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# A guide for an anatomically sensitive dentine microsampling and age-alignment approach for human teeth isotopic sequences

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## Abstract

**Objectives:** Stable isotope analysis of sequential dentine samples is a potentially powerful method to reveal insights into early life-histories of individuals in the past. Dentine incremental growth structures are complex, however, and current approaches that apply horizontal sectioning of demineralized tooth halves or quarters risk combining multiple growth layers and may include unwanted cementum or secondary dentine. They also require destruction of large parts of a tooth. Here, we present a less destructive and relatively straightforward protocol that reduces damage, increases temporal resolution, and improves the accuracy of age-alignment between individuals.

**Material and methods:** We outline a protocol that includes the sampling of small (1 mm diameter) cylindrical plug transects from a thin section, along with an age-alignment scheme predicated on average growth rates for dentine areas.

**Results and discussion:** The proposed protocol is readily applicable and more anatomically sensitive than horizontal slicing. Micro-samples are smaller (in both length and depth), hence minimizing temporal overlap and avoid directions that may contravene growth pattern. They completely avoid areas where secondary and tertiary dentine or cementum can be deposited. Age-alignment is improved by using growth ratios of anatomical tooth zones.

**Conclusion:** This method minimizes destruction, enables finer temporal resolution and facilitates data comparison. It can be readily combined with fluorescence imaging-based or other pre-screening methods of dentine collagen preservation.

## KEYWORDS

carbon and nitrogen isotope analysis, dental growth, dietary life-histories, juvenile feeding-practices

## 1 | INTRODUCTION

Well preserved bones and teeth allow for the study of human subsistence and life ways due to a range of bioarcheological approaches, including stable isotope analysis. Bone collagen isotope composition, represented by single bulk measurements, provides a measure of an individual's average diet, often represented as the last decade although this is uncertain due to bone's variable turnover rate through the life span (Hedges, Clement, Thomas, & O'Connell, 2007). This tells us little about dietary change during an individual's lifetime, especially during infancy and childhood. Consequently one of the major advances in recent years has been the attention paid to primary dentine analysis, as it not only reflects earlier life history but also potentially allows the recording of sub-annual variation in dietary practices because it does not remodel once deposited (e.g., Dean & Scandrett, 1995). Thus, sequential dentine isotope analysis is recognized as a valuable approach to address questions about life-histories from a dietary perspective, especially those related to infant diets and care, and past identity. The majority of dentine-based sampling protocols for stable isotope analysis currently apply sequential sampling of approx. 1 mm wide horizontal dentine sections from crown to root using a scalpel on half or a quarter longitudinally-cut demineralized tooth (e.g., Beaumont, Gledhill, Lee-Thorp, & Montgomery, 2013; Beaumont & Montgomery, 2016; Eerkens, Berget, & Bartelink, 2011; Eerkens, Bartelink, Bartel, & Johnson, 2019; Eerkens, Sullivan, & Greenwald, 2016; Henderson, Lee-Thorp, & Loe, 2014; Sandberg, Sponheimer, Lee-Thorp, & Van Gerven, 2014; Scharlotta et al. 2018; Tsutaya, Ishida, & Yoneda, 2015).

This protocol has the advantage of being straightforward and easily replicable. Its major disadvantage is that it neglects the complexity of the dentine deposition pattern (see Eerkens et al., 2011), which follows an inward and rootward direction (Nanci, 2008), analogous to "dome-like" layers that progressively increase in convexity toward the apex. As a result, 1 mm wide horizontal micro-dentine sections taken from a half or a quarter tooth include numerous bi-directional dentine incremental layers cut in a way that does not strictly correspond to successive discrete growth (see Dean et al., 1995; Eerkens et al., 2011; Guiry, Hepburn, & Richards, 2016). Consequently, adjacent sequential micro-dentine samples, especially from roots where increments become much more convex, include material from several growth-layers and thus do not represent separate time-spans. Furthermore, there is a risk of cutting through cementum on the outer surface of the root or areas with secondary and tertiary dentine deposits such as pulp or radicular channels (Hillson, 1996). Those tissues are formed later than dentine and may confound attempts at estimating early life history dietary data.

Sampling smaller sections achieves finer temporal resolution and circumvents some of the methodological limitations described above (e.g., Beaumont, Gledhill, & Montgomery, 2014). More complex cutting approaches can take dentine growth patterns into account (Czermak, Schermelleh, & Lee-Thorp, 2018). Yet the ability to minimize incremental overlapping is still limited. Moreover, practical restrictions related to minimum sample sizes, collagen yields and mass

spectrometry performance have to be taken into account (e.g., Burt & Amin, 2014).

Moreover it must be acknowledged that current dentine micro-sampling procedures are inherently destructive. Problems regarding preservation and loss of precious archeological material for further analysis are therefore implicit. Minimizing sample destruction should be a priority (Austin, Sholts, Williams, Kistler, & Hofman, 2019). Here, we outline a less destructive and yet more anatomically sensitive protocol of sequential human dentine microsampling for stable isotope analysis, coupled with a new approach for improving age-alignment resolution. We also suggest good practice recommendations for recording and preserving as much information as possible prior to sampling teeth, and improving mass spectrometry performance when working with small, single samples.

## 2 | METHODS

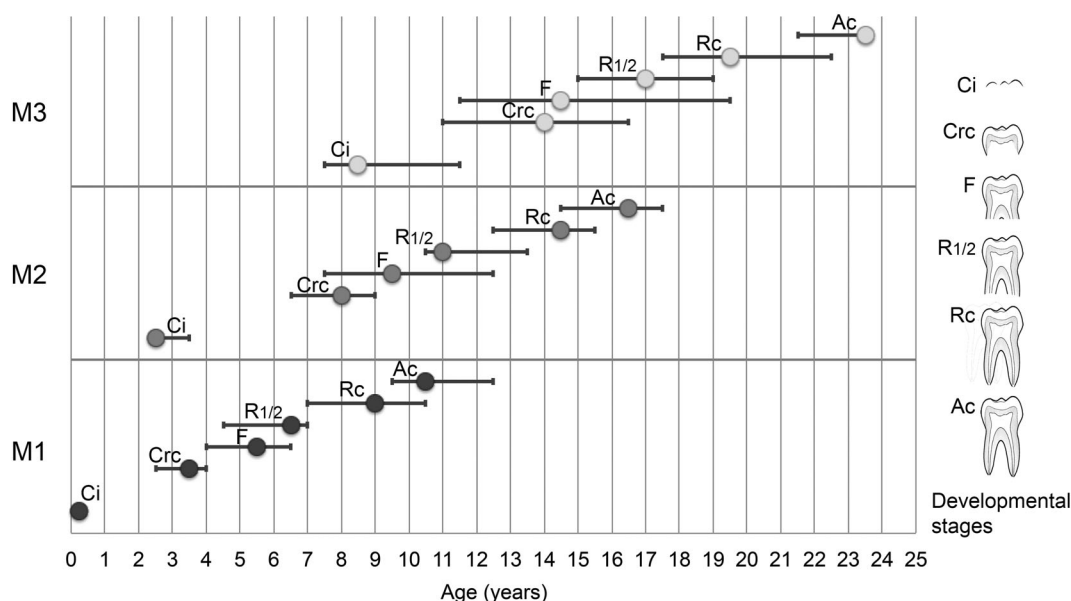
### 2.1 | Sample selection

Teeth tend to be one of the best preserved skeletal elements in the archeological record and they are informative for a wide range of analyses, including aDNA, dental micro-wear, pathologies, morphometric and biological affinity analyses, as well as diet and mobility based on isotopic composition. Thus, it is incumbent on researchers who apply destructive methods to adequately record the teeth, and to minimize damage as far as possible (Forshaw, 2014). It goes without saying that sample selection and sampling strategy must follow curatorial concerns and restrictions, and justified on a cost-benefit basis. The research questions—whether about nursing, weaning practices, child-rearing practices, adolescent diet, etc.—self-evidently require that the formation ages and the stage of development of the respectively selected teeth (e.g., molars,<sup>1</sup> single-rooted or deciduous teeth) are considered (e.g., Beaumont et al., 2018; Burt & Amin, 2014; Eerkens et al., 2016). It is worth pointing out, that this is not necessarily straightforward because of the variation in development (Figure 1), making comparisons between individuals difficult or targeted events hard to identify.

Although intact or well-preserved teeth are preferred, this is not always possible. Carious lesions and exposed dentine likely lead to collagen damage. Thus, fluorescence screening can be useful for assessing collagen preservation if poorly preserved areas are suspected (Czermak, Schermelleh, & Lee-Thorp, 2019).

### 2.2 | Minimizing information loss

Advances in high-resolution photogrammetry and scanning mean that it is relatively straightforward to record teeth in detail for 3D modeling or printing, in addition to standard photography or impressions of the crown. It is good practice to record all appositional, later grown tissues such as secondary and tertiary dentine and marked cementum layers, and tooth decay and attrition (e.g., Beaumont et al., 2013).



**FIGURE 1** Interval of the “age-of-completion” of the used developmental stages of first, second and third molars from the mandible after AlQahtani et al. (2010) (median, minimum and maximum age). Ci: initial cusp formation; Crc: crown completed, pulp roof defined; F: visible bifurcation; R 1/2: half of the root completed; Rc: root length completed; Ac: apex closed

Dental calculus, if present, can be removed and preserved for future analysis.

### 2.3 | Cutting protocol

After cleaning, the tooth is partially embedded in Herculite II (a gypsum molding material), leaving one root's mesial or distal surface exposed to direct the cutting. Using a Buehler Isomet low-speed saw with a micrometer gauge, an abrasive diamond wafering blade cooled with water bath (workflow see Figure 2) a 2 mm wide longitudinal central slice is cut either using two blades with a spacer, or by adjusting the micrometer in two consecutive cuts. We found that this depth is suitable for relatively well-preserved archeological teeth. The tooth slice and the remaining material are easily removed from the mold mechanically by dissolving the gypsum in water or using a small chisel and hammer.

Either of the two outer remaining tooth “halves” (one of which would otherwise have been destroyed in conventional sequential sampling protocols), may then be used for further analyses such as radiocarbon dating, or all of it can be returned to the museum.

### 2.4 | Demineralization

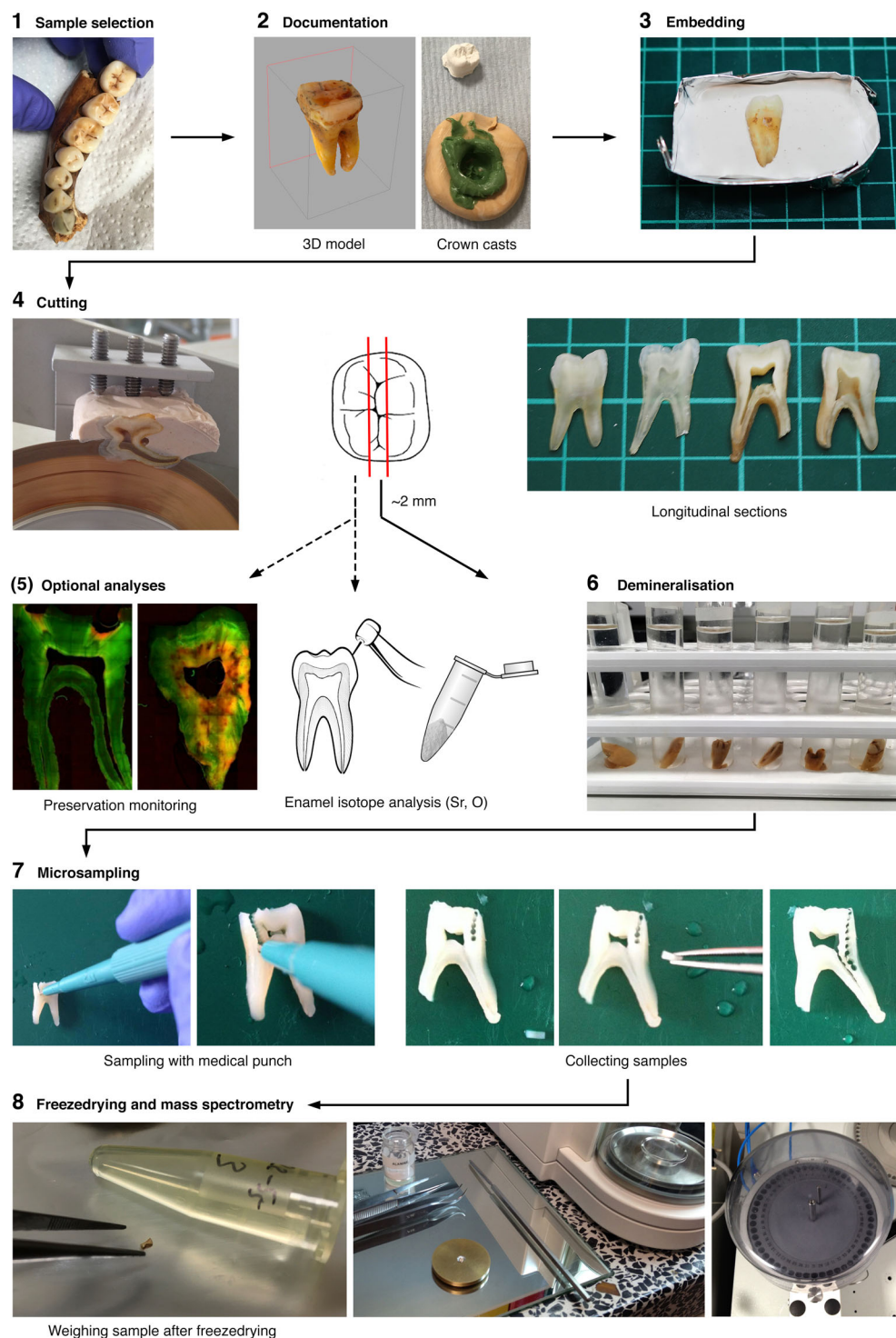
Demineralization is carried out following the “chunk” method modified after (Sealy, Johnson, Richards, & Nehlich, 2014). The 2 mm longitudinal slice is demineralized in 0.5 M HCl solution at 4°C until reaction is complete (ca. 7–10 days), and then rinsed three times in deionized water. This leaves a pseudomorph of the original slice. If the

presence of humates in the dentine is suspected, the standard 0.1 M NaOH treatment can be carried out at room temperature for 30 min and subsequent thorough rinsing (at least 3x) in deionized H<sub>2</sub>O. It is advisable to follow the NaOH-treatment with a rinse in 0.5 M HCl solution for 15 min and further rinsing (3x) in deionized water (Brock, Higham, Ditchfield, & Bronk-Ramsey, 2010). However, we caution that even this gentle humic acid extraction procedure decreases collagen yields (Szpak, Metcalfe, & Macdonald, 2017), and due to the small masses of the micro-samples obtained, this step needs to be carefully considered.

### 2.5 | Sequential microsampling

The demineralized longitudinal mid-tooth section will still retain its original shape if the collagen is well preserved. It can then be sampled sequentially on either its mesial or distal side from crown cusp to root apex, using a 1 mm diameter biopsy punch with plunger (KAI Medical).<sup>2</sup> The pulp chamber, radicular channels and exterior surfaces of the dentine and other areas where collagen may be exteriorly exposed should be avoided, as noted above. On average 20 cylindrical dentine micro-samples per molar may be obtained in this manner.

The resulting 1 × 2 mm cylinder-shaped dentine micro-samples are transferred to 1.5 ml micro-tubes and labeled in numerical sequence from cusp to apex indicating their anatomical location. That is—*crown*, from initial cusp to complete crown,<sup>3</sup> *neck*, from complete crown to root furcation, *superior half of the root*, from root furcation to root ½, and *inferior half of the root*, from root ½ to apex. This information is required for age-alignment post analysis (see Figure 3 and Graphical Abstract). The remainder of the demineralized 2 mm slice



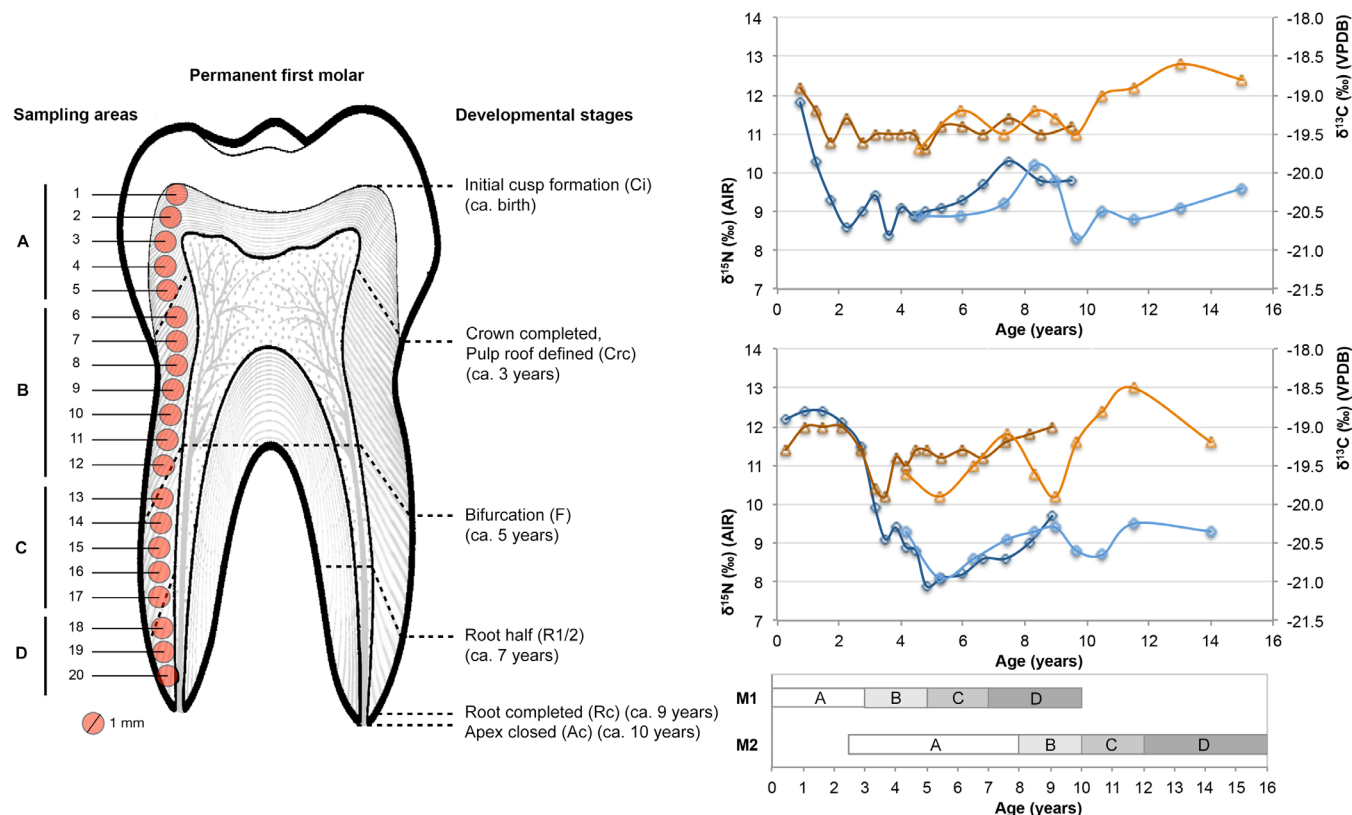
(i.e., central crown area and distal/mesial side) is stored in a freezer as a “hard-copy”.

## 2.6 | Freeze-drying and stable isotope determination

The demineralized 1 × 2 mm cylindrical micro-samples are freeze-dried before being weighed into tin capsules for mass spectrometry.

Masses generally vary from ca. 0.5 to 1 mg. The variation is largely the result of variable collagen preservation, despite quality control measures that show the collagen is intact (Czermak et al., 2019; Dobberstein et al., 2009). The lower size limit of samples that can be analyzed for stable isotopic composition will be determined by the analytical capabilities of system used. In our case, samples were analyzed on a SERCON 20/22 Isotope ratio mass spectrometer (IRMS) coupled to a SERCON GSL elemental analyzer. The effective lower weight limit on dentine micro-samples on this system was found to be





**FIGURE 3** Sampling map and exemplary display of isotope analyses. Left panel: Scheme for taking microsamples from anatomical areas (A–D) from a first permanent molar with corresponding age of completion (AlQahtani et al., 2010). Right panel: Examples for age alignment of isotope values measured of samples taken from anatomical areas indicated in the left panel. Two exemplary diagrams show age-aligned values of two teeth from the same individual matched according to the age of completion of tooth developmental stages. Data plots of dentine collagen  $\delta^{13}\text{C}$  (orange triangles) and  $\delta^{15}\text{N}$  (blue squares) profiles of first (M1, darker color) and second (M2, lighter color) molars from two individuals (original data from Fernández-Crespo et al. (2018): sample LHUE17c, top data plot; LHUE34, lower data plot). The bottom graph illustrates the correspondence between anatomical areas and tooth developmental ages used to assign age to each dentine sample

0.4 mg. If samples are smaller, then consecutive samples can be merged, although this will result in a lower temporal resolution.

To maximize analytical performance of the instrument, we sorted samples into weight-based groups and measured them in separate analytical runs (e.g., from 0.45 to 0.64, from 0.65 to 0.84, from 0.85 to 1 mg). In this way the size of standard reference materials used in each run can be weight-matched to the samples in that particular run. Both working standards (in our case Alanine) and in-house collagen standards (cow and seal) traceable back to USGS40 and USGS 41 glutamic acid standards are systematically interspersed among samples in each run in order for scale compression effects to be adequately corrected for.

Repeated measurement of sample aliquots is rarely feasible with this method due to small sample sizes. Precisely replicating a sample from elsewhere on the same tooth is also impractical due to the complexity of dentine growth (Dean, 2017; Hillson, 1996; Nanci, 2008). However, a “control” comparison may be conducted with samples with sufficient yield ( $\geq 1$  mg), with these samples being divided in two subsamples and measured in duplicate.

For calibration and analytical accuracy, we followed Szpak et al. (2017), for collagen quality we used criteria such as collagen

yield, atomic weight C:N ratio, %C and %N (Ambrose, 1990; DeNiro, 1985; van Klinken, 1999). In our dentine samples we observed the last two parameters often to be near the maximum limit.<sup>4</sup> It is worth pointing out, however, that the published collagen preservation criteria are based on bones in which the amino acid profiles differ subtly from dentine collagen. Therefore slightly higher %C and %N may reflect the norm for good dentine collagen preservation (Fernández-Crespo, Czermak, Lee-Thorp, & Schulting, 2018). Given the lack of duplicate measurements, we suggest a conservative approach regarding atomic weight C:N ratios, using a constrained C:N range of 2.9–3.4 (Czermak et al., 2019; Fernández-Crespo et al., 2018, 2020).

## 2.7 | Age-alignment, analysis and plotting of sequential isotope data

We assigned each sample to a putative age by comparing the samples' anatomical location to the stage of dental development following “The London Atlas” (AlQahtani et al., 2010). The London Atlas has been shown to be more accurate, at least for modern individuals, than

other widely-applied dental age estimation methods and has been systematically used in dentine isotope analysis (Beaumont & Montgomery, 2015; Crowder, Montgomery, Gröcke, & Filipek, 2019; van der Haas, Garvie-Lok, Bazaliiskii, & Weber, 2018; Scharlotta et al., 2018). Previous methods for age-alignment have opted for dividing the global time-span of tooth formation by the total number of samples obtained in each tooth to assign their corresponding age (e.g., Beaumont & Montgomery, 2015). Here we propose a more explicit use of the anatomical markers as provided in The London Atlas, that is, *crown*, *neck*, *superior half of the root*, and *inferior half of the root*, as temporal markers for growth rates (see above and Figure 3) (Czermak et al., 2018).<sup>5</sup> This approach minimizes the error introduced by distinct rates of dentine secretion in age-alignment that is required in order to compare individuals (e.g., as in Fernández-Crespo et al., 2020). For example, assuming that the crown formation of the first permanent molar starts around birth and ceases at 3 years of age on average, according to AlQahtani et al. (2010), and that five samples are taken from this area, each sample should cover approximately a fifth of that timespan. To compare data and to be consistent with other methods (e.g., Beaumont & Montgomery, 2015), the mid-point of each time-span can also be calculated.<sup>6</sup> However, because of variation in initiation and rates of growth any age-alignment of samples will inevitably be an estimate (Figure 1).

In circumstances that leave age-gaps, such as where samples are missing, or require merging due to small yields, or that do not meet the established collagen preservation criteria, we reflect these in age-alignment tables and graphs by leaving gaps or by decreasing temporal resolution.

### 3 | RESULTS AND DISCUSSION

Compared to previous methods, the sequential dentine microsampling approach presented here significantly reduces destruction of the sample material and is anatomically more sensitive than horizontal sectioning due to:

1. Microsamples being far smaller (in both length and depth), thus minimizing temporal overlapping.
2. The microsamples do not follow any particular direction that contravenes growth complexity.
3. Microsamples are not taken from areas where later grown tissues, as secondary and tertiary dentine or cementum, may be present, and areas identified as poorly preserved based on for example, fluorescence screening can be avoided.

Due to the small sizes and variable masses of the samples obtained, we propose standardized practices for collagen extraction that exclude denaturation, and a simple system to improve mass spectrometry performance by organizing samples according to mass groups. Means for refining age-assignment and thus resolution, and for age-alignment of samples from multiple individuals follow AlQahtani et al. (2010) in some detail.

Although there is a well-known reduction in the rate of growth in the coronal and apical areas of permanent teeth (e.g., Dean & Cole, 2013), previous sampling methods have tended to ignore this problem by assuming a relatively constant rate of dentine secretion throughout permanent teeth formation and arguing that any error introduced is not significant (e.g., Beaumont et al., 2014).

We argue that its relatively straightforward to take variations in average longitudinal growth extension rates into account, based on a more detailed reading of AlQahtani et al. (2010) that allows an age-alignment protocol based on the tooth's main anatomical areas (i.e., crown, neck, superior and inferior halves of the root) and the age of completion of these areas. This greatly minimizes the error in age-assignment and improves age-alignment of multiple tooth isotope profiles (see, e.g., Fernández-Crespo et al., 2020). We suggest the use of mid-points (i.e., median ages at which the stages of tooth formation are completed) instead of an actual time-span (Figure 1) for the sake of simplicity of age-calculations, even though it is acknowledged as introducing errors due to inter-individual variability in dental growth (Dean, Liversidge, & Elamin, 2014; Liversidge, 2015). This circumstance has not been explicitly addressed in previously published methods, as individual growth rates and its impact on group age-alignment cannot be accurately assessed for past populations. However, it has become increasingly evident that direct age-alignment is not as straightforward as previously assumed, especially when matching isotope profiles across multiple teeth, and that furthermore variability in the rate of dentine secretion plays a key role (e.g., Scharlotta, Goude, Herrscher, Bazaliiskii, & Weber, 2018). Exemplary diagrams (Figure 3) show age-aligned results from two teeth of one individual matched according to the age of completion of tooth developmental stages. Values that are taken at approximately the same age from different teeth are almost congruent at most stages. This alignment strategy also makes it more straightforward to compare individuals.

Using anatomical areas' average growth rates may not satisfactorily reduce the potential impact of variability in the rate of dentine secretion, but it does at least allow the comparison of dentine isotope data between individuals at the same developmental stage. Statistical comparisons between single samples from different individuals may be inaccurate due to tooth length inter-variability. Comparison of average isotope data within anatomical areas between individuals or groups of individuals is biologically coherent and has been shown to generate coherent results using Mixed Model Nested ANOVA tests (Fernández-Crespo et al., 2020).

### 4 | CONCLUSION

The dentine microsampling approach presented in this article is straightforward, easily applicable and anatomically more sensitive than previous microsampling strategies. It minimizes destruction and enables construction of finer temporal resolution sequences as well as higher sample throughput. The proposed age-alignment of microsamples is biologically more coherent and facilitates comparisons between individuals or groups of individuals.

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## AUTHOR CONTRIBUTIONS

**Andrea Czermak:** Conceptualization; investigation; methodology; visualization; writing-original draft; writing-review and editing. **Teresa Fernández-Crespo:** Conceptualization; investigation; methodology; visualization; writing-original draft. **Peter Ditchfield:** Methodology; writing-review and editing. **Julia Lee-Thorp:** Conceptualization; methodology; writing-review and editing.

## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## DATA AVAILABILITY

Primary data used in this manuscript are taken from <https://doi.org/10.1002/oa.2659>. Data is available from the corresponding author on request. There are no restrictions on data availability.

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## ENDNOTES

<sup>1</sup> We have focused on the analysis of molars for the following practical reasons: (1) The larger size of molars enables easy cutting of a central tooth section, which minimizes the risk of sampling overlapping growth layers, allows finer resolution of the dietary intake, and leaves enough material for additional analyses. (2) Sampling first, second and third molars enable to record the dietary intake from birth to early adulthood. Molar sampling is the common approach in comparable studies. (3) Molars' double roots provide a "backup" in case the analysis needs to be repeated. (4) The distinct bifurcation area allows straightforward assignment of anatomical areas according to AlQahtani, Hector, and Liversidge (2010).

<sup>2</sup> Biopsy punches for microsampling dentinal collagen were firstly used by Kirsanow, Makarewicz, and Tuross (2008) on ovicaprid teeth and by Burt and Garvie-Lok (2013) on human deciduous tooth.

<sup>3</sup> For high-resolution sampling of the crown center see Czermak et al. (2018).

<sup>4</sup> In intact collagen %C should be ca.  $34.8 \pm 8$  wt%, and %N 11–16 wt% (van Klinken, 1999, Oxford  $^{14}\text{C}$  database). The calculated C/N ratio for intact collagen is 3.2 (Szpak, 2011), although C/N ratios of 2.9–3.6 are considered acceptable in the literature (e.g., Ambrose, 1990; DeNiro, 1985; Madden, Man Wai Chan, Dundon, & France, 2018; Van Klinken, 1999).

<sup>5</sup> The median age of completion of each developmental stage was used as a "terminus ante quem": As dentine has formed to a certain stage by a

specific age, any sample from before this stage represents dietary intake by this age.

<sup>6</sup> In this case, a midpoint of 0.3 years of age (i.e.,  $0.6/2$ ) would be used for the first sample, despite its isotope signal theoretically reflecting the average diet consumed between 0 and 0.6. Similarly, the second sample would be assigned to a midpoint of 0.9 years of age (i.e.,  $0.3 + 0.6$ ), the third sample to a midpoint of 1.5 years of age (i.e.,  $0.9 + 0.6$ ), etc.

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