GC/MS ANALYSIS OF RESIDUES REVEALS NICOTINE IN TWO LATE PREHISTORIC PIPES FROM CA-ALA-554

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Three pipes were recovered during excavations at CA-ALA-554. Ethnographically, Native Californians smoked a range of plants, the most common of which is tobacco, in pipes. In order to determine the specific uses of these pipes, we extracted organic residues from the pipes and identified compounds using Gas Chromatography-Mass Spectrometry. Results show that the pipes have strong nicotine signatures, indicating they were used to smoke tobacco. Biomarkers for other plants are not present. Although tobacco use was introduced to California in ancient times, the precise date is unknown. The presence of nicotine in these pipes has implications for the timing of when tobacco entered California, and for indigenous horticultural practices.

Though archaeological pipes are typically assumed to be associated with tobacco (*Nicotiana* sp.) smoking, a wide variety of smoke plants were used for ritual and medicinal purposes in ethnographic North America. In fact, at least 55 genera of plants were smoked by ethnographic Native Americans (Moerman 1998). The most commonly smoked plants contain psychoactive alkaloids that are detectible via Gas Chromatography-Time of Flight / Mass Spectrometry (GC/MS) or similar techniques. Nicotine compounds, for example, found in wild and domesticated species of tobacco plants (*Nicotiana* spp.), have been successfully extracted from pipes in a handful of recent experimental studies, including two from the northeastern United States (Rafferty 2002, 2006) and five from sites in the southern Pacific Northwest Coast of North America by the U.C. Davis Archaeological Metabolomics Group (AMG) (Palazoglu et al. 2010; Tushingham et al. 2010). Such studies have the potential to contribute to our understanding of the spread of tobacco and use of smoke plants in ancient North America.

The current study includes nondestructive analysis of two pipe artifacts from ALA-554 for alkaloid plant residue characterization employing GC/MS analysis. The pipes include a dark-colored steatite complete piece from Burial 149 (13.2 cm long x 2.4 cm in external diameter), and a yellow fine-grained sandstone pipe from Burial 47 (18.5 cm long x 4.7 cm in maximum external diameter). The latter was broken about 4.5 cm from the mouth piece, and the interior contained dark sediment. For this latter pipe we sampled the bowl separately from the mouth piece and also attempted to extract alkaloids from the associated sediment.

The work is modeled on our pilot studies involving residue analysis of tobacco in ancient and experimental pipes where evidence for smoke plant residues in pipes was provided by detecting nicotine compounds and combustion decay products in five small pipe fragments analyzed from archaeological sites in northwestern California (Palazoglu et al. 2010; Tushingham et al. 2010).

To our knowledge this is the first study of its kind conducted on artifacts from central California. As we show below, both pipes tested positive for nicotine, demonstrating that inhabitants of the site either cultivated or had access to tobacco. Because so little is known about the timing of the introduction of tobacco use to California, the study provides important information on the minimum age for when this use was introduced into the region.

MATERIALS AND METHODS

This section describes sample preparation, extraction, and chemical characterization methods employed in the study. As the results of the ALA-554 study build on previous work involving chemical characterization of a suite of smoke plants used by ethnographic Native Americans and analysis of ancient and experimental pipes, the methods for the pilot smoke plant project are also summarized here.

CA-ALA-554

In 2011 archaeological consultant William Self Associates, Inc. (WSA) conducted testing and data recovery prior to the development of a shopping center in Pleasanton, California. A prehistoric village site, ALA-554, located within the project borders, contained intact archaeological deposits and human remains. WSA conducted pre-trenching of underground utilities within the boundaries of the previously recorded site in order to minimize disturbance to the resource. Archaeologists excavated 25 features and the remains of 187 prehistoric individuals within a 50 to 150 cm thick cultural layer associated with site habitation (Estes et al. 2012:1). The cultural strata were in relatively good condition, because the majority of skeletal elements were present and articulated, with grave goods maintaining their original placement at time of interment (Estes et al. 2012:361).

Evidence from absolute and relative dating techniques indicate that the site was occupied continuously for approximately 600 years from the end of the Middle–Late Transition period (A.D. 1000) into the beginning of the Late 2 period (A.D. 1500–1650). Although three main occupation strata were identified within the midden, over 90 percent of the burials recovered during the project were within Stratum II. This layer is a Late 1 period occupation, dating between A.D. 1000 and 1500. The assemblage is characterized by Augustine pattern cultural materials, including Stockton series projectile points, show mortars, Type M and K *Olivella* bead series, and “banjo” type *Haliotis* ornaments (Estes et al. 2012:xxviii).

Burials with Pipes

Burial 47 was found in the flexed ventral position, and was a 25-to-35-year-old male within Stratum II (Blake and Minturn 2012:162-165). Burial goods include 976 complete Type M1a, M1b, M1c, M2, M4, and spire-lopped *Olivella* beads concentrated around the neck area. Eight Type K2bII or AF4b, six Type S, and six Z series *Haliotis rufescens* ornaments were recovered around the cranium of the individual. Two of these pendants were placed on top of the orbits. The modified shell assemblage is representative of the Late 1 period (Estes et al 2012:154, 179). A complete strategically shaped sandstone pipe was recovered immediately east of the cranium, and 13 Type A5a stingray spine tips were placed 15 cm to the south of the cranium. Eight faunal bone whistles were placed on the north side of the cranium and four were placed on the south posterior scapula. A modified elk cranium bowl, canine tooth, and bird-bone pin were placed near the feet of Burial 47. Ochre-stained soil was concentrated immediately north of the left humerus and radius at mid-shaft. Burial 47 also contained a complete mica ornament and a concentration of fragmented mica sheets adjacent to the right humerus in the south sidewall of the burial pit (Estes et al. 2012:154, 179, 263, 283).

A radiocarbon date on collagen extracted from a small bone fragment of Burial 47 produced a date of 630 ±25 ($\delta^{13}C$ = -19.78). This date is well within what was expected, given the suite of bead types. Using a mixing model developed by Eric Bartelink for the region, we estimate that 14 percent of the carbon in this sample is from marine sources. Including this information, and calibrating the radiocarbon date, we estimate this individual lived between A.D. 1400 and 1430 (2 sigma range = A.D. 1324-1439).

Burial 149 is a partially cremated adult in Stratum II. The burial contained a total of 1,344 complete Type A1a, A1b, A4, B2, M1a, M1b, M1c, M2, M4, and spire-lopped *Olivella* beads indicative of the Late 1 period (Blake and Minturn 2012:310; Estes et al. 2012:165). The cremation matrix contained two obsidian Stockton projectile points and two obsidian indeterminate projectile point fragments. A

Table 1. Analyzed samples from site ALA-554.

Sample number	Artifact type	Description
ALA-554-47-Bowl	Pipe	Bowl section of sandstone pipe
ALA-554-47-Mouth	Pipe	Mouthpiece section of sandstone pipe
ALA-554-47-Sediment	Pipe	Sediment inside sandstone pipe
ALA-554-149	Pipe	Complete steatite pipe

Figure 1. Broken sandstone pipe associated with Burial 47.

complete strategically shaped steatite pipe was recovered within the cremation (Estes et al. 2012:223, 225, 263).

A radiocarbon date on collagen extracted from a small bone fragment from Burial 149 produced a date of 695 ±25 ($\delta^{13}C$ = -19.37). This also is well within what was expected, given the suite of bead types. Using the same mixing model developed by Eric Bartelink for the region, we estimate that 16 percent of the carbon in this sample is from marine sources. Including this information, and calibrating the radiocarbon date, we estimate this individual lived between A.D. 1319 and 1406 (2 sigma range = A.D. 1304-1415).

Methods

Initial preparation took place at the U.C. Davis Archaeometry Laboratory in Davis, California. The artifacts were photographed and inspected for possible visible smoking residue. Four residue samples were extracted from the two pipes. The pipe from Burial 149 was extracted as a whole in a single event, while the pipe from Burial 47 was extracted separately for the mouth and bowl sections. As well, an extraction was attempted on the associated sediment from the latter pipe. Samples are listed in Table 1. Figures 1 and 2 show images of the pipes included in the study.

Pyridine, chloroform, methanol, methoxyamine hydrochloride, and N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) were obtained from Sigma Aldrich, USA. All standards are of analytical grade and were also purchased from Sigma Aldrich, USA.

One end of the hollow pipe or pipe fragment was covered with a circular Teflon fitting that was sealed to the pipe with silicon jelly to prevent leakage of fluid material. Ethyl acetate was then poured into the pipe, and the open end was similarly sealed with parafilm and silicon jelly. The now-filled and sealed pipe was then placed in water and sonicated for 5 minutes. The pipe was then reopened and the extract poured off into a separate container for GC/MS analysis. This step was repeated twice. Extracts

Figure 2. Complete steatite pipe associated with Burial 149.

were evaporated under nitrogen. Importantly, the pipe remained complete during extraction, and the physical structure was not compromised in any way.

Dried extracts were then submitted to a two-step derivitazation with methoxyamine hydrochloride followed by silylation with N-methyl-N-trimethylsilyl trifluoroacetamide. A fatty acid methyl ester (FAME) mixture of C8 through C30 was added to the derivatized samples acting as retention indices markers. Derivatized samples were analyzed by GC/MS on an Agilent 6890 GC-LECO Pegasus III TOF equipped with a Cooled Injection System (CIS4), an Automated Linear Exchange system (ALEX), and a Multi Purpose Sampler (MPS, all Gerstel).

The injector was run with an initial temperature of 50º C and ramped to 275º C at a rate of 12º C/second. The injection volume was 0.5 microliters. GC conditions were set with a programmed oven temperature of 50º C, held there for 1 minute, then ramped to 330º C at a rate of 20º C/minute, held at 330º C for 15 minutes. The Gerstel injector was run with a carrier gas helium flow rate of 1.0 milliliters/min.

The column was an RTX-5 MS, 30 m long, 0.25 mm i.d. x 0.25 μm film. The transfer line and ion source temperature were set at 280º C and 250º C, respectively. Solvent delay was adjusted to 4.5 minutes, and MS acquisition was optimized to 17 spectra per scan, using a mass range of 50 to 600 m/z. The Multichannel Plate (MCP) detector voltage was adjusted to 1,850 volts.

Mass spectral data were processed by the open-source software BinBase. After peak picking, alignment, and annotation of data files, the resulting spectra were analyzed for chemical content. Our focus here was to determine whether nicotine was present. In the future, we plan to investigate whether other alkaloid compounds can be detected that are found in plants commonly smoked by ethnographic Native Americans and to see if overall chemical content of ancient pipes match any of our experimental pipes.

Pilot Smoke Plant Study

As a wide variety of smoke plants were used by Native Americans, not just tobacco, the AMG characterized a suite of 23 commonly smoked plants via GC/MS (Table 2). Dried and live native plant specimens were obtained from the U.C. Davis Arboretum, native plant nurseries, and Joseph Winters, a tobacco scholar and professor at the University of New Mexico. Plant samples were all dried and crushed to a fine powder. Additionally, samples of *Angelica arguta*, *Angelica hendersonii*, *Arbutus menziesii* (Pursh.), *Arctostaphylos uva-ursi* (L.) Spreng., *Artemisia douglasiana*, *Lomatium californicum*, *Nicotiana glauca*, *Nicotiana quadrivalvis* (Pursh.) var. *quadrivalvis*, *Nicotiana rustica* (Oneida), *Nicotiana tabacum*, and *Nicotiana* sp. were “smoked” in experimental clay pipes.

Table 2. Native smoke plants characterized in the AMG pilot study.

SCIENTIFIC NAME	COMMON NAME	ORGAN TYPE
Nicotiana sp.	American spirit tobacco (modern)	Leaves
Angelica arguta	Lyall's angelica	Root
Angelica hendersonii	Henderson's angelica	Root
Arbutus menziesii Pursh	madrone	Leaves
Arctostaphylos glandulosa Eastw.	Eastwood's manzanita	Leaves
Arctostaphylos uva-ursi [(L.) Spreng.]	bearberry/ kinnikinnick	Leaves
Arctostaphylos uva-ursi var. *A. nummularia*	Emerald carpet kinnikinnick (hybrid)	Leaves
Artemisia douglasiana	Mugwort	Leaves
Datura innoxia	Sacred datura	Leaves
Datura wrightii Regel	Sacred thornapple	Leaves
Datura wrightii Regel	Sacred thornapple	Seeds
Gaultheria shallon Pursh	Salal	Leaves
Lomatium californicum [*Leptotaenia californica* Nuttall]	Indian celery	Root
Nicotiana glauca	Tree tobacco	Leaves
Nicotiana trigonophilla	Coyote tobacco	Leaves
Nicotiana attenuata	Mountain tobacco	Leaves
Nicotiana quadrivalvis var. *quadrivalvis*	Mandan tobacco	Leaves
Nicotiana rustica	Lakota tobacco	Leaves
Nicotiana rustica	Oneida tobacco	Leaves
Nicotiana tabacum	Cherokee tobacco (commercial)	Leaves
Phoradendron sp. Nutt.	Common mistletoe	Leaves
Salvia sonomensis [*Ramona humilis* Greene]	Creeping sage	Leaves
Taxus brevifolia Nutt.	Yew	Needles

Experimental pipes were composed of clay and smoked by pulling the plunger on a 60-cc syringe connected to the pipes with heat shrink tubing. After smoking, clay pipes were sectioned into 11 equal parts, and residue from six out of 11 sections was analyzed. In total, there were 22 species of plant samples analyzed and 11 species of plants smoked in experimental pipes. Each plant sample and experimental pipe sample was analyzed in six replicates.

ANALYSIS AND RESULTS

A wide range of compounds was identified in the pipe samples; the majority of these are common lipids present in a wide range of plants and animals. However, nicotine, a biomarker for tobacco plants (associated with wild and domesticated species of tobacco or *Nicotiana* spp., including *N. trigonophilla*, *N. attenuata*, *N. quadrivalvis* var. *quadrivalvis*, *N. rustica*, and *N. tabacum*) was identified in two of the samples: the whole pipe from Burial 149, and the bowl section of the pipe associated with Burial 47. Figures 3 and 4 show chromatographs for masses 161 (in orange) and 162 (in green) for both these samples. Nicotine is expected to elute from the column at 8.27 minutes. Both these samples show peaks in the chromatograph at this location.

Further, both figures contain insets showing the mass spectra at 8.27 minutes (grey inset) and reference mass spectra for nicotine (white inset). The reference mass spectra for nicotine show characteristic peaks for molecular ions with masses of 84, 133, 161, and 162. The compound eluting off our column at 8.27 minutes for both pipe samples shows the characteristic ions with masses of 84, 133, 161, and 162, indicating the presence of nicotine in both pipe fragments.

By contrast, the mouth-piece and sediment collected from inside the pipe associated with Burial 47 did not produce a signature for nicotine. Figure 5 shows the lack of a peak for the mouth piece. This result is consistent with our experimental pipes, where sections closest to the mouthpiece have much lower quantities of nicotine than sections near the bowl. Likewise, scrapings of the surface material and sediment samples we have examined from other experimental and ancient pipes fail to produce significant

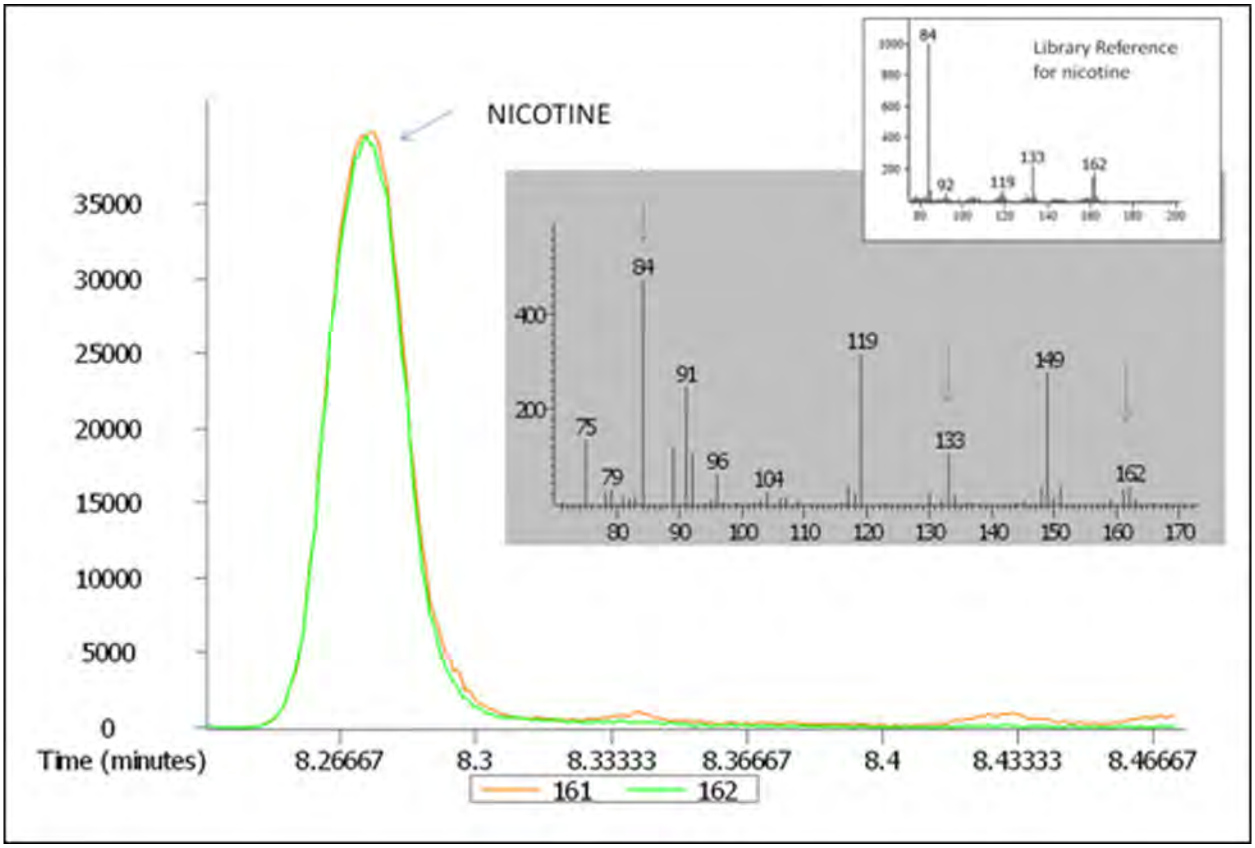

Figure 3. Section of gas chromatograph for pipe associated with Burial 149, with insets of mass spectra at 8.27 minutes (in grey) and reference mass spectra for nicotine (white).

amounts of nicotine. These findings suggest that the best place to sample ancient pipes is from the bowl section and that sediments from inside pipes are either not associated with smoking activities or have completely oxidized or degraded organic remains.

CONCLUSION

In sum, both pipes contained the alkaloid nicotine. From this, we conclude that both pipes were definitely used for smoking tobacco, though only the bowl section of one produced the biomarker nicotine. In line with our previous analyses, mouth pieces of pipes are less likely to preserve nicotine or other alkaloid biomarkers.

Radiocarbon dates on collagen from the two ALA-554 burials place the presence of tobacco in central California no later than A.D. 1415, and perhaps as early as A.D. 1305. Because these are among the first dates on tobacco in central California, additional dating of materials associated with pipes containing nicotine residues will undoubtedly push the date of tobacco even earlier.

The presence of nicotine in these two pipes, of course, does not preclude the smoking of other substances. In the future, we plan to search the spectra from these pipes for other alkaloid compounds that we were able to detect in the pilot study, including arbutin (*Arbutus menziesii* Pursh, or madrone; *Arctostaphylos glandulosa*, or Eastwood's manzantia; *A. uva-ursi* or bearberry/kinnikinnick) and anabasine (*Nicotiana glauca*, or tree tobacco) and scopolamine (*Datura innoxia*, or sacred datura; *D. wrightii* Regel, or sacred thornapple).

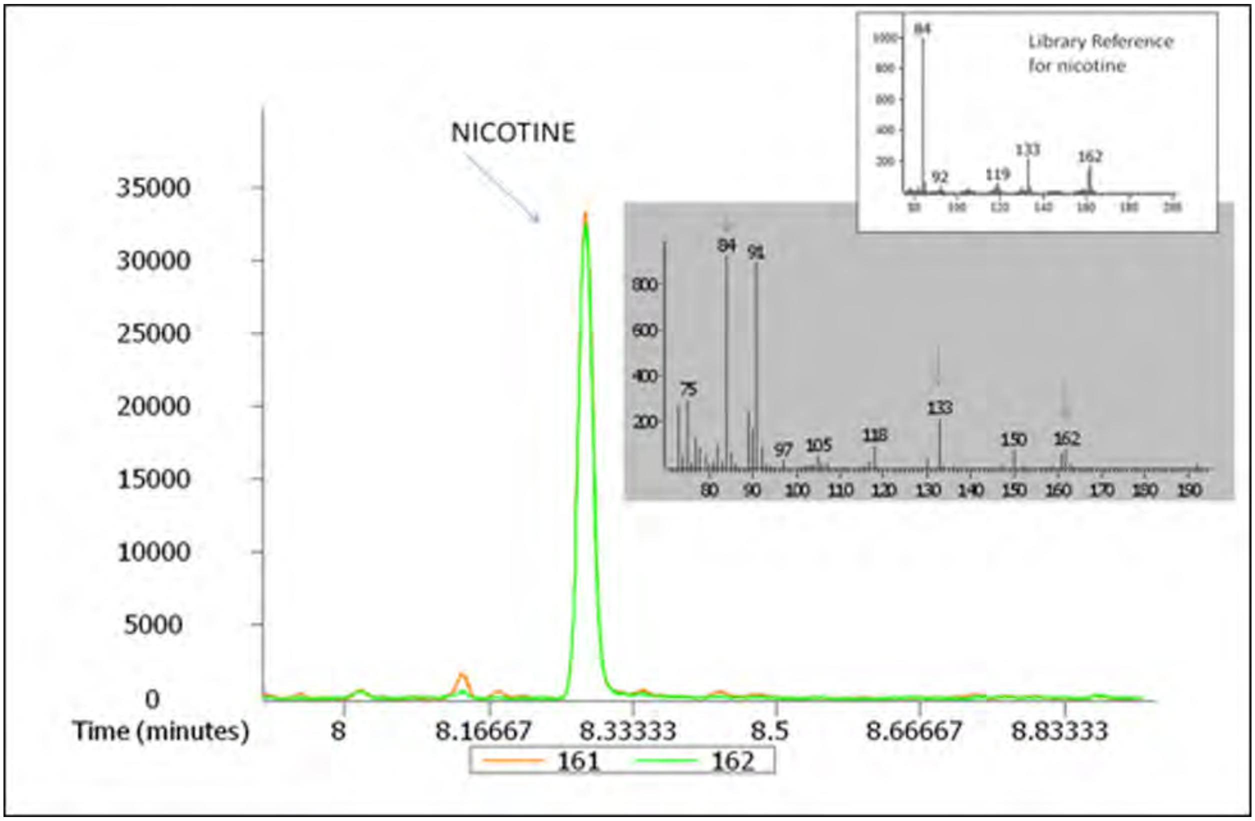

Figure 4. Section of gas chromatograph for bowl of pipe associated with Burial 47, with insets of mass spectra at 8.27 minutes (in grey) and reference mass spectra for nicotine (white).

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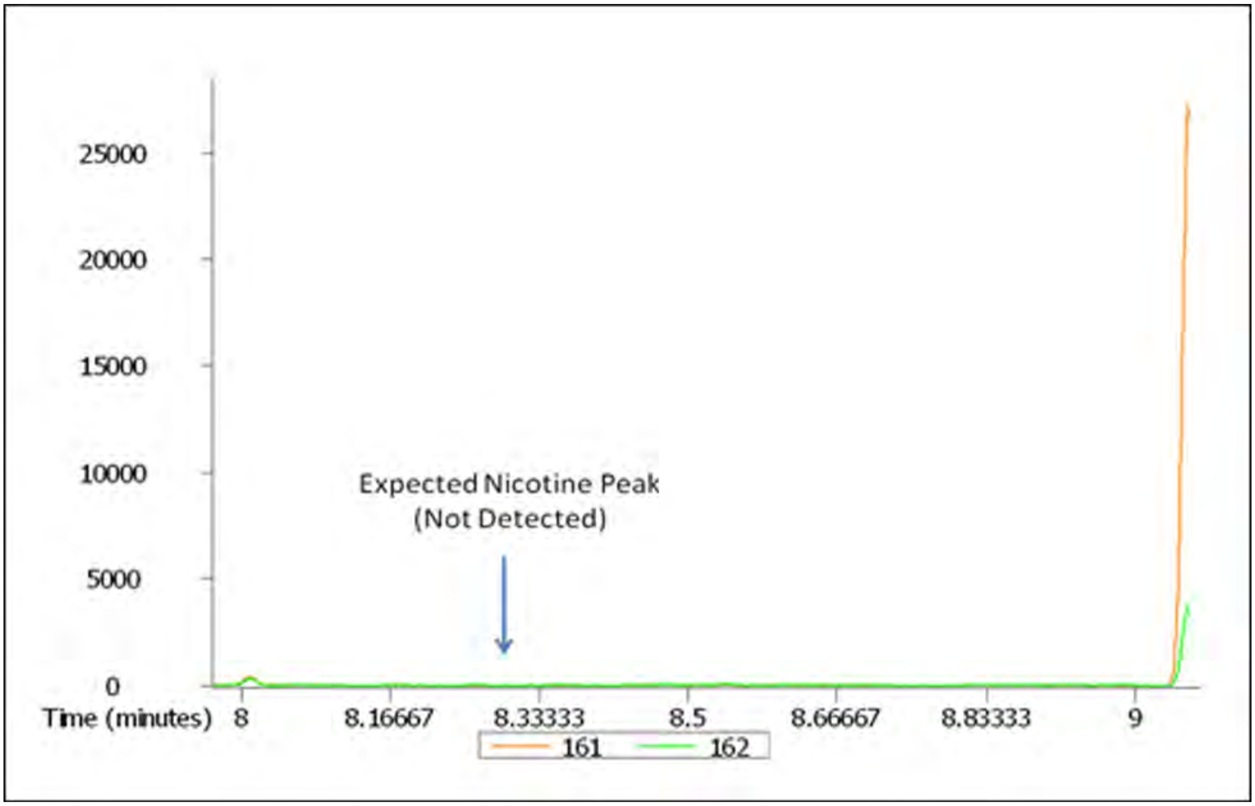

Figure 5. Section of gas chromatograph for mouthpiece associated with Burial 47, showing no peaks for molecular ions with masses 161 and 162 at 8.27 minutes, indicating no nicotine.