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ORIGINAL ARTICLE

Testing sample selection criteria and loss of biomarkers during cleaning of archaeological unglazed pottery to maximize organic residue quantities

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Abstract

As the number of studies in organic residue analysis (ORA) of ancient pottery—a sensitive but as of today also a destructive method—increases, archaeologists are interested in knowing which samples promise the biggest abundance of analytes in order to avoid unnecessary loss of artefacts. Another frequently asked question is whether the routinely performed cleaning procedure should be omitted for samples intended for ORA to preserve the availability of analytes. We tested several selection criteria commonly accessible to archaeologists (texture, position, shape) for lipid quantities in ancient pottery in order to determine the most productive sherds for analysis. Moreover, we monitored loss of lipids during the water-and-brush cleaning process. Beside the usually targeted straight-chain fatty acids (FA), less abundant biomarkers such as α,ω-dicarboxylic acids (DCA), ω-(*o*-alkylphenyl)alkanoic acids (APAA) and hydroxy fatty acids (HFA) were also screened. The highest concentrations of analytes were observed in rims of coarse-textured plates and cooking pots, demonstrating the usefulness of the proposed criteria. The washing procedure applied here did not lead to a loss of bulk FA, although the effect on minor components was not uniform.

KEYWORDS
ancient pottery, archaeological science, fatty acid methyl esters, GC-MS, hydroxy fatty acids, α,ω-dicarboxylic acids, *ω*-(*o*-alkylphenyl)alkanoic acids

INTRODUCTION

Organic residue analysis (ORA) is a field of growing importance, greatly facilitated by innovations in chromatographic separation and mass spectrometry (MS) (Kałużna-Czaplińska et al., 2016). One of its goals is the reconstruction of dietary practices throughout human (pre) history by analysing biomarkers, that is, biomolecules that are sufficiently specific for a taxon, found on ancient artefacts. In this process, an increasing number of studies rely on molecular and compound-specific stable isotope data, usually targeting lipids such as fatty acids (FA) extracted from ancient pottery and analysed as their fatty acid methyl esters (FAME) (Evershed et al., 2008). Recently, researchers were able to prove that dairy products were processed already in the earliest settlements of early European farmers (Casanova et al., 2022). Meanwhile, integration of biomarker studies with archaeogenetics led to the conclusion that selection pressure rather than consumption of milk was the main driver of the European lactase-persistence genotype (Evershed et al., 2022). The use of grain in Neolithic cooking—a previously elusive topic—was demonstrated for the first time by isolating the plant-specific substance class of alkylresorcinols (Hammann et al., 2022).

In the light of these advancements in the science of heritage and human evolution, many archaeologists curating unglazed ancient pottery are interested in insights from biomarker analysis. As samples intended for ORA require special measures to avoid contamination with exogenous lipids, some crucial rules and guidelines have emerged in the almost 50 years since the first application of this technique (Condamin et al., 1976). Recommendations include avoiding contamination from skin, cosmetics, food, beverages, adhesives and plastic bags, as well as using freshly excavated, unwashed samples (Historic England, 2017; Whelton et al., 2021). Yet, a critical question remains: How does one select the most suitable samples? Due to the destructive nature of ORA and the unique character of individual archaeological objects, the goal should be to select only samples that are most likely to lead to appreciable lipid quantities and avoid the destruction of those that do ultimately not afford any useful data.

Estimating the suitability of samples prior to analysis is still very difficult, but previous studies and reference experiments are available that have investigated the (chemical/physical) characteristics and shapes of ancient pottery as well as different sampling positions. The porosity of pottery walls was assessed and correlated to the quantity of extracted lipids, showing that porous surfaces absorb significantly more analyte than low-porosity ceramics (Correa-Ascencio & Evershed, 2014). Meanwhile, a high proportion of pores with sizes < 1 μm enhances preservation since they are impermeable to microorganisms (Drieu et al., 2019). Yet, these measurements require complex instruments such as surface imaging applications, which are mostly unavailable to archaeologists. In addition, multiple sampling of individual pottery objects demonstrated the dependence of longitudinal lipid distribution on vessel function, with rim sherds generally recommended for the sampling of cooking pots (Charters et al., 1993, 1997). Finally, it was revealed that FA accumulate in Ca oxide granules as water-insoluble Ca salts, suggesting that Ca-rich pottery preserves analytes more efficiently (Hammann et al., 2020). It has not yet been tested whether Ca concentration data can help to directly select pottery that promises high lipid quantities.

Another concern expressed by archaeologists refers to the washing of pottery with water and brushes. While washing is a prerequisite of routine archaeological examination, allowing

subsequent characterization and classification, it is generally discouraged for samples designated for ORA due to concerns that this step could lead to a significant loss of analyte or introduce contaminants (Historic England, 2017). However, to the best of our knowledge, no quantitative results have been published to support this argument.

Besides the bulk lipid (FAME) content, diagnostic minor components such as α,ω-dicarboxylic acids (DCA), ω-(*o*-alkylphenyl)alkanoic acids (APAA) and hydroxy fatty acids (HFA) can offer further information on lipid source and vessel use. These products of oxidative decomposition of unsaturated FA, which is favoured under elevated temperatures as well as the presence of metal ions and reactive oxygen species, have been detected in marine, animal, and plant oils from archaeological cooking vessels and oil lamps (Bondetti et al., 2021; Copley et al., 2005; Hansel & Evershed, 2009). Crucially, DCA and HFA can preserve the original position of the double bond and allow one to deduct the mechanism of formation, although HFA can also occur as endogenous metabolites in organisms (Cahoon & Li-Beisson, 2020; Kokotou et al., 2020). On the other hand, formation of APAA is accompanied by rapid migration of double bonds, and therefore they are usually detected as a series of eight isomers. They have been used as a proxy of polyunsaturated FA contained amply in marine products, but are also formed readily from oleic acid (Bondetti et al., 2021; Taché et al., 2019). To this day, very little is known about sampling and sample treatment strategies geared towards these compounds.

In our studies, we wanted to examine the practical applicability of the aforementioned findings in the literature, many of which were acquired from replica pottery, to selection criteria accessible to archaeologists using a statistically evaluable set of genuine artefacts, and also to address concerns about washing. For this, we tested the following hypotheses:

Hypothesis 1. Lipid quantity is higher in:

a. coarse-textured pottery than in smooth ceramics, since texture is a function of porosity
b. rims of vessels than in lower areas, since fats are less dense than water
c. sherds of pots rather than of bowls, jugs, and plates, since lipids are most soluble in water when it is heated.

Hypothesis 2. Determining the Ca concentration in ancient pottery can be used to identify samples with high lipid concentrations prior to destructive analysis since enrichment of FA was observed around Ca inclusions in the ceramic matrix.

Hypothesis 3. Washing pottery with water and brushes leads to reduction in bulk and minor lipid concentrations in the studied object.

MATERIALS AND METHODS

Archaeological samples

Pottery sherds ($n = 37$) originated from a Roman stone cellar (mid-second to mid-third centuries CE) near Hermeskeil (Lk. Trier-Saarburg, Germany) excavated in 2020. Sherds were air-dried and stored in plastic bags until further processing in 2022. Skin contact was avoided by wearing nitrile gloves. Samples were selected according to the following criteria: Texture (coarse $n = 8$, smooth $n = 23$, not assessed $n = 6$), position of the sherd on the original vessel (base $n = 14$, body $n = 6$, rim $n = 17$), and shape (bowls $n = 5$, jugs $n = 11$, plates $n = 4$, pots $n = 17$) (Table 1; and see Figure S1 in the supplemental data online for photographic documentation).

TABLE 1 Overview of sampled pottery sherds and associated soil as well as the properties used for selection.

Sample ID	Texture	Position	Shape	Cleaning comparison	Soil extracted
284	Smooth	Rim	Pot	Yes	No
290	Smooth	Body	Pot	Yes	No
214	Coarse	Rim	Pot	Yes	Yes
384	Coarse	Rim	Pot	Yes	Yes
304	Smooth	Rim	Pot	Yes	Yes
441	Smooth	Body	Jug	Yes	No
286	Coarse	Rim	Pot	Yes	Yes
320	Coarse	Base	Pot	Yes	Yes
258	Smooth	Rim	Jug	Yes	No
259	Smooth	Body	Jug	Yes	No
241	Smooth	Body	Jug	Yes	No
313	Smooth	Base	Jug	Yes	No
267	Smooth	Base	Plate	Yes	No
238	Not assessed	Rim	Plate	Yes	No
346	Smooth	Rim	Plate	Yes	Yes
LF	Smooth	Rim	Plate	Yes	No
182	Coarse	Body	Bowl	Yes	No
312	Smooth	Rim	Bowl	Yes	Yes
316	Smooth	Rim	Bowl	Yes	No
375	Coarse	Rim	Bowl	Yes	No
396	Smooth	Base	Bowl	Yes	No
188	Smooth	Base	Pot	No	No
239	Smooth	Base	Pot	No	No
246	Smooth	Base	Jug	No	No
250	Smooth	Base	Jug	No	No
278	Smooth	Body	Jug	No	No
282	Not assessed	Base	Pot	No	No
293	Not assessed	Base	Jug	No	No
381	Smooth	Rim	Pot	No	No
383	Smooth	Rim	Pot	No	No
388	Coarse	Rim	Pot	No	No
389	Not assessed	Base	Pot	No	No
395	Not assessed	Base	Pot	No	No
401	Smooth	Rim	Jug	No	No
410	Smooth	Base	Jug	No	No
417	Not assessed	Base	Pot	No	No
418	Coarse	Rim	Pot	No	No

Texture was defined as coarse if tempering was visible with the eye and physically noticeable on the pottery surface, and as smooth if the pottery was tempered with fine quartz or not tempered at all (Gose, 1950). Samples of soil were stored in the same bags as the respective associated sherds. Of these, seven were extracted and analysed as described below.

Chemicals

Methanol (GC Ultra Grade), *n*-hexane (UV/IR grade) and sulfuric acid (H_2SO_4, 96%, p.a.) were acquired from Carl Roth GmbH Co. KG (Karlsruhe, Germany). Ethyl acetate (≥ 99.5%) was purchased from Fisher Chemical (Hampton, NH, USA). *N*,*O*-*bis*-trimethylsilyl)trifluoroacetamide (BSTFA) with 1% (v/v) trimethylchlorosilane (TMCS), pyridine (99%), 37 component FAME mix (Lot LRAC3241), *n*-nonacosane (100%), d_{31}-palmitic acid (99%, 98.5 atom% D) and *n*-octacosane (99.4%) were from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation

From pottery sherds, two adjacent pieces weighing 3–5 g each were separated using a Micromot 50/E modelling drill (Proxxon, Föhren, Germany) with diamond cutting discs, resulting in 74 total pottery samples (Figure 1). Of these, 42 underwent comparisons of selection criteria as well as cleaning. One of the two separated pieces of a sherd was cleaned with deionized water in a single-use Al tray using a medium-strength toothbrush while wearing nitrile gloves, then dried in an oven at 40°C and stored in Al foil. The remaining pieces ($n = 32$) were included in the analysis in order to increase the statistical power of the considerations concerning selection criteria. Since none of the duplicates in this subset was washed prior to analysis, they also acted as a control group for the cleaning experiment. Exactly 1 mm of surface (measured using a micrometer to ensure comparability) was removed from all samples using the modelling drill equipped with diamond grinding bits. Finally, the pottery was ground to a fine powder in a solvent-washed mortar and pestle (see Methods 1 in the supplemental data online).

Elemental analysis via portable X-ray fluorescence spectrometry (pXRF)

Before mortar grinding, a minimum of one pXRF measurement was taken of each sample's inner and outer side using a handheld Niton XL3t XRF Analyzer (Thermo Scientific, Waltham, MA, USA) to correlate Ca concentration to lipid quantity. The device was operated in ore mode. Filter settings were set to 120 s for light and 60 s for main, low and high filters, respectively. Calibration was performed against a standard soil sample for 30 s and Ca concentrations were standardized against internal K concentration, affording Ca/K ratios.

Acidified methanol extraction

Simultaneous extraction and derivatization of FA in acidified methanol was performed in accordance with Correa-Ascencio & Evershed (2014). For this, 0.125–2.0 g of powdered sample were weighed out in a glass test tube and spiked with 20 µg of d_{31}-palmitic acid as internal standard 1 (IS1). After the addition of 5 mL of 2% (v/v) H_2SO_4/methanol, the tube was capped, and the suspension was heated to 70°C for 1 h under frequent mixing. The liquid was transferred to a clean tube after centrifugation (2500 rpm, 3 min) and 2 mL of deionized water (Barnstead Nano-pure, Thermo Scientific, Waltham, MA, USA) were added. Lipids were extracted from the supernatant with 3 × 2 mL *n*-hexane and from the ceramic residue with 2 × 1 mL *n*-hexane. The combined organic phases were concentrated under N_2 flow, spiked with 20 µg of *n*-nonacosane as IS2, and reconstituted in 2 mL *n*-hexane. Aliquots were transferred to GC vials for analysis. To monitor the level of laboratory contamination, at least two blanks were extracted per batch, which yielded FAME concentrations of 186 ± 137 ng mL^{-1} (mean ± SD; $n = 14$) (see the supplemental data online). This corresponds to a mass concentration of

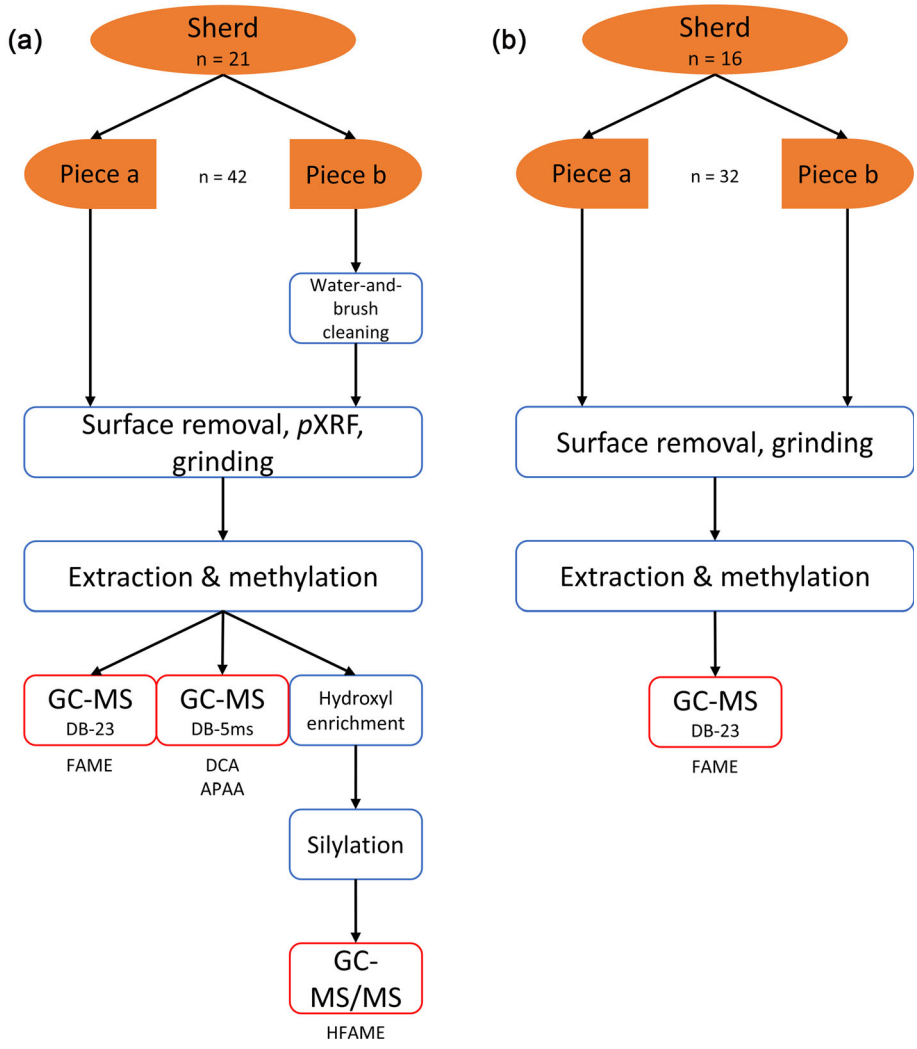

FIGURE 1 Graphical summary of the workflows performed on the sample subsets used to test the effects of (a) selection criteria as well as water-and-brush cleaning; and (b) selection criteria only. pXRF, portable X-ray fluorescence spectroscopy; GC-MS, gas chromatography-mass spectrometry; GC-MS/MS, GC tandem-MS; FAME, fatty acid methyl ester; DCA, α,ω-dicarboxylic acid; APPA, ω-(o-alkylphenyl)alkanoic acid; HFAME, hydroxy FAME.

0.37 μg g^{-1} of pottery—a concentration below the threshold of interpretability accepted for ORA (5 μg g^{-1}) (Evershed, 2008). No noticeable contaminants were introduced during the water-and-brush cleaning.

Enrichment of minor components and trimethylsilyl (TMS) derivatization

For the enrichment of hydroxyl group-containing biomarkers in analogy to Hammann et al. (2022), 1 ml of lipid extract was added to a glass column (0.5 cm i.d.) filled with 150 mg of an aminopropyl stationary phase (Supelclean LC-NH2, Sigma-Aldrich), previously equilibrated with 10 mL of 96:4 (v/v) n-hexane/ethyl acetate. The sample was added and FAMEs were eluted

with 10 mL of 96:4 (v/v) *n*-hexane/ethyl acetate. The desired hydroxylated fraction was collected with 10 mL of 90:10 (v/v) ethyl acetate/methanol, concentrated under N_2 flow, reconstituted in 200 µL of *n*-hexane and transferred into GC vials for derivatization. Here, the solutions were again concentrated under N_2 flow, after which silylation was carried out by adding 25 µL of pyridine and 50 µL of BSTFA/TMCS, capping, and heating at 70°C for 1 h. The reagents were then removed under N_2 flow and analytes were reconstituted in 150 µL *n*-hexane containing 10 ng μL^{-1} *n*-octacosane as IS3 before analysis.

GC-MS analysis of FAME

Methylated extracts were analysed using a 6890 N GC coupled to a 5975B inert XL MS detector equipped with a DB-23 column (30 m length, 0.25 mm i.d., 0.25 µm film thickness; all Agilent Technologies, Santa Clara, CA, USA). Samples (1 µL) were injected by a 7683B autosampler into a split/splitless inlet maintained at 240°C (split factors between 1:5 and 1:125, determined by pre-measurements on a GC coupled to flame ionization detection—GC-FID) and He was used as a carrier gas at a flow rate of 0.8 mL min^{-1}. Oven temperature was held at 50°C for 2 min, then increased to 165°C at a rate of 30°C min^{-1} and to 175°C at 4°C min^{-1}, where it was kept for 6.5 min. After that, it was changed to 230°C at 4°C min^{-1} and upheld for 10 min. Analytes were ionized in electron ionization mode at 70 eV. For targeting FAME, a single ion monitoring (SIM) method was developed using the 37 component FAME mix (see Table S1 and Figures S2 and S4A/B in Methods 2, 3 and 5 in the supplemental data online). Additionally, full-scan spectra of washed samples were recorded. GC-MS performance was monitored by a measurement of the standard mix before each sequence.

GC-MS analysis of DCA and APAA

Methylated extracts were also analysed using a 7890B GC coupled to a 7000D triple quadrupole MS equipped with a DB-5ms column (30 m length, 0.25 mm i.d., 0.25 µm film thickness; all Agilent Technologies). Samples were injected by a 7650A autosampler into a split/splitless inlet maintained at 300°C (splitless or split factor of 1:10, determined by pre-measurements on a GC-FID, pressure pulse at 25 psi until 1.5 min) and He was used as the carrier gas at a flow rate of 1.5 mL min^{-1}. The starting oven temperature of 80°C was held for 1.5 min, then increased to 300°C at a rate of 10°C min^{-1} and held for 8 min. After that, it was increased to 325°C at 25°C min^{-1} and held for 5 min. Analytes were ionized in electron ionization mode at 70 eV. Data were collected in SIM mode (see Table S1 and Figure S4C/I in Methods 4 and 5 in the supplemental data online). Additionally, full-scan spectra of unwashed samples were recorded.

GC-MS/MS analysis of minor components

Silylated fractions were analysed using the same 7890B/7000D system described above. The starting oven temperature of 50°C was held for 2 min, then increased to 200°C at a rate of 10°C min^{-1} and held for 10 min. After that, it was increased to 300°C at 5°C min^{-1} and held for 5 min. Finally, it was changed to 325°C at 10°C min^{-1} and maintained for 5 min. N_2 was employed as the collision cell gas (1.5 mL min^{-1}) and He as the quench gas (2.25 mL min^{-1}). Data were collected in full-scan and dynamic multiple reaction monitoring (dMRM) modes (see Table S1 and Figure S4D–F in Methods 4 and 5 in the supplemental data online).

RESULTS AND DISCUSSION

FAME 16:0 and 18:0 were the most abundant detected lipids, followed by FAME 14:0, 17:0 and 20:0 (Figure 2) (see Liebisch et al., 2020, for shorthand lipid nomenclature). Unsaturated and branched (*iso* and *anteiso*) FAME were also observed in low amounts. The latter are microbial biomarkers present in soil and ruminant lipids (Dunne et al., 2012; Evershed et al., 2002; Taormina et al., 2020). Out of 37 sherds sampled, 31 showed lipid contents > 5 μg g^{-1} (see Tables S2–S3 in the supplemental data online). Concentrations > 1000 μg g^{-1} were obtained from six sherds, including sherd 375 which contained 19,300 ± 300 μg g^{-1} (individual value ± standard error). The seven examined soil samples largely reflected the FAME signature found in the corresponding pottery sherds, albeit at much lower concentrations. Nevertheless, chromatograms of soil were distinct due to higher proportions of minor components such as *iso*-FA and long-chain DCA (see Figure S3 and Tables S4–S5 in Methods 6 online).

In addition, despite the fact that samples were stored in plastic bags, no traces of plasticizers were detected in MS scans when using the DB-23 column. Although dioctyl phthalate was present during analyses on the DB-5ms column, we cannot exclude that this may be due to accumulation during storage of extracts in storage vials. The initial absence of phthalates may be due to either the air-drying of pottery after excavation, thus reducing the risk of transferring substances from the bags to the samples via residual humidity, or the fact that pottery was cleaned in the laboratory after storage, thereby removing potential microplastic dust from the surfaces. In ORA practice, the use of plastic bags is routinely advised against (Historic England, 2017).

Lipid quantities in relation to selection criteria

Quantitative analysis revealed a decisive dependency of FAME concentration on the texture and position of the sherds, as well as the shape of the original vessel. Pieces of coarse pottery

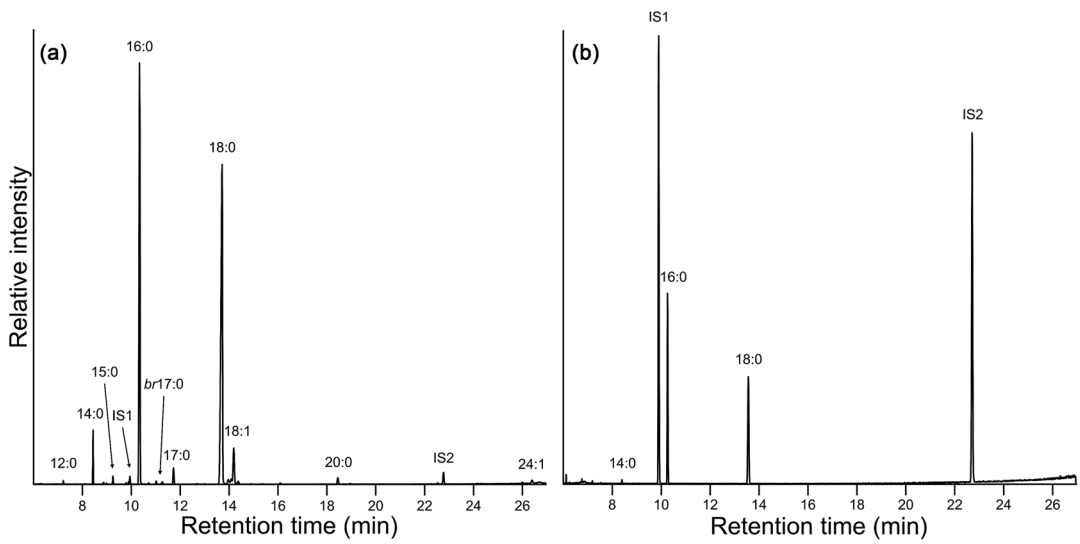

FIGURE 2 Exemplary total-ion chromatograms of washed pottery samples, recorded in full-scan mode on a DB-23 column (Agilent Technologies, Santa Clara, CA, USA), showing commonly encountered fatty acid methyl esters; numbers above peaks denote the chain length and degrees of unsaturation: (a) sample 384 (coarse, rim, pot); and (b) sample 396 (smooth, base, bowl). IS1, d_{31}-palmitic acid (detected as the methyl ester); IS2, *n*-nonacosane; *br*, branched.

(median, IQR (μg g^{-1}) = 335, 164–2035; n = 16) contained considerably more analyte than smooth ceramics (47.3, 7.20–526; n = 46) (Figure 3A). This could be caused by higher porosity which promotes the absorption of lipids (Correa-Ascencio & Evershed, 2014; Drieu et al., 2019), or exposure to fattier foodstuffs, leading to a higher initial lipid load. Pieces of rims (539, 120–2514; n = 34) showed the highest concentrations in regard to position and included five out of six samples yielding > 1000 μg g^{-1} (Figure 3B). Meanwhile, lipid contents in base pieces (72.7, 12.4–281; n = 28) were relatively low and body parts (4.69, 4.14–7.90; n = 12) showed very little analyte. This is in analogy with published experiments (Charters et al., 1993, 1997). Median (IQR) lipid concentration (μg g^{-1}) as a function of vessel shape decreased in the following order: plates (2820, 768–4000; n = 8) > pots (220, 38.1–531; n = 34) > bowls (29.4, 11.0–5320; n = 10) > jugs (10.1, 3.93–75.9; n = 22) (Figure 3C).

Thus, it can be inferred that Hypotheses 1a–b are confirmed by our findings. Rims of coarse pottery are more likely to contain sufficient lipids for ORA due to higher porosity and exposure to fats which float on top of water. This is subject to the condition that an aqueous medium was predominant in the vessel (used for cooking foodstuffs or storing beverages, etc.) rather than a fatty one (roasting, storage of oils, etc.). Although an ethnographic study of pottery from Bedik country (eastern Senegal) has shown some promise for body areas, it was performed on a small and contemporaneous sample set with rather low suggested fill heights (Drieu et al., 2022).

Interestingly, Hypothesis 1c was incorrect, as sherds of plates yielded the most FAME by a very high margin. Caution is advised during the interpretation of this finding, as it might be caused by a relatively low sample size (n = 8 duplicates) and a cultural phenomenon. Roman age plates in particular have been observed to have burn marks, leading to the assumption that they were sometimes used not only for serving meals but also for heating them (Gose, 1950; Hunold, 1997). Investigations of plates from a different origin may not lead to similar conclusions. Despite that, the wide spread of data points suggests either a rather inhomogeneous distribution of lipids on plates or differences in their use. Pots, meanwhile, exhibit a much narrower and thus more reliable range of results, which can be explained by the presence of water as a homogenizing medium during vessel use. In general, very different results can be

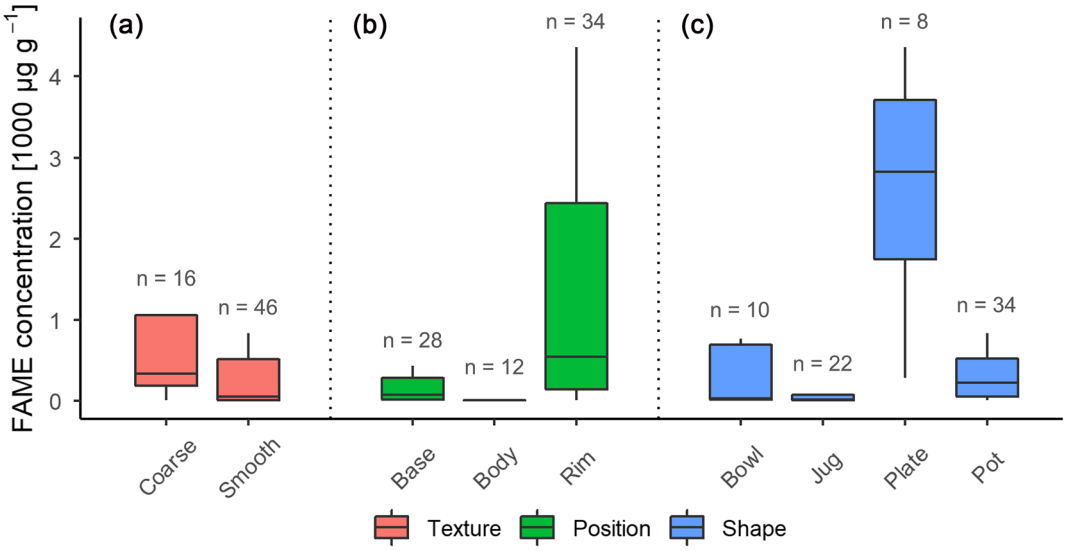

FIGURE 3 Distribution of FAME among sampling categories; washed and unwashed sherds included: (a) ceramic texture; (b) sherd position; and (c) vessel shape.

expected when sampling objects that have been intended for different purposes, emphasizing the importance of archaeological context in sample selection and interpretation.

Also to be rejected is Hypothesis 2, as Ca/K ratios were not correlated with FAME concentration (Spearman's $\rho = 0.23$, $n = 42$) (Figure 4). Except for two objects, high-FAME samples were inconspicuous when plotted against Ca/K values. However, this does not definitively exclude Ca content itself as a predictor for the presence of analyte. The low expressiveness of the results may be due to the limitations of pXRF, which is a relative, not an absolute technique. Although standardization against K concentration increases the robustness of the data, a coincidence of high K content could mask high Ca, making the measurements less useful. Therefore, pXRF does not seem to have value as a quantitative approach to non-destructive sample prospecting. High-resolution analytical methods such as micro-invasive inductively coupled plasma mass spectrometry (ICP-MS) or scanning electron microscopy coupled to energy dispersive X-ray spectroscopy (SEM-EDX) would not have similar limitations but may be less available to archaeologists. In any case, in-sample heterogeneity, which is characteristic of ceramic materials, would have to be accounted for.

Hence, with our data we cannot confirm or reject the observation made by Hammann et al. (2020) about the accumulation of FA as Ca salts. The question of the role of Ca in FA preservation could be approached via experimental archaeology involving replica pottery with varying concentrations of Ca.

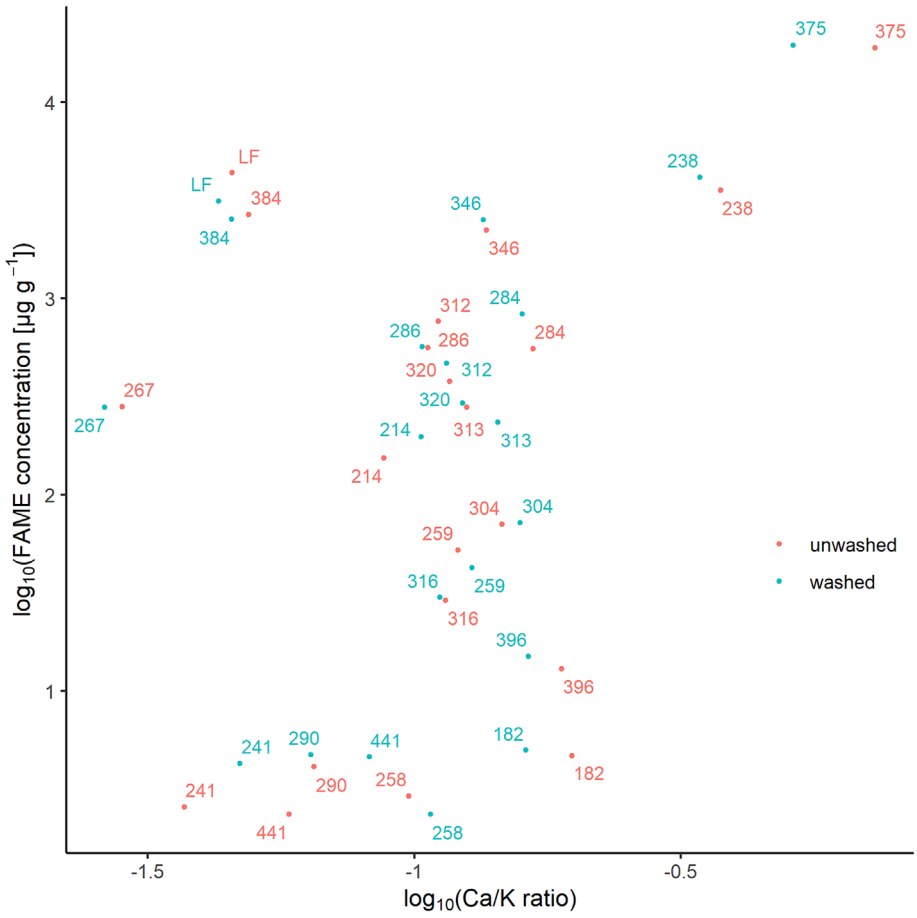

FIGURE 4 Log–log plot of total concentrations of fatty acid methyl esters (FAME) versus calcium-to-potassium (Ca/K) ratios measured in washed and unwashed samples; numbers correspond to sample IDs.

Water-and-brush cleaning

Comparison between washed and unwashed samples revealed no significant difference in bulk lipid content as a result of washing (Wilcoxon's $W = 216$, $p = 0.92$, $n = 21$) (Figure 5), causing us to reject Hypothesis 3. In fact, the mean of relative standard deviations within the sample group intended for the cleaning comparison (10%) was lower than in the additional sample subset that did not undergo any washing (25%) (see Table S3 in the supplemental data online). Most probably, water-and-brush cleaning does not influence bulk fats due to their absorption into deeper layers of the ceramic matrix where they are not easily accessed. Previously, in replica potteries used for experimental cooking, the highest concentrations had been observed in the section between 0.5 and 3 mm from the inside vessel wall (Hammann et al., 2020). These results may only be applicable to a chemically gentle and sterile cleaning procedure, while the still not uncommon acidic or soapy washings are likely to influence the molecular composition of organic residues in the sherd.

However, differences were observed when pottery objects were considered individually. Changes in FAME concentrations in washed pieces relative to unwashed ones ranged between –39% and +96% (mean change = –8.0% ± 31%) and between –37% and +820% (mean change = 96% ± 196%) in the additional sample subset (see Table S2 in the supplemental data online). An increase of FAME in washed relative to unwashed samples further contradicts Hypothesis 3. Fluctuations of FAME 18:0/16:0 ratios were also discovered, with percentage changes ranging from –21% to +27% (mean change = –2.1% ± 11%) in the washing comparison samples and from –40% to +140% (mean change = 19% ± 43%) in the additional sample subset. Thus, the spread ranges are actually widest in the latter. This indicates that the composition of lipids on pottery surfaces can vary despite a very small distance between sampling areas and independently of whether or not the sherd has been cleaned. No correlation was shown between changes in concentration and FAME 18:0/16:0 ratios, which shows that the fluctuations of these two variables are not related to each other (Spearman's $\rho = 0.18$, $n = 21$). Ratios of various FA found in potsherds have been used in the past to estimate the taxonomy of the lipid source (Copley et al., 2005; Eerkens, 2005), but in recent years this methodology has been

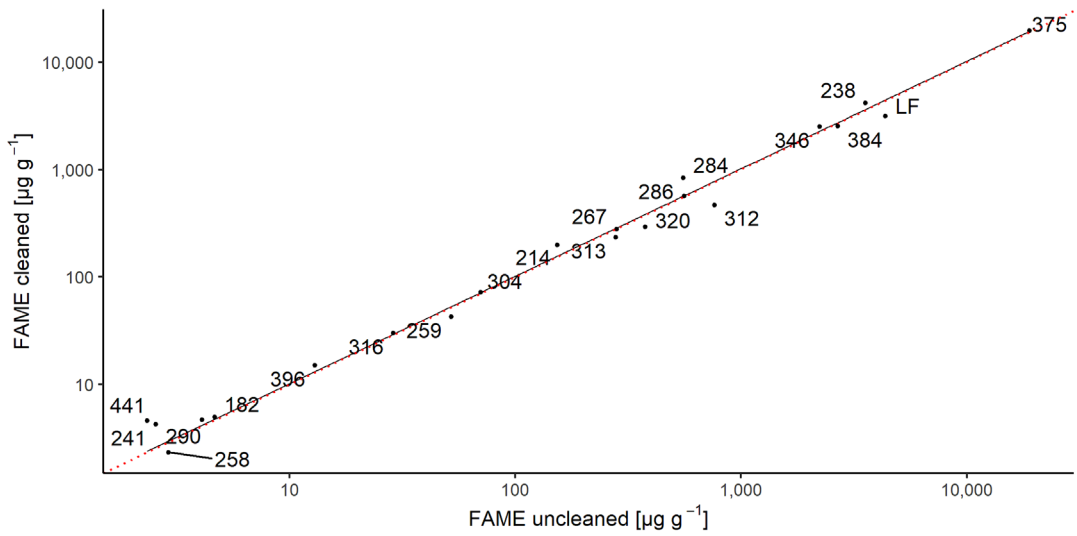

FIGURE 5 Log–log plot of fatty acid methyl ester (FAME) concentrations in washed versus unwashed pottery pieces, including the curve of the simple line fit (black line, fixed intercept, slope = 1.019); numbers correspond to the sample ID; red dotted line = theoretical scenario of no change for comparison (slope = 1).

advised against due to its diagnostic unreliability (Whelton et al., 2021). Nevertheless, it may still be a valuable marker of heterogeneity in lipid composition across pottery surfaces.

Under the assumption that cleaning is the only source of variation, stable FAME 18:0/16:0 ratios would be expected, since there are no significant differences in solubility and extractability among these lipids for the composition to be influenced by a single washing event using deionized water. In addition, the act of food preparation itself involves at least partial homogenization of the ingredients and in the case of boiling, generates an organic phase in which various lipids are easily intermiscible. The fact that ratios are distributed inhomogeneously in individual sherds could reflect different recipes being employed or different foodstuffs being stored throughout the lifetime of the vessel, possibly at differing fill heights from one use event to another. This, in turn, might influence the results of compound-specific stable isotope analysis obtained from various areas of a single sherd. Further experiments employing replicates (duplicates or triplicates) are needed to investigate whether variance of FAME ratios is correlated with variance of isotopic data. As of yet, multiple sampling of individual vessels on a regular basis is not a common practice in ORA and the question, therefore, difficult to answer. Furthermore, results from cooking experiments in replica pottery can be used to assess whether the assumption is at all valid that lipids would be well homogenized when employing a single recipe.

The percentage changes in FAME concentrations, as well, are better explained by physical and chemical heterogeneities typical of a ceramic matrix. For example, significant differences in lipid contents between rim, body, and base pieces of the same vessel have already been demonstrated (Charters et al., 1993). Such vertical gradients can be caused by phase separation between organic and aqueous layers, which transports fats toward the rim, or differing fill levels as mentioned above. Although we cut pottery pieces in such a way that samples were as horizontally adjacent as possible, uneven edges could have introduced different parts of these *vertical* gradients. High standard deviations of *horizontal* lipid distributions have also been reported previously, albeit using extracts collected from 120° intervals (Charters et al., 1997). Finally, the often observed heterogeneity in thickness, porosity and elemental composition of ceramic materials (Mikerov et al., 2020; Volzone & Zagorodny, 2014) can impact both the absolute mass of extracted lipids as well as mass concentrations, especially since the latter directly depend on the relation between the surface area and mass of a sample.

Minor constituents

Enrichment and GC-MS/MS analysis revealed the presence of HFA (present as trimethylsilylated methyl esters). Monohydroxy FAME (mHFAME) with chain lengths 16 and 18 were represented by series of regioisomers (see Figure S5 in the supplemental data online). In addition, *vic*-dihydroxy FAME (dHFAME) were present as *erythro* and *threo* isomers of FAME 18:0;9OH,10OH as well as one isomer of FAME 18:0;11OH,12OH.

Irrespective of FAME concentration change, normalized peak areas of mHFAME in all washed samples were much lower than in the corresponding unwashed ones (mean change = –77% ± 17%; $n = 20$) (see Table S6 in the supplemental data online). Over the entire data set, a reduction by 93% was observed (Figure 6). This implies that mHFA were easily removed by water-and-brush cleaning. Since these analytes were not abundant in soil samples but correlated strongly with FAME concentrations in pottery (see Figure S6 online), we assume that mHFA had formed as hydroxylation products of dietary unsaturated FA, probably at the interface between pottery wall and acidic soil (see Discussion 1 online). In contrast, dHFAME were less prone to removal by cleaning, which was visible on an individual level (mean change = 10% ± 97%; $n = 20$) (see Table S6 online) as well as—much more clearly—a data-wide level (reduction by 21%) (Figure 6). Also, dHFAME correlated strongly with FAME concentrations (see

Figure S6 online). Therefore, dHFA as well resulted from oxidation of dietary unsaturated FA, but appear to have been preserved deeper within the ceramic matrix than mHFA and could be extracted with fewer losses despite cleaning. A reason for this difference could be that cooking gives rise to dHFA, while mHFA are formed after burial. The primary diagnostic value of these species is the fact that they preserve the position of double bonds at the point of formation.

Similar to bulk fats, HFA were most abundant in rims of plates and pots with a preference toward coarse-textured pottery. Also, the ratio between dHFAME and mHFAME was highest in these vessels. This supports the notion that dHFA are generated under elevated temperatures and can act as stable biomarkers of cooking (Copley et al., 2005; Hansel & Evershed, 2009).

GC-MS analysis also afforded dimethyl esters of DCA in 19 samples. They were present as homologues with carbon chain lengths between seven and 28 and could be grouped into short-chain (C7–9), mid-chain (C10–18) and long-chain (C19–28) homologues according to the differences in their abundance, which might indicate distinctive origins or mechanisms of formation. Here as well, high variability of peak area percentage changes was revealed, ranging between –72% and +130% (mean change = 1.6% ± 48%; $n = 19$) (see Table S6 in the supplemental data online). Overall, DCA normalized peak areas were reduced by 9% (Figure 6) and correlated strongly with FAME concentrations (see Figure S6 online), suggesting that DCA were situated in the deeper ceramic layers together with bulk fats, which largely protected them from removal during cleaning. The highest observed correlations, meanwhile, were between short-chain DCA and HFAME. Presumably, oxidative conditions favour the generation of these substance classes on a similar scale. In contrast, long-chain DCA were so sparse that no significant correlations could be calculated. They were only detected in high amounts in samples of soil and therefore probably did not originate from foodstuffs (see Discussion 2 and Figure S3 in the supplemental data online).

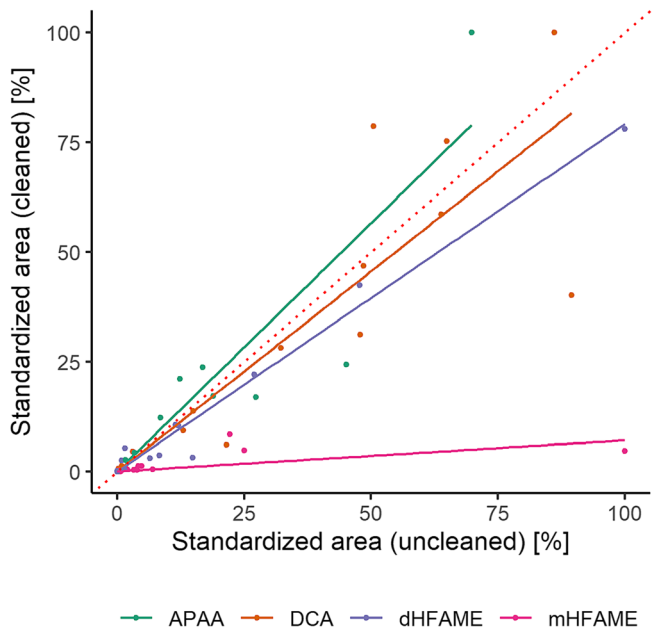

FIGURE 6 Scatter plot of standardized peak areas (100% ≙ highest normalized peak area in the respective category) of *ω*-(*o*-alkylphenyl)alkanoic acids (APAA), *α*,*ω*-dicarboxylic acids (DCA), *vic*-dihydroxy fatty acid methyl esters (dHFAME) and monohydroxy FAME (mHFAME) in washed versus unwashed pottery pieces, including the curves of the corresponding simple line fits (fixed intercepts, slopes = 1.131 (APAA), 0.901 (DCA), 0.791 (dHFAME), 0.071 (mHFAME)); red dotted line = theoretical scenario of no change for comparison (slope = 1).

At last, methyl esters of APAA (isomers C–H) were detected in eight samples. Interestingly, overall normalized peak areas increased by 13% (Figure 6) and area percentage changes ranged between –46% and +71% (mean change = 16% ± 40%; $n = 8$) (see Table S6 in the supplemental data online). This can only be explained by very sharp gradients of APAA distribution on the pottery surfaces. In fact, seven of the eight samples containing these compounds were rim pieces, and out of three corresponding soil samples in which APAA were also identified, two showed considerably higher peak areas. We assume that abrasion from the sherds heavily contaminated the soil (see Methods 6 online). Since abrasion affects surfaces quite evenly, areas with the highest abundance of APAA contributed to the results. In addition, our results revealed a strong negative correlation with FAME as well as weak negative correlations with other minor components (see Figure S6 and Discussion 3 online where this is addressed further).

CONCLUSIONS

The analysis of our sample set allowed us to investigate several aspects of sample selection for ORA and confirm some of our hypotheses.

While washing of pottery prior to analysis is oftentimes discouraged, we did not observe a general loss of bulk analytes under cleaning conditions applied in this study. This is especially relevant when stable isotope analyses are planned. However, the effect of cleaning on minor components is more diverse and appears to depend on their mechanisms of formation and diagenesis, which in many cases are poorly understood. While DCA, APAA and dHFA largely remained preserved, mHFA were almost entirely lost. Nonetheless, whenever cleaning allows a better assessment of selection criteria and contextual information about the pottery, the risk of losing some low-abundant biomarkers may be acceptable. In general, we advise archaeologists working with artefacts intended for use in ORA studies to ensure conditions which are as sterile as practically possible during handling to avoid introduction of contaminants. If pottery is washed in bulk under field conditions, distilled water and long rubber gloves may be used, followed by thinner gloves (not latex) during examination and documentation. Soil samples should be stored separately from the objects, as ceramic dust can reduce their informative value. Noteworthy, we did not observe adverse effects (such as abundant plasticizers) of the use of plastic bags for storage.

Confirming previous reports and reference experiments, pottery texture (as a function of porosity), position of the sherd, and shape had considerable influence on the presence of extractable lipids. Hence, this should always be considered when samples are selected for destructive analysis. Our results show that focusing on rims of coarse-textured cooking vessels is most likely to afford interpretable concentrations of hydrophobic analytes. In contrast, body sherds were found to be the least promising. However, not all selection criteria are equally impactful. While both kinds of texture examined (smooth and coarse) were likely to yield interpretable quantities of lipids and the selection of pottery shapes may be subject to the particular research question, the position of the sampling area on the original vessel is both the most deciding factor and the criterion over which researchers have the most control. It is also worth knowing that base pieces can be a viable alternative to rims, since it is not unusual for archaeological sites to be discovered accidentally during earthworks (construction or farming), which often results in the destruction of rims in particular.

On the contrary, consideration of Ca contents appears to be unnecessary. Given that XRF analysis can take non-negligible amounts of time and require additional instrumentation, obtaining elemental data for the sole purpose of prospecting samples for ORA is a waste of effort.

In summary, our experiments confirmed some previous observations and recommendations for selection of ceramic samples to maximize extractable lipid quantities while putting some much needed perspective on the relative importance of these criteria. Based on our experiments

and in contrast to recommendations by the Historic England guideline cited above, previously washed ceramic samples are not inferior to unwashed ones if an appropriate cleaning procedure was applied.

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DATA AVAILABILITY STATEMENT

All raw data as well as data analysis files are available on figshare (https://doi.org/10.6084/m9.figshare.22592602).

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