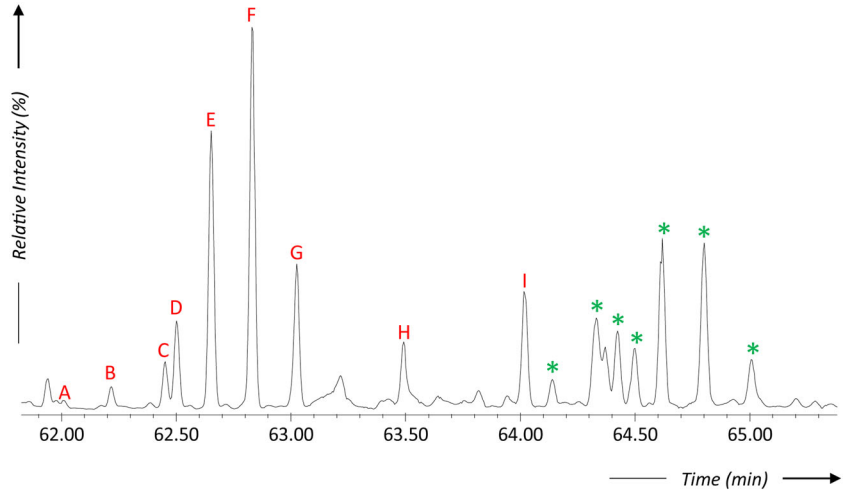

Figure 2 *Partial selected ion monitoring (SIM) chromatogram (m/z 105 ion) of cooked Viviparus shellfish showing the distribution of the APAAs with 18 (A–I, corresponding to the isomers) and 20 (*) carbon atoms.*

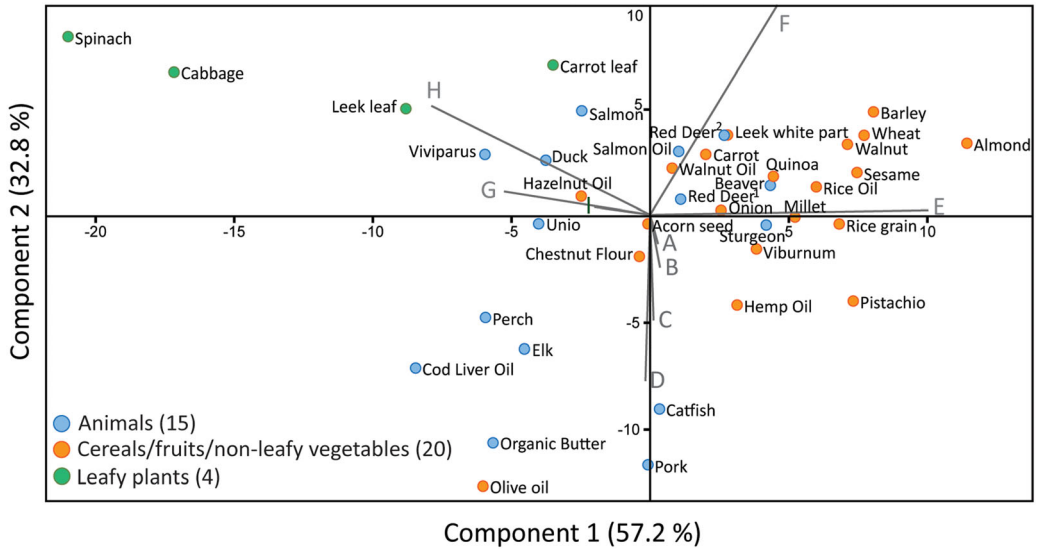

Figure 3 *Principal component analysis (PCA) scatter plot of the first two principal components (PCs) based on the APAA-C_{18} isomeric distribution derived from different foodstuffs subjected to heating in the laboratory with the ceramic powder at 270°C for 5 h.*

Based on the PCA, we calculated the E/H ratio and were able to effectively separate three groups of food products (Fig. 4); (1) cereals/fruits/non-leafy vegetables, (2) leafy vegetables, such as cabbage and spinach, and (3) animal products, including aquatic and terrestrial animals. The distribution of E/H ratios in the first group ($n = 20$; $\bar{x} = 5.3 \pm 1.6$) is significantly different to the leafy vegetables ($n = 4$; $\bar{x} = 1.2 \pm 0.7$; Mann–Whitney test: $U = 0$; $z = 3.1$; $p < 0.01$) and/or animal

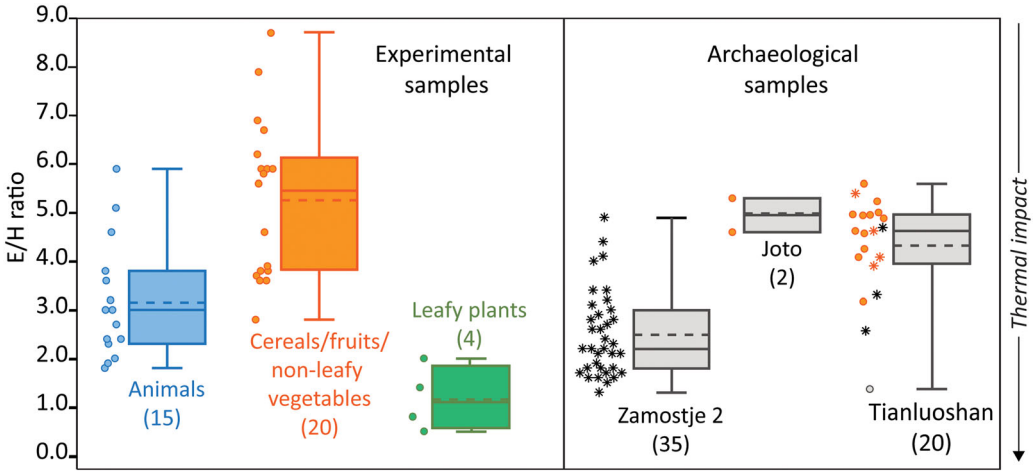

Figure 4 *Box plots of the E/H ratio of modern references thermally degraded in the laboratory and archaeological samples. Archaeological samples with aquatic biomarkers are indicated by an asterisk; samples with plant and beeswax biomarkers are in orange and grey, respectively. Plots represent the median (solid line), mean (dashed line), ranges and quartiles. The arrow (thermal impact) shows the effect of increasing temperature on the E/H ratio.*

products ($n = 15$; $\bar{x} = 3.2 \pm 1.2$; Mann–Whitney test: $U = 40$; $z = 3.7$; $p < 0.01$) (Fig. 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 UFAs 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; *c*.6600–4000 cal BCE), a riverine hunter–gatherer site located in Russia; and two early agricultural sites: Joto (Yayoi period; *c*.20–230 cal CE) in Japan and Tianluoshan (Neolithic; *c*.5000–4000 cal BCE) 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 (*c*.5000–4000 cal BCE) 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 ($\bar{x} = 10.7‰ \pm 2.2‰$), 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 scanning electron microscopy (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 ($\delta^{15}N_{mean} = 0.6‰ \pm 1.8‰$; $C{:}N_{mean} = 17.9 \pm 4.6$) (Shoda *et al*. 2011; Yoshida *et al*. 2013). A total of 20 samples were obtained from Tianluoshan, the majority ($n = 12$) being 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 ratios ($\delta^{15}N_{mean} = 4.8‰ \pm 1.7‰$; $C{:}N_{mean} = 16.0 \pm 3.6$; 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, a further four samples had both criteria, demonstrating some degree of mixing.

The E/H ratio of vessels (Fig. 4) from these three sites shows good correspondence with the presumed contents based on previous analysis (Shoda *et al*. 2011, 2018; Bondetti *et al*. 2020). The vessels used for animal fats from Zamostje 2 and Tianluoshan had mean E/H ratios of 2.5 (± 0.9) and 3.0 (± 1.1), respectively, while the E/H ratios for vessels focused on plant processing from Joto (5.0 ± 0.48) and Tianluoshan (4.7 ± 0.6) are 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 ratio ($\bar{x} = 4.5 \pm 0.8$) compared with samples with starchy plant biomarkers only.

The analysis of UFAs $C_{18:1}$, $C_{18:2}$, α-$C_{18:3}$ undertaken here on both pure compounds (see Table S1, A, and Fig. S3 in the additional supporting information) and previously published data (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 (see Tables S1A and B, S3 and S4 in the additional supporting information), 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 and H (see Table S1, A and B, and Fig. S3 in the additional supporting information). 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} versus 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., 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} (see Table S1, B online). 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)

(see Table S1, B online). 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., IFAs, 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, the 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 ($n = 9$; $\bar{x} = 0.21 \pm 0.03$) is significantly higher than both terrestrial plants ($n = 5$; $\bar{x} = 0.02 \pm 0.00$; Mann–Whitney test: $U = 0$; $z = 2.93$, $p < 0.01$) and terrestrial animals ($n = 5$; $\bar{x} = 0.04 \pm 0.00$; *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 > 0.06 ($n = 3$; $\bar{x} = 0.10 \pm 0.00$) following six months of 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 ($n = 32$; $\bar{x} = 0.11 \pm 0.04$) and Tianluoshan ($n = 7$; $\bar{x} = 0.08 \pm 0.04$), meeting the criteria for aquatic lipid identification, mostly fall within the range of modern aquatic data (Fig. 5), confirming their use for processing aquatic resources (Shoda *et al*. 2018; Bondetti *et al*. 2020).

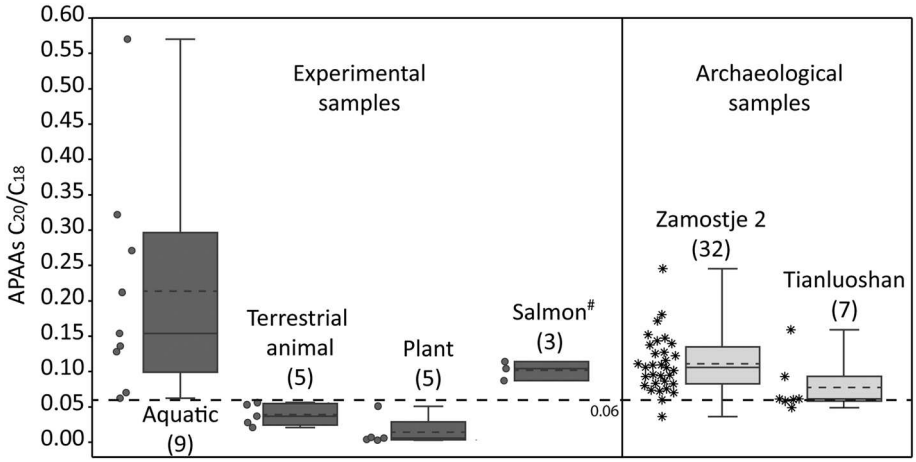

Figure 5 *Box plots of the APAA C_{20}/C_{18} ratio of modern references, heated either in the laboratory or during field experiments after 6 months of burial (#), and archaeological samples containing aquatic sources (*). Plots represent the median (solid line), mean (dashed line), ranges and quartiles.*

CONCLUSIONS

The thermal degradation of a wide range of commodities brought new insights with regards 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 after about 1 h 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 because it implies that they could theoretically form during a single cooking event rather than from many hours of protracted heating and extensive reuse 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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PEER REVIEW

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Summary of the results of the thermal degradation of rapeseed oil and mono- and unsaturated fatty acids C_{18} (A) and various foodstuffs (B) carried out in laboratory and the experimental parameters.

Table S2. Summary of the results of the simulated cooking in replicate pots of various foodstuffs on an open fire and the field experimental parameters. The layer of fats and oils formed from salmon and red deer during the cooking was skimmed and gathered in another pot and then placed back onto the fire to continue their cooking and concentrate the lipids. These samples are noted with an asterisk (*).

Table S3. Summary of the proportion of unsaturated fatty acid $C_{18:x}$ detected in products before being heated.

Table S4. Spearman test showing the correlation between the E/H ratio and initial fatty acid content of commodities used for the experiments. The test reveals a low correlation between the isomeric distribution observed in the foodstuffs after heating and their initial fatty acid content.

Figure S1. APAAs-C_{18} A–I isomeric distribution of rapeseed oil subjected to different heating conditions, time (a) and temperature (b), under either evacuated (line patterns) or air (diamond patterns).

Figure S2. Partial selected ion monitoring (SIM) chromatogram of rapeseed oil cooked under open air showing ω-(*o*-alkylphenyl)alkanoic acids C_{18} isomers (*m/z* 290 ion) with or without ceramic powder.

Figure S3. APAAs-C_{18} A–I isomeric distribution of pure unsaturated fatty acid (UFA), spinach and cabbage heated in an open glass tube at 270°C for 5 h with ceramic powder. For each UFA, the experiments were duplicated and the distribution of APAAs-C_{18} given corresponds to the average of these two analyses along with the error bars.