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Incorporating terpenes, monoterpenoids and alkanes into multiresidue organic biomarker analysis of archaeological stone artefacts from Liang Bua (Flores, Indonesia)

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ABSTRACT

A sensitive and selective gas chromatography-tandem mass spectrometry (GC–MS/MS) method for the detection and quantification of terpenes, monoterpenoids and alkanes was developed and validated, to complement an existing analytical workflow set up for organic biomarker analysis of stone artefacts. This method was applied to seven stone artefacts—six of which contained potential use-residues based on a previous study using non-volatile low molecular weight lipid biomarkers—recovered from Liang Bua, an archaeological cave site on the Indonesian island of Flores. Following localised solvent extractions of the artefacts, aliquots of the solvent extracts were directly assayed using the optimised method. Identification of an analyte was considered positive when three criteria were met: (1) the retention time was the same as observed for a reference standard; (2) the three selected multiple reaction monitoring transitions for a reference standard compound were observed for the analyte; and (3) the qualitative ions (relative to the quantitative ion) were present in the expected ratios consistent with the reference standard. Alkane chemical profiles indicated that the presence of plant residues cannot be excluded, but of particular interest was the detection of camphor on one of the artefacts. Camphor-containing plants are found throughout Asia, including Indonesia, and are known historically to have been used for medicinal and culinary purposes. The likelihood of resource processing was high for three of the artefacts, and medium for the remaining four artefacts, based on the specificity, quantity and combination of the analytes identified.

1. Introduction

Liang Bua is a large limestone cave on the Indonesian island of Flores that was utilised in various capacities by humans over the past ~190 thousand years (ka) (Morwood and Jungers, 2009; Sutikna et al., 2016). Until about 60–50 ka ago, the cave was used by *Homo floresiensis*, an extinct human species more closely related to modern humans than to chimpanzees and bonobos but whose ancestral lineage likely diverged from that of modern humans, Neandertals, and Denisovans sometime between about 1.5 and 3.0 million years ago (Brown et al., 2004; Morwood et al., 2005; Tocheri et al., 2007; Sutikna et al., 2016; Dembo et al., 2016; Argue et al., 2017). Unequivocal evidence of modern humans is found throughout the site's Holocene deposits (i.e., sediments dated to between 11.7 ka ago and the present day) (Morwood and Jungers, 2009) and new evidence is emerging that suggests modern humans were using the cave by at least 41 ka ago (Morley et al., 2017).

Stone artefacts are present throughout the ~190-ka-old sedimentary deposits at Liang Bua and offer great potential for comparing the behavioural repertoires and subsistence strategies of *H. floresiensis* with those of modern humans. Thus far, detailed analyses of the stone artefacts at Liang Bua suggest there are three main characteristics that distinguish between the stone artefact assemblages of these two hominin species: raw material selection, exposure to fire, and presence of edge-gloss (Moore et al., 2009). In the *H. floresiensis* assemblage,

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artefacts are made predominantly from silicified tuff (a volcanic rock), have rarely been exposed to fire (< 0.5%), and none show any form of edge-gloss (Moore et al., 2009). In contrast, in the Holocene modern human assemblage, chert is the most predominant raw material, exposure to fire is relatively common (approximately 18%), and edge-glossed flakes are present (Moore et al., 2009). Despite these important behavioural distinctions between *H. floresiensis* and modern humans, major questions still remain about how these stone artefacts were used by these two different human species in their day-to-day lives (e.g., for resource processing). Before investigating such questions with confidence, however, sensitive, robust and repeatable methods are required for detecting and analysing organic residues that are adhered to ancient stone artefact surfaces and are the direct or indirect consequence of how such artefacts were actually used.

In a previous study, we developed a comprehensive analytical workflow to monitor and quantify non-volatile low molecular weight lipids on stone artefacts from Liang Bua using gas chromatography-tandem mass spectrometry (GC–MS/MS) (Luong et al., 2017). In addition to an in-house optimised and validated instrumental method, this procedure used a sampling strategy that correlated potential use-residue chemical profiles to specific locations on the artefacts. It also collected chemical profiles contributed by the rocks and surrounding sediments that are most likely unrelated to artefact use. Discriminating between use-residues and sedimentary 'background' residues is critical, because although lipids are useful biomarkers for residue source determination due to their relative stability (i.e., in comparison with proteins and nucleotides), many are abundant in nature and not diagnostic for source identification when used alone (i.e., without the presence of other biomarkers). Of the 14 stone artefacts from Liang Bua studied previously using this analytical workflow, seven showed potential traces of wear based on provisional low-magnification microscopy, but only six of these contained plant and animal biomarkers not typically found in the surrounding sediments (Luong et al., 2017). These results suggested that the latter six artefacts were potentially used as implements for the processing of plant and/or animal material.

In this paper, we extend our previous work by adding another methodological sequence to the analytical workflow, to detect and quantify volatile organic compounds and to maximise the discriminating power of the potential use-residue chemical profiles. This methodological addition collects further chemical evidence to evaluate whether the residues are, in fact, related to artefact use and also provides a means to differentiate between unrelated sources of plant material. We developed and optimised a sensitive and selective GC–MS/MS method for the identification of 46 terpenes, monoterpenoids and alkanes, with method validation carried out for 29 of these analytes. We interpret the resulting biomarker data in combination with the non-volatile low molecular weight lipid data (Luong et al., 2017) and assign the likelihood of tool use to each of the potentially used stone artefacts, based on the specificity, quantity and combination of the analytes identified.

2. Materials

2.1. Reference standards and chemical reagents

Reference standards of myrcene, (*R*)-(+)-limonene, (+)-α-pinene, γ-terpinene, farnesene, squalene, (−)-carvone, (*1S*)-(−)-verbenone, citral, camphor, linalool, geraniol, eucalyptol, a C_8–C_{40} alkanes calibration standard (500 μg/mL in dichloromethane), squalane (99%) and 2,2,4,4,6,7,7-heptamethylnonane (HMN, 98%) were sourced from Sigma Aldrich (Castle Hill, NSW, Australia). All terpenes were analytical standard grade, except for linalool (certified reference material, TraceCERT®), camphor (96%), squalene (≥98%) and farnesene (mixture of isomers). Methanol (HPLC grade) was purchased from Thermo Fisher Scientific (Scoresby, VIC, Australia). Chloroform (HPLC grade) was sourced from VWR International (Tingalpa, QLD, Australia).

3. Experimental methods

3.1. Preparation of reagent blank and calibrator standards

A mixed stock standard (316.4–390.0 μg/mL) was prepared by diluting 20 μL aliquots of the neat liquid (terpenes and monoterpenoids) and camphor solid in chloroform/methanol (3:1 v/v). A working solution (12.656–15.600 μg/mL) was made by dilution of the stock standard. Using the working solution, calibrator standards were then prepared by serial dilution (a minimum of five levels for each analyte, ranging between 0.137 pg/mL and 15,600 ng/mL). For the alkanes, calibrator standards (2–2000 ng/mL, with at least five concentration levels) were prepared by dilution of the commercial standard. With the internal standards, stock solutions of HMN (3172 μg/mL) and squalane (3240 μg/mL) were prepared and further diluted to make working solutions at 15.86 μg/mL and 16.20 μg/mL, respectively. To prepare the calibration set containing the reagent blank and calibrator standards, 50 μL of each of the appropriate working solutions (or just chloroform/methanol (3:1 v/v) solvent for the reagent blank) were fortified with 2.6 μg/mL of HMN and 2.7 μg/mL of squalane, mixed with a vortex mixer and injected directly into the GC–MS/MS.

3.2. Preparation of quality control standards for method validation

Quality control (QC) standards were prepared independently from the calibrator standards. Two QC levels were evaluated for each analyte, using five replicates at each level, at approximately 350 ng/mL and 3500 ng/mL for the terpenes and monoterpenoids (see Table 1 for exact QC concentrations for each analyte), and 50 ng/mL and 500 ng/mL for the C_{10}–C_{26} saturated alkanes. As with the calibrator standards, 50 μL of each of the working solutions were spiked with internal standards, mixed and analysed by GC–MS/MS. Method validation consisted of the assessment of inter-day and intra-day accuracy and precision, as well as the limit of detection (LOD), limit of quantification (LOQ) and upper limit of quantification (ULOQ) of the method.

3.3. Stone artefact recovery and residue extraction

Seven stone artefacts (Fig. 1) recovered during archaeological excavations at Liang Bua in 2015 were analysed (field recovery numbers: XXIV-67, XXV-3931, XXV-3954, XXV-3956, XXV-4025, XXVI-4411 and XXVI-4414). These artefacts were initially chosen for chemical analysis because they show evidence of manufacture by hard-hammer percussion (i.e., stone flaking) and provisional low-magnification microscopy of the unwashed artefacts indicated the presence of observable wear along the edges (Luong et al., 2017). Artefacts XXIV-67, XXV-3931, XXV-3954, XXV-3956 and XXVI-4414 are flakes, XXV-4025 is a broken retouched flake and XXVI-4411 is a retouched flake. The long axis length range of these artefacts is 3–7 cm. Residues were first removed from the surfaces of each artefact in a localised manner by first immersing the potentially used edge(s) in the extraction solvent (Fig. 1). Subsequently, each artefact was totally submerged in the extraction solvent. Details related to the recovery and solvent extraction volumes associated with these artefacts, as well as a complete explanation of the strategy behind the residue extraction procedure, are provided elsewhere (Luong et al., 2017). Sample extracts were filtered through 0.22 μm hydrophobic syringe filter units (MicroAnalytix: Taren Point, NSW, Australia) and aliquots (50 μL) of each of the solvent extracts were spiked with HMN and squalane internal standards as described for the calibration set. The filtered sample extracts were then analysed directly by GC–MS/MS.

3.4. GC–MS/MS analysis

An Agilent Technologies 7890 GC system fitted with an Rxi®-5Sil MS 1,4-bis(dimethylsiloxy)phenylene dimethyl polysiloxane fused