Nitrogen content variation in archaeological bone and its implications for stable isotope analysis and radiocarbon dating

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Abstract:

The collagen component of ancient bones is routinely isolated for radiocarbon dating and stable isotope studies. However, it is impossible to tell the state of collagen preservation from visual inspection of bones. At the Oxford Radiocarbon Accelerator Unit (ORAU), the percent nitrogen by weight (%N) of a ~5 mg sample of bone powder is measured on a mass spectrometer and used as a proxy for protein content. A previous study showed that samples with %N >0.76 are considered likely to produce sufficient collagen for radiocarbon dating (Brock et al., 2010b). However, the extent of variation between bone %N and collagen yield is unclear, as is the intra-bone variation in %N. Here, we report a series of tests performed on Palaeolithic bones known to have variable collagen preservation. This new study shows significant variation in %N within the same bone and that there is sometimes a lack of correlation between %N and collagen yield. These results suggest that for bone samples from difficult environments or from Pleistocene contexts, it may be worth sub-sampling for %N in different locations of the bone (if possible) and then attempting to extract collagen from marginally preserved bones (%N around 0.2-0.7%), as they may still yield sufficient collagen for isotope and dating studies.

Keywords: bone diagenesis; collagen; nitrogen content; radiocarbon dating; isotope studies

1. Introduction

Bone is a key archaeological material for radiocarbon dating and stable isotope analyses (Makarewicz and Sealy, 2015; Wood, 2015) but bones must have sufficient endogenous carbon preserved for measurement. The collagenous portion of bone is preferred to its mineral component for measurement, as the latter can exchange carbonates with depositional groundwater (Zazzo and Saliège, 2011). Collagen, however, is prone to degradation over time, including arid environments. This means that bones from old, arid sites may not yield sufficient collagen. Higham et al. (2014), for example, surveyed bones from some 40 late Middle Palaeolithic sites, and found over a dozen sites that contained no bone with sufficient collagen for radiocarbon dating.

Both radiocarbon dating and stable isotope measurements require chemistry pretreatment protocols to isolate collagen and remove contaminants that would affect the measurements. As this process is time consuming and destructive, it is helpful to know the preservation state of collagen before investing in pretreatment. At the Oxford Radiocarbon Accelerator Unit (ORAU), bone samples are screened for nitrogen content (the ratio of sample nitrogen mass to sample mass, hereafter %N), as a proxy for collagen presence (Brock et al., 2010a, 2012). Previous work at the ORAU has established that, using a %N threshold of 0.76, 84% of bones are correctly identified as to whether they will produce sufficient collagen for radiocarbon dating (>1% of original sample mass) (Brock et al. 2010a, 2012). Within the 16% of false identifications are bones that fail to produce sufficient collagen despite producing %N < 0.76. These estimates apply to samples treated with ORAU's routine protocol, which includes ultrafiltration (Brock et al., 2010a; Bronk Ramsey et al., 2004). Stable isotope pretreatment does not always use ultrafiltration, but issues of sample preservation remain for obtaining sufficient amounts and quality of collagen (Ambrose, 1990; Jørkov et al., 2007; Sealy et al., 2014).

Questions remain about the relationship between %N and protein content.,The 0.76 %N cut-off sometimes produces false positives and negatives, which in some cases may be due to localised variation in collagen content within a bone. Since %N measurement requires only ~5 mg of bone powder, variation in bone could be missed. The aims of this study were to quantify the rate of false positives and negatives from bones from a difficult Pleistocene context and to study whether there is significant variation in %N results across individual bones.

2. Background

2.1. Isolating bone collagen

Modern bone is 3.5-4.5% nitrogen by mass (Stafford et al., 1998). By dry mass, 60-70% of bone is mineral and the rest organic. The hydroxyapatite has been shown to exchange carbonates with groundwater, making it a problematic material for carbon dating and isotope measurement (Zazzo and Saliège, 2011). Collagen (85-90% of the total organic component) is instead routinely targeted. Strictly speaking, current conventional pretreatments do not isolate collagen, but a more complex gelatinous mix that includes collagen (Brock et al., 2013a). These 'protein remnants' (Brown et al., 1988) make up what is usually analysed and referred to as collagen (Van Klinken, 1999).

Pretreatment chemistry is designed to isolate autochthonous carbon and remove exogenous carbon from bone. Many types of contaminants may be present, including degraded proteins from soil or bone (van Klinken and Hedges, 1995), humic substances, salts, museum preservatives, and others (Brock et al., 2013a).

Many different radiocarbon pretreatment protocols for bone have been attempted over the years (see Wood, 2015 and references therein). The ORAU currently uses an ABA treatment with gelatinisation and ultrafiltration (Longin, 1971; Bronk Ramsey et al., 2000, 2004, Brock et al., 2007, 2010a, 2013b). For low-collagen samples, ultrafiltration increases the risk of not getting enough material for analysis compared to the ABA-gelatinisation method alone. The protocol also takes at least four days to produce a collagen product and determine whether there is sufficient material for radiocarbon dating.

Stable isotope pretreatment protocols vary between laboratories, though most do not use ultrafiltration (Ambrose, 1990; Jørkov et al., 2007; Sealy et al., 2014). Samples are usually subjected to both acid and base washes, though there is significant variation in concentrations, timings, sample sizes, and whether samples are filtered before freeze-drying. However, the principle of extracting collagen and removing contaminants is the same as for radiocarbon dating. A trade-off emerges between the intensity of the pretreatment and the resulting size of the collagen yield, particularly for poorly-preserved samples (Sealy et al., 2014).

For both analyses, a pre-screening step can save time and consumables by predicting which samples are worth pretreatment. It is known that an individual site may contain bones with

variation in preservation; pre-screening enables chemists and archaeologists to choose the best-preserved samples, thereby saving time, money, and sample destruction.

2.2. Methods of screening

At the ORAU, collagen content is estimated by measuring bone weight %N. As mentioned earlier, a sample must usually have %N >0.76 to pass, and after chemical pretreatment the collagen must represent at least 1.0% of the initial sample weight. In practice, however, bones as low as 0.5% N may be passed, especially if the sample is of special significance or from a site that produces no higher %N samples (Brock et al., 2012). Nitrogen exists in the proteinaceous component of bone rather than the mineral, so it is thought to be a reliable proxy for collagen. Brock et al. (2010b) tested potential bone quality indicators (i.e. colour, hardness, C:N ratio, etc.), and found %N to have the strongest predictive value at 84% likelihood with a %N threshold of 0.76. However, when a larger data set and a threshold of 0.7% N was used, this success rate was lower (73%), particularly for bones >25 ka BP (68%) (Brock et al., 2012). The authors suggest this is due to a greater proportion of collagen being degraded into short-chain fragments which are lost during ultrafiltration.

There remain uncertainties with %N measurement on whole bone. It will not, for example, differentiate between autochthonous and exogenous protein and contaminants which contain nitrogen. Bones with acceptable %N may also contain degraded proteins that can pass through ultrafilters and be lost. This may explain why 58% of older Pleistocene samples with a %N of 0.75-0.99 still failed to produce 1% collagen (Brock et al., 2012).

As mentioned earlier, it is also unclear how %N—and, by proxy, collagen content—varies across a bone. Less than 5 mg of bone powder is required for %N measurements, usually drilled from a single spot on the bone. The amount of bone needed for radiocarbon pretreatment at the ORAU depends on sample preservation, but in general samples may be ~700 mg and poorly preserved samples as large as >1 g (Brock et al., 2010a). It is possible that, in bone of variable preservation, remaining collagen could be present in different locations in the bone. This potential variation in %N from the same bone has not yet been studied in detail.

3. Materials and methods

3.1. Zafarraya

Bones for this study come from the Pleistocene cave site of Zafarraya in Malaga, Spain. The site contains a Mousterian sequence with Neanderthal remains, though the precise chronology remains contested (Wood et al., 2013). Previous work showed significant variation in %N between bones (Wood, 2011; Wood et al., 2013). Of 30 samples screened in these studies, only three passed the 0.76% threshold and 18 had %N <0.2. For this new study, we increased the bone corpus and analysed 229 samples. The bones come from the entire Pleistocene sequence (layers UA to UE). While samples used here were mostly unidentifiable fragments, previous analyses found several mammal species, including carnivores and herbivores (Barroso Ruiz, 2010; Barroso-Ruiz and Bailon, 2003; Geraads, 1995; Geraads et al., 2013). Challenges with reliable radiocarbon dating have been due, in part, to the difficulty in finding bones with sufficient collagen preservation: only three dates were produced with robust ultrafiltration treatment in Wood et al. (2013) (Table 1).

OxA	Age BP	Error	Species	Context
21810	46300	2500	*Capra*	Mousterian
21813	>49300		*Capra ibex*	Mousterian
23198	>46700		*Capra pyrenaica*	Mousterian

Table 1: Three radiocarbon dates obtained on bones from Zafarraya using the ultrafiltration protocol (Wood et al., 2013). Age BP refers to conventional radiocarbon age (Stuiver and Polach, 1977).

3.2. Measuring %N

The %N values of 229 bones were measured using the same methods as described in Brock et al. (2012). Samples were first surface cleaned by air abrasion with fine aluminium oxide powder to remove contaminants, and then a cortical bone surface was drilled using a tungsten carbide spherical burr drill bit and the powder discarded. A small amount of bone powder (3-5 mg) was then drilled from this cleaned spot and collected for analysis. The bone powder was weighed into clean tin capsules and the %N and %C contents were measured using an automated carbon and nitrogen elemental analyzer (Carlo Erba EA1108). An in-house alanine standard (Merck, 05129, UK) was used for instrument calibration and quality control.

142 *3.3. Measuring intra-bone %N variability*

143 Eight bones were selected for sub-sampling to assess %N variation within bones. The samples
144 chosen were large enough to take 4 to 11 %N measurements per bone. Drill spots were spaced
145 across a bone and their locations recorded (Figure 1). The bone powder was then analyzed for
146 %N as above.

147

148 Figure 1: Example of a bone sub-sampled in six locations for %N values.

149 *3.4. Collagen extraction*

150 Collagen was extracted from a subset of fifty bones of sufficient size (>1 g). As much as
151 possible, two bones were selected from each 0.1% bin of %N results to extract collagen from.
152 Some bins had zero or one bone, in which case the one bone was selected. Collagen was
153 extracted using ABA plus gelatinisation and ultrafiltration, the routine protocol described in
154 Brock et al. (2010a). Collagen mass was weighed and the %yield calculated as the ratio of
155 collagen to starting bone sample mass.

156 **4. Results**

157 *4.1. %N screening*

158 As expected from previous work (Wood et al., 2013), the majority (87%) of Zafarraya bones
159 measured for %N failed to produce values over the threshold of 0.76% (Figure 2). Only 6% of

bones had %N values >1% N. The distribution of results did not show multimodality, which might have suggested two subpopulations of bones in terms of preservation. Comparison with samples' contexts did not reveal a spatial trend in %N results, either horizontally by grid square or vertically by depth. Rather, there seems to be general variability in %N across the site.

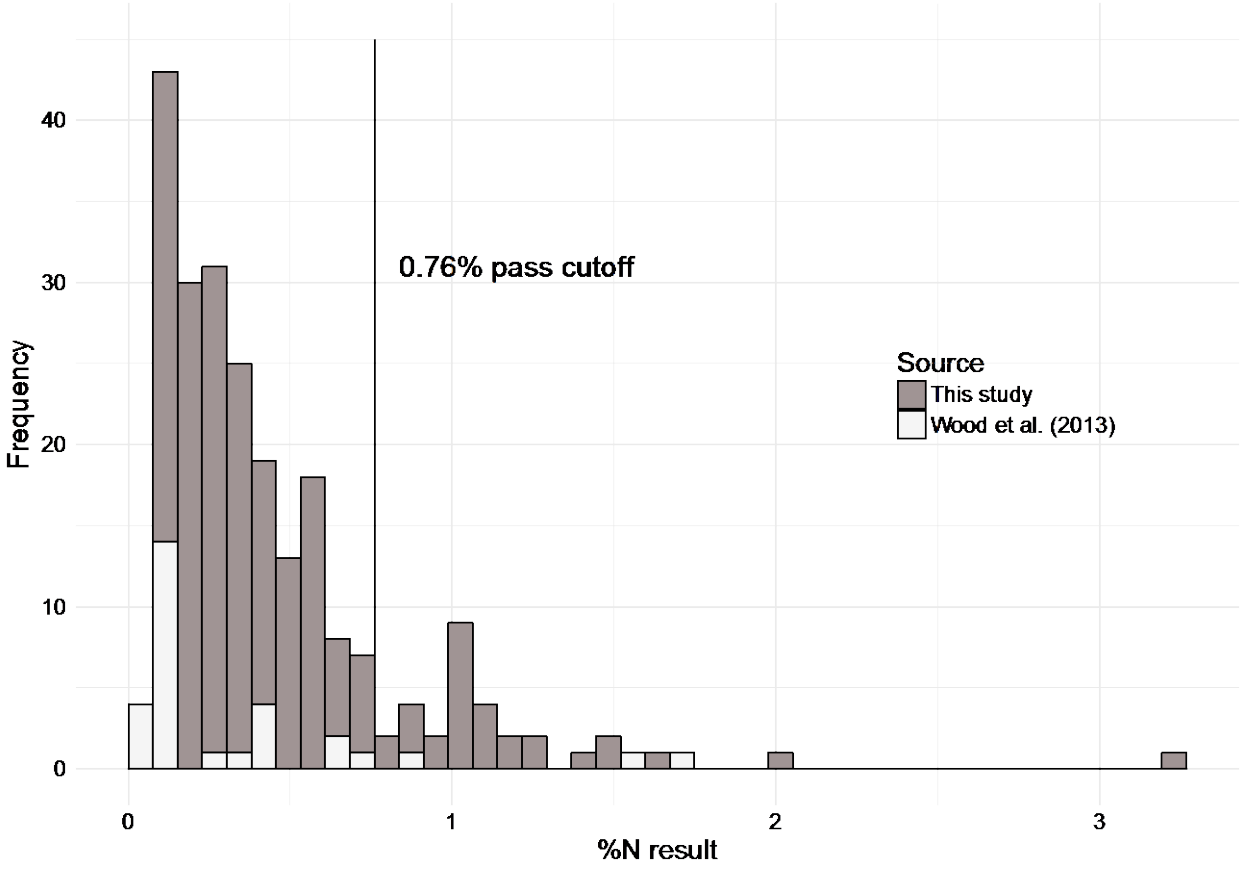
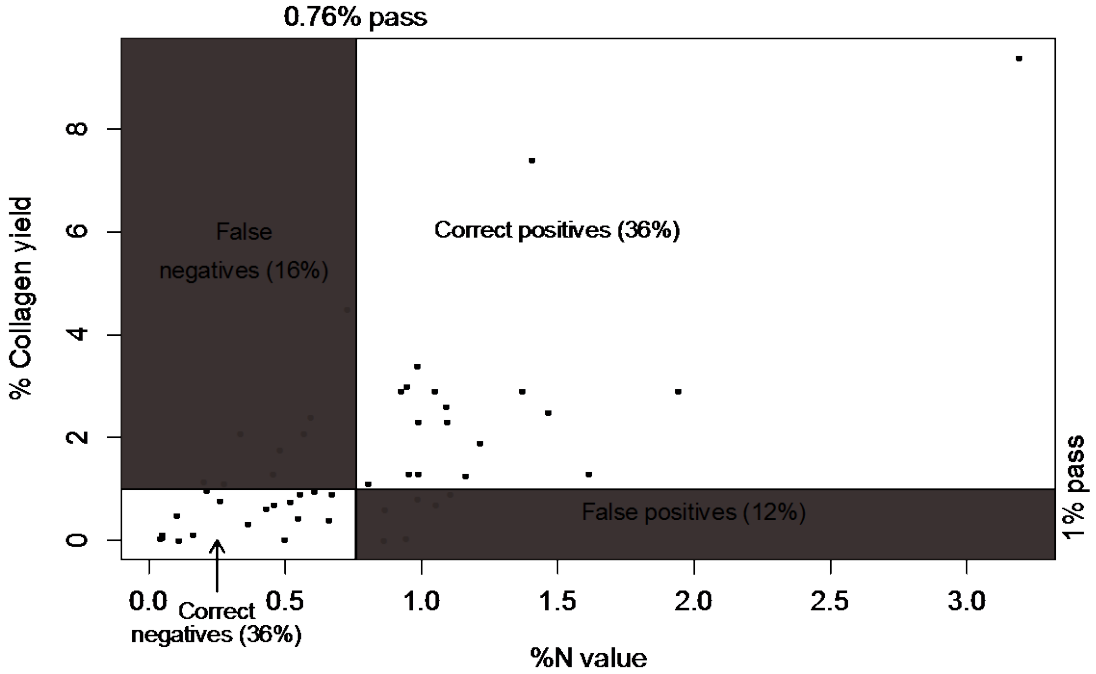


Figure 2: Histogram showing %N values for 259 samples from Zafarraya: 229 new to this study and 30 previously measured in Wood et al. (2013).

4.2. %N versus collagen yields

In total, 36 of the 50 selected bones (72%) produced collagen yields correctly predicted by their %N values (Figure 3). These bones were divided evenly between correctly predicted passes (>1% collagen yield) and fails. However, 14 bones (28%) produced incorrectly predicted collagen yields. Eight bones below the 0.76% N threshold produced >1% collagen (false negatives) and six bones over 0.76% N produced <1% collagen (false positives). The more lenient 0.5% N cut-off made more incorrect predictions overall (34%) due to increased false positives.



175

176 Figure 3: Graph showing the relationship between %N and ultrafiltered collagen yields from 50
177 bones. The lines on the graph represents the cut-offs used at the ORAU (%N >0.76 and
178 collagen yield >1%).

179 *4.3. Inter-bone variation in %N*

180 Eight large bones were sub-sampled at between 4 to 11 points to characterise %N variation. As
181 shown in Figure 4, the intra-bone variation of some bones was significant. The largest range in
182 a bone was 0.75% (Bone 2); given a modern bone is about 4% nitrogen, this represents some
183 19% of a bone's potential nitrogen content. Measurement error on the mass spectrometer is
184 ~0.3‰ and so cannot explain most of this variation.

185 Three of the bones (Bones 1-3) had samples that spanned the 0.76 %N cut-off. If only sampled
186 once, each bone could have failed or passed the screening depending on where it was drilled.
187 This suggests that the preservation of some bones is being incompletely characterised by a
188 single %N measurement, leading to false passes and failures.

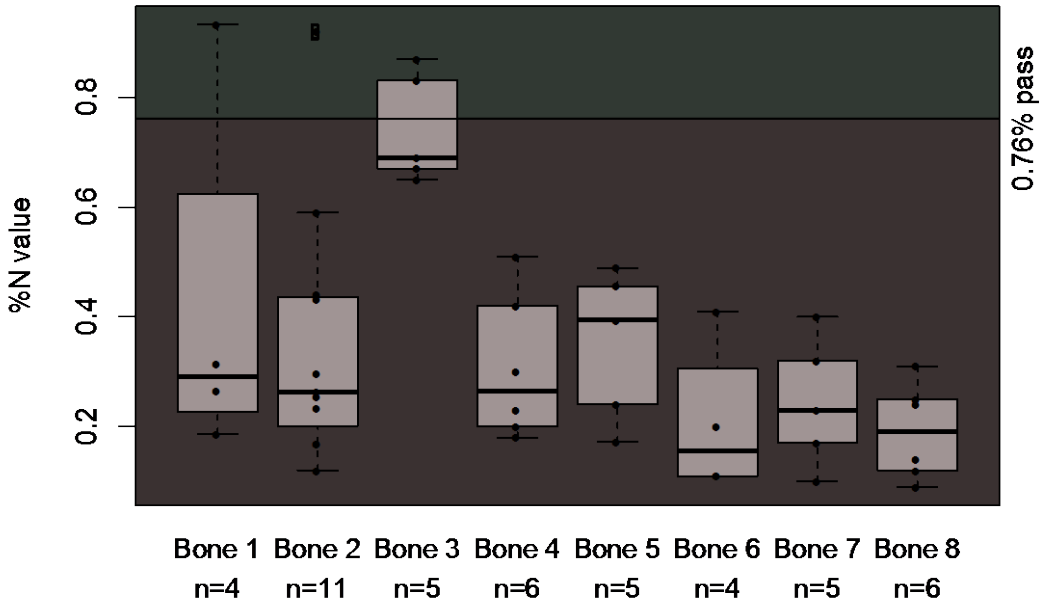


Figure 4: Results of sub-sampling eight bones with %N results as box plots. The number of samples taken from each bone is listed below the box plot. Bone 4 is pictured in Figure 1.

5. Discussion

This study found that %N correctly predicted collagen yields 72% of the time, similar to the 73% likelihood from previous results (Brock et al., 2012). Of the 28% incorrectly identified bones, there are examples where %N failed to predict both high and low collagen yields. There are multiple and complex processes involved in bone diagenesis, which makes explaining these results challenging (Collins et al., 2002; Dobberstein et al., 2009; van Klinken and Hedges, 1995). That bones with low %N may produce collagen is surprising. Brock et al. (2012) found all bones with <0.24% N failed to produce sufficient collagen, suggesting that %N screening is not missing bones with regions of collagen preservation. However, this study produced one sample with a %N = 0.207 and a collagen yield >1% weight. One explanation is that these bones contained variation in collagen preservation not revealed by a single %N measurement. The subsampling results suggest that significant variation in %N can exist in bones. Some bones

may be rejected based on a single %N measurement, when in fact a measurement taken from a different point in the bone may have passed.

Another possibility is that deamidation of collagen has removed nitrogen while significant partially degraded protein remains. This process removes the nitrogen-containing amide group from asparagine and glutamine and has been observed in ancient bones (Cleland et al., 2015; van Doorn et al., 2012; Wilson et al., 2012). Van Doorn et al. (2012) found that deamidation was accelerated in particular burial conditions, such as hydrological activity and low soil pH. In these accelerated cases deamidation could reduce nitrogen content before other protein-destroying processes are complete.

Other bones in this study produced a high %N but little collagen. There are three hypotheses that might explain these false positives. First, some of the high %N results may be due to contaminated regions, perhaps from humics, nitrates, bacteria, or fungi. Humics are complex large molecules that can contain nitrogen and carbon. They are soluble in basic solutions and the base wash in ABA is meant to remove them (Head, 1987; van Klinken and Hedges, 1995), but they may be a source of nitrogen in the initial %N screening. Presence of nitrates has been used to explain misleadingly high nitrogen values in bones from the Neolithic settlement of Çatalhöyük (Brock et al., 2012). Microbes and fungi that produce collagenase have also been isolated from archaeological bone (Child, 1995). If still present in the bone matrix, they may contribute to the %N value before pretreatment. Second, the samples that go on to pretreatment may contain collagen-free areas, despite a seemingly good %N screen. A larger bone sample (~1 g) was used for collagen extraction than %N measurement (~5 mg) and so there may be less protein in the extraction fraction than the %N fraction. Third, the quality of the collagen itself may be problematic. In Pleistocene bones, large chains of collagen may degrade into smaller fragments. These will still contain nitrogen but may be lost during ABA-gelatinization and ultrafiltration. Ultrafiltration in particular could allow small-chain collagen fragments to pass through the 30 kDa filter (Brock et al., 2013). Material from Zafarraya is Pleistocene in age, and so the quality of the collagen, not just its abundance, may be part of the variation in results. Any of these explanations may be relevant to a given site, depending on the post-depositional context of the bones.

Nitrogen variability exists within individual bones as well. While the above diagenetic processes may be occurring at locales within a bone, little is currently known about intra-bone nitrogen variation. Further exploration of the correlation between sub-sampled %N and collagen yields

would be useful, as the size of these higher nitrogen regions and their link to collagen yields is unclear. This could include mapping bones to understand which bone or part of the bone is more likely to have collagen preserved, similar to the way DNA is optimally preserved in the petrous bone (Pinhasi *et al.* 2015). One potential method is to use non-destructive FTIR to characterise sources of nitrogen in a sample (Lebon et al., 2016), but this has not yet been widely applied to difficult archaeological samples such as low-collagen bones or bones treated with organic conservation materials.

6. Conclusions

This study reveals that using %N as a simple pre-screening metric can be problematic, and there are likely unexplained processes that can undermine the predictive value of %N. Particularly in difficult Pleistocene sites where bones are producing marginal (>0.2% N) or variable values, it may be worth attempting pretreatment in hopes of finding some false negatives. Some of the Zafarraya material discarded by Wood et al. (2013) based on %N values, for example, may be worth revisiting.

This study also reveals significant variation in %N values when a bone is drilled in multiple locations. Again, in sites where preservation is likely to be problematic and for particularly important samples, it can be worth taking multiple %N measurements in the hope of finding a location to drill for dating where the collagen might be differentially better preserved than in other areas.

While %N continues to be a useful screening tool for radiocarbon dating and isotope analysis—it is cheap, rapid, and requires little sample destruction—there are situations where it can be worth relaxing use of the 0.76% cut-off. Indeed, this is already done at the ORAU when site conditions and overall preservation warrants trying lower-%N bones (Brock et al., 2010a). This requires sample destruction and may not always be worth the risk, but for archaeologists working with difficult samples it is useful to be aware of these frequent exceptions and the scale of variability that can exist in a single site and even a single bone.

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