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Tiina Väre

IMPROVING THE TEMPORAL REPRESENTATIVITY OF DENTIN SERIAL SAMPLES IN STABLE ISOTOPE STUDIES

Tiina Väre, Laboratory of Chronology, Finnish Museum of Natural History, P.O. Box 64, 00014 University of Helsinki & Archaeology, P.O. Box 1000, 90014 University of Oulu, Finland. tiina.vare@helsinki.fi ORCID 0000-0002-6558-5359

Väre, T. 2024. Improving the temporal representativity of dentin serial samples in stable isotope studies. *Fennoscandia Archaeologica* XLI: 135–139. https://doi.org/10.61258/fa.152128

INTRODUCTION

Dentin micro-sampling techniques and stable isotope analyses have been extensively utilized in studies exploring childhood diets within archaeological populations (e.g., Eerkens et al. 2011; Beaumont et al. 2013; Henderson et al. 2014). This is possible because dentition develops during early life and as the isotopic composition of teeth is inert, it reflects the dietary conditions of this period – apart from that of the later forming secondary and possibly tertiary dentin (Meinl et al. 2007; Smith et al. 2012).

The methodology offers a tool to assess past breastfeeding and weaning schedules. For example, Beaumont and colleagues (2013) analyzed the $\delta^{15}N$ and $\delta^{13}C$ values of transverse, 1 mm dentin segments cut successively from bisected and gelatinized first permanent molars (later M1).[1] I have used this method to tentatively address the breastfeeding customs among a few Finnish archaeological populations (Väre et al. 2022a; 2022b; 2023). The years spent utilizing the methodology have taught me that it only allows very crude estimations of the developmental periods for the transverse dentin segments: similar-sized sections naturally take different times to develop depending on the size of the tooth. But this is far from where the troubles end.

First, permanent molars develop during infancy (see AlQahtani et al. 2010), which is why their dentin isotope composition provides information about diet during infancy – including breastmilk consumption that leads to elevation of particularly the $\delta^{15}N$ value (e.g., Fogel et al. 1989; Fuller et al. 2006). As dentin develops from the crown toward the root, dietary changes can be traced following the changes

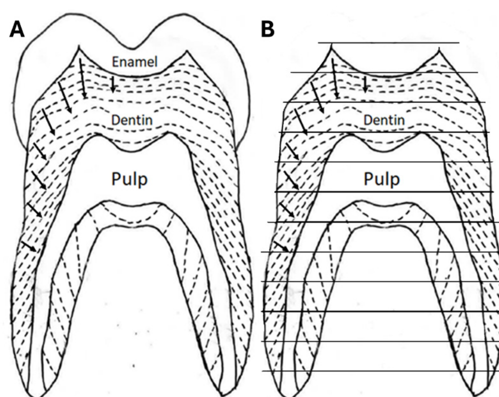

Figure 1.A. The schematical sagittal cross-section of the first permanent molar illustrates the direction and internal pattern of dentin growth increments (image modified by T. Väre after Eerkens et al. 2011, Fig. 2). As a result of the uneven, diagonally oriented growth, dentin does not accumulate evenly following the longitudinal axis of the tooth. B. In many earlier studies, the gelatinized dentin of bisected molars has been cut transversally resulting in parallel dentin segments of equal width. Such a way of cutting does not respect the true direction of dentin development and leads to increased overlapping of growth from different periods in segments toward the root.

in the delta values in the series of subsequent samples. The height of the analyzed segment defines the duration of the period represented by its delta values. It has been estimated that every 1 mm segment takes approximately half a year to develop (or if the various reviewers who have estimated my work are to be trusted, contains dentin grown during 3 to 9 or even 12 months, see also Eerkens et al. 2011; Beaumont et al. 2013; Beaumont & Montgomery 2015; 2016).

THE TROUBLESOME DENTIN SEGMENT DEVELOPMENT PERIOD ESTIMATIONS

The dentin growth rate is not even across the tooth, and the growth direction is not vertical – i.e., dentin does not accumulate in layers perpendicular to the longitudinal axis of the tooth (Fig. 1). Thus, the dentin in the central parts of a transversally cut segment has developed later than the dentin of its outer edge – and this difference is emphasized during the growth of the tooth, as the diagonal pattern becomes steeper towards the lower parts of the root. Consequently, the first couple of segments are the easiest ones to date accurately as the growth of dentin closest to the dentin-enamel junction is primarily directed toward the root, while the development of dentin in the root segments temporally overlaps much more. This causes the values of successive samples to partially reflect the diets of the same periods, time-averaging the values. This produces a rolling average of values that do not accurately represent the diets of limited, subsequent periods. (cf. Eerkens et al. 2011; Henderson et al. 2014; Beaumont et al. 2016.) For example, it is unclear whether the stabilization of values often seen in breastfeeding profiles after the very first segments is a sign of ceased breastfeeding (Fig. 2), or more an effect of the sampling technique causing several samples to contain large amounts of simultaneously developed dentin.

IMPROVEMENTS TO AGE-ESTIMATION ACCURACY

In recent years, new methods have been introduced to improve the accuracy of estimating which periods are reflected by the stable isotope values of dentin samples. These improvements are for a large part an effect of developments in mass spectrometry, enabling the isotope relations of ever smaller samples to be accurately measured. Czermak and colleagues (2018) approached the problem by visualizing the site of the dentin increments using transmission light microscopy on a longitudinal ~70-µm-thinsection cut from the mid-part of the tooth and mounted on a microscopy slide. They used images taken of this sample as a visual reference for the correct location of the lines in a demineralized, 1.5 mm thick longitudinal dentin that was sampled with the aid of a dissecting microscope. Czermak also led another study (2020) in which a 2 mm wide longitudinal central slice was cut from a molar, demineralized, and micro-sampled sequentially from the crown cusp to the root apex with a 1 mm diameter biopsy punch with a plunger. Both these techniques avoid mixing

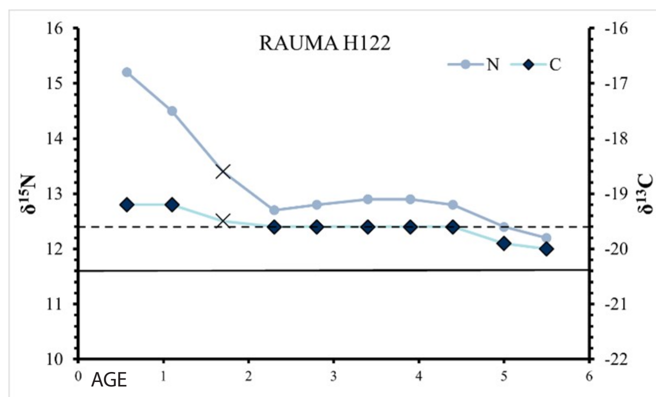

Figure 2. In early childhood dietary profiles, the initial elevation of the δ15N and δ13C values is followed by gradual stabilization near the maternal levels here represented by the population averages (black line δ15N and dashed line δ13C, also note that in the sample marked with X, the collagen quality was insufficient). The declining pattern of values is supposedly caused by first the introduction of weaning foods and then the cessation of breastfeeding. This is when the infant is lowered in the food chain from above the mother to her level (provided they consume isotopically similar diets). This example is from the 19th-century population of Rauma, Southwestern Finland (Väre et al. 2022a). Image: T. Väre.

significant amounts of simultaneously developed dentin between samples and make estimating the developmental periods easier. They have also already been applied to childhood dietary studies (cf. Fernández-Crespo et al. 2018; 2020). Curtis and colleagues (2022) have also introduced a novel way of cutting the incremental samples according to the dentin development lines. They sectioned a tooth-halve into a 1.5 mm thick longitudinal section, which was demineralized and lyophilized before being cut along visible incremental structures using MicroMill software producing as many as dozens of samples from a single tooth.

The new methods compared to the traditionally used 1 mm protocol certainly improve the temporal accuracy and representativity of childhood diet studies (cf. Cheung et al. 2022). The incremental micro-sampling technique by Curtis and colleagues (2022) sounds particularly promising. This method, however, is highly technical requiring specialized tools and software that are not found in every laboratory working with stable isotope samples. During my work, I have noticed that the temporal representativity of the dentin serial samples could be enhanced with much smaller efforts, which I will shortly introduce.

Restricting the sampling to the outer perimeter of the transverse, gelatinized dentin slices cut according to the protocol of Beaumont and colleagues (2013), and thus leaving the internal parts out of the analyses would reduce temporal overlapping between samples. This would make the values measured in subsequent segments align more accurately in chronological order and they would still form a temporally continuous series. Moreover, discarding the dentin surrounding the pulp chamber from analyses would solve the problem caused by the age-bound secondary dentin formation and its time-averaging effects (cf. Smith et al. 2012). As schematically demonstrated in Figure 3, after sectioning the gelatinized dentin of the sagittally bisected tooth halves into subsequent segments, only the outer "rim" of these roughly semicircular dentin slices would be cut out with a scalpel – and subsequently denaturized, lyophilized, weighed, and analyzed.

The $\delta^{15}N$ and $\delta^{13}C$ values are analyzed from dentin collagen. This is why the amount of collagen extracted from these modified

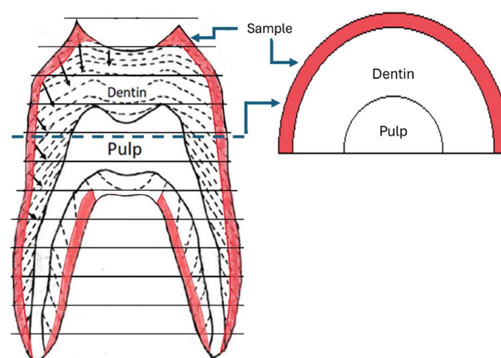

Figure 3. Sagittal cross-section of a molar and its schematic axial illustration presenting the sampling sites in red. The samples are cut from the perimeter of the semicircular, transverse segments of the gelatinized tooth-halves. This way, the periods during which the dentin in each sample has formed overlap much less (see Fig. 1). Image: T. Väre.

samples, comprising only of the outer rim of micro-slices, may be worth considering. In the protocol following Beaumont et al. 2013, the first segment sectioned from the half of M1 consists merely of the cusps, meaning that the amount of dentin is minuscule. Nevertheless, the amount of extracted collagen has almost always been sufficient for packing at least one 0.4–1.0 mg (depending on the laboratory) sample which has yielded reliable results. Unlike in the ultrafiltration method (Brown et al. 1988), the yield of collagen cannot be calculated using this method (whole sample demineralization). However, according to Sealy and colleagues (2014), the weight-% of carbon and nitrogen as well as their atomic ratio but not the yield are the most important determinants of collagen quality in stable isotope analyses. Nevertheless, samples with extremely low collagen yield as well as low carbon and nitrogen concentrations should still be discarded (Guiry & Szpak 2021). Based on my previous experience of visually abundant (albeit not weighed) yields of collagen from even just the cusps, I find it likely that outer-rim samples cut from the segments smaller than the standard 1 mm would contain enough collagen provided the preservation was sufficient.[2] The roots may make an exception to this if they are very narrow near the apex: particularly as it is advisable to only sample one of the roots of bifurcated teeth

to avoid mixing dentin grown during different periods. This is difficult to coordinate between the roots.

The suggested improvement does naturally not remove all the problems of the previous method. For example, without following the dentin growth lines, it is not possible to consider the variation in the growth rate of dentin and to accurately estimate the exact age at which the sample was developed. Moreover, even when the analyzed parts of dentin are rather small, the sampling still destroys half of the tooth. Thus far, the discontinued funding of my breastfeeding studies has prevented me from testing the method in practice. It will, however, be interesting to see whether this improvement changes the pattern seen in the early childhood dietary profiles.

ACKNOWLEDGEMENTS

I want to thank Jenny and Antti Wihuri Foundation.

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NOTES

[1] According to the protocol of Beaumont and colleagues (2013) the cleansed and bisected teeth are ultrasonicated in ultrapure water and their detachable enamel removed before submerging them in 0.5 M hydrochloric acid (HCl) for approximately 1 to 2 weeks. The demineralized tooth halves are rinsed with ultrapure water and sliced in parallel transverse segments (of 1 mm) beginning from the crown and proceeding to root tip. These segments are denaturalized in 0.001 M HCl solution in separate microcentrifuge tubes at 70°C for 24 hours, the solution is centrifuged, frozen, and lyophilized before weighing the resulting dry collagen samples in to tin cups for IRMS-analysis.

[2] The issue of archaeological samples containing carbon from humic acids of soil should be considered (particularly in Finland). NaOH-treatment removes acid (and lipids) from the sample (Ambrose 1990), but the treatment can reduce the amount of collagen (Chisholm et al. 1983), which may be a problem particularly with poorly preserved samples. The Beaumont et al. 2013 -protocol does not originally include NaOH-treatment.