University of Wollongong

Research Online

Faculty of Science, Medicine and Health -
Papers: part A

Faculty of Science, Medicine and Health

1-1-2017

Viability of Raman microscopy to identify micro-residues related to tool-use and modern contaminants on prehistoric stone artefacts

Luc Bordes

University of Wollongong, lfb437@uowmail.edu.au

Linda C. Prinsloo

University of Wollongong, lprinslo@uow.edu.au

Richard Fullagar

University of Wollongong, fullagar@uow.edu.au

Thomas Sutikna

University of Wollongong, tsutikna@uow.edu.au

Elspeth H. Hayes

University of Wollongong, ehayes@uow.edu.au

See next page for additional authors

Follow this and additional works at: <https://ro.uow.edu.au/smhpapers>

Part of the [Medicine and Health Sciences Commons](#), and the [Social and Behavioral Sciences Commons](#)

Recommended Citation

Bordes, Luc; Prinsloo, Linda C.; Fullagar, Richard; Sutikna, Thomas; Hayes, Elspeth H.; Jatmiko, -; Saptomo, E Wahyu; Tocheri, Matthew W.; and Roberts, Richard G., "Viability of Raman microscopy to identify micro-residues related to tool-use and modern contaminants on prehistoric stone artefacts" (2017). *Faculty of Science, Medicine and Health - Papers: part A*. 4895.
<https://ro.uow.edu.au/smhpapers/4895>

Research Online is the open access institutional repository for the University of Wollongong. For further information contact the UOW Library: research-pubs@uow.edu.au

Viability of Raman microscopy to identify micro-residues related to tool-use and modern contaminants on prehistoric stone artefacts

Abstract

Analyses of ancient micro-residues and usewear preserved on stone artefacts can potentially provide detailed information about how prehistoric humans used the artefacts to process materials such as food, pigments and/or adhesives. However, ancient micro-residues are likely degraded, and there are multiple potential sources of contamination, such as contact with sediments, groundwater, recent handling, storage materials or laboratory conditions, any of which can inhibit reliable identification of micro-residues and other traces of prehistoric use. In this pilot study, five stone tools from the archaeological site of Liang Bua (Flores, Indonesia) were used to evaluate the viability of Raman spectroscopy to identify ancient micro-residues preserved on stone artefact surfaces that are due specifically to prehistoric use as opposed to some form of ancient or modern source of contamination. Inorganic and organic deposits that occur commonly in the cave environment, including iron oxide, manganese oxide and biofilms, were identified in both the sediment and on the artefacts. Protein and saturated fatty acid micro-residues were identified on edges of all artefacts and may partially originate from modern handling. Proteins, plant fibres and other micro-residues associated with calcium nitrate are possibly archaeologically significant. Detection of plant fibres and starch grains may indicate either modern contamination or prehistoric contact with plant material that was transferred incidentally or during tool manufacture and/or tool use. These results demonstrate the viability of Raman microscopy to screen, at an early stage of archaeological residue analysis, for modern contaminants and micro-residues related to tool manufacture and/or tool use. This approach serves as a base for planning strategies and analytical protocols for future work that targets larger samples of artefacts, integrates Raman microscopy with GC-MS/LC-MS and includes more comprehensive studies of usewear.

Disciplines

Medicine and Health Sciences | Social and Behavioral Sciences

Publication Details

Bordes, L., Prinsloo, L. C., Fullagar, R., Sutikna, T., Hayes, E., Jatmiko, , Saptomo, E. Wahyu., Tocheri, M. W. & Roberts, R. G. (2017). Viability of Raman microscopy to identify micro-residues related to tool-use and modern contaminants on prehistoric stone artefacts. *Journal of Raman Spectroscopy*, 48 (9), 1212-1221.

Authors

Luc Bordes, Linda C. Prinsloo, Richard Fullagar, Thomas Sutikna, Elspeth H. Hayes, - Jatmiko, E Wahyu Saptomo, Matthew W. Tocheri, and Richard G. Roberts

Viability of Raman microscopy to identify micro-residues related to tool-use and modern contaminants on prehistoric stone artefacts.

^aLuc Bordes, ^aLinda C. Prinsloo, ^aRichard Fullagar, ^{a,b,c}Thomas Sutikna, ^aElsbeth Hayes, ^{b,c}Ako Jatmiko, ^{b,c}E. Wahyu Saptomo, ^{d,e}Matthew W. Tocheri and ^{a,c}Richard G. Roberts.

^a*Centre for Archaeological Science, School of Earth and Environmental Sciences, University of Wollongong, Wollongong, NSW 2522, Australia.*

^b*Pusat Penelitian Arkeologi Nasional, Jl. Raya Condet Pejaten No. 4, Jakarta 12510, Indonesia*

^c*ARC Centre of Excellence for Australian Biodiversity and Heritage, University of Wollongong, Wollongong, NSW 2522, Australia*

^d*Department of Anthropology, Lakehead University, Thunder Bay, P7B 5E1, Canada.*

^e*Human Origins Program, Department of Anthropology, National Museum of Natural History, Smithsonian Institution, Washington DC, 20560, USA.*

ABSTRACT

Analyses of ancient micro-residues and usewear preserved on stone artefacts can potentially provide detailed information about how prehistoric humans used the artefacts to process materials such as food, pigments and/or adhesives. However, ancient micro-residues are likely degraded and there are multiple potential sources of contamination, such as contact with sediments, groundwater, recent handling, storage materials or laboratory conditions, any of which can inhibit reliable identification of micro-residues and other traces of prehistoric use.

In this pilot study, five stone tools from the archaeological site of Liang Bua (Flores, Indonesia) were used to evaluate the viability of Raman spectroscopy to identify ancient micro-residues preserved on stone artefact surfaces that are due specifically to prehistoric use as opposed to some form of ancient or modern source of contamination.

Inorganic and organic deposits that occur commonly in the cave environment, including iron oxide, manganese oxide and biofilms, were identified in both the sediment and on the artefacts. Protein and saturated fatty acid micro-residues were identified on edges of all artefacts and may partially originate from modern handling. Proteins, plant fibres and other micro-residues associated with calcium nitrate are possibly archaeologically significant. Detection of plant fibres and starch grains may indicate either modern contamination or prehistoric contact with plant material that was transferred incidentally or during tool manufacture and/or tool use.

These results demonstrate the viability of Raman microscopy to screen, at an early stage of archaeological residue analysis, for modern contaminants and micro-residues related to tool manufacture and/or tool use. This approach serves as a base for planning strategies and analytical protocols for future work that targets larger samples of artefacts, integrates Raman microscopy with GC-MS/LC-MS and includes more comprehensive studies of usewear.

KEYWORDS: stone tools, Liang Bua, proteins, fatty acids, plant fibres

INTRODUCTION

Studying stone artefacts from archaeological sites spanning the last 3.3 million years¹ provides important clues about how people lived in the distant past. Archaeologists usually classify stone artefacts by their shape, raw material, function and the techniques used to make them, to address questions related to tool use, cultural phases, human evolution and cognitive capacity. Analysis of ancient micro-residues and wear preserved on artefacts can provide detailed information, such as the specific plant, animal and/or other materials that were stored in pots or processed by tools, the pigments used for decorative applications and the adhesives used for particular hafting techniques.^{2,3,4} Identification of ancient organic micro-residues is not a trivial task, as the micro-residues are likely degraded. Furthermore, micro-residues found on stone tools may originate from multiple agencies other than contact material transferred during use. For example, both organic and inorganic micro-residues can be transferred to stone tool surfaces via contact with sediments, groundwater, bacteria, insects and fungi. Contamination after excavation is also not negligible and can occur through handling by archaeologists during excavation and the type of storage material, or can be introduced by laboratory conditions and contact with analytical instruments and facilities used to study the tools.

Most previous spectroscopic analyses have focussed on stone tools with macro-residues visible to the naked eye or at low magnification.^{5,6} In such cases, the role of spectroscopic analysis is to confirm the identification of micro-residues. The study of stone artefacts with no visible macro-residues is more challenging, because micro-residues without recognisable structures must be correctly identified^{7,8} and their relation to tool use or other prehistoric tasks requires additional evidence. Indeed, because of their small size and often scattered distributions, micro-residues are difficult to distinguish from modern contaminants and the effects of post-depositional processes.⁹ To confidently determine that micro-residues are archaeologically significant, multiple lines of evidence are required,¹⁰ including micro-residue abundance and meaningful distributions.¹¹ Identification of a single micro-residue deposit or micro-residue class is not sufficient for determining the function of a stone tool, because use-related micro-residues are often preserved in relatively high abundance in predictable locations on tool surfaces, based on archaeological and experimental analyses.^{2,11}

Raman microscopy can help relate data from usewear/micro-residue and spectroscopic analyses, both of which are minimally destructive and ideally suited for many archaeological applications.¹² Here we examine the viability of Raman microscopy to screen for modern contaminants and micro-residues related to tool manufacture and/or tool use in a sample of five stone artefacts recovered during archaeological excavations at Liang Bua, Flores, Indonesia (Table 1).^{13,14}

ARCHAEOLOGICAL BACKGROUND

Liang Bua is a limestone cave located on the island of Flores, Indonesia (Fig. S1, Supporting Information), with a cultural sequence spanning the past ~190 thousand years.¹³ During this time, the cave was occupied successively by at least two human species, initially by *Homo floresiensis* and later by *Homo sapiens* (modern humans), currently with no evidence of temporal overlap.¹⁴

Previous work on the stone artefacts excavated at Liang Bua¹⁵ found little variation in manufacturing techniques between modern humans and *Homo floresiensis*, based on the lithic reduction sequence. Nevertheless, a noticeable shift to chert as the preferred knapping material, artefacts more frequently exposed to fire, and the abrupt appearance of flakes with prominent edge gloss were documented within the artefact assemblages of modern human.¹⁵ Our long-term objective is to compare tools made and used by modern humans with those of *Homo floresiensis* to reconstruct variation in resource-use and other behaviours that may, or may not, be reflected in the tool technology or reduction sequence, specifically.

For this pilot study, five stone artefacts recovered in 2004 during the archaeological excavations of Sector XI (Fig. 1) were selected for an initial assessment of the viability of Raman microscopy for micro-residue analysis. Three of these artefacts derive from Holocene sediments and two derive from Late Pleistocene sediments (Table 1 and Fig. S2, Supporting Information). More than a decade has elapsed since these artefacts were excavated and during this time they have been studied by other researchers and temporarily stored in other laboratories/institutions. Therefore, exposure to contamination sources that are unknown to us may have occurred. But as the artefacts were still covered by a fine sediment layer, which required removal before most micro-residues were detectable by Raman spectroscopy, contamination during this time period was probably minimal. All artefacts were first inspected under optical microscopes to document potential traces of use, including micro-residues, polish and edge rounding (Table 1). For all samples, sediment attached to the artefacts was also studied.

ANALYTICAL AND EXPERIMENTAL METHODS

Sample handling and cleaning

To avoid additional contamination (including further human contact), the artefacts were handled with nitrile gloves (latex, powder and protein free). Each artefact was placed on a support fashioned by Blu-Tack[®] (a synthetic rubber compound) to accommodate its shape. This enabled the positioning of each sample under the Raman microscope with the incident light (laser) normal (i.e., perpendicular) to the point of analysis. The support was covered with a piece of nitrile glove to

prevent contamination from the Blu-Tack. Basic precautions, such as storing the samples in clean bags and boxes, were taken before and after analysis.

A systematic Raman microscopic analysis of the artefacts proved challenging. Even though the samples appeared macroscopically clean, sediments adhering to the artefacts were observed under the microscope objectives, which obscured the artefact surfaces and contributed to a high fluorescence background. To circumvent this problem, we developed an optimal cleaning procedure consisting of two steps: (1) a short 5 s ultrasonication in deionised water to remove surplus sediment prior to a first in-depth Raman analysis, and (2) a longer 15 min ultrasonication before a second Raman analysis. In between these steps, the sediment removed by ultrasonication was collected, dried overnight (12 hr at 70°C), placed on slides and analysed using Raman microscopy. This procedure enabled comparison of micro-residues retained in the sediment to those remaining on the artefacts. This comparison was helpful in evaluating whether the micro-residue was strongly or loosely affixed to the artefact.

In an attempt to obtain a Raman signature of contamination due to modern handling, experimental stone flakes (flint) were heated in a furnace at 600°C for 3 hr to remove any organic material present. Only plastic boxes and nitrile gloves came in contact with these flakes before 5 min of deliberate handling using clean hands (washed with soap and tap water and air dried). The handled flakes were then examined under the Raman microscope.

We are also compiling Raman spectral references of experimental tools made and used to work plant material about 30–35 years ago for a study using optical microscopy.¹⁶ These tools, which were stored in plastic bags, can potentially give an indication of degradation processes during this time period.

Analysis

Raman spectra were recorded with a WITec® alpha 300R confocal Raman microscope (WITec® Instrument Corp., Germany) equipped with two UHTS300 spectrometers and two CCD detectors: (1) a visible DV401 detector for use with 532 nm excitation, and (2) a DV401 detector for 785 nm excitation. The excitation sources were two diode lasers operated at 532 nm and 785 nm wavelengths with 38 mW and 120 mW maximum power output respectively. Zeiss® microscope objectives (20X and 50X magnifications) were used, achieving a sub-micron spatial resolution. The samples were placed on a piezo-driven, feedback-controlled scanning stage.

Each artefact was analysed in three steps: (1) an initial scan before cleaning, followed by (2) systematic micro-residue observations and analysis after a 5 s ultrasonication, and (3) a second analysis after a further 15 min ultrasonication. Residual sediment samples were also analysed after

drying. On each artefact, all edges were investigated systematically along a strip, approximately 200 μm from the edge, where we expected to find a possible concentration of residues attributable to the use of the tool. Flake surfaces further than 200 μm from the edges were not investigated systematically, but were randomly probed focussing on particular features (e.g., stained areas or dark spots). Systematic spectral mapping of the artefacts was not possible due to uneven surfaces and high fluorescent backgrounds, which required quenching in order to obtain spectra. In general, the green 532 nm laser line was used as the excitation source for both mineral and organic micro-residues, because a better signal-to-noise ratio was obtained than for 785 nm excitation. Furthermore, the risk of burning the micro-residue was less for green excitation than red, and a larger spectral range (0–3500 cm^{-1}) was coverable in one analysis. Consequently, the red laser was used only when the fluorescence was too high, as with plant fibres, for example. Care was taken to increase the laser power progressively in order to collect the spectra while remaining under the threshold for damage due to laser heating. In some instances, unavoidable over-heating helped to distinguish between organic and inorganic micro-residues.

RESULTS

Potential contamination sources

A key problem in studying archaeological residues is determining the origin of the residue and whether it relates to past use, incidental contact, taphonomic processes or modern contamination.² For micro-residues, this issue is even more critical. Consequently, Raman spectra were collected for the nitrile gloves used to handle samples, mini Ziplock® plastic storage bags and Parafilm® (which we used initially to prevent direct contact between artefacts and the Blu-Tack support). The spectral fingerprint of the nitrile gloves, with two unique bands at 1533 and 2242 cm^{-1} (Fig. S3, 2c Supporting Information), is easily distinguishable from that of most organic micro-residues. However, materials like Ziplock® bags and Parafilm® (Fig. S3a and S3b, respectively, Supporting Information) are more problematic, because the main bands of their spectra are similar to those of unsaturated fatty acids and misinterpretation is possible in spectra with low signal-to-noise ratio. To minimise this issue, we replaced the Parafilm® cover over the Blu-Tack® support with nitrile gloves. These artefacts had been stored in Ziplock® storage bags, however, so each time a new compound was detected on the artefacts its assignment was checked against these reference spectra.

Other sources of identified contamination include water (used during ultrasonication) and plastic boxes (in which the artefacts were placed to dry after cleaning). Airborne particles that can settle on artefacts and laboratory equipment used during analysis and manipulation were also identified as major sources of contamination. To evaluate airborne contamination, we placed several glass microscope slides at different positions in the laboratory (including locations above the

Raman microscope and under the ventilation/air conditioning ducts) for one week. Particles detected included synthetic glass fibre, polyester, proteins, modern cotton fibres, modern dyed fibres, natural plant fibres and starch grains. Similar sources of contamination have been observed by usewear and micro-residue analysts in optical microscope laboratories.^{11, 17}

Mineral background

To distinguish between Raman signals of micro-residues attached to the artefacts and signals originating from the minerals in the stone, spectra were recorded for parts of the artefacts where no micro-residues were recognised. Spectra recorded on artefacts XI-45, XI-182, XI-228 and XI-250 consisted mainly of an α -quartz signal (Fig. S4b, Supporting Information), but spectra recorded on XI-337-2 had a different Raman signature (Fig. S4a, Supporting Information), consisting of peaks at 288, 480 and 515 cm^{-1} typical of feldspar, as well as bands at 113, 232 and 419 cm^{-1} characteristic of cristobalite, a high temperature phase of α -quartz.¹⁸ The sediments attached to the artefacts consisted mainly of α -quartz and feldspar, but other common minerals (e.g., calcium carbonate, goethite and anatase) were frequently observed (TableS1).

Micro-residues detected on stone artefacts

A summary of all mineral and organic phases identified as mineral background, in the sediments adhering to the artefacts, and as micro-residues on the artefact surfaces are summarised in Table S1 (Supporting Information). Three different classes of micro-residue were identified on artefact surfaces: 1) mineral and organic micro-residues also found in sediment, 2) micro-residues from contamination sources, and 3) potentially archaeologically significant micro-residues.

Manganese oxide

Late Pleistocene artefacts XI-250 and XI-337-2 (to a lesser extent) was covered by an extended layer of black to steel-grey mineral, with a metallic lustre (Fig. 2A, 2B). Raman spectra obtained for this layer show three broad bands centred around 500, 563 and 621 cm^{-1} , consistent with manganese dioxide (MnO_2) (Fig. 2C).¹⁹ This manganese oxide surface deposit did not show any recognisable distribution pattern on either artefact and was also found in the adhering sediments and in sediment layers elsewhere in the cave;²⁰ thus, it is unlikely to be a micro-residue linked to the use of either artefact.

Biofilm

All of the artefacts have some surface areas covered with biofilm to differing degrees, with the thickest covering occurring on XI-337-2. Biofilms can consist of bacteria, fungal colonies or

other living organisms and regularly occur on stone monuments.²¹ Due to a large fluorescence background with green excitation, the spectra in Fig. S5A (Supporting Information) were collected using a 785 nm laser line. The main vibrational bands in the spectra at 1236, 1304, 1416 and 1530 cm^{-1} (Fig. S5A, Supporting Information) are similar to the Raman fingerprint of fungi collected *in situ* on Neolithic paintings.²² Similar spectra were also obtained on individual fungi filaments encountered on the edges of the other four artefacts (examples in Fig. S5B and C, Supporting Information).

Iron oxides

Another common micro-residue observed on all samples with a quartz mineral background (artefacts XI-182, XI-228, XI-250 and XI-45) is a black iron oxide/hydroxide micro-residue coated with organic matter. Under low laser power excitation, the Raman signal obtained from these micro-residues corresponds to unspecified organic material with two broad bands centred at 1592 and 1380 cm^{-1} (Fig. 3C,a), respectively known as the G (sp^2 C-C bonds) and D (sp^3 C-C bonds) bands typical of amorphous carbon.²³ Increasing the laser power (and thereby burning the organic phase) resulted in spectra of different iron oxides: maghemite (Fig. 3C,b), with two bands at 681 and 719 cm^{-1} , and hematite (Fig 3C,c), with its characteristic vibrational bands at 231, 298 and 415 cm^{-1} .²⁴ In some cases, the spectra represented a mixture of these minerals, with the maghemite contribution to the hematite spectrum at 667 cm^{-1} (Fig 3C,d) recognised as a broadening of this band.² Mixtures of hematite, maghemite and organic matter were also observed (Fig. 3C,e). These results indicate that the organic matter is a superficial coating on the iron oxide grains. Their widespread distribution on the surfaces of the artefacts and strong presence in the sediment suggest that it is a naturally occurring micro-residue at Liang Bua. It may originate from the activities of micro-organisms such as fungi or bacteria. Previous studies have suggested that Mn- and Fe-rich crusts may form through the action of bacteria in caves.²⁶

Apatite

Small white rods (10–20 μm in length) were observed loosely attached to the surface of artefact XI-337-2 (Fig. 4A) and in the sediment removed from the artefact during sonication (Fig. 5B). The Raman spectrum of the rods consists of a strong PO_4^{4-} vibrational mode at 968 cm^{-1} and two weaker bands at 433 and 592 cm^{-1} (Fig. 4C,a), comparable to a spectrum (Fig. 4C,b) and published data of geological apatite.²⁷ In addition to the Raman peaks, strong broad bands occur in the spectrum of the rods at 1146, 2083 and 3291 cm^{-1} (564, 600 and 645 nm). These can be attributed to luminescence from the rare earth element samarium (Sm^{3+}) with a possible contribution from praseodymium (Pr^{3+}), as their luminescence bands overlap.²⁸ The spectrum

recorded for the geological apatite (of Australian origin) also shows small luminescence bands that can be attributed to europium. Apatite $\text{Ca}_5(\text{F,OH,Cl})(\text{PO}_4)_3$ as well as many other natural crystals usually includes rare earth elements.

The Raman spectra of the rods and geological apatite are compared to the spectrum of a small piece of bone removed from the surface of the same artefact during sonication (Fig. 4C,c) and a modern bone sample (Fig. 4C,d). The spectra differ from that of bone apatite (Fig. 4C,c–d) with the totally symmetric P-O stretch vibration at a higher wavenumber (968 cm^{-1}) than in bone (965 cm^{-1} , Fig. 4C,d) and the 9 cm^{-1} full width at half maximum (FWHM) of the 968 cm^{-1} band, half the 20 cm^{-1} FWHM of the 965 cm^{-1} band in modern bone. Bone apatite is considered to be hydroxylapatite with some substitution of phosphate ions by carbonate ions and contains collagen. Incorporating collagen and carbonate ions into the crystal lattice causes line broadening and the shift to lower wavenumbers, indicating some disorder in the structure. However, with time bone degrades and the first step is the loss of collagen. Therefore peaks attributed to collagen (1254 , 1451 and 1675 , 2880 and 2943 cm^{-1}) present in the spectrum of the modern bone sample (Fig. 4C,d) are absent in the spectrum of the bone fragment (Fig. 4C,c). The collagen loss is reflected in the decrease of the FWHM of the 964 cm^{-1} band to 17 cm^{-1} and is a further indication of degradation. Furthermore, the band at 1074 cm^{-1} attributed to carbonate in bone (Fig. 4C c-d) is also absent in the spectra of geological apatite. Upon further bone degradation the carbonate ions are replaced with Cl^- and F^- , the calcium ions by other cations including rare earth elements and complete recrystallization can take place.

These analyses show that Raman spectroscopy can play an important role in distinguishing between different kinds of apatite micro-residues and degradation processes in bone minerals. However, at this stage it is not possible to link the small fragment of bone or the apatite rods (which might be recrystallised bone) to tool use.

Proteins and lipids

Large numbers of particles were identified as proteins and lipids on all edges of the Liang Bua artefacts. Their frequency and widespread distribution make their classification as archaeological micro-residues linked to tool-use questionable; instead, their presence is probably due to modern contamination from handling. To test this hypothesis, a simple experiment was undertaken (see paragraph on sample handling and cleaning) and the results showed that, even after heating stone flakes to temperatures above 600°C , some protein and lipid micro-residues remained on the flakes (see Table S2, Supporting Information). The proteins and lipids detected using Raman spectroscopy had distinctive forms (as observed under the 50X microscope attached to the WiTec Raman instrument) and, in the case of lipids, sometimes appeared smeared. Images of protein and lipid

micro-residues observed on the experimental stone flakes after handling and on artefacts from Liang Bua are shown in Fig. S3 (Supporting Information).

The Raman spectrum of a protein is characterised by amide I ($1600\text{--}1690\text{ cm}^{-1}$) and amide III ($1230\text{--}1300\text{ cm}^{-1}$) vibrations of the peptide backbone. A strong broad peak at $\sim 1454\text{ cm}^{-1}$ corresponds to CH_2 and CH_3 bending modes.²⁹ Side-chain phenylalanine and tyrosine bands are observed at $1007\text{--}1008\text{ cm}^{-1}$ and $856\text{--}859\text{ cm}^{-1}$, respectively. The Raman spectrum of a typical micro-residue found on the Liang Bua artefacts is compared with the protein spectrum recorded for our handling experiments in Fig. 5A, a and b, respectively. The two spectra are similar, but they differ in the relative intensities of the amide II (1656 cm^{-1}), phenylalanine ($\sim 1007\text{ cm}^{-1}$) and tyrosine ($\sim 859\text{ cm}^{-1}$) peaks. The S-S vibrational band at 513 cm^{-1} is seen only in the archaeological artefact spectrum. Unfortunately, these differences vary according to the spot analysed on the artefact, making it difficult to distinguish systematically between different types of protein. Furthermore, archaeological micro-residues are most likely mixtures, and the spectral range between 500 and 1000 cm^{-1} is commonly noisy due to fluorescence, making protein discrimination even more challenging.

Raman spectra of lipid micro-residues detected on artefacts are characterised by very strong CH_2 and CH_3 stretching vibrations ($2800\text{--}2950\text{ cm}^{-1}$), bending CH_2/CH_3 vibrational bands at 1463 , 1443 cm^{-1} , a CH_2 twisting mode at 1300 cm^{-1} , and C-C stretching at 1133 , 1105 and 1067 cm^{-1} ,³⁰ all related to saturated fatty acids. Spectra recorded on the Liang Bua artefacts (Fig. 5B,a) and on the stone blank from the handling experiments (Fig. 5B,b) were compared to stearic and palmitic acids (Fig. 5B,c–d). The spectra differ only slightly, such as the presence of a shoulder at 1424 cm^{-1} and a more intense 1177 cm^{-1} band observed after the handling experiments. Unfortunately, these differences are hard to discern in low signal-to-noise spectra, making it difficult to distinguish systematically between contamination by modern handling and fatty acid micro-residues due to prehistoric use or handling. In some spectra, only a very strong band at 2882 cm^{-1} was detected, so the micro-residue was then classified simply as lipid. However, the visual appearance of lipid micro-residues can help distinguish between modern contamination and archaeological micro-residues. For example, a lipid micro-residue was found smeared on a small area of the polished edge of artefact XI-250 (Fig. S6D). This feature was not observed in our handling experiments and its smeared aspect and association with usewear suggests that it may have resulted from prehistoric tool use.

Micro-residues associated with calcium nitrate

Some Raman spectra of protein micro-residues on the artefacts show an additional sharp band at $\sim 1048\text{ cm}^{-1}$ (Fig. 6a,b). When the intensity of this band was high, we also observed two weaker

bands at $710\text{--}715\text{ cm}^{-1}$ and $738\text{--}740\text{ cm}^{-1}$, identifying this compound as calcium nitrate.³¹ The presence of calcium nitrate has been reported as a degradation product from burials,^{31,32} which could be a possible source of CaNO_3 in cave environments such as Liang Bua. However, calcium nitrate was found associated with pure protein or protein mixed with fatty acids (Fig. 6a–c), but not in any micro-residues originating from contamination experiments or in the sediment. It was also not found on the rock surface immediately around the micro residues but only associated with them. Furthermore, calcium nitrate was also detected in association with protein on some of the experimental stone tools used to work plant material made 30–35 years ago (Fig. S7), but not on micro-residues on tools used in new experiments on similar plant materials. Taking all of this into account, we suspect that the protein and protein/fatty acid mixtures containing calcium nitrate could be a result of alteration of plant material associated with the prehistoric use of a tool. This degradation and formation of CaNO_3 could have occurred *in situ* or during storage of the artefacts after excavation.

Plant fibres and starch grains

Several plant fibres were visually identified by their shape, as shown in Fig. S8 (Supporting Information). Raman spectra were mostly recorded using 785 nm excitation due to a high fluorescence background, but 532 nm excitation was used in cases where the fibre protruded from the mineral surface. The main chemical components of plant fibres are cellulose (including hemicelluloses), moisture, lignin and pectins, which vary in abundance between species and depending on growth conditions.

Raman spectra (Fig. 7A a,b) of plant fibres found on the tip of artefact XI-182 (Fig. S8A,B) closely resemble that of cellulose, with main bands at 1093 and 1121 cm^{-1} (C-O and O-C-O stretching modes) and the C-H deformation mode at 903 cm^{-1} .³³ Low wavenumber bands of cellulose were visible at 378 , 436 and 510 cm^{-1} . The presence of lignin was observed as a weak band at 1600 cm^{-1} .³⁴ The fibre on the surface of the tip has an intense calcium nitrate band at 1052 cm^{-1} (Fig. 7A), suggesting a possible archaeological origin. Because of their close locations on the tip of XI-182 (Fig. 8) and similar Raman spectral fingerprints, these two micro-residues possibly originate from the same plant.

Two plant fibres found on artefact XI-45 are located at opposite ends of the artefact and have distinct spectra. The fibre found on the striking platform (Fig. S8C) has a strong cellulose signal with no lignin band present (Fig. 7B,a), whereas the fibre found on the left distal edge (Fig. S8D) does have some lignin content and is associated with calcium nitrate, as indicated by the band at 1051 cm^{-1} (Fig. 7B,b). Furthermore, the latter plant fibre is folded in a hole in the surface, and the presence of the quartz band at 464 cm^{-1} indicates its proximity to the mineral surface. This

particular location could indicate an ancient origin. Other differences are difficult to evaluate, as the fibres from the striking platform and distal edge were analysed using 532 and 785 nm excitation, respectively, to maximise the signal-to-noise ratios. The observed differences between these two micro-residues suggest that they originate from different plants.

An isolated starch grain was found on a polished edge of artefact XI-337-2 (Fig. S9, Supporting Information). The Raman bands at 482 cm^{-1} (C-C-C bending, C-O torsion), 1462 cm^{-1} (CH, CH₂, C-O-H bending) and very strong band at 2906 cm^{-1} (C-H stretching) are diagnostic of starch (Fig. S9B). Other vibrational bands observed at 582, 863, 942, 1124 and 1340 cm^{-1} are also characteristic of starch³⁵ and the spectrum is compared to that of a spinifex starch grain recorded as a reference in Fig. S9B.

Unidentified black micro-residue

A black micro-residue was found in the middle of the right distal edge of artefact XI-228, after being through the ultrasonication cleaning process twice (total duration 2 hr, after cleaning this artefact we reduced the ultrasonication times). Similar micro-residues were not found in the sediment removed from the sample collected from the layer in which the artefact was found. The micro-residue appears as a patch of black droplets on the polished edge (Fig. S10A, Supporting Information). This particular position could potentially links it to some prehistoric tool-use, but Raman analysis showed that it is not the case. Indeed, the Raman signal was dominated by two bands at 1591 and 1383 cm^{-1} , with less intense bands at 1620, 1545, 1247, 1178, 917, 809 and 611 cm^{-1} (Fig. S10B,a, Supporting Information). The visual appearance of the micro-residue and its strong attachment to the artefact suggests that it could be a resin-like material, but its Raman spectrum does not match any in our current database of Australian resins or, as far as we can establish, any resin described in the literature.^{36,37} In fact, the spectrum is quite similar to that of crystal violet (Fig. S10B,b, Supporting Information), a modern synthetic dye, so we conclude that this black residue is probably a modern contaminant.³⁸

DISCUSSION

Our study of stone artefacts from Liang Bua using Raman spectroscopy has identified mineral micro-residues originating from the cave environment, such as manganese oxide, apatite and iron oxides, the latter associated with organic material. Proteins, lipids (mostly saturated fatty acids) and protein/fatty acid mixtures were found in abundance, some in association with calcium nitrate. A starch grain, several plant fibres and an unknown micro-residue were also found adhering to the artefact surfaces (Table S1, Supporting information). Furthermore, we have shown that contamination through modern handling and exposure to laboratory conditions has contributed to

the suite of micro-residues on these artefacts (Table S2, Supporting information). To accurately distinguish between modern contaminants and micro-residues related to prehistoric tool-use, other criteria must be taken into account.

Comparing the presence of micro-residues on artefacts with the chemical constituents in the sediments removed from the artefacts helps distinguish whether a micro-residue is archaeologically significant. For example, the maghemite/hematite associated with an organic phase, the manganese oxide, and the mineral phases abundant in the sediment (calcite, goethite, anatase, feldspar) (Table S1, Supporting information), were ruled out as associated with usewear. However, weathering cycles may cause some micro-residues initially attached to artefacts to dislodge and become part of the adjacent sediment.

Stone tools used repeatedly to scrape, cut or saw particular materials sustain diagnostic usewear, including polish associated with changes in surface micro-topography that can be visually identified under a light microscope.¹⁶ It has been shown experimentally that micro-residues are commonly found in areas with usewear.¹¹ In this study, protein and saturated fatty acid micro-residues were found in abundance on all five Liang Bua artefacts. The protein micro-residues, however, were distributed along the artefact edges, whereas the saturated fatty acid micro-residues were frequently concentrated on use-polish (e.g., the distal right edge of artefact XI-182; Fig. 8). This indicates a possible link with tool use, perhaps as the final degradation product of a worked material. We related calcium nitrate associated with proteins and plant fibres to degradation processes, making it possible to separate them from modern contaminants, but this criterion alone is not sufficient to confirm that these micro-residues are archaeologically significant. Indeed, protein and mixed protein/fatty acid micro-residues show no clear spatial distribution on polished edges, although they are frequently associated with calcium nitrate. Stone tool experiments using fresh materials indicate, however, that some micro-residues are initially located away from the used and polished edges.³⁹ Such micro-residues may be archaeologically significant, but their relative preservation on these different areas of the artefact after thousands of years is still unknown. The presence of saturated fatty acids smeared over a large area of artefact XI-250 suggests that they were acquired during prehistoric use.

Raman spectra of plant fibres had low signal-to-noise ratios and fluorescent backgrounds, which may reflect their antiquity compared to the high signal-to-noise ratios typical of spectra for modern fibres.⁴⁰ Two fibres with similar Raman signals were located on the tip of artefact XI-182. On another artefact, XI-45, Raman spectra were used to discriminate between two fibre micro-residues found on different edges. One of these was folded in a surface hole and showed lignin and calcium nitrate Raman peaks, suggesting a prehistoric origin, whereas the other fibre was found loosely attached to the edge and was composed only of cellulose. Plant fibre analysis shows the

potential of Raman spectroscopy to discriminate among some contaminants, but plant fibres are too few in this set of artefacts to reliably infer any plant-related use in the past.

Prehistoric use of artefacts should appear either as a group of micro-residues or as micro-residues on more than one area of the tool. Although the isolated starch grain found on artefact XI-337-2 highlights the capability of Raman spectroscopy to identify individual starch grains *in situ* among mineral grains that look similar, the common occurrence of starch grains from airborne and other laboratory contamination sources necessitates a cautious interpretation.^{2,17}

Finally, micro-residues strongly adhering to artefact surfaces do not necessarily indicate prehistoric use. For example, the black micro-residue strongly adhering to artefact XI-228 (even after two ultrasonifications) was not detected in the sediment that covered it and appeared as a cluster of resin-like droplets. However, close resemblance of its Raman spectrum to that of the modern pigment crystal violet (possibly derived from black modern ink) casts doubt on its classification as a prehistoric micro-residue.

CONCLUSIONS

The results of this pilot study examining five stone artefacts recovered from archaeological excavations at Liang Bua demonstrate the utility of Raman microscopy for identifying mineral and organic micro-residues, including proteins, lipids, plant fibres and starch grains. Contaminants from various sources clearly hamper interpretations of prehistoric tool use and function. Future work must eliminate contamination sources as far as possible, while also developing a database of Raman spectra of possible micro-residues on ancient artefacts; these include pure lipids, amino acids and natural materials of archaeological importance, such as resins and animal fats. Ideally, stone artefacts should be collected covered sufficiently in a pedestal of sediment, such that they arrive at the laboratory with *in situ*, buried surfaces untouched by either excavation equipment or recent handling, as important information is gained by analysing the surrounding sediment. This pilot study confirms that Raman microscopy is a powerful technique to screen the chemical nature of micro-residues attached to artefact surfaces prior to other analyses. Future studies include a larger sample of artefacts from various stratigraphic units at Liang Bua and integrate Raman microscopy with GC-MS/LC-MS and usewear analyses to more comprehensively identify micro-residues related to prehistoric human activities.

ACKNOWLEDGEMENTS

This study was supported by the Australian Research Council through Australian Laureate Fellowship FL130100116 (R.G.R.), with additional funding from the University of Wollongong

(T.S.), the Canada Research Chairs Program and the Smithsonian Institution's Humans Origins Program (M.W.T.). Fieldwork was authorised by Pusat Penelitian Arkeologi Nasional (Jakarta, Indonesia) and Pemerintah Daerah Kabupaten Manggarai (Flores, Nusa Tenggara Timur). We also thank I Made Geria, S. Wasisto, V.N. Sene, I Dewa Kompiang and the entire Liang Bua Team from Teras, Golo Manuk and Bere.

For Peer Review

REFERENCES

- 1 S. Harmand, J. E. Lewis, C. S. Feibel, C. J. Lepre, S. Prat, A. Lenoble, X. Boes, R. L. Quinn, M. Brenet, A. Arroyo et al., *Nature*. **2015** ; 521 , 310–315.
- 2 L. Wadley, M. Lombard, *J. Archaeol. Sci.* **2007** ; 34 , 1001–1010.
- 3 M. Lombard, *J. Archaeol. Sci.* **2008** ; 35 , 26–41.
- 4 R. Fullagar, In *Archaeology in Practice: A Student Guide to Archaeological Analyses*, ed. J. Balme and A. Paterson (Blackwell Publishing), **2014** , 232–263.
- 5 S. Vahur, A. Kriiska, I. Leito, *Estonian Journal of Archaeology*. **2011** ; 15 , 3–17.
- 6 M. Carciumaru, R. Ion, E. Nitu R. Stefanescu, *J. Archaeol. Sci.* **2012** ; 39 , 1942–1950.
- 7 G. H. Langejans, *S. Afr. Archaeol. Bull.* **2012** ; 67 , 200–213.
- 8 G. F. Monnier, J. L. Ladwig, S. T. Porter, *J. Archaeol. Sci.* **2012** ; 39 , 3284–3300.
- 9 G. H. Langejans, *J. Archaeol. Sci.* **2010** ; 37 , 971–985.
- 10 M. Lombard, L. Wadley, L. In *Archaeological Science Under a Microscope: Studies in Residue and Ancient DNA Analysis in Honour of Thomas H. Loy*, ed. M. Haslam, G. Robertson, A. Crowther, S. Nugent and L. Kirkwood (ANU Press), **2009** , 11–28.
- 11 V. Rots, E. Hayes, D. Cnuts, C. Lepers, R. Fullagar, *PLoS ONE*. **2016** ; 11(3) , e0150437.
- 12 G. F. Monnier, T. C. Hauck, J. M. Feinberg, B. Luo, J. L. Tonsor, H. A. Sakhel, *J. Archaeol. Sci.* **2013** ; 40 , 3722–3739.
- 13 M. J. Morwood, W. L. Jungers (eds), *J. Hum. Evol.* **2009** ; 57 , 437–648.
- 14 T. Sutikna, M. W. Tocheri, M. J. Morwood, E. W. Saptomo, Jatmiko, R. D. Awe, S. Wasisto, K. E. Westaway, M. Aubert, B. Li et al., *Nature*. **2016** ; 532 , 366–369.
- 15 M. Moore, T. Sutikna, Jatmiko, M. Morwood, A. Brumm, *J. Hum. Evol.* **2009** ; 57 , 503–526.
- 16 R. L. K. Fullagar, *Use-Wear and Residues on Stone Tools: Functional Analysis and its Application to Two Southeastern Australian Archaeological Assemblages*, PhD thesis, Department of Archaeology, La Trobe University, Melbourne, **1986** ; 382 pp.
- 17 A. Crowther, M. Haslam, N. Oakden, D. Walde, J. Mercader, *J. Archaeol. Sci.* **2014** ; 49 , 90–104.
- 18 K. J. Kingma, R. J. Hemley, *Am Mineral.* **1994** ; 79 , 269–273.
- 19 F. Buciuman, F. Patcas, R. Craciun, D. R. T. Zahn, *Phys. Chem. Chem. Phys.* **1999** ; 1 , 185–190.
- 20 M. W. Morley, P. Goldberg, T. Sutikna, M. W. Tocheri, L. C. Prinsloo, E. W. Saptomo, Jatmiko, S. Wasisto, R. G. Roberts, *J. Archaeol. Sci.* **2017** ; 77 , 125–142.
- 21 P. D. Martino, *The Open Conference Proceedings Journal*. **2016** ; 6 , 14–28.
- 22 A. Hernanz, M. Iriarte, P. Bueno-Ramirez, R. de Balban-Behrmann, J. M. Gavira-Vallejo, D. Calderan-Saturio, L. Laporte, R. Barroso-Bermejo, P. Gouezin, A. Maroto-Valiente et al., *J. Raman Spectrosc.* **2015** ; 37 , 492–497.

- 23 M. B. Tzolov, N. V. Tzenov, D. I. Dimova-Malinovska, D. Y. Yankov, *Appl. Phys. Lett.* **1993** ; 62 , 2396–2398.
- 24 F. Froment, A. Tournié, P. Colomban, *J. Raman Spectrosc.* **2008** ; 39 , 560–568.
- 25 M. Hanesch, *Geophys. J. Int.* **2009** ; 177 , 941–948.
- 26 S. Sebel, M. Miler, S. Skobe, S. Torkar, N. Zupan, *Facies*. **2015** ; 61 , 1–13.
- 27 B. Wopenka, J. D. Pasteris, *Mater. Sci. Eng: C*. **2005** ; 25 , 131–143.
- 28 M. Czaja, S. Bodyl, P. Gluchowski, Z. Mazurak, W. Strek, *J. Alloys Compd.* **2008** ; 451 , 290–292.
- 29 A. Rygula, K. Majzner, K. M. Marzec, A. Kaczor, M. Pilarczyk, M. Baranska, *J. Raman Spectrosc.* **2013** ; 44 , 1061–1076.
- 30 K. Czamara, K. Majzner, M. Z. Pacia, K. Kochan, A. Kaczor, M. Baranska, *J. Raman Spectrosc.* **2015** ; 46 , 4–20.
- 31 M. Pérez-Alonso, M. K. Castro, I. Martinez-Arkarazo, M. Angulo, M. A. Olazabal, J. M. Madariaga, *Anal. and Bioanal. Chem.* **2004** ; 379 , 42–50.
- 32 M. Bradtmoller, A. Sarmiento, U. Perales, M. C. Zuluaga, *J. Archaeol. Sci: Reports*. **2016** ; 7 , 1–13.
- 33 U. P. Agarwal, S. A. Ralph, *Appl. Spectrosc.* **1997** ; 51 , 1648–1655.
- 34 U. P. Agarwal, J. D. McSweeney, S. A. Ralph, *J. Wood Chem. Technol.* **2011** ; 17 , 1–26.
- 35 B. H. Holder, *Characterization of Starch by Vibrational Spectroscopy*, MSc thesis, Department of Food Science and Technology, University of Nebraska, Lincoln, **2012** ; 84 pp.
- 36 C. Daher, C. Paris, A. Le Ho, L. Bellot-Gurlet, J. Echard, *J. Raman Spectrosc.* **2010** ; 41 , 1494–1499.
- 37 H. Edwards, D. Farwell, L. Daffner, *Spectrochim. Acta, Part A*. **1996** ; 52 , 1639–1648.
- 38 F. Pozzi, J. R. Lombardi, M. Leona, *Heritage Science*. **2013** ; 1 , 1–8.
- 39 H. Xhaufclair, A. Pawlik, H. Forestier, T. Saos, E. Dizon, C. Gaillard, *Quaternary International*. **2017** ; 427B , 80–93.
- 40 K. Kavkler, A. Demsar, *Spectrochim. Acta, Part A*. **2011** ; 78 , 740–746.

Table 1: Sample field recovery numbers, estimated ages and initial optical microscopy observations

Field recovery number	Estimated age (thousands of years)	Forms of wear	Likely function based on usewear
XI-45	3	Use-polish, scarring, alignments, striations	Scraping siliceous plant
XI-182	5–8	Use-polish, scarring, edge-rounding	Scraping plant
XI-228	8–12	Unstable edges, slight edge rounding	Not used or possible use only
XI-250	60–100	Use-polish, striations, scarring	Scraping woody plant
XI-337-2	60–100	Unstable edges, scarring, slight edge rounding	Not used or possible use only

Figure Captions

Figure 1: The five artefacts in the pilot study, showing ventral (left) and dorsal (right) views: a) XI-182, b) XI-228, c) XI-337-2, d) XI-45 (inset shows the location of polish in f, e) XI-250, f) Polish from use on a rounded edge of XI-45. Dashed lines indicate the locations of possible utilised edges. Scale bars for a-e are 1 cm, and the scale bar for f is 0.05 mm.

Figure 2: Manganese oxide layer on artefact XI-250: A) Black aspect, B) Metallic aspect. C) Typical Raman spectrum of manganese dioxide obtained on artefact XI-250.

Figure 3: Images of black micro-residues found on artefacts A) XI-228 and B) XI-250. C) Raman spectra recorded on the residues: a) recorded with low laser power and b-e) iron oxide compositions obtained after laser heating. Maghemite (b), haematite (c), haematite + maghemite (d), haematite + maghemite + organic matter (e).

Figure 4: Images of apatite rods: A) on the edge of artefact XI-337-2 and B) in the sediment washed of the artefact. C) Raman spectra recorded on an apatite rod found in sediment attached to artefact XI-337-2 (a), geological apatite reference (b) bone fragment found in sediment washed from artefact XI-337-2 (c) and bone containing collagen (d).

Figure 5: A) Raman spectrum of a typical protein micro-residue obtained on Liang Bua artefacts (a) compared to the Raman spectrum obtained from protein originating from handling the artefacts (b). B) Raman spectrum of a typical fatty acid micro-residue found on Liang Bua artefacts (a) in comparison to contamination from handling (b), stearic acid (c) and palmitic acid (d).

Figure 6: Spectra of proteins and protein / fatty acid mixtures associated with calcium nitrate (characteristic peak at 1048 cm^{-1}): a) Protein micro-residue with high saturated fatty acid content, b) Protein micro-residue with low saturated fatty acid content and c) pure protein micro-residue.

Figure 7: A) Raman spectra of two plant fibres on artefact XI-182: tip surface (a) and on edge (b) (excitation 785 nm). B) Plant fibre micro-residue found on artefact XI-45 striking platform (a) (excitation 785 nm) and left distal edge (b) (excitation 532 nm).

Figure 8: Polished edge and residue distribution on artefact XI-182. Analyses residues had been summarised for each edge. Number of each type of residue for each edge are indicated between ().

Figure 1: The five artefacts in the pilot study, showing ventral (left) and dorsal (right) views: a) XI-182, b) XI-228, c) XI-337-2, d) XI-45 (inset shows the location of polish in f, e) XI-250, f) Polish from use on a rounded edge of XI-45. Dashed lines indicate the locations of possible utilised edges. Scale bars for a-e are 1 cm, and the scale bar for f is 0.05 mm.

140x114mm (150 x 150 DPI)

Figure 2: Manganese oxide layer on artefact XI-250: A) Black aspect, B) Metallic aspect. C) Typical Raman spectrum of manganese dioxide obtained on artefact XI-250.

Figure 3: Images of black micro-residues found on artefacts A) XI-228 and B) XI-250. C) Raman spectra recorded on the residues: a) recorded with low laser power and b-e) iron oxide compositions obtained after laser heating. Maghemite (b), haematite (c), haematite + maghemite (d), haematite + maghemite + organic matter (e).

Figure 4: Images of apatite rods: A) on the edge of artefact XI-337-2 and B) in the sediment washed of the artefact. C) Raman spectra recorded on an apatite rod found in sediment attached to artefact XI-337-2 (a), geological apatite reference (b) bone fragment found in sediment washed from artefact XI-337-2 (c) and bone containing collagen (d).

Figure 5: A) Raman spectrum of a typical protein micro-residue obtained on Liang Bua artefacts (a) compared to the Raman spectrum obtained from protein originating from handling the artefacts (b). B) Raman spectrum of a typical fatty acid micro-residue found on Liang Bua artefacts (a) in comparison to contamination from handling (b), stearic acid (c) and palmitic acid (d).

Figure 6: Spectra of proteins and protein / fatty acid mixtures associated with calcium nitrate (characteristic peak at 1048 cm⁻¹): a) Protein micro-residue with high saturated fatty acid content, b) Protein micro-residue with low saturated fatty acid content and c) pure protein micro-residue.

Figure 7: A) Raman spectra of two plant fibres on artefact XI-182: tip surface (a) and on edge (b) (excitation 785 nm). B) Plant fibre micro-residue found on artefact XI-45 striking platform (a) (excitation 785 nm) and left distal edge (b) (excitation 532 nm).

Figure 8: Polished edge and residue distribution on artefact XI-182. Analyses residues had been summarised for each edge. Number of each type of residue for each edge are indicated between ().

152x114mm (150 x 150 DPI)