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# Testing the validity of stable isotope analyses of dental calculus as a proxy in paleodietary studies

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ABSTRACT

Stable isotopic analyses ($\delta^{13}C$, $\delta^{15}N$) of dental calculus have been suggested as a proxy for the study of diet of ancient populations but questions about their validity have been raised. Here we test this question, introducing significant improvements in the analysis of $\delta^{13}C$ and comparing our results for $\delta^{13}C$ and $\delta^{15}N$ of calculus with corresponding analyses of associated well-preserved bone which are widely believed to provide reliable paleodiet values. The content of organic material in calculus is decreased by ~75% compared with modern calculus, resulting in diagenetic changes to $\delta^{13}C$ and $\delta^{15}N$ of organic matter. Neither $\delta^{13}C$ nor $\delta^{15}N$ analyses of the organic component of calculus provide accurate estimates of paleodiet. Although $\delta^{15}N$ values of dental calculus are correlated with $\delta^{15}N$ values of bone collagen from the same individual, it is clear that they have been greatly affected by diagenesis, as shown by a correlation between C/N ratio and $\delta^{15}N$. The inorganic (mineral-bound) carbon component of calculus, analyzed separately from the organic component, gave $\delta^{13}C$ values slightly offset from $\delta^{13}C$ values of $CO_3$ in bone mineral. Thus it alone appears to have potential as a dietary proxy.



## 1. Introduction

Dental calculus, or mineralized plaque, is common in archaeological remains and has been utilized in dietary research using a variety of methods. Recently, dental calculus in modern and archaeological remains has been analyzed isotopically to evaluate its use as a proxy to bone in paleodietary studies (Dorio, 2012; Scott and Poulson, 2012; Poulson et al., 2013; Salazar-García et al., 2014; Eerkens et al., 2014). Dorio (2012) found that the stable carbon isotope ratios ($\delta^{13}C$) of modern dental calculus samples of living individuals were significantly correlated with those of hair and nails, while the $\delta^{15}N$ values were not. Scott and Poulson (2012) found that $\delta^{13}C$ and $\delta^{15}N$ values of 12th to 19th century Basque and Spanish skeletons and one prehistoric Alaskan Inuit skeleton resembled values in bone collagen from European sites of varying time periods, and suggested the use of calculus isotopes as a paleodietary proxy. Poulson et al. (2013) showed that $\delta^{13}C$ and $\delta^{15}N$ values of dental calculus from archaeological sites in northern Chile dating from the Archaic to the Late Intermediate period were comparable to values from bone collagen and tooth dentin.

Eerkens et al. (2014), studying multiple archaeological sites in California and one in Africa, found a positive correlation between bone collagen $\delta^{15}N$ values and dental calculus $\delta^{15}N$ values of the same individual, although the calculus isotope values exhibited a greater range of variation (lower $\delta^{13}C$ values and higher $\delta^{15}N$ values) compared to bone.

Salazar-García et al. (2014) found, however, that $\delta^{15}N$ and $\delta^{13}C$ values of dental calculus from a medieval cemetery in Spain did not correlate with those of bone and dentin collagen samples taken from the same individuals, although average values were similar to those of collagen. They suggested that dietary interpretations based on the stable isotope analysis of dental calculus must be done with caution.

## 2. The composition and formation of dental calculus

Dental calculus is the result of mineralization of the dental biofilm, plaque (Hillson, 1996, 2005; Lieverse 1999). In deposits six months in age or older the mineral component is hydroxyapatite (Hillson 1996, 2005; Jin and Yip, 2002; Hayashizaki et al., 2008;

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Bosshardt and Lang, 2015). Organic components include proteins, glycoproteins, lipids, and carbohydrates, plus trapped food particles (Mandel, 1990; Hillson, 1996). Supragingival calculus formation begins with the deposition of an organic layer that is colonized by microorganisms, which obtain their nutrients primarily from saliva (Mandel, 1990; Marsh, 2015). Saliva is highly supersaturated with respect to hydroxyapatite (Larsen, 1975; Ekstrand et al., 2015), and hydroxyapatite crystals form on the bacterial surfaces, and within the internal structure of the bacteria (Zander et al., 1960). Dental calculus is appositional, accreting alternating layers of gram-positive and gram-negative bacteria (Mandel, 1990; Jin and Yip, 2002; Warinner et al., 2014). Modern and ancient calculus contains thousands of phylotypes of bacteria (Adler et al., 2013; Warinner et al., 2014; Weyrich et al., 2017). The rate of accumulation of calculus is controlled by variation in diet, mineral content of drinking water (Gaare et al., 1989), and mineral concentration and rate of production of saliva (Lieverse, 1999), among other factors.

Modern supragingival calculus contains about 16 wt% organic matter (Jin and Yip, 2002). Dorio (2012), in a study of 33 randomly selected dental patients in Reno, Nevada showed that the average C/N ratio was $5.4 \pm 0.4$.

### 2.1. Goal of this research

In all previous studies of the isotopic composition of calculus, an elemental analyzer (EA) was used to analyse bulk dental calculus samples. Analysis in an EA results in all of the C atoms of the sample (both organic molecules and apatite-bound $CO_3$) being converted to $CO_2$ by heating in the presence of oxygen.

During this process N atoms are also converted to $N_2$ gas, and analyses of $\delta^{13}C$ and $\delta^{15}N$ are carried out on these gases. As we have seen above, dental calculus is a composite material made of an organic component (plaque) plus trapped food particles, as well as an inorganic mineral phase hydroxyapatite, containing carbonate ($CO_3$). In an EA analysis of bulk calculus the $CO_3$ is liberated as $CO_2$ which mixes with the $CO_2$ formed by oxidation of the organic component. As a result, the $\delta^{13}C$ values obtained are a blended value for the apatite-bound carbonate and organic components. We would expect that the $\delta^{13}C$ of the $CO_3$ in dental calculus would resemble values for bone mineral since the $CO_3$ component of both is precipitated from the same serum-derived pool of bicarbonate either in the extracellular fluid of bone or in the saliva (Dawes, 1970). It would therefore differ by about 12‰ from the $\delta^{13}C$ of the diet (Prowse et al., 2004) whereas the $\delta^{13}C$ of the organic component could be offset by a different, smaller amount from diet. It would therefore be preferable to analyze these two components separately. Thus two important objectives of this research were to: 1) determine if organic and inorganic components of dental calculus could be extracted and analyzed separately for $\delta^{13}C$; and 2) compare the $\delta^{13}C$ values measured in these components with corresponding components in bone (i.e. bone collagen and bone bioapatite) samples taken from the same individuals. As well, we would revisit the comparison between $\delta^{15}N$ values of the organic component of calculus and the $\delta^{15}N$ value of bone collagen from the same individual.

The overall goal is to determine if isotopic analyses of calculus can be considered possible proxies for paleodiet. This would be true if we could show that the δ-values of the organic and inorganic components of calculus were offset by a constant amount from the δ values of corresponding components (collagen and mineral) of associated bone, which have already been shown to be reliable proxies for paleodiet. The study will focus on one particular archaeological site, but we will show later that problems identified here are very likely to be present in other published studies.

## 3. Methods and materials

The samples used in this study derive primarily from the Kalfata-Budjaka necropolis associated with the ancient Greek colony of Apollonia Pontica, founded in 610 BC by colonists from the city of Miletus in Asia Minor (Turkey). Representing a portion of the more than 1500 burials that have been excavated to date, the samples come from five different locations within the necropolis and span the mid-5th to mid-3rd centuries BC. Additional details on Apollonia and its necropolis may be found in Nedev and Panayotova (2003) and Hermary et al. (2010).

Archaeological evidence from Apollonia, including food remains found in fireplaces that were associated with some of the graves suggests that the population ate a varied diet of fish, meat, shellfish, grains and nuts (Panayotova, 1998). Previous $\delta^{13}C$ and $\delta^{15}N$ analyses of bone collagen from the site indicate that these individuals consumed a mixed diet of terrestrial ($C_3$) and marine resources (Keenleyside et al., 2006).

We analyzed calculus deposits removed from the teeth of 27 individuals: 13 females, 13 males, and one individual of indeterminate sex (Table 1). All but one was adult. Samples of calculus were mainly removed from a single tooth representing each individual (see Supplemental Material, Table S1). For nine individuals, however, this proved inadequate, and material was removed from two to four teeth and pooled into a single sample to provide sufficient material for analysis. Three subsamples were taken from the $RM_2$ of individual Ap 451 to test for variation of $\delta^{13}C$ of $CO_3$ in the mineral component of the calculus within an individual. Also, to test for the reliability of the analyses of $\delta^{13}C$ of the mineral component, two samples were analyzed from a pooled sample of dental calculus taken from four teeth of individual Ap 5525-461.

### 3.1. Preparation of bone samples

Collagen was extracted from the bone samples following the procedures outlined in Chisholm et al. (1983). The bone samples were cleaned ultrasonically; fragments 15–25 mm across and weighing approximately 5 g were taken of each bone. The samples were soaked in 0.25 M hydrochloric acid (HCl) until all of the mineral was dissolved. The remaining collagen samples were soaked in 0.1 M sodium hydroxide (NaOH) for 20 min to remove any humic contaminants. The samples were then solubilized in 0.1 N HCl at 90 °C, filtered, and evaporated in a drying oven. A 100 mg subsample of bone to be analyzed for $\delta^{13}C$ of bioapatite was prepared using a modified version of the protocol of Koch et al. (1997). The sample was crushed into a fine powder using a mortar and pestle, and treated for three days with 4 ml of 2.5% sodium hypochlorite (NaOCl; bleach) to remove collagen. The sample was then spun down on a centrifuge, washed 5X with deionized water, and treated for 24 h with 4 ml of 1 M acetic acid-acetate buffered solution (pH ~ 4.5). Lastly, the samples were spun down in a centrifuge, rinsed with deionized water (DIW) 4X, and dried at 37 °C.

### 3.2. Preparation of dental calculus samples

The isotopic and chemical analyses of the calculus were done in two stages: one for the organic component of the calculus and the other for the analysis of the mineral component. These separate components will be referred to by the terms “organic calculus” and “mineral calculus”. In preparation to obtaining analyses of these two components, the calculus samples were pre-treated using techniques similar to those used on the bone samples.

For analysis of organic calculus, calculus which had been scraped off one or more teeth was broken into 2–3 mm pieces, and