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Assessing use and suitability of scanning electron microscopy in the analysis of micro remains in dental calculus

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ABSTRACT

Dental calculus is increasingly recognized as a major reservoir of dietary information. Palaeodietary studies using plant and animal micro remains (e.g. phytoliths, pollen, sponge spicules, and starch grains) trapped in calculus have the potential to revise our knowledge of the dietary role of plants in past populations. The conventional methods used to isolate and identify these micro remains rely on removing them from their microenvironment in the calculus, thus the microenvironment that traps and preserves micro remains is not understood. By using scanning electron microscopy and energy-dispersive X-ray spectroscopy (SEM–EDX) on modern chimpanzee calculus from the Taï Forest, Côte d'Ivoire, and human calculus from the Chalcolithic site of Camino del Molino, Spain, we present the first reported observations on characteristics of the matrix setting that are conducive to the survival of starch in dental calculus. We also assess the potential for SEM–EDX to detect starch and differentiate it from structurally and molecularly similar substrates. We demonstrate that SEM–EDX may offer a non-destructive technique for studying micro remains in certain contexts. Finally, we compare traditional optical analytical techniques (OM) with less invasive electron microscopy. The results indicate that SEM–EDX and OM are both effective for observing micro remains in calculus, but differ in their analytical resolution to identify different micro remains, and we therefore recommend a sequential use of both techniques.

1. Introduction

Dental calculus, or dental plaque calcified by salivary calcium phosphate, was first noticed as a reservoir of dietary information when Armitage (1975) recognized plant remains on the teeth of archaeological ungulates. Dobney and Brothwell (1986, 1988) later demonstrated the value of calculus in the study of human diets. Analysis of plant and animal micro remains in archaeological dental calculus is a rapidly growing field in dietary reconstruction (e.g. Boyadjian et al. 2007; Henry, 2012; Liu, 2012; Mickleburgh and Pagán-Jiménez, 2012; Warinner et al. 2014). Researchers have reported starch, phytoliths, pollen, diatoms, chrysophycean cysts, sponge spicules, and mineral particles in human calculus up to tens of thousands of years old (e.g., Boyadjian, 2012; Dobney and Brothwell, 1988).

Despite this interest in dental calculus as a source of dietary information, there are still many questions about the mechanisms by which plant micro remains, particularly starch grains, are preserved within the calculus. Native starch grains (i.e., starches in their original, unaltered state) are the major focus of many recent and ancient dietary studies (Leonard et al., 2013). Starch is a foremost nutritional component in many human and non-human primate diets, and it can also survive in the archaeological record over long periods of time due to its semi-crystalline polysaccharide structure (Hardy et al. 2009; Henry et al. 2011; Mercader et al. 2008; Salazar-García et al. 2013). The means by which starch embeds and preserves in calculus is still unclear. The mouth is a hostile environment for starch preservation because of the action of

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salivary digestive enzymes and bacterial metabolic activity (Lukacs and Largaespada, 2006). Calculus forms gradually as bacteria-rich plaque biofilms mineralize from calcium phosphate in the saliva over a period of days to years (Abraham et al. 2005). During this formation and mineralization process, the starch grains are exposed to α-amylase, which is present in the saliva of humans and several orders of mammals (Butterworth et al., 2011). Amylase quickly digests starch by breaking down the polysaccharide crystalline structure into various simple and complex sugars through hydrolysis (Lukacs and Largaespada, 2006). Theoretically, starch may avoid oral digestion and survive in protected niche areas in calculus, but this has not yet been empirically confirmed.

In addition to the difficulties with starch preservation in the oral cavity, there is also the possibility that the starches that have been recovered from calculus are actually the result of modern contamination. Modern starches are abundant in the air, water, and working surfaces of most facilities, making environmental contamination a strong possibility. Archaeological and field site contexts suffer from sources of contamination such as airborne starch rain, but the greatest risk of contamination comes from excavation and post-excavation handling in the presence of food or due to the use of gloves powdered with corn or other starches (Laurence et al. 2011; Loy and Barton, 2005; Newsom and Shaw, 1997).

Currently, the standard methodology for starch grain recovery from calculus is too destructive to confirm whether observed starch came from the calculus or from contamination. This method involves mechanically or chemically removing calculus from the tooth, grinding or dissolution to break up the sample, and finally examining the particles using optical light microscopy (OM) (Henry and Piperno, 2008). Furthermore, to the untrained eye, several other calculus components, such as cysts, mineral grains, fungal spores, wood cells, and air bubbles may be confused with starch grains when viewed only under OM. Some have proposed confirming starch presence by measuring amylase activity on treated samples (Hardy et al. 2009), but this enzyme destroys the starch in the process. One common and reliable means to detect starch is to apply iodine potassium iodide (IKI) solution, which binds to the amylose molecule, and look for the characteristic blue–black stain. However, this temporarily obscures the starch's diagnostic surface features. Furthermore, it is impractical to apply a staining solution to an intact calculus matrix because objects within the mineralized matrix are protected from moisture. Accordingly, there is a great need for more sophisticated and non-destructive methods to confirm the successful detection of starch grains in dental calculus. Some researchers have suggested the possibility of using scanning electron microscopy (SEM) to study plant micro remains in calculus (Dobney and Brothwell, 1986; Reinhard et al. 2001; Tromp, 2012). Despite the success of this method in locating phytoliths (Arensburg, 1996; Charlier et al. 2010; Lalueza-Fox et al. 1996; Kucera et al. 2011; Tao, 2011; Tromp, 2012), the detection of starch grains through SEM has not yet been attempted.

In this study, we present SEM coupled with energy-dispersive X-ray spectroscopy (EDX) as a novel means for identifying starch and other micro remains in intact human and chimpanzee dental calculus. This system provides us with the ability to identify micro remains, including starch grains, by their morphology and elemental composition *in situ* in the calculus, thus ruling out contamination. It also allows us to explore the kinds of environments within the calculus that may permit starch preservation. Furthermore, we examine the potential of EDX to detect starch by comparing the elemental makeup of native starch to those of saliva-hydrolysed starch and other non-starch saccharides to learn whether EDX distinguishes starch from other polymers of similar elemental makeup. This identification allows us to positively show that starch grains survive in calculus. Finally, we compare the results from SEM–EDX to those from OM on the same human calculus samples to determine whether these techniques offer comparable or complimentary results. Due to time constraints, we were unable to conduct this portion of the analysis on the chimpanzee samples and instead only used human dental calculus samples.

2. Materials and methods

2.1. Study groups

The calculus samples were obtained from two groups, modern wild chimpanzees from the Taï Forest (Côte d'Ivoire) and humans from the Chalcolithic collective burial of Camino del Molino in Spain (Table 1). We chose these two test groups for multiple reasons: 1) individuals from both have abundant calculus on their teeth; 2) they represent modern (chimpanzee) and archaeological (Chalcolithic humans) timeframes; and 3) both groups maintained very different dietary strategies and should therefore have different microfossil profiles.

The sample of chimpanzee calculus came from the Taï Chimpanzee Osteology Collection curated at the Max Planck Institute for Evolutionary Anthropology (MPI-EVA) in Leipzig, Germany. The behaviour of the wild chimpanzees living in the Taï Forest has been monitored and documented since the commencement of the Taï Chimpanzee Project in 1979 (Boesch and Boesch-Achermann, 2000). Taï Forest data collection complied with the requirements and guidelines of the Ministère de l'Enseignement Supérieure et de la Recherche Scientifique, and adhered to the legal requirements of Côte d'Ivoire. The osteology collection contains 77 chimpanzees. We chose calculus samples from individuals who had comprehensive observational records documenting diet, sex and age. After their death, the remains of these individuals were interred for defleshing and later exhumed and curated. We collected calculus from molars or canines of six individuals; two females and four males. The Taï Chimpanzees consume native starch from wild nuts and seeds such as the Gabon nut (*Coula edulis* Baill.) and Kola nut (*Cola nitida* (Vent) Schott et Endl.) (Hohmann et al. 2010; N'guessan, 2012), and unlike humans, they consume no cooked or processed foods. Our preliminary reference collection of Tai Forest

Table 1
Calculus samples analysed using SEM–EDX and OM. Sex and age classification of the Camino del Molino remains are preliminary (Haber Uriarte et al., 2013).

Lab identifier	Individual number	Type	Tooth	Sex	Age	Weight
SJ-13-32	Sujeto 6	Camino del Molino	P1 mandible	F	26–28	1.76 mg
SJ-13-33	Sujeto 8	Camino del Molino	C maxilla	M	22–24	0.51 mg
SJ-13-36	Sujeto 11	Camino del Molino	I2 mandible	?	?	6.06 mg
SJ-13-37	Sujeto 17	Camino del Molino	M2 mandible	M?	43–55	10.0 mg
SJ-13-38	Sujeto 113	Camino del Molino	M2 mandible	F	24–28	1.88 mg
SJ-13-39	Sujeto 151	Camino del Molino	C mandible	M	30–35	1.09 mg
Venus	15001	Taï chimpanzee	M3 maxilla	F	27	0.72 mg
Leo	15012	Taï chimpanzee	M3 mandible	M	19	1.19 mg
Fanny	11780	Taï chimpanzee	M3 mandible	F	25	3.34 mg
Goma	15004	Taï chimpanzee	M3 mandible	F	28	2.40 mg
Rubra	15023	Taï chimpanzee	M3 mandible	F	38?	3.88 mg
Castor	13439	Taï chimpanzee	M3 mandible	F	22	2.25 mg

chimpanzee foods shows that ten of the 82 foods we have analysed are starch-rich. However, these 82 species represent less than a third of plants this population is known to consume, and we are still building this reference collection. Chimpanzees also produce salivary amylase, though likely at much lower quantities than do humans (Behringer et al. 2013; Perry et al. 2007).

Camino del Molino is a Chalcolithic collective burial pit found during construction work in the city of Caravaca de la Cruz (Murcia, southeast Spain). Radiocarbon dates from bone collagen samples spanning the burial sequence indicate that the site was in continual use over a span of 300–400 years during the first half of the third millennium B.C. The site contained a minimum of 1300 individuals, likely the remains of 16–20 generations of one population buried at one place (Lomba Maurandi et al., 2009). Approximately 30% of the individuals are classified as juvenile (<14 yrs.), and the rest are adults spanning from young to old (Haber Uriarte et al., 2013). We collected dental calculus preferably from lower molars for standardisation from the teeth of six individuals; two female, two male, and two individual of unknown sex (Table 1). There are no archaeobotanical studies from Camino del Molino or from the broader region of Murcia contemporary to the site. However, studies of Late Neolithic and Chalcolithic deposits in neighbouring regions suggest that the number of cultivated species is low and consists mainly of naked wheat (*Triticum* sp.), barley (*Hordeum vulgare* L), some lentil (*Lens culinaris* Medikus) and common vetch (*Vicia sativa* L) (e.g. Pérez Jordà, 2005; Pérez Jordà and Carrión Marco, 2011). There is no published study from the site on culinary practices, in part because it is a necropolis and not a habitation site. Despite this, its Chalcolithic age indicates that this population consumed cooked food, because cooking is widespread across the European Neolithic and Bronze Age societies (Halstead, 2012; Thissen et al. 2010).

2.2. Calculus sampling

We selected teeth encrusted with a prominent band of calculus present on the enamel surface. We sampled only supragingival calculus (above the gum line), since it is unclear if subgingival calculus (below the gum line, on the neck of the tooth) preserves food remains. We photographed the calculus before sampling, and then brushed the sample tooth gently with a dry, sterile toothbrush to remove surface contaminants. We then used a dental scaler to remove small areas of supragingival calculus (~4 mm area), from the enamel. We conducted all calculus sampling in a positive pressure hood at the archaeological science laboratories at the MPI-EVA. We then weighed each of the samples and transferred them to microcentrifuge tubes for storage until further use. Following sampling, the teeth and surviving calculus were photographed again. Additionally, we collected control samples, including the packing material in which the teeth had been stored.

2.3. Electron microscopy analysis

We conducted the SEM–EDX analysis at University College Dublin's Nano-Imaging and Materials Analysis Centre (NIMAC) in Dublin, Ireland. The calculus samples were mounted on stubs using double-sided carbon tape, and sputter coated with gold for 20 s using an Emitech K575X Sputter Coating Unit, to prevent surface charging by the electron beam. We then examined the calculus using a FEI Quanta 3D FEG DualBeam (FEI Ltd, Hillsboro, USA) SEM with an attached EDAX ED APOLLO XV Silicon Drift Detector with a 5–10 kV accelerating voltage. EDX detected and documented most elements of interest excluding hydrogen, which is non-detectable with this method. We omitted the gold elemental peak from each spectrum since the gold was added during sputter coating. We photographed and documented every tentative micro remain and later described our observations.

2.4. Optical microscopy analysis

We performed optical microscopy on the ancient human remains at the Plants Working Group laboratory in the MPI-EVA, Leipzig. We removed the gold plated calculus samples from the SEM mounting stubs, and then ground them in a 5 ml Eppendorf microcentrifuge tube with a micro pestle containing ~30 mL of a 25% glycerine solution to reduce sample loss due to static electricity. The samples were then centrifuged at 1691× *g* (Heraeus MEGAFUGE 16 with TX-400 Swinging Bucket Rotors) for 10 min. All of the resulting pellets were mounted on glass slides and examined under brightfield and cross-polarized light on a A1 Zeiss Axioscope microscope at 400× magnification. Larger samples were mounted on several slides. Each micro remain was photographed and described.

2.5. Carbohydrate reference standards and partially hydrolysed controls

We used a variety of reference standards (see Table 2) to assess the accuracy of EDX reads on the experimental sample types of starch. Starches from a variety of plants were selected to represent major starch types such as corn starch, potato starch, and common dietary components for each population (Boesch and Boesch, 1983): wheat (*Triticum aestivum* L.), Gabon nut (*Coula edulis* Baill.), Xylia (*Xylia evansii* Hutch.) and Kola nut (*Cola nitida* [Vent] Schott et Endl.). The nuts were ground, dried and weighed to derive nut flour suitable for use. Wheat, potato and corn were purchased from local distributors in Germany (Table 2).

Laboratory-grade fructose, sucrose, maltose and glucose (Roth, Germany) were included as standards because they have nearly identical elemental compositions as starch but with structurally different molecular arrangements (e.g. sucrose has 2.1 wt % (mass fraction) more carbon than fructose, but 2.1 wt % less than starch).

To compare EDX element signatures for the different types of saccharides, we took EDX measurements from five individual grains of fructose, sucrose, maltose, glucose, wheat starch, corn starch, Kola nut starch, Xylia starch and potato starch. This allows the comparison of monosaccharides, a disaccharides, and a polysaccharide (starch).

Finally, to assess whether EDX signatures and detection accuracy is affected by the salivary modification (hydrolysis) of starch, we experimentally hydrolysed the native starches from the wheat

Table 2
List of reference samples analysed using EDX.

Reference sample	Part	Type	Source
Fructose	N/A	Lab-grade	Roth – 4981.1
Sucrose	N/A	Lab-grade	Roth – 4621.1
Maltose	N/A	Lab-grade	Roth – 8951.1
Glucose	N/A	Lab-grade	Sigma – G7528
Maize (*Zea mays* subsp. *Mays* L.)	Grain	Cornstarch	Speisestärke, RUF Lebensmittelwerk
Kola nut (*Cola nitida* (Vent) Schott et Endl.)	Nut	Bulk plant	Collected in Taï National Park
Xylia (*Xylia evansii* Hutch.)	Nut	Bulk plant	Collected in Taï National Park
Gabon nut (*Coula edulis* Baill.)	Nut	Bulk plant	Collected in Taï National Park
Potato (*Solanum tuberosum* L)	Tuber	Flour	Kartoffelmehl, RUF Lebensmittelwerk KG
Wheat (*Triticum aestivum* L.)	Grain	wheat starch	Weizella, Hermann Kröner GmbH

flour and both nut varieties using salivary amylase derived from human saliva – a simulation of the effects of oral digestion on starch that can occur. One of us (R.C.P.) provided the saliva used in all experiments, which was collected on a single occasion. We split each of the individual plant samples into nine subsamples of approximately 2 mg each: three subsample per plant remained untreated (control), three were exposed to amylase (35 μL of saliva) for 30 min, and three were exposed to amylase (35 μL of saliva) for 90 min. We also similarly partitioned the wheat flour into nine aliquots into three subsamples of 2 mg each for identical amylase treatment. We ceased hydrolysis by displacing the saliva with alcohol and centrifugation at 1691× *g* to remove as much liquid from the sample as possible and stop hydrolysis. Then the remaining alcohol was evaporated at 35° Celsius in a drying oven. We performed measurements using SEM–EDX in triplicate on one starch grain from each subsample, creating nine readings per category (e.g. wheat 30 min hydrolysed). A summary of these analyses is provided in Figs. 1 and 2.

3. Results

3.1. Standards

The EDX spectrum of starch is distinct from other saccharides but not sufficiently to permit reliable identification (Fig. 1; S1.1). The EDX results from all the samples indicate that oxygen is underrepresented. Though carbon comprises roughly 40–50 wt % of these saccharides, the EDX spectra indicates carbon at 60–90 wt % (Fig. 1). Comparing the short-chain saccharides to the starches there is a difference but some type of starch overlaps with each short-chain saccharides. This indicates that some starch may be distinguishable from short-chain saccharides through EDX. There was far more variability in carbon values in starch than in short-chain saccharides. Starch is composed of oxygen, hydrogen and carbon $(C_6H_{10}O_5)_n$, where *n* ranges from 300 to 1000, so starch is approximately 42.1% = carbon, 6.5% = hydrogen, 51.4% = oxygen (Newman et al., 1996). Thus maize starch comes closest to the expected values of starch. This variability possibly reflects the heterogeneous nature of the starch. Starch varies in both proportion of amylose and amylopectin and minor compounds such as proteins and lipids (Belitz et al. 2009). We see further evidence of this elemental variability in the native starch samples in Fig. 2, which had a higher variability of both carbon and oxygen than the hydrolysed starches. The EDX profiles of hydrolysed starches fall within the range of their native counterparts, yet they show noticeable less variation and reduced oxygen values (Fig. 2). The reduction in variation and lower oxygen levels in these samples may be either from the result of the added ethanol reducing oxygen in the starch or the ethanol washed off debris on the starch surface. A few of the damaged starches have slightly increased oxygen percentages, but this is not consistent across all hydrolysed subsamples. We found no evidence that saliva-activated hydrolysis could obscure starch's EDX elemental signature. Thus when large starch shaped objects are present under SEM, it is possible to test whether these particles have a molecular make-up that is similar to starch and other saccharides.

3.2. Calculus samples

Examination of the SEM images of the calculus confirmed that this matrix has a heterogeneous texture, with many pores, cracks and crevices. Most of the pores appear to be the result of rod bacterial pseudomorphs, which are shallow and measure only between 0.3 and 1 μm in width (bottom left in Fig. 4 and widely scattered in Fig. 5). Although these pores are too small to shelter and preserve micro remains, the cracks and crevices often contained them (Fig. 4). Further examination of the calculus revealed several types of inclusions within the matrix. In some cases, these inclusions were consistent with the overall size (15–40 μm) and shape (ovoid to pyriform) of certain starch grain types, and inconsistent with other micro remains such as yeast and bacterial cells (Fig. 3). The supposed starch clusters were clearly embedded in the matrix, with grains occluded by overlying deposits of the matrix material. Interestingly, the starch grains were not evenly

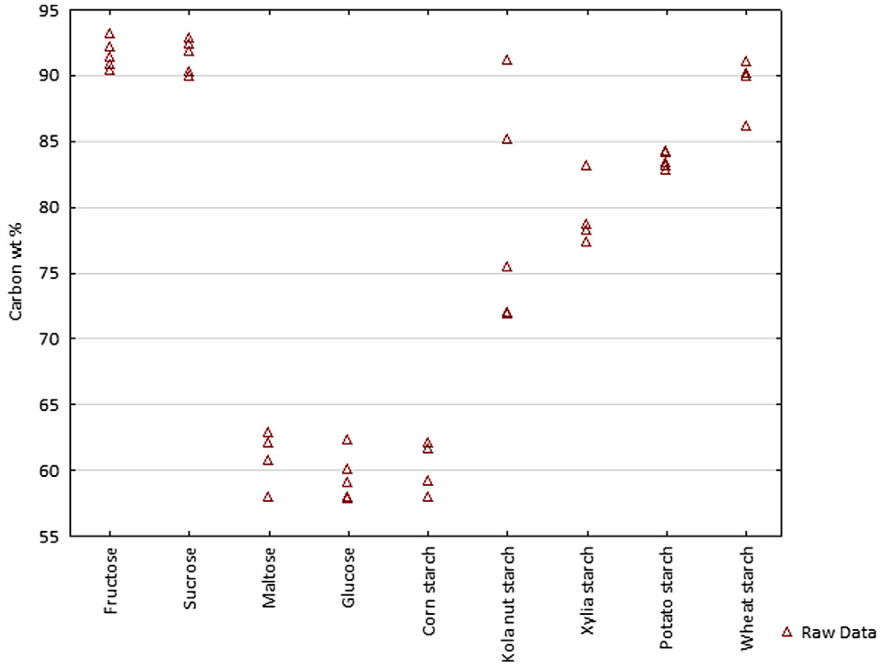

Fig. 1. Plot of carbon wt % (mass fraction) from five individual grains of fructose, sucrose, glucose, maltose and various starches detected with EDX. Percent values exclude largely minor contaminating elements such as potassium from sweat (S1.1).

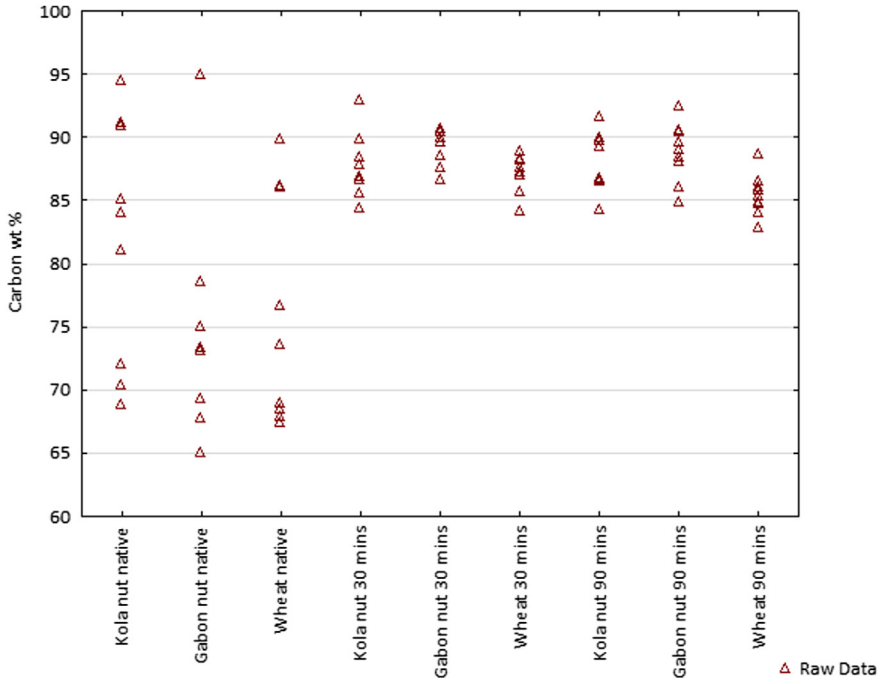

Fig. 2. Plot of carbon wt % comparing native starch versus samples that were hydrolysed with amylase for 30 and 90 min at room temperature. Three starches were sampled with triplicate readings. Percent values exclude largely minor contaminating elements such as potassium such as potassium from sweat (S1.2).

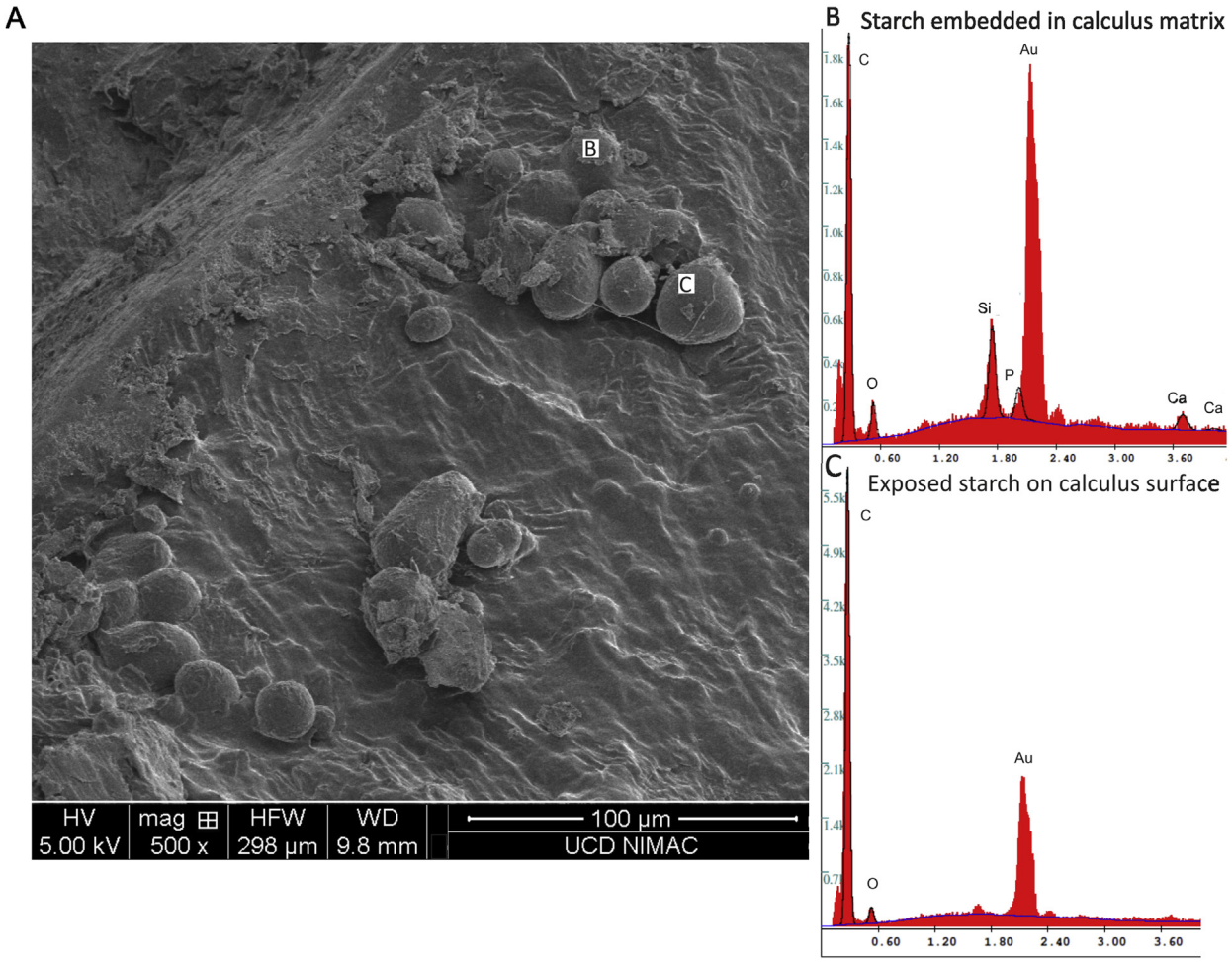

Fig. 3. (A) SEM image showing a group of starches trapped in the matrix of one of the chimpanzee dental calculus samples (Venus), with the corresponding EDX spectrum (right) showing a calcium phosphate and silicon mantle covering a carbon rich starch (B) and solely a carbon-rich starch (C).

Fig. 4. SEM image showing a concentration of pollen (A) and sponge spicules (B) in SJ-13-39 from Camino del Molino. Micro remains were often found clustered.

distributed in the matrix, but often appeared in clumps (Figs. 3 and 4). This could be explained in two ways; i) plant micro remains are deposited in groups originating from clumps in food lumps, or ii) micro remains are only preserved in localised niches, such as larger cracks and crevices, in the calculus matrix.

The EDX spectra of the calculus matrix from all of our samples indicate that it is mostly composed of calcium and phosphorus (S1.3), with trace amounts of aluminium, magnesium, silicon, sodium and manganese. These elements confirm our supposition that the majority of our samples consist of calculus, a mixture of hydroxyapatite and other minerals, rather than contaminating exogenous matter (Charlier et al. 2010; Salazar-García et al. 2014). In some instances, silicon was locally abundant in the calculus (Fig. 3, S1.3), which may be important for the preservation of starch grains. In contrast to the mineral matrix, the suspected starch clusters, such as on chimpanzees Venus and Castor, had significant carbon peaks (Fig. 3). Additionally, the starches often had calcium and phosphorus peaks, reinforcing visual observations that they were indeed embedded in calculus (Fig. 3). The combination of shape and elemental data (Fig. 3) is strongly suggestive of *in-situ* findings of micro remains preserved in the dental calculus environment. This is possible as starch is morphologically distinct from other carbon rich particles such as fungal filaments, *Candida albicans* cells, cellulose and sugars. We also note that the starch we located with SEM–EDX was undamaged (Fig. 3) and we did not locate any gelatinized or hydrolysed starch.

In addition to the starches, we also identified a variety of other plant and animal micro remains preserved in the calculus using SEM–EDX, including phytoliths, sponge spicules, diatoms and pollen (Table 3). These micro remains were identified by their diagnostic morphology using conventional methods (e.g. Nadel et al. 2013; Power et al. 2014; Rosen, 2010; Torrence and Barton, 2006), and this identification was confirmed by their EDX spectra. For example, spicules were easily identified based on their long rectangular shape and high level of regularity (Figs. 4 and 5) unlike smooth long-cell phytoliths, and EDX readings confirmed their biogenic silica composition (S3).

OM also demonstrated the presence of a rich assemblage of plant micro remains (Table 3). Some of these micro remains were also seen during the SEM analysis, such as the abundant monoaxon spicules (Fig. 4), but some, such as multi-cell long-cell phytoliths, unsilicified plant cells and calcium oxalate (Fig. 6), were only detected with OM.

A comparison of the micro remains observed under SEM–EDX with those seen in OM revealed important differences (Table 3). We observed more starch micro remains using OM than SEM–EDX. This is probably because the sample preparation for OM breaks down the calculus matrix, freeing starch micro remains that were trapped in the middle of the calculus chunk. Yet paradoxically, other micro remains, such as sponge spicules, were more commonly seen in SEM–EDX than in OM of the same samples.

Based solely on the SEM results (we did not perform OM on the chimpanzee samples), the two groups we studied did present some differences. The chimpanzee samples were rich in starch grains and diatoms, while the human samples had an abundance of unsilicified plant cells and sponge spicules (Table 3).

4. Discussion

Analysis of calculus samples by SEM–EDX and OM provides data that validates the study of micro remains recovered from this biological material. By SEM–EDX, we were able to identify the elemental constituents of starch, and confirm its position *in situ* in calculus particles. This is the first time that starch has been identified by its elemental signature while still embedded within the

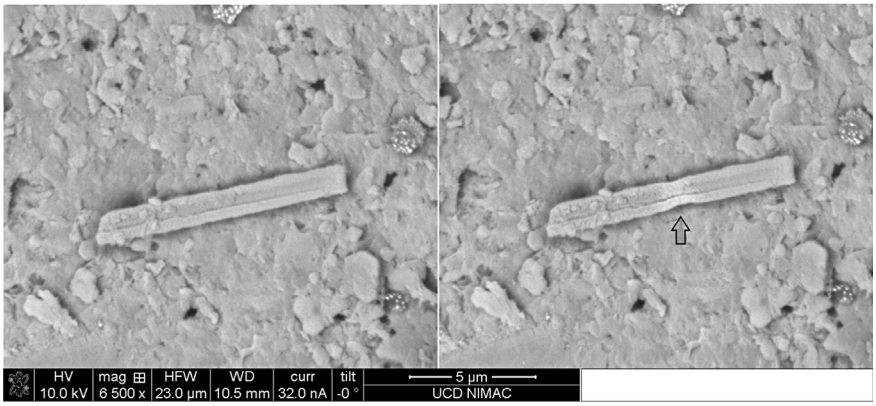

Fig. 5. SEM image showing localised damage that arises from higher primary voltage SEM (10 kV) and EDX on a spicule in calculus from Camino del Molino. Before (left); after (right).

Table 3
Counts of recovered micro remains using both microscopy approaches.

		Scanning electron microscopy												Optical microscopy					
		Taï chimpanzees						Camino del Molino						Camino del Molino					
		Venus	Leo	Fanny	Goma	Rubra	Castor	SJ-13-32	SJ-13-33	SJ-13-36	SJ-13-37	SJ-13-38	SJ-13-39	SJ-13-32	SJ-13-33	SJ-13-36	SJ-13-37	SJ-13-38	SJ-13-39
Starch		29	2	3	4	40	22	3		1		1	6	1		8	10	1	3
Phytoliths	Single-cell long-cell			1				1		1	1	2		3		5	10	1	3
	Multi-cellular long-cell													1			11	1	
	Short-cell			1										3	1		2		3
	Parallelepipedal							1		1				1		1	6	1	
	Bulliform													1			2		
	Plate							1		1								1	
	Rugulose spheroid		2			1													
	Smooth spheroid							3		2			1					1	1
	Hair										2	1			1	1		1	
	unidentified							1			1		3	2					3
Unsilicified plant cell																	15		
Prism calcium oxalate																5	8		2
Annular ring															2				
Monaxon spicule								30	1	5	1	15	46	8	5	14	18	11	10
Quartz grain			1	2															
Pennate diatom						20													
Other diatoms				2														2	
Echinate pollen					1		1					1	3					3	
Other pollen										3		1	1	3	1	1	2		
Chrysophycean cyst				4															
Fungal filaments			[a]	[a]															
Fibre		[a]						1											
Invertebrate		1																	
Other						2													

[a] Several unquantified microremains.

calculus matrix, and confirms that starch can be preserved in calculus, and can therefore be a reliable source of dietary information.

The analysis suggests that certain features of the calculus may promote the preservation of microfossils, and starch grains in particular. While the pores caused by bacteria were too small to provide a protected niche for starches, larger cracks and crevices were full of micro remains, possibly because these areas provided a protected environment. Furthermore, the silicon we detected in the dental calculus may be significant. Silicic acid can induce spontaneous precipitation of calcium phosphate in the saliva, which is the precursor mineral necessary for calculus formation. Silicic acid may be consumed directly via water or indirectly via plants, as it enters plants along with groundwater. Consuming polysilicic acid and silica increases calculus formation, thereby regulating this process (Damen and Ten Cate, 1989; Jin and Yip, 2002; Roberts-Harry and Clerehugh, 2000). Our observations of silicon concentrations adjacent to embedded starch clusters (Fig. 3) corroborates these reports, suggesting that dietary exposure to silica or silicic acid enables enhanced calculus formation and thus the preservation of native starch in dental calculus.

By following the SEM analysis with an OM examination of the same samples, we are able to compare the effectiveness of each for specific micro remain types. Sponge spicules were easily visualized under SEM, but were seen less with OM. This may be because the spicules are relatively fragile and are damaged when the calculus is processed, possibly explaining why spicules are rarely reported in dental calculus studies (Dudgeon and Tromp, 2012; Tromp, 2012). Because these particles, as well as diatoms and Chrysophycean cysts, are highly dependent upon water sources, they may indicate source type and provenance of consumed water, making them powerful potential ecological markers for primatology and archaeology studies (Dudgeon and Tromp, 2012). In contrast, calcium oxalate crystals were only visible under OM, and not SEM. These crystals, which may occur as druses, raphides or other similar forms, are a potentially useful marker of plants. They may be more visible using OM because they have high interference colours that are visible under cross polarized light (Fig. 6). For reasons that remain unclear, calcium oxalate is rarely reported or discussed in calculus literature. Some research indicates that calcium oxalate does not survive due to acidity in the mouth (Tromp, 2012), but given their sheer abundance in plants and the relatively neutral oral pH, it is likely that calcium oxalates do survive and are simply overlooked. On the other hand, starch grains were clearly visible using both SEM and OM. However, we did note that within individuals, the starches that we observed under OM typically did not match the size and morphology of those seen in SEM–EDX. This contrasts with the spicules, which often matched size and shape.

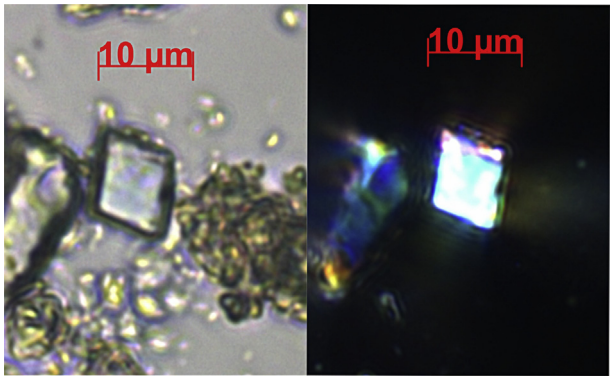

Fig. 6. A calcium oxalate prism observed with optical microscopy in SJ-13-37; under brightfield optical microscopy (left), and polarising optical microscopy (right).

This is likely due to the small number of starches but high number of spicules. We did also observe pollen grains embedded in the calculus using SEM (Fig 4) and OM. Although this type of pollen grain were too small to analyse with the EDX, we do believe that SEM–EDX may be appropriate for identifying many larger types of pollen grains, since these plant remains are composed of potassium, magnesium, sodium and calcium (Szczęsna, 2007) and should be easily visible in the EDX spectra.

Finally, The SEM analysis accurately reflected some stark differences between our study groups. The differences in microfossil number and types between the chimpanzee and humans likely reflect the dietary behaviour and the age of the remains. The chimpanzees consumed only raw plants, while the human group potentially cooked much of their food. The chimpanzees therefore consumed many more native, undamaged starch grains, and so there is greater opportunity for the preservation of native starch grains in dental calculus. Though the humans may have consumed more starch overall, many of these starches would have been gelatinized through cooking, disrupting the semi-crystalline structure and reducing the potential for starch preservation in the mouth (Holm et al. 1988). Cooking, combined with higher levels of salivary amylase in humans relative to chimpanzees (Behringer et al. 2013; Perry et al. 2007) may have greatly reduced the relative proportion of starch entering the human calculus matrix during its formation. Furthermore, the chimpanzee samples are modern and likely to be well-preserved while starch in the human calculus may have depleted due to digenesis over thousands of years.

Overall, SEM–EDX does allow us to visualize and identify micro remains embedded in dental calculus, but this technique is not without limitations and constraints. Internal features of starch grains that are vital for identifying the taxonomic origin of the starch are not visible under SEM. We found that when using EDX combined with higher primary voltage (10 kV), the beam moved or damaged fragile micro remains such as spicules (Fig. 5). EDX can only give reliable data on objects $\geq$4 µm due to the penetration of the beam, making it impossible to measure very small micro remains including smaller starches. We found other techniques such as backscatter detection to be of little additional advantage in detecting starch, though this method may be useful in certain contexts such as examining calculus for embedded phytoliths (Tromp, 2012). It is possible to examine only the surface portion of intact calculus matrix using SEM–EDX, and so this is not a viable method for visualizing interior dental calculus structure and micro remains. Sample preparation may also be destructive since samples must be gold-plated and mounted, but use of SEM without the plating may cause the sample image quality and identification power to deteriorate.

5. Conclusions

The visual identification and subsequent elemental testing of micro remains embedded in the dental calculus of humans and chimpanzees suggests that these important dietary markers are indeed trapped and preserved in calculus during the lifetime of the individual. Clearly, this matrix has a protective quality that shields fragile and degradable components, namely starch, from the enzymatic oral environment.

SEM–EDX and OM have different sensitivity to different micro remains. SEM–EDX offers a means to confirm the presence of starch by combining morphological and elemental information without having to destroy either the calculus, as required in processing for OM, or the starch grains themselves, as proposed when using enzymatic reactions. Even if starch is gelatinized it should preserve an elemental signature that is suggestive of starch. We applied

SEM-EDX to intact calculus to witness micro remains *in situ*, but this technique is equally viable for more finely processed calculus samples mounted on plates, or even to calculus still attached on the original tooth. However, it is important to note that diagnostic features of starch grains, such as the hilum and lamellae, are only visible using OM.

Our study indicates that SEM–EDX is a viable alternative to OM analysis of calculus, but researchers should choose their analytical method based on the questions they seek to answer, and the plant micro remains that they intend to study. Furthermore, on very sensitive osteological remains, it may be possible to use SEM–EDX to study calculus using entirely non-destructive means to examine embedded micro remains directly on the tooth; a useful technique if the tooth is not firmly attached in the mandible or maxilla. We prefer to consider SEM–EDX a complimentary rather than replacement technique in the study of dental calculus micro remains. A sequential workflow that first examines calculus under SEM–EDX and then under OM may be the optimal solution for highest resolution of micro remains, though we recognize that this approach is time intensive and can be costly. We believe that further exploration and experimentation of SEM techniques is important in the field of archaeological and paleodietary reconstruction. The continued refinement and expansion of dental calculus analysis techniques is an important focus in order to maximize the information we can harvest from this ephemeral and fragile material.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jas.2014.04.016.

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