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**Refining 14C dating of bone >30,000 BP : establishing an accurate chronology for the Middle to Upper Palaeolithic transition in France**  
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## 6. A comparison of bone pretreatment methods for AMS dating of samples >30.000 BP

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The focus of this paper was to find the best pretreatment method to minimise or remove modern contamination in bone samples older than 30,000 cal BP.

The study involves two different bone samples. The first one is a Mammoth rib from North Sea (lab code S-EVA 2000, Figure 6.1) and the second is a bison bone, also from North Sea (lab code S-EVA 2001, Figure 6.2)



*Figure 6.1 Mammoth rib sample involved in this work*



*Figure 6.2 Bison sample involved in this work*



## A COMPARISON OF BONE PRETREATMENT METHODS FOR AMS DATING OF SAMPLES >30,000 BP

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**ABSTRACT.** Bone is a commonly used material for radiocarbon dating, yet at ages close to the limit of the method (>30,000 BP), it is a substantial challenge to remove contamination and produce accurate ages. We report here on the preliminary results of a dating study of 2 bones older than 30,000 yr, which were each treated with a suite of pretreatment procedures, including ultrafiltration (Brown et al. 1988). Substantial differences in the  $^{14}\text{C}$  ages were observed, which is most likely linked to crucial steps in the removal of contamination both in the bone and in the laboratory. Using a comprehensive sequence of pretreatment procedures, including ultrafiltration, we obtain generally older ages.

### INTRODUCTION

Bone is one of the most important archaeological materials used for radiocarbon dating, but at the same time it is also one of the most difficult materials to date as bone can be susceptible to contamination and is also often degraded. Prior to 1970, whole bone was generally used for dating, and often included bone carbonate, which itself could be postdepositionally contaminated. Longin (1971) proposed a method to isolate bone collagen, which was seen to be a much more stable material and less susceptible to contamination than bone mineral. The main challenge in chemically pretreating bones for  $^{14}\text{C}$  dating is therefore to extract collagen and then confirm that the extracted collagen is indeed largely intact and free from contamination, usually through the measurement of a suite of indicators, including C:N ratio, collagen yield, and %C and %N in the collagen extract.

Most laboratories use some variation of the Longin (1971) method, but other laboratories have specialized in bone dating and have dramatically improved collagen extraction and purification techniques. For instance, in 1992 the Oxford Radiocarbon Accelerator Unit (ORAU) published the HPLC technique, which is one of the most sophisticated methods to carefully extract and purify amino acids from bone collagen (Van Klinken et al. 1994). Another way to purify the bone samples was proposed earlier by a Canadian research team (Brown et al. 1988), to separate the high-molecular weight (>30 kD) from the low-molecular weight fraction using ultrafiltration, and then produce dates using only the >30-kD fraction. In 2000, the ORAU adopted this ultrafiltration method (Bronk Ramsey et al. 2000, 2004) for their extracted gelatin, and also included a primary filtration step using an Ezee-Filter™ (Elkay Laboratory Products Ltd., UK). After various tests of potential contamination from both the ultrafilters and the Ezee-Filters, the ORAU lab found that both of the filters require careful cleaning in an ultrasonic bath (Brock et al. 2007). The ultrafilter has a humectant coating (glycerol) added to maintain the flexibility of the filter. As glycerol can be manufactured from either plant or animal extracts, or alternatively from petroleum processing byproducts, this glycerol needs to be removed as it could add young or old carbon to the sample. Bronk Ramsey et al. (2004) suggested that the best way to eliminate this glycerol without damaging the filter is repeated washes and sonification, and after 3 washes showed that the humectant was effectively removed as shown by no measurable carbon in the eluent. Despite the apparent beneficial role in removing the low-molecular weight fraction of the collagen (Higham et al. 2006), there is an ongoing discussion in the  $^{14}\text{C}$  community about the utility and accuracy of ultrafiltration. Hüls et al.

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(2007) tested 2 different ultrafilters, a Vivaspin™ 15R and a Vivaspin 20, using bones of various ages. They showed that in spite of careful cleaning of the ultrafilter, small amounts of contaminant young carbon remained, which probably came from glycerin; hence, they recommended caution in the use of the ultrafilter. In at least one example, dates of mammoth bone samples have been obtained without ultrafiltration, where the ages compare well with contemporary peat and wood (Hajdas et al. 2007).

The  $^{14}\text{C}$  activity of the humectant has been shown to be bimodal, i.e. essentially modern or of fossil origin (Brock et al. 2007). For bones older than 30,000 BP, contamination by modern carbon will alter the true age much more dramatically than the addition of  $^{14}\text{C}$ -dead carbon of a similar size.

At our institution (MPI, EVA Leipzig, Germany), we developed techniques to extract collagen from Pleistocene-age bone for stable isotope analysis and the resultant collagen was often sent to accelerator mass spectrometry (AMS) facilities for  $^{14}\text{C}$  dating. In 2005, well-preserved bison and mammoth bones from the North Sea of unknown age were adopted as the long-term quality control material, especially to test for background contamination in the sample preparation, with the assumption that these 2 bones were at least Pleistocene in age, and ideally older than 50,000 BP. Initial  $^{14}\text{C}$  results from 2 AMS laboratories on collagen prepared at MPI showed the bones to be in the age range of 30,000 to 45,000  $^{14}\text{C}$  BP, but we observed large discrepancies in the  $^{14}\text{C}$  ages between different  $^{14}\text{C}$  labs. These inconsistencies could have been caused by deficits in the pretreatment methods that we had established for collagen extraction, by insufficient removal of contamination in the samples, in the AMS measurements themselves, or all three.

We therefore designed a study to investigate the source of these inconsistent dates, and we also compared the results of our pretreatment methods against results from the methods from two of these AMS labs.

#### METHODS

We obtained 2 bones, the first a mammoth (S-EVA 2000) and the second a bison (SEVA 2001) from the Pleistocene North Sea plain. Both bones have relatively well-preserved collagen. Bone powder was drilled from the 2 bones and pretreated in 3 different ways (methods A–C) as outlined in Table 1. Method A was the pretreatment method employed when we first ran stable isotope analysis and later on dated the mammoth and bison samples. The bone is decalcified in HCl over several days while refrigerated ( $\sim 4^\circ\text{C}$ ) until no effervescence is observed. The gelatinization step is at pH 3 at  $70^\circ\text{C}$  for 48 hr. The ultrafilter (Vivaspin 15, 30 kD) is rinsed in NaOH (1 $\times$ ) and  $\text{H}_2\text{O}$  (4 $\times$ ). Method B differs only in the cleaning of the ultrafilter, which was modified by removing the NaOH cleaning, and adding a 1-hr ultrasonic bath after the third  $\text{H}_2\text{O}$  rinse. In initial tests, we also used Millipore ultrafilters but in agreement with ORAU (Brock et al. 2007), we found Vivaspin 15 to be better suited for our purposes.

In Method C, the decalcification is at room temperature for 4 hr, with an additional NaOH step to remove humics and followed by an HCl step to remove potential contamination from modern  $\text{CO}_2$  taken up by the NaOH. The gelatinization is done at pH 3 but at  $75^\circ\text{C}$  for 20 hr. The ultrafilter is cleaned by centrifuging in  $\text{H}_2\text{O}$  (5 $\times$ ) without the NaOH rinse, and kept for 1 hr in ultrasonic bath after the third  $\text{H}_2\text{O}$  rinse. The cleaning is checked by monitoring the carbon level in the eluent.

We also submitted unpretreated samples (2 pieces of each bone) directly to the Oxford and Kiel laboratories, which were then pretreated in each laboratory following standard in-house pretreatment methods (**Method D<sub>K</sub>**, **Method D<sub>O</sub>**). The specifics of these methods are given below.

Table 1 Pretreatment steps for all 3 methods.

Method	Decalcification				Cleaning of the ultrafilter			
	HCl	NaOH	HCl	Gelatinization	NaOH centrifuge	H <sub>2</sub> O centrifuge	Ultrasonic bath	H <sub>2</sub> O centrifuge
A	Several days at ~4 °C	No	No	pH 3, 70 °C for 48 hr	1×	4×	No	No
B	Several days at ~4 °C	No	No	pH 3, 70 °C for 48 hr	No	3×	1 hr	2×
C	4 hr at room temperature	Yes	Yes	pH 3, 75 °C for 20 hr	No	3×	1 hr	2×

**Method D<sub>K</sub>, Kiel-Leibniz Laboratory:** Demineralization of bone pieces 0.5 to 2 mm in size with 1% HCl at room temperature, keeping pH < 1, and then subsequent washes with Milli-Q™ water (Millipore Corp.) until pH > 4. Then, an extraction was done for 1 hr at room temp with 1% NaOH, followed by washing with Milli-Q until pH < 9, and then again an extraction with 1% HCl for 1 hr at room temperature followed by a water rinse. The resulting extract is then hydrolyzed overnight at pH = 3 at 85 °C in a water bath. The gelatin solution is then filtered through a precleaned 0.45- $\mu\text{m}$  silver filter. This laboratory normally does not use ultrafiltration because they are still investigating potential contamination by the filters.

**Method D<sub>O</sub>: Oxford Laboratory:** This method is described fully in Brock et al. (2007).

## RESULTS

The stable isotope data (C:N,  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , and %C and %N content) for mammoth and bison collagen is presented in Table 2 for all methods. We do not observe significant differences in these values between the different preparation methods and the parameters indicate well-preserved collagen (DeNiro 1985; Ambrose 1990).

Table 2 Atomic C:N ratio and stable isotope analysis of collagen from the mammoth (S-EVA 2000) and bison (S-EVA 2001) bones. The collagen was prepared according to methods A–C. For  $\delta^{13}\text{C}$ , the standard is VPDB; for  $\delta^{15}\text{N}$ , the standard used is IAEA N1 and N2. Typical analytical precision is 0.1‰ for  $\delta^{13}\text{C}$  and 0.2‰ for  $\delta^{15}\text{N}$ .

	Method	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	%C	%N	C:N
<b>Mammoth</b>						
2000	A	-21.1	7.0	41.9	15.4	3.2
2000-XXII	B	-21.2	6.8	39.4	13.9	3.3
2000 D	C	-21.4	6.8	44.2	16.2	3.2
<b>Bison</b>						
2001	A	-20.8	3.1	38.8	13.9	3.3
2001-XII	B	-20.5	2.4	40.5	14.5	3.3
2001 C	C	-20.5	2.6	41.4	15.2	3.2

Dating results for the mammoth (S-EVA 2000) are given in Table 3a. The collagen prepared at the Max Planck Institute (MPI) by methods A–C was submitted to both Kiel and Oxford for dating. For methods A and C, separate preparations had to be made to obtain enough material for analysis. For Method B, collagen yields allowed us to split the same collagen sample to be sent to the different labs. The resulting mammoth ages from the different methods and different labs range between

31,660 and 35,280  $^{14}\text{C}$  BP with  $1\sigma$  errors between 200 and 510 yr. The youngest results are associated with Method A, while methods B and C tend to give older ages (Figure 1). Within each method, the results of methods A, B, and C agree within  $1\sigma$  between the AMS labs of Kiel and Oxford, but for Method D the results are statistically different. For these samples submitted as bone to the 2  $^{14}\text{C}$  labs for pretreatment in their laboratories (methods  $\text{D}_\text{K}$  and  $\text{D}_\text{O}$ ), the difference between the dates is 2620 yr and the results in the labs do not agree statistically with their respective results of Method C (Figure 1).

Table 3a  $^{14}\text{C}$  results of mammoth samples prepared using methods A to D. Missing values were not reported. The  $\delta^{13}\text{C}$  reported is derived from the AMS sample combustion procedure. OxA-V indicates that the material was combusted and graphitized/dated in Oxford, but the chemical pretreatment of the bone was done at MPI.

S-EVA mammoth	Method	Lab code	% yield of collagen	C content (%)	pMC	$^{14}\text{C}$ age	Error $\pm 1\sigma$	$\delta^{13}\text{C}$ (‰)
2000	A	KIA-28337		46	$1.94 \pm 0.06$	31,660	240	-21.2
2000	A	KIA-29434		45.5	$1.90 \pm 0.12$	31,820	510	-22
2000	A	OxA-V-2166-47				31,910	200	-19.7
2000-XXII <sup>a</sup>	B	KIA-35978	3.3	48.8	$1.59 \pm 0.06$	33,280	320	-19.1
2000-XXIIa <sup>a</sup>	B	OxA-V-2281-52	3.3	45.3	$1.51 \pm 0.05$	33,670	200	-21.2
2000 XXIII	C	KIA-44753	4.2		$1.53 \pm 0.06$	33,560	320	-23.7
2000 D <sup>a</sup>	C	OxA-UnK;54	9.4		$1.50 \pm 0.05$	33,733	257	-19.2
2000 D <sub>-</sub> <sup>a</sup>	C	MAMS-10399	9.4		$1.27 \pm 0.04$	35,075	260	-23.3 <sup>b</sup>
2000	$\text{D}_\text{K}$ <sup>c</sup>	KIA 29435		38.6	$1.71 \pm 0.07$	32,660	350	-20.6
2000	$\text{D}_\text{O}$	OxA-15908			$1.24 \pm 0.05$	35,280	340	-20.5

<sup>a</sup>Split collagen.

<sup>b</sup> $\delta^{13}\text{C}$  AMS.

<sup>c</sup>No ultrafiltration step.

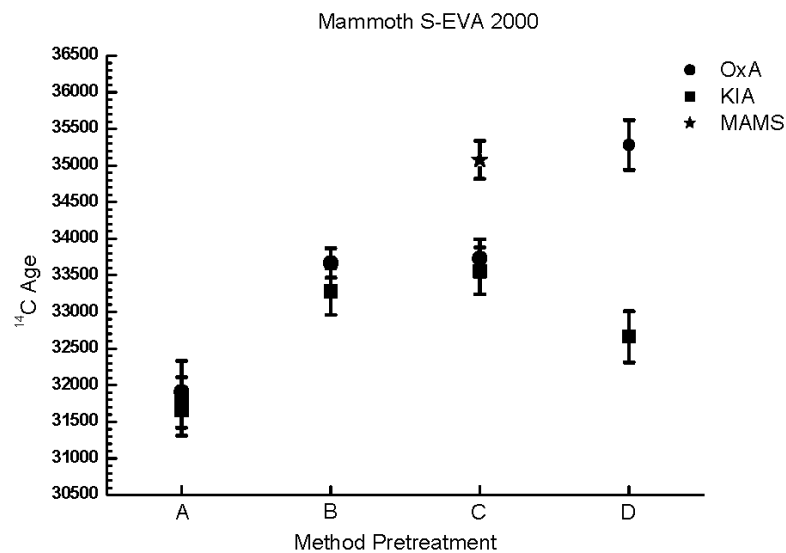


Figure 1  $^{14}\text{C}$  ages of mammoth bone obtained from methods A to D



The bison bone (S-EVA 2001)  $^{14}\text{C}$  ages range between 37,090 and >44,800  $^{14}\text{C}$  BP with errors from 360 to 1630 yr (Table 3b). The results of Method B agree between the 2 AMS labs involved, but they are more than 2000 yr younger than the results of the other methods (except for Method  $\text{D}_\text{K}$ , which is the youngest of the results). The results of methods A and C agree statistically at the  $2\sigma$  level (Figure 2).

Table 3b  $^{14}\text{C}$  results of bison samples prepared using methods A to D. Missing values were not reported. The  $\delta^{13}\text{C}$  reported is derived from the AMS sample combustion procedure. OxA-V indicates that the material was combusted and graphitized/dated in Oxford but the chemical pretreatment of the bone was done at MPI.

S-EVA bison	Method	Lab code	% yield of collagen	C content (%)	pMC	$^{14}\text{C}$ age	Error $\pm 1 \sigma$	$\delta^{13}\text{C}$ (‰)
2001	A	KIA 28338		45.5	$0.49 \pm 0.05$	42,660	790	-21.7
2001	A	KIA 29436		45.3	$0.39 \pm 0.09$	44,480	1630	-20.4
2001	A	OxA-V-2166-48			$0.28 \pm 0.03$	47,300	900	-19.3
2001-XII <sup>a</sup>	B	KIA 35982	2.5	49.6	$0.69 \pm 0.05$	40,200	640	-22.1
2001-XIIa <sup>a</sup>	B	OxA-V-2281-53	2.5	44.5	$0.64 \pm 0.03$	40,630	360	-20.8
2001 XIII	C	KIA-44754	7.1		$0.34 \pm 0.05$	45,740	1420	-21.3
2001 C <sup>a</sup>	C	OxA-UnK;53	3.7		$0.43 \pm 0.03$	43,674	450	-18
2001 C <sup>a</sup>	C	MAMS-10398	3.7		$0.29 \pm 0.04$	47,000	1250	-21.6 <sup>b</sup>
2001	$\text{D}_\text{K}$ <sup>c</sup>	KIA 29437		39.5	$0.99 \pm 0.07$	37,090	570	-19.3
2001	$\text{D}_\text{O}$	OxA-15909				>44,800	—	-20.1

<sup>a</sup>Split collagen.  
<sup>b</sup> $\delta^{13}\text{C}$  AMS.  
<sup>c</sup>No ultrafiltration step.

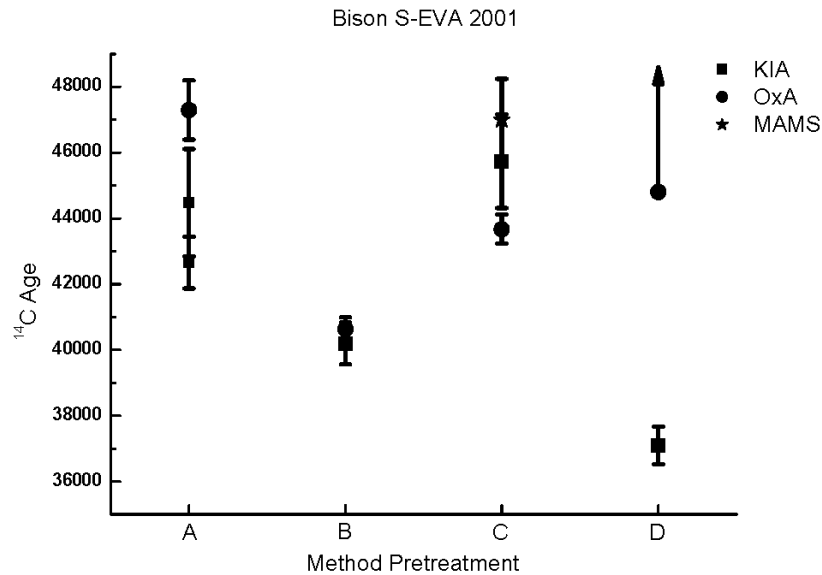


Figure 2  $^{14}\text{C}$  ages of bison bone obtained from methods A to D

As an additional test of possible contamination in the *graphitization* stage, we also sent the collagen prepared using what we believe is the most stringent preparation method, Method C, to a third AMS lab, Mannheim (lab code MAMS), to compare the results between the 3 labs. Surprisingly, the Mannheim lab for both the mammoth and bison samples had older ages than both Kiel and Oxford, for the same collagen extract. The combination of Method C preparation in our lab and then subsequent graphite production and AMS measurement in the Mannheim lab resulted in the same, or very similar, older ages as the bone samples prepared and measured entirely in the Oxford lab (Table 3a,b, Figures 1 and 2).

#### DISCUSSION AND CONCLUSION

To obtain reliable bone dates, good quality collagen is required, which can be confirmed through the measurement of the C:N ratio, yield, %C, and %N. As shown in Table 2, collagen from both of our samples is well preserved for all of the pretreatment methods. There was very little difference in the collagen isotope values and preservation criteria indicators between the 3 methods.

With the pretreatment methods we used, almost all of these criteria remained the same, with the exception of collagen yield. We observed a significant increase in yield when the decalcification in HCl was performed relatively quickly at room temperature (Method C) compared to Method A that required decalcification for several days at a constant temperature (5 °C). For example, we obtained 50% more for the bison and up to 3 times more collagen for the mammoth.

The true ages of the 2 bone samples is unknown, but the influence of contamination at ages older than 30,000 yr BP is very asymmetric, with young contamination dominant relative to the original  $^{14}\text{C}$  content of an old sample, even at low percent levels of exogenous carbon, contrary to relatively small effect that the addition of  $^{14}\text{C}$ -free (dead) carbon has (Mook and Stuiver 1981). We know that in the recent shipments of the filters the humectant is modern (Brock et al. 2007), so there is good reason to consider the oldest ages the real ones, and younger ages resulting from modern contamination. In a case when modern contamination is substantial in the collagen to be dated, one might even obtain the paradoxical result that the measured ages appear more homogeneous compared to the case when samples of very low  $^{14}\text{C}$  activity, without added contamination, are analyzed.

The age results indicate that the 2 bone samples need to be discussed separately. For the mammoth bone (Table 3a, Figure 1), the picture would be consistent if only the results of methods A to C for Oxford and Kiel are considered. They indicate incomplete removal of modern contamination in the collagen of Method A, and the lack of any measurable effect of the NaOH step, which is the main difference between methods B and C. But the results for Method D, where each of the labs are in full control of all procedures, are clearly incompatible, both among the 2 labs but also compared to the results of these labs with collagen of methods C and B. This was one of the reasons we then sent an aliquot of the collagen prepared in our lab using Method C to a third AMS lab (Mannheim), which resulted in an age identical to the older ages of Oxford with Method D.

We must conclude that either the ages of Oxford ( $D_0$ ) and Mannheim (C) are true or the 4 results younger by about 1500 yr are the correct ones. As explained above, a shift to older ages compared to the true age is much less probable than the opposite effect of modern contamination. We therefore conclude that the true age of the mammoth is most likely around 35,000  $^{14}\text{C}$  BP, and the younger ages as being caused by modern contamination in the AMS labs, e.g. during graphitization.

The bison sample is older than the mammoth; therefore, we expect the effects of contamination to be more severe for this bone (Table 3b, Figure 2). Again, methods C and  $D_0$  show the oldest ages,

but Method A has 2 similarly old results as well. An explanation could be that the filters may contain a variable amount of humectant, which might have been removed by the basic cleaning used in Method A. If this is the case, then this means that regular checks on the cleaning efficiency of the ultrafilters are necessary and this is part of methods C and B. The latter method does not include the NaOH step to remove humics; therefore, for bones of this antiquity, contamination by humics could alter the  $^{14}\text{C}$  age, and thus, the NaOH step becomes important.

When samples are close to the lower age limit of  $^{14}\text{C}$  dating, the low  $^{14}\text{C}$  activity level and difficulty of obtaining sufficient and well-preserved collagen means that bone is an especially problematic material to accurately date. In this study, as in earlier exercises (Higham et al. 2006; Hajdas et al. 2007; Hüls et al. 2007), we observed that by using elaborate pretreatment procedures that eliminate both modern laboratory contamination and contamination from degenerated proteins and humic acids we were able to obtain older ages. We still observe discrepancies between the results of different AMS labs, highlighting the many challenges of  $^{14}\text{C}$  dating at very low  $^{14}\text{C}$  activity.

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