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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.

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

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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

By dry mass, 60–70% of bone is mineral and the rest organic. 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).

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.

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).

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