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Development and application of a comprehensive analytical workflow for the quantification of non-volatile low molecular weight lipids on archaeological stone tools

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Abstract

Source determination of use-related residues on prehistoric stone tools is especially challenging, due to issues related to preservation, contamination and the contribution of residues from multiple sources. To increase confidence in this process, an analytical workflow was developed to include: (1) a sampling strategy that retains spatial information of the recovered residues and enables monitoring of environmental contamination; and (2) a sensitive and selective gas chromatography-tandem mass spectrometry (GC-MS/MS) procedure to quantify non-volatile low molecular weight lipids on stone artefacts. This workflow was applied to 14 stone artefacts excavated from deposits at Liang Bua, a limestone cave on the Indonesian island of Flores. These artefacts range in age between ~14 000 and 1000 years old, and were preliminarily classified as either potentially showing traces of use ($n = 7$) or not ($n = 7$) using low magnification microscopy. Residues were sampled by direct solvent extraction off the surface of the artefacts. The aliquots were spiked with internal standards and derivatised. The trimethylsilyl derivatives of 40 saturated fatty acids, sterols, di- and tri-terpenoids and their analogues were quantified using optimised multiple reaction monitoring (MRM) transitions. Six of the potentially used artefacts contained sterols, phytosterols and terpenoids, either individually or in combination, whereas none of these compounds was commonly found on the seven artefacts preliminarily classified as unused. This suggests that these six artefacts may have been used as implements to process resources, and provides scope for further investigation. This workflow can also be adapted for the analysis of other archaeological objects.

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Development and application of a comprehensive analytical workflow for the quantification of non-volatile low molecular weight lipids on archaeological stone tools

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Abstract

Source determination of use-related residues on prehistoric stone tools is especially challenging, due to issues related to preservation, contamination and the contribution of residues from multiple sources. To increase confidence in this process, an analytical workflow was developed to include: (1) a sampling strategy that retains spatial information of the recovered residues and enables monitoring of environmental contamination; and (2) a sensitive and selective gas chromatography-tandem mass spectrometry (GC-MS/MS) procedure to quantify non-volatile low molecular weight lipids on stone artefacts. This workflow was applied to 14 stone artefacts excavated from deposits at Liang Bua, a limestone cave on the Indonesian island of Flores. These artefacts range in age between ~14,000 and 1,000 years old, and were preliminarily classified as either potentially showing traces of use (n = 7) or not (n = 7) using low magnification microscopy. Residues were sampled by direct solvent extraction off the surface of the artefacts. The aliquots were spiked with internal standards and derivatised. The trimethylsilyl derivatives of 40 saturated fatty acids, sterols, di- and tri-terpenoids and their analogues were quantified using optimised multiple reaction monitoring (MRM) transitions. Six of the potentially used artefacts contained sterols, phytosterols and terpenoids, either individually or in combination, whereas none of these compounds was commonly found on the seven artefacts preliminarily classified as unused. This suggests that these six artefacts may have been used as implements to process resources, and provides scope for further investigation. This workflow can also be adapted for the analysis of other archaeological objects.

Introduction

Organic biomarker analysis of lipids and lipid analogues is a potentially valuable tool for gaining insights into past human activities and human-environment interactions. Organic residues are often complicated mixtures containing nucleotides, proteins, carbohydrates and lipids.^{1, 2} Although nucleotides and proteins are more taxonomically specific compared to lipids and carbohydrates, they are prone to degradation processes such as denaturation and hydrolysis. Their polar nature increases their solubility in water, making them susceptible to being washed into or out of unconsolidated archaeological deposits. In contrast, lipids are relatively hydrophobic, more resistant to microbial attack and abiotic degradation processes,³ and occur in the fats, oils, resins and natural waxes related to terrestrial and marine animals and plants.⁴ This combination of relative persistence and potential for source identification superior to carbohydrates makes lipids and their analogues suitable markers for use in archaeological contexts.

Stone artefacts result from the use or modification of stone by hominins (i.e., modern humans and extinct human species) but stone tools are defined specifically as those that have been used or modified for some task. Although stone artefacts have been the focus of several residue studies,⁵⁻⁹ ceramics (e.g., pottery) have typically received more attention.^{3, 10-30} Residue analysis of ceramics is often successful, as the porous matrix of the material can trap and protect residues from microbial degradation.² It is more challenging to determine whether the presence of residues on stone artefacts is archaeologically significant or the result of post-depositional processes (i.e., contamination). Most types of stone selected by hominins for tool manufacture and/or use consist of hard, non-porous materials, which provide the necessary requirements for efficient tool function but significantly reduce the likelihood of residues being trapped and protected internally. Micro-cracks can form when the stone is used or modified, however, and these may preserve residues in favourable environmental conditions;⁷ residues may also adsorb onto stone tool surfaces when it is made or used. Determining whether residues detected on ancient stone artefacts reflect behavioural details related to tool manufacture and/or use, or are the result of subsequent contamination, is a major priority of research in archaeological science.

For archaeological chemistry investigations, gas chromatography is the separation technique of choice for most lipid studies (where low molecular weight lipids are the analytes of interest), with mass spectral acquisition carried out mainly in full scan mode,^{2-4, 10-12, 15, 17, 18, 20, 21, 23, 24, 26, 27, 31-40} but also selected ion monitoring mode (SIM)^{9, 10, 34, 36} and multiple reaction monitoring (MRM)⁴¹ mode. In these studies, validation data are typically not presented alongside the quantitative results associated with archaeological objects and the deposits from which they were recovered. Reliable data are a prerequisite for source determination and specifications such as linear range, limit of detection (LOD), limit of quantification (LOQ), inter-day and intra-day accuracy and precision of the analytical method provides a point of reference for the sensitivity, measurement of uncertainty and overall performance of the method. Validation data are critical to mitigate the unique set of challenges associated with the analysis of archaeological specimens. The suite of organic biomarkers found in use-related residues is highly variable depending on the quantity of source material and the preservation conditions; as such, analyte abundances can vary over many orders of magnitude, with certain compound classes more prominent due to the environmental conditions favouring their persistence (or vice versa). Archaeological sites are also rarely sterile and artefact surfaces are likely exposed to a wide range of pre- and post-depositional events. While lipid biomarkers are favoured

due to their persistence, some are ubiquitously found in nature, contributing to the analyte abundance profiles of use-residues and complicating the source determination process. Moreover, residues may undergo site-specific degradation processes over time,^{11, 13, 42, 43} thereby presenting a complex series of potential chemical pathways that should be considered when interpreting the results. Following chemical transformation, molecular analogues can be used as biomarkers for the parent compounds that were originally present, and are diagnostic of the types of reactions that have occurred at the site. However, working backwards to piece together the types of compounds originally deposited and more importantly, their relative abundances to attempt to identify origin of the use-residue, is not straightforward. It is not always a reliable approach to make linkages between chemical profiles of use-residues and reference materials that are often 'modern-day', and subjected to minimal degradation, particularly if significant diagenesis is known to occur at the archaeological site where the stone artefacts were retrieved. Due to these specific challenges for analysis of archaeological objects, developing a sensitive, specific and validated assay is necessary and the first step to place confidence in the characterisation of manufacture or use-related residues on stone tools.

In this study, an analytical workflow for the analysis of non-volatile low molecular weight lipids on stone objects was developed and applied to stone artefacts excavated from cave deposits at Liang Bua on the Indonesian island of Flores. This site was chosen for our study because the tropical climate and cave environment presents particularly challenging conditions for chemical preservation. Although Liang Bua preserves the skeletal and cultural remains of the primitive hominin species *Homo floresiensis*, dated to 100,000–60,000 and 190,000–50,000 years ago, respectively,^{44–47} the artefacts analysed here derive from younger sediments (~14,000–1,000 years old) and are the handiwork of our species, *Homo sapiens*.⁴⁸ First, a surface extraction procedure was established for the removal of residues from ancient stone artefacts. Then, a targeted gas chromatography-tandem mass spectrometry (GC-MS/MS) method was developed, optimised and validated for the analysis of non-volatile low molecular weight lipids recovered from the artefacts. This enabled a more selective approach for target compound monitoring, and increased sensitivity for the quantitative analysis. However, full scan acquisition was also incorporated into the methodology to aid retrospective analysis of compounds that were not included in the targeted method. These data were used to determine if any of the artefacts was potentially used as a tool to process resources.

Materials

Reference standards

Reference standards of saturated fatty acids (C_{10} – C_{31} , ≥ 98 –99%), saturated mono-hydroxy fatty acids (97–98%), saturated dicarboxylic acids (96–99%), di-/tri-terpenoids (75–99%), sterols and stanols (as well as their oxy-derivatives) were sourced from Sigma Aldrich (Castle Hill, NSW Australia), as was stearic acid- D_3 (99%). Cholesterol- D_7 ($> 99\%$) was purchased from Avanti Lipids (Alabama, USA). The purity of the sterol analogues was ≥ 95 –99%, except for campesterol (approximately 65%) and β -sitosterol ($\geq 85\%$). The specific reference standards are: decanoic acid, lauric acid, tridecanoic acid, myristic acid, pentadecanoic acid, palmitic acid, heptadecanoic acid, stearic acid, nonadecanoic acid, arachidic acid, heneicosanoic acid, behenic acid, tricosanoic acid, tetracosanoic acid, hexacosanoic acid, octacosanoic acid, hentriacontanoic acid, 12-

hydroxydodecanoic acid, 16-hydroxyhexadecanoic acid, azelaic acid, sebacic acid, thapsic acid, abiestic acid, lupeol, betulin, oleanolic acid, betulinic acid, ursolic acid, 5 α -cholestan-3 β -ol, 5-cholesten-3-one, 7-ketocholesterol, 7-dehydrocholesterol, 25-hydroxycholesterol, β -sitosterol, campesterol, cholesterol, coprostanol (5 β -cholestan-3 β -ol), ergosterol, stigmasterol and 5 α -cholestan-3-one.

Chemical reagents

Acetonitrile (LC-MS grade), chloroform and toluene (HPLC grade) solvents were obtained from VWR International (Tingalpa, QLD, Australia). HPLC grade methanol was sourced from Thermo Fisher Scientific (Scoresby, VIC, Australia). N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) + 1% trimethylchlorosilane (TMCS) silylation reagent was purchased from United Chemical Technologies (Bristol, Pennsylvania, USA).

Stone artefacts

The sample set consisted of 14 stone artefacts recovered during archaeological excavations at Liang Bua in 2015. All of these artefacts show evidence of manufacture by hard-hammer percussion (i.e., stone flaking). Each sampled artefact was targeted specifically for residue analysis. Thus, each was collected on a sediment pedestal, not washed or cleaned prior to analysis and, based on documentation of use-wear using low magnification microscopy, preliminarily classified as either potentially showing traces of use ($n = 7$) or not ($n = 7$) (Table 1). Microscopy was carried out using an Olympus SZ61 stereomicroscope with an external fibre optic light source. Low magnification microscopy was used as a screening method since high magnification microscopy requires artefacts to be cleaned, and the cleaning process would jeopardise the experimental goals of our study.

Experimental methods

Preparation of standards and method validation

Mixed stock standards (0.65–1 mg/mL) of the acid analogues, sterol compounds and di- and tri-terpenoids were prepared using volumetric flasks by dissolving the solids in chloroform/methanol (3:1 v/v) and made up to the mark. Stock standards were diluted to make calibrator standards (a minimum of five levels for each analyte, ranging between 0.1–10,000 ng/mL). Quality control (QC) standards were prepared independently from the calibrator standards. Five replicates were prepared for each QC level. One QC level (3000 ng/mL) was used for hentriacontanoic acid, 7-ketocholesterol, 5 α -cholestan-3-one and 5-cholesten-3-one. For the other analytes, two QC levels were utilised at approximately 50 ng/mL and 3000 ng/mL for the C₁₀–C₂₀ saturated fatty acids, coprostanol, campesterol, cholesterol, abiestic acid and ursolic acid, and approximately 500 ng/mL and 3000 ng/mL for the C₂₁–C₂₈ saturated fatty acids, mono-hydroxy acids, dicarboxylic acids and the rest of the sterol analogues and terpenoids. Please refer to Tables 2 and 3 for the exact concentrations of the QC standards prepared for each analyte. Individual 200 μ g/mL stearic acid-D₃ and cholesterol-D₇ internal standards (stock solutions) were prepared in the same manner as the target compound stock standards, and then further diluted to make 10 μ g/mL working solutions. To prepare a calibration set containing a reagent blank, calibrator and QC standards, 1 mL of the calibrator solutions were spiked with 100 ng/mL of each internal standard. Samples were mixed with a vortex mixer, dried down under a gentle stream of nitrogen and derivatised at 70°C with

MSTFA/1% TMCS (40 μ L) and acetonitrile (10 μ L) for 1 hr on a heating block. The samples were subsequently dried down a second time and the trimethylsilyl derivatives reconstituted in toluene (50 μ L) for GC-MS analysis.

Extraction of residues and preparation of extracts for GC-MS analysis

A simple 20 min solvent extraction with chloroform/methanol (3:1 v/v), assisted by ultrasonication, was used to remove residues from the stone artefact surfaces. The seven artefacts that showed no preliminary evidence of use were placed into the smallest diameter beaker possible and covered in the minimum volume of extraction solvent (16–50 mL) required for total submersion. A double/triple extraction was carried out for the other seven artefacts, all of which displayed preliminary evidence of use. The first stage involved extraction from the edge(s) of the artefact that had been preliminarily identified as potentially used. Tweezers were used to hold the specimen in the desired position, so that only the edge of interest was in contact with the extraction solvent (1–20 mL). The second stage consisted of total submersion of the artefact in the minimum volume of solvent required (7–100 mL), as was done with the artefacts that did not display any preliminary evidence of use.

Extracts were filtered through a 0.22 μ m hydrophobic syringe filter unit (MicroAnalytix: Taren Point, NSW, Australia). Aliquots (1 mL) of each extract were prepared for GC-MS analysis, as described for the samples in the calibration sets.

GC-MS analysis

An Agilent Technologies 7890 GC system coupled to a 7000 GC/MS triple quadrupole mass spectrometer was employed for instrumental analysis of the artefact extracts, calibrator standards, QC standards and reagent control samples. Chromatographic separation was carried out using an Rxi®-5Sil MS 1,4-bis(dimethylsiloxy)phenylene dimethyl polysiloxane fused silica column (20 m $\times$ 0.18 mm $\times$ 0.18 μ m; Restek: Bellefonte, Pennsylvania).

Samples were injected in pulsed split mode (split ratio = 10:1) with a 1 μ L injection volume. With the front inlet temperature maintained at 270°C, the inlet and injection pulse pressures were set at 30.177 psi and 35 psi, respectively. The septum purge flow and helium carrier gas flow were kept at 3 mL/min and 1.4 mL/min, respectively. A temperature gradient was utilised; the starting temperature of 80°C was held for 3 min, and then increased at a rate of 20°C/min until 315°C was reached. The temperature was then held at 315°C for 4 min for a total run-time of 18.75 min. The auxiliary heater and source temperature were maintained at 270°C and 280°C, respectively. Mass spectrometric acquisition was carried out after a 3-min solvent delay, with a standard electron ionisation (EI) energy of 70 eV and a gain factor of 1. Both full scan and MRM data were obtained for each sample. For the full scan analysis, the scanning mass range was set at m/z 50–900, with a scan time of 600 ms (1.8 cycles/s). For the MRM acquisition, three MRM transitions (one quantifying transition and two qualifying transitions) were selected for each analyte (Tables 4 and 5). Three time segments were employed, starting at 2.2 min, 14 min and 16.1 min (1.6 cycles/s scan rate for each time segment). Each sample was injected in triplicate, with a single injection in full scan mode and duplicate injections in MRM mode.

Data processing

Agilent MassHunter qualitative and quantitative software packages were used for processing the data obtained. For MRM analyses, positive identification of a compound was made if three criteria were satisfied: (1) the analyte retention time corresponded to the expected retention time of the compound compared to the reference standard; (2) three ion transitions expected for the compound were observed for the analyte (Tables 4 and 5); and (3) qualitative ions were present in the expected ratios relative to the quantitative ion monitored, as observed for the reference standards. Average analyte concentrations were reported for duplicate injections. The full scan data acquired for each sample served as a means to both monitor known compounds that were not incorporated into the targeted methods, and make retrospective analyses of potential compounds of interest that may have been present in the sample but were unknown at the time of analysis. In this acquisition mode, analytes were identified using the Wiley 7n database.

Results and discussion

Selection of analytes for targeted GC-MS/MS analysis

The compounds were chosen after taking into consideration their expected stability and diagnostic values. Although ubiquitous, saturated fatty acids are found in both animals and plants in varying abundances, with analyte ratios used to differentiate between animal and plant residues.^{13, 24} Long-chain fatty acids (C₂₀–C₃₀) are found in significantly higher amounts in plant waxes, such as the surfaces of leaves and skins of fruit, than in animal fat or plant oil.⁵ Also, the presence of odd-chain fatty acids can be used as an indicator of bacterial activity typical of ruminant origin. Phytosterols such as β -sitosterol, campesterol and stigmasterol as well as terpenoids can be used to identify plant material. Relatively non-volatile terpenoids, such as abietic acid, have been shown to survive for centuries or longer in ceramic pottery.¹³ Cholesterol was selected as a biomarker to support the identification of residues of animal origin. Ergosterol (fungi-specific sterol³⁸) and selected hydroxy acids, oxysterols and stanol derivatives (such as 5 α -cholestanol) were included as environmental markers that may potentially provide insights into the chemical environment surrounding the stone artefacts. Dicarboxylic acids, such as azelaic acid, were also targeted as a proxy for unsaturated fatty acids that have undergone oxidation.⁴

Analytical workflow rationale

Signal contributions from environmental contamination (i.e., the sedimentary background) were monitored using artefacts showing no preliminary evidence of use. These artefacts were extracted using a single extraction to obtain a representative abundance of analytes, while the potentially used artefacts were extracted using a double/triple extraction, to preserve the spatial resolution of any residues that may be present.

The artefacts were excavated on sediment pedestals to reduce handling during excavation as well as post-excavation contamination. Removal of the artefacts from their respective pedestals took place in a clean laboratory environment. For ceramics, one solution to minimise environmental contamination is to remove its outermost portion prior to further preparation for instrumental analysis.^{3, 10, 49-51} Unfortunately, this is not ideal for stone artefacts because the residues may be adsorbed or adhered onto their surfaces. Any modification that alters the topography of the artefact surface also prevents any post-extraction analysis of any potential microscopic wear patterns. Although wear analysis is often conducted first in the analytical sequence due to its non-destructive

nature, fine detail present on artefact edges is sometimes obscured by sediment. In these cases, a thorough wear analysis can be performed only after removal of the sediment. Furthermore, lipids are present inside rocks due to natural processes;¹ thus, removal of the outer surface is not an adequate measure for eliminating lipid contributions not related to use. The analysis of sediment around archaeological objects such as stone and bone has also been suggested as a monitor for environmental contamination,²⁰ but the extraction efficiency is expected to differ between matrices. To keep the specimen preparation procedure relatively simple and easily adaptable to stone artefacts of different sizes, shapes and materials, we utilised a solvent extraction with chloroform and methanol (3:1 v/v). Environmental contamination was monitored through the analysis of artefacts that showed no preliminary evidence of use.

Single extractions involving the total submersion the artefacts preliminarily classified as unused were carried out to obtain chemical profiles as baseline data for comparisons with those classified as potentially used. For the latter, a double or triple extraction was performed to preserve the spatial resolution of any residues that may be present. It was expected that the final extract (total submersion extract) would provide baseline data for the specific sedimentary environment in which the artefact was deposited, and these data can be subsequently compared to those of the potential residue(s) in the first/second extract(s), for a given artefact, assuming that potential residues are localised in the areas identified by wear patterns. Ideally, a constant solvent volume would be employed for all extractions, but this was not applicable because of the different sizes and surface area-to-volume ratios of the artefacts, and the need to use different extraction volumes to preserve the spatial resolution of potential residues. Instead, as our standard procedure, we used the minimum volume of extraction solvent required to extract potential residues from the surfaces of interest, under the assumption that there is a proportional relationship between solvent extraction volume and stone volume.

Quantitative analysis is not straightforward for surface analyses of heterogeneous objects using solvent extraction and GC-MS. For ease and consistency, the analytes were first quantified in the solvent extracts rather than on the artefact itself. The two possible fortification methods for internal standards were to directly spike them onto the stone artefact edge prior to extraction, or into the organic extract that was obtained after extraction. Since stone edges are highly variable between specimens, the latter method was used to avoid the possible bias associated with choosing a deposition location directly on the artefact. In addition to the natural variation of the stones, variability among potentially used artefacts may result from the creation of uneven edges and distribution of micro-fractures during use and/or manufacturing processes. Flakes are typically made from low porosity materials, such as chert or silicified tuff,^{52, 53} whereas grinding stones and milling tools are more often made of higher porosity materials, such as sandstone and andesite.¹ Differences in porosity of the raw materials would likely result in inconsistent adsorption and recovery of the internal standards. Solvent-only calibrator standards were used as it was not possible to prepare matrix-matched calibrator standards that would be universally applicable for ancient residues on stone. Residue matrices are complex and non-standardised, and may be intact, such as animal or plant fibres, or incorporated with sediment. Furthermore, the masses and location of deposited residues are highly variable: they can be visible or invisible, and deposited in micro-cracks or smeared across the edge of an artefact. Quantification of the analytes on the stone artefacts was carried out in two steps. For each artefact, (1) the concentrations of the analytes (expressed as ng/mL) in the solvent extract were calculated, and if the values fell within the linear

range for the given analytes (obtained from the validation data, Tables 2 and 3), then (2) the quantities (masses) of the analytes on the sampled area of the artefact were determined by multiplying its concentration by the extraction volume (Table 1). Since the assessment of analyte recovery and matrix effects was not the focus of our study, the quantitative results should be considered as approximate rather than true quantities of the analytes. For residue identification purposes, the relative quantities of each of the analytes within a sample is the most useful. However, a quantitative approach was taken rather than solely comparing peak area abundances, to confirm that the analyte signals fell within their respective quantifiable ranges. This ensured that the resulting lipid profiles reflect what is in each of the samples, and was not skewed because of an analytical issue (e.g. saturation of the MS detector; a matter to consider when the quantity of the original source material is always unknown).

GC-MS/MS method development

The analytes of interest in our study are not amenable to GC-MS in their native form due to the presence of hydroxyl (-OH) groups. To increase volatility, -OH groups were converted to trimethylsilyl (TMS) groups via reaction with the MSTFA/TMCS silylation reagent. In the presence of heat, MSTFA introduces TMS groups onto the lipid molecules. TMCS acts as a catalyst by increasing the donor strength of MSTFA, but a small volume of acetonitrile was also added to further facilitate the reaction. Besides converting the lipids to a more volatile form, the derivatisation step allows more characteristic MS ions to be produced. This was useful during the selection and optimisation of the MRM quantitative and qualitative ions for each of the analytes.

For the GC oven temperature gradient used to analyse the derivatised analytes, elution occurred in three general groups, corresponding to three time segments to maximise signal intensity: the 'acids' (saturated fatty acids, dicarboxylic acids and hydroxy acids), the sterol analogues, and the di- and tri-terpenoids. It was difficult to achieve baseline separation on a non-polar polysiloxane stationary phase for some of the sterols and terpenoids, where the compounds are very similar in structure or isomers of each other. To encourage peak separation, we conducted experiments using a lower temperature ramp rate. This was successful to a small extent, but baseline resolution was not attained and the peak shape suffered in some cases. Difficulties with baseline resolution has been demonstrated previously for such compounds.⁵⁴ The latter study used a silica column similar to the one used in our study and a 90.4 min run-time (3–4°C/min ramp rate), obtaining trimethylsilyl (TMS) derivatives of oleanolic acid, betulinic acid and ursolic acid elutions at 74.02, 74.35 and 74.77 min, respectively (0.75 min range). Some degree of baseline resolution was achieved, but not a significant improvement considering the run-time is almost 5 times longer than that used in our study; in an 18.75 min run, TMS derivatives of oleanolic acid, betulinic acid and ursolic acid eluted at 16.58, 16.70 and 16.87 min, respectively (0.29 min range). As speed is an important consideration, careful selection of the MRM transitions for these analytes was relied upon to ensure correct identification.

Optimisation of the MS parameters mainly involved the selection of appropriate MRM transitions for each analyte, taking into account the abundance and specificity of the ions. Reference standards were analysed in full scan mode to obtain the characteristic spectrum for each analyte. In most cases, the highest abundance m/z ions were chosen as the precursor ions for collision induced dissociation (CID) experiments. Generally, higher mass precursor ions were selected so that they were more characteristic. The precursor to product ion reaction that resulted in the highest

abundance product ion was assigned as the quantitative transition, with the second and third highest abundance product ions selected as the two qualitative transitions. This helped improve the sensitivity of the method. This approach, however, was not possible for several of the co-eluting analytes with similar MS fragmentation behaviour; in these cases, MRM transitions were preferably chosen if they were more specific for an analyte, or if the transition peak was as baseline-resolved as possible from co-eluting analytes. This was particularly important for the selection of the quantitative transitions.

Method validation

A minimum of five evenly spaced calibration levels was utilised to obtain data that were fitted with linear regression curves with correlation coefficients of ≥ 0.99 . As the calibrator concentration ranges covered multiple orders of magnitude (to reflect the large range of analyte abundances associated with use-residues), a $1/x$ weighted least-squares model was applied to compensate for the heterogeneity of variances expected for a relatively large calibration range.⁵⁵ The LOD and LOQ was determined using signal-to-noise ratio criteria of ≥ 3 and ≥ 10 , respectively. The latter also corresponded to the concentration of the lowest calibrator standard (lower limit of the linear range). The ULOQ was taken to be the concentration of the highest calibrator standard (upper limit of the linear range). These validation results, alongside the inter-day and intra-day accuracy (expressed as percent mean relative error, % MRE) and precision (expressed as percent relative standard deviation, % RSD) data (within $\pm 20\%$ for all analytes), is presented in Tables 2 and 3.

Analysis of stone artefacts

Table 1 lists the excavation locations (including depths from the present cave surface), approximate ages, preliminary microscopic assessments and solvent volumes used to extract volatile and non-volatile compounds from the 14 artefacts studied.

Baseline chemical profiles for the stone artefacts

Although the sample size is small, the baseline chemical profiles for the stone artefacts were assessed using the artefacts preliminarily classified as showing no evidence of use-wear ($n = 7$). The signals corresponding to these 'blank' stones include decanoic acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0) in various relative abundances. Except for one sampled artefact (XXV-4253), the dominant analytes are C16:0 and C18:0, and their ratio of C16:0 to C18:0 ranges from 1.7 to 4.1 ($n = 6$, $3.0 \pm 29\%$ RSD). Pentadecanoic acid (C15:0), arachidic acid (C20:0) and cholesterol were also found on four of these artefacts (Figure 1). Odd-carbon numbered tridecanoic acid (C13:0), C15:0 and heptadecanoic acid (C17:0) were also found at detectable levels. Fatty acids have been found in sediments, from the earliest rocks on Earth to recent deposits, with even-carbon numbered fatty acids generally present at higher abundances than odd-carbon numbered fatty acids.⁵³ The presence and distribution of fatty acids on these artefacts is, therefore, not surprising given the depositional context. No other acid analogues, terpenoids or sterols were found, except for cholesterol on artefact XXIV-79. At this stage, we attribute the latter to the leaching of cholesterol from neighbouring faunal remains or as a residue related to human use of this artefact; we consider the former more likely, based on preliminary low magnification use-wear analysis.

For the seven artefacts preliminarily classified as unused, no obvious patterns are apparent between the chemical profiles observed, the stratigraphic locations of the artefacts and the extraction solvent volumes. However, differences in chemical profiles are observed for artefacts excavated from the same stratigraphic location. For artefacts XXIV-79 and XXIV-42, which were both found in Sector XXIV between 55 and 65 cm depth from the present cave surface, in the compounds extracted with the same volume of solvent (16 mL), cholesterol was found on XXIV-79 and arachidic acid on XXIV-42. Different chemical profiles were also observed for artefacts found in different stratigraphic contexts, as expected. XXV-4130 and XXV-4253 were excavated from the same 2 x 2 m square but different depths, and the compounds extracted using the same volume of solvent (Table 1). The distribution of analytes is similar (Figure 1), whereas the C16:0 to C18:0 ratios are 4.08 and 0.78, respectively. Conversely, artefacts XXV-3951 and XXVI-4413 were excavated from different squares and depths and different volumes of solvent were used (Table 1), but their chemical profiles are similar (Figure 1) with C16:0 to C18:0 ratios of 3.12 and 3.32, respectively.

For purposes of comparison with the artefacts preliminarily classified as showing evidence of wear, the 'background' chemical profile consists of C₁₀–C₂₀ saturated fatty acids, with even-carbon numbered fatty acids found at significantly higher abundances than the odd-carbon numbered fatty acids. Owing to the limited sample size, no definitive trends can be identified in the analyte distributions, but the presence of these saturated fatty acids on the artefacts, together with the absence of other analytes, was used to distinguish likely unused artefacts from those that have residues of potential archaeological significance.

Stone artefacts with preliminary evidence of wear

Two questions were addressed using the data obtained for the seven potentially used artefacts: (1) could the total submersion extract be used as a direct background for the extracts from the used edge(s) of each artefact? and (2) are residues of probable archaeological significance preserved on these stone artefacts?

Following localised extractions from these seven artefacts (Figure 2a), the relative concentrations of the C₁₀–C₂₀ saturated fatty acids (excluding palmitic and stearic acids) in the extracts were compared (Figure 2b–h). Palmitic acid was present as the highest abundance analyte in all edge-of-interest and total submersion extracts, as expected, except in three cases. Two of these are total submersion extracts obtained after the extractions from two potentially used edges on each artefact (XXV-4025 and XXV-3954). The third exception is the extract from the first edge of interest on artefact XXV-3954, which also contained other analytes (Figure 2h); the differences in this extract may thus be due to a different depositional environment or the presence of other residues.

The total submersion extracts from artefacts XXV-4025 (Figure 2g) and XXV-3954 (Figure 2h) contained only lauric acid and lauric/stearic acids, respectively. The lack of other saturated fatty acid analytes differs from the baseline profiles for the artefacts preliminarily classified as unused, so we do not consider them reliable baseline indicators. The same note of caution applies to the other total submersion extracts: although more analytes are present, the relative abundances of decanoic acid and arachidic acid are reversed compared to those determined for the artefacts classified as unused. Also, use of the total submersion extracts as background for the edge of interest may be inaccurate if the artefact was used as a tool, because use-residues may not be confined to the area used for processing (e.g., cutting or grinding). This was the case for artefact XXIV-67, where

taxonomically more specific analytes were found in both the edge-of-interest and total submersion extracts. The latter is useful to collect for each artefact, because it provides spatial information, but it cannot be reliably used as a representative blank for the local depositional environment of an artefact.

To identify residues not associated with the sedimentary background, we extended our search for markers to sterols, terpenoids, longer-chain saturated fatty acids and acid analogues. Sterols and terpenoids are more taxonomically specific and can aid in the differentiation of plant and animal residues. For artefact XXIV-67, a large range of non-volatile compounds was found in the extract from the edge showing evidence of wear (Figure 3a). Abietic acid was also found in the total submersion extract, which suggests that the residue is not confined to only one edge. Cholesterol, oleanolic acid and ursolic acid (Figure 3b–d) were observed at quantifiable concentrations, and betulinic acid (Figure 3d) at detectable levels. The presence of cholesterol indicates a probable residue of animal origin. Some plant lipids, however, contain a small amount of cholesterol,¹⁰ so a mixed residue containing compounds from both animal and plant sources cannot be ruled out. Of the seven potentially used artefacts studied here, only XXIV-67 contained a suite of terpenoid residues. Comparatively low extraction volumes were used for this artefact (Table 1), which is small and has a correspondingly smaller surface area than the other artefacts. The low extraction volumes, therefore, are proportional to the size of each artefact. These terpenoids indicate the presence of residue with a plant origin: abietic acid is found widely in plants and associated with pine resin, with the other compounds distributed widely in many different parts of plants (leaves and bark) and in herbs, waxes and peels of fruit.^{2, 13, 28}

The presence of azelaic acid, sebacic acid and thapsic acid on XXIV-67 provides further evidence of plant oils representing the unsaturated fatty acids that were deposited on this artefact prior to oxidation into these dicarboxylic acids. In particular, azelaic acid has been used in archaeological chemistry as a proxy for fatty acids with unsaturation at the C₉ position, such as oleic acid, which is found in high abundances in plant-derived residues.⁵⁶ In addition to the dicarboxylic acids, the mono-hydroxylated fatty acids found on XXIV-67 indicate that oxidative processes occur at the site. These processes, and warm and humid environments in general, are widely viewed as detrimental to residue preservation.^{11, 13, 42, 48} As we detected both parent compounds and their mono-hydroxylated analogues, it suggests that the preservation of organic biomarkers is still possible under these adverse conditions. Saturated fatty acids C20:0, C21:0, C23:0 and C24:0 were also found on artefact XXIV-67. The latter three were not found on the artefacts preliminarily classified as unused and may, therefore, indicate an archaeologically significant residue. Odd-carbon numbered saturated fatty acids have been associated with ruminant lipids,¹⁰ but their abundances in XXIV-67 compared to the even-carbon numbered fatty acids is not significantly higher, contrary to the outcome expected for ruminant contributions. Based on non-volatile low molecular weight lipid biomarker evidence, artefact XXIV-67 was likely used for the processing of plant material and possibly animal remains.

Using the same logical approach, plant and animal residues were also found on artefact XXV-3954. Taxonomically specific residues were found mostly in the extracts corresponding to the edges preliminarily identified with wear. The analytes include cholesterol, abietic acid, β -sitosterol and a suite of saturated fatty acids (C13:0, C19:0, C20:0 and C22:0), as well as azelaic acid.

A summary of findings for the other five potentially used artefacts is given in Table 6. Of the seven artefacts exhibiting preliminary evidence of wear, six contained sterols, phytosterols and terpenoids, either individually or in combination. None of these compounds was commonly found on the seven unused artefacts. This suggests that these six artefacts may have been used as implements to process resources, and provides scope for further investigation. In addition to the targeted analyses, the full scan data were also examined for these artefacts. Unsaturated fatty acids were not incorporated into the targeted methods due to its susceptibility to degradation and unexpected survival. However, the trimethylsilyl derivative of oleic acid (library score = 94.38) on the edge of interest for artefact XXVI-4411 was found in the full scan data.

Conclusions

We have developed, validated and applied a robust extraction method and GC-MS/MS method for the analysis of non-volatile low molecular weight lipids to 14 stone artefacts excavated from an archaeological cave site in eastern Indonesia. Residues that were potentially deposited as a result of human processing of plant and animal resources were found on some of these artefacts. As supporting data, these results should be corroborated by complementary use-wear analysis of the artefacts, which is currently underway as part of a separate study. Preliminary results of the high magnification use-wear analysis indicate that, in general, there is agreement between the use-wear and GC-MS data, but differences in use scores were observed for selected artefacts.

To gain further insights into resource processing practices from residues deposited on artefacts, additional classes of compounds that are useful for use-residue source determination (such as terpenes and alkanes) should be incorporated into the workflow, to maximise the discriminating power of the chemical profiles obtained for each sample. Also, a larger sample of stone artefacts should be examined. Other aspects of method validation such as analyte recoveries and matrix effects were not assessed because the focus of our study was to identify the potential commodities (i.e., animal, plant and aquatic source materials) that may be present on the stone artefacts. It is very challenging to evaluate these parameters given the age of the samples and where they were recovered from. More meaningful assessments can be derived from experiments where modern-day analogues of potential source materials are applied onto experimental stone flakes, buried in suitable sediments and then extracted, rather than using solvent-only standards. However, the great number of permutations of stone tool type and raw material, and sediment type (even considering what is found at just one archaeological site) means that a very large number of experiments will have to be performed. Even with these experiments, modern-day analogues are not entirely representative of the degraded ancient residues encountered on archaeological implements, complicating evaluation of analyte recovery and matrix effects in archaeological contexts. Use-residue matrices are complicated mixtures, and highly variable from one stone tool to another depending on recovery location and age. Perhaps the use of standard addition for quantification purposes, to minimise matrix effects, is an alternative that should be explored in future studies.

Based on the findings of our study, the degree of preservation of organic residues is better than expected for stone artefacts recovered from a tropical setting, but the mechanisms governing their preservation and destruction remain poorly understood. Further studies are needed to expand our knowledge in these areas and make reliable inferences about human activities in the distant past.

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Tables

Table 1: Recovery locations and preliminary microscopic assessments of the stone artefacts, together with solvent volumes used to extract the edge(s) with traces of use (Extract 1) and the rest of the artefact (Extract 2).

Artefact	Sector	Depth (cm)	Age range (ka) ^a	Evidence of wear ^b	Extract 1 ^c (mL)	Extract 2 ^d (mL)
XXIV-67	XXIV	79	11–5	1	1	7
XXV-3931	XXV	513	14–13	3	8	80
XXV-3954	XXV	523	14–13	3	4 ^e	40
XXV-3956	XXV	521	14–13	2	8	40
XXV-4025	XXV	526	14–13	2	20 ^e	50
XXVI-4411	XXVI	541	14–13	3	20	100
XXVI-4414	XXVI	539	14–13	3	8	40
XXIV-42	XXIV	62	5–1	0	n/a	16
XXIV-79	XXIV	63	5–1			16
XXV-3951	XXV	516	14–13			50
XXV-3957	XXV	522	14–13			30
XXV-4130	XXV	539	14–13			20
XXV-4253	XXV	562	14–13			20
XXVI-4413	XXVI	538	14–13			30

^aAge ranges based on radiocarbon dating of charcoal samples from sediments stratigraphically above and below the stone artefacts used in this study (ka, thousand years).

^bGiven a score after low magnification analysis only; 0 = no use, 1 = possible use, 2 = probable use and 3 = definite use but unsure of worked material.

^cMinimum volume used to extract the artefact edge containing wear marks.

^dMinimum volume used to extract the whole artefact (total submersion).

^eTwo edges of interest were identified, each extracted with the same volume of solvent.

Table 2: GC-MS/MS MRM method validation results for the targeted saturated fatty acids, dicarboxylic acids and mono-hydroxy fatty acids.

Analyte	Linearity range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	QC concentration (ng/mL)	Intra-day accuracy (% MRE)	Intra-day precision (% RSD)	Inter-day accuracy (% MRE)	Inter-day precision (% RSD)
decanoic acid	10-10000	<0.1	10	50	3.6	17.8	3.3	19.6
				3000	3.8	10.4	4.4	16.5
lauric acid	5-10000	<0.1	5	50	17.0	13.8	6.8	14.4
				3000	11.9	7.9	3.6	13.1
tridecanoic acid	10-10000	1	10	50	-16.2	18.6	-4.8	15.7
				3000	-2.8	13.3	-3.3	13.2
myristic acid	0.1-5000	<0.1	0.1	50	17.0	17.9	9.5	17.8
				3000	-5.9	5.5	-0.6	10.9
pentadecanoic acid	0.4950-4950	<0.099	0.4950	49.5	14.9	15.3	12.1	16.0
				2970	-6.4	14.0	-5.5	18.4
palmitic acid	10-10000	<0.1	10	50	10.9	14.5	9.8	12.1
				3000	-4.5	5.1	4.7	13.6
heptadecanoic acid	0.1-5000	<0.1	0.1	50	-9.1	16.9	10.7	12.6
				3000	-12.7	5.2	-10.1	5.5
stearic acid	10-10000	<0.1	10	50	19.4	19.1	16.6	12.5
				3000	3.9	5.1	4.0	7.0
nonadecanoic acid	0.1-5000	<0.1	0.1	50	-7.7	14.5	-3.2	12.3
				3000	-2.5	2.3	5.7	10.6
arachidic acid	0.1-5000	<0.1	0.1	50	-10.1	18.3	2.5	16.9
				3000	14.7	7.4	-9.5	3.25
heneicosanoic acid	100-5000	<0.1	100	500	11.6	17.4	-3.2	19.0
				3000	14.1	11.8	14.2	7.76
behenic acid	100-5000	<0.1	100	500	2.3	11.3	8.8	19.3
				3000	3.1	9.1	-1.2	11.2
tricosanoic acid	100-5000	<0.1	100	500	-2.7	8.0	-7.7	19.4
				3000	11.7	9.3	8.8	12.8
tetracosanoic acid	100-5000	<0.1	100	500	-13.3	16.3	0.8	19.2

				3000	12.8	12.8	8.7	14.9
hexacosanoic acid	100-5000	<0.1	100	500	19.0	15.8	9.7	18.2
				3000	13.0	14.2	7.7	10.6
octacosanoic acid	100-5000	<0.1	100	500	-11.3	12.4	8.5	17.5
				3000	5.1	15.3	4.6	13.1
hentriacontanoic acid	1000-5000	100-1000	1000	3000	4.0	15.0	8.7	17.4
azelaic acid	100-5000	10-100	100	500	9.6	10.2	-8.2	19.4
				3000	7.2	9.4	6.1	11.1
sebacic acid	99-4950	9.9-99	99	495	-12.5	16.6	-17.5	16.4
				2970	9.1	9.2	-5.6	15.4
thapsic acid	96-4800	9.6-96	96	480	-7.6	17.4	-12.6	17.6
				2880	-10.1	9.9	-4.6	15.1
12-hydroxydodecanoic acid	97-4850	9.7	97	485	-10.4	11.1	-6.4	13.6
				2910	13.4	6.8	10.3	13.0
16-hydroxyhexadecanoic acid	98-4900	9.8	98	490	-7.4	9.5	2.7	16.8
				2940	7.9	5.3	9.0	16.7

Table 3: GC-MS/MS MRM method validation results for the targeted sterol analogues and di- and tri-terpenoids.

Analyte	Linearity range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	QC concentration (ng/mL)	Intra-day accuracy (% MRE)	Intra-day precision (% RSD)	Inter-day accuracy (% MRE)	Inter-day precision (% RSD)
cholesterol	0.1-10000	<0.1	0.1	50	11.1	15.0	0.76	15.4
				3000	8.6	10.3	-6.8	11.8
5 α -cholestanol	100-10000	10-100	100	500	-10.6	11.7	1.5	15.3
				3000	8.4	7.9	10.4	10.4
coprostanol	7.2-7200	3.6	7.2	36	-6.1	12.3	1.3	15.7
				2160	-11.5	13.5	-2.3	12.8
ergosterol	100-10000	10-100	100	500	11.4	10.7	19.4	11.1
				3000	-13.9	12.1	-1.4	13.8
β -sitosterol	100-10000	1	100	500	12.2	13.9	18.5	10.7
				3000	4.2	7.3	10.3	7.7
campesterol	6.5-6500	3.25	6.5	32.5	-12.4	13.5	-15.3	8.5
				1950	-9.2	8.2	-18.7	10.4
stigmasterol	95-9500	9.5-95	95	475	-15.0	10.5	-15.3	17.9
				2850	-10.8	8.6	-16.3	12.1
7-ketocholesterol	1000-10000	100-1000	1000	3000	-4.6	8.9	0.9	11.6
7-dehydrocholesterol	100-5000	10	100	500	6.2	12.9	-4.4	15.4
				3000	-19.2	13.1	-19.1	7.8
25-hydroxycholesterol	100-10000	10-100	100	500	3.6	15.6	-5.6	19.9
				3000	-3.2	13.7	-1.8	11.5
5-cholesten-3-one	1000-10000	100	1000	3000	-12.1	12.9	-4.1	14.0
5 α -cholestanone	1000-10000	100-1000	1000	3000	3.9	12.8	1.9	12.2
abietic acid	0.75-7500	0.075	0.75	37.5	-7.4	14.7	-10.6	19.1
				2250	12.5	10.5	4.5	15.5
lupeol	80-8000	8	80	400	-2.5	11.4	6.0	17.6
				2400	-10.7	8.7	5.0	17.4
betulin	100-5000	10-100	100	500	14.5	15.8	11.0	11.2
				3000	7.7	11.5	-2.3	19.0

oleanolic acid	100-5000	10	100	500	7.3	10.8	19.0	19.3
				3000	9.3	13.5	-5.5	16.9
betulinic acid	100-5000	10-100	100	500	10.3	13.9	7.4	17.0
				3000	3.6	14.1	4.7	16.0
ursolic acid	0.5-5000	<0.1	0.5	50	8.2	9.4	-1.9	16.3
				3000	-14.9	8.1	-12.4	16.0

Table 4: MRM ion transitions and optimised collision energies for the targeted saturated fatty acids, dicarboxylic acids and mono-hydroxy fatty acids.

Analyte	Transition 1 (quant.)	Transition 1 collision energy (eV)	Transition 2 (qual. 1)	Transition 2 collision energy (eV)	Transition 3 (qual. 2)	Transition 3 collision energy (eV)
decanoic acid	229->75	15	229->131	10	229->81	10
lauric acid	257->75	15	257->131	10	257->95	10
tridecanoic acid	271->75	15	271->131	10	271->95	10
myristic acid	285->75	20	285->131	10	285->95	10
pentadecanoic acid	299->75	15	299->131	10	299->95	15
palmitic acid	313->75	25	313->131	10	313->95	10
heptadecanoic acid	327->75	15	327->131	10	327->95	10
stearic acid	341->75	15	341->131	10	341->95	10
nonadecanoic acid	355->75	25	355->131	10	355->95	15
arachidic acid	369->75	20	369->131	15	369->95	10
heneicosanoic acid	383->75	25	383->131	15	383->95	15
behenic acid	397->75	25	397->131	15	397->95	20
tricosanoic acid	411->75	25	411->131	20	411->81	20
tetracosanoic acid	425->75	25	425->131	15	425->95	15
hexacosanoic acid	453->75	25	453->131	15	453->95	20
octacosanoic acid	481->75	25	481->131	15	481->95	15
hentriacontanoic acid	523->75	25	523->131	15	479->143	15
azelaic acid	201->83	5	317->149	10	317->123	10
sebacic acid	331->149	10	215->55	10	331->95	10
thapsic acid	217->55	10	415->149	15	415->95	15
12-hydroxydodecanoic acid	345->55	25	345->255	10	345->329	10
16-hydroxyhexadecanoic acid	311->75	15	401-> 385	10	401-> 311	10
stearic acid-D ₃ ^a	344->75	30	375->213	15	344->95	15

^aInternal standard used for the 22 acidic analytes targeted in the study.

Table 5: MRM ion transitions and optimised collision energies for the targeted sterol analogues and di- and tri-terpenoids.

Analyte	Transition 1 (quant.)	Transition 1 collision energy (eV)	Transition 2 (qual. 1)	Transition 2 collision energy (eV)	Transition 3 (qual. 2)	Transition 3 collision energy (eV)
cholesterol	329->95	25	368->353	15	368->339	15
5 α -cholestanol	403->73	45	460->215	15	355->91	45
coprostanol	370->215	15	370->355	15	403->73	45
ergosterol	363->69	15	363->143	15	253->197	15
β -sitosterol	357->95	15	396->381	15	396->367	15
campesterol	343->95	25	343->121	25	382->145	45
stigmasterol	255->105	25	394->83	15	394->211	15
7-ketocholesterol	233->73	20	454->439	10	454->233	20
7-dehydrocholesterol	351->143	25	325->119	15	456->351	15
25-hydroxycholesterol	327->95	25	456->131	15	327->105	35
5-cholesten-3-one	456->209	15	456->196	15	456->441	15
5 α -cholestanone	429->167	20	458->429	20	401->195	20
abietic acid	256->241	10	256->213	10	374->256	20
lupeol	189->119	15	369->203	10	498->189	20
betulin	496->216	10	496->173	20	189->95	10
oleanolic acid	320->203	5	320->133	10	189->95	10
betulinic acid	189->119	15	203->133	10	320->133	10
ursolic acid	203->133	10	320->133	10	189->119	15
cholesterol-D ₇ ^a	336->95	20	375->213	15	375->255	15

^aInternal standard used for the 18 native sterol analogues and di- and tri-terpenoids targeted in the study.

Table 6: Summary of findings based on non-volatile low molecular weight lipid analysis of the stone artefacts preliminarily classified as possessing characteristics of wear.

Artefact	Potential source(s) of residue	Interpretation/reasoning
XXIV-67	Plant/animal	See main text for discussion.
XXV-3931	More information required	No sterols or terpenoids were found; since these compounds were found on XXVI-4414 and XXV-3956 (same Sector and adjacent spit; similar environment) using the same extraction volume, they would have been detected if present on the artefact. Less taxonomically specific C13:0, C15:0 and C17:0 saturated fatty acids were found on the edge of interest, but not elsewhere on the artefact. The presence of archaeologically significant residues cannot be excluded at this stage.
XXV-3954	Plant/animal	See main text for discussion.
XXV-3956	Plant	β -sitosterol (indicative of plant matter), C17:0 and C22:0 saturated fatty acids were detected in the extract from the edge of interest, but in not the total submersion extract. There may be potentially significant plant residues present.
XXV-4025	Plant/animal	Cholesterol was found at a relatively high abundance compared to other artefacts, with 1.4 μ g extracted from this artefact. β -sitosterol, C13:0 and C17:0 saturated fatty acids were also detected. This suggests that a mixed plant/animal residue is present.
XXVI-4411	Plant	Campesterol was detected in the extract from the potentially used edge. Oleic acid-TMS (library score = 94.38) was also found in this extract using the full scan data. These results indicate that this artefact may have been used to process plant-based resources.
XXVI-4414	Plant/animal	Cholesterol, β -sitosterol and stigmasterol were detected, in addition to C17:0, C19:0, C:20 and C22:0 saturated fatty acids. The presence of a mixed plant/animal residue is proposed.

Figures

Figure 1: Relative concentrations of analytes (normalised to 100% for the most abundant analyte) detected in the extracts from the artefacts preliminarily identified as having no traces of use.

Note: Palmitic acid and stearic acid are not included in this figure, so that the differences between analytes found at significantly lower abundances can be seen.

Figure 2: (a) Image of artefact XXV-3956 showing the sampled artefact edge; the dashed line shows the solvent level for the first extraction. Relative concentrations of saturated fatty acids (normalised to 100% for the most abundant analyte) in the extracts from artefacts (b) XXV-3956, (c) XXV-3931, (d) XXIV-67, (e) XXVI-4414, (f) XXVI-4411, (g) XXV-4025 and (h) XXV-3954. *Note: Palmitic acid and stearic acid are not included in this figure, so that the differences between analytes found at significantly lower abundances can be seen.*

Figure 3: (a) **TIC** (total ion chromatogram) trace of the lipids found in the extract from the edge of interest of artefact XXIV-67, with overlays of the MRM extracted ion chromatograms for (b) cholesterol ($R_t = 14.85$ min), (c) oleanolic acid ($R_t = 16.58$ min) and (d) betulinic acid ($R_t = 16.68$ min) and ursolic acid ($R_t = 16.87$ min). *Note: TIC peaks at 7.3 min and 11.0 min are not analytes of interest. Qt = quantifying ion and Ql = qualifying ion. Y-axis = counts (abundance) and X-axis = acquisition (retention) time.*