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Publication Date

2018-04-01

DOI

10.1016/j.jasrep.2018.02.004

Peer reviewed

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Journal of Archaeological Science: Reports 18 (2018) 509–515

Contents lists available at ScienceDirect

Journal of Archaeological Science: Reports

journal homepage: www.elsevier.com/locate/jasrep

Dental calculus as a source of ancient alkaloids: Detection of nicotine by LC-MS in calculus samples from the Americas

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ABSTRACT

Dental calculus has been shown to be a repository of a variety of exogenous organic materials, including bacterial DNA, proteins, phytoliths, and starch grains. Here we show that certain alkaloids, nicotine in this case, can also be trapped and preserved in ancient dental calculus. We present Ultra-Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) results of analyses of ten archaeological calculus samples from eight individuals from Central California. Two samples tested positive for the presence of nicotine, including one from an individual buried with a pipe. UPLC-MS analyses of dental calculus could provide an alternative means to trace the spread and consumption of tobacco and other plants with distinctive alkaloid products in ancient societies. As shown here for the first time in ancient samples, the ability to detect nicotine alkaloids in calculus has enormous potential to help us better understand the consumption of intoxicant plants by ancient humans on the individual level, for example by giving us the ability to test assumptions about the age, sex, and status of individual tobacco users in the past.

1. Introduction

Until recently, dental calculus was largely ignored by archaeologists as a source of information about ancient human biology and behavior (Lieverse, 1999). In the last 10 years, there has been a notable uptick in archaeological studies that exploit ancient dental calculus to reveal new information about the past. Some of these studies have focused on bulk samples of calculus employing analytical techniques such as scanning electron microscopy (Charlier et al., 2010; Power et al., 2014), X-Ray Diffraction (Klepinger et al., 1977), or stable isotopes (Scott and Poulson, 2012; Poulson et al., 2013; Eerkens et al., 2014; Salazar-García et al., 2014). Other approaches attempt to break calculus down into constituent parts, for example, to identify microfossil starch grains or phytoliths (Dudgeon and Tromp, 2014; Hardy et al., 2009; Henry et al., 2010; Piperno and Dillehay, 2008), or specific biomolecules, such as DNA (Adler et al., 2013; Preus et al., 2011; Warinner et al., 2014a, 2014b; Weyrich et al., 2015) or proteins (Warinner et al., 2014c).

The mouth is an interface between the internal human body and the external environment. Materials that enter the oral cavity and become embedded and preserved in calculus can inform on both the individual, including their health, behavior, or genetic ancestry, as well as interactions with their immediate environment, including plants, animals, and bacteria. While ecofacts (animal bones, charred seeds) and artifacts (e.g., pipes, arrowheads) can also inform on activities in ancient environments, these items are typically separated from the specific individuals who made or used them. In short, the ability to link certain aspects of human behavior to particular individuals makes analysis of dental calculus a particularly promising area of research.

Dental calculus is mineralized plaque that adheres to the surfaces of teeth and is often present in archaeological cases (Hillson, 1996). Supragingival calculus, the subject of most archaeological studies, commonly forms on the lingual surfaces of anterior mandibular teeth and the buccal surfaces of maxillary molars (White, 1997:513). The majority of calculus, by weight, consists of inorganic minerals such as hydroxyapatite, octacalcium phosphate, and whitlockite. However, calculus also serves as a trap or repository of organic materials, including plant fibers, glycoproteins, proteins, lipids, carbohydrates, and DNA (Kani et al., 1983; Lieverse, 1999). Using sensitive modern

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https://doi.org/10.1016/j.jasrep.2018.02.004
Received 2 November 2017; Received in revised form 1 February 2018; Accepted 5 February 2018
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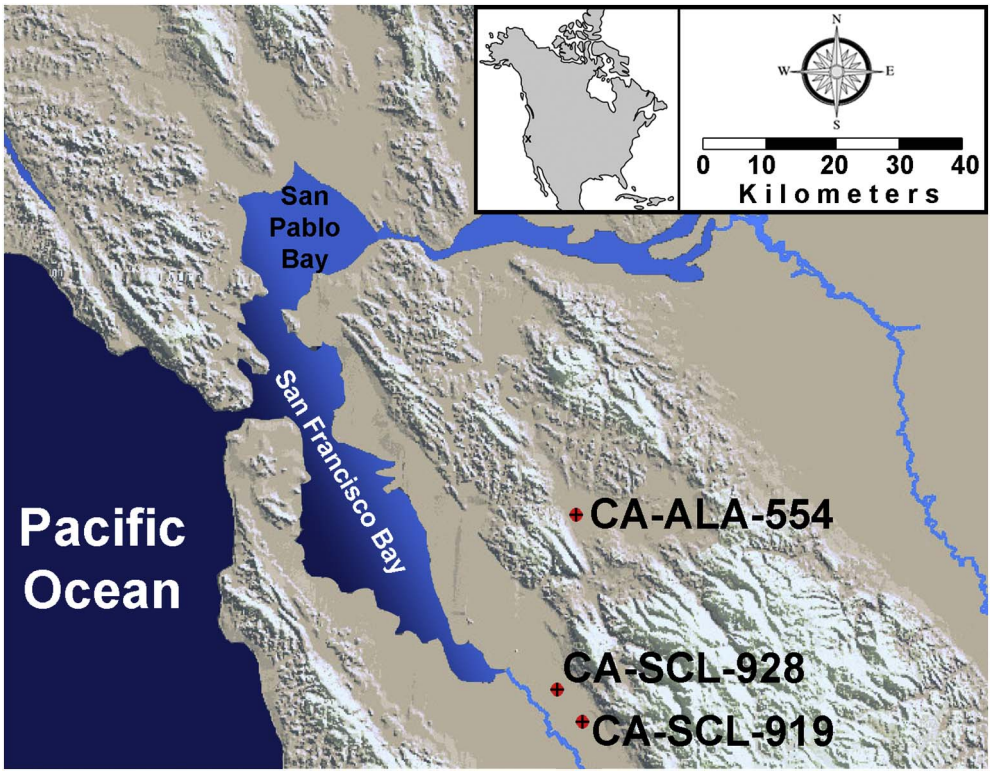

Fig. 1. Map of Central California showing San Francisco Bay and the location of the three sites included in this study.

analytical instrumentation, we can now detect and characterize trace amounts of these organic compounds.

This study focuses on the detection of nicotine, an alkaloid of tobacco plants (*Nicotiana* sp.), in dental calculus. Because nicotine is relatively stable and can survive over archaeological time scales (Rafferty, 2002, 2006; Rafferty et al., 2012; Tushingham et al., 2013), and is typically introduced to the body via the mouth, we hypothesized that it may preserve in dental calculus. Tracing the ancient spread of tobacco in the Americas has traditionally relied on the presence of pipes and/or charred tobacco seeds (e.g., Adair, 2000; Adams and Toll, 2000; Carrasco et al., 2015; Echeverría et al., 2014; Gili et al., 2017; Haberman, 1984; Pauketat et al., 2002; Wagner, 2000; papers in Bollwerk and Tushingham, 2016, Rafferty and Mann, 2004), and more rarely preserved leaves, and/or quids (e.g., Adams et al., 2015; Fewkes, 1912:143; Jones, 1935; Jones and Morris, 1960) and pollen (e.g., Cummings, 2000). However, these items are rare in the archaeological record and are not linked to particular individuals, unless associated with specific burials, and even then, association with an individual is an inference rather than a direct measurement. Because the leaves are smoked, the distinctively-shaped seeds are rarely charred and incorporated into the archaeological record. Further, tobacco was commonly chewed (Kroeber, 1941; Turner and Taylor, 1972) or smoked using organic materials such as cane or rolled in leaves as "cigarettes" (Adams, 1990; Fewkes, 1912:142–143; Harrington, 1942:28) that are exceedingly rare in the record. As a result, tobacco use is difficult to document archaeologically and its importance is certainly under-represented in archaeological studies (Tushingham and Eerkens, 2016).

Because dental calculus is common and nicotine is a stable and long-lasting compound, analysis of the former has the potential to allow us to trace the history of tobacco use across a broader social context and geographic area, and much further back in prehistory. At European contact, domesticated species of tobacco were grown by farming communities across much of North America. While some have suggested that tobacco use was late, appearing in southeast North America only around 1000 CE, and as late as the historic period in the American southwest (Ford, 1981), more recent work suggests a much greater antiquity. Data from eastern and southwestern North America suggests use of tobacco by 2000–3000 years ago, though the particular species of tobacco is not known (Adams, 1990; Haberman, 1984; Pauketat et al., 2002; Rafferty, 2002, 2006; Rafferty et al., 2012; Winter, 2000a). As well, there seems to be consensus that *Nicotiana rustica* reached the Eastern USA and Canada from South America by 3000 and 2000 years ago. *Nicotiana tabacum* appears to spread to parts of the southwestern United States and the Caribbean sometime afterward (cf. Winter, 2000a, 2000b; Rafferty, 2006). Recent DNA analyses of several quids from Antelope Cave, Arizona, also shows that wild tobaccos were commonly chewed as long as 2000 years ago (Adams et al., 2015).

Much less is known about tobacco use in western North America. Ethnographic studies show that hunting, gathering, and fishing communities of this region also used an array of wild or "indigenous" tobacco species (Tushingham and Eerkens, 2016). Tobacco was actively managed and cultivated by many groups as far north as British Columbia (Kroeber, 1941; Lepofsky and Lertzman, 2008; Turner and Taylor, 1972). The timing and trajectory of tobacco use, however, is widely debated. Some believe tobacco was introduced by Euro-American traders in the contact period (e.g., Dixon, 1933; Kroeber, 1941:14). Others posit much older origins, perhaps being used in parts of the arid west as early as the Late Pleistocene (e.g., Heiser, 1969:16; La Barre, 1970; Siegel, 1989:3; Winter, 2000a). Recent chemical studies of pipes associated with hunting and gathering communities from California and the Pacific Northwest has revealed traces of nicotine in pipes as old as 1100 BP (Eerkens et al., 2012; Tushingham et al., 2013; Tushingham and Eerkens, 2016). Although there remains much to be learned, a growing number of studies are addressing the spread and use of tobacco throughout the region, and archaeologists are increasingly recognizing the potential these studies offer to our field (Tushingham and Eerkens, 2016).

In addition to providing new evidence about the history and spread of tobacco, detection of nicotine in calculus has the potential to link tobacco use to particular individuals. This level of information could

facilitate an evaluation of the social context(s) surrounding the plant, its exploitation, and why it spread over such a large geographic region. For example, by linking tobacco use to particular individuals we can evaluate whether all members of society used tobacco, or only adults, or only males or females. As such, the ability to identify nicotine and other alkaloids in calculus has tremendous potential to open new windows on the ancient consumption of intoxicants by humans.

2. Samples

For this study, we examined 10 dental calculus samples from eight pre-contact individuals from three archaeological sites in Central California (Fig. 1). The sites were chosen to represent a range of archaeological ages. Ethnographic accounts (e.g., Harrington, 1932; Kroeber, 1941) indicate that tobacco was both smoked in pipes and combined with lime and either chewed or eaten in pre-contact California. Our previous research with clay and stone pipes shows that tobacco was smoked in Central and Northern California by at least 1200 years ago (Tushingham et al., 2013). Analysis of calculus presents the opportunity to push back the date of tobacco use even further, and moreover, allows us to evaluate tobacco use on an individual-by-individual basis.

Site CA-SCL-919 is a late pre-contact period village near San Francisco Bay (see Eerkens et al., 2016a). Radiocarbon dates from bone collagen of seven burials, including the two sampled for calculus here, are all between 450 and 200 cal BP. Calculus from the third molars of two adult individuals (Burial 12, a male, and Burial 15, a female) was included in the analysis.

CA-ALA-554 is a slightly older archaeological site in the Amador Valley to the east of San Francisco Bay (see Eerkens et al., 2016b). Bone collagen radiocarbon dates from 55 individuals, including one from this study, are consistently between 1800 and 450 cal BP. Four adult male individuals from CA-ALA-554 were included in this study, including two people buried with pipes. The pipe with Burial 47 previously tested positive for nicotine (Eerkens et al., 2012; Tushingham and Eerkens, 2016), while the other pipe (with Burial DN-2) did not show evidence for the presence of nicotine (unpublished data). For the two individuals buried with a pipe, we analyzed calculus from two different teeth, one from the anterior (incisor) and one towards the posterior (premolar or molar) of the dental arcade. Calculus from the incisors of Burial 47, as well as enamel on some of the anterior teeth, exhibited a dark staining.

Site CA-SCL-928 is a Middle Holocene site near CA-SCL-919 (Eerkens et al., 2016a). The site contains minimal accumulation of midden, but three individuals were encountered during excavations. Radiocarbon dates on all three produced calibrated ages ranging between 5590 and 6210 cal BP. Calculus from two individuals (Burial 1, female, and Burial 2, male) was included in the current study.

3. Methods

All solvents used for extraction and analyses were purchased from Sigma Aldrich (St. Louis, MO, USA) and were of liquid chromatography-mass spectrometry grade. Authentic standards of anabasine, atropine, caffeine, cotinine, nicotine, theobromine, and theophylline were also purchased from Sigma Aldrich. The limit of detection (LOD) and limit of quantification (LOQ) for these standards ranged between 10 and 50 nM (nanomolar). The LOD for anabasine, atropine, caffeine, cotinine, nicotine, theobromine, and theophylline are 30, 10, 20, 40, 30, 40, and 40 nM, respectively. The LOQ for anabasine, atropine, caffeine, cotinine, nicotine, theobromine, and theophylline are 40, 20, 30, 50, 40, 50, and 50 nM, respectively.

3.1. Extraction of calculus samples

Calculus samples (sample weights given in Table 1) were gently powdered in an agate mortar and pestle, placed in sterile glass vials, immersed in a 500 μL mixture of acetonitrile: 2-propanol: water [3:2:2] (APW), and sonicated for 10 min at room temperature. The APW supernatant (extracts) were then transferred to 1.5 mL tubes and freeze-dried for 3 days. The vials containing the calculus samples were allowed to dry in a fume hood. The freeze-dried APW extracts were then re-suspended with 50 μL of 0.10% formic acid/water: acetonitrile [1:1], vortexed, and centrifuged at 10,000*g* for 10 min at 4 °C. An amount of 40 μL was placed in a glass insert within a sample vial for analysis.

3.2. Ultra-performance liquid chromatography analysis

Ultra-performance liquid chromatography (UPLC) was conducted on a Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA) with photodiode array (PDA) detection ranging between 210 and 400 nm. One microliter of APW extract samples were injected through a 2.0 μL sample loop using the full loop injection mode. Flow rate through a Waters Acquity UPLC T3 column (HSS T3, 1.8 μm, 2.1 × 100 mm) was 0.32 mL min^{-1} with 0.10% formic acid/water (A) and 0.10% formic acid/methanol: acetonitrile [2:3] (B) as solvents. The elution gradient was as follows: initial conditions (97% A:3% B) at time 0, then gradient to 15% A:85% B at 12.00 min, 3% A:97% B at 12.10 min, maintained at 3% A:97% B until 14.00 min, returned to the initial conditions of 97% A:3% B at 14.10 min, and held until 16.00 min to reequilibrate the column. Total analysis time per sample was 16.00 min. The autosampler chamber and column temperature were 8 °C and 35 °C, respectively.

3.3. (Ultra-performance) liquid chromatography-mass spectrometry analysis

The same chromatographic conditions for UPLC-MS/MS analysis were as outlined above for UPLC analysis, using the same Acquity UPLC instrument coupled inline to a mass detector, a Waters Synapt G2-S HDMS Q-TOF with a LockSpray Exact Mass Ionization Source (Waters Corporation, Milford, MA, USA). The Synapt instrument was operated in ESI positive mode and resolution mass mode. The capillary voltage, sampling cone voltage, and source offset voltage were 3.0 kV, 60 V, and 60 V, respectively. The source temperature was 100 °C with a cone gas (nitrogen) flow rate of 50 L h^{-1}. The desolvation temperature was 250 °C with a desolvation gas (nitrogen) flow rate of 900 L h^{-1}. The nebulizer gas (nitrogen) flow was 6.0 bar. The lock mass compound was leucine encephalin (reference mass of 556.2771 *m/z* $[M-H]^+$).

The scan range was from 100 to 1200 *m/z* with a scan time of 0.3 s. Mass spectral data were collected in profile mode using MS^E, a form of tandem mass spectrometric (MS/MS) data collection, with a high collision energy (ramp 15 to 40 V) for fragmentation. Under our UPLC-MS/MS conditions, nicotine (163.12 *m/z* $[M + H]^+$) eluted at 1.08 min. The UPLC-MS/MS data were processed in MassLynx (Waters Corporation, Milford, MA, USA). We also scanned the resulting LC-MS/MS chromatograms for ions indicative of other alkaloids, including anabasine (163.12 *m/z* [M + H] +), atropine (290.18 *m/z* [M + H] +), caffeine (195.09 *m/z* [M + H] +), cotinine (177.10 *m/z* [M + H] +), theobromine (181.08 *m/z* [M + H] +), and theophylline (181.08 *m/z* [M + H] +), which eluted at 1.50, 3.85, 3.80, 1.50, 2.65, and 3.15 min, respectively. Many of these alkaloids are active ingredients of other smoke plants documented in California (Tushingham et al., 2013).

4. Results

This study used UPLC-MS/MS to analyze calculus samples for the presence of nicotine. Samples were compared against a nicotine standard to confirm the presence and absence of nicotine in the calculus samples extracted with a solvent mixture of acetonitrile, 2-propanol and water (at a ratio of 3:2:2 and called APW). Of the 10 calculus samples evaluated, two showed unambiguous evidence for nicotine, including calculus removed from the incisor from Burial 47 at CA-ALA-

Table 1
Dental calculus samples included in this study and associated demographic and radiocarbon data.

Site	Burial #	Tooth	Sample Wt. (mg)	Sex	Age	Assoc. pipe?	Radiocarbon date (uncalibrated)
SCL-919	12	RM3	3.4	Male	40–49	No	465 ± 30 BP
SCL-919	15	LM3	6.0	Female	35–44	No	420 ± 25 BP
ALA-554	47	LLI1	0.4	Male	30–35	Yes	630 ± 25 BP
		LRM1	0.4				
ALA-554	63	M3	2.3	Male	45+	No	730 ± 25 BP
ALA-554	DN-2	LLI2	2.8	Male	45+	Yes	
		LLP2	1.4				
ALA-554	DN-6A	Lower molars[a]	4.0	Male	20–25	No	
SCL-928	1	RM1	1.1	Female	25+	No	4940 ± 60 BP
SCL-928	2	RM3	1.7	Male	20+	No	4940 ± 35 BP

Notes: Assoc. = associated in burial pit.
[a] Calculus from several lower molars was combined.

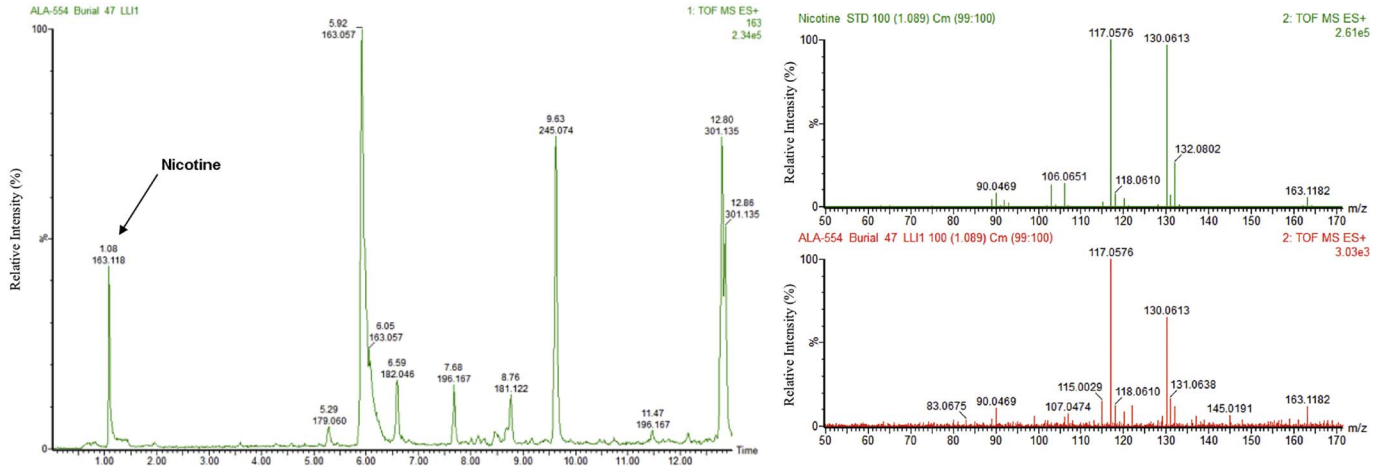

Fig. 2. UPLC-MS/MS chromatogram (left) from Burial 47 at CA-ALA-554 calculus sample, along with MS/MS spectra (right) of nicotine standard (top) and the compound corresponding to the black arrow at in the chromatogram 1.08 min and identified as nicotine (bottom).

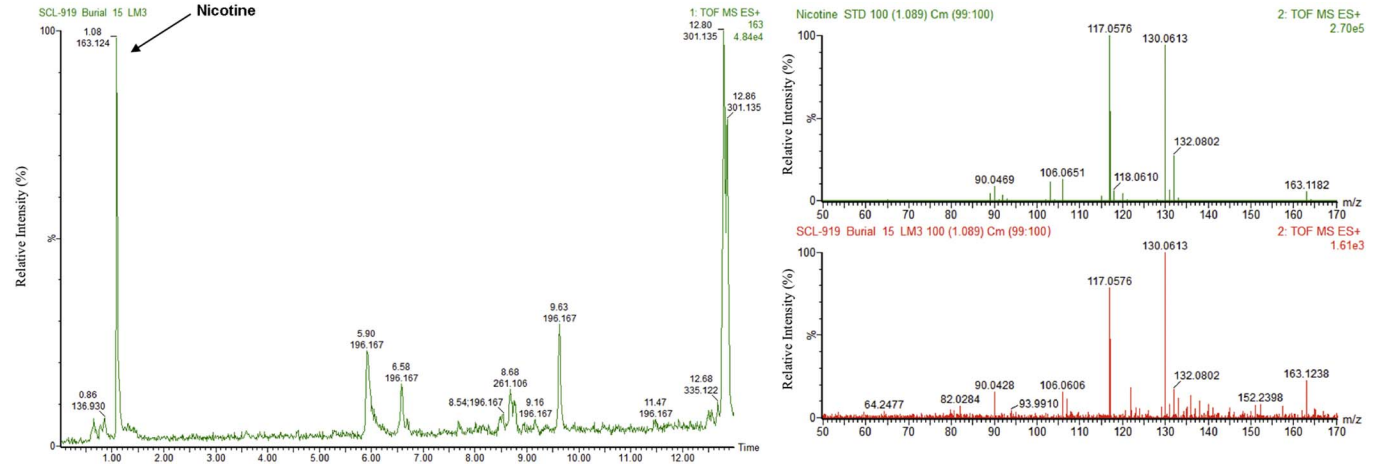

Fig. 3. UPLC-MS/MS chromatogram (left) from Burial 15 at CA-SCL-919 calculus sample, along with MS/MS spectra (right) of nicotine standard (top) and the compound corresponding to the black arrow in the chromatogram at 1.08 min and identified as nicotine (bottom).

554 (an individual buried with a pipe), and the third molar of Burial 15 at CA-SCL-919 (not buried with a pipe). In these two samples, a compound eluted from the column at 1.08 min that displayed major ions of *m/z* 117 and 130 (Figs. 2–3). This retention time and associated ions matches those of the nicotine standard. Other compounds were detected in these extracts, as indicated by other peaks shown in the chromatograms (Figs. 2–3), but these were not consistent between samples and were not nicotine derivatives. These compounds were not identified; without authentic matching standards, or a suggestion as to what the compounds could be, such as from the use of other plants as might be indicated by artifacts associated with the burial, it is very difficult to identify unknown compounds in samples.

We did not detect nicotine in the remaining eight dental calculus samples. Interestingly, this includes a second calculus sample from Burial 47 at CA-ALA-554, but taken from a different tooth, a molar. It is possible, of course, that nicotine is present in these eight calculus samples but is below instrument detection limits. We also scanned the resulting LC-MS/MS chromatograms for ions indicative of other

alkaloids, including anabasine, atropine, caffeine, cotinine, theobromine, and theophylline. However, we were unable to detect evidence for any of these other alkaloid compounds in any of the calculus samples, including the two testing positive for nicotine.

5. Discussion

In modern studies of smokers' teeth, variable amounts of residual nicotine can be detected via HPLC-MS (and similar techniques, such as UPLC-MS/MS) in the calculus and plaque on root surfaces (Cuff et al., 1989; Katti et al., 2012). However, the studies reported to date have focused on heavy smokers who have lost teeth due to periodontal disease. This report outlines the first study to demonstrate that nicotine can survive in detectable amounts in ancient samples, and in what appear to be more casual users of tobacco. Two of 10 dental calculus samples from pre-contact California revealed the presence of nicotine. In one of these cases (Burial 47 from CA-ALA-554), the individual had been buried with a stone pipe that previously also tested positive for nicotine (Eerkens et al., 2012). Two calculus samples from this person were analyzed, one from an incisor and one from a molar, but only the incisor tested positive for nicotine. Calculus from the incisor was visibly stained a dark color while calculus from the molar was not obviously stained. This suggests that identification of ancient tobacco use via (UP) LC-MS/MS analysis of calculus may depend on the particular tooth that is sampled for calculus.

The data presented in this report support the hypothesis that for a smoker, it is calculus from the anterior teeth (incisors and canines) that is more likely to yield nicotine. By contrast, a person that frequently chews tobacco may be more likely to incorporate nicotine into calculus on more posterior teeth, such as premolars and molars. Modern studies show that tobacco use is strongly correlated with high rates of periodontal disease and tooth staining (e.g., Watts and Addy, 2001), as well as calculus deposition (Bergström, 1999). Furthermore, calculus buildup varies in different parts of an individual's mouth, with mineralized deposits most frequent on the lingual sides of mandibular anterior (lower front) teeth, especially central incisors, followed by lateral incisors and canines. Thus, tobacco residues are more likely to build up in these areas of the mouth (Corbett and Dawes, 1998). While sampling, we did not systematically record the side of the tooth (e.g., lingual vs. vestibular/buccal) from which calculus was removed. Instead, we combined calculus from all sides into a single analytical sample due to concerns related to sample size and detection limits. Thus, we are unable to evaluate whether lingual or buccal sides, or both, test positive or negative for nicotine. However, the observed pattern for Burial 47 does seem to support the idea that tobacco users produce significant calculus buildup in anterior teeth (as also shown by modern periodontal studies) and this may be related to greater nicotine uptake and/or detection in calculus associated with incisors.

By contrast, a second individual from this same site who was also buried with a pipe (Burial DN-2) did not have measurable nicotine in calculus removed from two different teeth, an incisor and a second premolar. However, in this case, the pipe he was buried with did *not* test positive for nicotine. It is possible that this person did not smoke tobacco, and an unused pipe was symbolically placed with him in preparation for an afterlife. Alternatively, the pipe may have been used to smoke non-tobacco products. It is also possible that the pipe was used for smoking tobacco, but not enough to deposit significant amounts of nicotine in the pipe, nor in his calculus. In any case, our studies suggest that associations between pipes and definitive tobacco use are not simple.

One surprise is the discovery of nicotine in calculus from a third molar of a middle-aged woman from CA-SCL-919. Ethnographic interviews with native Californians in the late 1800s and early 1900s suggest that tobacco smoking was largely limited to males of all ages, older women (Fig. 4), and female shamans (Harrington, 1932; Winter, 2000a). Both male and female shamans, or curers, smoked tobacco and regularly used this plant in doctoring activities, and as offerings. Indeed, in general, ethnographic records indicate a strong and widespread association between tobacco use and shamanism across Western North America (Kroeber, 1941:20), including among Ohlone in the San Francisco Bay area where this sample is from (Harrington, 1942). Shamans commonly smoked tobacco as part of curing rituals, but the plant was widely consumed as part of other shamanistic activities and presented by shamans in offerings. Ethnographic records indicate that Ohlone shamans were “mostly men” (Harrington, 1942:39). In this case, there is no specific evidence to suggest she was a shaman, by the presence of special burial-associated grave goods, for example. While we cannot rule out her status as a shaman, the limited evidence suggests she was not a shaman but simply consumed tobacco as part of other ritual or recreational activities. Additional analysis, currently underway, may shed further light on this issue.

Fig. 4. Yokuts woman smoking pipe - Maggie Icho 1945 - Wikchamni - Clifford Relander photo.

More broadly, in most tobacco-using societies globally there is a trend showing fewer female smokers than males. This is typically attributed to gender inequalities or cultural preferences. However, recent research by Hagen et al. (2016) and Roulette et al. (2016) offer an alternative explanation: that female avoidance of plant toxins, including those contained in tobacco and similar plants, is an evolutionary response to protect fetuses and nursing infants from harmful biochemicals. If true, this avoidance mechanism should be most prominent among women of childbearing age, while smoking rates among older, post-menopausal women should increase (assuming other cultural practices do not simultaneously limit female access to tobacco). This seems to fit the pattern observed for ethnographic Native Californians, though it may be hard to tease out whether there were underlying evolutionary mechanisms at play. Indeed, for our tobacco-using woman from CA-SCL-919, osteological analyses determined she was a “middle-aged adult” most likely between 35 and 44 years. Based on osteological analyses of skeletal populations from archaeological sites, the average life expectancy of pre-contact native Californians was under 30 years, when all individuals including sub-adults are included in the calculation (e.g., Bartelink, 2009; Jurmain et al., 2009; Schwitalla et al., 2014). Life expectancy still averages around 40 years when subadults are removed from the analysis. In either case, at 35–45 years Burial 15 at SCL-919 was approaching or past the average life expectancy and was likely at or past the end of her reproductive years. Given generation times (ca. 20–25 years), there is a good chance she was a grandmother. While no broad conclusions can be made with this single case, her age, sex, and association with tobacco use is intriguing. Moreover, it highlights the potential of this type of individual-level analysis to test

hypotheses about intoxicant use in the past.

6. Conclusions

The ability to detect drug use via identification of alkaloids from human remains has generally been limited to hair (e.g., Echeverría and Niemeyer, 2013; Springfield et al., 1993; Wilson et al., 2013) and coprolites (Reinhard et al., 1991). Unfortunately, hair and coprolites are rare in the archaeological record and only preserve under unusual circumstances, such as in highly arid or frozen environments, or in caves. Dental calculus, on the other hand, is a much more common biomaterial that preserves under a wider range of burial conditions, and more importantly, is associated with particular individuals from the past.

Our results show that dental calculus can preserve nicotine over archaeological time scales. It remains to be determined if other alkaloids are also preserved, though we did not detect any in this study. The ability to link nicotine use with specific individuals opens new windows into testing patterns about ancient human behaviors. Ethnographic accounts from the Americas, for example, suggest tobacco use was usually associated with males and older/higher status individuals. Analysis of calculus from such individuals, as well as females and younger/lower status individuals, would allow us to test such notions in more ancient contexts. Likewise, tobacco use in the pre-contact Americas is often associated with shamanism, medicine, and doctoring activities, and less with recreational or every-day behavior. Analysis of individuals who were buried in contexts, or with artifacts, connected to shamanism or doctoring, again provides the opportunity to test this association and antiquity of such patterns. These analyses can also challenge entrenched notions about the gender, age, sex, and social status of drug users.

Furthermore, while previous chemical residue studies of tobacco have largely been dependent on the presence of pipes, there were many places where tobacco was chewed (with lime, in quids) or smoked in perishable cigarettes or cigars. Testing of dental calculus could reveal tobacco use in such contexts as well, and allow archaeologists to increase the spatial and temporal context in which tobacco was used.

Acknowledgements

We thank VTA environmental staff Lauren Bobadilla and Samantha Swan for supporting this research. We thank Far Western Anthropological Research Group, William Self Inc., Jennifer Blake, and Christina Alonso for facilitating access to the collections. This research is based upon work supported by the National Science Foundation under Grant No. BCS-1419506. The authors would like to thank the two anonymous reviewers whose comments helped to improve the manuscript.

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