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New methods for old challenges: A sampling protocol for sequential stable isotope analysis ($\delta^{13}C$ and $\delta^{15}N$) of dentine collagen in high-crowned teeth

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ABSTRACT

Intra-tooth or sequential stable isotope analyses are nowadays widely implemented in zooarchaeological research. Sequential isotopic analyses have been mainly restricted to the mineral fraction of the enamel, while a wider implementation in dentine collagen has been generally eluded, despite conforming an essential organic compound for paleodietary studies. In high-crowned teeth, dentine grows oblique to the vertical axis of growth and some challenges arise when trying to isolate dentine increments during a sequential sampling. Previous sampling strategies slice the whole dentine thickness and provide sequences where isotopic variation is largely attenuated. In this study, we show a new proposal for performing carbon and nitrogen isotope analyses of dentine collagen which better fits dentine's growth. We implemented it in mandibular second molars of an experimental modern sheep flock. Our sampling largely reduces the isotopic "damping" of previous approaches and provides short time-span samples. Sequential $\delta^{13}C$ values obtained in dentine tissue yielded similar resolution to those obtained in enamel tissue of the same individuals. This proposal, especially suitable for caprine and other bovid species, broadens the implementation of dentine collagen signatures in the field of stable isotope analyses in zooarchaeology.

1. Introduction

Hypsodonty is a common morphological characteristic in dentitions of herbivorous-grazing mammals. It defines those dentitions with prolonged periods of tooth crown formation, still growing after eruption, and with elongated tooth crowns (i.e. high-crowned teeth) (Janis 1988). These teeth are susceptible to provide a long record of the isotopic variation occurring during dental development by sampling their main growth direction in height (i.e. longitudinally), and this feature has been widely exploited in paleoecological and zooarchaeological studies since the 90s (e.g. Fricke and O'Neil, 1996; Sharp and Cerling 1998; Wiedemann et al., 1999). These approaches have been mainly focused on the analysis of phosphates and carbonates compounds of tooth enamel-hydroxyapatites, especially since enamel shows high resistance to diagenetic alteration in fossil remains (Lee-Thorp and van der Merwe 1991; Schoeninger and DeNiro 1982). Conversely, in zooarchaeological studies, intra-tooth isotopic analyses of dentine have not undergone such popularity during the last decades, despite the potential of this tissue to track the isotopic fluctuations of collagen recorded along several months of tooth formation: the organic fraction (mainly collagen type I) forms up to ~20% of total dentine by weight (Carlson 1991; LeGeros 1981) and dentine do not undergo regeneration once mineralized, except for pathological factors (Bada et al., 1990; Carlson 1991; Frank and Nalbandian 1989).

Dentine develops incrementally, in a two-step process where the collagenous organic matrix (predentine) is first deposited and then mineralized, at a constant rate of organic-matrix apposition and mineralization (Kahle et al., 2018; Nanci 2013). However, isolating different dentine increments during sampling may entail certain complexities. Despite the elongated crown, dentine in high-crowned teeth grows oblique to the vertical axis (Hillson 2005; Kahle et al., 2018) and this pattern makes it difficult to sample following growth direction while obtaining enough sample size to implement standard protocols of collagen isolation (Zazzo et al., 2006).

The possibility of obtaining an intra-tooth isotopic record of collagen through a sequential sampling of high-crowned teeth was first explored by M. Balasse and colleagues (Balasse et al., 2001; Balasse and Tresset 2002). These studies showed that $\delta^{15}N_{collagen}$ values obtained from

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sequentially sampled M_1 cattle molars may represent the shift between the suckling period and the later herbivorous diet, allowing to study weaning management in past animal populations. Since then, sequential isotopic analyses in dentine collagen have been implemented in several archaeological studies, not only linked to define weaning age in domestic calves (Gillis et al., 2013; Griffith and Mulville 2020; Stojanovski et al., 2020), but also to track other strictly herbivorous conditions such as seasonal feeding strategies of domestic herds (Makarewicz 2014, 2017; Makarewicz et al., 2018), or to reconstruct paleoenvironmental conditions from wild or free-range specimens (Hopper et al., 2018; Kirsanow et al., 2008).

The implementation of this sampling approach needs to deal with some dampening of the isotopic sequences, resulting from the admixture of different dentine increments during sampling (Díez-Canseco et al., 2022). The sampling strategy developed by M. Balasse and nowadays implemented, slices the lobe selected from the apex to the base, obtaining a series of samples along the vertical axis of the tooth which are susceptible to further treatments of collagen isolation, but which represent an isotopic pool of the whole dentine thickness. As a consequence, samples represent large time spans and sequences show an attenuation of the diet isotope signatures which complicates the interpretation of the final data. In order to obtain smaller and more time-accurate samples of dentine, some authors have proposed the possibility of performing a serial sampling by drilling, without any further treatment of the final powder sample, analysing thus "raw dentine" (Guiry et al., 2016, implemented in pig's tusks). However, this protocol entails discarding $\delta^{13}C$ values (which mainly come from the inorganic fraction), and the reliability of $\delta^{15}N$ values could be compromised by the presence of exogenous nitrogen-rich compounds in the samples. A modified version of this protocol where drilled, powdered dentine samples are demineralized with hydrochloric acid (Guiry et al., 2018), allows for obtaining $\delta^{13}C$ values coming mainly from collagen, but sample size does not allow additional treatments for the removal of base-soluble contaminants (Losey et al., 2020), potentially present after burial conditions. No further methodological proposals have been made relating to the implementation of sequential carbon and nitrogen isotope analyses of dentine collagen in faunal remains.

Likewise, sampling strategies developed in recent years for human dentine, although may be suitable for other animal species (e.g. Rogers et al., 2019), do not appear to be suitable to yield major improvements for high-crowned teeth, considering the differences existing between the growth geometries of both dentitions. Sampling after generating a thin section of the teeth, whether by slicing sections with a scalpel (Beaumont et al., 2013; Czermak et al., 2018) or by sampling with a biopsy punch tool (i.e. ~1 mm of diameter) (Czermak et al., 2020; Fernández-Crespo et al., 2018), does not guarantee to isolate dentine increments in high-crowned molars, where (1) increments are laid down in more oblique angles than in bunodont molars, (2) the whole dentine thickness may be cramped in less than 2 mm width, and (3) lobe-curved morphologies complicate generating a section thick enough for the micro-punches method. In addition, the time resolution targeted in humans with these methods is highly lower (i.e. several years) than the one that would be feasible for hypsodont teeth (i.e. several months).

In this study, we present a new sequential sampling strategy of dentine that allows for performing stable carbon and nitrogen isotope analysis and which better suits molar growth in hypsodont species. Since the inner dentine is not sampled through this strategy, it allows to take advantage of the longitudinal development in high-crowned teeth, while obtaining better resolution than previous sampling protocols. This method is tested in modern sheep specimens coming from an experimental program where diet conditions and isotopic inputs are known. Results are compared with standard methods of dentine and enamel sequential analyses performed in the same specimens. We evaluate the sampling resolution and its potential biases in the implementation in the archaeological record.

2. Materials

An experimental program with several flocks of modern sheep (*Ovis aries*, Linnaeus, 1758) was developed between 2017 and 2019 in the Farm Services and Experimental Fields facilities of the Autonomous University of Barcelona (UAB), located in Bellaterra (Spain). The aim of the program was to provide referential data sets for several paleodietary-related research studies (e.g. Díez-Canseco et al., 2022; Gallego-Valle et al., 2020). The experiment was approved by the ethics committee of animal experimentation of UAB, and it was carried out under the supervision of the Animal Welfare advisor in charge, together with the rest of the farm's veterinary staff. The sheep specimens selected for the present study come from a 12-female flock of Ripollesa breed. Ripollesa is an autochthonous sheep breed from north-eastern regions of Catalonia (NE Spain), traditionally oriented to lamb production and meat exploitation (Caja et al., 2010; Esquivelzeta et al., 2011).

The sheep flock was raised in a stall under controlled life and feeding conditions. All lambs were born in February–March 2017. Lambs were weaned at eight weeks of age, and weanlings were fed with a mixture of alfalfa hay and supplement pellet (based on maize, barley, soy and wheat) for the next two/three months. The flock was then fed following a schedule based on three monospecific diets alternated as follows: DIET1, based on alfalfa hay (*Medicago sativa*); DIET2, based on foxtail millet hay (*Setaria italica*); DIET3, based on barley (*Hordeum vulgare*) supplied in the form of straw and grain (Fig. 1). These three diets were scheduled in a C_3-leguminous–C_4–C_3 plant scheme, thus expecting major differences in the isotopic signature between these periods. Diet changes were abruptly performed on March 22nd and July 10th, when sheep were 12 and 16 months old, respectively. Fodder samples from the three monospecific diets (alfalfa, foxtail millet, barley-straw and barley-grains) were periodically collected from troughs from November 2017 until sheep's slaughtering –thus covering the last year of sheep's life– and were subjected to stable carbon and nitrogen isotope analysis. The isotopic data of fodder samples was previously published in Díez-Canseco et al. (2022); the main isotopic results from fodder analyses are shown in Table 1.

A total of five sheep specimens from this flock were selected for the present study (OV-07, OV-08, OV-09, OV-10, OV-11), all of them slaughtered at 20.5–21 months old (detailed information for each specimen is given in Supplementary Material 1). It was expected that Ripollesa sheep showed completed crowns of mandibular second molars (M_2) at the time of death. Mandibular second molars formation starts during the second month of life, and crown formation may complete at 12 months in improved breeds (Milhaud and Nezit 1991; Zazzo et al., 2010) or extend until 18 or 19 months of life in breeds with lower growth rates (Upex and Dobney 2012; Witzel et al., 2018). A delay of 4–6 months in the enamel mineralization has been observed in sheep (Balasse et al., 2012; Zazzo et al., 2010), so diet changes were expected to be recorded in the enamel of M_2 molars. A previous study showed that the three diet periods are also recorded in dentine (Díez-Canseco et al., 2022).

3. Methods

Mandibles from the five selected sheep specimens were separated from the skull, partially defleshed, and boiled in water (100 °C) for 2 h; the remaining soft tissues were then removed manually. M_2 molars were extracted from the right hemi-mandibles. At the time of death, M_2 showed a wear stage D following criteria in Payne (1973), and the crown was recently completed; roots were in an early stage of development, barely exceeding the enamel-root junction (ERJ). The posterior lobes of the right M_2 ($N = 5$) were selected for sequential stable isotope analysis of enamel and dentine tissues.

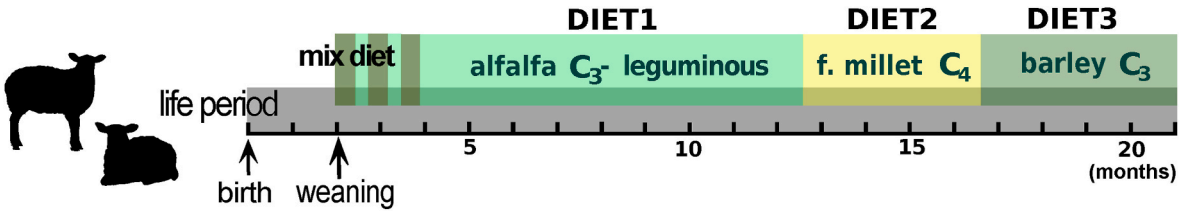

Fig. 1. Life period of the sheep selected for this study and diet periods scheduled during the experimental program.

Table 1
Mean and standard deviation of carbon and nitrogen isotopic results of sampled fodder supplied to sheep during the three monospecific diets. Data from Díez-Canseco et al. (2022).

	DIET1	DIET2	DIET3	
	Alfalfa (n = 3)	Foxtail millet (n = 9)	Barley-straw (n = 12)	Barley-grain (n = 16)
$\delta^{13}C$ (‰)	−28.9 ± 0.8	−13.1 ± 0.3	−28.2 ± 0.4	−25.6 ± 0.1
$\delta^{15}N$ (‰)	+1.2 ± 1.5	+11.7 ± 0.7	+3.6 ± 1.8	+7.5 ± 0.3

3.1. Enamel sequential sampling

Enamel sampling was performed on the buccal side of the posterior lobes of right M_2, using a Dremel tool coupled to a diamond bit. Between 23 and 29 samples were obtained mechanically in each sequence. Samples weighing 5.3 ± 1.2 mg were obtained each 1.5–2 mm along the crown's longitudinal axis. The position of samples was noted as the distance (in mm) from the ERJ. Powder samples were then chemically treated following protocols published by Balasse et al. (2002) and Tornero et al. (2013). Briefly, samples were treated for 4 h in 0.1 M acetic acid [CH3COOH] (0.1 ml solution/1 mg of sample), rinsed five times with Milli-Q water, and dried in an oven for 48 h at 70 °C. During chemical treatment, samples (N = 127) lost an average of 45.9 ± 9.2% of their weight.

3.2. Dentine sequential sampling

Posterior lobes were separated from the tooth mechanically. Lobes were demineralized in EDTA (0.5 M, pH = 8) for several weeks at room temperature following criteria from Tuross et al. (1988) and Balasse et al. (2001). Demineralized lobes were then intensively rinsed with Milli-Q water, including a one-week soak, and then placed in a 0.125 M NaOH solution at room temperature for 20 h, rinsed again several times and lyophilized. The buccal part was then sampled using a cylindrical diamond point of 1 mm of diameter, sampling each 1.5 mm along the tooth's vertical axis and obtaining powder samples weighing 1.0 ± 0.3 mg. Depth sampling was homogenized using the point diameter as reference, resulting in grooves of about 0.3–0.5 mm deep. Between 23 and 26 samples were obtained in each sequence.

The position of samples was noted as distance (in mm) from the base of the sampled lobe (i.e. root base). Since demineralized lobes slightly shrink during freeze-drying (3.3 ± 0.5 mm), the distances are corrected considering the total length of each lobe before and after freeze-drying as follows: $d_0=(d_1*l_0)/l_1$; where 'd_0' is distance from root-base before lyophilization, 'd_1' is distance from root-base after lyophilization, 'l_0' is length of the lobe before lyophilization, and 'l_1' is length of the lobe after lyophilization. The distance of the root base from ERJ was measured before demineralization, so the dentine samples position may be also expressed as distance from ERJ to allow comparisons between molars, and between enamel and dentine series.

Powder samples were treated with a 0.5 M HCL solution for 30 min following criteria in Czermak et al. (2020) to avoid potential contamination of precipitated atmospheric CO_2 (Hatté et al., 2001). Samples were then rinsed in Milli-Q water, and freeze-dried. Here, we refer as collagen to the EDTA insoluble residue obtained after this treatment protocol, although other minor non-collagenous tooth proteins may be present in the sample after the demineralization steps (Ambrose 1990; Cleland et al., 2021; Guiry and Szpak 2020; Masters 1987; Tuross et al., 1988).

To test the resolution of this sampling strategy, this method (hereinafter "drill-sampling" or DS) is compared with the standard dentine sampling previously implemented in zooarchaeological studies for sequential isotopic analysis, which obtains "chunk" samples by slicing the lobe into 2 mm sections once demineralized (Balasse et al., 2001). This standard method (hereinafter "slice-sampling" or SS) was previously tested in the same sheep individuals used in this study, but using the posterior lobes of left M_2 teeth (Díez-Canseco et al., 2022) instead of right M_2 teeth (this study).

3.3. Isotopic measurements

A total number of 127 enamel bioapatite samples and 125 dentine collagen samples were subjected to isotopic analysis. Each sample was measured once. Carbon isotope ratios of treated enamel bioapatite samples were measured using an automated Kiel-III Carbonate Device coupled to a Finnigan MAT 252 isotope ratio mass spectrometer (IRMS) at the Environmental Isotope Laboratory (Department of Geosciences) of the University of Arizona (USA). Samples weighting ~600 μg were reacted with dehydrated phosphoric acid under vacuum at 70 °C. Measurements' accuracy and precision were checked and calibrated using NBS-19 (theoretical $\delta^{13}C$ values = +1.95‰) international standard. All measured values of the standard are shown in Supplementary Material 2. The mean analytical precision varies by ±0.05‰ (1σ) for $\delta^{13}C$, determined within each run and from replicate measurements of standards.

For dentine samples, ~350 μg of collagen were weighted into tin samples. Carbon and nitrogen content (%), and $\delta^{13}C$ and $\delta^{15}N$ signatures were measured using a Thermo Flash 1112 elemental analyzer (EA) coupled to a Thermo Delta V Advantage isotope ratio mass spectrometer (IRMS) with a Conflo III interface at the Institute of Environmental Science and Technology (ICTA-UAB) (Barcelona, Spain). The international standard IAEA 600 (caffeine) (theoretical values: $\delta^{13}C = -27.77 \pm 0.04$‰, $\delta^{15}N = +1.0 \pm 0.2$‰) was used for data calibration. All measured values of the standard are shown in Supplementary Material 2. The mean analytical precision was ±0.08‰ for $\delta^{13}C$ and ±0.11‰ $\delta^{15}N$, determined within each run and from repeated measurements of standards. Isotope ratio is reported in δ notation: $\delta^{13}C$ values are expressed relative to Vienna PeeDee Belemnite (V-PDB) standard and $\delta^{15}N$ values relative to air N_2 (AIR).

4. Results

4.1. Results from sequential $\delta^{13}C$ analysis of enamel

Results from stable carbon isotope analysis of the enamel bioapatite samples are shown in Supplementary Material 3. Table 2 shows a summary with the minimum (Min.) and maximum (Max.) $\delta^{13}C$ values,

Table 2
Summary table with isotopic results from sequential analysis of enamel ($\delta^{13}C_{bioapatite}$) and dentine ($\delta^{13}C_{collagen}$ and $\delta^{15}N_{collagen}$). Minimum values (Min.), maximum values (Max.), and maximum range of variation (Range) are shown per specimen, with mean values and standard deviation for all specimens.

Specimen	$\delta^{13}C_{bioapatite}$ (‰)			$\delta^{13}C_{collagen}$ (‰)			$\delta^{15}N_{collagen}$ (‰)		
	Min.	Max.	Range	Min.	Max.	Range	Min.	Max.	Range
OV-07	−14,0	−3,5	10,4	−22,9	−12,1	10,8	+7,0	+16,3	9,3
OV-08	−13,3	−2,5	10,7	−22,4	−11,9	10,5	+7,4	+15,7	8,3
OV-09	−13,8	−3,3	10,5	−22,8	−11,4	11,5	+7,2	+16,4	9,2
OV-10	−14,2	−2,8	11,4	−23,0	−11,7	11,3	+7,0	+16,2	9,2
OV-11	−14,4	−3,1	11,3	−23,2	−11,6	11,5	+6,5	+15,8	9,3
Mean	**−13,9**	**−3,1**	**10,9**	**−22,9**	**−11,7**	**11,1**	**+7,0**	**+16,1**	**9,1**
SD	**0,4**	**0,4**	**0,4**	**0,3**	**0,3**	**0,4**	**0,3**	**0,3**	**0,4**

and the maximum range of variation per each sheep specimen. Enamel samples yielded $\delta^{13}C$ values ranging from −14.4 ‰ to −2.5‰. All teeth yield similar absolute carbon values, where Min. values range from −14.4‰ to −13.3‰, and Max. values range from −3.5‰ to −2.5‰. All specimens show a high intra-tooth variation in their $\delta^{13}C$ values: the mean amplitude is 10.9 ± 0.4‰. Inter-individual variation (considering Min. and Max. values) is less than 1.1‰.

The distribution of $\delta^{13}C$ values along the M_2 crowns is shown in Fig. 2. Every tooth shows a similar variation of the carbon isotopic values, where the highest and lowest values are located in similar positions of the tooth crown. The carbon values in all teeth vary in accordance with the dietary conditions induced in sheep during the last year of life, when three monospecific-forage periods were alternated in a C_3–C_4–C_3 plant scheme. The lowest $\delta^{13}C$ values of the first part of the sequence represent DIET1, while the increase of values at the middle of the sequence and the following decrease represent the changes into DIET2 and DIET3, respectively.

4.2. Results from sequential $\delta^{13}C$ and $\delta^{15}N$ analysis of dentine

Stable carbon and nitrogen isotope ratios, carbon and nitrogen content, and C:N ratios of dentine collagen samples are presented in Supplementary Material 2. All samples yield carbon content values (in wt %C) ranging from 40.8% to 45.1% (average 43.6 ± 0.8%) and nitrogen content values (in wt %N) ranging from 14.7 % to 16.8% (average 15.7 ± 0.4%). C:N ratios range from 3.10 to 3.44 (average 3.23 ± 0.07). Dentine collagen samples yield $\delta^{13}C$ values ranging from −23.2‰ to −11.4‰, and $\delta^{15}N$ values ranging from +6.5‰ to +16.4‰. All five M_2 dentine series present high intra-tooth variation, with a mean amplitude result of 11.1 ± 0.4‰ in $\delta^{13}C$ values, and 9.1 ± 0.4‰ in $\delta^{15}N$ values (Table 2).

The variation of $\delta^{13}C$ and $\delta^{15}N$ values along the tooth is shown in Fig. 2. Overall, all dentine sequences present small inter-individual variation, showing a similar fluctuation of isotopic values along the crown. When considering Min. and Max. isotopic values, all sequences differ inter-individually ≤0.8‰ in $\delta^{13}C$ and ≤0.9‰ in $\delta^{15}N$. The major discrepancy is present in OV-09, which slightly deviates in the $\delta^{13}C$ values along the first part of the crown. A higher resolution is expected in this part of the crown, as it presents faster tooth growth (Jordana and Köhler 2011; Kahle et al., 2018; Witzel et al., 2018), and inter-individual disparities (e.g. dental growth timings, or specific behavioral or metabolic differences affecting the final isotopic composition of collagen) may be here amplified.

Isotopic results from collagen samples are in accordance with the dietary conditions induced in sheep during the experimental program. In all teeth, two main diet changes (C_3–C_4–C_3 alternation) are represented in the second half of the tooth, in accordance with the timings of the three monospecific diets. Clear maximum peaks are present in the final part of the crown, in accordance with the DIET2 period (based on a C_4 plant highly enriched in ^{15}N), while the lowest isotopic values are located previous to the C_4 maximum peak, which fit with the previous DIET1 (alfalfa-based) period.

5. Discussion

5.1. Evaluation of the drill-sampling strategy for dentine sequential analyses

The incremental growth of dentine in high-crowned teeth is oblique to the vertical axis, so dentine continues developing in thickness while the teeth develop in height. The main advantage of sampling dentine by drilling is to obtain small-size samples where the inclusion of inner

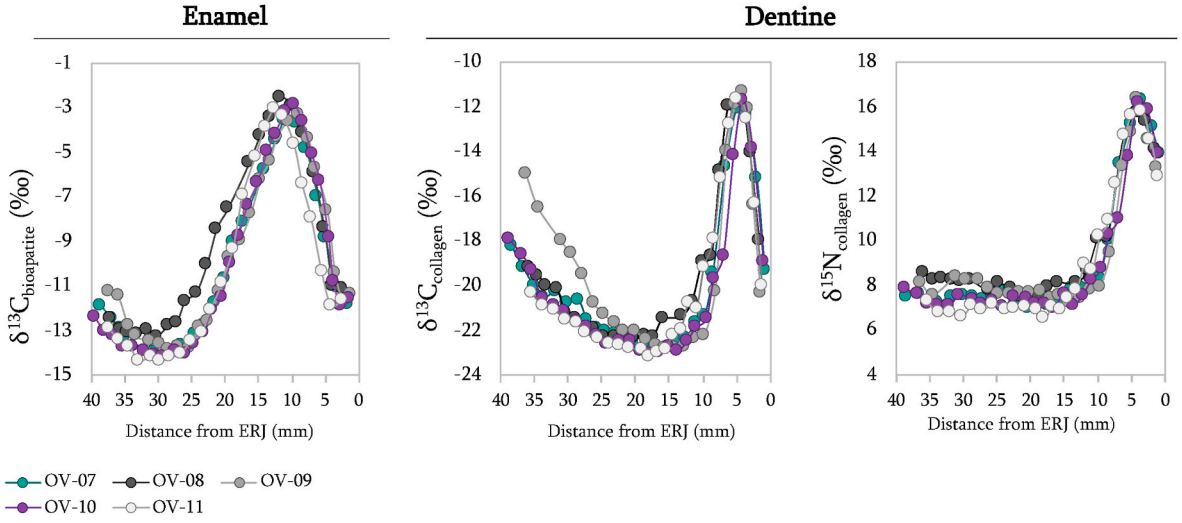

Fig. 2. Distribution of isotopic values obtained by sequential sampling of enamel ($\delta^{13}C$) and dentine ($\delta^{13}C$ and $\delta^{15}N$) along the M_2 crown of all specimens analyzed.

dentine increments, formed in later stages of dental development, is highly reduced. In Fig. 3 we compare the intra-tooth series of $\delta^{13}C$ and $\delta^{15}N$ values obtained in dentine by drill-sampling or slice-sampling in the M_2 of the same sheep specimens (right or left M_2, respectively). The main effects of the slice-sampling previously observed (Balasse et al., 2001; Díez-Canseco et al., 2022) are now largely reduced as follows.

(i) Drilled series show less attenuation of diet isotopic signatures. In both carbon and nitrogen signatures DS yields a higher range of variation than the SS series (11.1 ± 0.4‰ in front of 6.2 ± 0.3‰ in $\delta^{13}C$ values, and 9.1 ± 0.4‰ in front of 7.2 ± 0.4‰ in $\delta^{15}N$ values), therefore closer to the original isotopic amplitude existing between diets (DIET1-DIET2: ~15.8‰ in $\delta^{13}C$ and ~10.5‰ in $\delta^{15}N$). Also, DS yields a fluctuation of $\delta^{13}C$ values along the first part of the crown which is not recorded in the sliced series.

(ii) Abrupt diet changes are represented in shorter spans in the crown, reducing the over-representation of dietary changes along the sequences. In the DS series, the increasing trends of $\delta^{13}C$ and $\delta^{15}N$ values – i.e. isotopic change into DIET2 signatures– start in lower positions of the crown (14.0 ± 2.1 mm and 13.9 ± 2.4 mm from ERJ, respectively), while in sliced series they start in upper parts of the crown (22.2 ± 1.3 mm and 24.2 ± 3.0 mm from ERJ, respectively) or directly after the first samples (e.g. $\delta^{15}N$ in OV-11).

On the DS series, samples incorporate a small proportion of the total dentine thickness commonly sampled by the SS strategy. It entails obtaining a more faithful picture of the original isotopic variation recorded along a vertical axis of the tooth crown, since the sampling of large time-spans, which may involve different isotopic scenarios, is avoided. The more time-accurate samples of drilled series also engender that changes in diet –i.e. the shifts into new isotopic signatures– are not represented in such advanced positions of the sequences, but more accordingly to the real growth timings of the tooth crown (i.e. in height) (Fig. 4).

In both incremental dentine strategies, part of the isotopic variation observed in the sequences is the result of the sampling procedure rather than the actual collagen isotopic variation over time (i.e. the equilibration period between isotopic input and tissue synthesis) (Díez-Canseco et al., 2022). Considering the growth pattern of dentine in high-crowned teeth, it is expectable that the observed changes into new isotopic values mostly reflect the gradual admixture of subsequent dentine increments. Increments synthesized in earlier periods are progressively sampled in less proportion in favour of later increments, until the first period is no longer sampled. This is an important aspect to consider when interpreting the isotopic results, since the position of diet changes in the crown corresponds, if visible, to the final point of the variation observed in the sequences (commonly Max. or Min. values), instead of the starting point. However, high rates of dentine admixture may alter the position of diet changes in the sequences, as can be observed in slice-series where the minimum $\delta^{13}C$ values of DIET1 are completely blurred.

In general, our drill-sampling allows a better representation of the original isotopic signature recorded in dentine, especially in the case of existing several dietary scenarios with different isotopic signatures. Besides, it may provide a huge advantage when sampling molars in a population where different development stages are present in teeth: the drill method provides sequences that are independent of the addition of new inner increments during growth, and thus comparable between individuals of different ages. In contrast, the slice-sampling obtains bigger size samples, presumably easier to apply to archaeological assemblages where some preservation issues may occur, but the

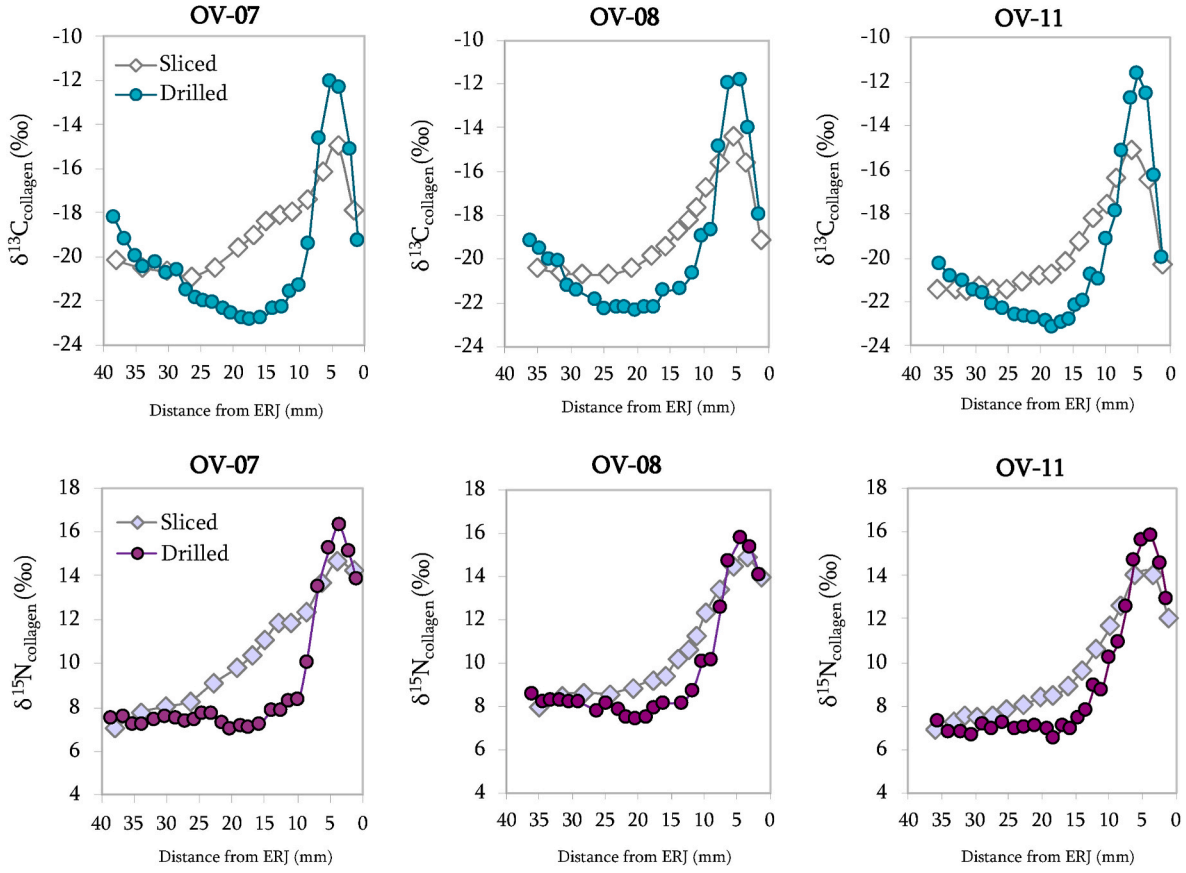

Fig. 3. Sequential $\delta^{13}C$ values (upper row) and $\delta^{15}N$ values (lower row) of dentine collagen obtained by slice-sampling (rhomboid symbol; data from Díez-Canseco et al., 2022) and drill-sampling (circle symbols; this study) in M_2 molars of same specimens.

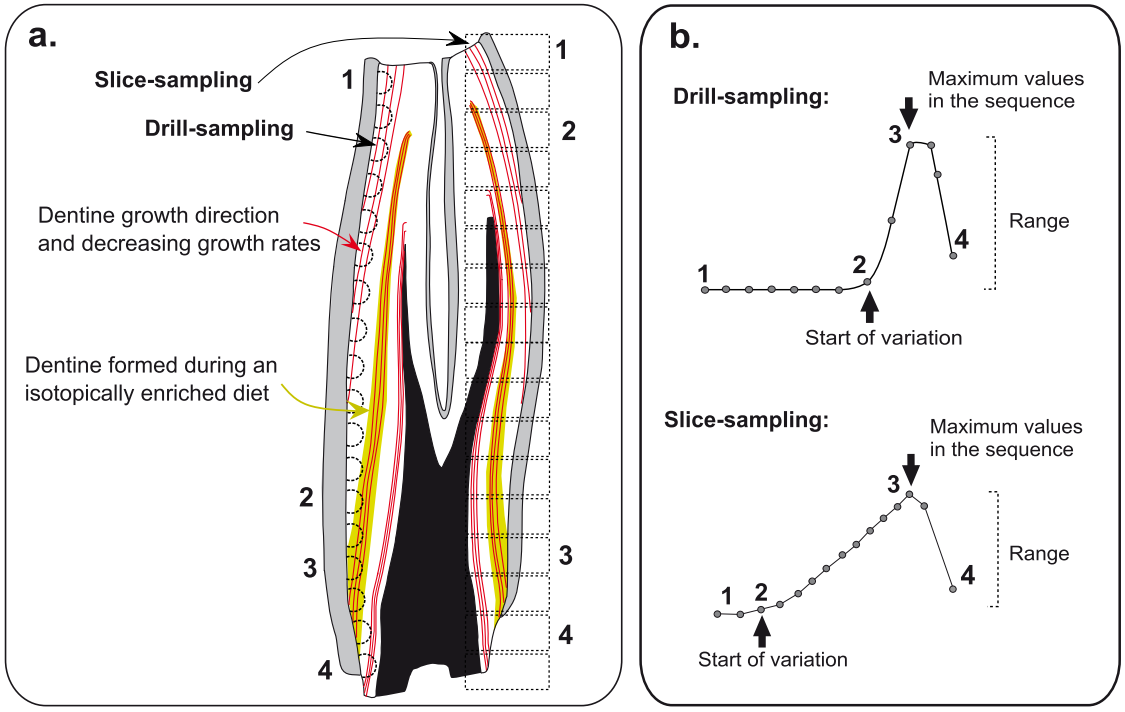

Fig. 4. Representation of diet changes in the isotopic results depending on the sampling strategy implemented in dentine. a) Scheme of a bucco-lingual section of a mandibular molar. Dentine growth direction and decreasing growth rates are shown: red lines represent different groups of increments laid down in equal time spans but in different parts of the crown (edited and modified from Kahle et al., 2018). The yellow band represents a hypothetical scenario of an isotopically enriched diet during the final months of tooth crown development. b) Intra-tooth isotopic variation obtained after slice-sampling (SS) or drill-sampling (DS) approaches: changes in diet are located more accordingly to growth timing in DS series, while Max. values fit with the moment of the new diet in both SS and DS approaches. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

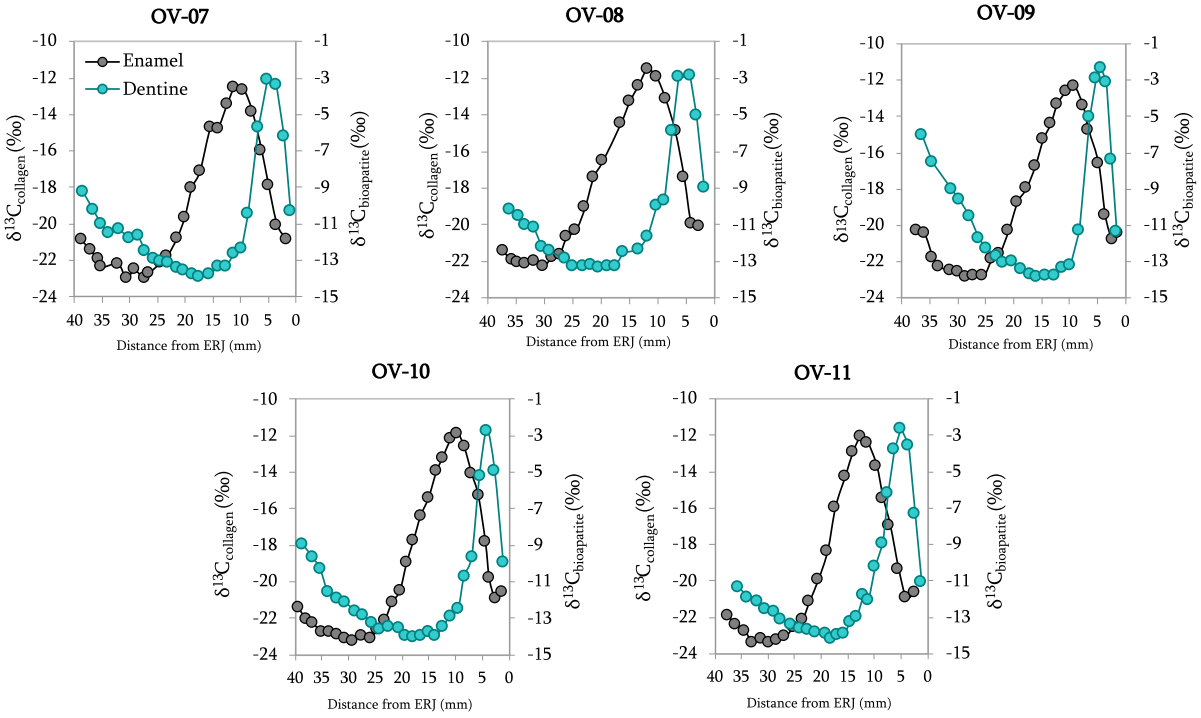

Fig. 5. Carbon isotope values of dentine (blue symbol, left axis) and enamel (grey symbol, right axis) obtained by drilling in the same specimen and same tooth (all sampled sheep individuals, right M_2). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

attenuation of the input isotopic signal should be highly considered when the slice method is applied: some isotopic variations could be highly blurred or could result indistinguishable, especially along the first part of the crown or in those teeth where dentine is well developed (older specimens). Different stages of development should be avoided when implementing the slice method in inter-individual comparisons.

5.2. A comparison with enamel sequential analysis: histological implications, time resolution, and comparability of isotopic results

In Fig. 5 we compare the carbon isotope results from the sequential sampling of enamel (bioapatite $\delta^{13}C$) and dentine drill-sampling (collagen $\delta^{13}C$) performed in the same tooth. Both approaches, dentine and enamel sequential sampling, show clear similarities in their pattern of distribution of $\delta^{13}C$ values, in accordance with the alternation of C_3–C_4–C_3 diets induced in sheep during the experimental program. However, it is quite surprising that dentine-drilled series, despite the admixture of dentine increments, have provided a similar range of intra-tooth variation in $\delta^{13}C$ values than the one obtained in enamel through the standard protocol of sequential analysis. All dentine collagen sequences present a mean $\delta^{13}C$ amplitude of 11.1 ± 0.4‰, in front of the 10.9 ± 0.4‰ represented in enamel (actual DIET1-DIET2 $\delta^{13}C$ spacing = 15.8‰), showing a similar attenuation of the original amplitude in both approaches. This data suggests that by drilling, dentine sequential sampling is capable to represent changes in isotope ratios along the tooth crown with similar resolution as enamel standard protocols, at least in terms of the range of intra-tooth variation.

Although both approaches seem to converge in similar damping of the isotopic sequences, different histological factors relating to dentine and enamel development are involved, and the time span represented in samples is not the same in both tissues. The complexities of the enamel mineralization process, proceeding in two phases —matrix formation and secondary mineralization or maturation— engender that the final and fully-mineralized enamel accumulates carbonate from several months, which results in a subsequent time-averaged isotope ratio (Balasse 2002, 2003; Passey and Cerling 2002; Zazzo et al. 2005, 2010). In sheep, this whole enamel developmental process covers about 5–6 months (Balasse et al., 2012). In the case of dentine, conversely, the attenuation of diet isotopic signatures strictly derives from the sampling performance, from mixing collagen from different increments and therefore synthesized in different moments.

Dental growth rates decrease progressively during the later stages of tooth formation (Witzel et al., 2018), so the time span represented in dentine samples is not constant along the crown. Previous studies focusing on dental growth rates in Soay sheep show that the mineral apposition rates of dentine vary from 21 μm to <2 μm per day in a first mandibular molar, decreasing along the vertical axis of growth, i.e. from cuspal to cervical, and from the enamel-dentine junction towards the pulp chamber, i.e. from outer to inner parts of the crown (Kahle et al., 2018). Considering these data, by performing a drill-sampling of ~500 μm depth, samples may involve time spans that vary from 24 days (in upper parts of the crown developed during first months of dental growth) to ≥8 months (later formed dentine of lower and inner parts of the crown). These decreasing trends in growth ratios entail that the time span involved in each dentine sample is *gradually* enlarged in a serial sampling, increasing while approaching cervical parts of the crown. Lower growth rates are also present in enamel in the final part of the crown (Witzel et al., 2018; Zazzo et al., 2010), but due to the extended length of the maturation front, a more constant isotope-mixing and time-average along the crown should be expected in enamel samples than in dentine. This feature should be considered when interpreting isotopic data from dentine sequential analyses.

The time frame involved in dentinogenesis and enamel mineralization processes also affects directly the position of the different diet scenarios along the isotopic sequences, i.e. along the crown. In dentine sequences the position of the Min. and Max. $\delta^{13}C$ values is uniformly located in lower positions of the crown than in enamel sequences: respectively, 13.7 ± 1.7 mm and 6.7 ± 0.8 mm closer to ERJ. As a consequence of the extended period of enamel maturation, the carbon isotopic composition from the mineral fraction of enamel represents mineralization moments occurring later in time than the development of that part of the crown (formed during a previous phase of enamel matrix formation). As a result, diet changes represented in enamel are "displaced", and we cannot infer the age at which a diet change occurred directly from the position of isotopic changes in the tooth (Balasse 2002, 2003). In contrast, the analyses of dentine collagen will represent the predentine apposition phase (organic-matrix formation), thus representing crown development with no delay. When analysing dentine collagen, isotopic signatures timing may be inferred directly from the position in the tooth. In this line, in agreement with Stojanovski et al. (2020), diet at death time could be also inferred from the last samples in the case of still-growing teeth. However, as discussed above, time resolution decreases in the final parts of the crown, and a larger attenuation of the isotope signatures should be considered here.

The comparison of both approaches suggests that dentine sequential analyses performed by a drill-sampling may be as capable as the standard protocols of enamel sequential analyses to represent the diachronic isotopic variation recorded along the tooth crown. Even when samples do not isolate short amounts of time, these analyses allow for representing life conditions and diet variations of specimens over time.

6. Conclusions

In this paper, we provide a new sampling protocol to implement sequential stable isotope analysis of dentine collagen in high-crowned teeth. This proposal highly reduces the isotopic attenuation caused by dentine admixture in comparison with previous sampling strategies, and yields similar resolution as enamel sequential analyses. A high uniformity between sampled individuals is shown in dentine isotopic results, indicating that the protocol is reproducible in different specimens while yielding comparable isotopic data. We highly recommend performing a dentine drill-sampling when possible, especially when comparing data between individuals of different ages. The method offers better guarantees than the slice-sampling procedure to represent the actual isotopic composition of collagen recorded along the tooth crown. Moreover, the isotopic variation is represented more accordingly to growth timings, providing data more susceptible to be accurately interpreted.

The implementation of this method may encourage future zooarchaeological studies to explore the uses and possibilities of stable isotope analyses of incremental dentine collagen. Tracking the isotopic variation of collagen through animals' life, offers numerous possibilities to study life conditions linked to environment, diet, mobility, or reproduction, especially in the case of nitrogen isotopes which have been barely explored through sequential analyses. The protocol may also be applicable to other bovid species, although more methodological work will be needed in this line. The resolution of the strategy could be higher when implemented in bigger size species than sheep, such as cattle.

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CRediT authorship contribution statement

Celia Díez-Canseco: Conceptualization, Methodology, Investigation, Writing - original draft. **Carlos Tornero:** Conceptualization, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jas.2023.105923.

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