DEVELOPMENT AND APPLICATION OF AN ANALYTICAL METHOD FOR RADIOCARBON DATING BONES USING THE AMINO ACID HYDROXYPROLINE

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Abstract

Archaeological bones are usually dated by radiocarbon measurement of extracted collagen. However, low collagen content, contamination from the burial environment or museum conservation work have previously lead to inaccurate results, especially for old bones, compromising the ability to reconstruct reliable past chronologies. It is reported, for example, that up to 70% of Palaeolithic radiocarbon dates on bones are likely to be underestimates of the real age, blurring the picture of modern human dispersals and Neanderthal extinction.

In this thesis, a method for isolating and radiocarbon dating the collagen amino acid hydroxyproline is described. Hydroxyproline consists of about 10% of bone collagen but is not found in significant amounts elsewhere in nature. The hydroxyproline dating method uses a mixed-mode (i.e. ion-exchange combined with hydrophobic chemistry), semi preparative HPLC methodology. The amino acids do not require derivatisation, and no organic solvents are used, thereby avoiding addition of carbon. The hypothesis of this thesis is that the hydroxyproline can be used as a bone specific biomarker, improving dating accuracy and making it possible to obtain radiocarbon determinations where previously it has been impossible.

It was calculated that on average 3.3±1.4μg of contaminant carbon are added to each sample in the process of isolating the hydroxyproline, a low level suitable for ^{14}C dating. It was investigated whether a deliberately contaminated bone and 'naturally' contaminated archaeological bones, yielding erroneous dates when dated using the normal pretreatment method, could be dated accurately using this method. In addition, a hydroxyproline date was obtained for a bone with too little surviving collagen to be dateable by the bulk collagen method. Finally, using the hydroxyproline dating method, the earliest direct ages for the presence of anatomically modern humans on the Russian Plain were obtained. The method proved to be a powerful tool that can help resolve longstanding archaeological questions.

Table of contents

List of Figures

List of Tables

List of Abbreviations

aa	Amino acid
ABA	Acid Base Acid, a standard protocol for collagen extraction
AF	the ORAU lab code for bones treated with ABA, gelatinisation and ultra-filtration prior to combustion
AG	the ORAU lab code for bones treated with ABA and gelatinisation prior to combustion
AI	the ORAU lab code for bones treated with ABA, gelatinisation and ion exchange prior to combustion
Ala	Alanine
AMS	Accelerator Mass Spectrometer
Arg	Arginine
Asp	Aspartic acid
BP	Before Present (uncalibrated radiocarbon age in radiocarbon years before present, "present" defined as 1950)
Cal BP	Calibrated, calendaric years before present ("present" defined as 1950)
CH	Chalk Hill
CI	Campanian Ignimbrite
C:N	Molar ratio of carbon to nitrogen in a sample
CSRA	Compound specific radiocarbon analysis
$\delta^{13}C$ and d13C	Ratio of ^{13}C to ^{12}C in a sample relative to an international standard
$\delta^{15}N$ and $d^{15}N$	Ratio of ^{15}N to ^{14}N in a sample relative to an international standard
Da	Dalton (unit of mass measurement)
EA	Elemental Analyser
EA-IRMS	Elemental Analyser attached to IRMS
Gla	γ-carboxyglutamic acid
Gly	Glycine
Glu	Glutamic acid
His	Histidine
HPLC	High performance liquid chromatography

Hyp	4-hydroxyproline
IEC	Ion Exchange Chromatography
IPC	Ion Pair Chromatography
Ile	Isoleucine
IRMS	Isotope ratio mass spectrometry
Ka	kilo annum (1000 years)
kD	Kilodalton (1000 Daltons)
LC	Liquid chromatography
LC/IRMS	Liquid chromatography isotope ratio mass spectrometry
Leu	Leucine
LM	Lemon Mine
Lys	Lysine
Met	Methionine
MR	Mary Rose
MWCO	Molecular weight cut off
NCP	Non collagenous protein
ORAU	Oxford radiocarbon accelerator unit
OxCal	Program intended for radiocarbon calibration and analysis of archaeological and environmental chronological information
PDB	Pee Dee Belemnite, reference standard for $\delta^{13}C$ measurements
Phe	Phenylalanine
pMC	percent modern carbon
PNumber	lab code assigned to each dating attempt
Pro	Proline
RPC	Reversed-phase chromatography
Ser	Serine
St. Dev.	Standard deviation
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
VPDB	standard Vienna Pee Dee Belemnite standard, the common reference standard for $\delta^{13}C$ measurements
Ya	Years ago

Chapter 1: Introduction

1.1 Preface

Bones have been radiocarbon dated for the past six decades. Judging by archaeological expectation (e.g. based on dates of other material from the same stratigraphic layer), most bones yield accurate dates[1]. Some bones, however, are clearly difficult to date accurately; one reason for this is that the state of preservation of fossil "collagen" determines the accuracy and precision of the analysis. The reliability of bone ^{14}C dates has increased greatly since the introduction of pretreatment protocols designed to remove secondary carbon components, namely, carbonaceous material of post-depositional source. Nevertheless, extracting contaminant-free indigenous carbon, and in sufficient amounts for AMS analysis, can prove difficult in some cases. At the same time, some of the most important dates are required from heavily contaminated bones. Subsequently bone dates have been ranked among the most unreliable of all radiocarbon measurements (Bronk Ramsey, 2004a).

Radiocarbon dating bone is carried out on its major organic fraction – the most significant component of which is the protein collagen. In archaeological bone, however, the amount of collagen is often decreased, as a result of either microbial attack or chemical degradation (Collins et al., 2002). In addition, there is a greater risk of measuring organic matter that is actually mainly contamination acquired during burial and/or curation, such as glues, preservatives and fumigants added to 'protect' the bones (see section 1.2.6). Three categories of archaeological bone samples raise serious difficulties for radiocarbon dating:

[1] The question of what is a correct date is a philosophical one; unless the object to be radiocarbon dated is also historically dated, one can never be absolutely sure of the correctness of the date.

(1) Bones with a low concentration of collagen. As the original organic material diminishes, a larger sample needs to be processed, increasing the amount of exogenous material in the sample. In poorly preserved bone, it is desirable to extract highly specific fractions, significantly increasing the quantity of bone required. In addition, purifying indigenous material becomes harder as even the surviving protein might be contaminated.

(2) Bones which are severely contaminated. Any bone is liable to contain exogenous soluble and insoluble organic materials- ranging from rootlets and soil particles to humics (decay products of plants and animals found in the ground; see section 1.2.5) and other molecules mobilised to groundwater (and in addition museum glue and the like). It is particularly problematic to differentiate between the indigenous collagen and exogenous organic material of a different ^{14}C age being co-extracted.

(3) Older bones add a further complication, as the sensitivity to contamination by modern carbon increases exponentially with age. For bone older than 8 ka serious systematic errors arise from 1% of modern contamination. For such errors to be insignificant, contamination should be kept at a level less than 0.2-0.1%. Even as much as 0.5 pMC (percent modern carbon) corresponds to an age limit of about 44 ka (as opposed to ca. 50 ka, which is the radiocarbon limit of detection) (van Klinken and Hedges, 1992) (see also chapter 3).

Radiocarbon labs use criteria such as the level of remaining collagen (as indicated by nitrogen percentage of the whole bone) and the C:N ratio of the collagen fraction for

prescreening samples, in order to eliminate those which underwent substantial diagenetic processes and therefore their collagen carbon cannot be considered original. These bones will generally not be datable using normal pre-treatment procedures. If a bone specific molecule could be isolated, it would guarantee that the radiocarbon date is obtained from the original material. 4-hydroxyproline (Hyp) serves as an ideal candidate, consisting of about 10% of bone collagen but not found in significant amounts in other proteins.

The backbone of the chronology of all late Quaternary archaeology is radiocarbon dating (Bowman, 1995). However, geological and museum derived contamination becomes more and more important as the limit of the technique is approached, both because of the lower amount of residual collagen in older bones and because of the lower abundance of the ^{14}C, increasing their sensitivity to modern contamination. It is thought that perhaps 70% or more of the bone dates from the Middle and early Upper Palaeolithic are likely to be underestimates of the true age (Higham, 2011). The hypothesis of this thesis is that the isolation of Hyp will enable the accurate dating of poorly preserved bones and bones suspected of being contaminated, provided that sufficient quantity of Hyp can be extracted. In order to test this hypothesis some technical issues needed to be addressed first. These included finding the best conditions for the separation of Hyp and, at the same time, minimizing the addition of background carbon added in the process of isolation (see chapters 2 and 3).

1.2 Radiocarbon dating bones

1.2.1 Introduction: dating and archaeology

Establishing reliable chronologies is a prerequisite for constructing a picture of the human past. How are sequences within a site or culture and contemporaneity between geographically separate cultures determined? Before the advent of the radiocarbon dating technique (Libby et al., 1949), dating in archaeology was achieved by comparing the stratigraphic relationship between excavated objects within a site, and by linking cultures based on artifactual similarities, relating non-literate societies to the literate world (e.g. Egypt, Mesopotamia or China, which had written their own chronologies). Radiocarbon dating has brought a revolution to the discipline of archaeology, making it possible to assign absolute dates to archaeological objects (of organic source) and bones.

1.2.2 Radiocarbon dating[2]

Radiocarbon (^{14}C) dating is possibly the most important technique for studying the timing of events over the past 50,000 years. After an organism dies, the radioactive isotope ^{14}C in its body decays as a function of time, without being replenished by new ^{14}C from atmospheric CO_2. The amount of ^{14}C left in organic materials can therefore be used to determine the time that had passed since their death.

Carbon has three naturally occurring isotopes: ^{12}C and ^{13}C, which are stable, and ^{14}C, which is radioactive. These isotopes do not occur equally: carbon consists of 99% ^{12}C, 1% of ^{13}C, and only one part in million million of modern carbon is ^{14}C. ^{14}C is

[2] A lot of this part is based on Bowman, 1995 and Aitken, 1990, chapters 3 and 4, and the reader is referred to these books for more extensive background on radiocarbon dating.

continually being formed in the upper atmosphere, by interactions of neutrons produced by cosmic rays and nitrogen atoms; being unstable, ^{14}C atoms also continuously decays back to ^{14}N. If the production rate is constant, there's a dynamic equilibrium between formation and decay, resulting in a constant ^{14}C concentration in the atmosphere. After formation, ^{14}C quickly combines with oxygen to form carbon dioxide. This $^{14}CO_2$ mixes throughout the atmosphere, dissolves into the oceans, and enters all living organisms via photosynthesis. Since ^{14}C levels in living organisms are in equilibrium with the atmosphere, they should theoretically be constant as well.

When a plant or an organism dies, it ceases to assimilate new carbon, and its ^{14}C levels decay exponentially with time. Time elapsed since death could be calculated, therefore, by measuring the remaining ^{14}C levels. This notion was first formulated by Libby in 1946, and its successful application for dating carbonaceous materials followed shortly afterwards (Libby, 1946; Anderson et al., 1947; Arnold and Libby, 1949; Libby et al., 1949). Determination of radiocarbon age is calculated using the equation:

$$\text{Age} = -8033 * \ln(A/A_0)$$

Where 8033 is the average life expectancy of a ^{14}C atom as estimated by Libby and co-workers back in the 1940s (and since been corrected but is still used as a convention); A is the activity (or number of ^{14}C atoms) left after time t and A_0 is the initial activity (or number of ^{14}C atoms) at time zero. The relationship between ^{14}C activity (expressed here in decay per gram per min) and time can also be demonstrated graphically, where one half life ($T_{1/2}$, 5570 radiocarbon years) corresponds to the time in which half of the original ^{14}C atoms remain:

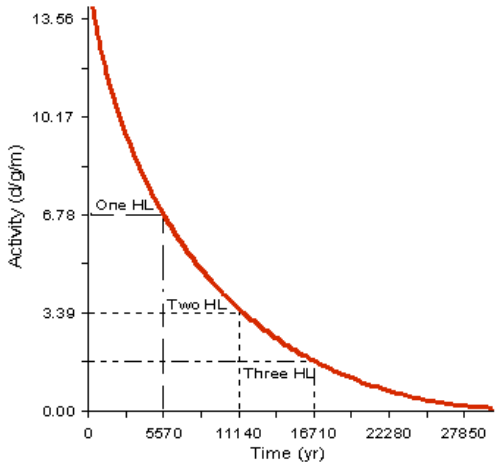


Figure 1.1. The exponential decay graph for radiocarbon atoms.
(taken from http://www.geo.arizona.edu/palynology/geos462/10radiometric.html)

The ^{14}C content of a sample could be either evaluated by measuring its radioactivity (β-counting), or measured directly – as a ratio between carbon 14 and carbon 12 (or carbon 13), using accelerator mass spectrometry (AMS). In the latter case a nuclear accelerator is applied, making the throughput of the samples much higher and the sample sizes required smaller by several orders of magnitude (as little as 10µg instead of 1g or more). The smaller sample sizes required for dating by AMS make it possible to date specific compounds, and even single molecules (see more below, '*The radiocarbon revolutions'*, and in section 1.4).

Assumptions

For the radiocarbon dating technique to work at all periods of times concerned, ^{14}C levels should be approximately uniform over time and space. This requires the biosphere and the atmosphere to be in equilibrium, globally. In addition, production rate needs to be constant, sizes of reservoirs should be invariable and there should be rapid mixing, exchange and transfer rate. All of the above assumptions are only true

to a certain extent, and the divergence from them must be accounted for in order to produce accurate dates.

Calibration

In their paper from 1949, Arnold and Libby published the first set of archaeological radiocarbon dates, showing they corresponded with their actual calendar (known) dates (known as "the curve of knowns", Arnold and Libby, 1949). Yet as early as the 1960's it was realized that the assumption of constant $^{14}CO_2$ levels in the atmosphere is not valid. What causes these atmospheric $^{14}CO_2$ variations? ^{14}C production rate in the stratosphere is affected by fluctuations in the earth's magnetic field strength (when the magnetic moment is high production of ^{14}C falls); variations in sunspot activity (causing short term variations: when sunspot activity increases ^{14}C production decreases); glaciation and inter-glaciation periods (in warm periods there is a decrease in solubility of CO_2 and release of aged carbon from ice); the fossil-fuel effect (burning of large quantities of fossil fuel from the beginning of the 20th century released 'dead' carbon that diluted the atmospheric ^{14}C concentrations and changed both the size and the isotopic composition of the atmospheric carbon reservoir); and finally- nuclear weapons testing (production of large quantities of ^{14}C in the 1950s and 1960s) (Bowman, 1995; Aitken, 1990).

The fact that atmospheric ^{14}C concentrations is not constant over time, and the revision of the ^{14}C half life (which is actually 5730 years and not 5568 as previously thought) means that raw radiocarbon dates can diverge from real calendar years by hundreds of years. This led to the need to apply a calibration curve, based on known age samples, in order to link a radiocarbon age with its solar age (Aitken, 1990).

Internationally-agreed *radiocarbon calibration curve* estimates are being built using information from tree rings, corals, lake sediments, ice cores and other sources[3]. New curves are being published approximately every 6 years, the latest one being IntCal09 (Reimer et al., 2009). In order to convert a radiocarbon age into calendar years, softwares such as OxCal can be employed (Bronk Ramsey, 2001; Bronk Ramsey, 2009), in which a raw radiocarbon date is inserted and, using the calibration curve and Bayesian statistics , a likelihood distribution of calendaric dates is produced. When an uncalibrated radiocarbon age is reported it is calculated by convention using the old Libby ^{14}C half-life, and is given the suffix BP, meaning "before present", where "present" is defined as 1950. A calibrate date is given the suffix Cal BP.

Reservoir effects

Global mixing rates between atmosphere and the terrestrial biosphere are rapid. But the situation is very different for deep oceans, where radiocarbon can be decaying for thousands of years before mixing with the surface layers. Mixing rates in deep oceans are so slow, that deep water organisms show radiocarbon age older by up to a few millennia than contemporaneous terrestrial organisms. The upwelling of deep water carrying ^{14}C-depleted compounds means that the surface water has an apparent radiocarbon age of about 400 years relative to the atmosphere. This radiocarbon age offset is called the marine reservoir effect. Other local environments that can affect significantly the radiocarbon age of the carbon assimilating organisms are hard-water and volcanic areas.

[3] A calibration curve for the past 12 ky BP was constructed based on absolutely dated tree-ring chronologies; before that and back to 50 ky BP, measurements of ^{14}C on U/Th dated or varved samples from lacustrine, marine and speleothem records as well as foraminifera shells from sediments of Cariaco Basin and corals, all corrected for corresponding local reservoir age, were used (Reimer et al., 2009).

Fractionation

Although all carbon isotopes are chemically interchangeable, in any biological process there will be a tendency for the lighter isotopes to be taken up more readily. In the process of photosynthesis, for example, the uptake of 'heavy' $^{14}CO_2$ is discriminated against compared to 'light' $^{12}CO_2$. As a result the actual ^{14}C content is lower by 3-4% in plants compared to the atmosphere. This is equivalent to making plants appear 240-320 years too old. The preferential uptake of one isotope, causing a variation in radiocarbon content between contemporaneous specimens (or even different parts of the same individual) is called fractionation, and needs to be taken into account in order to get correct dates.

Fractionation effects for ^{14}C are assumed to be twice as much as those for ^{13}C. In order to correct radiocarbon ages the sample's $^{13}C/^{12}C$ ratio first needs to be measured and compared to a standard (this is often VPDB – Vienna Pee Dee Belemnite). The difference from the standard is expressed as:

$$\delta^{13}\mathrm{C} = \left(\frac{\left(\frac{^{13}\mathrm{C}}{^{12}\mathrm{C}}\right)_{\mathrm{sample}}}{\left(\frac{^{13}\mathrm{C}}{^{12}\mathrm{C}}\right)_{\mathrm{standard}}} - 1 \right) \times 1000\ {}^{o}/_{oo}$$

The deviation of the $\delta^{13}C$ of the sample from a standard value of -25‰ (approximately the value of wood) is then calculated: the more the sample value is shifted from the -25‰, the larger the age correction needed, with 16 years added or subtracted for every 1‰ difference (Aitken, 1990). Table 1.1 illustrates the ranges of isotopic fractionation in various materials:

Sample material	$\delta^{13}C$ (‰)
Wood, charcoal, peat	-25 ± 3
Beet sugar	-25 ± 2
Bone collagen, amino acids	-20 ± 2
NBS Oxalic acid	-19 ± 1
Succulent plants	-17 ± 2
Freshwater plants	-16 ± 2
Arid zone grasses, sedges	-13 ± 2
Marine plants	-12 ± 2
Cane sugar	-11 ± 2
Maize, millet	-10 ± 2
Bone apatite	-10 ± 2
Atmospheric carbon dioxide	-9 ± 2
Non-marine carbonates	-5 ± 5
Marine carbonates	0 ± 3

Table 1.1. Typical $\delta^{13}C$ ranges (^{13}C to ^{12}C ratio compared to the VPDB standard) of different materials. Taken from Aitken, 1990.

The radiocarbon revolutions

As already noted, radiocarbon dating had had a truly revolutionary effect on the field of archaeology, providing a worldwide timescale for the last 40-50 ky. Direct dates can be assigned to artifacts and remains and chronometric relationships can be established without the need to rely on assumptions about cultural processes as before (Taylor, 1995). In retrospect, the introduction of radiocarbon to the discipline of archaeology was named by Colin Renfrew 'the first radiocarbon revolution', the 'second revolution' being the recognition in the early 1970's that radiocarbon dates need to be calibrated to get 'real' calendar dates. The revised radiocarbon dates revolutionised common understanding about the introduction of important innovations

in prehistoric Europe such as megalithic chamber tombs and European metallurgy (Renfrew, 1974).

The third radiocarbon revolution took place in the 1980s, with the development of the AMS. AMS requires sample sizes much smaller than the β-counting methods opening new and diverse areas of research. Study could now be carried out on new materials (e.g. single grains, fragments of parchment and linen) (Damon et al., 1989; Bonani et al., 1992). Moreover, more stringent chemistry could be applied to samples, ensuring environmental contamination is removed. Examples for the important contribution of AMS-based ^{14}C dates to the critical evaluation of archaeological data include dating early plant domestication in the Near East (e.g. Wendorf et al., 1984) and European Upper Paleolithic and Mesolithic materials (e.g. Hayden et al., 1993).

Perhaps not a revolution yet, but in the last two decades radiocarbon analysis of molecules specific to the material dated from 'problematic' or mixed samples has been carried out (termed "compound specific radiocarbon analysis", or CSRA; see section 1.3.1). CSRA became possible due to both advances in separation methods and instrumental development enabling the analysis of samples in the sub-milligram range. It has opened up new areas of application in environmental studies such as dating sediments (e.g. Eglinton et al., 1996) and aerosols (Currie et al., 1997), and in archaeology, such as dating lipids absorbed in archaeological potsherds (Stott et al., 2001).

1.2.3 Radiocarbon dating bones

Bones are widely found in archaeological and geological sites, being rather resistant to decay and easily recognised. Before the development of AMS dating, human skeletal remains were often dated by contextual association with something less valuable, such as charcoal. However, as human remains are often buried in sediment, their age-association with other materials is not certain. Any associated material that is found, could itself be stratigraphically mobile and so direct dating of bones, directly associated with the archaeological event, if a preferable way to determine archaeological chronology. This has been avoided in the past however due to the destruction of large amounts of bone for β-counting. However since the application of AMS, direct dating of bone can now be carried out with sampling a few grams of the bone at most.

Bones are generally classified as long bones (e.g. tibia, femur) short bones (e.g. ankle), flat bones (e.g. skull) and irregular bones (e.g. vertebrae). Bone tissue is either compact (sometimes referred to as dense or cortical) or cancellous (spongy). The less porous compact bone is preferred for ^{14}C dating since it is generally less altered by diagenesis (Tisnérat-Laborde et al., 2003). In living organisms, bone contains a number of specialized cells responsible for the production of bone tissue (osteoblasts), tissue maintenance (osteocytes) and destruction (osteoclasts). Blood is supplied to the bone tissue by minute canals, called "Haversian canals", supplied with blood vessels (Furneaux, 2004, Ch. VI, The composition and structure of bone, pp. 40-45). Bone tissue is a composite material, made of a bioapatite (mineral) fraction, which gives it rigidity and solid structure, and an organic (mainly collagen, but also other proteins and lipids) fraction, which provides strength and some degree of flexibility (Pollard

and Heron, 2008). Apart from collagen, which constitutes around 22% by weight of modern bone, bone also contains lipids, and proteins that are classified as 'non-collagenous-proteins' (NCPs). There are two hundred or more NCPs (Delmas et al., 1984), but together they generally comprise less than 10% of the total protein content. Of these, the most abundant is the calcium binding protein osteocalcin, making 10-20% of the NCPs (Hauschka, 1986).

Radiocarbon dating bones is routinely performed on the collagen fraction of the bone, whereby removing diagenetic organic contaminants such as humic and fulvic acids from decomposing soil organic matter (see section 1.2.5), rootlets etc., and inorganic contaminants such as groundwater carbonates and sedimentary carbon (Ambrose and Krigbaum, 2003). It is recognised that in order to obtain a reliable biogenic signal, for both stable isotope and radiocarbon, analysis should be conducted only on the endogenous material. In order to be able to do so, at least some understanding of the bone diagenesis processes is required.

1.2.4 Bone diagenesis

Bone contains information at many different levels (isotopic, molecular, biochemical, structural) (Hedges, 2002), and these can all be altered in buried bone, through a process termed diagenesis. Successful recovery of biomolecular information relies on understanding of the mechanisms of biomolecular deterioration. The history of the bone between death and burial (e.g. cooking, de-fleshing, exposure or deep burial) after burial (e.g. temperature, aridity, pH, groundwater consistency, etc.) and even after excavation (application of conservation materials such as resin) may all change its nature. These changes may include:

- uptake of cations and organics from the ground, exchange of some ions
- breakdown of collagen and other proteins to polypeptides and amino acids, the more soluble component leaching out
- alteration and leaching of mineral matrix, infilling with mineral deposits
- contaminant molecules such as amino acids, amino acids bound to humidified materials, or non amino compounds such as humic acids and polysaccharides entering the bone, being adsorbed onto hydroxyapatite surfaces or reacting with indigenous polypeptides e.g. through condensation reaction between sugar residues and amino groups. Condensations and polymerisations can also take place with endogenous molecules ('auto-humification')
- bacterial or fungal attack could produce further chemical exchange

(Hedges and Law, 1989; van Klinken, 1999; Hedges, 2002)

The degree of change, the speed of change, and which of the processes will be dominant are all determined by conditions such as climate and soil content but also by the micro-environment, and so are very much unpredictable.

As already mentioned, the isotopic analysis of bone (^{13}C, ^{15}N and radiocarbon) is normally carried out on the collagen fraction. Collagen abundance, and the fact that it is a chemically defined fraction that is easily isolated, have made it an ideal material for biomolecular analysis (Hedges and Law, 1989). But it could also potentially be continuously in exchange with its environment, and so diagenetically altered collagen should be avoided if a reliable biogenic signal is to be extracted.

1.2.5 Collagen diagenesis

The collagens are a family of fibrous structural proteins, distributed throughout the connective tissues of the body. The basic collagen I molecule, the most abundant collagen in mammals, is approximately 300kDa in size, and consists of two α1 polypeptide chains and one α2 chain. The following sequence of three amino acids is repeated extensively in collagens:

- Gly-X-Y-Gly-X-Y-

where Gly is the amino acid glycine and X and Y are one of the other 17 amino acids found in collagen, but often proline (Pro) and hydroxyproline (Hyp). Most kinds of human collagens constitute (by number of residues), on average, of 32-34% glycine, 7-13% proline, and 9-13% hydroxyproline; the other 13 amino acids make up less than half of the total residues (Pollard and Heron, 2008, ch8, Amino acid stereochemistry and the first Americans). Figure 1.2 shows the distribution of the different amino acids in human type I collagen, and the percentage of carbon contributed by each of them to the total carbon content in collagen.

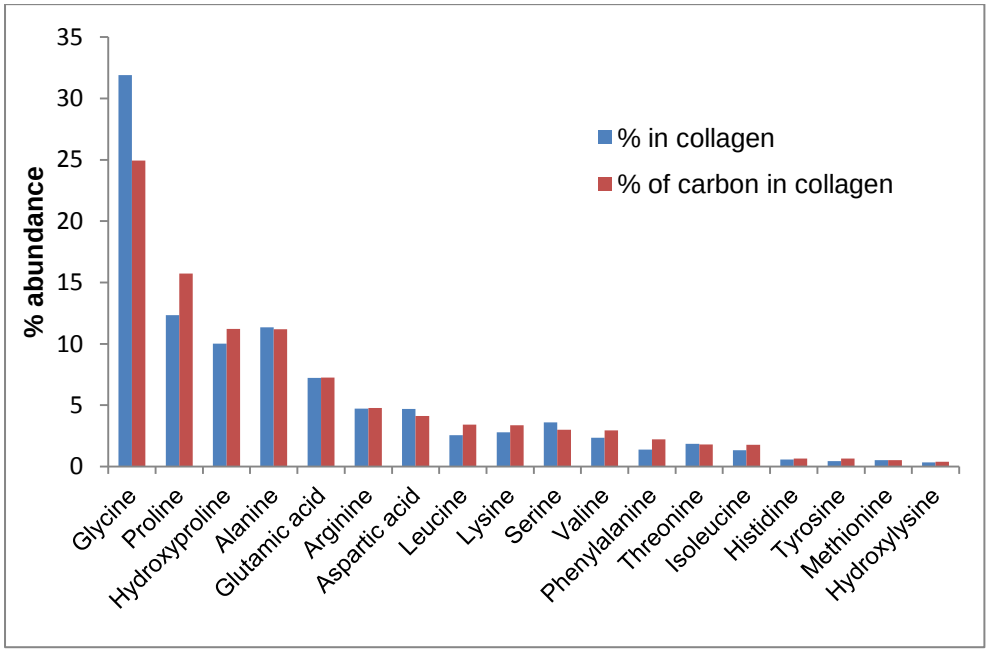


Figure 1.2. Amino acid composition of human bone collagen, in percentage of residues (blue), **and the percentage of carbon each of them makes out of the total collagen carbon content** (red)**.** Adapted from Eastoe, 1955. Hydroxyproline accounts for 10% of collagen residues, and around 11% of collagen carbon comes from hydroxyproline.

Each amino acid in the amino acid chain is twisted to the left, such that a complete turn is achieved every three residues, aligning the glycines above each other. Three chains are twisted together in a right-hand helix to make a rope-like structure (figure 1.3). The tertiary structure of the triple helix is held by a "ladder" of hydrogen bonds between the glycines of one chain and Xaa in an adjacent chain (Bella and Berman, 1996), and through stereoelectronic effect conferred by the hydroxyprolines (Holmgren et al., 1998). Covalent bonds between hydroxylysines in the Yaa position of α chains are thought to form inter- and intra-molecular cross-links (Orgel et al., 2000).

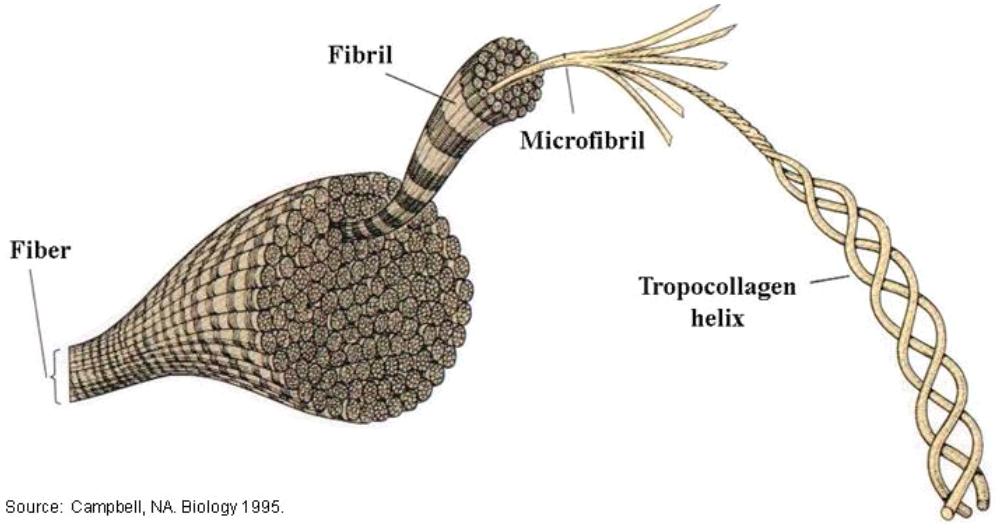


Figure 1.3. Structure of collagen fibers.

The fact that collagen can survive in bones after tens of thousands of years is surprising, given its enormous surface area. Collagen is thought to deteriorate by two mechanisms. One is chemical hydrolysis, the rate of which depends on time, temperature and pH. The other mechanism, and probably the most common one, is biodegradation, in which microorganisms and fungi capable of degrading collagen attack the bone (Collins et al., 2002). It has been suggested that the presence of the mineral and the close packaging of the collagen fibrils are the main factors helping to keep the collagen intact (Kronick and Cooke, 1996). The key factor influencing collagen survival is the thermal history of the sample, elevated temperatures leading to its loss (Collins et al., 2002). Apart from collagen loss, collagen is liable to be diagenetically altered by reacting with humic substances.

Humic substances are the decay products of the biota in soil and organic waters. They are formed through aerobic and anaerobic decomposition of plant and animal remains, as well as secondary microbial synthesis (Stevenson, 1994). This process, termed

humification, produces a dark coloured, acidic, predominantly aromatic, hydrophilic humic material, ranging from a few hundred to several thousand Daltons in mass. The diversity among humic substances (arising from differences in age, genesis and origin) makes their classification difficult. It is customary to divide them into three operationally defined classes, broadly based on their solubility characteristics: fulvic acids (soluble in weak alkali), humic acids (also soluble in alkali but precipitate on re-acidification) and humin (not soluble in weak acid or base) (van Klinken and Hedges, 1995). It is unknown whether influx of humics from the soil or *in situ* humification of buried bone collagen is the main sources for humics in archaeological bones. Their residence time in soil varies from 800 to >2000 years (Stevenson, 1994) and so they could be older, younger or the same age as the bone itself. The initial uptake mechanism of humic acids from the soil to the bone is thought to involve fast complexation of the collagen and humic acids through hydrogen bonds. Later on condensation reactions (Maillard reactions) will take place, in which a sugar and a free amino group react, and after rearrangements and polymerisations will result in melanoidins, polyphenolic substances (van Klinken and Hedges, 1995). Figure 1.4 depicts the chemical structure of a typical humic acid molecule:

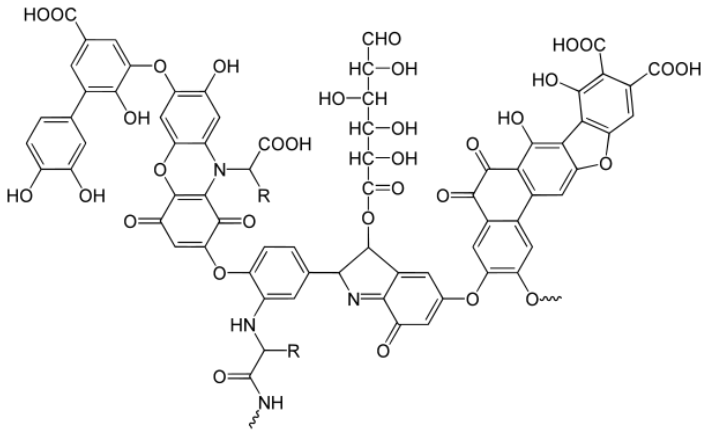


Figure 1.4 Example of a humic acid molecule (Stevenson, 1994).

In theory, only exchange with exogenous carbonaceous material would affect the date when analyzing diagenetically changed bone. However, when dating degraded or contaminated collagen, even though not necessarily compromised isotopically, the sample size will have to be increased, inevitably increasing also background contamination, either bone or lab-derived (van Klinken, 1999).

1.2.6 Conservation and curation derived contamination

Surprisingly, radiocarbon dating bones with conservation related 'contamination' could sometimes be as complicated as dating naturally contaminated ones. In many cases a record of the materials applied is not kept, and so their removal demands some guess work. Bone 'consolidants' are applied to the bone by either brushing externally or by immersing the whole bone in a solution. The consolidant can sometimes invade the bone cortex through the Haversian canals (see section 1.2.3) and form a tight coating against the hydroxyapatite. Bone consolidants and adhesives could either be radiocarbon 'dead' petroleum-based chemicals (e.g. paraffin), or have a modern ^{14}C content (e.g. shellac), and are difficult or impossible to remove with the standard chemical sample pretreatment techniques used in many radiocarbon laboratories (Bruhn et al., 2001). Some commonly used consolidants are listed in table 1.2.

Type	Common examples
Solvent soluble adhesives	Paraloid B-72 (ethyl methacrylate co-polymer, formerly called Acryloid) Butvar B-76 (polyvinyl butyral, or PVB) Butvar B-98 (polyvinyl butyral or PVB) McGean B-15 (polyvinyl acetate or PVAC, formerly called Vinac B-15) "White glue" dispersions and emulsions (e.g. Elmers, Rhoplex, Lascaux)
Reaction adhesives	Various brands of epoxies (e.g. Devcon, Epo-Tek) Various brands of cyanoacrylates (e.g. Aron Alpha, Paleo-bond)
Synthetic Adhesives	Duco cement Elmer's glue-all Five minute epoxy Cellulose Nitrate polymers (e.g. Glyptal) Model aeroplane glue Ambroid Natural organic materials
Natural waxes	Shellac Gum arabic Animal protein glues

Table 1.2. Common types of bone adhesives applied during conservation and curation. Adhesives are used for joining, consolidating, coating and gap filling. Consolidants are generally dilute forms of adhesives that penetrate the bone and adhere internally. Many of the synthetic adhesives and natural waxes are not recommended for use nowadays.
(Source: http://preparation.paleo.amnh.org/47/adhesives-and-consolidants of the American Museum of Natural History- PaleoPortal Fossil Preparation).

1.2.7 Assessment of collagen quality

The quality of collagen is central to accuracy of the ^{14}C analysis of bone. But how is contaminated or diagenetically altered collagen detected? Several criteria have been suggested in the past for prescreening samples, in order to eliminate those which underwent substantial diagenetic processes or are severely contaminated and their collagen therefore cannot be considered endogenous: the level of remaining collagen (as expressed by % weight of collagen from total bone); the C:N ratio (collagen value is 3.2, a value between 2.9-3.6 is in the acceptable range; higher values indicate high proportion of exogenous carbon) (DeNiro, 1985; Ambrose, 1990); amino acid composition to determine to what extent the composition is still characteristic of

collagen (DeNiro and Weiner, 1988a) (it was specifically suggested by Hare that Gly/Glu ratios would be used as a non-collagenous composition index) (Hare, 1980); degree of racemisation; porosity of the bone; histological alterations; stable isotope ratio; IR spectrum (DeNiro and Weiner, 1988a); the amino acid composition of collagenase digested collagen – collagenous composition is expected from most bones in which at least 5% of the original protein appears to have survived; and the %N of the whole sample as an estimation for the amount of the surviving collagen (DeNiro and Weiner, 1988b; Hedges and Law, 1989; van Klinken and Hedges, 1992). Recently, a new non-destructive method for estimating the collagen survival was proposed, using micro-computed tomography (Tripp and Hedges, 2009). At the Oxford Radiocarbon Accelerator Unit (ORAU), all samples that yield less than 1% weight collagen after pre-treatment and those with a collagen C:N ratio that does not fall in the range of 2.9-3.5 are rejected prior to AMS dating. The nitrogen content of the whole bone is used as a pre-screening tool for bones thought to have poor preservation, as it was shown that bones with less than 0.76% N are likely to yield less than 1% collagen (Brock et al., 2010b).

Interestingly, even in microbially deteriorated bone, ‘islands’ of unaltered collagen may persist (Collins et al., 2002; Hedges, 2002). This suggests that pre-screening the whole bone for collagen integrity might lead to rejection of a bone with a potential of being accurately dated. In contrast, it must be noted that none of the above mentioned criteria is sensitive enough to identify the presence of minute amounts of contamination, that could have a great effect in on the date (for example, addition of 0.5% carbon would only shift the C:N ratio from 3.2 to 3.35, still well in the

acceptable range). However, they can be used to eliminate the worst cases and to detect problematic bones, especially when a combination of these parameters is employed (van Klinken, 1999).

1.3 Collagen pretreatment methods

There are two approaches to sample pretreatment that have been adopted in the literature. The first is chemical cleanup, aiming to remove potential contaminants. The other approach is isolation of the building blocks of the organic material to be dated, thereby avoiding the potential contaminant altogether (Hedges and van Klinken, 1992). The chemical cleanup is the most common approach for ^{14}C dating. In the case of bone it is assumed that there is an intact original collagen "core" that can be purified by applying a sequence of chemical steps, taking advantage of the fact that collagen is insoluble in dilute acid. Since the employment of AMS, the ability to chemically pre-treat samples has improved, as targets in the mg range rather than gram are required, so the pretreatment can be more rigorous and fractions can be selected from samples that are not homogenous.

Standard sample preparation protocols for dating bones generally follow an acid-base-acid (ABA) protocol, based on the Longin method (Longin, 1971), involving a decalcification step to mobilize hydroxyapatite, followed by a dilute NaOH wash to remove humic and fulvic acids, and then re-acidification. After washing, the extracted collagen is usually gelatinised at pH 3 at a temperature of 60-70ºC so it can be separated from other insoluble materials (Hedges and Law, 1989). Some laboratories, including ORAU, add an ultra-filtration step to remove low molecular weight material, retaining >30,000 Da peptides for dating (Brown et al., 1988; Bronk

Ramsey, 2004a). Ultra-filtration has been shown to yield older ^{14}C dates, because it selects high molecular weight proteins which are more likely to be from the original collagen present in the bone, and remove contaminants likely to be both low molecular weight and younger (for example, Brown et al., 1988; Jacobi et al., 2006; Jacobi and Higham, 2008). This step will not remove high molecular weight humic acids, or humics cross linked to partially degraded collagen. Ion exchanging the gelatin using AGMP-50 has also produced older dates (Hedges and Law, 1989).

If a bone is known to be treated with a specific conservation material, a suitable organic solvent can be used to remove it before the collagen is extracted. At ORAU, if it is suspected that a bone was treated (either from its appearance or from its unusual C:N ratio for example), a suit of organic solvents (acetone, methanol, chloroform) is applied. However, the organic solvents themselves could contribute carbon (which has no radiocarbon activity), and have to be removed completely. At the radiocarbon laboratory in Leibniz a computer-controlled soxhlet extraction is used, in which hot extraction with tetrahydrofurane, trichlormethane, petroleumether, acetone and methanol are used prior to the normal collagen extraction procedure (Bruhn et al., 2001).

However, even when the greatest care is taken to isolate a contaminant-free fraction or to eliminate contaminated samples, practice has shown that the cleanup methods may not be effective when the collagen has undergone severe contamination or substantial diagenesis, involving covalent bonding with contaminants. For example, the anatomically modern human from the site of Kostenki in Russia (further discussed

in section 1.4.4 and in chapter 4) is estimated to be Upper Palaeolithic, but yielded direct radiocarbon dates of between ~3.7 and 13.6 ka BP (Sinitsyn, 2004; Hoffecker, 2011), probably due to residual conservation material. Even if chemical clean-up methods may occasionally yield accurate dates, there are no known chemical criteria for predicting when they will. For poorly preserved collagen bones (with collagen content <2%), for old bones (>20ky) and for other bones suspected of contamination, a better approach would be to isolate a specific compound, which is better defined at the molecular level, and whose structure unambiguously represents its archaeological source.

1.4 Compound specific radiocarbon dating bone using single amino acids

1.4.1 Compound specific radiocarbon dating

Dating a sample of organic material with heterogeneous composition, or a sample that is made of complex mixture of organic and inorganic carbon compounds, could lead to a misleading interpretation (Ingalls et al., 2004). Examples include soils, sediments, fresh and marine waters, atmospheric particulates, and bone and shell fossils (e.g. Douka et al., 2010a). This problem could be resolved by focusing on a biomarker, specific to one compound, and analyzing its radiocarbon content. The dating of single classes of molecules has been named Compound Specific Radiocarbon Analysis (CSRA), and is a field that grew in parallel with the development of micro-scale techniques, enabling measurements of samples smaller than 0.5mg carbon (Pearson et al., 1998). CSRA was applied in geoscience studies investigating the heterogeneity of carbon sources in environmental samples (e.g. by isolating and dating sedimentary lipids; Eglinton et al., 1996; Eglinton et al., 1997), and also in other disciplines such

as biology and archaeology (Santos et al., 2007). In archaeology, CSRA was employed to directly date time of use of potsherds by isolating and radiocarbon dating lipid residues absorbed into pots walls (Stott et al., 2001; Stott et al., 2003; Berstan et al., 2008), to date cellulose by isolating the constituent monosaccharides (Hodgins et al., 2001; Nemec et al., 2010) and to radiocarbon date archaeological bone (see below). The two major challenges of CSRA are the isolation of sufficient material, which requires application of rather sophisticated purification methods that are time consuming, involving multiple chromatography separation steps (preparative capillary gas chromatography or preparative HPLC), and the risk of introducing contamination in the process (e.g. via the mobile phase, derivatisation reagent, and stationary phase bleed). The 'blank' contribution associated with purification procedures must be carefully evaluated before radiocarbon dates can be interpreted (see Shah and Pearson, 2007).

1.4.2 Compound specific radiocarbon dating bone

In an article from 1969, Ho and co-workers radiocarbon dated bones with severe contamination using only the amino acid fraction isolated via liquid-chromatography (Ho et al., 1969). The idea was that the amino acid fraction, being well defined chemically, would yield a more reliable date in this case than the bulk collagen. Dating the total amino acids fraction hydrolyzed from the extracted collagen was also attempted several times later on (Bada et al., 1984; Gillespie et al., 1984; Hedges et al., 1989), where additional purification steps (using XAD resin, charcoal and ion exchange column) were sometimes applied (e.g. Gillespie et al., 1986; Hedges et al., 1989). However this was abandoned as it was not clear it offered advantage over the standard method that had been adopted by then (namely ABA and gelatinisation,

discussed in detail in section 1.2.7). Moreover, it was shown that condensation products of the amino acids and impurities such as carbohydrates could be formed during hydrolysis (Hedges and van Klinken, 1992).

Dating individual amino acids isolated from the collagen fraction has the potential to improve accuracy beyond that of dating the total 'collagen' fraction, in cases were contamination is known to have occurred. However, it was postulated that amino acids found in bone may not all be endogenous, some originating from microorganisms and humic acids in the bone's depositional environment. When different individual amino acids were separated from a non-collagenous Mammoth bone (using XAD2 RP resin to adsorb humics and AG50X8 cation exchange resin to separate Pro from Hyp), they yielded dates that were up to 9,000 years too young, which was interpreted as evidence that all or most of the original collagen was replaced with more recent organic matter (Stafford Jr. et al., 1991) (an alternative explanation is possible, though, that the procedure itself is responsible for the modern contamination). Hedges and van Klinken posed the paradox in their paper from 1992 (Hedges and van Klinken, 1992): when the fraction dated is most characteristic of bone (e.g. collagen), its purity cannot be guaranteed; but when the purity can be guaranteed (single amino acids separated by HPLC), it is not uniquely characteristic of bone!

As a way to circumvent this, it was suggested to radiocarbon date peptides of collagen rather than single amino acid. van Klinken et al. (van Klinken and Hedges, 1992; van Klinken et al., 1994; van Klinken and Hedges, 1995) used HPLC to isolate the tri-peptides (Gly-Pro-Hyp) produced by the digestion of collagen by collagenase,

following DeNiro and Weiner, who used collagenase to prepare archaeological bone samples for stable isotope analysis (DeNiro and Weiner, 1988b). The tri-peptides are only characteristic to bone, and are produced in a very specific enzymatic reaction. As a way of testing the method humic acids were artificially added to bones. It had been shown that the tri-peptide method (and also the ninhydrin method, see below) succeeded in removing the contaminant, while standard pretreatment procedures failed (van Klinken and Hedges, 1995). Although this method seems very appealing, a major drawback was its low yield (it should be noted that this was developed before the advance of micro-scale AMS). Not only that Gly-Pro-Hyp consists only 10% of the collagen to begin with, but also, yields strongly depended on the state of the collagen (probably because the collagenase is very sensitive to steric conditions) (ibid).

A different approach to overcome the problem of securing the origin of the amino acids was taken by Nelson in 1991, using a ninhydrin preparation method (Nelson, 1991). The method chemically extracts the carboxylic carbon of the peptide bond, by reaction with ninhydrin, thereby ensuring that the carbon dated is coming from the collagen backbone and not free amino acids in the bone leached in from external sources. This method was not commonly adopted though, because of its complexity and the large variations in CO_2 yields obtained (van Klinken and Hedges, 1995).

Other molecules that were used as a bone specific biomarker are the non-collagenous bone proteins (NCPs; e.g. Masters, 1987; Tuross et al., 1989), in particular osteocalcin (Ajie et al., 1991). The possibility of using γ-carboxyglutamic acid (Gla) (an amino acid found almost uniquely in osteocalcin) as a bone specific biomarker was

investigated by Burkey and co-workers in 1998. Dating Gla was attempted based on the suggestion that the non collagenous proteins, osteocalcin among them, are preferentially retained and more resistant to diagenetic contamination than collagen (Hare, 1980; Ajie et al., 1990). However, it was found that Gla and collagen concentrations decreased in concordance, and that Gla yielded erroneous radiocarbon dates (Burky et al., 1998).

The amino acid hydroxyproline (figure 1.5) accounts for about 10% of collagen (figure 1.2), but is not found in significant quantities elsewhere in nature, making it an ultimate candidate for bone specific radiocarbon dating. Most of the research on compound specific radiocarbon dating of bones had concentrated on the amino acid hydroxyproline.

1.4.3 Single amino acid dating using hydroxyproline

The isolation and dating of Hyp was first suggested by Ho and co-workers in 1969 as a way of ensuring that all dated carbon is derived from bone collagen in contaminated bones (Ho et al., 1969). Hydroxyproline is a proline residue with an extra hydroxyl group on at the fourth carbon position (figure 1.5). About half of the proline residues in human collagen α1 chain are hydroxylated in position 4. Infrequently, proline residues in collagen are also hydroxylated in position 3. Proline hydroxylation takes place after translation of the protein sequence in the nucleus of osteoblasts and is therefore known as a post-translational modification (Gorres and Raines, 2010). The attachment of the hydroxyl occurs by the action of an enzyme, prolyl-4-hydroxyase, which requires molecular oxygen, 2-oxoglutarate, ascorbic acid (vitamin C) and Fe^{2+} as co-factors in the reaction (Prockop et al., 1976). The peptidyl proline needs to be in

the context -Gly-Xaa-Pro- (Hutton et al., 1967; Vanderrest and Garrone, 1991). Hydroxyproline is mostly degraded to glutamate (Meister, 1965). The fact that the metabolism of hyp requires a specific enzyme, co-factors and a specific context is of significance, because it indicates that the hydroxylation cannot happen spontaneously in diagenetic bone. Hyp found in archaeological bone, therefore, is likely to originate from endogenous collagen.

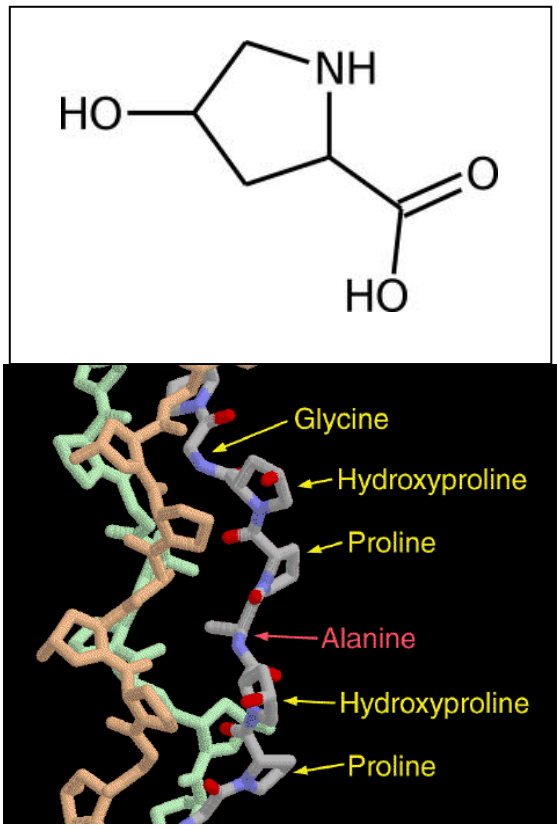


Figure 1.5. Hydroxyproline chemical structure (top); the collagen backbone model (bottom). Taken from http://www.rcsb.org/pdb/101/motm.do?momID=4

Radiocarbon dating single amino acids from hydrolyzed collagen requires large scale separation. Liquid chromatography (LC) was used in some cases (e.g. Gillespie et al., 1984; Stafford Jr. et al., 1991), but high performance liquid chromatography (HPLC) is preferred because the on-line detection enables to monitor the quality of the

separation, the separation is better, retention time is more reproducible (and therefore peaks can be directly identified) and the extraction time is shorter (e.g. Wand, 1981; Gillespie et al., 1986; van Klinken and Mook, 1990; O'Connell and Hedges, 2001; Tripp et al., 2006; McCullagh, 2007). Several different chromatographic techniques are reported in the literature for collecting amino acids. The success of an HPLC separation (developed for accurate ^{13}C analysis for example), is no guarantee of reliability for radiocarbon dating, because of the increased sensitivity of the latter to contaminating ^{14}C (originating for example from derivatisation reagents or organics from mobile phase).

The 'natural' choice for separating amino acids is ion exchange (IE) chromatography, and indeed cation exchange chromatography was applied when Hyp was first isolated from fossil bone in 1981 (Wand, 1981) and later on isolated and AMS dated by Gillespie and co-workers in 1984 (Gillespie et al., 1984) and Stafford and co-workers (Stafford Jr. et al., 1982; Stafford Jr. et al., 1987; Stafford Jr. et al., 1991) (for an explanation on the principals of the different chromatographic methods see appendix A). Results showed that the method provided dates that, at the time, were as accurate as conventional bulk collagen dates if not better (Gillespie et al., 1984; Gillespie et al., 1986) see also figure 1.6 and table 1.3). However, it has since been shown that IE produces a significant in-peak fractionation, which could affect the radiocarbon date if not all the peak is collected (van Klinken and Mook, 1990). In addition, it has proven difficult to obtain baseline separation of all the amino acids, which is desirable. Moreover, if strong acid or base is used as a mobile phase, they will need to be removed before combustion, and could also increase HPLC column bleed, affecting the radiocarbon date (McCullagh et al., 2010, and chapter 2).

Reverse phase (RP) seemed like a better choice, and was attempted by the Oxford lab (O'Connell and Hedges, 2001). RP proved to be a suitable approach for stable amino acid analysis of single amino acids (although, again, only ten of the amino acids of the 20 that comprise collagen could be separated), but not for AMS dating because the large amounts of carbon added as protecting groups for the amino acids and not all mobile phase could be effectively removed, causing erroneous dates (O'Connell and Hedges, 2001).

The Oxford lab went on to adopt a two-step separation method, where a simple RP method was used to separate the non-polar amino acids, using water as mobile phase; the un-retained peak, comprising the more polar amino acids, was subsequently separated by Ion-Pair chromatography. This method does not require derivatisation of the amino acids, avoiding the possibility of contamination from this source. A few radiocarbon measurements from amino acids from the same historical bone (the Mary Rose pig bone) collected by this method were produced, and the resulting dates were compared to each other and to the historical known-age bulk date, in order to test the method. It was found that the non-polar amino acids (mostly the essential ones), separated on the RP column, gave accurate dates, indistinguishable from the bulk dates. However, the polar (non essential) amino acids, that were further separated on the IP column, were all too old up to several thousand years, probably as a result of remaining IP reagent (Tripp et al., 2006; McCullagh, 2007).

Subsequently, a new method was developed, combining both RP and IE retention mechanisms. Separation of 15 underivatized amino acids from collagen using an acidic aqueous mobile phase and mixed mode chromatography was reported by

McCullagh and co-workers in 2006 (McCullagh et al., 2006). The method used a reversed-phase column (Primesep A) having an additional charge on the C_{12} stationary phase alkyl chain via a carboxyl group. The mechanism of retention was therefore a combination of hydrophobic and ion exchange interactions. Embedded carboxyl groups are negatively charged at all working pHs, but the charge state of the amino acids in the mobile phase solution is influenced by the pH of the mobile phase. The retention time of the different amino acids is affected by applying a gradient with decreasing pH. The method was tested, again, by comparing single amino acid dates (including Hyp) produced from the Mary Rose pig bone to each other and to the known date. All the amino acids dates were statistically indistinguishable from each other and from the bulk date (albeit being slightly too old). The method has advantages over previous separation methods in being the simplest and the most precise, and providing the largest number of baseline separated amino acids (McCullagh et al., 2006; McCullagh, 2007). In addition, the fact that the mixed mode HPLC column used is silica based rather than resin based and that the mobile phase chosen is free of organic material both potentially minimize the background carbon added in the process.

With notable exceptions (see table 1.3), molecular level dating methods in general, and Hyp dating specifically, are rare in the literature despite the obvious theoretical benefits over conventional techniques. One of the main reasons for this has been the complexity they bring to the sample preparation process, adding many more steps to the normal pretreatment protocol, and therefore making them unfavorable as routinely used methods. In addition, any method dating a fraction of the collagen means larger quantities of bone are needed. In the case of Hyp, at least 10 times as much bone is

required. Moreover, it has been documented that during diagenesis the amino acid composition of collagen changes. In 'non-collagenous' bones (where the amino acid composition is not collagen-like any more), there is an increase in the concentrations of serine, aspartic and glutamic acid and decreases in arginine, hydroxyproline and glycine levels. Below 5% protein content a total loss of hydroxyproline and often proline had been observed (Bada et al., 1984; DeNiro and Weiner, 1988a; Stafford Jr. et al., 1991). To allow for Hyp loss, in some cases the amount of collagen needed to be extracted will be an order of magnitude bigger, and in a number of cases, it is expected that no Hyp will be left. The requirement for big samples in the range of tens of milligrams or a few grams (as opposed to a few milligrams for normal AMS dating) is of course a limitation of the method as (1) not always such big samples will be available, and (2) the bigger the sample that needs to be handled, the larger the risk of contamination. The isolation of Hyp should override the problem of environmental contamination, being a bone specific substance, but not the increased risk of lab derived contamination. This is further complicated when old bone is dated, as old samples will be more sensitive to modern ^{14}C contamination, and again, the whole process of Hyp extraction could be adding background carbon to the measurement. In none of the few reported Hyp dating methods such sample blanks were stated, making it difficult to judge their suitability for radiocarbon dating. Table 1.3 depicts the different fractions of bone used in the past for radiocarbon dating.

Fraction isolated	Reference	Isolation method	Bones treated	Results
High MW collagen peptides				
>30kDa fraction of collagen	(Brown et al., 1988)	Ultrafilters	Wolf <200 BP, human 5,000 BP and bison 10,000 BP	Older dates for the >30kDa fraction of collagen suggesting removal of low MW contamination
>30kDa fraction of collagen	(Bronk Ramsey, 2004a; Brock et al., 2007) and many others; e.g. (Jacobi and Higham, 2008)	Ultrafilters (cleaned from glycerol coating)		Considerable advance in removing environmental contamination; best method for older bones
NCPs				
NCPs	(Masters, 1987)	Hydrolysis of whole bone, ion exchange column (Dowex 5OW-X8)	a series of bones exhibiting different amounts of residual collagen	dates consistent with ages estimated from archaeological information.
IgG, osteocalcin, and albumin	(Tuross et al., 1989)			-
osteocalcin	(Ajie et al., 1990; Ajie et al., 1991)	Gel filtration of acid soluble fraction -> DEAE ion exchange column	a series of bones exhibiting different amounts of residual collagen	Collagen and osteocalcin produce same dates for well preserved bones; for bones with very little collagen left only the osteocalcin samples gave the correct dates
γ-carboxyglutamic acid (Gla) from osteocalcin	(Burky et al., 1998)	Ion exchange (Dowex-50w)		Discordant ^{14}C values on Gla and collagen
Peptide bonded carbon				
	(Nelson, 1991)	Reaction of free aa with 2,2-dihy- droxy-1,3-indandione (ninhydrin)	bison (>10ky) and mammoth (>20ky) (from northern Canada) and cave bear from Yugoslavia (>30ky)	Dates entirely consistent with independent measurement; background is minimal as measured on a very old bone
	(van Klinken and Hedges, 1995)	(as in Nelson 1991)	bones deliberately contaminated with humics	contamination successfully removed (conventional chemistry failed)
Tripeptides				
Gly-Pro-Hyp/ Gly-Pro-Ala	(van Klinken and Hedges, 1992; van Klinken et al., 1994; van Klinken and Hedges, 1995)	Collagenase digestion; RP HPLC separation	A suit of bones with ages ranging between 1300y to background; bones deliberately contaminated with humics	The dates for Gly-Pro-Hyp and Gly-Pro-Ala are the most reliable, in agreement with archaeological expectation; contamination successfully removed (conventional chemistry failed)
Collagen amino acid mixture				
Hydrolyzed collagen	(Ho et al., 1969)	Ion exchange column (Dowex50-wx8)	Petroleum-contaminated from Rancho La Brea tar pits of Los Angeles, California (12.5ky-21.5ky BP)	Dates in accord with dates for wood from the same stratigraphic levels
Hydrolyzed collagen	(Bada et al., 1984)	Ion exchange column (Dowex50-wx8)	Californian Palaeoindian skeletons (Holocene)	All dates Holocene (as opposed to what initially thought based on aa racemisation)

Hydrolyzed collagen	(Gillespie et al., 1984)	Charcoal (for removal of humic acids); Ion exchange column (AG50X8)	A woolly rhinoceros (>20ky); Red deer bone (ca. 7ky BP)	Dates at least as good as produced by conventional methods
Ion exchanged gelatine	(Hedges et al., 1989)	Apply gelatin to ion exchange column (AGMP-50)-> hydrolyze -> apply free aa to ion exchange column (50W-X8 resin)		Uncertainty as to whether this is an improvement over standard pretreatment; condensation products of the amino acids and impurities such as carbohydrates could be formed during hydrolysis
Collagen individual amino acids				
Hyp	(Wand, 1981)	deamination with nitrous acid, HPLC Partisil 1OSCX cation exchanger		-
Hyp, Pro Asp, Glu, Thr, Gly, Ala	(Stafford Jr. et al., 1982; Stafford Jr. et al., 1987; Stafford Jr. et al., 1988; Stafford Jr. et al., 1991)	Reverse phase column to adsorb humates (XAD2) -> deamination (aqua regia) -> cation exchange chromatography (AG50X8) to separate Pro from Hyp	Modern and fossil bone	-collagenous composition bones dated correctly (single aa dates same as XAD2 treated gelatin); non-collagemous bone dated 9,000 years too young; background carbon not measured
Hyp, Pro	(Gillespie et al., 1984; Gillespie et al., 1986)	Charcoal (for removal of humic acids); Ion exchange column (AG50X8); deamination (Nitrous acid); cation exchange HPLC	A woolly rhinoceros (>20ky) an Anglo-Saxon burial (ca. 1,200y BP); Red deer bone (ca. 7ky BP)	Single amino acid dates older than either collagen or total amino acid dates (see figure 1.6)
Gly, Hyp, Glu, Thr, Ala	(van Klinken and Mook, 1990)	HPLC (IE; Rogel-S, Alltec h) -> desalting with ion exchange resin (AG 11 A8)		-
Phe, Leu+Ile, Val, Gly	(Tripp et al., 2006)	Two step HPLC separation: waters symmetry C18 column (RP) -> Supelco Discovery HS F5 (IP)	Mary Rose Pig bone (311 BP)	Dates of non polar aas, separated on the RP column, in agreement with bulk and historical date; aas separated on the IP column too old, probably because of remaining IP reagent
Hyp, Gly+Glu, Pro	(McCullagh 2007)	Mixed mode separation: Primesep A	Mary Rose Pig bone (311 BP)	Dates indistinguishable from bulk and historical date, however all slightly too old

Table 1.3. Review of the isolation of different fractions of bone, the separation method, the bone treated and the radiocarbon analysis outcome (if attempted).

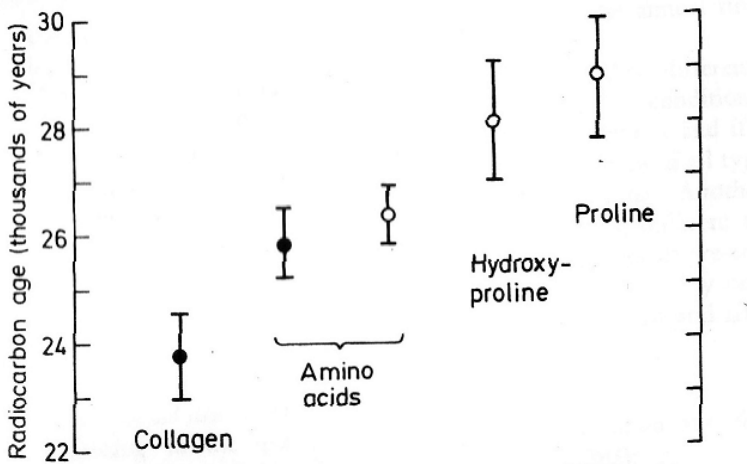


Figure 1.6. Radiocarbon ages for different fractions of Rhinoceros bone. Open symbols indicate AMS dating (at Oxford) and closed symbols conventional beta-decay counting (at the British museum). The older dates given by the proline and hydroxyproline, are of the expected range, and therefore suggest modern contamination in the other fractions was removed (figure taken from Aitken, 1990, based on Gillespie at al., 1984).

1.4.4 Application of Hyp dating in an archaeological context - the potential of the method

For which bones is the hydroxyproline dating method likely to be advantageous? As mentioned above, there are several criteria which radiocarbon labs use as part of the pre-screening process, in order to avoid dating bones that are likely to yield erroneous date due to contamination or low collagen (e.g. van Klinken, 1999). Some of those rejected bones are most interesting archaeologically. For example, it is likely that a significant number of Upper Pleistocene bone samples from Europe and the Near East would be characterised by low or trace amounts of collagen (Taylor, 1995). If sufficient hydroxyproline can be found in (some of) these bones, then a date could be assigned to them securely, helping resolve long standing archaeological questions about the spread of early anatomically modern humans out of Africa and into Europe and Eurasia.

Another set of bones that the hydroxyproline dating method is expected to be beneficial for are bones that were contaminated by consolidants applied for curation and preservation purposes, which cannot be fully removed. Ironically, some of the most important bones, such as Upper Palaeolithic modern humans skeletal remains, especially the ones that were excavated in the mid 20th century or before, are likely to have museum related material that could be hard to remove completely using the standard methods. A very good example is the Kostenki 14- Markina Gora Anatomically modern human burial, discovered in 1954. The stratigraphic context and direct radiocarbon dates of material from the same cultural level suggest that the age of the human must be at least 30ka BP but less than ~35ka BP. Direct radiocarbon dates of the specimen, however, yielded surprisingly young dates, between ~3.7 and 13.6 ka BP (Sinitsyn, 2004; Hoffecker, 2011). The elevated C:N ratios of the extracted collagen (around 4) suggest that the problem might be related to conservation material. In their paper from 2010, Krause et al. conclude it seems "impossible to get a reliable radiocarbon date directly from the Kostenki 14 skeleton" (Krause et al., 2010). This appeared an ideal case study to test the hydroxyproline dating method.

Another Upper Palaeolithic site from Russia with problematic radiocarbon dates is the Sungir site, first excavated in 1955. Direct radiocarbon dating of three of the skeletons thought to be contemporaneous was attempted previously (Pettitt and Bader, 2000; Kuzmin et al., 2004; Dobrovolskaya et al., 2011), but the results were highly inconsistent, both between the laboratories and between the different individuals dated, ranging between 19-27 ka BP. It is to be suspected, again, that carbonaceous conservation material had been applied to the bone, which the various chemistry

pretreatment methods performed on the sample were unable to remove. As opposed to the Kostenki site, the ‘true’ age for the Sungir bones is unknown; however successful elimination of the contaminant is likely to yield an older and consistent age for all the bones. Accurate dates for the aforementioned important sites will contribute considerably to our understanding of human dispersals in this period. The results of the application of the method to the Sungir and Kostenki bones are reported in chapter 4.

Old (>20ky) but well preserved collagen bones are also likely to benefit from the method, as they are more susceptible to modern carbon contamination which may not be detectable (Hedges and Law, 1989; see also chapter 3). The hydroxyproline method promises a ‘gold standard’ for dating bones and should be used for important or controversial bones.

An interesting possibility rose from the observation that a number of bones, albeit having little or no collagen left when extracting with weak acid, still have some residual degraded collagen (Elster et al., 1991). When looking at the weak-acid soluble fraction of 'non-collagenous' bone (a bone with 0.1%-1% collagen), in some cases amino acids can be found, probably originating from collagen in the process of being leached away (Hedges and Law, 1989; Hedges and van Klinken, 1992). It was possible, therefore, that bones which would normally be rejected for radiocarbon dating on the grounds of having insufficient amounts of collagen, would be suitable for single amino acid dating, by extracting hydroxyproline (and/or other specific amino acids) from this soluble fraction (see chapters 3 and 4).

1.4.5 Thesis objectives

In the project reported in this thesis the mixed mode chromatographic method described above was further developed and applied to obtain radiocarbon dates from the amino acid hydroxyproline from a number of archaeological bones. Chromatographic method development procedures were used to obtain optimal conditions for separation of Hyp from other eluting amino acids, and at the same time shortening the chromatogram so that a run will be completed in a reasonable time, within the limitations of the HPLC column and pressure module. These procedures included experimenting with gradient length and timing, the flow rate of mobile phase applied, the maximum amount of hydrolyzed collagen that could be injected without overloading, and choice of mobile phase and means of removing excess of it. The most important aspect of method development was to establish background carbon levels (the 'procedure blank'). A lot of effort was devoted to characterizing the blank (background carbon content) and finding ways of minimizing this. As mentioned above, a blank assessment is missing from all the previous Hyp dating attempts, but is essential for interpretation of the radiocarbon data (Mollenhauer and Rethemeyer, 2009).

The questions addressed in this thesis are:

(1) Does the method add significant amounts of carbon and does any such carbon affect the radiocarbon date? Before the method could be applied to archaeological bones it had to be determined whether it can provide radiocarbon dates that are both accurate and precise for bones of all ages. This was tested both by assessing the sample processing blank and by dating known age standard bones.

(2) Will contaminated bones date accurately using the method? This was approached by deliberately contaminating a radiocarbon dead bone with a modern contaminant. When it was established that the method was capable of removing the contamination, it was applied to archaeological bones suspected of being contaminated by conservation, which modern bulk radiocarbon dating protocols have failed to date accurately.

(3) Could the method be applied to low collagen content bone? For poorly preserved bone, the quantity of bone required is bigger, and so is the risk of dating non-indigenous material. In addition, even the surviving protein could be contaminated. The Hyp fraction was isolated from an archaeological bone which had very low percentage of nitrogen and therefore failed the prescreening criterion for dating using the normal chemical methods. In addition, experimental weathering of bone was attempted: bone fragments were subjected to heat trying to mimic diagenesis effect on collagen preservation. It was investigated whether Hyp could be extracted and dated from collagen that became soluble.

Chapter 2: Materials and Methods

The methods applied in the project described in this thesis are detailed in the following chapter. To begin with the chemicals and standards used are detailed, and then the protocols and instrumentation involved in the process leading from a bone to a radiocarbon date are depicted. To conclude, the issues addressed when the method was developed are listed. An additional discussion of the method development stages is provided in appendix B.

2.1 Chemicals and standards

All reagents used in the following protocols and procedures were of chromatography grade and water was from a Milli Q reverse osmosis system (typically 18.2MΩ resistivity and <4ppb carbon). Reagents and materials were purchased from Sigma-Aldrich Ltd (Gillingham, UK) and Fisher Scientific UK Ltd (Loughborough, UK).

2.1.1 Standard amino acids

Two standard mixtures of amino acids were created and used to develop chromatographic separation methods: a 'collagen-like amino acid standard', made with similar concentrations of amino acids to those found in bone collagen (according to Eastoe, 1955), and a second mixture of the first seven eluting amino acids. The amino acid composition of the standards can be found in table 2.1.

Amino Acid	% Molar in collagen	Molecular weight	Calculated % weight	'Collagen like amino acid standard' mg/100mL	'Amino acid standard' mg/100mL
Alanine (Ala)	11.4	89	9.3	9.3	13
Arginine (Arg)	4.7	174	7.5	7.5	-
Aspargine (Asn)	-	132	-	-	-
Aspartic acid (Asp)	4.7	133	5.7	5.7	8
Cysteine (Cys)	-	121	-	-	-
Glutamine (Gln)	-	146	-	-	-
Glutamic acid (Glu)	7.2	147	9.7	9.7	13
Glycine (Gly)	31.9	75	21.9	21.9	31
Histidine (His)	0.6	155	0.9	0.9	-
Hydroxyproline (Hyp)	10.0	131	12.0	12.0	17
Isoleucine (Ile)	1.3	131	1.6	1.6	-
Leucine (Leu)	2.6	131	3.1	3.1	-
Lycine (Lys)	2.0	146	3.8	3.8	-
Methionine (Met)	0.5	149	0.7	0.7	-
Phenylalanine (Phe)	1.4	165	2.1	2.1	-
Proline (Pro)	12.3	115	13.0	13.0	18
Serine (Ser)	3.6	105	3.5	3.5	3.5
Threonine (Thr)	1.8	119	2.0	2.0	-
Tryptophan (Trp)		204			-
Tyrosine (Tyr)	0.5	181	0.8	0.8	-
Valine (Val)	2.4	117	2.6	2.6	-

Table 2.1. Standard amino acid mixtures' composition. Molar percentage of amino acids in collagen taken from Eastoe, 1955.

2.2 The protocol for radiocarbon dating Hyp from archaeological bone

An overview of the protocol for dating Hyp from bone to a radiocarbon date is provided in figure 2.1.

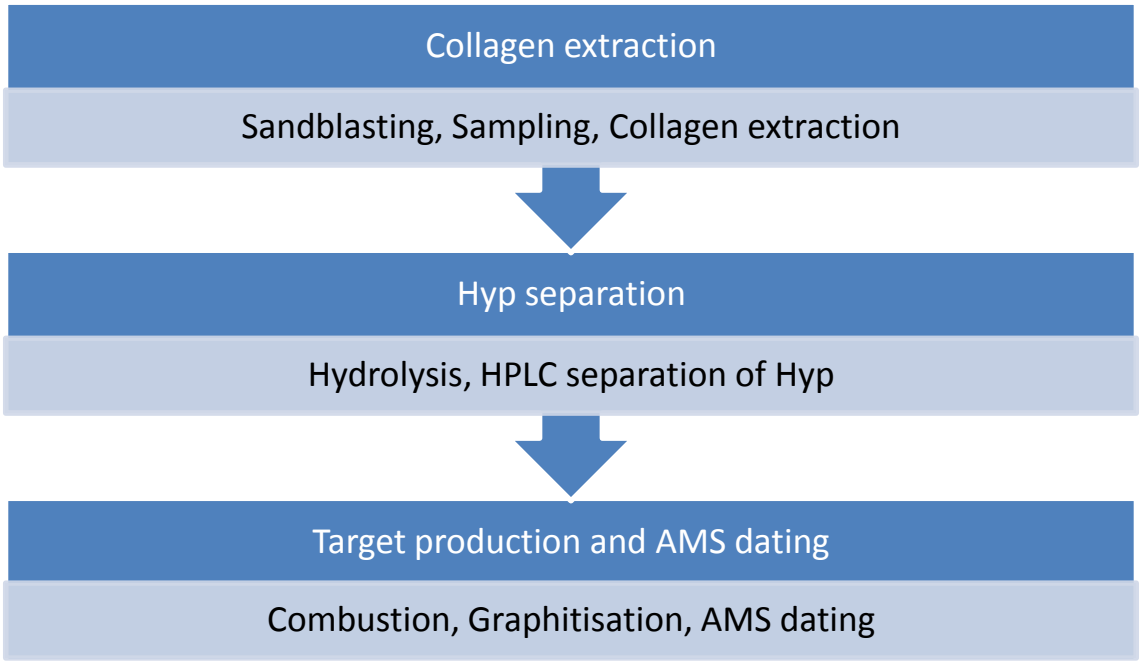


Figure 2.1. An overview of dating Hyp from bone to a radiocarbon date.

2.2.1 Collagen extraction

Bone samples were surface cleaned by air abrasion with aluminium oxide powder before being broken into small chunks or drilled using tungsten carbide drills at low speed and mechanical drilling in preparation for collagen extraction (Brock et al., 2010a).

2.2.1.1 Standard methods for collagen extraction

Bone collagen extraction followed the modified method (Longin, 1971) of Richards and Hedges (Richards and Hedges, 1999). The extraction of bone collagen required de-mineralisation followed by gelatinisation of the bone sample. Depending on the expected collagen yield, about 500mg of bone was treated with 10ml of cold 0.5M HCl in a test tube and left in a fridge for several days covered with aluminum foil. It was shaken twice daily and the acid was changed every two days until the sample was soft or floated. The supernatant liquor was then decanted and bone residues were rinsed in distilled water. The

remaining collagen pellets were heated in dilute HCl solution (1 mM, pH 3.0) at 75°C for 24-48 hours at which point the collagen dissolved, leaving behind all acid insoluble material. The supernatant liquor, which now contained gelatin, was filtered off using Ezee-filters™ (Elkay Laboratory Products (UK) Limited, Basingstoke, UK) and freeze-dried. It was then ready for use.

Although the method mentioned above was the standard method used in this project for extracting collagen, and is routinely used for stable isotope work, in some cases the more elaborate method "acid-base-acid" (ABA), routinely used at ORAU for radiocarbon analysis, was used[4] (e.g. when the separation of Hyp was made on previously ABA extracted collagen, or when the Hyp method was compared with the standard AMS method, where bones were deliberately contaminated for example). In addition, as Hyp dates were compared with bulk dates that were achieved using the ABA method, it was crucial to understand the different steps involved. In the ABA method the demineralisation step was carried out at room temperature for 2-4 hours, changing the acid once after 1-2h, and then over-night at <10°C if the process hasn't finished. The demineralised bone was then rinsed three times with water, and subjected to NaOH 0.1M for 30 min, to remove humic and fulvic acids, followed by three water rinses. The collagen was then re-acidified with 0.5M HCl for 15 min to remove dissolved CO_2, and gelatinised in 1mM HCl at 75º for 20h. Subsequently the gelatin was filtered off using an 'Ezee-filter' that was previously ultra-sonicated in MilliQ water for 20 mins, and then freeze-dried. The sample was then either combusted directly (given the lab code AG), ion-exchanged using the AGMP-50 (given the lab code AI; a method routinely used before the ultra-filtration was adopted), or

[4] The 'less stringent' protocol for collagen extraction was adopted because the chromatographic separation of Hyp from the collagen will undoubtedly remove any contaminant that the ABA and ultra-filtration steps are aimed to eliminate, and more.

ultra-filtered (Vivaspin™ 15–30 kD MWCO) (given the lab code AF). Before use the filters were rinsed inside and out, and then rinsed twice with a filter volume of MilliQ water by centrifuging for 15-20 min at 2500-3000rpm, until all water has spun through. The column was then ultra-sonicated in MilliQ water for 1h, rinsed inside and out and centrifuged three times with filter volume MilliQ water for 15-20 min. After rinsing the filters inside and out again, the gelatin was transferred to the ultra-filter and centrifuged at 2500-3000rpm, until mostly filtered (liquid in filter <0.5ml), typically 20-60 min. The >30kD solution was pipetted out. The top of the filter was rinsed with 1ml MilliQ water and added to the solution, which was then freeze-dried. For bulk AMS dating approximately 2.5mg of extracted protein was wrapped in a clean tin capsule and combusted for graphitisation (see below, sections 2.2.3.1 and 2.2.3.2) (Bronk Ramsey, 2004a; Brock et al., 2007).

2.2.1.2 Soluble and insoluble collagen extraction

In chapters 3 and 4 the extraction of 'soluble' collagen (organic material from the acid soluble fraction that normally gets discarded) is mentioned. The protocol used was different from the standard methods described above, in that the demineralisation step was carried out in a dialysis tube, allowing the inorganic dissolved material to pass through the tubes while retaining the bigger molecules, including, presumably, soluble collagen. The protocol is based on the protocol published by Elster and co-workers (Elster et al., 1991). The surfaces of the bones were cleaned by air abrasion with aluminium oxide powder before being crushed or drilled (crushing was preferred because chunks are easier to move in and out of the dialysis tubes, and in most cases the whole bone will have been used). 10 ml of water and 10g of crushed bone were

vortexed, and the supernatant was collected. This was repeated 4 times, yielding 40 ml 'leached fraction' (LF) that was then evaporated and Ezee filtered. The bone, with 10 ml water, was placed in a dialysis tube (Spectra/Por® 3, 1kD MWCO) that was previously soaked over-night in water. The bag contents were then dialyzed against 1.5L of 0.3M HCl at room temperature. Decalcification was complete after a few hours, as indicated by the flotation of the dialysis bags. The samples were then dialyzed against 3 changes of water, a change every hour, and then left dialyzing against water over night. The soluble and insoluble contents of the bag (representing soluble collagen and acid-insoluble, 'normal', collagen) were separated by centrifugation (5 min, 3000 rpm). The insoluble fraction was then vortexed with 10ml water, and the supernatant added to the soluble fraction. This was repeated 3 times. The resulting 40ml soluble fraction (SF) was evaporated and filtered. The insoluble fraction was gelatinised by applying 1mM HCl (pH3) at 75°C for 24-48 hours at which point the collagen dissolved, leaving behind all acid insoluble material. The supernatant liquor, which now contained gelatin, was freeze dried. This represented the 'collagen fraction' (CF).

The SF was purified further by passing through a Dowex® 50W-X8 resin (Dow Chemical Co. Ltd) ion exchange column. The Dowex resin was soaked for 15 min in 2M HCl, which was thereafter replaced with water and left to stand for 10 min. The water was changed a few times till the water pH was 5. The resin slurry was then poured into glass columns, flushed several times with water, and equilibrated with 15ml 10mM HCl. The soluble fraction was passed through the resin column, and then washed with 15ml 10mM HCl. The flow-through collected should have contained primarily the NCPs and humic

substances (Elster et al., 1991). The "collagen rich" fraction was eluted with 15ml of 6M HCl, and was directly subjected to hydrolysation in the acid eluate (see section 2.2.2.1).

2.2.2 Hydroxyproline separation

2.2.2.1 Collagen Hydrolysis

The dried collagen was stirred with 6M HCl and left for 24 hours at 110°C under a nitrogen atmosphere. After the reaction was complete, the aqueous acid was removed on a gyro-vacuum evaporator (EZ-2 evaporator, HCl compatible, Genevac Ltd. Ipswich, UK), and the residue dissolved in MilliQ water was filtered using a 0.2µm PTFE filter (Chromacol, from Fisher Scientific UK Ltd, Loughborough, UK) to remove precipitates. Hydrolyzates in this condition were then used immediately for HPLC separation and analysis or frozen (at -40^{O}C) for later use.

2.2.2.2 HPLC separation

HPLC (High Performance Liquid Chromatography) is a liquid chromatographic system which utilises high pressure pumps and a sensitive detector in order to separate and purify substances. Individual components are separated as each one of them interacts differently with the mobile and stationary phases. A mixture is injected, and the separated components elute off at different retention times, yielding distinctive peaks by the detector, and collected in fractions. A photograph of the apparatus used in the research reported in this thesis can be found in figure 2.2.

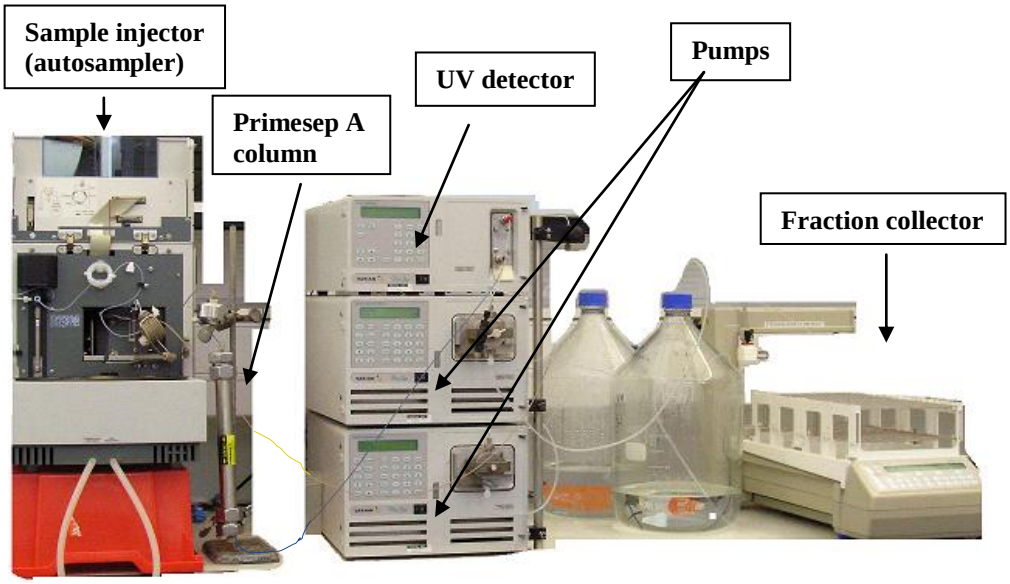


Figure 2.2. The HPLC system used to generate the data presented in this thesis.

In the setup used here, the hydrolyzed collagen was separated to individual amino acids using a semi-preparative scale HPLC. The chromatography did not only separate the amino acids from one another, but also served to purify them, eliminating contaminants introduced during the bone's depositional history. Because the amino acid peak of a known retention time was selected, species in the solution that were non-chemically bound were removed, and amino acids that were modified did not get selected, as they shifted the retention time. The elution times for each amino acid were determined by injection of uncontaminated amino acid standards. The successful separation of an amino acid standard mixture back to its individual amino acids was verified by Electrospray Time of Flight Mass Spectrometry (ESI-TOF-MS).

The preparative HPLC separations were performed on a Varian ProStar HPLC system consisting of two 210 isocratic pumps, a 410 autosampler, a 320 dual-path length UV detector, and a 701 fraction collector, all controlled by Star Workstation ver 5.5 PC

software. The autosampler had a 1 mL syringe and 2 mL sample loop. Up to 1 mL of sample (at a concentration of up to approximately 10-15 mg/mL of amino acid mixture) could be injected. Amino acid fractions were collected using an automated Varian 710 fraction collector controlled via the Star software.

The amino acids were separated and collected using preparative mixed-mode chromatography, employing a Primesep A column (250 mm x 22 mm, particle size 5 µM, SIELC Technologies Inc., Illinois, USA). The retention mechanism of the stationary phase is based on both hydrophobic interactions, provided by C_{12} alkyl groups bonded to the surface of the silica backbone, and ion exchange interactions, provided by carboxylic acid groups on the stationary phase surface. The embedded carboxylic groups of the Primesep A are ionised under all operating pH conditions, whereas the analyte amino acids change their charge state as a result of changes in mobile phase pH as mobile phase composition changes according to the gradient program. Below a certain pH, depending on their individual pKa and hydrophobic properties, ionic interactions between the amino acids and the stationary phase can no longer compete with an increasing concentration of hydrogen ions and they are eluted (figure 2.3).

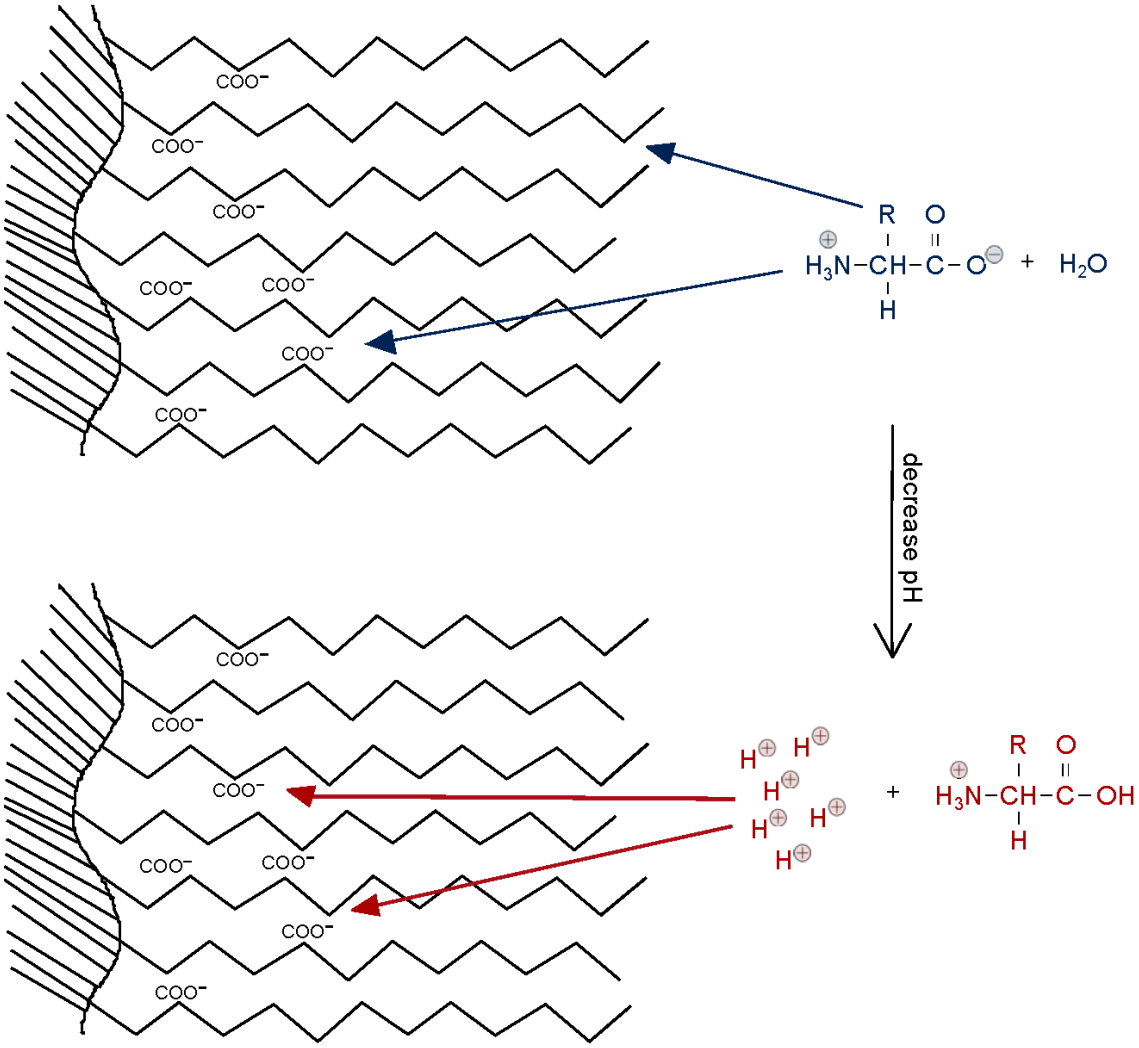


Figure 2.3. Amino acids' interaction with the Primesep A stationary phase in changing pH conditions.

In order to separate single amino acids the collagen hydrolysate was injected into the HPLC and a linear gradient program was applied: pump A (100% water) was pumped isocratically for the first 35 min; Then, from 35 to 65 min a linear gradient from 100% A to 100% B (0.3% ortho-phosphoric acid by volume) took place. From 65 min till the end of the run at 599 min, B was pumped isocratically. An isocratic run of 100% A for 35 min followed, in order to re-equilibrate the column. The flow rate of the mobile phase was maintained at 6ml/min throughout the run. For each sample 3 (and on some occasions 4) injections were made and overlaid in order to obtain enough amino acid.

The concentration and volume of sample that could be injected per chromatographic run were limited by the column's loading capacity and injection loop size (1mL). The UV absorbance was set to 205nm. Typically, Hyp eluted ca. 60 min after injection, and 54mL of eluent was collected as the peak eluted; Ala eluted around 85 min after injection, and 72mL of eluent was collected; Pro eluted around 105 min after injection, and 162mL of eluent was collected. Examples of separations of a synthetic standard mixture of amino acids with a collagen-like composition and a hydrolyzed collagen sample are presented in figures 2.4 and 2.5. Excess water was removed using the EZ-2 evaporator, and the samples were run again on the HPLC in order to remove the acid. This involved re-injection of the amino acid/H_3PO_4 mixture using isocratic elution conditions (100% MilliQ water) at a flow rate of 15 ml/min. Typically 45mL of eluent was collected for Hyp, 225mL of eluent was collected for Ala, and 225mL of eluent was collected for Pro. An example of a re-injection of Hyp is shown in figure 2.6. The individual amino acids were finally evaporated to dryness using the EZ-2 evaporator (for details on the method development see section 2.3 and appendix B).

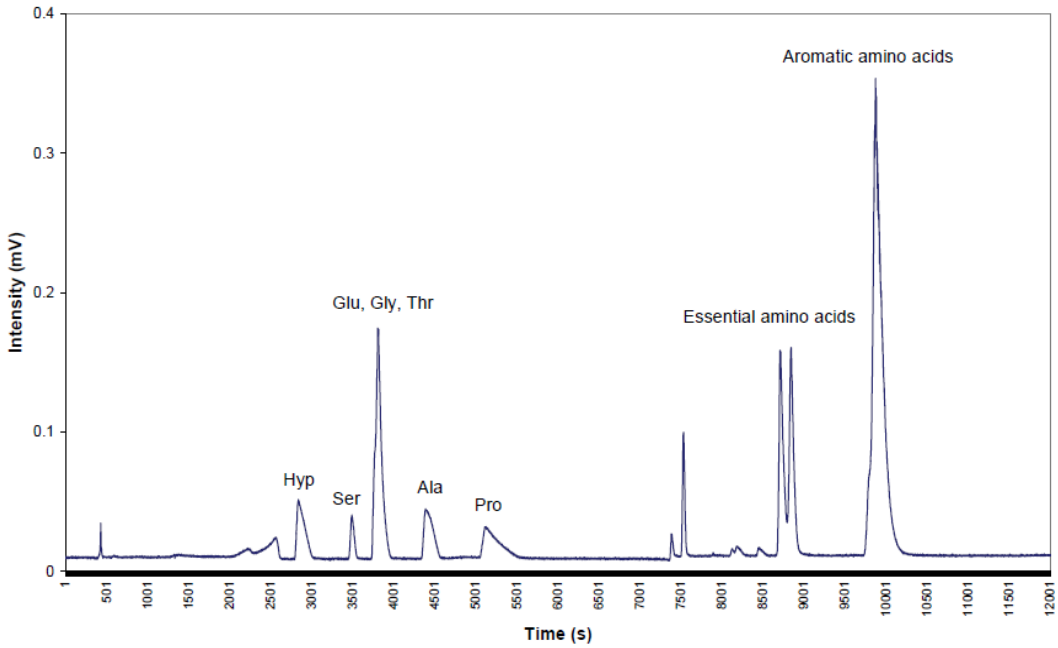


Figure 2.4. Separation of a standard amino acid mixture (collagen-like composition), 20mg/mL. Note, peak height and area relative to UV absorbance at 205nm.

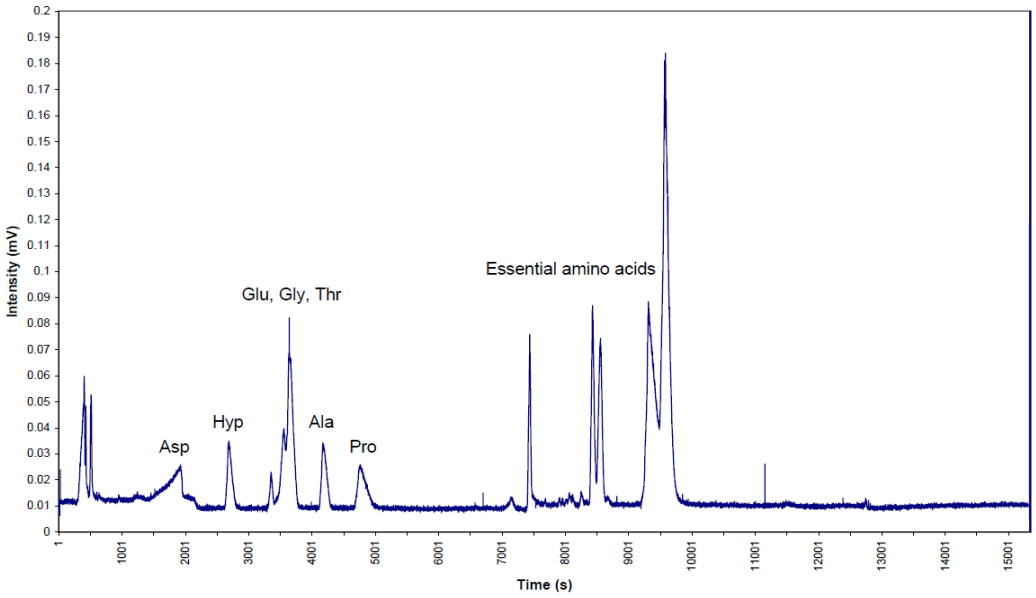


Figure 2.5. Separation of bone collagen hydrolyzate, 15mg/mL (Mary Rose pig bone collagen). Note, peak height and area relative to UV absorbance at 205nm.

As a rule, whenever an amino acid was collected for dating, a 'blank' sample that assesses the background carbon addition of the whole analytical process was also

collected (see chapter 3). Figure 2.6 summarises the procedure undertaken for the isolation of individual amino acids such as Hyp.

Procedure for collecting Hyp and Hyp-blank

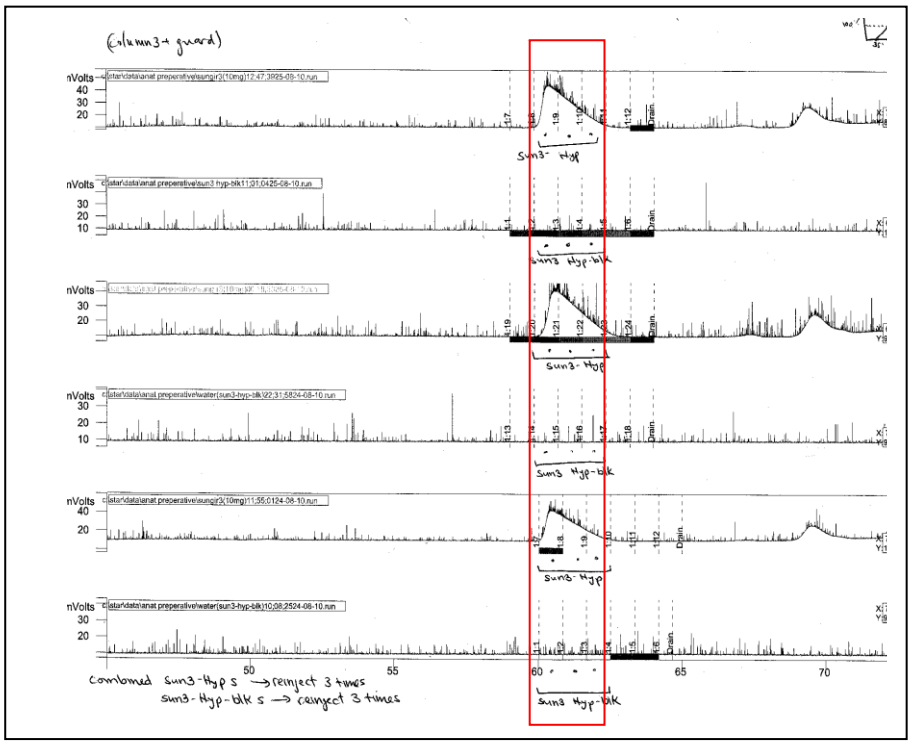
(1) Before the sample was injected to the HPLC, water was injected and the separation method applied

- to eliminate compounds that might come off the column under these conditions.

(2) Hydrolyzed collagen sample was injected and the separation method was applied without collection

- if cross contamination occurs, at least with material of the same age.

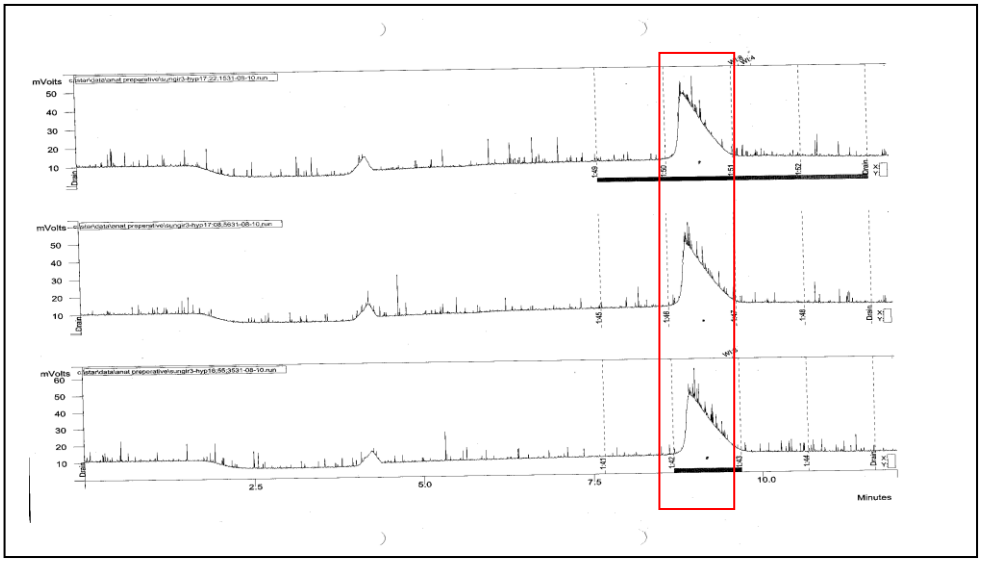
(3) Sample (10-15mg) injected and separation method applied ➔ Hyp fractions collected; water injected, separation method applied again, and the same fractions were collected (Hyp blank). Repeated step 3 three times:



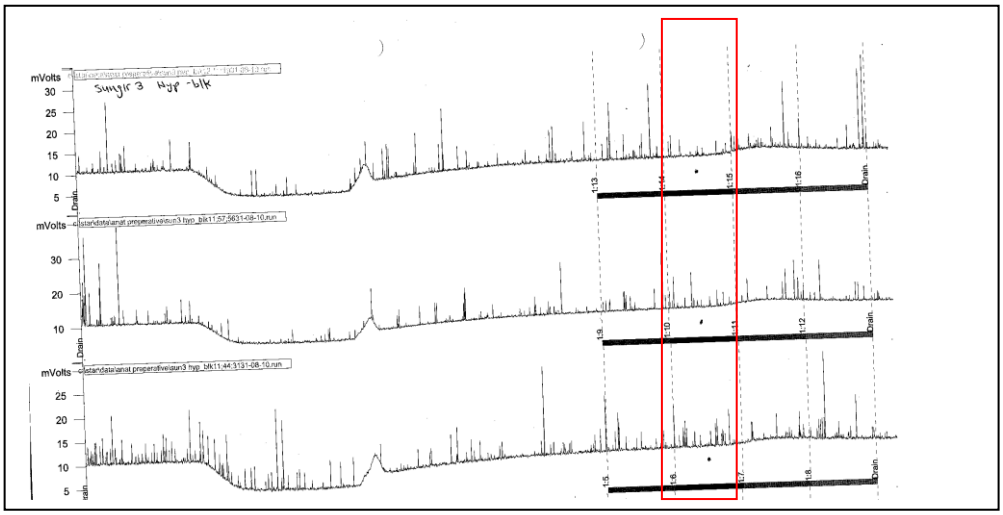
(4) The collected Hyp and Hyp-blank fractions were each combined and their volumes reduced by evaporation to 3ml each. Water was injected and the separation method was applied to the second column without collection.

- to eliminate compounds that might come off the column under these conditions.

(5) 1ml of Hyp in acid was injected to 2nd column and separation method was applied ➔ Hyp fractions were collected. Repeated three times:



(6) The same was done for the Hyp-blank fractions: 1ml of Hyp-blank in acid was injected to 2nd column and separation method was applied ➔ blank fractions (where the Hyp fractions were collected) were collected. Repeated three times:



(7) The collected fractions of Hyp and Hyp blank were evaporated to dryness and combusted. The Hyp was graphitised; the Hyp blank was tested for carbon content on the IRMS.

Figure 2.6. The procedure for collecting Hyp and Hyp-blank. The chromatograms of the separations of the Sungir 3 sample are used as an example. The collected fractions are framed. Note that the baseline is noisy, due to the UV lamp nearing the end of its lifetime**;** the lines do not represent eluting compounds.

2.2.3 Target production and AMS dating

2.2.3.1 Combustion

Materials being combusted were enclosed in cylindrical tin containers. Liquid samples tend to escape from the container through capillarity, but can be immobilised by absorption onto Chromosorb™ (an inert silica material used as a combustion substrate: W/AW, Mesh Size 30-60, Phase separations Ltd). In order to recover as much as possible of the separated amino acid, after evaporation the dry amino acid was reconstituted in 30µL of 1mM HCl and put onto a pre-cleaned tin, packed half full with pre-baked Chromosorb™. The tin capsules were cleaned by soaking in cyclohexane for 2 min, followed with Acetone for 2 min. They were then ultra-sonicated with distol acetone for another 2 min. Excess solvent was removed by baking for 10-20 min in 100ºC. Chromosorb™ was baked for 3h at 500º in aliquots. It was then sealed to avoid re-absorbance of atmospheric CO_2. Once open, an aliquot was re-baked before being used again.

Samples for either bulk stable isotope measurements or AMS were combusted using a carbon and nitrogen elemental analyzer (EA) (Carlo Erba NA 2000) coupled to a gas source isotope ratio mass spectrometer (IRMS) in continuous flow mode (Sercon 20/20 or Europa Geo 20/20) (a diagram of an EA-IRMS can be found in figure 2.7). A carbon and nitrogen containing sample was loaded into a tin boat. It was thereafter dropped into a furnace at 1000ºC while in an atmosphere of oxygen. The tin ignited and burned exothermically, and the temperature rose to about 1800ºC, oxidising the sample. Water was removed via a chemical trap and the N_2 and CO_2 were separated in a GC column packed with Carbosieve™ (Supelco G60/80 mesh; Bellefonte, Pennsylvania, USA)

packing medium. A helium carrier gas stream of 100 mL/min was used throughout. Samples were admitted into the mass spectrometer via a static 50:1 splitter with N_2 and CO_2 gas pulses being analyzed sequentially for each sample.

Bulk collagen and individual amino acid samples were prepared in a similar way for IRMS analysis. IRMS protocols at Oxford recommend that appropriate sample weights for analysis should contain approximately 5mg bone powder, or 2.5mg collagen and between 3mg and 4mg for most amino acids. Isotopic ratios for samples are calculated relative to the results of a nylon or an alanine standard that is run in duplicate for every 7 unknown samples. Typical measurement error for analytical replicate samples is ±0.3‰ for $\delta^{13}C$ and ±0.4‰ for $\delta^{15}N$ (1sd)[5]. $\delta^{13}C$ values are reported with reference to the VPDB standard (Vienna Pee Dee Belemnite standard, the common reference standard for $\delta^{13}C$ measurements) and $\delta^{15}N$ results are reported with reference to the 'air' standard for nitrogen (Brock et al., 2010a).

[5] The precision of the measurements depends on the age of the chemicals in the EA, age of the filament in the mass spec and the type and size of the samples. The error term is calculated from the standard deviation of the Alanine standards measured within each IRMS run from their known $\delta^{13}C$ and $\delta^{15}N$ values. These errors are typically smaller than cited in the Brock et al., 2010a.

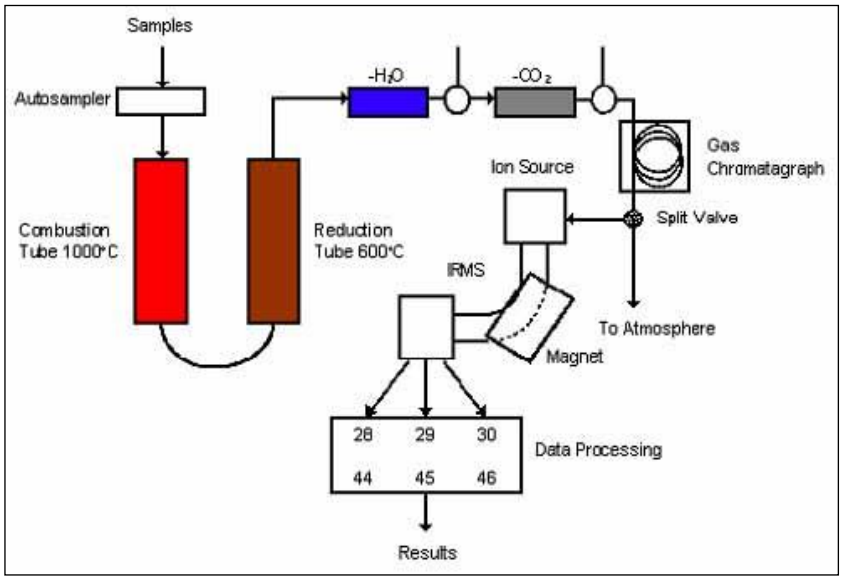


Figure 2.7. Schema showing the layout of a typical EA-IRMS system.

2.2.3.2 Graphitisation

Samples were graphitised according to Dee and Bronk Ramsey (Dee and Bronk Ramsey, 2000). Briefly, CO_2 was formed via combustion of the samples and was trapped in liquid nitrogen by the gas collection system and then transferred to the specifically designed 10mL graphitisation rig (Bronk Ramsey and Hedges, 1997). The rig was connected to two tubes: a graphitisation tube and a water tube. The sample CO_2 was reduced over 2-2.5mg iron catalyst (Aldrich iron powder) placed in the graphitisation tube, in an excess H_2 atmosphere (the H_2/CO_2 ratio is 2.2) at 560°C for 6h. Water produced by the reaction was condensed out by applying <10ºC to the water tube[6] (Hedges et al., 1992; Dee and Bronk Ramsey, 2000). Pre-reduction of the iron with H_2 at 450ºC for 1h is necessary to remove as much modern carbon contamination from the catalyst as possible. Prior to being loaded on the AMS, the residual pressure was measured to ensure more than 95% conversion has occurred, and the graphites

[6] When very low graphites are expected (i.e. <0.5mg C), the water tube is packed half to two-thirds full with magnesium perchlorate covered with a layer of silica wool, in order to reduce rig volume and promote better graphitization yields (Santos et al., 2007).

were pressed into aluminium targets for AMS at 350 kgf for 20 seconds (Brock et al., 2010a).

2.2.3.3 Accelerator Mass Spectrometer dating

The ^{14}C content of the graphites was measured on an Accelerator Mass Spectrometer (High Voltage Engineering Europa AMS, described in Bronk Ramsey, 2004b) (figure 2.8) at the Oxford Radiocarbon Accelerator Unit (ORAU). The procedure for AMS dating on the Oxford instrument is described bellow: C ions are liberated from the graphite sample by bombarding it with positively charged cesium ions (Cs^{+1}). The resulting negative carbon ions (carbanions) are focused into a fast-moving beam. Under such conditions nitrogen does not form a negative ion, and so the confusion of ^{14}N for ^{14}C (both having the same weight) is avoided. A magnet selects ions of mass 14 (this will include large number of $^{12}CH^{2-}$ and $^{13}CH^{-}$ ions and a very few $^{14}C^{-}$ ions). The ions then enter an accelerator. As they are accelerated they collide with gas molecules in a central `stripper canal'. Molecules will break apart in this stripping stage, and several of their electrons will get stripped off, converting them into positively charged ions. Most of the carbon ions will have four electrons removed, producing C^{3+} ions. The positively charged carbon ions are then accelerated down the second half of the tandem accelerator. A second magnet selects ions with the momentum expected of ^{14}C ions. Finally the filtered ^{14}C ions enter a detector where their velocity and energy are checked so that the number of ^{14}C ions in the sample can be counted (http://c14.arch.ox.ac.uk).

For each sample a ratio of $^{14}C/^{13}C$ is calculated and compared to measurements made on standards with known ratios. A radiocarbon age (BP) is assigned to the AMS measured sample after the combustion, graphitisation and AMS backgrounds are

subtracted and isotopic fractionation is corrected for (see chapter 3 and Wood et al., 2010). Statistical analyses and radiocarbon date calibrations (yielding a Cal BP probable date range) are performed using OxCal v4.1.7 (Bronk Ramsey, 2001; Bronk Ramsey, 2009), employing the IntcCal09 calibration curve (Reimer et al., 2009).

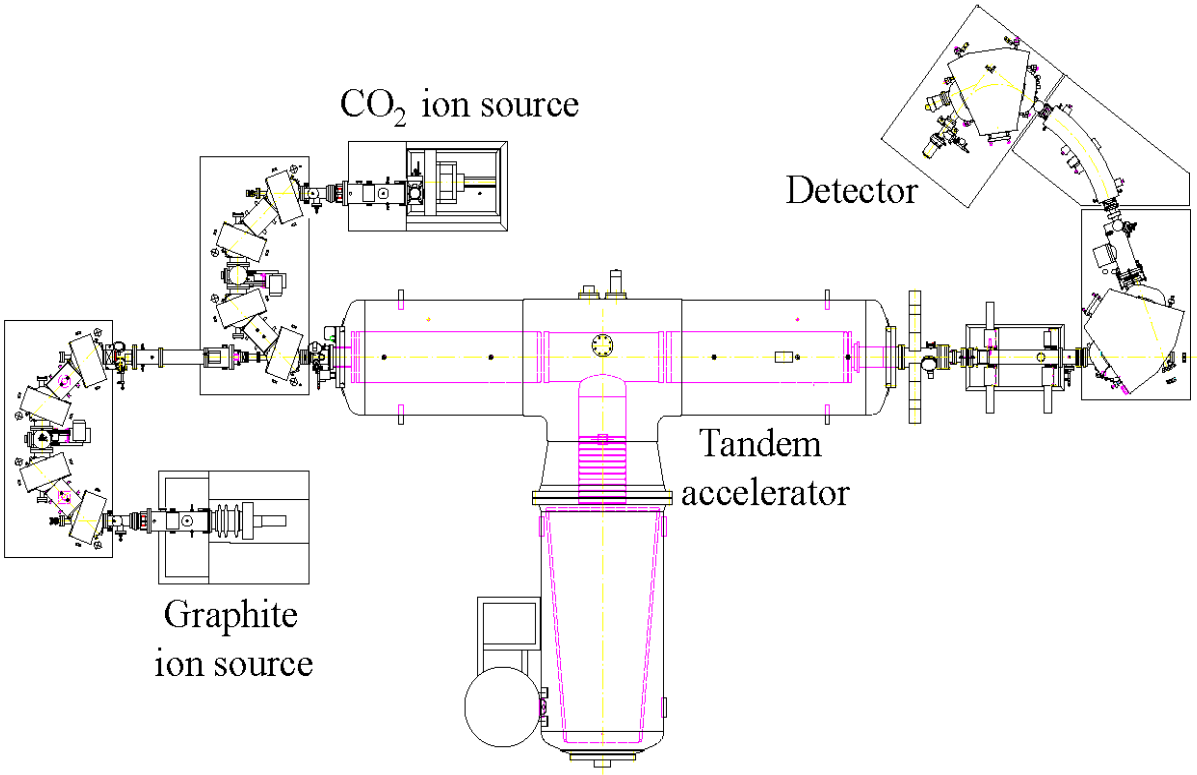


Figure 2.8. Schema of the Accelerator Mass Spectrometer at Oxford.

2.2.4 How is Hyp dated: the workflow

The diagram below (figure 2.9) summarises the process of amino acid dating from the injection of a hydrolyzed collagen sample onto the HPLC to production of an AMS radiocarbon date.

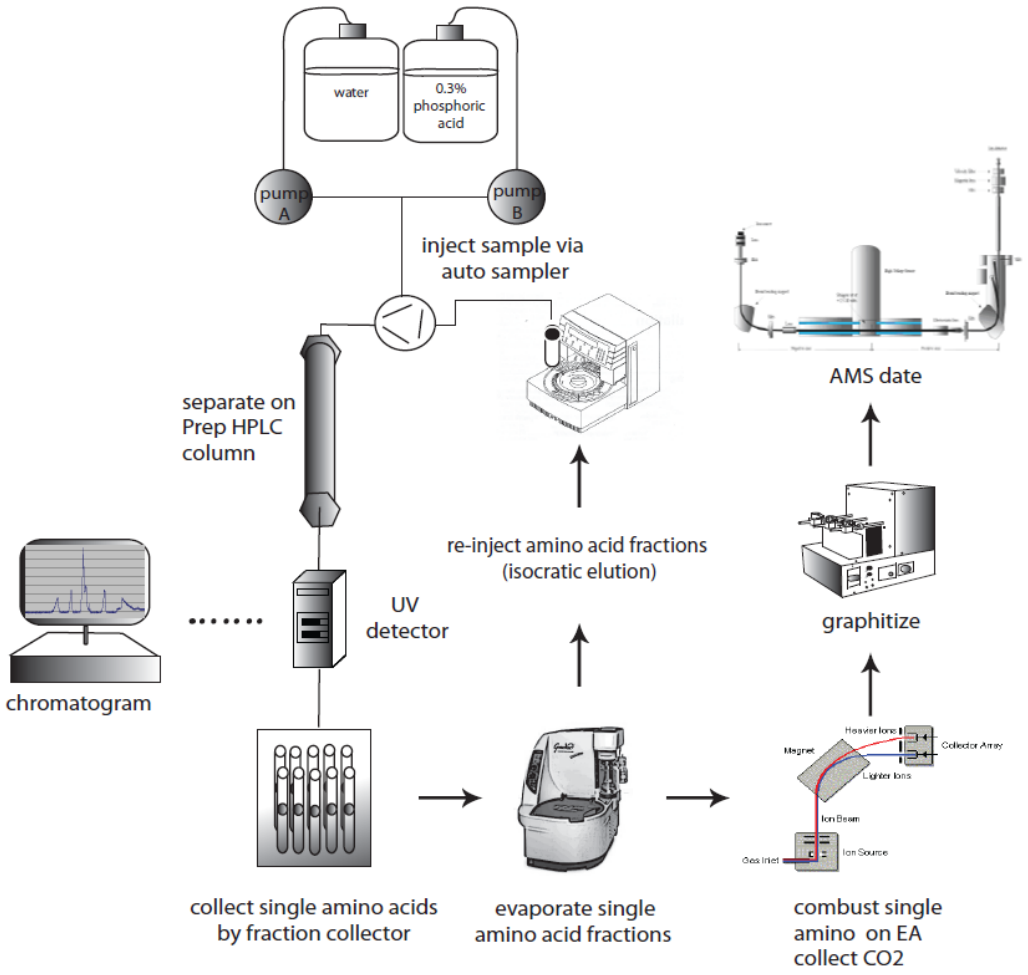


Figure 2.9. Flow diagram for the method to radiocarbon date individual amino acids that integrates a number of methodologies to create a workflow for compound specific dating.

2.3 Method development

The major effort in the development of the method was devoted to the measurement, characterisation and reduction of the procedural background (see chapter 3). Another key issue was the efficiency of the process, and thought was put in trying to increase yields. Optimizing chromatography conditions was also fundamental. The preparative chromatographic method is based on the analytical method developed for the separation of underivatized amino acids from bone collagen for stable isotope analysis (McCullagh et al., 2006; McCullagh, 2007; McCullagh et al., 2008) and on preliminary preparative separations of single amino acids for radiocarbon dating (McCullagh, 2007). Obtaining

baseline resolved, semi-preparative chromatography for underivatized amino acids using inorganic mobiles phases is challenging. Chromatographic method development was undertaken, mainly to determine the best conditions (e.g. gradient length and timing, mobile phase composition and flow rate, maximum amount of hydrolyzed collagen injected) for the separation of Hyp from the neighbouring amino acids. Reducing the considerable run time was also an important factor. The attempts to reduce the HPLC run time and to remove the mobile phase are provided in appendix B.

Chapter 3: Evaluation of the method

3.1 Introduction

Before the Hydroxyproline dating method could be applied to archaeological bones, its suitability for radiocarbon dating needed to be evaluated. It needed to be determined that the process of isolating of single amino acids using mixed mode chromatography, developed by McCullagh (McCullagh, 2007) and further developed in the project reported in this thesis, itself does not contribute contaminating carbon, and is successful in removing sample contamination. It was also claimed that the hydroxyproline dating method could be advantageous in dating bones in which the collagen had started degrading. It would be useful, therefore, to demonstrate that Hyp can be extracted from bone with low collagen content, either from the organic fraction ('collagen', the acid insoluble fraction) or from the mineral fraction (the acid soluble fraction).

3.2 Hydroxyproline dating and contamination

Contamination affecting the radiocarbon date must be a result of exogenous carbon, at a different age from the original collagen, that has been introduced from the depositional environment, conservation or the laboratory during sample preparation or analysis (e.g. mobile phases, impurities in reagents used etc.). In order to evaluate the method with respect to contamination, two questions needed to be answered: (1) does the method remove contamination present in the sample? (2) does the method add any significant level of background carbon contamination?

3.2.1 The effect of contamination on the date

Before addressing these questions we will first look at what affect contaminating carbon will have on the radiocarbon date. This depends on the amount of contamination present, and the relative ages of sample and the contaminant. The overall ^{14}C activity

(the measured activity) is composed of the ^{14}C activity of the contaminant, combined with that of the true sample, according to their relative proportions, as described by equation 1:

(1) $$A_m = f*A_x + (1-f)*A_s$$

Where f is the fraction of the contaminant, A_m is the measured activity, A_x is the activity of the contaminant and A_s is the activity of the sample.

A contaminant in which all the radiocarbon has decayed (or is 'radiocarbon dead') has the same affect on the date as if a part of the sample was missing. For each percentage of 'dead' contamination an offset of around 80 years is introduced, regardless of the sample's age (Bowman, 1995). Modern contamination, on the other hand, has a more complex effect. For a sample which is 17,000 years old, for example, the addition of 1% of new carbon will make the radiocarbon age 600 years younger. For a 34,000-year-old sample the same percentage of modern contamination causes an error of 4,000 years, making it appear 30,000 years old. For a 50,000-year-old sample the apparent age will be 36,000 years. A radiocarbon 'dead' sample, beyond the limits of detection of radiocarbon measurement, will never exceed the age 38,000 years in the presence of 1% modern carbon (see figure 3.1) (Bowman, 1995).

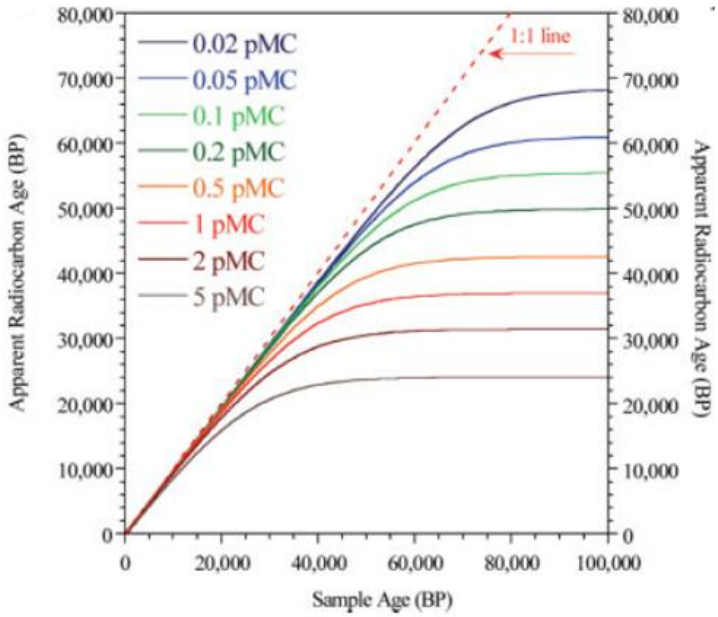


Figure 3.1. The effect of modern contamination on apparent radiocarbon age.
Taken from Grun, 2006.

3.2.2 Procedural blank assessments

The process of isolating hydroxyproline should remove through chromatography *in situ* contamination (diagenesis effects, conservation, handling etc) as well as contamination added during sample preparation prior to HPLC separation (e.g. condensation reactions might take place during hydrolysis) (Stafford Jr. et al., 1988; Shah and Pearson, 2007). Dating Hyp could therefore produce more reliable dates than analysis of bulk protein. However it is possible (as with all analytical procedures), that the process of hydroxyproline separation could add contaminating compounds and hence background carbon, in addition to the standard bone pretreatment procedure blank[7]. It was therefore necessary to perform an assessment of all possible contributions of exogenous carbon that may result from sample processing during and after chromatography. The

[7] In fact, previous attempts to radiocarbon date Hyp, although using a different separation method, seem to have suffered from high background carbon, as the resulting dates suggest (Stafford et al., 1991).

assessment of the sample processing blank is essential for interpretation of radiocarbon data, especially if samples to be dated are small (Shah and Pearson, 2007; Mollenhauer and Rethemeyer, 2009). As hydroxyproline originally consists of only 10% of the collagen, and might comprise even less of an archaeological bone, the end product will inevitably be a "small" graphite target (at ORAU a small target is defined as 0.5-1mg carbon[8]). Any background ^{14}C levels (introduced during pre-treatment, combustion, graphitisation, or AMS analysis[9]) would have a much more significant effect on the date of smaller samples, especially when older samples are contaminated with modern carbon. It is important to establish where any background carbon is coming from, its magnitude and radiocarbon date, and ways to reduce its presence or affect. If the background carbon addition is constant, it can also be used to make an age correction if necessary.

3.2.2.1 Possible sources for background carbon

Any AMS measurement is liable to have some background carbon, which could be introduced during pretreatment, combustion, graphitisation, and AMS measurement. This is a result of the extremely sensitive nature of the measurement and the abundance of carbon. To assess the AMS and graphitisation background (assumed to be modern), a radiocarbon 'dead' gas sample could be measured (thus leaving out the pretreatment and combustion background addition). At the Oxford Radiocarbon Accelerator Unit (ORAU), this is done by running in each AMS batch 2 anthracite gas samples, graphitised using the same procedure as all other samples but combusted in bulk by the

[8] Small graphites are run on separate wheels than 'normal' (1.7mg) graphites, so as to ensure size consistency between standards and samples.
[9] The AMS machine background is unlikely to be simply 'carbon', but more likely atomic/molecular ions that make it into the detector and electronic noise.

CIO Groningen laboratory. The graphitisation and AMS blank of the ORAU measured this way is reported to be around 0.15% of modern levels (52 ka BP) and in optimal conditions can be as low as 0.1% (55 ka BP) (Bronk Ramsey, 2004b).

The amount of modern carbon contamination added through combustion is assumed to be 0.7±1 μg carbon (1sd), as measured from the activity of a Nylon standard (which has no ^{14}C activity). The possible effect of any ^{14}C deficient background added through combustion is minimized by measuring HOXII standards (Oxalic acid, a standard representing modern ^{14}C levels; HOXII samples are also used to check the internal consistency from one sample to the next in a run, as they should yield the same activity levels). The combustion background (equivalent to ~0.1% pMC) sets a maximum age of 54,000 BP for all samples combusted within a CHN elemental analyzer (see figure 3.1). The pretreatment background is monitored by dating with each batch 2 known-age standards of similar material, size and age as the unknown samples. The ultra-filtration pretreatment (equivalent to ~0.2% pMC) adds an extra background term resulting in a background age of 49,900 BP for the analysis of bone samples (see figure 3.1). When assigning a date to the AMS measured sample, the combustion, graphitisation and AMS backgrounds are subtracted (also the ultra-filtration background, if this pretreatment step was taken) (Bronk Ramsey, 2004b; Wood et al., 2010).

Studies that have focused on radiocarbon measurement using small graphites (i.e. in the sub-milligram range) have reported a significant offset for sample weights below 200-300 micrograms of graphitic carbon (Vogel, 1987; Kirner, 1996; McCullagh, 2007). As samples decrease in size, the constant amount of contamination added makes an

increasing percentage of the total carbon content of the sample, leading to larger age offsets (Kirner, 1996). This applies for modern contamination making older dates increasingly younger as well as for contamination with no detectable ^{14}C activity (introduced for example by pump oil and vacuum grease) making recent samples look older. Another explanation has been also suggested: a mass dependent isotopic fractionation different for the ^{13}C isotope and for ^{14}C during graphitisation and/or during transmission through the AMS occurring in smaller samples (Kirner, 1996; Mollenhauer and Rethemeyer, 2009). The problem of mass dependence can be compensated for by measuring sub-milligram samples against similarly sized standards, providing these background offsets are reproducible. In light of this data, to avoid these small sample effects altogether, the aim in this project was to date graphites in the range of 500 micrograms and larger. It has been shown that samples above 500 micrograms are much less affected by the constant modern and 'dead' contaminations introduced during pretreatment onwards (estimated by Kirner and co-workers to be 1 microgram of each using their analytical procedures).

For the standard bone pretreatment protocol, the ultra-filtration step is thought to be the major contributor of background carbon, and to dominate the error term of the ^{14}C date (assuming contamination, if was present, is now eliminated), adding on average 25±8 μg modern carbon to each sample (Wood et al., 2010). The hydroxyproline dating method does not include an ultra-filtration step. However contamination could occur during and after passage through the HPLC. The HPLC step itself can contribute ^{14}C contamination from column bleed, mobile phase composition, co-elution of exogenous compounds with the Hyp (or other single compounds isolated for dating), and carry-

over of chromatographic impurities from the total collagen sample (and/or previous runs). It is also possible that contamination may come from sample handling and/or from ^{14}C contamination from chemical reagents or compounds absorbed onto glassware.

To minimize these sources of contamination all reagents used were analytical grade or above and glassware was baked out at 500°C before use. All HPLC lines were metal, where appropriate, and the system was free of organic solvents. Tin capsules were washed and cleaned and Chromosorb^{TM} was baked at 500°C (see chapter 2 and section 3.2.2.2 below). In order to make sure there was no carryover of potentially contaminating material from previous separations, the column was subjected to a water wash by applying the separation method with the injection of MilliQ water before each injection; then a low concentration of the hydrolyzed collagen sample was injected to be separated. This was to ensure that if any cross contamination occurred between samples (no evidence was found for this) it would be of the same radiocarbon age. In addition, after each separation water was injected and fractions were collected at the elution time of the amino acids so the background blank carbon value could be monitored. The second column was also washed using a water injection and subjected to the separation method, to remove any compound that might come off the column under these conditions. When more than one amino acid was collected a "rinsing" method was applied between injections (see chapter 2, figure 2.6).

As mentioned, another possible source of carbon was co-elution of undesired compounds with the Hyp fraction. As a result of the detection mode of our HPLC setup,

a single peak as detected by UV absorption at 205 nm (in which all amino acids absorb UV light) could in theory be a response to more than one compound. In order to test this, HPLC collected single amino acid fractions and 'blanks', fractions of mobile phase collected at the same positions as for eluting amino acids but after injection of MilliQ water only. Amino acid analysis was made of these fractions (at the Protein Characterisation Facility of the department of Biochemistry or at the Mass Spectrometry facility of the department of Chemistry at the Chemistry Research Laboratory, both at the University of Oxford). The collected amino acid fractions came out as expected, i.e. no more than one amino acid was found in each collected peak, and the amino acid found was the one expected by retention time[10]; the blank samples came out indistinguishable from unprocessed MilliQ water, confirming that no carryover occurred.

3.2.2.2 Background carbon measurements

Assessing the amount and source of background carbon added during the different stages of isolating single amino acids is essential for two reasons: one is to determine how it can be eliminated, or at least minimized, and the other is to be able to create a model to correct the radiocarbon measurements themselves, if it can be determined that the analytical protocol adds a constant amount of carbon with a constant ^{14}C activity. However, measuring blanks associated with the entire sample treatment (procedure blank), or with individual steps of the sample preparation process is not trivial (Mollenhauer and Rethemeyer, 2009). In fact most of the time spend on this project was devoted to establishing and characterizing the blank levels. It should also be noted that

[10] The amino acid retention times were identified previously using standards and spiking natural samples; LC/MS analysis of selected amino acid fractions was used to confirm identification of peaks by mass (McCullagh et al., 2006).

these blanks will never be constant from one AMS laboratory to the next even if the same procedures are followed. They are dependent inherently on the materials used which include the analytical instrumentation itself as well as the process followed; metal lines, pumps and combustion materials will all differ slightly in volume, length and composition. They will therefore provide a unique contribution to the overall blank. The procedure blank can even change over time within the same lab, as each of the exogenous carbon sources may vary independently in time (Shah and Pearson, 2007; Mollenhauer and Rethemeyer, 2009). For example, as HPLC columns age, the composition and amount of their carbon bleed may change.

As already mentioned, amongst others, the mobile phase itself can add background ^{14}C during the process of isolating hydroxyproline. Although the mobile phase chosen for the HPLC separation was intentionally carbon free (phosphoric acid), it could still contain residual carbon in the form of dissolved organic and inorganic compounds at low abundance. In order to test for these, concentrated phosphoric acid samples (corresponding to the amount of acid in 10 milliliters of mobile phase) were put onto tins with Chromosorb^{TM} and combusted using a continuous flow isotope ratio mass spectrometer coupled to a Carlo Erba elemental analyzer (Micromass, Manchester, UK). The carbon content was then compared with that of Chromosorb^{TM} plus tin alone. The results presented in figure 3.2 show that the acid itself did not add carbon, as there was no more carbon in the 'acid' samples than there was in the Chromosorb^{TM} control. On the contrary, the tin plus Chromosorb^{TM} alone were slightly enriched in carbon compared to the acid samples on average. This was reasonable, based on the fact that under acidic conditions CO_2 will dissolve less in aqueous solution and it was assumed

that CO_2 dissolution was one source of exogenous carbon contamination. Interestingly, Chromosorb^{TM} itself seems to absorb carbon from the atmosphere fairly quickly: after baking Chromosorb^{TM} at 500^{o} C its background carbon levels dropped from 4.7±1.6 µg carbon to 1.2±0.6 µg carbon (indistinguishable from a cleaned tin on its own); but after it was left to stand for several weeks open to the atmosphere it apparently re-absorbed atmospheric CO_2, as the same material showed an average background of 3.3±1.2 µg C, close to the level prior to baking out (figure 3.3).

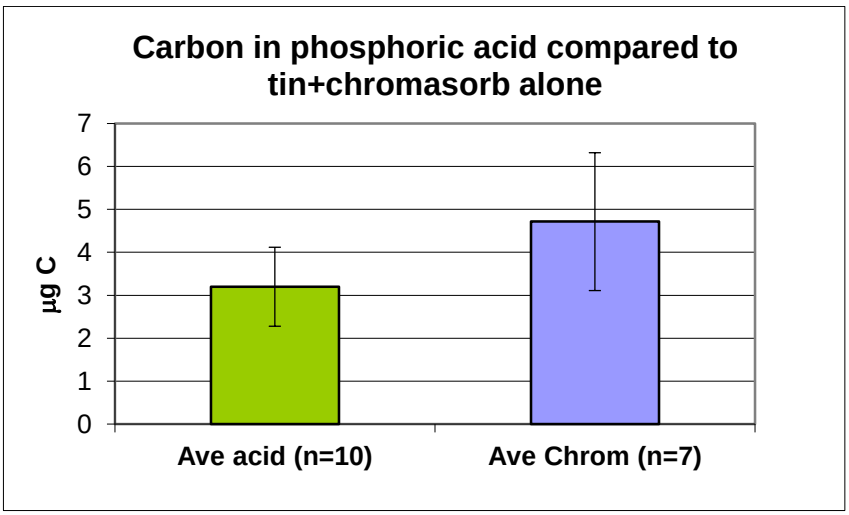


Figure 3.2. Background carbon measurement of phosphoric acid combined with tin & Chromosorb^{TM} versus tin+Chromosorb^{TM} only. Note that the tins were not cleaned in these instances.

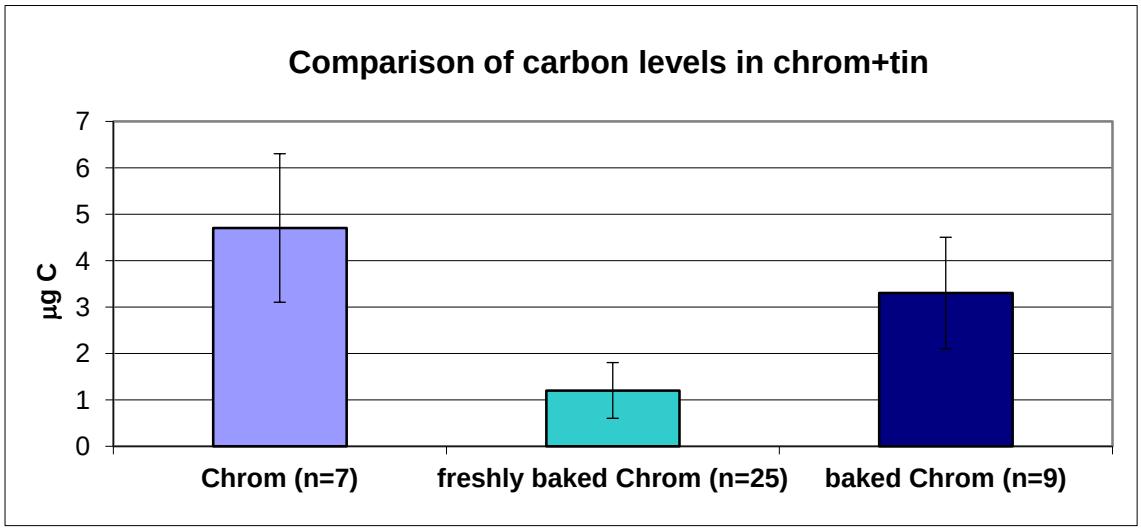


Figure 3.3. Background carbon for Chromosorb^{TM}+tin, Chromosorb^{TM}+tin measured immediately after baking at 500^{o}C and after a few days.

Additional potential sources of carbon contamination as a result of sample processing are the evaporator (Genevac EZ2 Personal Solvent evaporator, acid compatible) and the freeze dryer, both used to remove excess water from the HPLC eluent. However, there was no indication that either of the instruments was contributing carbon. Acidification of the water prior to loading onto Chromosorb^{TM}, thereby preventing from CO_2 to dissolve in the water (as seen also by combustion of Chromosorb^{TM} samples containing phosphoric acid), reduced the carbon levels measured from an average of 4µg carbon to 2µg (table 3.1). Since during the process of Hyp extraction, the samples are in acidic aqueous solution at all times, it seems unlikely that the backgrounds observed are a result of dissolved CO_2. However, the method was slightly modified to use 1 mM HCl rather than MilliQ in the last stage of re-constituting the dry amino acids to be moved to the tin capsule for combustion, in order to avoid the possible addition of carbonates. It should be noted that degassing the mobile phase solution using N_2 was also tried, and acidification was found to be more efficient in reducing background levels. Table 3.1 summarises the contribution of various sources of background carbon examined:

	treatment	**C content (in µg)**
Tin	cleaned	1.0±0 (n=25)
Chromosorb^{TM}+tin		4.7±1.6 (n=7)
	freshly baked out	1.2±0.6 (n=25)
Water (+Chromosorb^{TM}+tin)	left standing*	4.0±2.0 (n=30)
	acidified	2.0±1.0 (n=12)

Table 3.1. Different sources of background carbon identified.* left standing for several weeks open to the atmosphere.The error is based on the standard deviation of the replicated values.

Another plausible source for background carbon in the Hyp isolation process is the HPLC protocol itself. If the major component of the carbon blank is contributed by the HPLC protocols, it is expected that a linear relationship between the size of the carbon

blank and the volume of HPLC eluent collected will be found. In addition, pooling repeated HPLC injections, as the Hyp isolation procedure demands, should be effectively combining the background carbon contributions from each injection, magnifying the effect of the carbon blank. Is the Primesep A HPLC column adding background carbon? Both the C_{12} alkyl groups bonded to the surface of the silica backbone and the incorporated cation-exchange sites (carboxylic acid groups) of the stationary phase could potentially bleed carbon, contaminating the amino acid fraction collected (e.g. Teutenberg et al., 2006; Luo and Carr, 2008). Dissolved compounds in the system lines could also be contributing carbon. Another potential source is 'sample handling'. The method of separating the Hyp fraction involves the use of many collection tubes, evaporation and merging tubes contents. Although not reported in the literature, it seems reasonable that sample handling may also introduce contamination.

In order to quantify the procedure blank to the point of graphitisation, whenever amino acids were collected for dating, a "blank" fraction (4 tubes of 18mL) was collected in places in the chromatogram where no amino acids were eluting. These were concentrated using the EZ2 evaporator, re-injected (via 3 injections) onto the HPLC column, and again eluent fractions were collected (12 tubes of 15mL water). These were then combined and evaporated again, put onto tinned Chromosorb^{TM} and combusted using the Carlo Erba elemental analyser which was coupled to an OPTIMA stable isotope ratio mass spectrometer, in order to detect the carbon content. On average, the blank samples contained 10 micrograms of carbon that could be attributed to bleed, handling and combustion. This contamination would make-up 1-2% of the final amount of carbon dated (depending on the volume collected and the size of the

graphite). This value could be significant, especially when trying to date very old bones, depending of course on the age of the contamination. It should also be noted that the carbon background levels measured were very variable, ranging between 2μg and 16μg C, as shown in table 3.2:

Sample	μg C per 180mL HPLC 2nd column eluent	μg C per mL HPLC 2nd column eluent
CH11 blank (27/1)	10	0.05
SCO2 blank (1/4)	8	0.04
LM blank (27/3)	10	0.05
MR blank (26/3)	16	0.08
SCO3 blank (31/3)	2	0.01

Table 3.2. Pretreatment plus combustion background carbon, as measured on 'blanks' of various separations of single amino acids from hydrolyzed bone collagen. 72mL eluent was collected from the first injection and 180mL was collected from the second injection. These volumes represent the average amino acid elution volume.

Blanks were also assessed in an alternative way: water was injected instead of an amino acid mixture on the first column, and the eluent, over the elution time for Hyp, was collected and concentrated using the EZ2 evaporator to around 3 mL. The reduced volume sample was then re-injected in 3x 1mL injections and again the eluent where Hyp elutes was collected. This was finally evaporated to dryness, reconstituted with water and put onto tinned Chromosorb™ to be combusted using the elemental analyzer. This procedure was repeated 8 times. The results show a carbon content which varies between 2 and 13 micrograms, with an average of 8.4±3.6 micrograms of carbon per Hyp equivalent blank fraction (~54mL of first injection, 30-90mL of second injection). Overall, there is an agreement between the two ways of assessing blanks; both in the average carbon addition and in its inconsistency. The reason for the variability is still unclear, but could be related to the variable volume of mobile phase collected for each sample, and where it was taken along the chromatogram (both correlating with the

amount of acid in the sample). Dates may therefore be subject to small, unpredictable differences in the amount of background carbon addition.

3.2.2.3 New background carbon levels

By using an LC-Isolink system coupled to an Delta V Advantage isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany) it was shown that the amount of carbon bleeding from the Primesep A column is directly proportional to the amount of acid present in the mobile phase (McCullagh et al., 2010). In addition, the higher the flow rate used the more column bleed expected. As mentioned, HPLC reversed-phase columns in general are known to bleed during chromatography, when the alkyl chain supported by the silica backbone of the column becomes detached. The hydrolysis of those alkyl chains from the stationary phase surface increases with lower pH and higher temperature (Teutenberg et al., 2006; Luo and Carr, 2008). It was therefore decided to modify the amino acid separation method, in the hope of reducing the background carbon added through column bleed.

Modification of the method involved a change in flow rate to 6mL/min throughout the run, and a maximum ortho-phosphoric acid concentration of 0.3% by volume, compared to 15mL/min previously and a final acid concentration of 2% when amino acids that were not of interest were eluting from the column (see chapter 2). The new background carbon levels were, again, measured in two ways: by collecting HPLC eluent during a separation of a sample in a place in the chromatogram where no amino acids were eluting (‘Sample blank’), and by injecting water and treating it like a real sample (‘Blank’). The background carbon measured using both approaches was

0.02±0.01 µg C per mL. Figure 3.4 shows how the new background carbon levels compare with those obtained with the previous method:

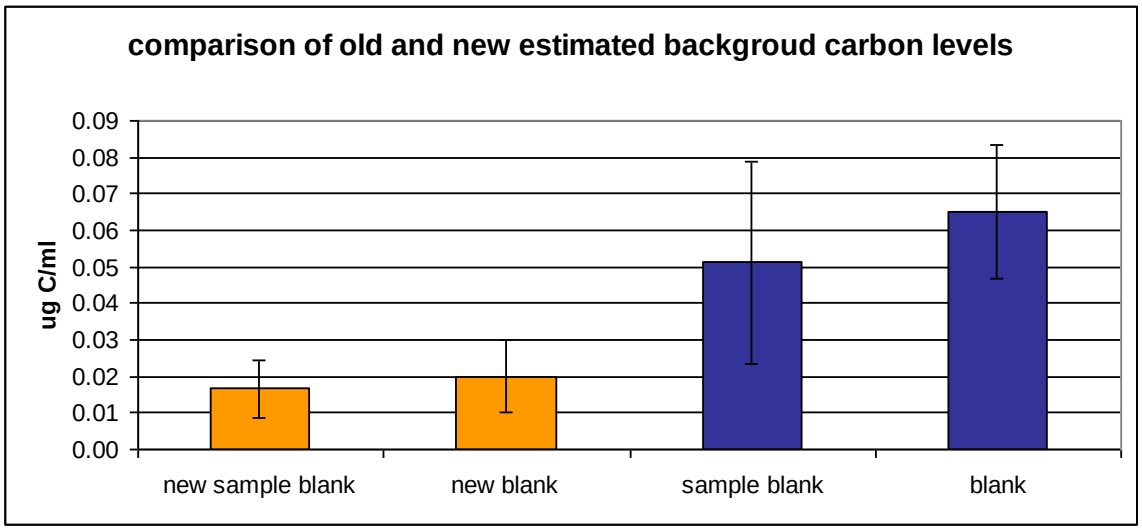


Figure 3.4. Background carbon levels collected with the previous method (blue) and with the new method (orange), in microgram per mL of eluent. 'Sample blank' = eluent collected during the separation of a sample at a position in the chromatogram where no amino acids eluted. 'Blank' = water injected and treated like a real sample.

The new levels of background carbon were also checked by collecting different volumes of HPLC eluent and combusting them to quantify the carbon content (figure 3.5). The masses of the combusted HPLC eluent samples showed a strong linear relationship with the volume collected:

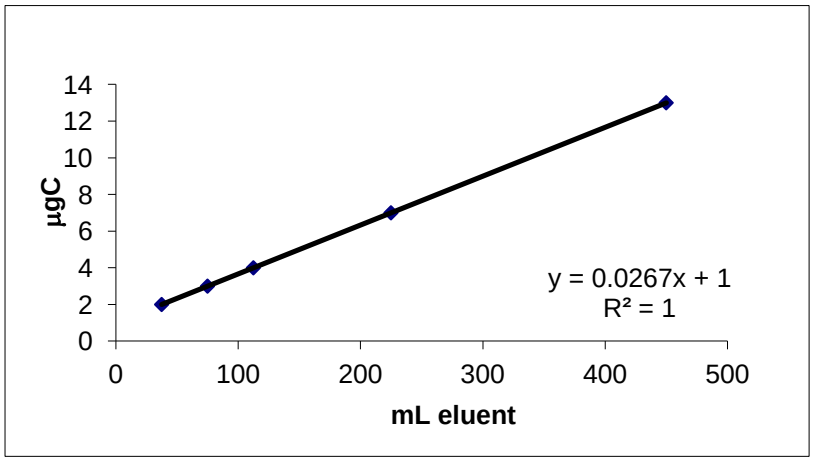


Figure 3.5. Amount of carbon in combusted HPLC eluent plotted against its volume.

This experiment supports the hypothesis that most of the background carbon is coming from column bleed, and that this is between 0.02-0.03µg carbon per mL eluent. The linear regression line crosses the Y axes at 1, indicating the combustion blank (including any contribution from the Chromosorb^{TM}, water, tin, and MS) is 1µg carbon. This is in remarkable agreement with the values reported by Shah and Pearson, which were 0.03µg C/mL, with a combustion blank of 1µg C (Shah and Pearson, 2007).

Background carbon addition was also estimated this time by injecting water before each separation, applying the separation method and collecting mobile phase where Hyp and also alanine eluted in the successive separation ("Hyp-blank" and "Ala-blank"), for both the first and second injections (see figure 2.6 in chapter 2, section 2.2.2.2). This seemed to be the most realistic way for evaluating the blank, as not only column bleed but also any carry-over between runs should be present in the eluent collected. The carbon addition measured this way was 0.02 ± 0.02 micrograms/mL (n=30). The average Hyp blank (n=18) was 1.7±0.7 micrograms C and the average alanine blank was 2.6±1.5 micrograms C (n=10) (1sd).

All the methods used to assess the new level of background carbon presented here were in agreement, showing that about 0.02-0.03±0.02 micrograms of carbon was added to each mL of HPLC eluent collected, an addition of ~1-3 micrograms C to the Hyp fraction. It was not confirmed, however, that the new, lower levels are indeed a result of less column bleed following the change in method. In fact, it seems reasonable to assume that any column bleed from the first separation will be removed by the second separation step, and therefore any column bleed seen would originate from the second

column. However this second injection step wasn't modified, albeit as a result of the modification of the first injection step, less acid was injected onto the 2nd column. The reason why background levels dropped is therefore unclear, and could also be associated with column age, a reduction in other potential sources of carbon such as the EZ2 evaporator, and the use of slightly different acidity of the 1mM HCl to remove carbonates in the last stage of reconstitution prior to combustion. Future work could try to tackle the origin of the background carbon in order to be able to knowledgably reduce it further.

In conclusion, the procedure blank is assessed to be 1-3 micrograms C for the Hyp fraction and around 3-7 micrograms to an alanine fraction. The radiocarbon age of this background carbon can have a significant effect on the dates. In order to test the background age, single amino acids from bones of known age were dated:

3.2.2.4 Single amino acid dates from known age bones

The aim of dating known age bones was to learn about the age and amount of the procedure blank by comparing the bulk date, produced by the "normal" method, and the single amino acid radiocarbon dates. To this end three standard well preserved bones were chosen[11]: a relatively modern historical pig bone from the Mary Rose ship wreck (historically dated), a bison bone from the Alaskan Permafrost with no detectable radiocarbon (the Lemon Mine bone) and a cattle bone from the site of Chalk Hill, which is about one radiocarbon half life. Apart from Hyp, additional amino acids were separated and radiocarbon dated as well. As these bones are not suspected of

[11] Two standards aren't enough, as the blank's radiocarbon age could be the same age as one of the bones and therefore be undetectable.

contamination, all their amino acids should be giving the same radiocarbon age. By dating several fractions from the same bone more data could be obtained on the background issues. The HPLC chromatograms of the separations of the Mary Rose, Lemon Mine and Chalk Hill bones are provided in appendix D, figure D.1, D.2, and D.3, respectively.

Mary Rose single amino acid dates

The Mary Rose (MR) was King Henry the VIIIth flag ship, which sank in AD 1545 (Bradford, 1982). Pig bone from the Mary Rose is routinely used at the ORAU as a historically known age standard to check for background carbon addition and therefore validate dates. Throughout this project Mary Rose hydroxyproline and alanine fractions were collected and dated in order to ascertain that the amount of added carbon in this procedure is low enough to produce accurate dates, that it is reproducible, and to learn about its age to enable correction. The dates produced for Mary Rose hydroxyproline and alanine fractions are presented in table 3.3 and in figure 3.6:

	Pnumber	Lab code	Radiocarbon Date BP (±1σ error)	$F^{14}C$	±	$\delta^{13}C$	Graphite size (mg C)
MR Hyp	24,705.0 NRC2 01	X-2316-57	329 ± 29	0.9601	0.0035	-25.7	0.54
	24,705.1 NRC 01	X-2386-24	351 ± 31	0.9573	0.0036	-24.7	0.48
	24,705.1 NRC1 01	X-2387-13	319 ± 31	0.9611	0.0038	-24.8	0.61
	24,705.2 NRC1 01	X-2395-10	337 ± 26	0.9589	0.0031	-22.3	0.90
MR Ala	24,705.1 NRC2 01	X-2387-12	419 ± 30	0.9492	0.0036	-17.7	0.68
	24,705.1 NRC3 01	X-2386-25	430 ± 40	0.9478	0.0048	-17.6	0.46
	24,705.2 NRC 01	X-2395-9	390 ± 35	0.9526	0.0041	-22.3	0.52
	24,705.0 NRC 01	X-2316-55	400 ± 31	0.9516	0.0037	-30.4	0.46
MR Bulk	(weighted average of tens different dates)[†]		311 ± 11				

Table 3.3. Mary Rose hydroxyproline and alanine raw data. . [†] The bulk dates were produced by ABA and ultra-filtration method (see chapter 2, section 2.2.1.1).

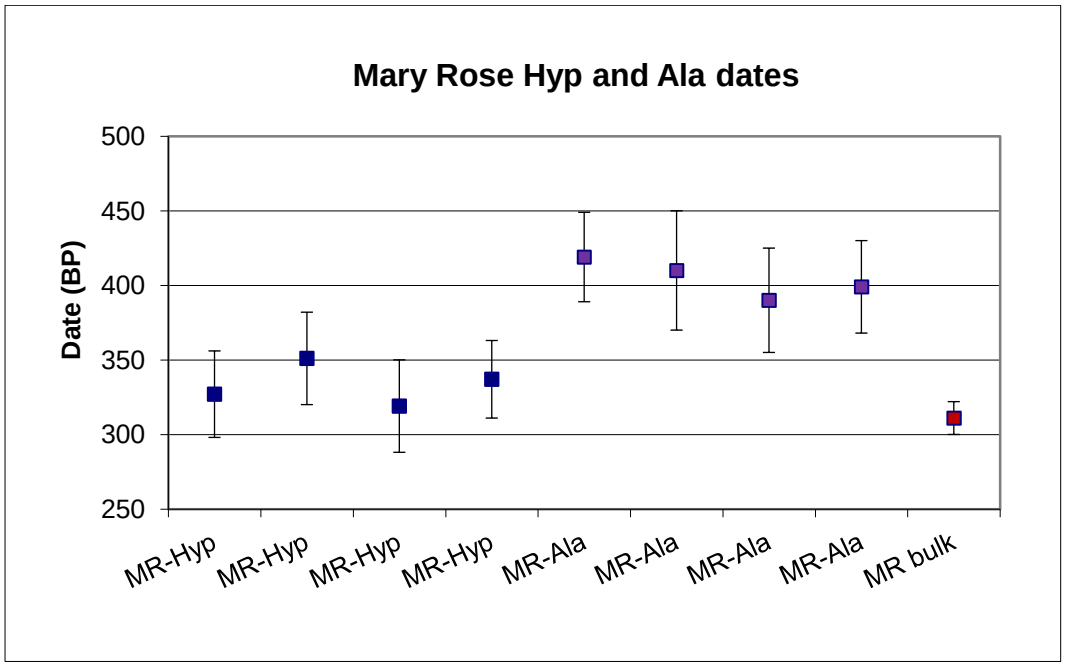


Figure 3.6. Mary Rose hydroxyproline and alanine dates (BP), compared to bulk values.

The Hyp dates show internal agreement: they are statistically indistinguishable, and produce a combined date (weighted average) of 334±17 BP [T=0.6 (χ^2 0.05=7.8)]. They are also indistinguishable statistically from the 311±11 BP bulk date [T=2.2 (χ^2 0.05=9.5)]. The alanines are also producing internally consistent dates, producing a combined date (weighted average) of 409±19 BP, passing a χ^2 test [T=0.8 (χ^2 0.05=7.8)], but failing it when combined with the bulk date [T=24.7 (χ^2 0.05=9.5)].The hydroxyproline dates are on average about 20 years older than the bulk date, and the alanine dates are about 100 radiocarbon years too old.

The Mary Rose dates suggested that the procedure blank contained a radiocarbon 'dead' component, which could be a result of column bleed originating from fossil carbon (Shah and Pearson, 2007; Mollenhauer and Rethemeyer, 2009). The hypothesis that the carbon background is radiocarbon 'dead', or partially 'dead', and HPLC related, is also supported by the experiment presented in figure 3.5, which showed that the masses of the combusted HPLC eluent samples were linearly dependant on the volume of eluent collected. If that is the case then one would also expect to see an HPLC eluent volume dependant shift in the Mary Rose dates. The fact that the alanine dates were consistently older than the Hyp dates, regardless of the final graphite sizes, implied that this is indeed the case, as alanine elutes in a broader peak than hydroxyproline, so 120-240 mL of mobile phase is collected, as opposed to 30-90 mL in the case of hydroxyproline.

Chalk Hill single amino acid dates

Another set of single amino acid dates was produced from a bone from the site of Chalk Hill (CH). Chalk Hill is a site on the western outskirts of Ramsgate, near Upper chalk

cliffs overlooking Pegwell Bay, UK (Whittle, Manuscript in preparation). A sample of well preserved bovine scapula (Chalk Hill 11) was used to extract gelatinised protein which was then subjected to bulk and single amino acid dating.

Different amino acids were isolated twice, once in April 2009, and this was one of the first sets of dates achieved in this project, and then again in November of the same year. The results are shown in table 3.4 and figure 3.7. Interestingly, the two sets differ from each other, and the second set is closer to the bulk date. Although the results of the first set demonstrate statistical agreement with each other when a χ^2 test is applied [T=0.1 (χ^2 0.05=7.8)], they fail it when combined with the bulk date, which is 4887±18 years [T=19.8 (χ^2 0.05=9.5)]. The first set combined date (weighted average) is 5001±20, which is about 100 years too old compared with the bulk. All the dates in the second set apart from the alanine were indistinguishable from the bulk, passing the χ^2 test [T=3.5 (χ^2 0.05=7.8)] and giving a combined date of 4892±16 (weighted average). If the alanine is included the dates do not pass χ^2 test [T=11.6 (χ^2 0.05=9.5)]. The older alanine date (~160 years too old) might be explained by it not being fully collected (the alanine peak is broad, and the tails from both ends might have been left out). In-peak fractionation of the eluting compounds is known to happen during chromatographic separations (e.g. Macko et al., 1987; van Klinken and Mook, 1990). If the amino acid peak is not fully collected, both the $\delta^{13}C$ and $\delta^{14}C$ values of the fraction collected could be different from their true values. This should not have an effect on the date, however, as fractionation of the $\delta^{14}C$ is corrected for according to the $\delta^{13}C$ value (see chapter 1, section 1.2.2), but that is only true if this fractionation of both isotopes is proportional, and there is no evidence that it is when large fractionations occur. The sample's unusually depleted $\delta^{13}C$ of -30‰, as opposed to the more commonly found -25‰ or

less might suggest fractionation has indeed occurred. The Chalk Hill alanine date needs to be repeated before it can be ignored as an outlier, but the fact that the alanine's date was only different from the other amino acids' dates in the second set suggests so. It is not clear why the second set of dates is different from the first. Is it a result of the column getting older and therefore bleeding less carbon? In any case, it is significant that the dates are now both precise and accurate.

	Pnumber	Lab code	Fraction	^{14}C date BP (±1σ error)	F^{14}C	±	δ^{13}C	Graphite size (mg C)
First set	24,706.0 NRC1 01	X-2323-22	CH-Ala	4995 ± 40	0.5370	0.0027	-26.4	0.44
	24,706.0 NRC3 01	X-2323-23	CH-Hyp	5010 ± 36	0.5360	0.0024	-24.8	0.61
	24,706.0 NRC4 01	X-2323-24	CH-GG*	4993 ± 35	0.5371	0.0023	-18.0	1.17
	24,706.0 NRC6 01	X-2323-25	CH-Pro	5005 ± 36	0.5363	0.0023	-22.4	1.00
Second set	24,706.1 NRC1 01	X-2358-31	CH-Ala	5056 ± 60	0.5323	0.0039	-31.2	0.52
	24,706.1 NRC2 01	X-2345-47#	CH-Pro1	4948 ± 34	0.5401	0.0023	-24.5	0.68
	24,706.1 NRC2 02	X-2345-47#	CH-Pro2	4865 ± 33	0.5457	0.0023	-24.3	1.03
	24,706.1 NRC 02	X-2345-42	CH-Hyp	4884 ± 34	0.5444	0.0023	-26.0	1.10
Bulk date	(weighted average of three different dates)†		CH Bulk	4887 ± 18				

Table 3.4. Chalk Hill single amino acid dates (BP). The first set was produced in April 2009, and the second in November of the same year. *GG – a mixture of Glycine, Glutamine and Threonine. #the two CH-Pro dates were combined to produce the date 4906±24 BP (weighted average), and given the OxA number X-2345-47. † The bulk dates were produced by ABA and ultra-filtration method (see chapter 2, section 2.2.1.1).

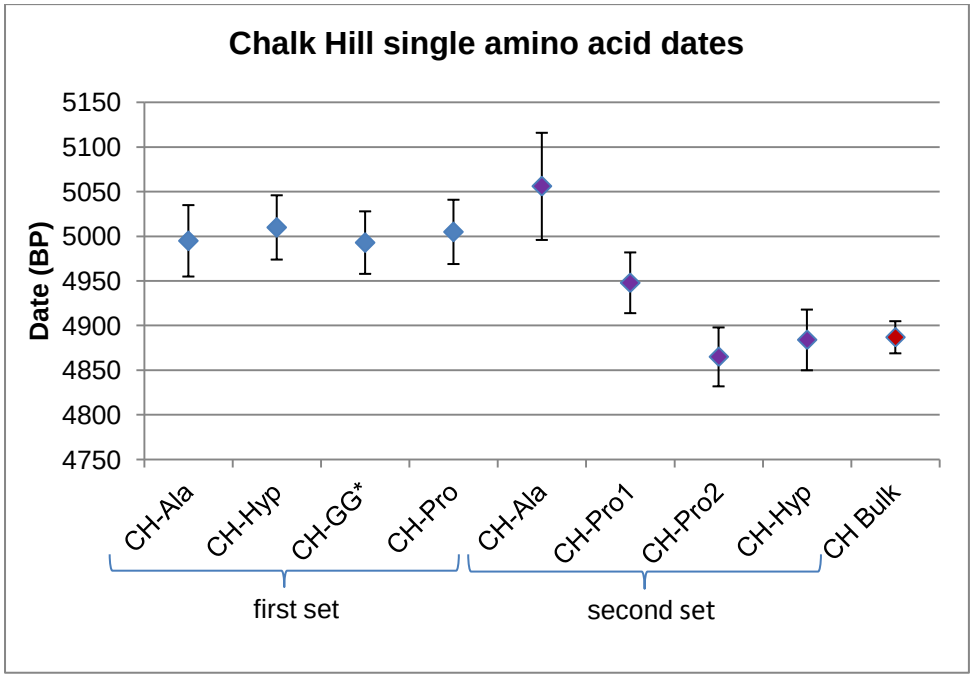


Figure 3.7. The two sets of single amino acid dates (BP) from the Chalk Hill 11 bone, compared to the bulk date.

Lemon Mine single amino acid dates

Any procedure blank will inevitably contain a contaminating modern carbon component, and as already discussed, a modern contaminant will have a significant effect on the date of a very old sample. Procedural blanks of charcoal and carbonate samples, assumed to be modern, can be monitored through the measurement of graphite and marble, respectively. Bone, however, does not have an equivalent ^{14}C depleted standard, and so an old bone, beyond the limit of ^{14}C, has to be used. When choosing a standard bone for ^{14}C background measurement it needs to be not only radiocarbon 'dead', but also as contaminant free as possible, not to obscure the results. Ideal for this is the Lemon Mine Alaskan permafrost bison bone, which has excellent collagen preservation yet its carbon is thought to have no radiocarbon activity, i.e. is radiocarbon 'dead'. This bone is routinely used at ORAU to check modern contamination levels. The hydroxyproline, alanine and proline fractions of a Lemon Mine (LM) Bison long

bone were isolated several times using the same procedure. The dates produced are shown in table 3.5:

Fraction	Pnumber	Lab code	^{14}C date BP (±1σ error)	F^{14}C	±	δ^{13}C	Graphite size (mg C)	Modern C (μg)
LM-Hyp	24,707.2 NRC 01	-	>45,900	0.0013	0.0010	-19.2	1.03	**1.4**
	24,707.3 NRC1 01	X-2395-11	>44,100	0.0016	0.0012	NM	0.83	**1.4**
	24,707.4 NRC 01	X-2395-13	>44,200	0.0020	0.0011	-23.7	0.99	**2.0**
	24,707.4 NRC1 01	X-2395-24	>45,100	0.0013	0.0012	NM	0.90	**1.2**
	24,707.5 NRC 01	X-2403-21	>41,000	0.0023	0.0019	-20.9	0.57	**1.3**
LM-Ala	24,707.2 NRC1 01	X-2358-25	39,000 ± 1,600	0.0078	0.0016	-20.8	0.68	**5.3**
	24,707.5 NRC2 01	X-2403-20	39,200 ± 2,000	0.0076	0.0019	-23.5	0.55	**4.2**
LM-Pro	24,707.1 NRC2 01	X-2345-46	43,900 ± 1,700	0.0042	0.0009	-25.0	1.22	**5.1**
	24,707.2 NRC2 01	-	45,500 ± 2,000	0.0035	0.0008	-24.0	1.24	**4.3**
LM-Bulk[†]	18,801.101 AF* 01	-	>49,000	0.0003	0.0010	-19.8	2.10	**0.6**

Table 3.5. Lemon Mine hydroxyproline, alanine and proline dates (BP). NM – Not measured.
[†]a characteristic LM bulk date, produced by ABA and ultra-filtration method (see chapter 2, section 2.2.1.1).

All the hydroxyproline dates produced for the Lemon Mine bone were older than 41 ka BP. The two alanine dates can be combined, giving the age of 39,080±1250 BP, and the two proline dates give a combined date of 44,690±1,300 BP [T=0.4 (χ^2 0.05=3.8)] (the combined dates are weighted averages). The different single amino acid dates for Lemon Mine cannot be combined to one radiocarbon date, failing the χ^2 test [T=8.6 (χ^2 0.05=7.8)].

The contaminant affecting the measured ^{14}C activity described by equation 1 could be treated mathematically as made up of a ^{14}C 'dead' fraction and a modern fraction:

(2) $$A_m = f_d*A_d + f_M*A_M + (1-f_d-f_M)*A_s$$

where A_m is the measured activity, f_d and A_d are the fraction 'dead' and its activity, which equals zero, f_M and A_M are fraction modern and its activity, which equals 1, and A_s is the 'real' activity of the sample. Therefore:

(3) $$A_m = f_M + (1-f_d-f_M)*A_s$$

For a radiocarbon 'dead' sample, in which A_s equals zero,

(4) $$A_m = f_M$$

For the Lemon Mine Hyp samples, the fraction modern (f_M, or $F^{14}C$) is therefore between 0.13% and 0.23% of the total sample. By multiplying the activity and the graphite size, one can get the actual modern carbon addition (see table 3.5), which is on average 1.46±0.31 micrograms modern carbon for the Hyp samples (the error is based on the standard deviation of the replicated values). The same applies for the alanine samples, where the $F^{14}C$ is around 0.8% of the sample, and the addition of modern carbon calculated in the same way is 4.73 ± 0.76 micrograms. For the Pro samples, the $F^{14}C$ is 0.3-0.4%, and the modern carbon addition is the same as that for Ala: 4.71 ± 0.60 micrograms.

3.2.2.5 Correction algorithm

The accuracy and precision of the dates shown above provide evidence that dating the hydroxyproline is a valid method for bones of all ages, from radiocarbon 'dead' to

modern. However, as the background carbon addition is fairly constant, it is possible to use these known age bones to obtain the procedure blank's modern and 'dead' components and create a correction algorithm that can then be applied to any single amino acid bone date produced in the same way.

Equation 3 can be rearranged to give:

(5) $$f_d = (f_M + A_s - f_M * A_s - A_m)/A_s$$

The amount of modern carbon added to samples by the procedure is assumed to be known, based on the LM measurements, and is 1.4 micrograms/graphite for Hyp and 4.7 micrograms/graphite for alanine (see section 3.6). The amount of 'dead' carbon can be therefore calculated, using equation (5) and the Mary Rose dates: the A_s for Mary Rose is known (0.96202), as is the A_m for each sample. f_d is calculated for each sample, and the 'dead' carbon in micrograms is then attained by multiplying f_d by graphite size (table 3.6 and appendix C, table C.1).

The amount of 'dead' and modern carbon now known, the weight and activity of the contaminant can be easily calculated. A_m can also be represented as follows:

(6) $$A_m = f_c * A_c + (1 - f_c) * A_s = W_c/W_T * A_c + W_s/W_T * A_s$$

Where f_c is the fraction of contaminant, A_c is its activity, W_c is its weight, W_T is the total weight of the sample+contaminant and W_s is the weight of the sample and the rest of the symbols are as above. The corrected activity of the sample is therefore:

(7) $A_s = (A_m - W_c/W_T * A_c)/(W_s/W_T)$

For each sample, equation (7) can be used to calculate the correction that needs to be applied to compensate for the date shift caused by the addition of the contaminant.
The error for the weight of the contaminant is:

(8) $\delta A_s(W_c) = \partial A_s/\partial W_c * \delta W_c = -A_c/W_s * \delta W_c$

The error for activity of the contaminant is:

(9) $\delta A_s(A_c) = \partial A_s/\partial A_c * \delta A_c = -W_c/W_s * \delta A_c$

And the total error is:

(10) $\sigma^2 = \sqrt{[\delta A_s(W_c)^2 + \delta A_s(A_c)^2 + \delta A_s(AMS)^2]}$

Where A_s(AMS) is the AMS measurement error

On average the addition of 'dead' carbon to any Hyp fraction is 1.81±1.10 micrograms, and so 3.24±1.14 micrograms C of contaminant are added to each Hyp sample (=weight of contaminant, W_c) on the whole. The contaminant activity is 0.44±0.18 (=activity of contaminant, A_c). The alanine samples had on average 6.7±1.93 micrograms of 'dead' carbon added, and so the W_c was 11.43±2.05 micrograms C and A_c was 0.41±0.10 (table 3.7). Table 3.6 shows the calculated 'dead' carbon and the corrected dates for the Mary Rose hydroxyproline and alanine samples (see calculation details in appendix C table C.2).

Pnumber	Fraction	^{14}C date BP (±1σ error)	f_d	Graphite size (mg)	Dead C (µg)	Corrected ^{14}C date BP (±1σ error)
24705.0 NRC2 01	MR - Hyp	327 ± 29	0.002	0.54	**1.1**	**301 ± 59**
24705.1 NRC 01	MR - Hyp	351 ±3 1	0.005	0.48	**2.4**	**321 ± 65**
24705.1 NRC1 01	MR - Hyp	319 ± 31	0.001	0.61	**0.6**	**296 ± 58**
24705.2 NRC1 01	MR - Hyp	337 ± 26	0.003	0.90	**3.0**	**322 ± 43**
24705.1 NRC2 01	MR - Ala	419 ± 30	0.014	0.68	**9.3**	**343 ± 39**
24705.1 NRC3 01	MR – Ala	410 ± 40	0.015	0.46	**7.1**	**318 ± 60**
24705.2 NRC 01	MR - Ala	390 ± 35	0.010	0.52	**5.3**	**288 ± 56**
24705.0 NRC 01	MR - Ala	399 ± 31	0.011	0.46	**5.2**	**285 ± 68**

Table 3.6. Calculation of the 'dead' carbon added by the procedure to the Mary Rose samples, and the dates (BP) after applying the correction algorithm. MR Bulk date is 311±11 (weighted average of tens different dates). Dates as in table 3.3.

The Chalk Hill dates also support a background activity of around one radiocarbon half life, as there was no difference in age between the amino acids (apart from the alanine in the second set of dates), and they were indistinguishable from the bulk date (table 3.4). Interestingly, the background levels estimated by collecting blanks in different ways (see section 3.5) were lower than the ones deduced from known age bones' age shift, especially for the alanines. A possible explanation could be that some material (e.g. "column bleed") gets eluted only when a sample is being separated or eluted.

Amino acid	Dead C added (µg)	Modern C added (µg)	Total C added (procedure blank, µg)	Activity of blank
Hyp	1.81±1.10	1.46±0.31	3.24±1.14	0.44±0.18
Ala	6.73±1.93	4.73±0.76	11.43±2.05	0.41±0.10

Table 3.7. Summary of the sample processing blank values as assessed by dates from bones of known age. The uncertainty is the propagated error of the different measurements.

Table 3.6 and in Figure 3.8 present Mary Rose Hyp dates before and after applying the correction algorithm. Note that the correction makes the dates closer to the bulk value, and the error bars are somewhat enlarged.

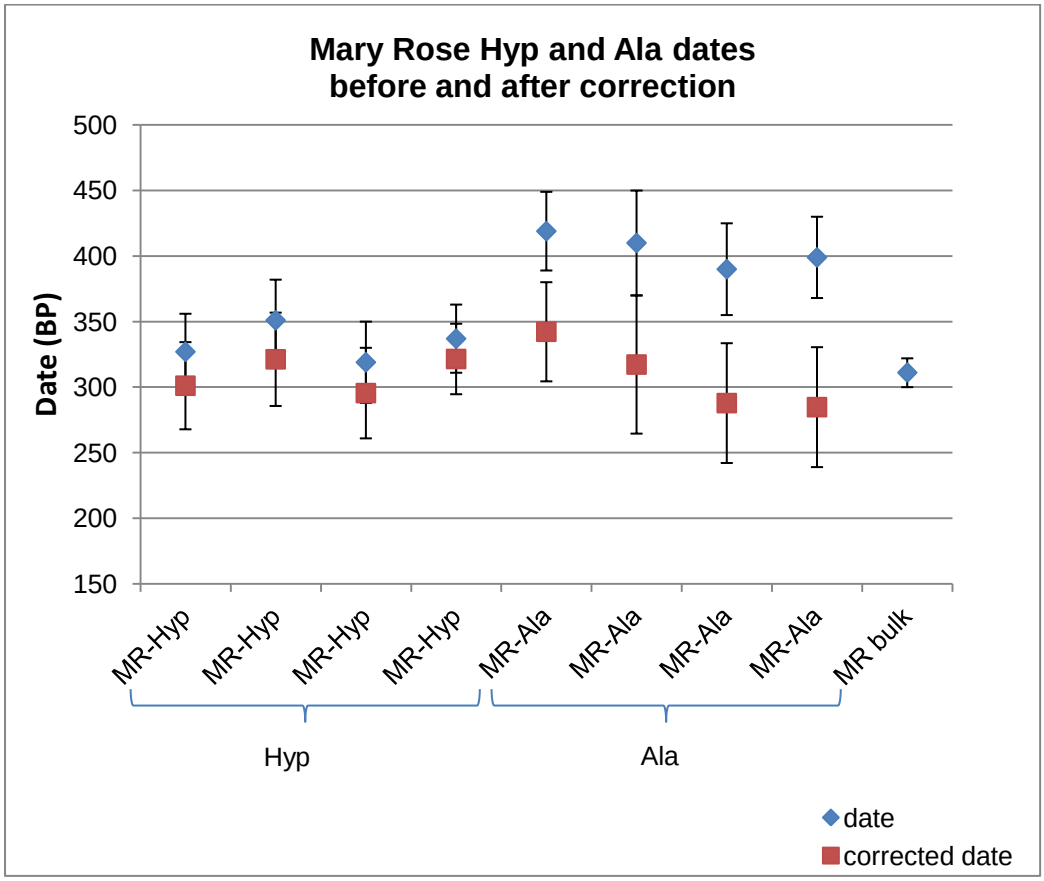


Figure 3.8. Mary Rose Hyp dates (BP) after applying the correction algorithm.

The same was done for the Lemon Mine hydroxyproline and alanine dates, this time $F^{14}C$, or the activity, is on the Y axe, as shown in figures 3.9 and 3.10 (see also appendix C table C.3 for more details). After correction, the activity oscillates closer to zero, as expected from a radiocarbon 'dead' sample.

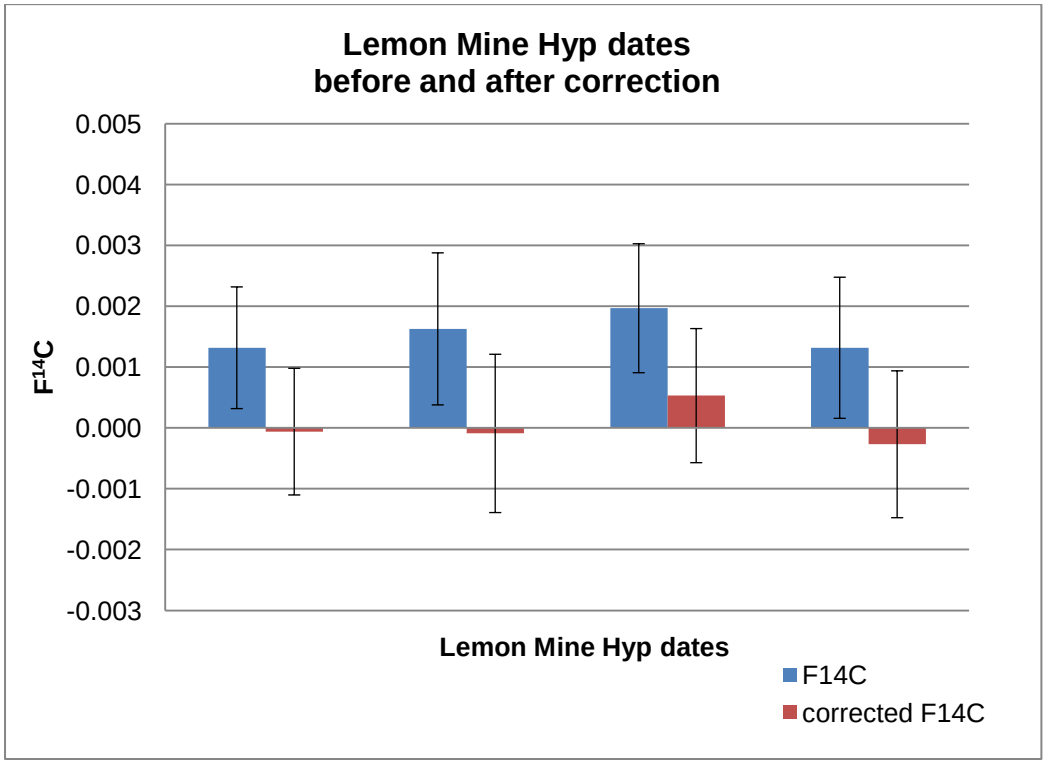


Figure 3.9. Four different Lemon Mine Hyp dates after applying the correction algorithm. Note that the activity now is closer to zero, as expected from a radiocarbon 'dead' sample.

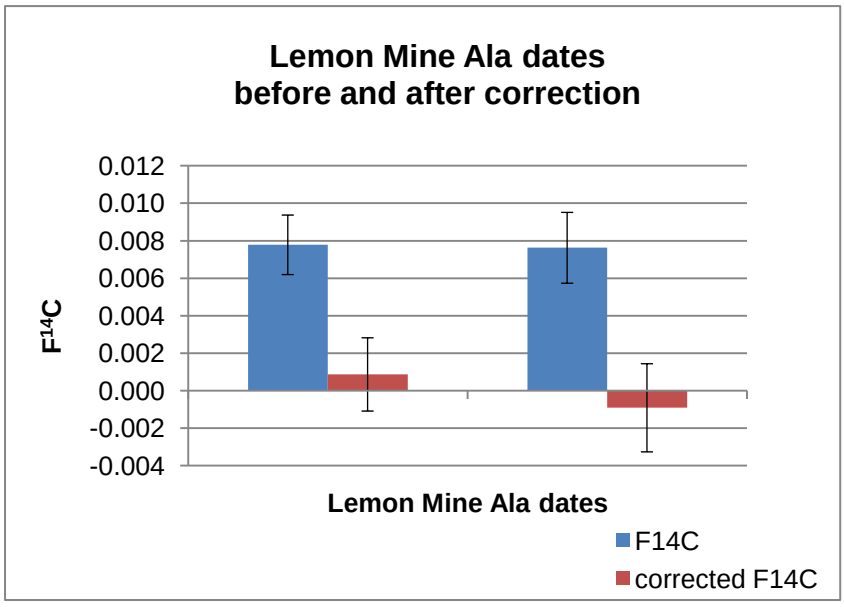


Figure 3.10. Two different Lemon Mine alanine dates after applying the correction algorithm. Note that the activity now is closer to zero, as expected from a radiocarbon 'dead' sample.

3.2.3 Bone contaminants: deliberately contaminated bones

It has so far been assumed that isolation of Hyp would deal with any contamination that might have occurred prior to chromatography, and the efforts were invested in trying to assess the procedural background addition as the only source for any measurement inaccuracy. But can the hydroxyproline dating method indeed remove effectively contaminating compounds?

In theory, the hydroxyproline dating method should be capable of isolating Hyp from almost all contaminants that get co-extracted with, or are chemically bound to collagen, regardless of the bond- type (excluding contamination that contains Hyp, e.g. collagen derived consolidant, such as hide glue). The only possibility in which the hydroxyproline dating method would fail is if some molecules co-elute with the Hyp fraction during chromatography (e.g. the contamination peak and the Hyp peak, although not bound, co-elute, or that the contamination is directly bound to the Hyp in a bond that is not broken by hydrolysis, and the Hyp and the Hyp plus contaminant peaks cannot be resolved, which is less likely).

To test the Hyp method's contamination removal efficiency, a known age bone can be artificially contaminated with a known age contaminant. It is normally assumed (using the conventional bulk dating protocol) that humic acids would be removed by the pretreatment alkali wash step, combined with gelatinisation and the selection of the >30 kDa molecules through ultra-filtration. However, in their paper from 1995, van Klinken and Hedges have shown that only the HPLC-purified tri-peptides and the ninhydrin methods were capable of completely removing humic acids artificially added to collagen, while conventional radiocarbon chemistries failed (van Klinken and Hedges,

1995). Here, tea was used as a source of modern polyphenols (as a proxy to humic acids) to artificially contaminate a radiocarbon depleted bone, the Lemon Mine bison (which is also thought to be contaminant free). The bone was crushed and de-calcified (see chapter 2), and then immersed in 15 mL of hot tea, vibrating for 1 hour. The sample was then rinsed repeatedly, until the water was not stained anymore. Subsequently it was repeatedly rinsed with alkali, until the wash water was colourless. It was now assumed that any contaminants left in the sample are bound to the collagen. After re-acidification a part of the sample was subjected to ultra-filtration. Lastly, the samples were freeze dried, and combusted on the IRMS to check their $\delta^{13}C$ and C:N values. The results are presented in table 3.8. The successful contamination was verified by colouration, C:N ratios (the contaminated sample had a slightly elevated value), and, finally, radiocarbon date. The tea contaminated bone treated with ABA and ultra-filtration (see chapter 2) yielded a radiocarbon date of 22,170 ± 140 BP, indicating that around 6% of the sample carbon constituted of a modern carbon contaminant.

The next step was to hydrolyze the contaminated sample (see chapter 2) and attempt to isolate a contaminant-free Hyp fraction. Interestingly, prior to the injection to the HPLC a filtration step was taken (so as to remove any undisolved material). This filtration step seemed to produce a clear hydrolysate, with a C:N ratio similar to the uncontaminated bone. It was therefore suspected that the filtration step might be sufficient in removing the contaminant completely. However, the AMS measurement yielded a radiocarbon date of 33,200 BP, demonstrating that around 1% of modern contaminant was still left in the sample. Next the hydrolyzed sample was separated on the HPLC. Figure 3.11 compares a chromatogram of a normal Lemon Mine with the tea contaminated one. In the contaminated one several extra peaks appear, but the Hyp shape and retention time

is unchanged. This peak was collected and run on the AMS, giving a contamination-free date of >44,100 BP, undistinguishable from the untreated LM Hyp date (table 3.8).

	Treatment	C:N	Graphite size (mg)	^{14}C date BP (±1σ error)	% modern ($F^{14}Cx100$)
LM	ABA UF (AF)	3.2	2.1	>49,000[12]	~0%
LM Hyp	**HPLC separated fraction**	**5.1**	**1.0**	**>44,200**	**0.20%**
LM+Tea	ABA	3.4	-	-	-
LM+Tea	ABA UF (AF)	3.4	2.4	22,170 ± 140	6.33%
LM+Tea	Hydrolyzed and filtered	3.2	0.9	33,200 ± 600	1.63%
LM+Tea Hyp	**HPLC separated fraction**	**5.0**	**0.8**	**>44,100**	**0.16%**

Table 3.8. The C:N ratios and radiocarbon dates of tea contaminated Lemon Mine collagen, after subjecting it to different treatments. LM is Lemon Mine, ABA stands for the Acid Base Acid protocol (see chapter 2), UF stands for ultra-filtration, and AF is the ORAU laboratory code for ABA+UF.

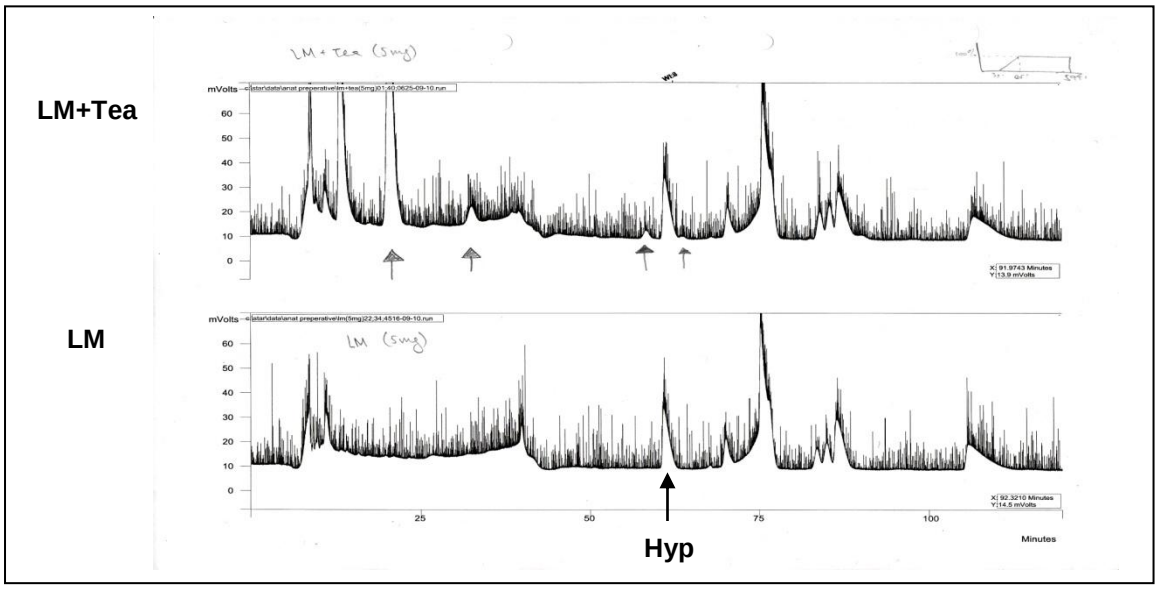


Figure 3.11. A comparison of the HPLC chromatogram of the tea contaminated Lemon Mine bone (top) and the normal Lemon Mine (bottom). Note the several extra peaks in the contaminated sample (marked with arrows). Also, the chromatograms' baseline is noisy**,** due to the UV lamp nearing the end of its lifetime; the straight lines do not represent eluting compounds. More chromatograms of the Tea contaminated bone can be found in appendix D, figure D.4.

[12] Note that the bulk date for the Lemon Mine (one characteristic date out of hundreds) is older than the Hyp ones. "AF" samples radiocarbon age is corrected for pretreatment contamination, while other samples are not. Additionally, the bulk samples are bigger, and bigger samples are subjected to bigger combustion contamination radiocarbon date correction. While these corrections could be legitimate, they could also be an over correction, yielding slightly older dates.

It could be rightly questioned how much tea actually represents naturally occurring bone contaminations or indeed museum related ones. Tea is polyphenolic, as are many humic-type compounds, and is similar to materials used for leather tanning (e.g. oak bark). It is therefore likely to bind to collagen, making it a good material to be used for mimicking the contamination of bone in the burial environment. The successful dating of the deliberately tea contaminated collagen can thus be seen as a 'proof of concept' that the hydroxyproline dating method is capable of removing contaminants when conventional radiocarbon pretreatment chemistries are unable to. The next section includes experiments which investigated whether using the method reliable dates could be obtained from poorly preserved collagen bones.

3.3 Low collagen bones

As discussed in chapter 1, one of the cases in which the hydroxyproline dating method could prove advantageous is when the bone to be dated has low collagen content. If the remaining collagen content of an archaeological bone is under the threshold for the normal chemistry (at ORAU, a nitrogen content of <0.8% of the whole bone is set as the cutoff point, assumed to predict bones with collagen content <1%; Brock et al., 2010b), but the sample itself is very big, a large amount of bone can be used. The extracted Hyp, if existing in sufficient levels, should provide an accurate date. This was tested on a low collagen Horse bone from the site of Kostenki (see chapter 4). Hyp could, at least theoretically, also be found in bones with deteriorated collagen that has already started degrading but is still found in the "collagen" fraction, and where it already started leaching out and might be found in the weak acid soluble fraction (soluble collagen). These two cases are examined in more detail below.

3.3.1 Degraded collagen

Some bones have sufficient initial levels of collagen, but it is of 'low quality' - a significant amount is degraded, which could indicate compromised integrity. At ORAU, when bone collagen is subjected to ultra-filtration as part of the standard pretreatment method aimed to eliminate low molecular weight contaminants (see chapter 2), this low quality collagen will pass through the ultra-filter. It will subsequently get discarded, resulting, in many cases, in samples that are too small for dating. If Hyp could be isolated from such bones, a date could be securely assigned, as one may assume that the Hyp is from the original source. As the hydroxyproline dating method does not require ultra-filtration, the Hyp will be extracted from 'low quality' bones using the same procedure as the other bones (see chapter 2), collecting Hyp from both intact and degraded collagen at the same time.

3.3.2 Soluble collagen - artificially weathered collagen

As mentioned in chapter 1, it had been reported in the literature that bones with little or no insoluble collagen left may still contain indigenous soluble amino acids, originating from degraded collagen. The broken down collagen will leach out of the bone in the presence of water, but in dry sites some may get (temporarily) retained in the mineral fraction. This fraction of degraded, soluble collagen has the potential for dating using the hydroxyproline dating method, where the normal methods fail[13].

As the degradation of bone in the burial environment is heavily influenced by temperature, and it has proven difficult to distinguish those changes caused by cooking

[13] Dating of carbonaceous material from the hydroxyapatite fraction of the bone is considered problematic, because of the increased potential for exchange with carbonates from the environment.

and diagenesis (e.g. loss of collagen, increased crystalinity, altered porosity, histological alteration) (Shipman et al., 1984; Roberts et al., 2002; Koon et al., 2003), cooking bones is widely used as a way of mimicking the degradation of bone in the burial environment.

In order to characterise the "soluble fraction" better, and to find out whether Hyp is retained in this fraction, experiments were undertaken, in which bones were cooked, as a proxy for diagenesis. A modern elephant bone was crushed and subjected to 90°C for different lengths of time (see chapter 2 for method details). The leached fraction (**LF**) (the fraction that was found in the water), the soluble fraction (**SF**) (the acid soluble fraction) and the collagen fraction (**CF**) (the acid insoluble fraction) were collected and characterised for yield, $\delta^{13}C$, $\delta^{15}N$, C:N ratio and amino acid content. The results are presented in figs 3.11-15. All of the results are in line with what Dobberstein et al. reported in their paper from 2009 (Dobberstein et al., 2009).

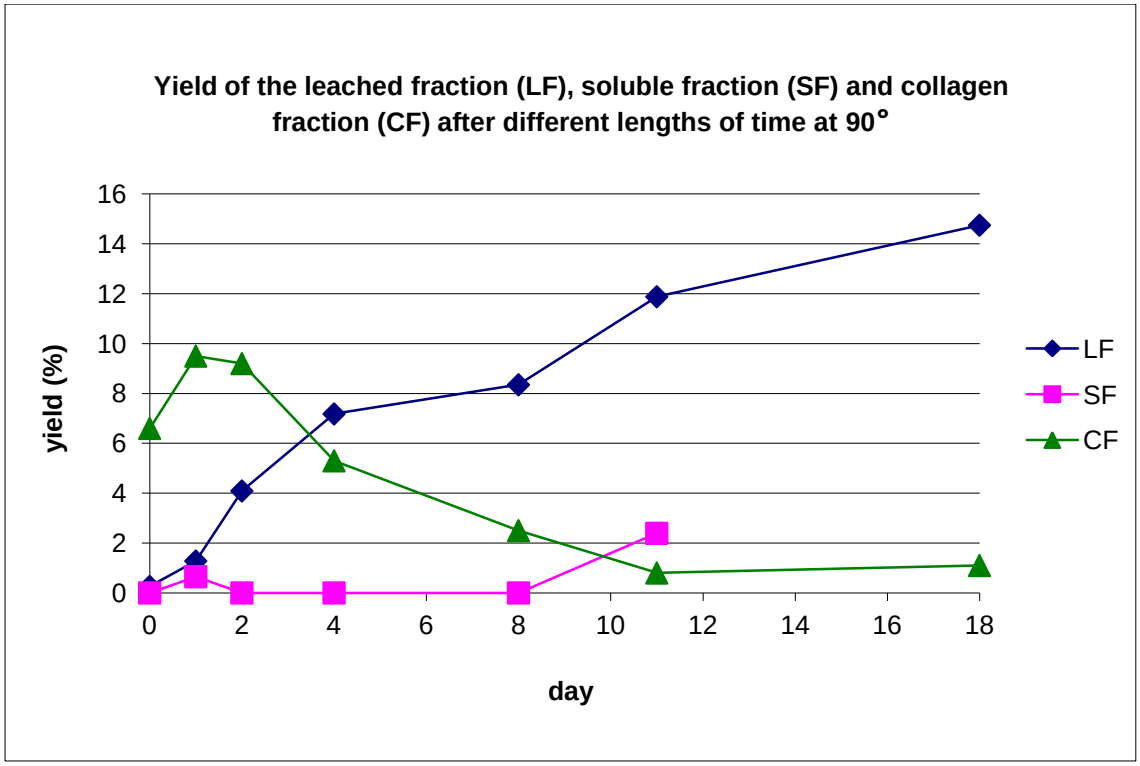


Figure 3.12. Cooking experiment as a proxy for diagenesis: yield of the leached fraction (LF), the soluble fraction (SF) and collagen fraction (CF), after different lengths of time at 90°C. The 18 days SF sample was lost.

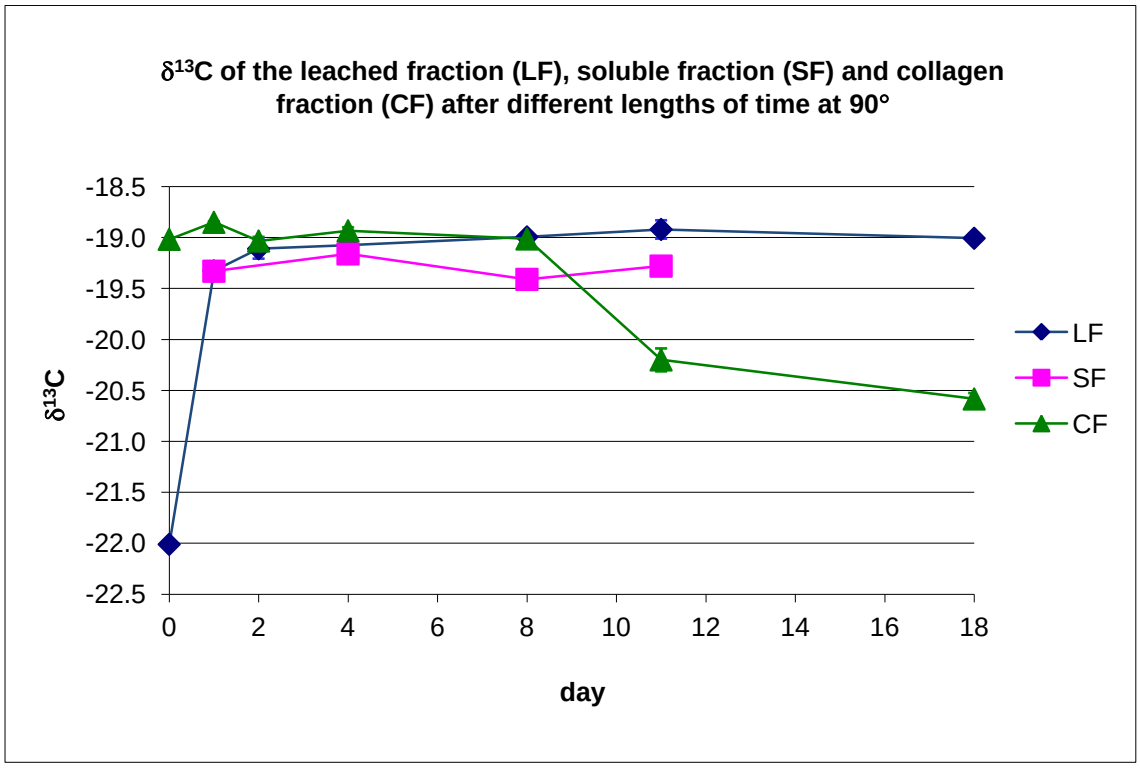


Figure 3.13. $\delta^{13}C$ of the leached fraction (LF), the soluble fraction (SF) and collagen fraction (CF), after different lengths of time at 90°C. The 0 and 18 days SF samples were lost.

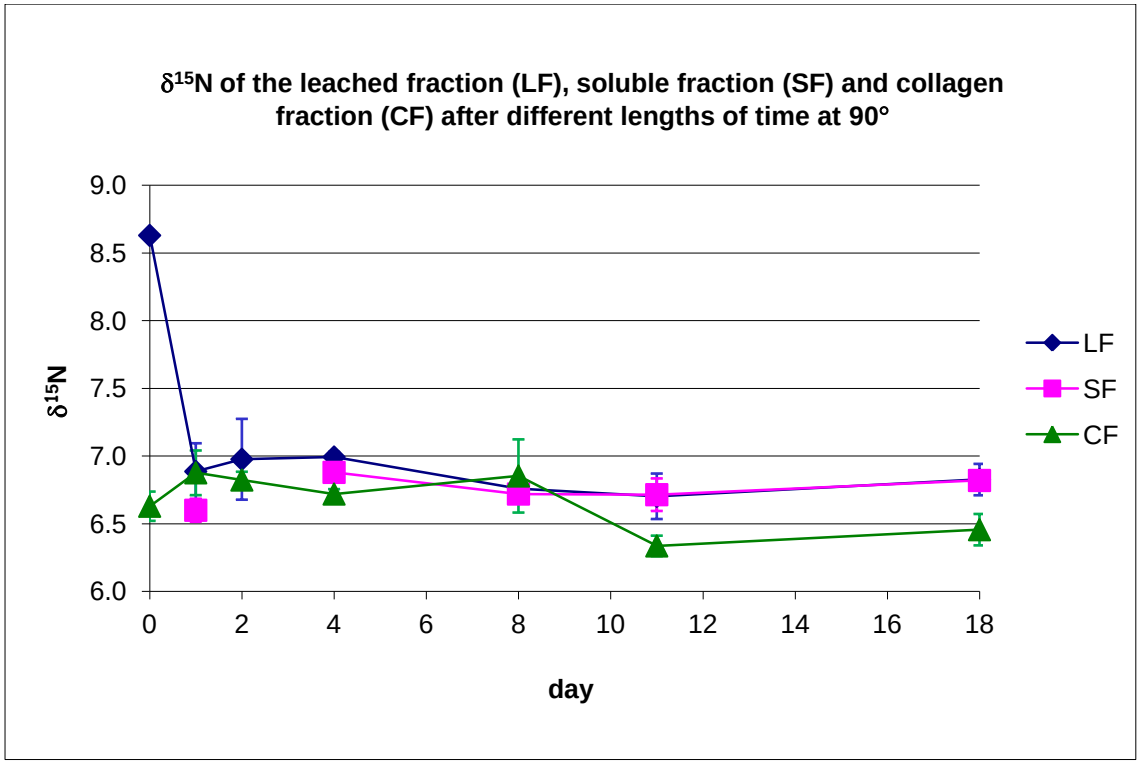


Figure 3.14. $\delta^{15}N$ of the leached fraction (LF), the soluble fraction (SF) and collagen fraction (CF), after different lengths of time at 90°C. The 0 and 2 days SF samples were lost.

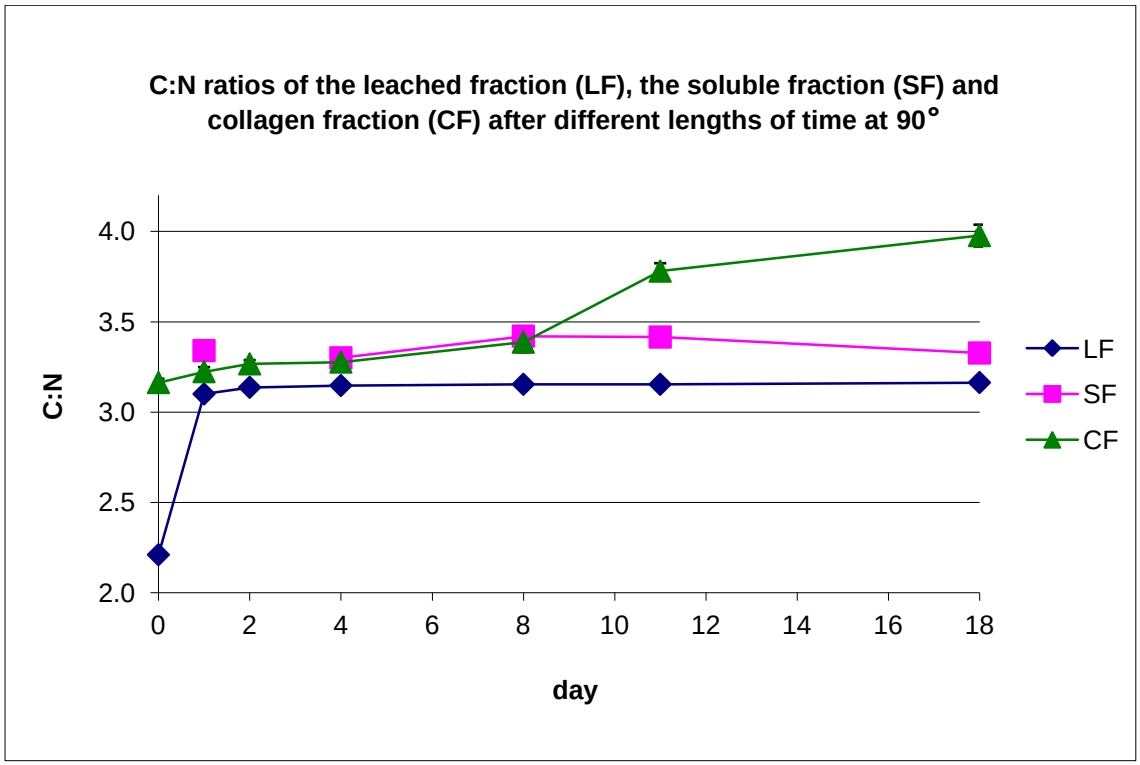


Figure 3.15. C:N ratios of the leached fraction (LF), the soluble fraction (SF) and collagen fraction (CF), after different lengths of time at 90°C. The 0 and 2 days SF samples were lost.

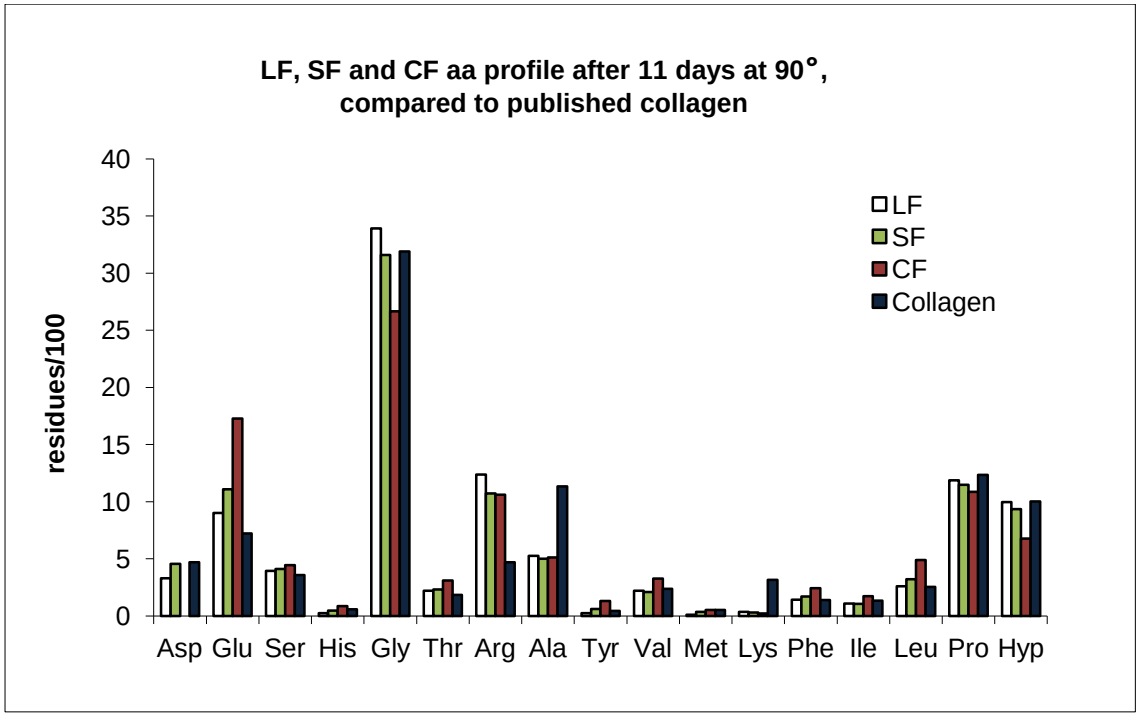


Figure 3.16. Amino acid profile (residues per 100) of the leached fraction (LF), the soluble fraction (SF) and collagen fraction (CF), after 11 days at 90°C, compared to published collagen amino acid profile.

After applying heat, the collagen in the insoluble fraction started breaking down in a sigmoidal pattern. At the same time the soluble collagen accumulated, both the fraction that leached into the water (LF) and the one that was retained within the mineral (SF), (figure 3.12). The measurements of $\delta^{13}C$, $\delta^{15}N$ and C:N suggested that until insoluble collagen levels fell below 2% (after 8 days), the collagen remained intact. When more than 98% of the collagen was lost, a change in the collagen occurred, but only in the insoluble fraction; the SF and LF fractions show similar characteristics, throughout the experiment, different from the CF. SF and LF $\delta^{13}C$ value stayed constant around -19‰ while the CF's was slightly depleted to -20.5‰ after 18 days (figure 3.13); their $\delta^{15}N$ stayed around 6.8‰ while the CF's got slightly depleted to 6.5‰ (see figure 3.14); and their C:N ratio stayed around 3.2 while the CF's was elevated to 4 after 18 days (figure 3.15). All these results are in accord with published data for both artificially degraded

bone and diagenetically altered archaeological bones (DeNiro and Weiner, 1988a; Dobberstein et al., 2009). The fact that the results are in clear support of some of the recent work is not trivial: there is a lot published on collagen degradation, and much of it is inconsistent and unclear. It seems that the artificial degradation does model successfully (at least to some extent) real diagenesis. But what do the different fractions consist of? How are they different from collagen? And can Hyp be found in the soluble fraction?

The amino acid profile of the different fractions at the point where only 1% by wgt of collagen was left (after 11 days of "cooking") was compared to published collagen (figure 3.16). The CF's amino acid profile was slightly different from what is expected from intact collagen, with lower levels of Hyp. However the "missing" Hyp can be found in the soluble collagen. In their paper from 1988, DeNiro and Weiner found that the break between good and bad prehistoric samples (samples with non-collagenous amino acid content) occurred at around 2% wgt collagen, correlating to what was found here (DeNiro and Weiner, 1988a). The "bad" bones were characterised by decreased levels of arginine, proline, glycine and hydroxyproline, and increased levels of serine, aspartic and glutamic acids. They also found, however, that the soluble collagen followed the same diagenetic pattern as the insoluble collagen (ibid). The marked high C:N ratio of the CF in the collagen depleted bone cannot be explained solely by the altered amino acid composition, and is probably a result of more heat resilient NCPs (as suggested, among others, by Hare, 1980 and Ajie et al., 1991, but see Burky et al., 1998, for a different view) and other, non-proteinaceous components (e.g. lipids), making a bigger fraction of the remaining material in the CF.

To conclude, assuming that the cooking experiment models real diagenetic alterations in archaeological bone, in low collagen bones Hyp may still be found in the mineral fraction, as soluble collagen.

3.4 Conclusions

In conclusion, it has been shown that dating the hydroxyproline fraction of collagen is valid for bones of all ages, from radiocarbon 'dead' to modern, as the dates it produced were both accurate and precise. The background carbon added by the procedure has been determined as 3.2±1.1 micrograms, as assessed both by measurement (see section 3.2.2.3) and by calculation from known age bones' slight age shift (section 3.2.2.4). It has also been shown that the activity of this background carbon is about half modern, or in other words, one radiocarbon half life of age (table 3.7). This is very encouraging, as it means that for any 1mg graphite only about 0.15% of both modern and radiocarbon 'dead' carbon is added. As seen in figure 3.1 and from the Lemon Mine results (section 3.2.2.4, table 3.5), the modern component will have an effect only on the very old bones (>40ky) and is close to the radiocarbon limit anyway. The 'dead' component will have a negligible effect (-14 years) on this size of graphite, no matter of what age. In addition, although the number of known age dates collected so far is still quite small, it seems that they are consistent enough for a correction algorithm to be applied (section 3.2.2.5). It is therefore concluded that the hydroxyproline dating method could be applied to archaeological bones. Dating the alanine fraction is slightly more problematic for several reasons. One is obvious – because it is an abundant amino acid in nature (as opposed to hydroxyproline which is almost only found in collagen at high abundance), there's a bigger risk of it being non indigenous. In addition, more background carbon

was found consistently in the alanine fractions collected for dating: it is assessed to be more than 3 times as much as was found in the Hyp fractions (table 3.7, section 3.2.2.5). The effect this increased background level will have on a date depends on its age – modern bones will appear older than they really are, while very old bones will appear more modern. Although applying the algorithm seems to correct the age offset introduced by the procedure blank, the dataset is still very small, and more dates need to be produced in order to increase precision. Even though dating the alanines does not seem to be as straight forward as dating the hydroxyprolines, the alanine radiocarbon dates themselves were useful, as they hint to the origin of the background carbon. The radiocarbon age shift the alanine fractions demonstrated was independent of graphite size. This is in accordance with the blanks coming from the HPLC separation (column bleed), as alanine is eluting off the HPLC column in much broader peaks compared to the hydroxyproline, requiring the collection of big volumes of mobile phase (120-240mL per sample, as opposed to 30-90 mL for Hyp).

The artificially contamination and "cooking" experiments show that, at least theoretically, the hydroxyproline dating method can be applied to bones which are either contaminated or poorly preserved (i.e. have low collagen content), demonstrating the potential of the method. In the next chapter, the results of applying the hydroxyproline dating method to several archaeological bones are presented. The HPLC chromatograms of their separations are presented in appendix D.

Chapter 4: Archaeological applications

4.1 Introduction

In the previous chapter it was established that the hydroxyproline dating method does not add significant amounts of background carbon, and could be useful for dating contaminated and low collagen bones. In section 4.2 the application of the hydroxyproline dating method to bones (or to the previously extracted collagen) from the archaeological sites of ***Flixton, Kostenki, Sungir*** and ***La Ferrassie*** is described. These bones have all been radiocarbon dated in the past, and produced dates that were improbable, most likely due to contamination (either environmental or conservation-related) not fully removed by normal pretreatment methods. The aim was to see whether the hydroxyproline dating method is able to eliminate the effects of contamination to provide new, more accurate results. Each site is briefly introduced, explaining the problems of the existing data. The new Hyp date is subsequently presented and discussed in its wider archaeological context.

In section 4.3 it was attempted to extract and date Hyp from low collagen bones. These bones could not be dated by the normal radiocarbon methods due to their low collagen content, as indicated by the percentage of nitrogen of the whole bones. An entire bone from the ***Kostenki*** site was crushed and the collagen was extracted in order to find enough hydroxyproline to obtain a date. In addition, the potential of the method to date badly preserved bones was explored by looking for Hyp in both the insoluble and soluble collagen fraction of bones from ***St. Cesaire*** and ***Akrotiri Aetokremnos***.

The Hyp dates of archaeological bones presented in this chapter are the ‘raw dates’, as applying the correction algorithm does not change dates of these age ranges appreciably; corrected dates are reported as footnotes.

4.2 Contaminated bones

4.2.1 Flixton horse

The palaeo-lake Flixton is a part of the Star Carr archaeological site, located in the Vale of Pickering, in North Yorkshire, UK. Star Carr is the most renowned Mesolithic site in England, occupied intermittently ca. 10,000-8000 BC. At the time this was a swamp fringed lake, inhabited by hunter-gatherers, the first settlers to move back into Britain after the end of the last ice age. Its importance lies in its unusually good preservation of organic materials, and exceptionally rare finds from the site include barbed points, stag head-dresses and a boat paddle, as well as the earliest evidence of carpentry in Europe, in the shape of wooden platforms, and evidence for a structure constituting "the earliest house" in Britain (the current excavation website: http://www.starcarr.com/; Schadla-Hall and Lane, in press).

Flixton was first excavated by John Moore between 1947-9 as part of a more general excavation of the vale. At Flixton 2 he had found 38 mostly incomplete horse bones and four teeth. The horse bones were recovered from a layer of fine detritus mud (layer H), and none of them showed traces of cut marks. Pollen record and stratigraphy studies suggest that the horse bones date to the Upper Palaeolithic. The pollen profile during the first half of the Holocene has changed from the establishment of a post-glacial mixed forest trees and sequences of wetland plants, with aquatic, reed swamp and fen environments following (Bahn, 2004; Schadla-Hall and Lane, in press).

A bulk sample of the nekron mud[14] associated with the horse bones, taken from around a horse astragalus (Find. No. 2713), yielded a radiocarbon date of 9850 ± 80 BP (CAR-1016). Another bulk mud sample, from the top of that layer, produced a radiocarbon date of 9,270 ± 210 BP (Q-66) (Schadla-Hall and Lane, in press). The reliability of these dates is questionable however, having been produced from the crude mud, liable to humic contamination.

Based on the pollen record it is thought that around 9.9-10Ky BP the climate in this area became warm leading to the spread of the forest. As a result horses became locally extinct. It is therefore believed that horses from this site could not be younger than 9.9-10Ky BP (Schadla-Hall and Lane, in press). A number of the horse bones had been radiocarbon dated over the years, some of them several times, with dates ranging from 10,150 to 9,160 BP, and C:N ratios ranging between 3.3 and 3.5 (table 4.1). Interestingly, the older and more likely dates, are the ones in which the samples were passed through an ion-exchange column as part of the pre-treatment (with the exception of sample OxA-6,329, that gave the date 9,160 ± 80 BP, see table 4.1)[15]. This suggests that perhaps ultra-filtration is less efficient in de-contaminating these samples than the ion-exchange protocol.

The collagen from the horse left astragal from Flixton2 that produced the seemingly too modern date of 9,290 ± 45 (OxA-21,175) was further separated using the hydroxyproline dating method. 100mg were hydrolyzed and a fraction of the hydrolyzate was sent to amino acid analysis. The amino acid profile was

[14] A rapidly accumulating, organic, muddy deposit, characteristic of eutrophic lakes.
[15] Ion exchange as part of the pretreatment process was routinely conducted in the past at the ORAU, before being replaced by ultra-filtration; see chapter 1 and Hedges and Law, 1989.

indistinguishable from published collagen, and so 30 mg of the hydrolyzate were injected to the HPLC (the separation chromatogram can be found in appendix D figure D.5). The collected Hyp fraction yielded the date 10,155 ± 55 BP[16], corresponding to between 12,390 and 11,260 Cal BP range (based on IntCal09 calibration curve, with 95.4% probability) (table 4.1, figure 4.1). This result matches perfectly the archaeological expectation and the ion-exchanged dated bones from the site (figure 4.1). It is concluded that the Flixton horse bone is contaminated (probably with humic acids), and that the contamination was removed by dating the hydroxyproline fraction, and mostly removed by ion exchange[17], but not by the normal pretreatment procedures.

Sample	**Lab code**	**Treatment**	**C:N**	**$\delta^{13}C$**	**^{14}C age BP (±1σ error)**
XB23 left astragal	**X-2395-14**	**Hyp**	**5.0**	**-24.6**	**10,155 ± 55**
	OxA-6,328	AI*	3.3	-20.2	10,150 ± 90
	OxA-20,322	AF	3.4	-21.3	9,626 ± 39
	OxA-20,356	AF*	3.5	-21.2	9,640 ± 40
	OxA-21,175	AF*	3.5	-20.5	9,290 ± 45
1st phalange	OxA-20,695	AF*	3.3	-20.5	9,920 ± 45
	OxA-6,318	AI	NM	-20.8	10,090 ± 90
bone	OxA-6,329	AI*	3.5	-20.3	9,160 ± 80
1st phalange	OxA-20,696	AF	3.3	-21.0	9,975 ± 45
	OxA-6,319	AI	NM	-20.8	10,150 ± 80
soil around bone	CAR-1016		NP	NP	9,850 ± 80
mud from top of an organic mud layer	Q66		NP	NP	9,270 ± 210[#]

Table 4.1. Radiocarbon dates and C:N ratios from different samples from Flixton2. Where samples were radiocarbon dated more than once the results are grouped together. AF designates ultra-filtration; AI designates ion exchange; * designates solvent wash. In blue – the Hyp sample from this study. All the dates apart from the last two (Schadla-Hall and Lane, in press) were done in Oxford. [#]The date is reported to be 8555± 210 BC, which is roughly equivalent to 9,270 ± 210 BP (Schadla-Hall and Lane, in press). NM- Not measured. NP- Not published.

[16] After applying the correction algorithm (section 3.2.2.5 in chapter 3), the date is 10,175± 60 BP, indistinguishable from the 'raw' date.

[17] The chemical explanation for the successful removal of humic acids by the ion exchange is that they have the same charge as the cationic column and hence are not retained with the amino acids.

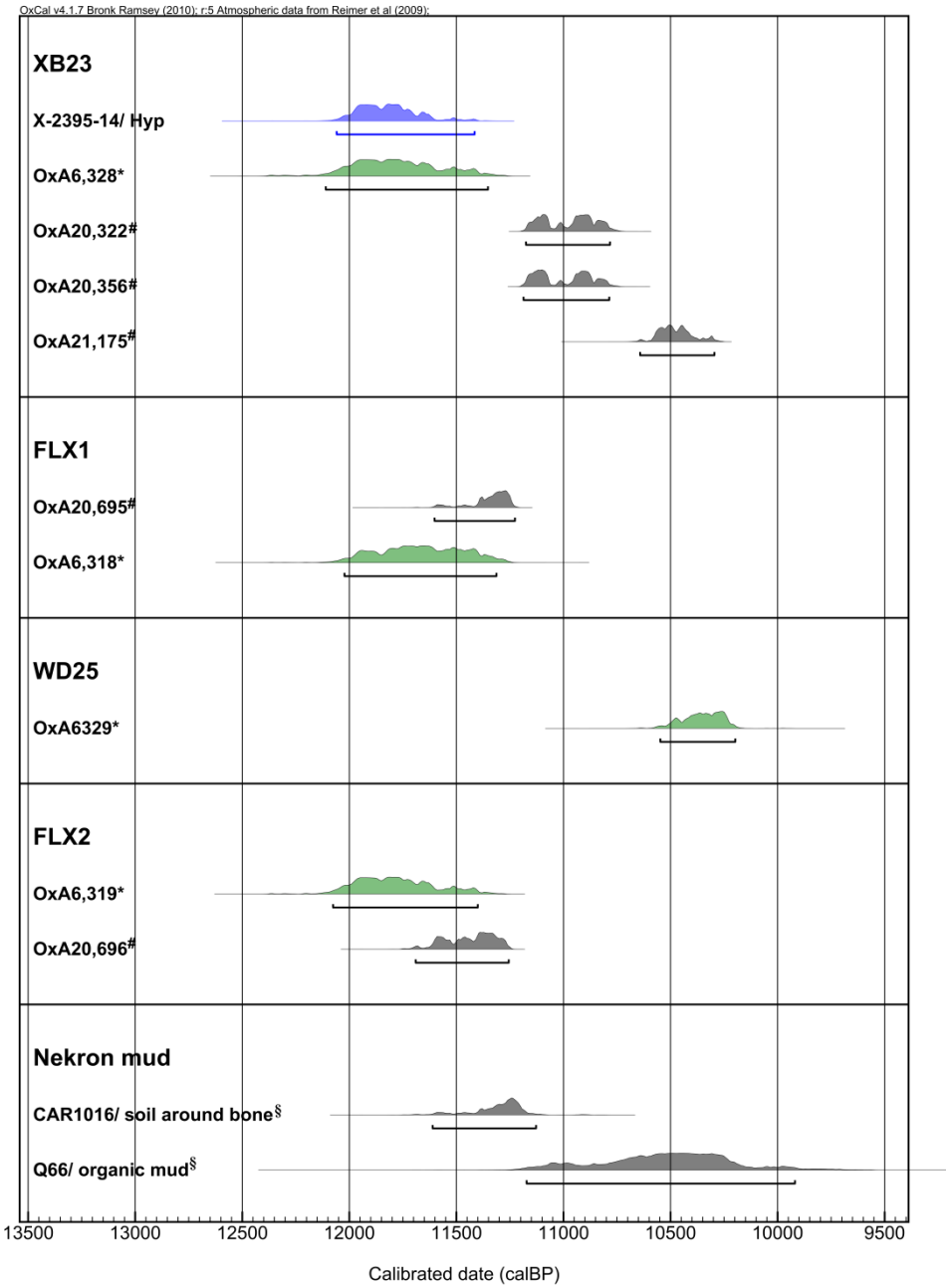


Figure 4.1. Calibrated radiocarbon dates for Flixton 2 horse bones, produced with OxCal v4.1.7 and IntCal09 calibration curve. (Bronk Ramsey, 2009; Reimer, 2009). 1σ error is marked on the likelihood distribution of the calibrated date range**.** * denotes samples pretreated with an ion exchange method (AI) and their likelihood distributions are coloured in green; # denotes samples ultra-filtered prior to AMS dating (AF); § denotes bulk date. The Hyp likelihood distribution is coloured in blue. Dates are as in table 4.1.

4.2.2 Kostenki 14 anatomically modern human

Kostenki, on the banks of the river Don in Southern Russia (figure 4.2), is an important archaeological site that has yielded large concentrations of finds from the Palaeolithic. Some of these date back to 45,000 years ago, demonstrating that a fully developed Upper Palaeolithic tool kit that included sophisticated stone tools, artifacts made of bone, antler and ivory, and small perforated shell ornaments, was present on the central East European Plain as early as anywhere in northern Euroasia. Such early arrival to the east may indicate several separate routes of dispersal for modern humans entering Europe (Anikovich et al., 2007).

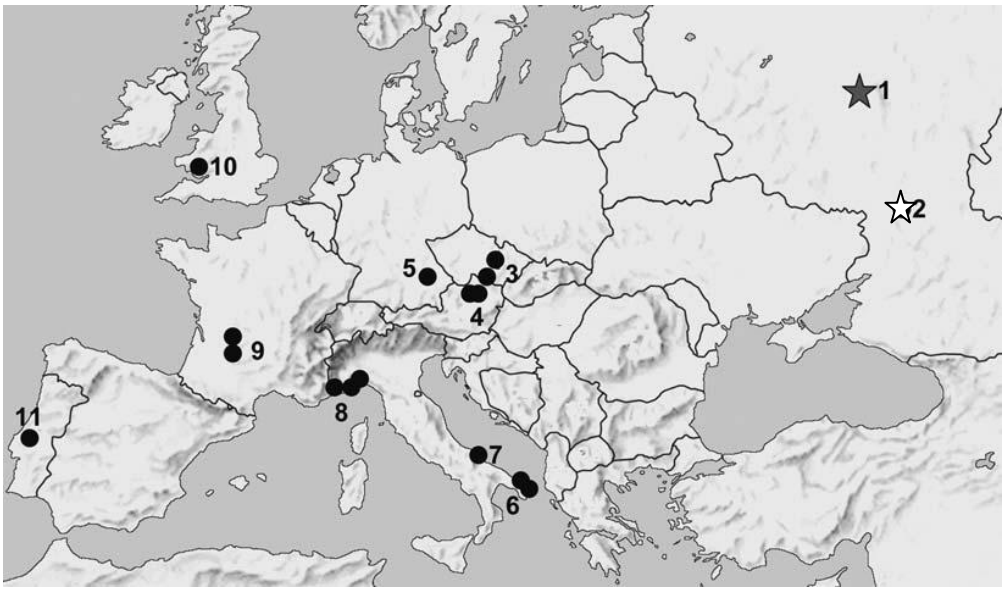


Figure 4.2. Map of Europe with the distribution of Mid Upper Paleolithic sites yielding human "red ochre" burials. 1: Sunghir; 2: Kostenki; 3: Moravian sites of Brno-Francouzská, Předmostí, Dolní Věstonice I and II, and Pavlov I; 4: Krems-Wachtburg and Miesslingtal; 5: Mittlere Klause; 6: Santa Maria di Agnano (Ostuni) and Veneri (Parabita); 7: Paglicci; 8: Ligurian sites of Arene Candide, Barma Grande, Bausu da Ture, Caviglione and Grotte-des-Enfants; 9: Cro-Magnon and Cussac; 10: Goat's Hole (Paviland); 11: Lagar Velho (taken from Dobrovolskaya et al., 2011).

Establishing global human dispersals in the past is increasingly based on the genetic variation of modern populations, and, to a lesser extent, genetic data derived from ancient human DNA. Genetic markers among the former suggest multiple population

movements across Africa, Eurasia and into Australia and Oceania during the Upper Palaeolithic (50,000–30,000 cal BP) (Hoffecker, 2011). Kostenki provides further evidence to support the early arrival hypothesis, in the shape of the mtDNA sequence of the Kostenki 14 (K14) - Markina Gora skeleton.

The K14 human skeleton was excavated near Voronezh, Russia in 1954 (Rogachev, 1955). The skeleton was found in an oval pit, lying on its left side, in an extreme crouched position (figure 4.3). The face was oriented downward with fists reaching for the mandible. The skeleton's position, together with the fact that it was covered in red ochre, suggests an intentional burial. The individual was identified as a male based on pelvic analysis, and the age was estimated, according to the teeth and crania, to be at least 20 years old (Rogachev, 1957; Sinitsyn, 1996). In their paper from 2010, Krause and colleagues published its complete mtDNA sequence (Krause et al., 2010). The sequence shows the five diagnostic substitutions defining haplogroup U2. The U haplogroup is widely distributed in living populations in Europe and is one of the oldest mitochondrial genetic markers in Europe. The mtDNA therefore suggests that the ancestors of the Kostenki 14 human were amongst the earliest humans to have migrated out of Africa, probably around 50,000 BP. This ties in well with the hypothesis that during Early Upper Palaeolithic time-span anatomically modern humans and their industry migrated over large areas, as opposed to long sequences of local cultural development (Hoffecker, 2011).

Figure 4.3. The K14 (Markina Gora) man burial.

What is the date of the Kostenki 14 burial? The burial lies under cultural layer III of Kostenki 14 (~28.3—31.7 ka BP) (Hoffecker, 2011), but no signs of a burial pit were observed from the level of this cultural layer, and so it was assumed by A.N. Rogachev, the excavator, that it must pre-date it (Rogachev, 1955). The pit containing the body had cut through the volcanic ash horizon at the site, identified as the Campanian Ignimbrite (CI), dated by $^{40}Ar/^{39}Ar$ to 39,280±110 y BP (De Vivo et al., 2001). The tephra was clearly visible in the walls of the burial pit but absent from the burial fill (Rogachev, 1957; Sinitsyn, 1996), and is considered in situ, so the K14 skeleton most likely post-dated it. The most probable context for the burial is therefore thought to be with the "cultural layer in volcanic ash" of Aurignacian attribution (between cultural layers III and IVa). The stratigraphic context and direct radiocarbon dates of material from the same cultural level provide a probable date for the human, between 32ka BP and 39ka BP (figure 4.4).

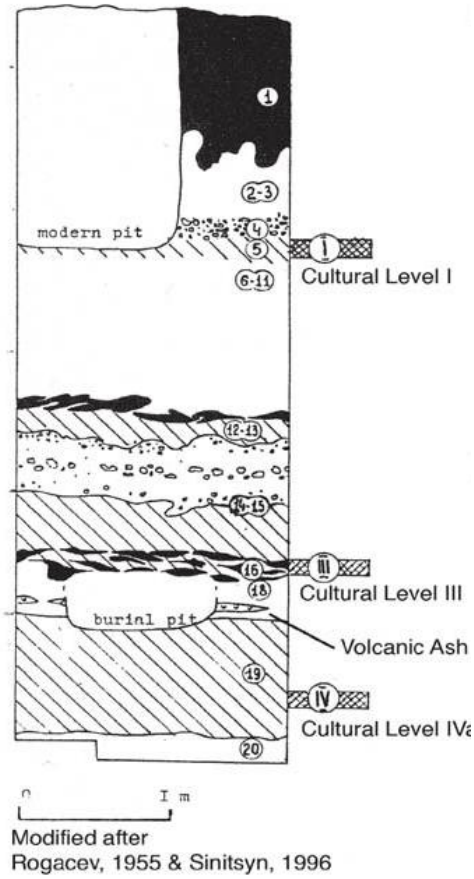


Figure 4.4. Section showing the stratigraphic location of the burial of K14 man (from Krause et al., 2010).

However, direct radiocarbon dating of the skeleton has yielded uncalibrated radiocarbon dates of 3,730 BP and 4,705 BP (Sinitsyn, 2004); and, recently, 13,610 BP (Hoffecker, 2011). An additional attempt at Leipzig, with pre-treatment including ultra-filtration step, failed (Krause et al., 2010). Those dates probably reflect contamination, as the abnormally high C:N ratio suggests (table 4.2). The contaminating carbon is most likely derived from conservation material applied to preserve the bone (BF glue, Professor A. Sinitsyn, Russian Academy of Sciences, Pers. Comm.). In addition, it should be noted that at Kostenki 14, even bones that are not likely to have been conserved exhibit inconsistencies and often yield a wide radiocarbon age range for the same layer, presumably due to humic acids contamination not fully removed.

Radiocarbon dates on charcoal are also problematic, as they appear to underestimate the age of the CI tephra by more than 1ky (careful pretreatment may resolve this – see Douka et al., 2010b). Optically stimulated luminescence (OSL) dates of the sediment underlying the CI, despite having very large standard errors, mostly yielded ages greater than 40ky BP_{OSL}, as expected (Hoffecker et al., 2008).

Fraction/treatment	**Lab code**	**C:N**	**^{14}C age, BP (±1σ error)**	**$\delta^{13}C$**	**Source**
Ultrafiltered collagen	OxA-7126	3.7	4,750 ± 40	-20.7	Sinitsyn, 2004
	GrA-9303	NP	3,730 ± 40	NP	Sinitsyn, 2004
	SR-7366/ UCIAMS-61666	NP	13,610 ± 40	NP	Hoffecker, 2011
Modified Longin; ultrafiltered collagen		4.1	NM	NM	Krause et al., 2010
Ultrafiltered collagen, hydrolyzed and filtered	**OxA-X-2395-16**	**3.0**	**31,080± 350**	**-18.4**	**This thesis**
HPLC separated Hyp fraction	**OxA-X-2395-15**	**5.1**	**33,250± 500**	**-23.2**	**This thesis (Marom et al., 2012)**

Table 4.2. C:N atomic ratios and radiocarbon ages from the Kostenki 14 (Markina Gora) skeleton (a tibia). Note that the C:N ratio of Hyp is 5. NP-not published. NM- not measured. In blue – dates from this thesis.

In an attempt to date again the skeleton at ORAU using conventional bulk dating techniques including ultra-filtration, 0.7g bone powder from the right tibia of the Markina Gora skeleton was sampled (figure 4.5). In order to avoid the contamination, the interior of the bone was sampled. However the resulting 40mg ultra-filtered collagen had a very high C:N ratio (3.8) and the sample was failed. As a part of this thesis, 40mg of the contaminated collagen were hydrolyzed. As hydrolysis (followed by filtering) was able to remove most of the contamination when bone was deliberately

contaminated with tea (see section 3.2.3 in chapter 3), it seemed sensible to also assess this for the K14 contaminated collagen. The resulting C:N ratio was 3.0[18], indicating that most of the contamination if not all of it may have been eliminated. This sample was thereafter run on the AMS to yield a bulk date of 31,080 ± 350 BP, closer to the expected date than any direct dating attempt so far. In addition, as more material was needed, another 0.5g of bone was crushed, and 50mg collagen was extracted and hydrolyzed[19]. 40mg of the hydrolyzate was separated and the Hyp fraction radiocarbon dated as described in chapter 2 (the HPLC chromatogram is presented in appendix D, figure D.6). The uncalibrated date obtained from the K14 skeleton Hyp fraction was 33,250 ± 500 BP[20] (table 4.2). The calibrated date (based on IntCal09 calibration curve, with 95.4% probability) is 39,080 - 36,680 Cal BP, in excellent agreement with its chrono-stratigraphic position (table 4.3).

Figure 4.5. The K14 man tibia (the hole shows where the aDNA samples were taken) (left) and skull (right).

[18]Or 3.3 when measured on a different MS when producing the graphite
[19] Interestingly, this time the collagen yield was 10%, as opposed to ca. 6% when the extraction was done with the base wash and ultra-filtration steps
[20] After applying the correction algorithm (section 3.2.2.5 in chapter 3), the date is 33,850 ± 520BP, both dates indistinguishable statistically: T = 0.7 (χ^2 0.05 = 3.8).

A Bayesian model was built using OxCal v4.1.7 (Bronk Ramsey, 2009), using dates within and below the CI, including the K14 Hyp date (figure 4.6 and table 4.3). The following assumptions were made:

- Dates are progressively younger
- The eruption date is taken as 39,280 ± 110 BP (De Vivo et al., 2001)
- The ABOx-SC treated charcoal from the Level of Volcanic Ash (LVA) at Kostenki 14 (OxA-19021: 35,080 ± 240 BP) is taken as an approximation for the pre-eruption date (Douka et al., 2010b)
- A date obtained from a charcoal found directly above the ash in cultural layer I at Kostenki 12 (GrN-7758: 32,700 ± 700 BP) post dates the eruption; the Kostenki 14 man is assumed to be from the same level

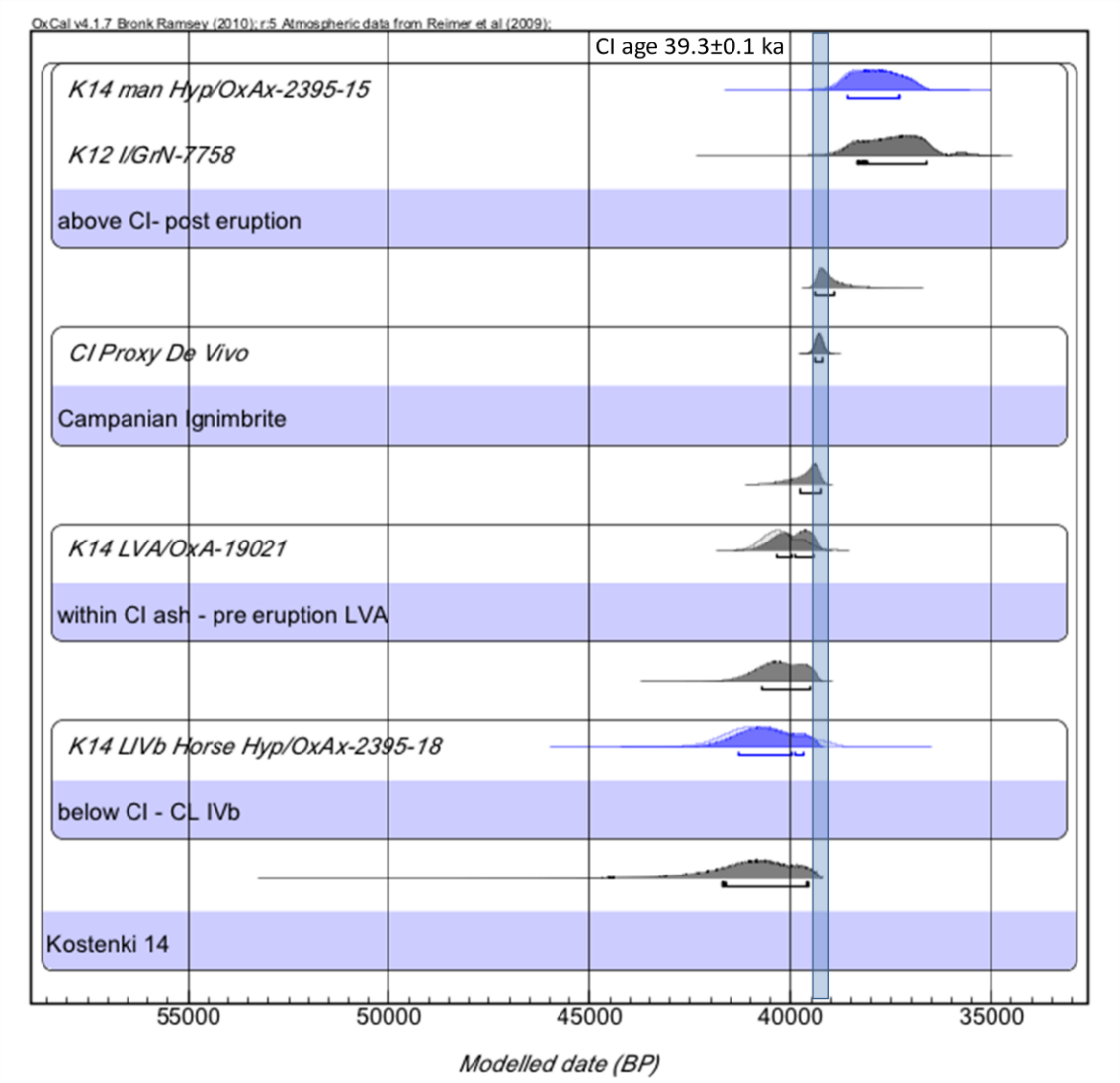


Figure 4.6. Bayesian model built with OxCal 4.1.7, using IntCal 09 (Bronk Ramsey, 2009; Reimer, 2009) to calibrate the Kostenki dates. 1σ error is marked on the likelihood distribution of the calibrated date range**.** The CI eruption date (blue line) is given here as the $^{40}Ar/^{39}Ar$ age of (De Vivo et al., 2001), at 39.28 ±110 Cal BP. The K14 man Hyp postdates the CI eruption, while the K14 horse Hyp predates it, as expected by their stratigraphic position (both Hyp likelihood distributions are in blue**).** Note that the figure includes a date for K14 Horse, discussed in section 4.3.1.

Radiocarbon ages BP		**Unmodelled calibrated dates (IntCal 09) Cal BP**				**Modelled calibrated dates (IntCal 09) Cal BP**			
Lab code	^{14}C date ±1σ	68.2%		95.4%		68.2%		95.4%	
		from	to	from	to	from	to	from	to
K14 man Hyp/ OxAx-2395-15	33,250±500	38,650	37,320	39,080	36,680	38,570	37,290	38,850	36,750

Table 4.3. Radiocarbon and calibrated dates (modelled and unmodelled) for the Kostenki man. Note that the model has not had a significant effect on the dates.

The OxCal model demonstrates that the date obtained for the Markina Gora human by extracting the hydroxyproline, falls, for the first time, where expected based on the archaeology of the site. The model does not change the calibrated age significantly, but narrows it to 38,850 – 36,750 Cal BP, with 95.4% probability (table 4.3).

4.2.3 Sungir 2 and 3 anatomically modern humans and a mammoth bone from the same occupation layer

At Sungir, a key Russian Upper Palaeolithic site discovered in 1955, several spectacular burials were excavated (Bader and Bader, 2000). In total, the remains of 8 individuals were found, ornamented with ivory spears, bracelets, brooches, numerous ivory beads and perforated fox teeth, attesting to the technical sophistication of its inhabitants. The cultural assemblage and the red ochre that coated the bodies associate the burials to the wider mid Upper Palaeolithic cultures of European anatomically modern humans, probably the first formal burial by modern humans in Europe (Pettitt and Bader, 2000). Sungir is the northernmost of them all (see figure 4.2). The lithics in the site suggests that it may be attributed to a "transitional" cultural phase related to the previous early Upper Palaeolithic (EUP) (Marom et al., 2012).

The most elaborate and impressive burials are those of Sungir 1, an older adult male, and the joined burials of Sungir 2 (an early adolescent) and Sungir 3 (a late juvenile) (figure 4.7). Sungir 2 and Sungir 3 were buried head-to-head, and the burial is exceptionally rich in artifacts. Sungir 1 and 2 appear to have been normal, while Sungir 3 has suffered from congenital deformities of the femora (Formicola and Buzhilova, 2004). The proximity and ritual similarity of the graves suggest they were also

contemporaneous. Direct radiocarbon dating of the three skeletons was attempted previously in Oxford, Arizona and Kiel (Germany) (Pettitt and Bader, 2000; Kuzmin et al., 2004; Dobrovolskaya et al., 2011). The bones were pretreated by the gelatinisation method (see chapter 2) (OxA-9036, OxA-9037, OxA-9038 and OxA-9039), by slow dissolution[21] (Kuzmin et al., 2004) (AA-36473, AA-36474, AA-36475 and AA-36476) or by ultra-filtration (chapter 2) (KIA-27006, KIA-27006, OxA- 15751, OxA- 15752, OxA- 15753, OxA- 15754, OxA- 15755) (table 4.4). The results were highly inconsistent, both between laboratories and between the different individuals dated, ranging between 19-27,000 BP. The inconsistency in the dates has lead to problems in placing the burials into an accurate archaeological context. Some of the treated samples had an unusually high C:N ratio (table 4.4), again indicating that carbonaceous material may have been applied to the bone, probably a mixture of tree sap (termed kanefol), PVB and phenol/formaldehyde, added together with ethanol, commonly used as a conservation material in the Gerasimov Laboratory in Moscow, where the Sungir bones are stored. The conservation material (traces of which could be observed by eye; Professor Tom Higham, Oxford University, Pers. Comm.) could not be removed completely by the various chemistry pretreatment methods performed on the samples.

[21] A method developed in Russia in the 1970s. Collagen was extracted from whole bones by reacting with dilute HCl under 2-3 °C for a few days. Washed collagen was carbonized prior to combustion (Kuzmin et al., 2004).

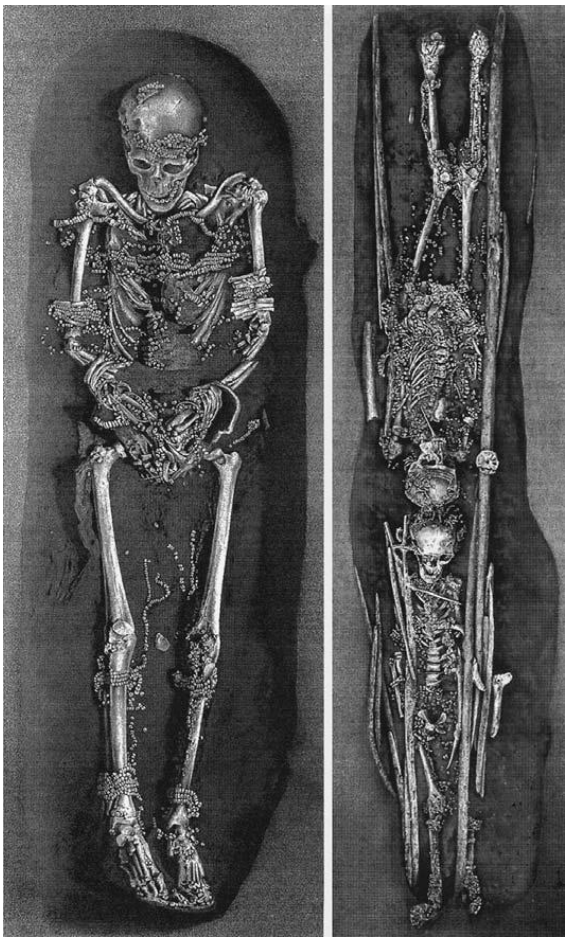


Figure 4.7. Drawings of the Sunghir Mid Upper Paleolithic burials. Left: Grave I with Sungir 1. Right: Grave II with Sungir 2 (above) and Sungir 3 (below). Drawing courtesy of N.O. Bader, Institute of Archaeology of RAS (taken from Dobrovolskaya et al., 2011).

Hyp was separated from 30mg of previously extracted and dated collagen from the Sungir 2 and 3 individuals. Unfortunately, there was not enough material to re-date Sungir 1 as well. In addition, Hyp was also extracted from a 30mg collagen sample of a mammoth bone that came from the same occupation area of the site (the separation chromatograms for the Sungir samples can be found in appendix D, figures D.7, D.8 and D.9). The new dates, shown in table 4.4, are remarkably similar, consistent with a single event. The fact that the two human dates are internally consistent further supports their accuracy because they were interred together in the same grave. Figure 4.8 shows the calibrated ages for the Sungir samples. Interestingly, for the mammoth bone, the ultra-filtered bulk dates (OxA-15752 and OxA-15755) are very similar to the Hyp date,

implying that it was not preserved and so the contaminant that made it appear more modern was effectively removed by the ultra-filtration.

The new dates for the Sungir burials yield a weighted average date of 30.1 ± 0.3 ka BP[22] [T=0 (χ^2 0.05 = 6.0)], between 35.2 and 34.1 ky Cal BP with 95.4% confidence (see table 3.5), appreciably older than previously assumed. Prior to this work, the earliest direct date for a red ochre burial was ~29 Ka BP for the 'Red Lady' of Paviland in the British Isles (Jacobi and Higham, 2008). The Kostenki 14 and Sungir burials may therefore suggest an earlier onset of Mid-Upper Palaeolithic technocomplexes and complex ritual burial behaviours in Eastern Europe compared with Western Europe, but more dating is required (Marom et al., 2012). In addition, they support the DNA evidence that suggests successive waves of movement of anatomically modern humans in the Upper Palaeolithic. The new Sungir dates together with the Kostenki 14 date are the earliest dates for anatomically modern humans found in the Russian plain, and probably represent one of those waves of migration. However it is most likely that they do not represent the first time anatomically modern humans populated this region, as the lowest occupation layers from Kostenki, associated with modern humans, date to 45,000-40,000 years ago (Anikovich et al., 2007). Moreover, new evidence suggests anatomically modern humans had already been living in west Europe around the same time (Benazzi et al., 2011; Higham et al., 2011).

[22] After applying the correction algorithm (section 3.2.2.5 in chapter 3), the date is 33.7 ± 0.3ka BP, both dates indistinguishable statistically: T = 2.1 (χ^2 0.05 = 3.8).

	Lab code	C:N	^{14}C age, BP (±1σ error)	δ^{13}C	Source
Sungir1	AA-36473 (vertebra fragment)	NP	19,160 ± 270	NP	Kuzmin et al., 2004
	OxA-9036*	NP	22,930 ± 200	-19.2	Pettitt and Bader, 2000
	KIA-27006†	3.1	27,050 ± 210	NP	Dobrovolskaya et al., 2011
Sungir2	AA-36474 (right side ribs)	NP	27,210 ± 710	NP	Kuzmin et al., 2004
	AA-36475 (left side ribs)	NP	26,200 ± 640	NP	Kuzmin et al., 2004
	OxA-9037*	3.5	23,830 ± 220	-19.0	Pettitt and Bader, 2000
	OxA- 15753†	3.3	25,020 ± 120	-19.1	Marom et al., 2012
	OxX-2395-6 Hyp fraction	**5.0**	**30,100 ± 550**	**-21.1**	**This thesis (Marom et al., 2012)**
Sungir3	AA-36476 (rib fragments)	NP	26,190 ± 640	NP	Kuzmin et al., 2004
	OxA-9038*	3.4	24,100 ± 240	-18.9	Pettitt and Bader, 2000
	OxA-15751†	3.2	25,430 ± 160	-19.1	Marom et al., 2012
	OxA-15754†	3.2	24,830 ± 110	-18.9	Marom et al., 2012
	KIA- 27007†	3.5	26,000 ± 410	NP	Dobrovolskaya et al., 2011
	OxX-2395-7 Hyp fraction	**5.0**	**30,000 ± 550**	**-26.6**	**This thesis (Marom et al., 2012)**
Sungir Mammoth bone	OxA-9039*	3.5	27,460 ± 310	-21.0	Pettitt and Bader, 2000
	OxA-15752†	3.1	29,640 ± 180	-20.8	Marom et al., 2012
	OxA-15755†	3.2	29,450 ± 180	-20.5	Marom et al., 2012
	OxX-2395-8 Hyp fraction	**5.1**	**30,100 ± 400**	**-30.5**	**This thesis (Marom et al., 2012)**

Table 4.4. C:N atomic ratios and radiocarbon ages from Sungir. *denotes samples pretreated with a gelatinisation method; †denotes samples ultra-filtered prior to AMS dating. NP – not published. Note that the C:N ratio of Hyp is 5.

	^{14}C age, BP (±1σ error)	Calibrated dates (IntCal 09) Cal BP			
		68.2%		95.4%	
Laboratory ID		from	to	from	to
Sungir1					
AA-36473	19,160 ± 270	23,230	22,490	23,580	22,260
OxA-9036	22,930 ± 200	28,080	27,060	28,270	26,890
KIA-27006	27,050 ± 210	31,450	31,200	31,600	31,090
Sungir 2					
AA-36474	27,210 ± 710	32,430	31,050	33,450	30,500
AA-36475	26,200 ± 640	31,260	30,360	31,780	29,550
OxA-9037	23,830 ± 220	29,010	28,340	29,300	28,070
OxA- 15753	25,020 ± 120	30,180	29,610	30,240	29,520
OxX-2395-6(Hyp)	**30,100 ± 550**	**35,260**	**34,000**	**36,270**	**33,410**
Sungir 3					
AA-36476	26,190 ± 640	31,260	30,350	31,760	29,550
OxA-9038	24,100 ± 240	29,250	28,640	29,470	28,400
OxA-15751	25,430 ± 160	30,550	29,830	30,640	29,640
OxA-15754	24,830 ± 110	29,870	29,450	30,170	29,420
KIA-27007	26,000 ± 410	31,050	30,440	31,300	29,800
OxX-2395-7(Hyp)	**30,000 ± 550**	**35,150**	**33,920**	**36,240**	**33,290**
Sungir Mammoth					
OxA-9039	27,460 ± 310	31,920	31,300	32,550	31,180
OxA-15752	29,640 ± 180	34,680	34,090	34,760	33,670
OxA-15755	29,450 ± 180	34,570	33,890	34,650	33,500
OxX-2395-8(Hyp)	**30,100 ± 400**	**35,130**	**34,480**	**36,170**	**33,610**
Sungir 2,3 and Mammoth combined date	**30,070 ± 280**	**34,940**	**34,560**	**35,190**	**34,070**

Table 4.5. Calibrated dates for the Sungir dates. Bulk calibrated dates for the Sungir 2, 3 and Mammoth range between ca. 28.1 ky Cal BP and 34.8 ky Cal BP, but the three Hyp fraction dates all fall between 33.3 ky Cal BP and 36.3 ky Cal BP (using the 2σ range), and can be combined with greater than 95% probability to a single calibrated date that falls between 34.1 and 35.2 ky Cal BP.

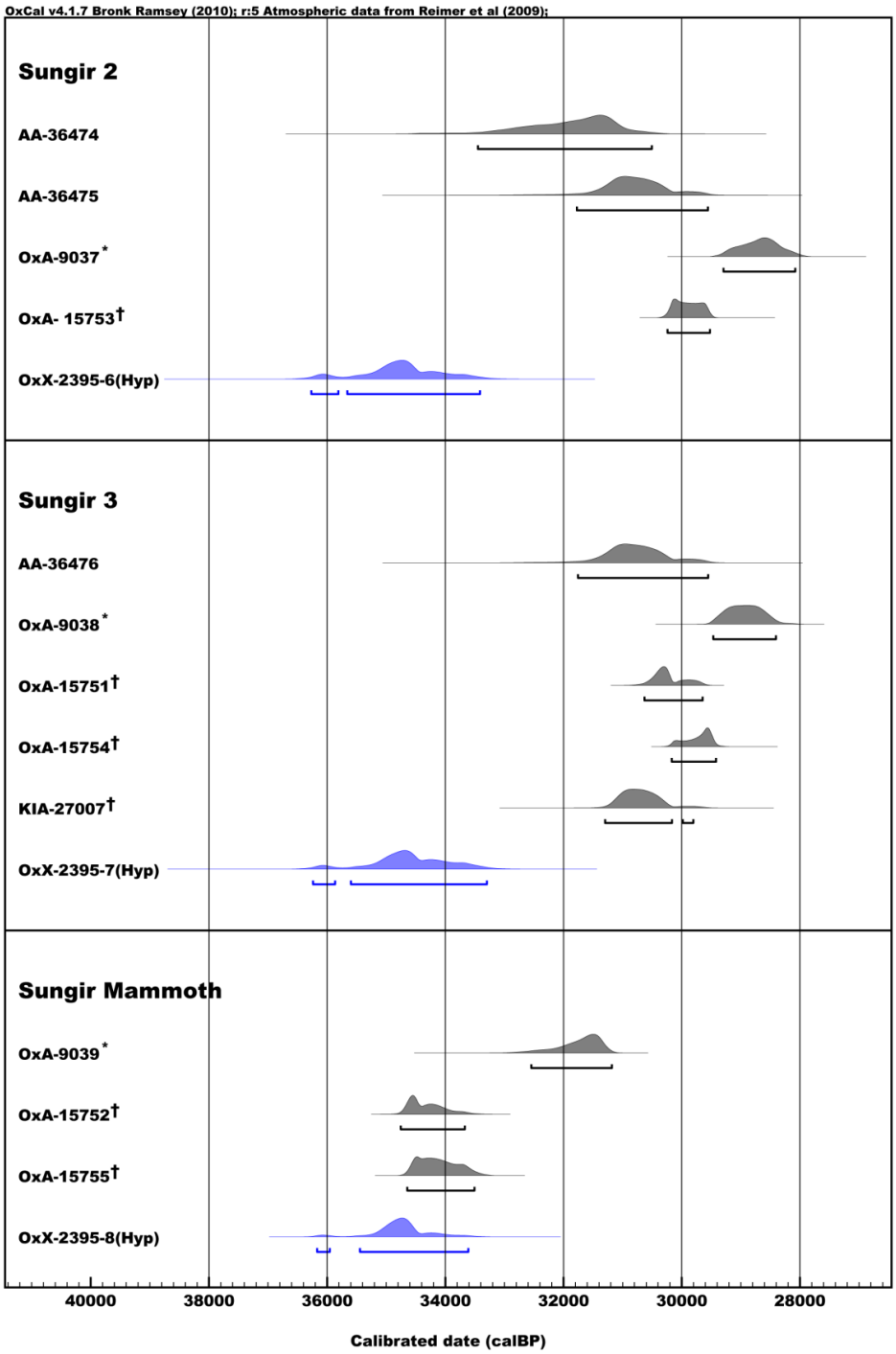


Figure 4.8. Calibrated radiocarbon dates for Sungir 2 Sungir 3 and the Sungir Mammoth bones, produced with OxCal v4.1.7 and IntCal09 Calibration curve (Bronk Ramsey, 2009; Reimer, 2009).*denotes samples pretreated with a gelatinisation method; †denotes samples ultra-filtered prior to AMS dating.

4.2.4 La Ferrassie Neanderthal bone

La Ferrassie is a Middle-Upper Palaeolithic rock shelter in Dordogne, France, in which the burials of two Neanderthal adults and five children and babies were found in the early 20th century. The Upper Palaeolithic material found in La Ferrassie has been a key for understanding the Upper Palaeolithic sequence in France. One of the most important individuals found at the site is La Ferrassie 1, the skeleton of an adult male. His skull, the largest and most complete Neanderthal skull ever found, has many of the typical Neanderthal traits such as the low, sloping forehead and large nasal opening (figure 4.9). The site also contains what is considered the earliest found art form in Europe, in the shape of cupules in a large limestone slab placed over one of the graves (Bahn, 2004) and the Smithsonian website (www.humanorigins.si.edu/evidence/human-fossils/fossils/la-ferrassie).

Both the distal left tibia and the distal right tibia of La Ferrassie 1 were sampled and dated at Oxford previously. The surface of the bones seemed to have been treated with preservation material that the sampling process attempted to avoid, by sampling the interior of the bone. The results were very different from each other, the left tibia yielding a date of 32,750 BP, and the right - 11,540 BP (table 4.6). These results, together with the high C:N values, suggested that both the bones were contaminated, with the right tibia to greater extent than the left[23].

30.7mg of the contaminated collagen that was previously extracted from the left tibia and the 19.8mg that was previously extracted from the right tibia, together with the

[23] The possibility that the bones do not belong to the same individual is highly unlikely, because the skeleton was found in articulation (see figure 4.9)

corresponding ultra-filtrates (the low molecular weight collagen that passed through the ultra-filter) were hydrolyzed and separated on the HPLC and the Hyp fraction was dated (see chromatograms in appendix D, figures D.10 and D.11). The Hyp dates were both older, with the left tibia now dating to 35,700 ± 1500 BP and the right tibia – 12,910 ± 90 BP. However the discrepancy between them was not resolved and the right tibia still yielded a date about 20Ky more recent than the left, indicating that it still contained around 23% modern carbon contamination. The hydroxyproline dating method did not yield the same date for the two bones, suggesting that it was unable to eliminate all of the contaminant in this case. The fact that the HPLC separated Hyp fraction had a typical C:N ratio and the elution time and shape of the Hyp peak were unaffected may both suggest that the remaining contamination is of collagenous origin.

A possible candidate is collagen-based glue, commonly used by curators in the past, customarily obtained from boiling collagen-containing animal parts such as hides and bones. If this is the case, then using a mass spectrometry method such as ZooMS (Buckley et al., 2009) should be able to show that apart from the Neanderthal collagen the sample also contains collagen from a different species, and also possibly identify it. Based on their age shift from the left tibia Hyp date, the presence of 23% collagen contamination in the right tibia and 1% in the left can be assumed, and the stable isotopes values of the contaminant can be calculated. The contaminant's $\delta^{13}C$ and $\delta^{15}N$ are -20.9‰ and 8.4‰, respectively, both depleted relatively to the $\delta^{13}C$ of -18.9‰ and $\delta^{15}N$ of 11.4‰ of the Neanderthal sample, and in accord with the contaminant being from collagen of a C3-eating herbivore (e.g. glue made of horse collagen).

	PNumber	treatment	C:N	$\delta^{13}C$	$\delta^{15}N$	^{14}C date BP (±1σ error)
Left tibia	22,719.0 AF* 01	Ultrafiltration	3.4	-18.9	11.4	32,750 ± 450
	22,719.1 NRC 01	**Hyp separation**	**4.9**	**-22.8**	**13.4**	**35,700 ± 1500**
Right tibia	22,720.0 AF* 01	Ultrafiltration	3.5	-19.3	10.7	11,540 ± 55
	22,720.2 NRC 01	**Hyp separation**	**4.9**	**-22.9**	**12.7**	**12,910 ± 90**

Table 4.6. C:N values and dates of La Ferrassie left and right tibia.

Figure 4.9. La Ferrassie 1 Neanderthal.
Left: The skeleton as found in the excavation site
Right: The displayed skeleton. Photo taken by Don Hitchcock, 2008, from the display at Musée National de Préhistoire, Les Eyzies (http://donsmaps.com/ferrassie.html)

4.3 Low collagen bones

4.3.1 Kostenki 14 Horse

Another bone from Kostenki 14 that was radiocarbon dated using the hydroxyproline dating method is a horse 2nd/3rd phalange, dug at the lowermost cultural level IVb1, just above Horizon of hearths (figure 4.4, figure 4.10). Its nitrogen percentage was 0.3%, as

opposed to 4.5% in modern bone, suggesting less than 1% of collagen was left (at ORAU bones with less than 0.8% N content are not dated; See chapter 1). A whole bone C:N ratio of <5 was proposed as indication that a bone is suitable for dating (Tisnérat-Laborde et al., 2003) (but Brock et al., 2012, suggest the threshold is 17[24]). The Kostenki horse bone had a C:N ratio of ~12, indicating, again, its highly degraded or contaminated collagen. This bone was never exposed to any conservation material. The whole bone (140g) was sand blasted, and the remaining 100g were crushed to extract collagen (see chapter 2).

Figure 4.10. Kostenki 14 horse phalange.

As mentioned in chapter 3, bones with less than 2% collagen are expected to be 'non-collagenous' (i.e. their amino acid profile stops being collagen-like) (DeNiro and Weiner, 1988a). Surprisingly, the resulting 1.2g collagen was seemingly of good quality by appearance. This impression was confirmed when it was measured on the IRMS, yielding values well within the expected (table 4.7). It was therefore decided to date not only the Hyp fraction, but also the bulk collagen (before and after hydrolysis), so as to

[24] Based on linear regression analysis, showing that if a threshold of a C:N ratio of >17 of the whole bone is used, there is a 71% success in predicting which bones will have >1% collagen.

see if there was any difference in the emerging dates. Some of it was thus reconstituted in water and ultra-filtered; another fraction was hydrolyzed and filtered, and both were combusted and graphitised as bulk. Finally, some of the latter was run on the HPLC and the separated Hyp was combusted and graphitised (the separation chromatogram can be found in appendix D, figure D.12). The values of the different fractions are shown in table 4.7:

Fraction/ treatment	**Lab code**	**$\delta^{13}C$**	**$\delta^{15}N$**	**C:N**	**^{14}C date BP (±1σ error)**
collagen		-19.9	5.0	3.2	NM
AF	X-2365-11	-20.5	4.7	3.0	34,200 ± 550
Hyd	X-2395-22	-19.4	5.0	3.2	34,800 ± 550
Hyp	X-2395-18	-20.0	10.4	5.0	35,700 ± 900
Combined date					34,740 ± 360

Table 4.7. $\delta^{13}C$, $\delta^{15}N$, C:N ratio and radiocarbon date values for different fractions of the K14 Horse bone. AF - Ultra-filtered, but not base washed; Hyd – hydrolyzed. NM- not measured.

The radiocarbon date of the Hyp is 35.7 ka BP[25], older than the hydrolyzed bulk, which, in turn, is slightly older than the ultra-filtered bulk date, probably indicating that the bone was not considerably contaminated (table 4.6). The age difference is not significant (due to the large errors), and the three dates can be effectively combined, to yield the date 34,740 ± 360 BP (weighted average). The horse bone was found in cultural layer VIb, below the Y5 CI tephra level, and above the horizon of hearths, dated by OSL to 44,880 ± 3,580, equivalent to 42,930 ± 3,580 Cal BP (UIC-748; Hoffecker et al., 2008). The horse date is therefore expected to be between 46.5 and 39.2 Ky Cal BP. It yielded the calibrated age 42.3-38.9 Ka Cal BP (table 4.8; based on

[25] After applying the correction algorithm (section 3.2.2.5 in chapter 3), the date is 37.0 ± 1ka BP, both dates indistinguishable statistically: T = 0.9 (χ^2 0.05 = 3.8).

IntCal09 calibration curve, with 95.4% probability) for the Hyp fraction, which is within the expected range, albeit on the younger side. A model was made using OxCal, assuming that the horse bone precedes the date of charcoal from the Level of Volcanic Ash (LVA), taken as an approximation for the pre-eruption date (Douka et al., 2010b). The modelled calibrated date was narrowed to 42.0-39.4 Ka Cal BP (see table 4.8 and figure 4.6), which is closer to the expected value.

Radiocarbon ages BP		**Unmodelled calibrated dates (IntCal 09) Cal BP**				**Modelled calibrated dates (IntCal 09) Cal BP**			
Lab code	^{14}C date ±1σ	68.2%		95.4%		68.2%		95.4%	
		from	to	from	to	from	to	from	to
K14 Horse Hyp/ OxAx-2395-18	35,700 ± 900	41,750	39,780	42,280	38,890	41,550	40,140	42,030	39,390

Table 4.8. Radiocarbon and calibrated dates (modelled and unmodelled) for the Kostenki horse Hyp. Note that the model has not had a significant effect on the dates.

It is perfectly plausible, therefore, that the Horse bone is older than 39.3 Ka Cal BP. However, there is another possibility, that the volcanic ash was re-deposited at the site, as there is evidence of re-deposition of the tephra in many locations at Kostenki (Hoffecker et al., 2008). Even if this was the case here, it is not expected to change the age of the level associated with the volcanic ash significantly. Anyway, the horse bone pre-dates the cultural levels that were found above it, and so serves as a proof that reliable dates could be measured using the hydroxyproline dating method, even from low collagen bones, considered un-datable by conventional bulk dating methods. Although the collagen extracted was surprisingly well preserved (both in terms of quality and level, which was not as low as expected from the %N of the whole bone), and the bulk date was similar to the one obtained from the Hyp fraction, it is claimed that the Hyp date is more trustworthy. The increased reliability of the Hyp date emerges from the fact that when such big bone samples are processed there is a bigger risk of

including contaminating material in the extracted ‘collagen’(van Klinken, 1999; Section 1.2.4 in chapter 1), a problem the Hyp fraction is less likely to be affected by.

4.3.2 Exploring the potential of the Hyp dating method to date soluble collagen from very low collagen bones

4.3.2.1 St. Cesaire bison

St. Cesaire is a prehistoric rock shelter in Southwestern France, excavated in the late 1970's to 1980's by F. Leveque. It has yielded stratified layers from the Middle to the Upper Palaeolithic periods. A nearly complete Neanderthal skeleton found in association with an artifact assemblage classified as Chattelperronian (the onset of the Upper Palaeolithic) was dated by OSL to 36.3 BP, and represents one of the latest Neanderthal dates (Mercier et al., 1991). It is considered a controversial find, however, as the Chattelperronian tool kit was believed until then to be produced only by early modern humans. The association was since questioned, though, as the layers could have been mixed (Lévêque et al., 1993; Bahn, 2004), Ch. 1, St. Cesaire and the debate on the transition from the middle to upper Palaeolithic.

A Bison left tibia from the Mousterian layer of the site (figure 4.11), having a C:N ratio of ca. 50 and nitrogen content of 0.12% was clearly a very low collagen bone, which could not be dated by the conventional bulk dating method. Being 74g in weight, however, the suitability of the hydroxyproline dating method was investigated. The sample was completely crushed and treated in the search for collagen in both the soluble and insoluble fractions (see chapters 1 and 2). The demineralisation step was carried out using dialysis, yielding 34.3mg insoluble collagen (0.05%) and around 320mg soluble collagen (note that this fraction never dried completely so the weight is

only an approximation). The latter was further cleaned using ion exchange, and a sample from each step was combusted on the MS to check their stable isotope and C:N ratio values (Figure 4.12).

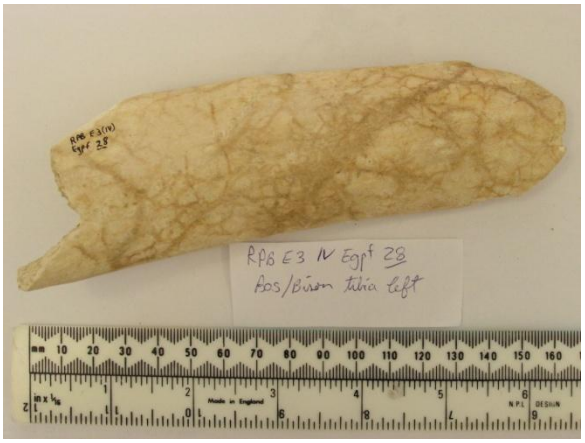


Figure 4.11. A bison left tibia from the St. Cesaire site

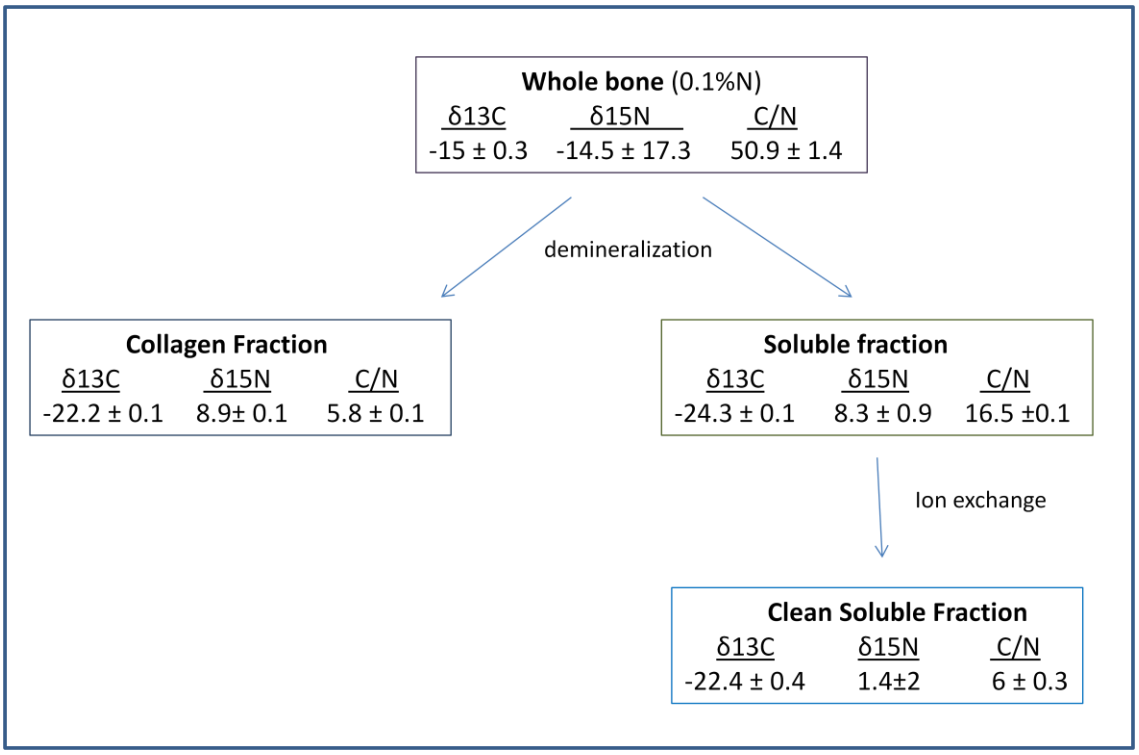


Figure 4.12. $\delta^{13}C$, $\delta^{15}N$ and C:N ratio values of the different fractions of the St. Cesaire bison bone. The expected values for clean bison collagen would be $\delta^{13}C$ ca. -20‰, $\delta^{15}N$ ca. 6‰ and C:N 3.2. It can be seen that both the collagen fraction and the clean soluble fraction have $\delta^{13}C$ values close to the expected; however the $\delta^{15}N$ values of the soluble fraction is too low (but could be due to very low yield, as the very high standard error also suggests), and the C:N ratio of both is still much too high.

Both the collagen fraction and the cleaned soluble fraction were thereafter hydrolyzed and run on the HPLC. Only the collagen fraction yielded a Hyp peak (see chromatogram in appendix D, figure D.13). However it was too small to be dated[26].

4.3.2.2 Akrotiri Aetokremnos pygmy hippo

Akrotiri-Aetokremnos, a site on the south central coast of Cyprus, probably holds the earliest evidence of human activity in Cyprus, dating to the 10th millennium B.C.E. Aetokremnos is a controversial site, due to the assumed association of cultural materials with extinct endemic Pleistocene fauna, especially the pygmy hippopotamus. The bones of over 500 pygmy Hippos were found in the site, together with remains of other species such as pygmy elephants. It is debated whether the site was permanently occupied, whether the Hippos were being hunted, and whether the humans contributed or directly caused their extinction. Reliable radiocarbon dates are crucial for resolving the last question, as they could prove beyond any doubt if the humans and the Hippos were contemporaneous or not (Ammerman and Jay Stratton, 2005). Several radiocarbon dates have been produced for the site however the bone dates are considered unreliable, being both too modern and incompatible with the charcoal dates.

A pygmy Hippo bone from the site was treated in hope of finding Hyp in its soluble fraction. Of the initial 6.5g there was only 0.02% nitrogen, indicating almost no collagen was left (in either fraction). Extraction of the leached fraction (LF), soluble fraction (SF) and insoluble fraction, or collagen fraction (CF) revealed most of the organic material had gone, and most of the remaining material was in the soluble fraction (figure 4.13).

[26] About 5 times more Hyp was needed to get a reasonably sized graphite; graphitization was however attempted, but failed.

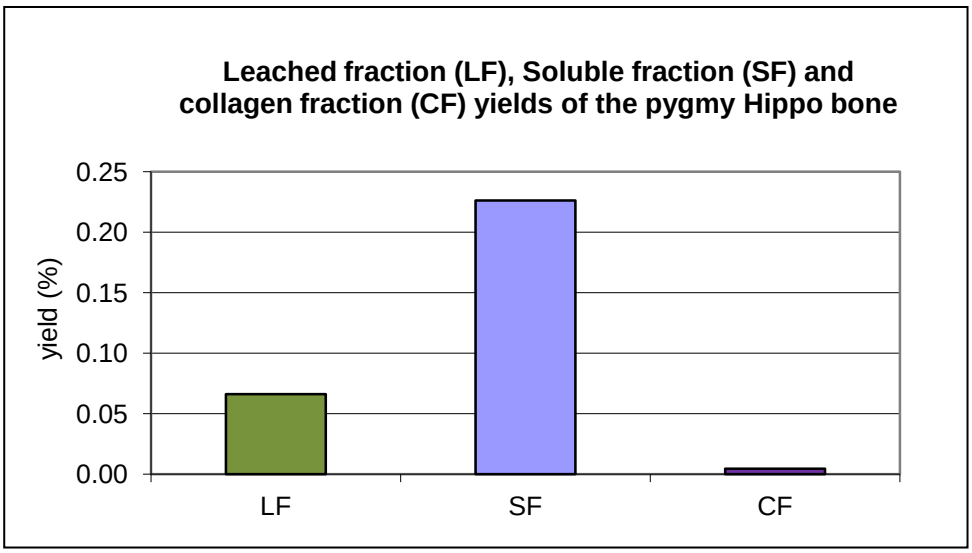


Figure 4.13. The dry weight percentage of the different fractions (leached, soluble and insoluble, or collagen) of the pygmy Hippo bone. CF (collagen) consisted only 0.005% of the whole bone, as opposed to 20% in modern bone.

The $\delta^{13}C$ and $\delta^{15}N$ values of the soluble fraction were -24.2±0.3 and 9.2±0.6, respectively, in the acceptable range for herbivores. The C:N value, however, was 17.7±0.9, a great deal higher than the expected 3.3 (see chapter 3). The amino acid profile of the Hippo soluble fraction was non-collagenous compared with that of standard collagen (figure 4.14). Some amino acids (aspartate, glutamate, arginine and proline) were enriched in this fraction, while most of the others were depleted or totally absent; only three amino acids (serine, leucine and valine) showed the amount expected from collagen (figure 4.14). Hyp was completely missing from this fraction, as might be expected from the literature (e.g. Knight 1968; Bada et al., 1984; DeNiro and Weiner, 1988a). These results contradict the results of the artificial degradation experiments, possibly because such low levels of collagen had not been examined (chapter 3; see also chapter 5 for discussion).

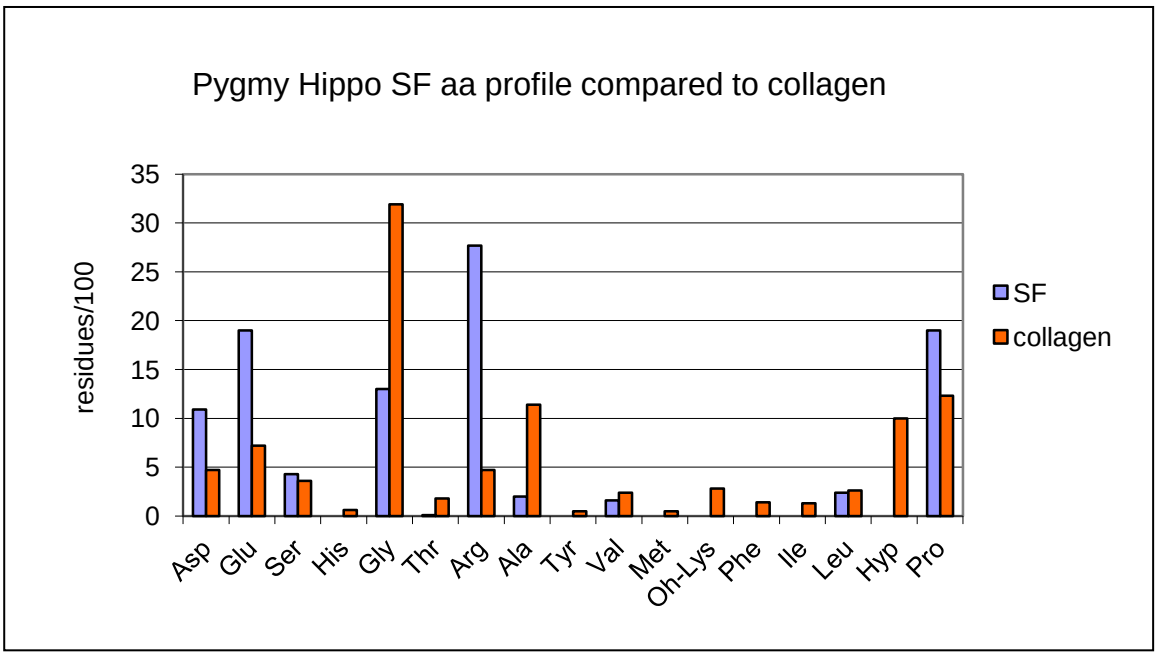


Figure 4.14. Amino acid profile of the pygmy hippo soluble fraction (purple), compared to that of standard collagen (orange).

4.4 Conclusions

In conclusion, it has been shown that hydroxyproline can be successfully extracted and provide reliable radiocarbon dates from archaeological bones that are contaminated, and in so doing prove useful in resolving some longstanding archaeological debates. It has been shown to be a powerful tool for dating bones that the conventional bulk methods are unable to date, or yield inconsistent dates, due to variable and limited success in the elimination of the contaminants. Consolidants that are collagen derived, however, will not get eliminated by this method, as presumably shown by the La Ferrasie Neanderthal dates. For low collagen bones it has been shown that the Hyp dating method is able to yield reliable dates when a large enough sample is available. For bones in which only trace amounts of collagen remains, the potential of the method is still unclear: from the experimental degradation of collagen it seems hydroxyproline could be found in the mineral fraction; however the attempt to isolate soluble hydroxyproline from

archaeological bones was unsuccessful, probably because the bones chosen had already lost most of their organic content.

Chapter 5: Discussion

5.1 Summary

In the research reported in this thesis a mixed mode chromatographic method for separating and radiocarbon dating the amino acid hydroxyproline from bones was further developed, and its suitability for dating contaminated bones and bones with low levels of collagen was verified. Although dating hydroxyproline has been suggested in the past as a way to reliably date bones, and was even attempted several times, until now the Hyp isolation procedural background was never reported, frustrating the evaluation of the different separation approaches. The procedure background for the method described here was calculated to be 3.2±1.1 µg carbon, of which it was determined that 1.4±0.3 µg is modern and 1.8±1.1 µg is ^{14}C-free. This is a very low and consistent background level, which will not have a significant effect on the date, regardless of the bone age. The hydroxyproline dating method was used to obtain radiocarbon dates from archaeological bones which were either contaminated or had little remaining collagen. In the following chapter the results of the application of the method are discussed with regard to its usefulness and prospects for future applications. I attempt to establish a set of circumstances under which the hydroxyproline dating method should be used. The chapter concludes with what should be done next in order to improve the method to make it more easily and widely available.

5.2 Contaminated bones

The Hyp method proved to be successful in dating a number of contaminated bones, as shown both by applying it to those that were deliberately contaminated (tea contamination experiment, chapter 3, section 3.2.3), and by dating archaeological bones from Flixton (probably contaminated by humic acids), Kostenki and Sungir (probably

contaminated by conservation material) (chapter 4, section 4.2). It had failed to yield accurate dates for the La Ferrasie bones, most likely due to collagenous consolidant.

How can bone contamination be detected? If a bone, or a number of bones thought to be contemporaneous yield variable dates (like the Sungir human bones), it is likely to be contaminated. The same applies for a bone that yields a date that is very different from what is expected (e.g. its date differs from what is found for specimens from the same stratigraphic level, or does not comply with secure dates for strata above and below, as the Kostenki 14 human bone). However these circumstances, in which contamination can be easily identified by bone dates, are fairly unusual. The carbon:nitrogen atomic weight ratio (C:N) can also provide an indication that contamination is present. Intact collagen will have a C:N ratio of 3.21 (Ambrose, 1990). Bones are considered 'datable' when their collagen C:N ratio is between 2.9-3.6 (DeNiro, 1985; Ambrose, 1990) or 3.1 and 3.5 (van Klinken and Hedges, 1995). High values (i.e. >3.5) may indicate diagenetic alteration has taken place, causing de-amination, and/or contamination by exogenous carbon-containing compounds. Evidently, the C:N ratio is far less sensitive to contamination than the ^{14}C measurement, and there is no guarantee that even bones with 'normal' C:N (e.g. 3.1-3.3, given the measurement error) are contaminant-free. For example, in the case of a sample containing 800 µg C, with a C:N ratio of 3.20, addition of another 30 µg C will result in a C:N ratio of 3.32, which is still perfectly adequate. Even an addition of 50 µg carbon, consisting more than 5% of the sample, will only shift the C:N ratio to 3.4, still in the considered acceptable range. The effect of this contamination on the date can be up to tens of thousands of years, depending on the sample and contaminant ages. If the contamination in this instance has no ^{14}C activity, it will cause the sample to appear

around 500 years too old. If the sample age is close to the radiocarbon detection limit, and the contaminating carbon is modern, than the apparent age for this sample will be ca. 24,000 (see figure 3.1 in Chapter 3). For bones like those of the Kostenki 14 human, the exceptionally elevated C:N ratios (around 4 in this case) leave no doubt that the bone is contaminated. But what about more marginal C:Ns? In the Flixton horse bones data, although none of the C:N ratios are greater than 3.5, a correlation can be observed when plotting the radiocarbon dates against the C:N ratios, with the samples showing higher C:Ns producing younger dates, as expected if the contaminating carbon is more modern than the bones[27] (figure 5.1; see also chapter 1). For the Sungir bones the trend is less clear, but it still can be seen that some of the bones, bound to be contaminated from the date they produce, have also elevated C:Ns. This suggests that a collagen C:N ratio higher than 3.3, although normally not considered an indication of contamination, could signify the presence of extraneous carbon.

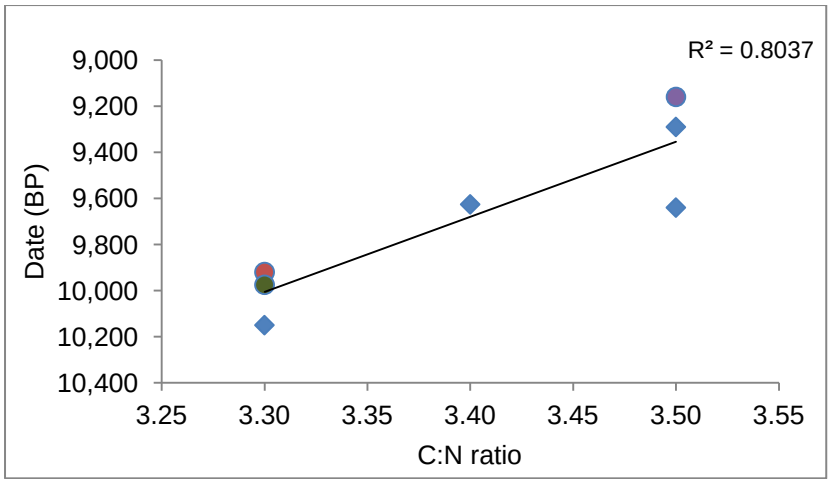


Figure 5.1. Flixton 2 horse bone radiocarbon dates plotted against their collagen C:N ratio. There seems to be a correlation in which the higher the C:N value is, the younger the radiocarbon date, indicating more recent carbonaceous contamination. The blue diamonds represent different dating attempts of the XB23 astragal; the different coloured circles represent different horse bones from the same layer. Although the different bones do not necessarily have the same dates, the fact that they fall on the same trendline suggests that they do.

[27] Interestingly, the Flixton samples that were dated at ORAU in the past, using the ion exchange method, are in agreement with the new Hyp date (except for sample OxA6329), suggesting that this pre-treatment may have been better at removing the contaminant in this case (see figure 4.1).

Analyzing the data from all the bones treated by ultra-filtration at ORAU so far (8438 bones, given the lab code AF or AF*) shows that 93% of them have C:N ratios between 3.1 and 3.5, and this percentage is essentially independent of the collagen yield (figure 5.2). The ~2% of bones showing a C:N ratio higher than 3.5 most likely represent contaminated bones, and so are good candidates for application of the hydroxyproline dating method. A similar percentage was found both for bones that were treated with solvent extraction prior to collagen extraction (given the lab code AF*) and for bones that were not. If bone samples with C:N ratios higher than 3.3 are treated as potentially contaminated, then 28% of all the bones treated in ORAU might benefit from the hydroxyproline dating method on that ground (figure 5.2). In fact it is plausible that it is not uncommon to have humic acid contamination of older bones making them appear younger by thousands of years without knowing.

It has to be noted though, that high C:N ratios are not always a sign of contamination: the reported C:N ratios are probably skewed upwards, because the measurements using an elemental analyzer tend to be inaccurately high when the sample combusted is considerably smaller than the standard it is compared with[28]. High C:Ns found in low collagen bones could also be a result of preferential degradation of some amino acids (e.g. glycine) as well as preferential degradation of collagen compared to NCPs or lipids, rather than contamination (Dobberstein et al., 2009, and Chapter 3). Elevated C:N ratios , >4.5 for a bone with less than 1% collagen, are commonly found in poorly preserved collagen bones, as artificial collagen degrading experiments have shown (ibid). Even genuinely high C:N ratios do not necessarily predict there will be an effect

[28] It is worth noting that even processed samples that contained only Hyp, as verified by amino acid analysis, and should therefore yield a C:N ratio of 5, gave at several instances values of 5.1 and 4.9

on the date, as the carbonaceous material contaminating the sample could have the same age as the bone itself. It is therefore not argued that 26% of the samples dated with the normal protocol yield erroneous dates, but it is suggested that there is a case for being suspicious of the date when samples exhibit C:N ratios higher than 3.3, and perhaps it is worthwhile for those samples to characterize the composition of the collagen by other means such as infrared spectra if a conventional bulk dating method is used.

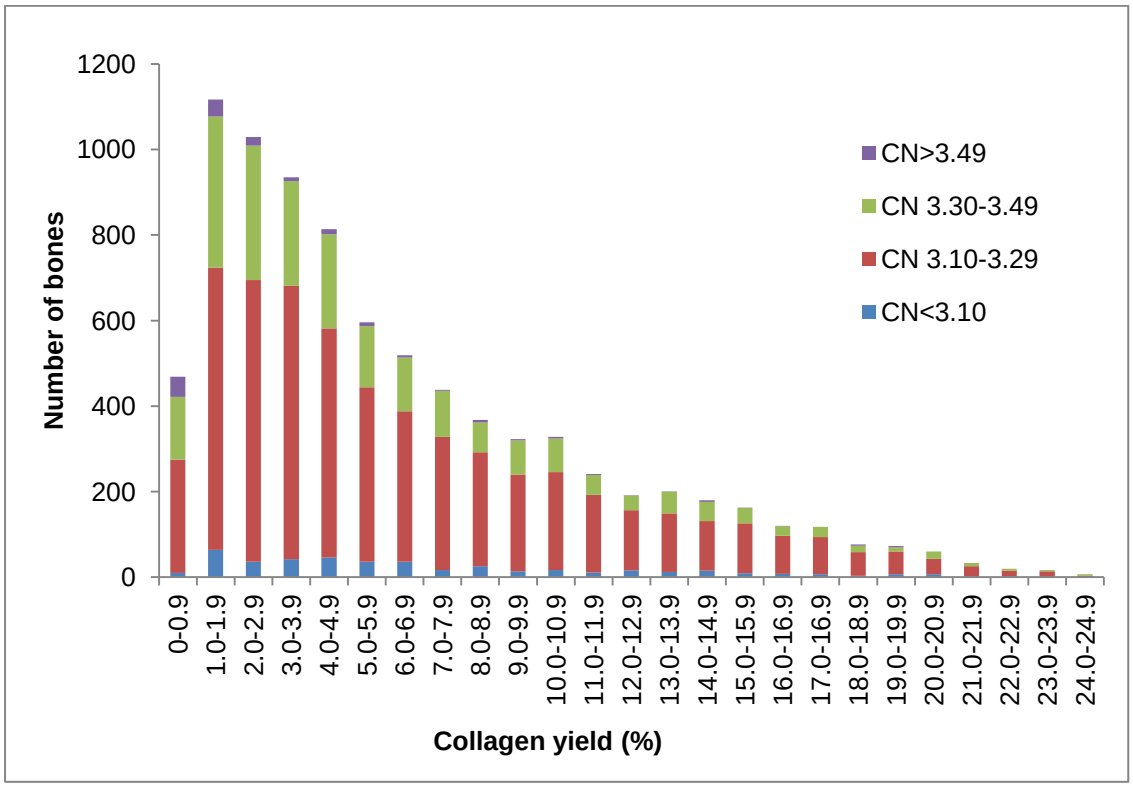


Figure 5.2. Number of bone samples treated by the ultra-filtration protocol at ORAU yielding C:N ratio <3.1, between 3.1 and 3.29, between 3.3 and 3.49 and >3.5 for different ranges of collagen yields. Note that bones with collagen yield lower than 1% are normally 'failed' so this histogram does not represent the real number of bones in this range.

Contaminated bones can be dated accurately in some cases, by applying ion exchange/ hydrolysis/ or ultra-filtration steps to the collagen before dating. However, only Hyp can guarantee the accuracy of a suspected date (unless it is contaminated by collagenous material!).

5.3 Low collagen bones

The dating of the Kostenki horse bone had shown that if sufficient bone is available, an accurate date could be obtained from low collagen bones using the Hyp method. It is commonly believed that bones with trace amount of collagen (<1%) cannot be reliably dated using the normal procedures, because they are thought to be diagenetically altered. In addition, the increased amount of bone that will need to be treated would also increase the risk of including contamination in the sample (van Klinken, 1999; section 1.2.4 in chapter 1). How many bones are failed due to low collagen yield? Figure 5.3 shows the distribution of high collagen (>5%), low collagen (5%<collagen yield<1%) and trace amounts of collagen (<1%) bones pre-treated at ORAU by ultra-filtration (AF and AF*). About 16% of all bones submitted for dating are failed due to low collagen yields. Note that this percentage does not represent all the bones that have <1% collagen, or the ones that were failed before collagen extraction (e.g. after failing the prescreening criterion), only the ones submitted and treated with the AF method.

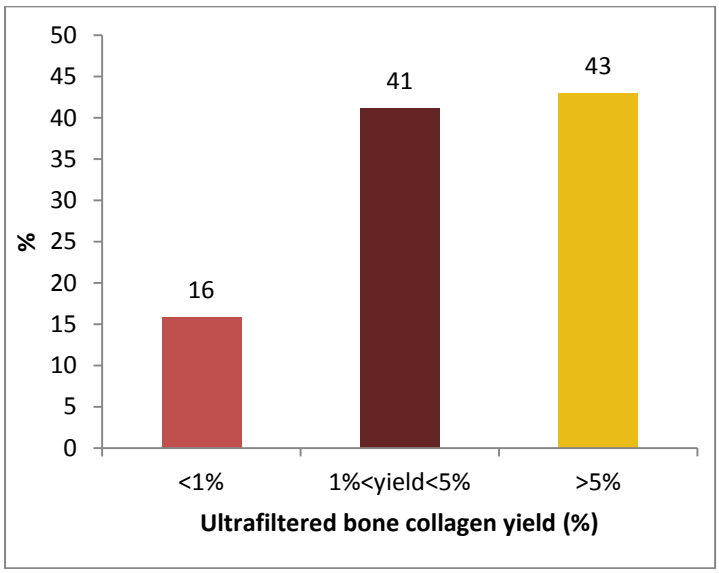


Figure 5.3. The percentage of high collagen, low collagen and trace amounts of collagen in the ultra-filtered bones at ORAU. The <1% collagen bones do not get dated.

Some bones that yield trace amounts of collagen have indeed lost most of their collagen. For example, as collagen survival is very much dependant on climate, a large proportion of the bones from many temperate and most tropical areas exhibit low (<5%) or trace (<1%) amounts of collagen. Even when minute amounts of organic material do remain, Hyp is reported to be lost (Bada et al., 1984; DeNiro and Weiner, 1988a; Stafford Jr. et al., 1991), the reason being Hyp is one of the most water-soluble amino acids, therefore likely to be among the first to be lost from degraded collagen bone (Meister, 1965). In fact, measurement of the remaining percentage of hydroxyproline in human collagen was suggested in forensic science as a way to determine time elapsed since death (Knight, 1968). It is expected, however, that some of the very low collagen bones still contain endogenous collagen, probably partially degraded. From its very nature, the ultra-filtration step will reduce yields significantly (e.g. the Kostenki human had yielded 6% collagen when ultra-filtration was applied, as opposed to 10% yield for the gelatinisation only method). The low yields are a result of the fact that everything smaller than 30kDa (about a third of the collagen α chain) will pass through the filter and will not be dated. This step is of course intended for the elimination of contaminants, but inevitably will also bring to the loss of more degraded collagen. For older bones (e.g. Palaeolithic), in which the collagen's quality has deteriorated, more is lost during ultra-filtration (Brock et al., 2010b). These bones, which still contain collagen but with compromised integrity, embody another group of bones that can benefit from the hydroxyproline dating method.

But how many of those low collagen bones that fail to get dated using the normal method are there? And how can the ones that could potentially get dated using the Hyp method be identified? At ORAU, bones from sites known to have variable or poor

collagen preservation are pre-screened in order to identify samples suitable for radiocarbon dating using conventional methods (samples that yield >1% wt collagen) by measuring the %N content of whole bones (Brock et al., 2007; Brock et al., 2010b; Brock et al., 2012). The %N of fresh modern bone is 3.5-4.5 (Stafford Jr. et al., 1988). Although when measuring the whole bone nitrogen content it is not only the nitrogen from collagen that is being measured, but also nitrogen from NCPs and potentially nitrogen from depositional soil (e.g. microbial proteins, humic acids) or conservation material, it has been shown that %N>0.7 is a good indicator for the survival of collagen, with ~70% prediction success (Brock et al., 2012). Figure 5.4 shows the analysis of 521 bones from a wide range of contexts and ages. 82% of the bones (428) had whole bone %N content higher than the 0.7% threshold. Out of those, 26% (112 samples, making 21% of the whole dataset) were 'false positives', i.e. yielded less than 1% collagen and were therefore failed. These represent either contaminated bones, or, most likely, bones with degraded collagen that passed through the ultrafilters (with up to 70% of the 'collagen' being <30kD) (Brock et al., 2012). A bone with a high nitrogen content, but low collagen yield, will fail to be dated using conventional methods but may potentially succeed using the Hyp method, if the Hyp content is sufficient and enough bone is available.

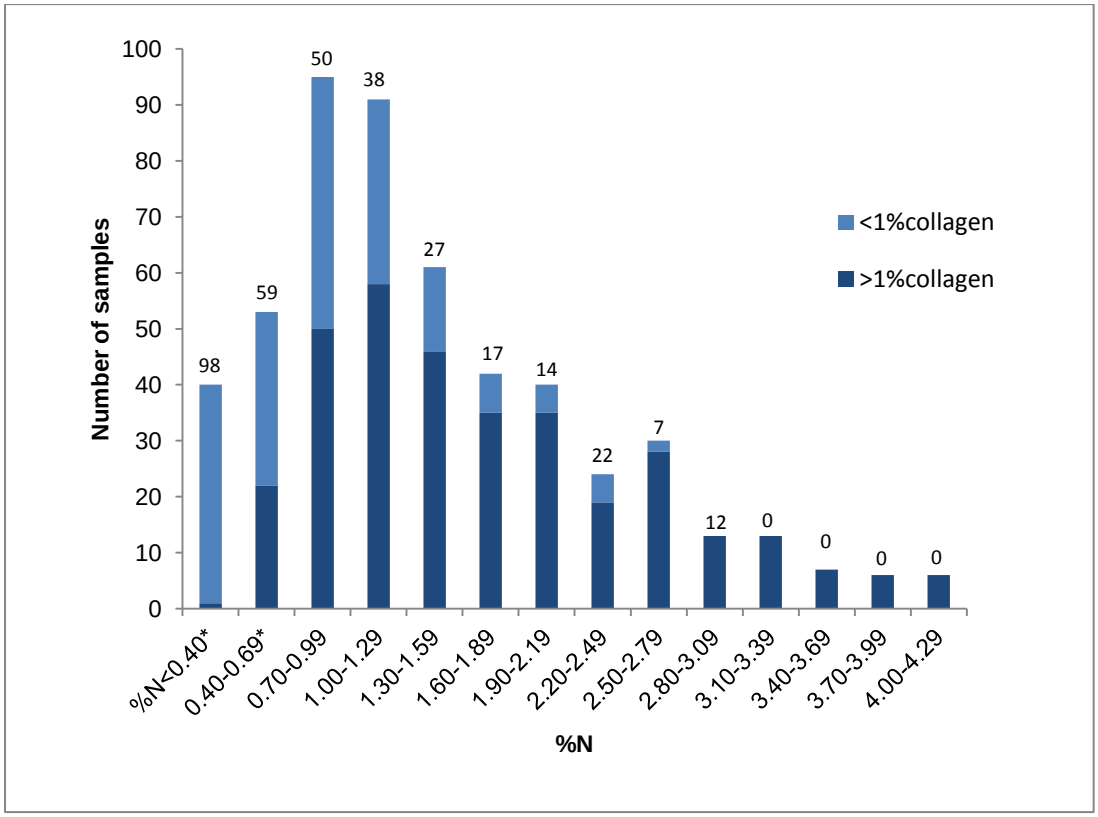


Figure 5.4. The number and percentage of bone samples that yielded <1% and >1% wt collagen for 0.3% ranges of whole bone %N content for a dataset of 521 bones from a wide range of contextes and ages. * The dataset is biased against the lower %N bones, as most of these samples are failed and so do not get analyzed. In addition, the screening for %N is mostly done for bones that are either considered to be poorly preserved or are from archaeological sites known for poor or variable preservation, meaning that the pattern presented here may not apply to all sites.

Figure 5.5 shows a case study in which the low molecular weight fraction of an archaeological bone, which passed the ultra-filter (<30kDa) was hydrolyzed and separated by the HPLC. At least in this case Hyp is found.

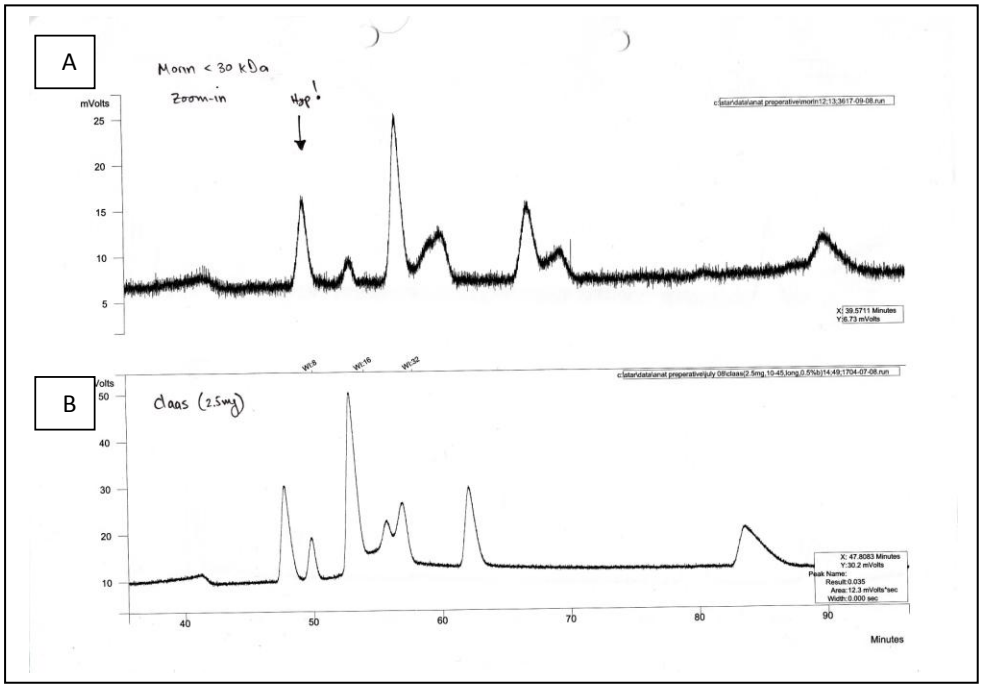


Figure 5.5. (A) Hydrolyzed low molecular weight collagen; (B) collagen-like amino acid standard mixture

Theoretically, low collagen bones could be dated by applying the Hyp method not only to the acid-insoluble ('collagen') fraction, but also to the organic material in the acid-soluble fraction, comprising of degraded collagen in the process of being leached away (Hedges and Law, 1989; Elster et al., 1991; Hedges and van Klinken, 1992; see also section 3.3.2 in chapter 3). However, the attempts to find Hyp in the soluble and insoluble fractions of archaeological low collagen bones from the sites of St. Cesaire and Aktorini failed (section 4.3.2 in chapter 4). Why was Hyp not found in those bones, even though the amino acid profiles of the soluble and insoluble fractions of experimentally degraded bones were both similar to that of published collagen (figure 3.16 in chapter 3)? The window of opportunity for finding Hyp in the collagen fraction in low collagen bones is probably temporally small: the amino acid composition of collagen is known to remain unchanged until values fall to less than 1% of the bone

weight; below that point they change very rapidly (e.g. Hare, 1980; Masters, 1987; Dobberstein et al., 2009). It appears that the process of collagen degradation is not random, and the acid insoluble collagen is either present as intact alpha-chains, or absent (Dobberstein et al., 2009). Moreover, soluble, degraded collagen will probably be retained, if at all, under special circumstances only, wet environments leading to its loss. A probable explanation for the absence of Hyp in the archaeological bones is that this window was missed in the bones chosen, as their minute %N content might suggest: the pygmy hippo bone from Aktorini and the bison from St. Cesaire had only 0.02% and 0.12% nitrogen, respectively.

It also cannot be expected that the experimental degradation of bones would be an accurate model of diagenesis (as was also found by Dobberstein and co-workers for Pleistocene bones Dobberstein et al., 2009). Although the features of cooked bones resemble those of diagenetic bones, unlike the sterile laboratory conditions, the taphonomic environment of archaeological bone includes soil particles (e.g. ions, minerals, humidified materials, polysaccharides, bacteria and fungi) that could interact and exchange with the bone (see section 1.2.4 in chapter 1). These interactions are likely to affect both the speed of collagen diagenesis and the chemical changes that take place; this could also of course be site specific (e.g. Brock et al., 2012, regarding the application of site-specific %N threshold values).

In spite of these reservations, it is still possible, that in some cases Hyp could be isolated from the soluble fraction of a big low-collagen bone with high %N and low collagen yield, and a reliable date could be obtained. The only way to find out if Hyp survives in the soluble fraction of a bone is to attempt isolating it; presumably this will

take place only with a sample whose date would be important enough to justify the extra investigation.

5.4 The amount of bone required for dating

One of the disadvantages of the Hyp method is that at best, only 12% (by wgt) of the collagen can be used, demanding that the starting material be at least 10 times bigger than that required for routine bulk collagen dating. How much bone is required to date using the hydroxyproline method? The answer depends on the state of preservation of the bone. To produce a graphite target of 1mg of carbon, around 2mg of hydroxyproline needs to be isolated, given that the Hyp carbon content is 45.8%. In modern and well preserved bones, the percentage of collagen is around 16-33% of the bone (DeNiro and Weiner, 1988a), and Hyp consists 12% of the collagen, and so around a 100mg of bone will be required. In a well preserved archaeological bone the collagen yield would be in the range of 1%-10%, but as already mentioned, some work indicates the Hyp levels will decrease with collagen deterioration (DeNiro and Weiner, 1988a; alternatively, for 'all or nothing' view on the mode of collagen degradation see Dobberstein et al., 2009). The initial bone weight needed will therefore be between 200mg and 20g, but normally a few grams are likely to suffice (see table 5.1). In bones with trace amount of collagen, below ~1% protein content, a total loss of hydroxyproline has been observed (Bada et al., 1984; DeNiro and Weiner, 1988a; Stafford Jr. et al., 1991)[29]. One should also take into account that the sample recovery is never 100%, and in fact was measured to be approximately 50% using the current protocol. The requirement for big samples in the range of a few hundred milligrams to tens of grams (as opposed to a few milligrams for

[29] As already explained, in some cases Hyp will be lost altogether from the collagen fraction, but it could be attempted to find it in the acid-soluble fraction. It is not known a priori how much bone will need to be processed in such a case, but probably at least in the range of 10s of grams.

normal AMS dating) is of course a limitation of the method, as such big samples will not always be available.

Figure 5.6 shows the distribution of low weight (<200mg), medium weight (200mg<bone weight<1g) and high weight (>1g) bones submitted to ORAU. Encouragingly, it is calculated that about 70% of all bones submitted for dating are bigger than one gram, and at least a third of those (about 20% of all bones) are bigger than 10g (the exact number is not known, because in many cases only the 600mg of bone sample required by ORAU is sent, although more of the bone is available; in addition, submitted bones bigger than 1g are sometimes not weighed, and some of those may well be bigger than 10g).

Table 5.1 summarises the collagen yields, the amount of hydrolyzed collagen used to separate Hyp and the sizes of the obtained Hyp graphites of the different archaeological bones treated in this D.Phil. A calculation of the amount of starting material needed to obtain a graphite 1mg carbon of size is presented. It is not claimed that all the bones that will be treated using the Hyp method will be the same as the ones dated in this D.Phil., but it can give an estimate as to the applicable range of bones samples.

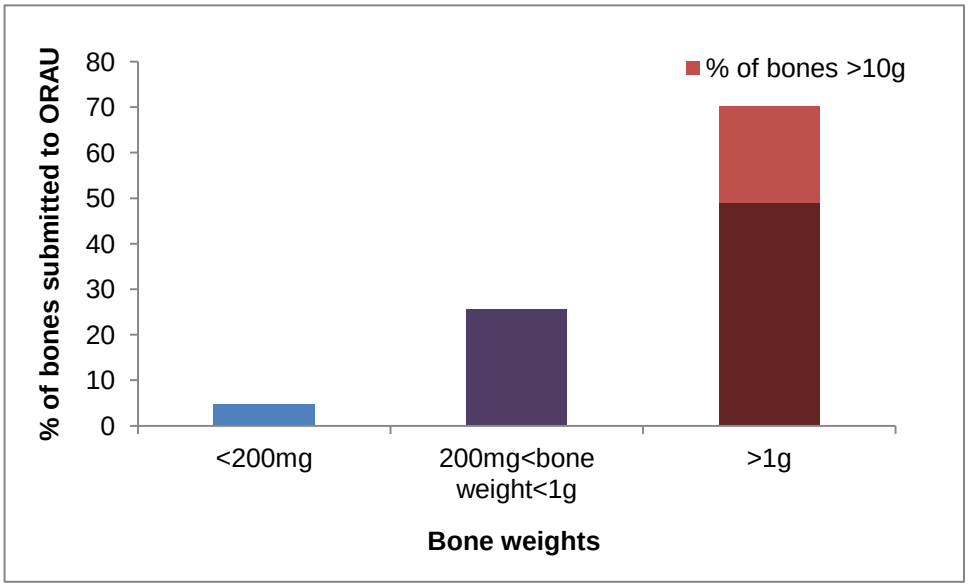


Figure 5.6. The distribution by weight of bone samples submitted to ORAU.

Bone	Collagen yield (%)	Amount run on HPLC (mg)	graphite size (mg)	Amount of bone required (in mg) to obtain 1mg graphite
Mary Rose	17	30	0.54	330
Lemon Mine	10	30	1.0	300
Chalk Hill	6.8	45	1.1	600
Kostenki man	5.7 (AF) 10	40	1.17	340
Flixton	9.8 (AF*)	30	0.81	380
Sungir 2	9.5 (AG)	30	0.65	490
Sungir 3	6.1 (AG)	30	0.67	730
Sungir mammoth	5.7 (AG)	30	0.88	600
Kostenki horse	0.8	30	0.84	4460

Table 5.1. Collagen yield, the amount of hydrolyzed collagen used to separate Hyp and the size of the obtained Hyp graphite for the different archaeological bones used in this PhD. In red – the calculated size of the bone that was theoretically need to be sampled in order to obtain a graphite of 1mg carbon. AG denotes acid-base-acid and gelatinisation treatment, but no ultra-filtration; AF denotes ultra-filtration. * denotes solvent extraction. When no lab code is assigned the collagen was extracted using demineralisation and gelatinisation only. Using the latter method collagen yields are generally higher, and so less bone will be required.

5.5 Under what set of circumstances should hydroxyproline dating be applied?

Although the Hyp method has proved to be successful for a number of applications, it is not suggested here that it should necessarily be used routinely. The main reason for this is the fact it takes more sample preparation time than conventional methods, and in addition, the need for larger quantities of starting material. However under some circumstances, detailed in figure 5.7, the application of the method will be highly advantageous, and could contribute greatly to the field of archaeology in providing dates for 'controversial' bones and for bones that fail to get dated using the normal method.

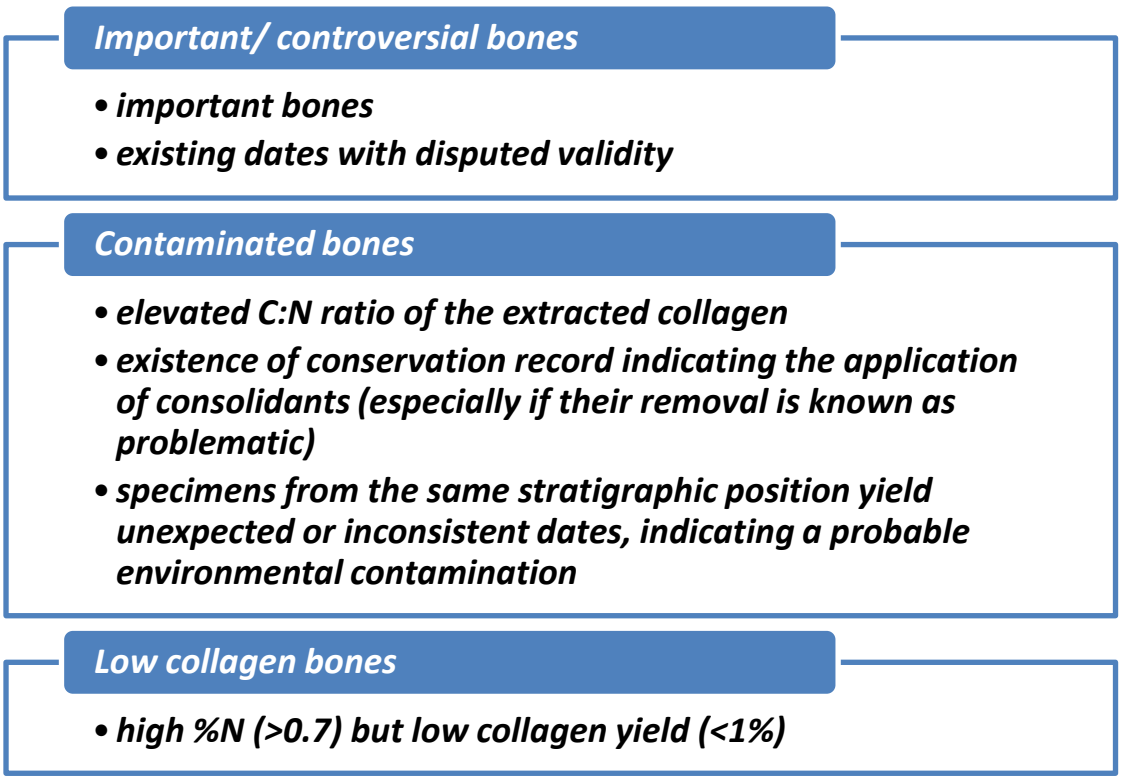


Figure 5.7. A suggested set of circumstances under which the hydroxyproline dating method should be applied.

5.6 Conclusions

The development of the radiocarbon measurements and AMS revolutionised the field of archaeology by making it possible to attribute absolute dates to objects and bones. However, contaminated or severely weathered bones are notorious for being hard to date accurately. The application of more rigorous ultra-filtration protocols has improved this situation recently (e.g. Jacobi et al., 2006), but still high molecular weight or cross linked contaminants do not get removed, affecting the date. In addition, bones in which the collagen levels have dropped to less than 1% of the bone weight are suspected of being diagenetically altered and do not get dated. As shown by the dates obtained in this thesis, the hydroxyproline dating method has the potential to have a major effect on the field of archaeology by enabling reliable dating of important and controversial bones, as well as bones considered 'un-datable'. The method makes use of a chemically characterizable, compound-specific molecule that eliminates the contamination that other methods cannot, and should therefore serve as a 'gold standard' for dating. Appendix E summarizes the recommended methodology for Hyp dating.

One area likely to benefit from the hydroxyproline dating method is that of dating the spread of early anatomically modern humans out of Africa and into Europe and Eurasia. The nature and timing of major demographic dispersals, Neanderthal extinctions and admixture across Eurasia is heavily based on radiocarbon chronologies. However, the direct dating of hominin fossils is likely to be problematic both because the important and scarce specimens are often conserved in museums and collections and so potentially contaminated with more modern carbon, and because geological and museum-derived contamination becomes increasingly important as the limit of

radiocarbon is approached. As already mentioned, it is suggested that perhaps ~70% or more of the bone dates from the Middle and early Upper Palaeolithic are liable to be underestimates of the true age (Higham, 2011). Setting aside the erroneous dates and obtaining new, reliable ones for these fossils is therefore crucial in order to be able to correctly reconstruct the history of Palaeolithic hominin migrations.

5.7 Future work

As mentioned, among the main drawbacks of the hydroxyproline dating method compared to the traditional bulk dating methods at present are its added treatment time and reduced efficiency. The extra costs also need to be taken into account. For a bone (corresponding to the set of circumstances outlined in figure 5.6) to be dated by the hydroxyproline method it needs therefore to be determined first if it is worth the additional time, cost and effort. The additional time required is mostly for sample hydrolysis (24h), and for the separation and evaporation of the mobile phases (38 hours). As a way for shortening the evaporation time, and probably also improving the efficiency, it was planned to connect the first HPLC column, used for the separation of the collagen amino acids, to the second column, used for the removal of the mobile phase, so that only the eluent from the second run would need to be evaporated. This was not achievable in the timeframe of this project, but could be explored in future work, together with other approaches to shorten the procedure, especially the separation time. When this is accomplished, it would make the method more easily and widely available, enhancing its potential to play a significant role in providing more accurate archaeological chronologies. Future work could also involve investigating Hyp fractionation during the HPLC separation (there is a possibility that carbon isotope fractionation is produced by incomplete recovery of the Hyp fraction as it is eluted);

what types of contamination the hydroxyproline dating method is able to remove (it would be interesting to artificially contaminate bone with other materials, or even add contaminating compounds during the hydrolysis step and check the method's success in eliminating them); what is the nature of background carbon contamination resulting from the protocol, and whether it can be reduced. Lastly, although not many samples have been dated so far using the hydroxyproline method, it has already proved to be a very powerful tool, and so the identification of other suitable bones and their dating using the method is greatly anticipated.

Appendices

Appendix A: Principles of chromatography methods used for separating amino acids

Reverse Phase Chromatography (RPC)

This is the most common chromatographic method utilizing a stationary phase composed of non-polar functional groups, commonly linear alkyls chains of 18 carbon atoms in length (C_{18}). The mobile phase used is an organic solvent (e.g. methanol, acetonitrile), and the retention of the analyte to the stationary phase is based on hydrophobic interactions. The mobile phase competes with the stationary functional groups and the analyte is desorbed according to its constituent's hydrophobicity, the most hydrophobic compounds being the last to elute.

Ion pair chromatography (IPC)

The column and mobile phase used for IP chromatography is similar to the ones used in RP separations. However in IP an ion-paring reagent is added to the mobile phase, in order to improve the separation. Negative molecules of the ion-pair reagent (e.g. hexane sulfonate) are attached to the stationary phase by hydrophobic interaction, and are in equilibrium with positive ions (e.g. Na+) from the reagent. A positively charged sample ion can now exchange with a positive ion from the reagent, resulting in the retention of the sample ion by an ion-exchange process.

Ion exchange chromatography (IEC)

Ion exchange chromatography is particularly useful in separating small and highly charged compounds. Columns used for IE are characterised by the presence of charged groups covalently attached to the stationary phase. The biggest impact that ion

exchange had was to offer the first efficient separation of all twenty biologically important amino acid residues, first explored for ^{13}C fractionation by Abelson and Hoering in 1961 (Abelson and Hoering, 1961). In order to get a chromatographic separation, the surface charge needs to be opposite that of the analyte of interest. An increase in salt or buffer concentration in IEC results in decreased retention, and the effect is greater for more highly charged sample compounds. Varying pH is usually the preferred way to change selectivity in IE separations. With cation exchange, decrease in pH favors the retention of bases, and the pH is gradually increased, whereas with anion exchange it is sequentially decreased.

Mixed-Mode chromatography

Mixed-Mode separation refers to the use of stationary phases that employ more than one retention mechanism, such as reversed-phase and ion-exchange together. Compounds with opposite charge to that embedded in the stationary phase are retained predominantly by ion exchange, and an increase in the mobile-phase ionic strength (or decrease in the mobile phase pH) will reduce their retention. Neutral compounds are retained predominantly by hydrophobic interaction with the stationary phase, and increase in organic solvent concentration will result in decreased retention. It is probably the case however that both mechanisms influence most types of compounds, albeit unequally. Mixed-mode separations have been mostly used for separating biological samples. Their more complicated production of the mixed-mode columns infers greater column-to-column variability. See more on the mixed mode column used in this thesis in the text (chapter 1, section 1.3.3).

Appendix B: Method development

Attempts to reduce HPLC run time

To achieve a reduction in the chromatographic run time (10h per injection), a stepped gradient separation method was developed: after the first 135 min in which a linear gradient between 40 min to 70 min occurred, and the non-essential amino acids eluted (Asp, Hyp, Ser, Thr, Gly, Glu, Ala and Pro), the composition of the mobile phase was adjusted from 0.3% to 2% phosphoric acid, and the flow rate raised from 6mL/min to 15mL/min in order to elute all the remaining amino acids (Val, Hyl, Met, Lys, His, Ile, Leu, Tyr, Arg and Phe). This approach was adopted because collecting essential amino acids (those later eluting) were not of interest for this research and their retention times were very long, increasing the sample to sample injection time significantly. Although this approach markedly reduced the run time it was subsequently abandoned due to evidence that the increase in acidity led to greater column bleed creating a higher carbon background (McCullagh et al., 2010). The method finally adopted is the one described in the chapter 2, section 2.2.2.2.

Removal of mobile phase

After the mobile phase eluent, containing individual amino acids is collected, most of the liquid is removed by rotary evaporation, and the remaining is loaded onto Chromosorb^{TM} in a tin capsule to be combusted and graphitised (see section 2.1.1). The loading capacity of the tin is limited both in volume – the maximum that can be loaded is 30µL, and in pH– high acidity will oxidize and perforate the tin container. However after the HPLC separation one is left with an amino acid in tens of milliliters of eluent (typically 54-90 ml for Hyp). If the mobile phase is non volatile, as is the case with phosphoric acid, evaporation will reduce the volume but also concentrate the acid.

Several approaches were tested in order to solve this problem: changing the mobile phase, precipitating out the phosphate using iron, and re-injecting sample in different mobile phase conditions.

If the mobile phase used was changed into a wholly volatile one, the mobile phase could be evaporated without concentration of acidity. One candidate was Acetonitrile (ACN), a typically used mobile phase in liquid chromatography. ACN was tried and abandoned, however, because its UV absorbance overlaps with that of the amino acids, obscuring their detection. In addition, using an organic as a mobile phase was avoided, as it could potentially contaminate the samples with residual carbon.

A different approach tried was precipitating out the phosphates in the eluent by using iron. Finely powdered iron was added to the concentrated analyte mixture. The iron-phosphates generated was then centrifuged and filtered, leaving the amino acid in water only. This was abandoned because the procedure proved to be very inefficient, mostly because the iron-phosphate tended to block the filters.

The method eventually adopted involved re-injection of the analyte/H_3PO_4 mixture using the same HPLC column but using only water as the mobile phase. This exploited the fact that the Primesep A column does not retain phosphoric acid, and can therefore be used to separate the amino acid from the phosphoric acid. The eluent from the first run was concentrated on the EZ-2 evaporator, and then re-injected to the column (three times, 1ml at a time), using isocratic elution conditions (100% MilliQ water) and a flow rate of 15 ml/min. The phosphoric acid eluted with the void volume, as determined by measuring the pH of the eluent throughout the run. The eluent collected at the retention

time for the amino acid (typically 45mL for Hyp, with pH ~5) could now be evaporated to dryness using the EZ-2 evaporator. An example chromatogram for the re-injection of Hyp is given in figure B.1:

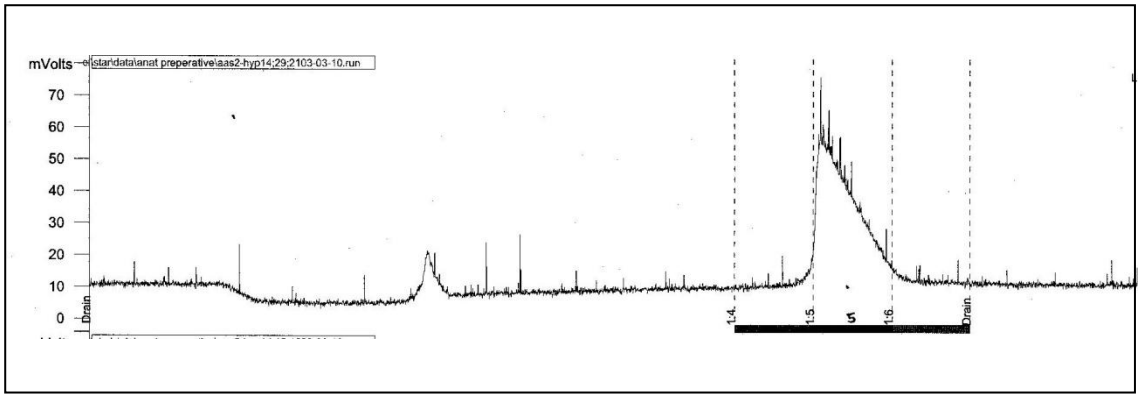


Figure B.1. Re-injection of Hyp. The Hyp fraction from an amino acid mixture was separated applying a phosphoric acid gradient, collected, concentrated and injected again to the HPLC, this time using 100% water as a mobile phase. The phosphoric acid elutes with the void volume, while the Hyp is retained, and elutes off after 10 min. The pH of the Hyp containing eluent is ~5.

The drawbacks of the re-injection method are lower yields, and possible increased background carbon, resulting from the extra handling and/or more passing through columns and tubes (see chapter 3).

Appendix C: Data for the calculation of the date correction

$A_m = f_d * A_d + f_M * A_M + (1-f_d-f_M) * A_s$
$f_d = (f_M + A_s - f_M * A_s - A_m)/A_s$

	Pnumber	date	±	A_m	±	f_M	A_s	f_d	dead C (µg)	Modern C (µg)
MR Hyp	24705.0 NRC2 01	327	29	0.9601	0.0035	0.0027675	0.96202	0.002	**1.1**	**1.4**
	24705.1 NRC 01	351	31	0.9573	0.0037	0.003125	0.96202	0.005	**2.4**	**1.4**
	24705.1 NRC1 01	319	31	0.9611	0.0038	0.002459	0.96202	0.001	**0.6**	**1.4**
	24705.2 NRC1 01	337	26	0.9589	0.0030	0.0016648	0.96202	0.003	**3.0**	**1.4**
MR Ala	24705.1 NRC2 01	419	30	0.94915	0.0036	0.0077827	0.96202	0.014	**9.3**	**4.7**
	24705.1 NRC3 01	410	40	0.94776	0.0049	0.0114471	0.96202	0.015	**7.1**	**4.7**
	24705.2 NRC 01	390	35	0.95262	0.0041	0.0102515	0.96202	0.010	**5.2**	**4.7**
	24705.0 NRC 01	399	31	0.95156	0.0037	0.0114471	0.96202	0.011	**5.2**	**4.7**

Table C.1. Calculation of f_M, f_d and 'dead' C for Mary Rose samples. On average the dead C addition to the Mary Rose Hyp samples was 1.8±1.1 ug C, and to the Ala samples 6.7±1.9 C. In total 3.2±1.1 of ug C were added to the Hyp samples, and 11.5±2.0 were added to the Ala samples

$A_m=(W_c*A_c+W_s*A_s)/(W_c+W_s)$
$A_s=(A_m*(W_c+W_s)-W_c*A_c)/W_s$

$\partial A_s/\partial W_M=(A_m-A_M)/W_s=(A_m-1)/W_s$
$\partial A_s/\partial W_d=(A_m-A_d)/W_s=A_m/W_s$
$\delta A_s(M)=\partial A_s/\partial W_M*\delta W_M$
$\delta A_s(d)=\partial A_s/\partial W_d*\delta W_d$
$\sigma^2=\sqrt{(\delta A_s(M)^2+\delta A_s(d)^2+\delta A_s(AMS)^2}$

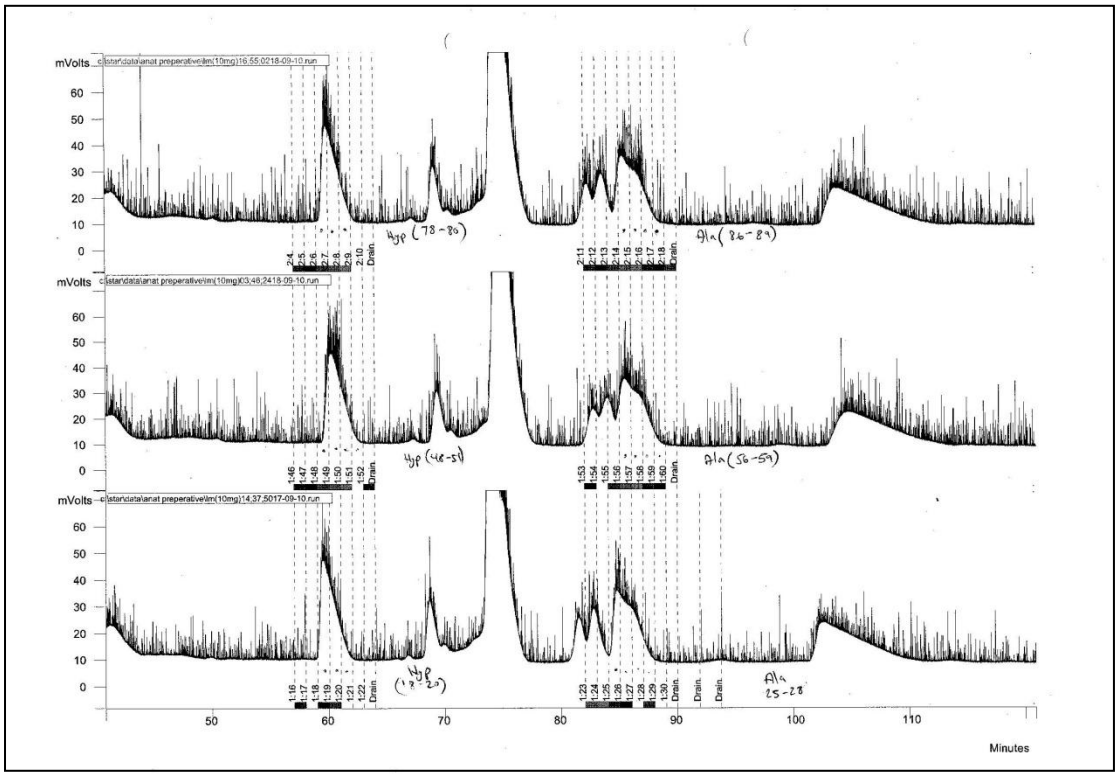
	Pnumber	W_c	W_s	A_c	A_s	δA_s(Modern)	δA_s(dead)	δA_s(AMS)	σ^2	cor. Date	±
MR Hyp	24705.0 NRC2 01	3.2	538.76	0.44	0.963209555	-2.22233E-05	0.00196024	0.00348	0.003994	**301**	**33**
	24705.1 NRC 01	3.2	476.76	0.44	0.960796208	-2.68752E-05	0.002208698	0.00365	0.004266	**321**	**36**
	24705.1 NRC1 01	3.2	606.76	0.44	0.963885391	-1.92284E-05	0.001742404	0.00377	0.004153	**295**	**35**
	24705.2 NRC1 01	3.2	897.76	0.44	0.9607678	-1.37342E-05	0.001174913	0.003	0.003222	**322**	**27**
MR Ala	24705.1 NRC2 01	11.4	669.57	0.41	0.958288379	-5.77176E-05	0.002735875	0.0036	0.004522	**342**	**38**
	24705.1 NRC3 01	11.4	451.57	0.41	0.961274841	-8.79208E-05	0.004050705	0.00486	0.006327	**317**	**53**
	24705.2 NRC 01	11.4	505.57	0.41	0.964801195	-7.12242E-05	0.003636601	0.00413	0.005503	**288**	**46**
	24705.0 NRC 01	11.4	451.57	0.41	0.965171026	-8.15253E-05	0.004066946	0.00372	0.005512	**285**	**46**

Table C.2. Calculation of A_s, the corrected date and the propagated error for the Mary Rose Hyp and Ala samples after applying the correction algorithm.

Pnumber	fraction	date	±	F14C	W_c	W_s	A_c	δA_s(Modern)	δA_s(dead)	δA_s(AMS)	σ^2	corrected $F^{14}C$
24,707.2 NRC 01	LM Hyp	45,900	>	0.00132	3.24	1030.76	0.44	-0.000290663	1.40867E-06	0.00100	0.001041	-5.8908E-05
24707.3 NRC1 01	LM Tea Hyp	44,100	>	0.00163	3.24	826.76	0.44	-0.000362271	2.16871E-06	0.00125	0.001301	-8.79336E-05
24,707.4 NRC 01	LM Hyp	44,200	>	0.00197	3.24	987.76	0.44	-0.000303119	2.19385E-06	0.00106	0.001045	0.000533196
24,707.4 NRC1 01	LM Hyp	45,100	>	0.00132	3.24	895.76	0.44	-0.000334469	1.62097E-06	0.00116	0.001207	-0.000266723
24707.5 NRC 01	LM Hyp	41,100	>	0.00228	3.24	566.76	0.44	-0.000528118	4.42515E-06	0.00191	0.001982	-0.000222316
24,707.2 NRC1 01	LM Ala	39,000	1600	0.00779	11.43	664.57	0.41	-0.001134688	2.26232E-05	0.00159	0.001953	0.000872354
24707.5 NRC2 01	LM Ala	39,200	2000	0.00763	11.43	538.57	0.41	-0.001400377	2.73426E-05	0.00189	0.002352	-0.000909445

Table C.3. Calculation of the corrected $F^{14}C$ and the propagated error for the Lemon Mine hydroxyproline and alanine samples.

Appendix D: HPLC chromatograms archaeological bones samples[30]



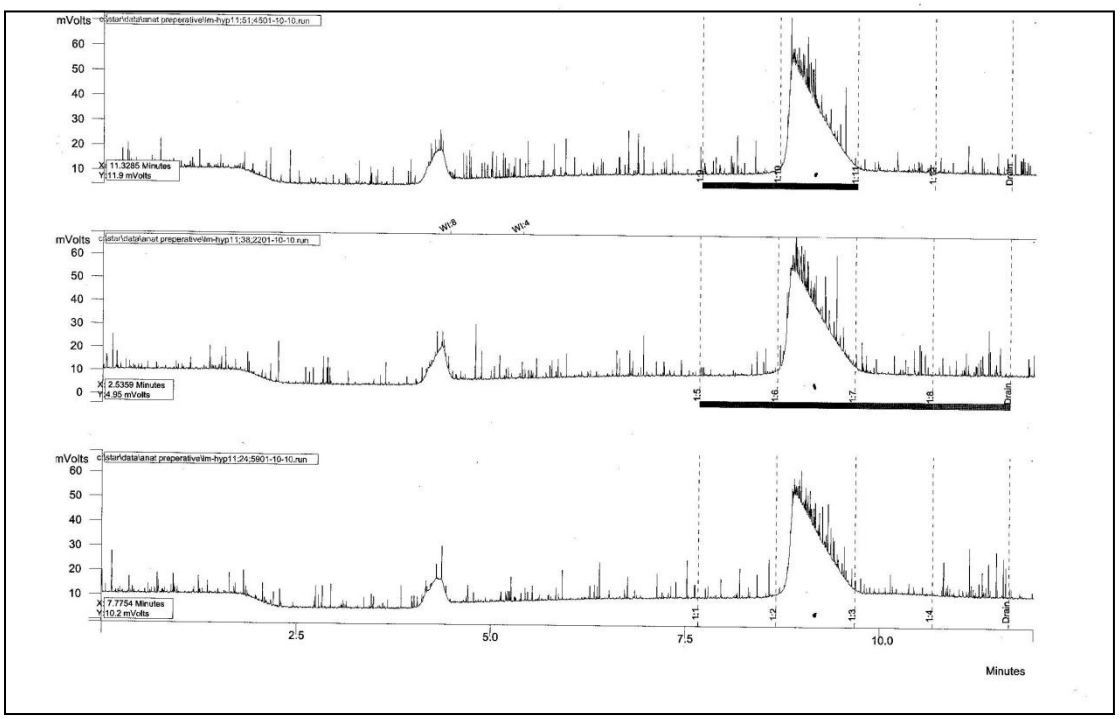


Figure D.1. Lemon Mine first injections (zoomed in) (top); Lemon Mine second injections (bottom).

[30] Note that in many cases the baseline is noisy, due to the UV lamp nearing the end of its lifetime. The vertical dashed lines represent the start and stop time for collection on the fraction collector.

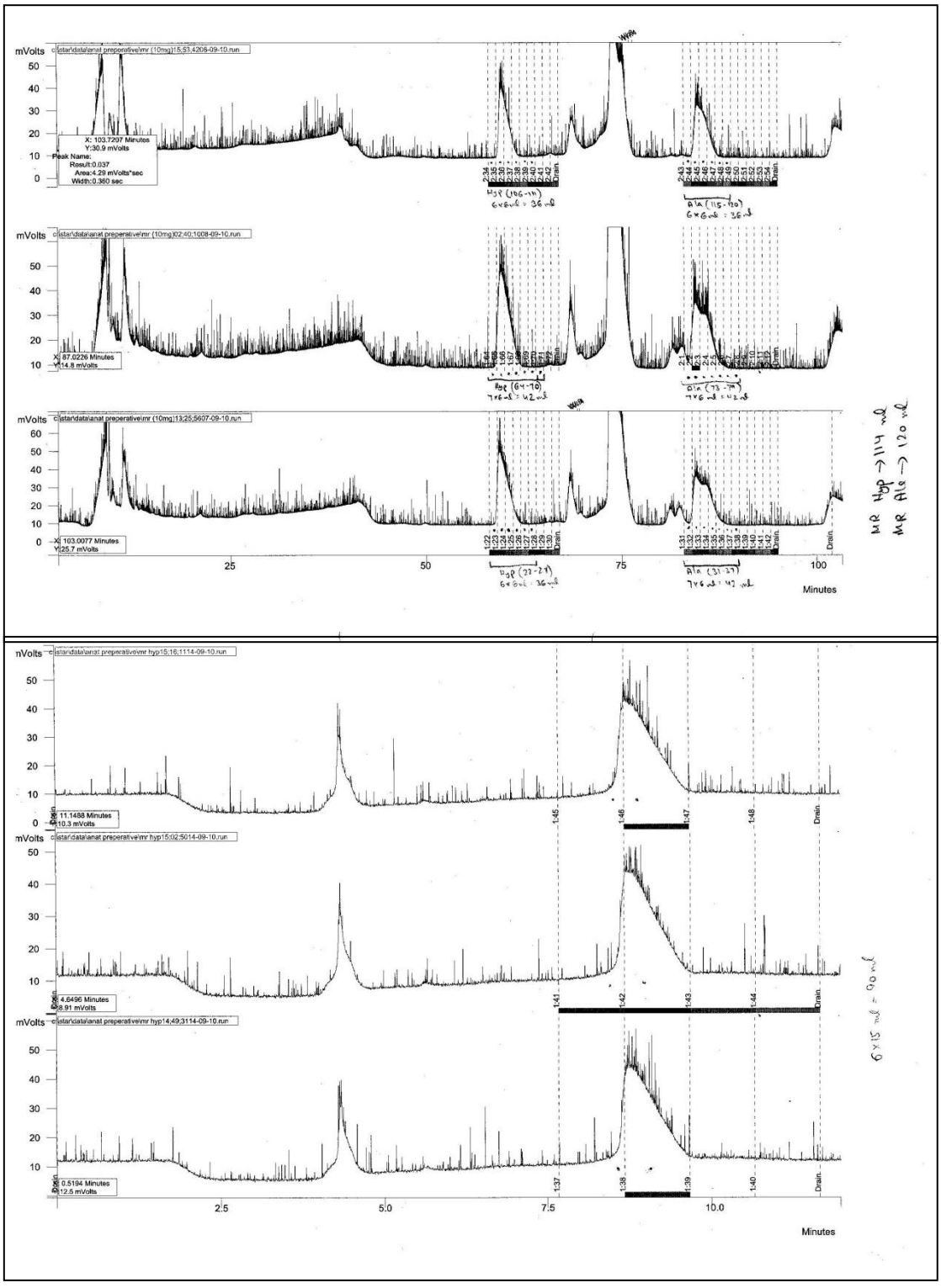


Figure D.2. Mary Rose first injections (zoomed in) (top); Mary Rose second injections (bottom).

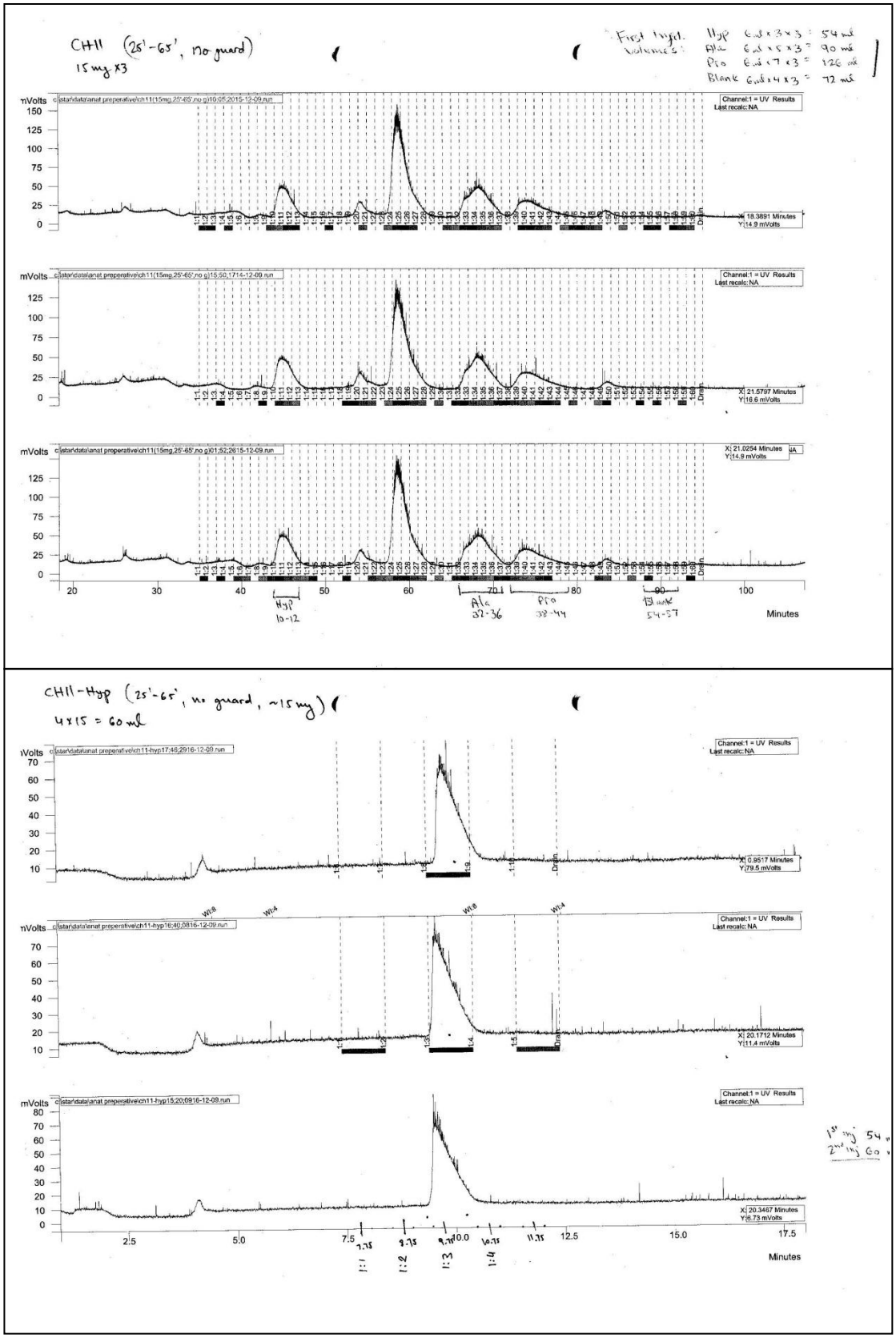


Figure D.3. Chalk Hill first injections (zoomed in) (top); Chalk Hill second injections (bottom).

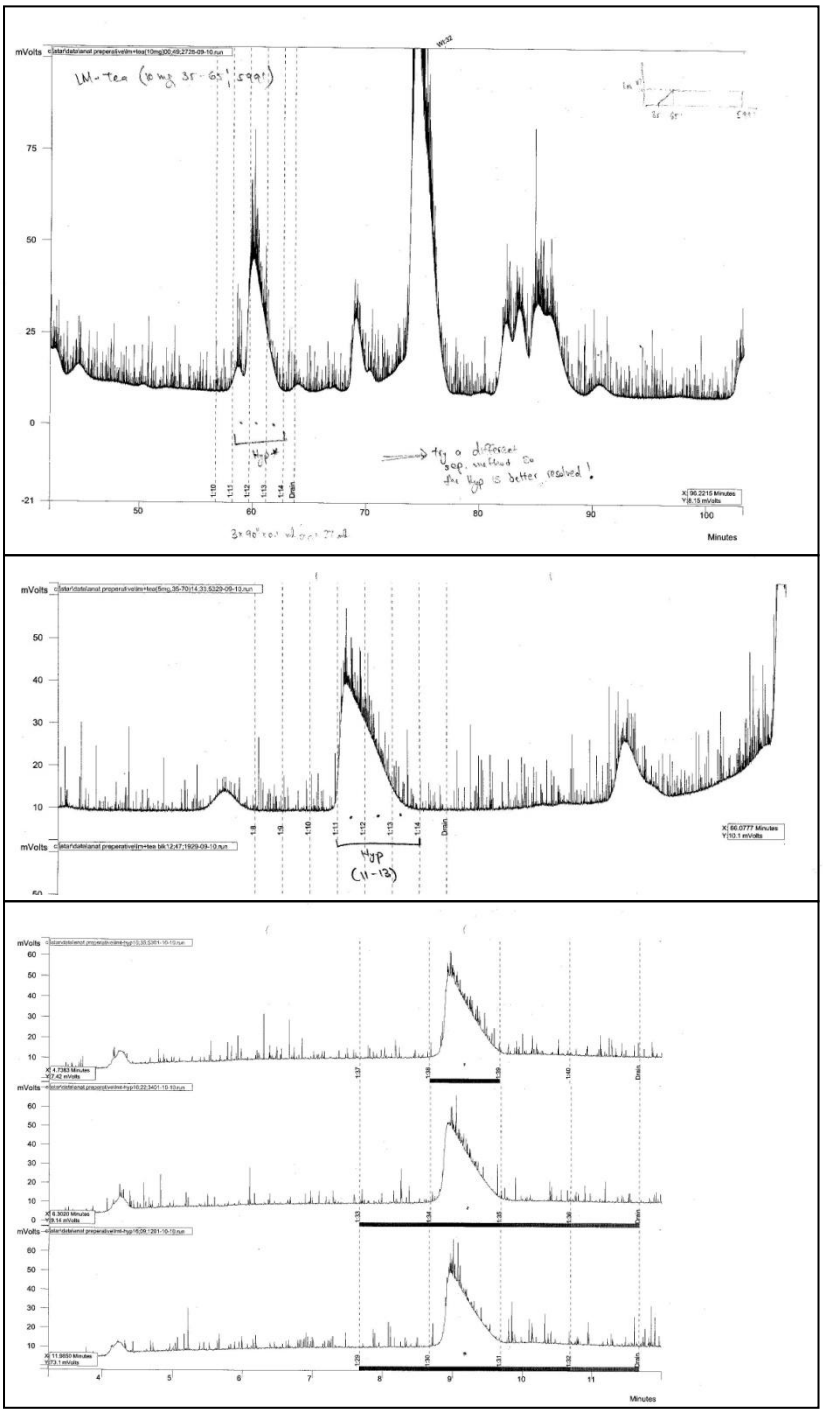


Figure D.4. Tea contaminated Lemon Mine first injection (10mg, gradient affected from 35' to 65'): Hyp is not totally resolved (zoomed in) (top); the same sample was separated using a slightly different method (5mg, gradient affected from 35' to 70') (middle); Tea contaminated Lemon Mine second injections (bottom).

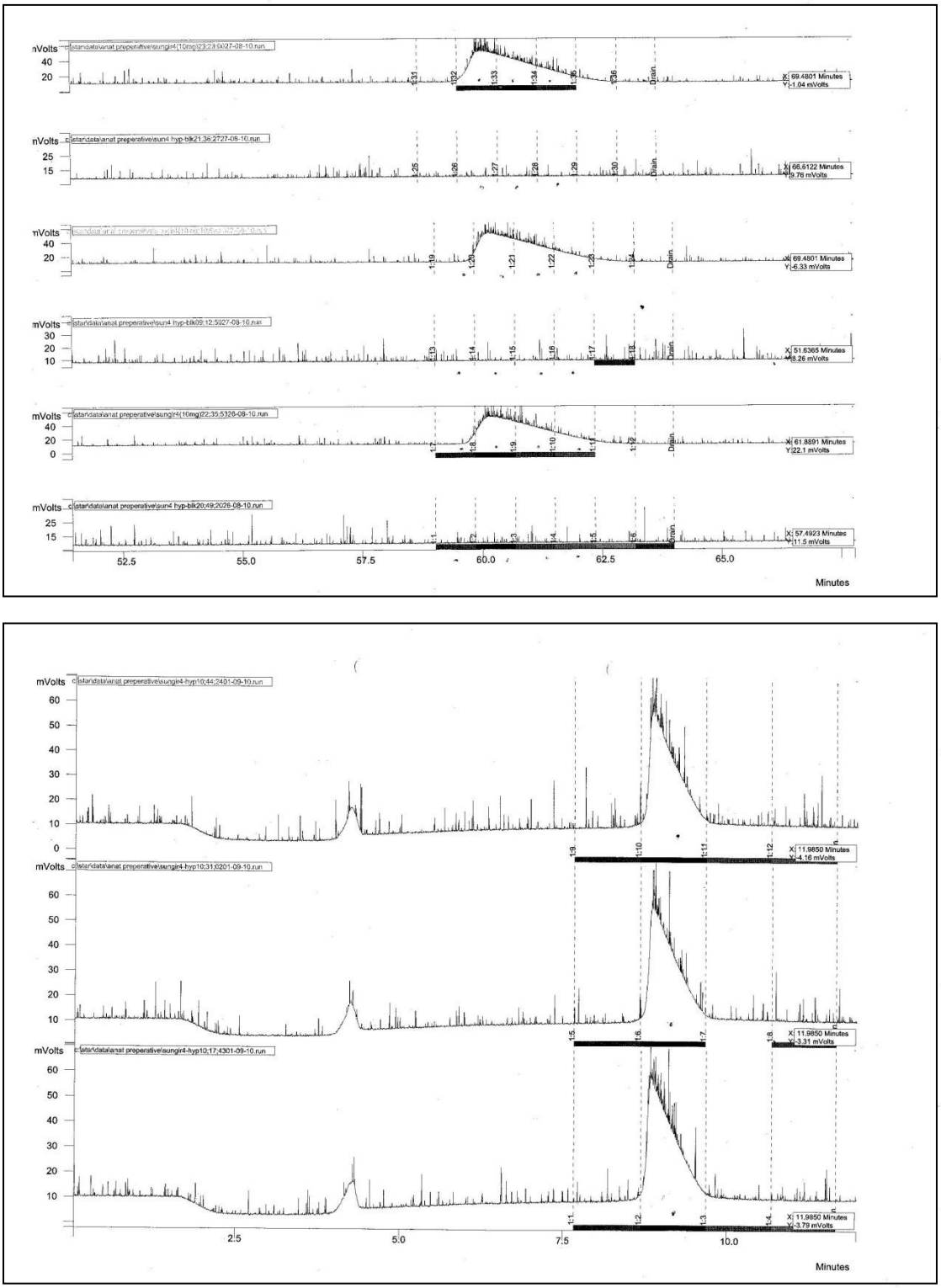


Figure D.5. Flixton first injections (zoomed in; each run is followed by a water injection) (top); Flixton second injections (bottom).

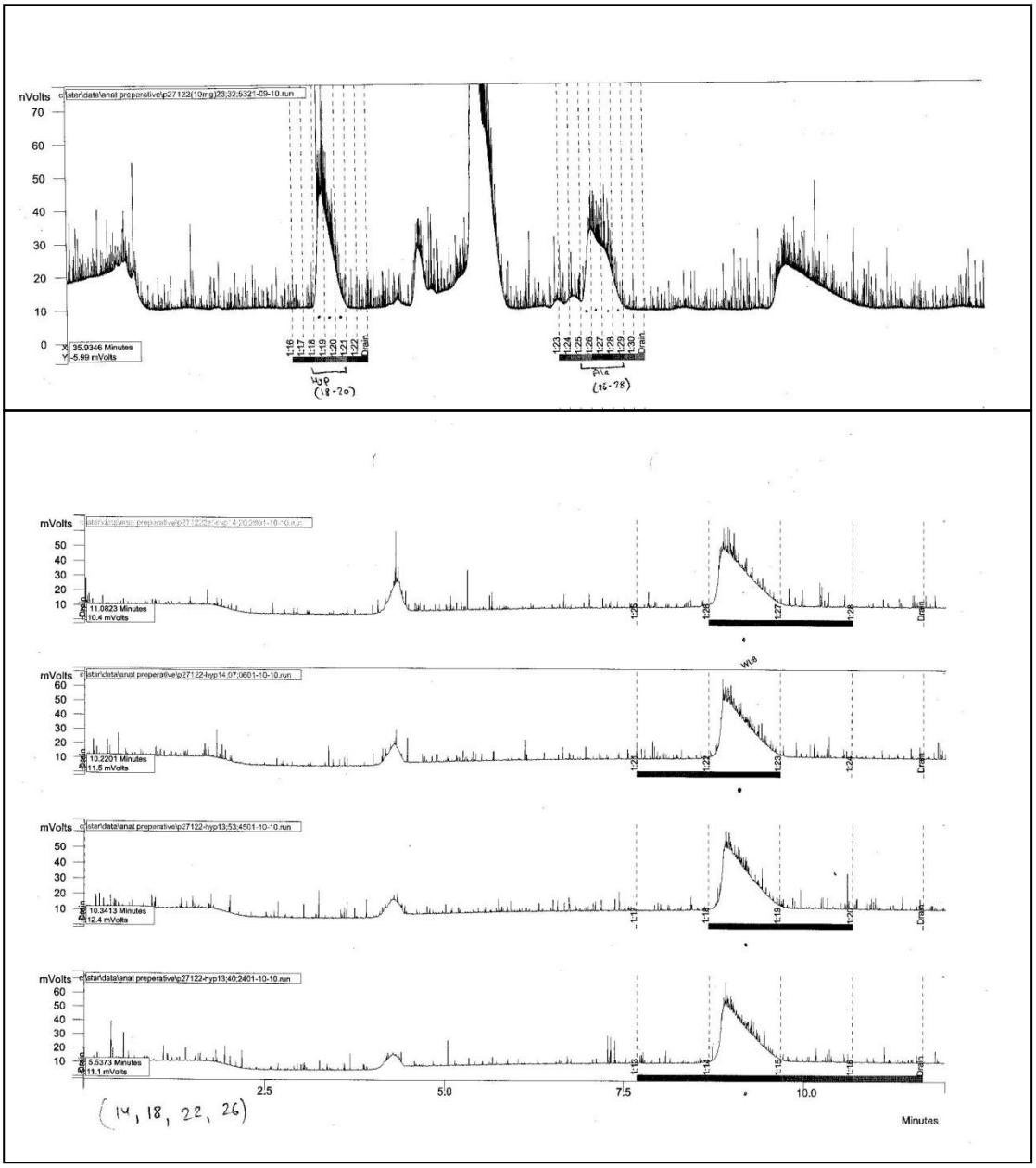


Figure D.6. Kostenki man first injection (zoomed in; one of four) (top); Kostenki man second injections (bottom).

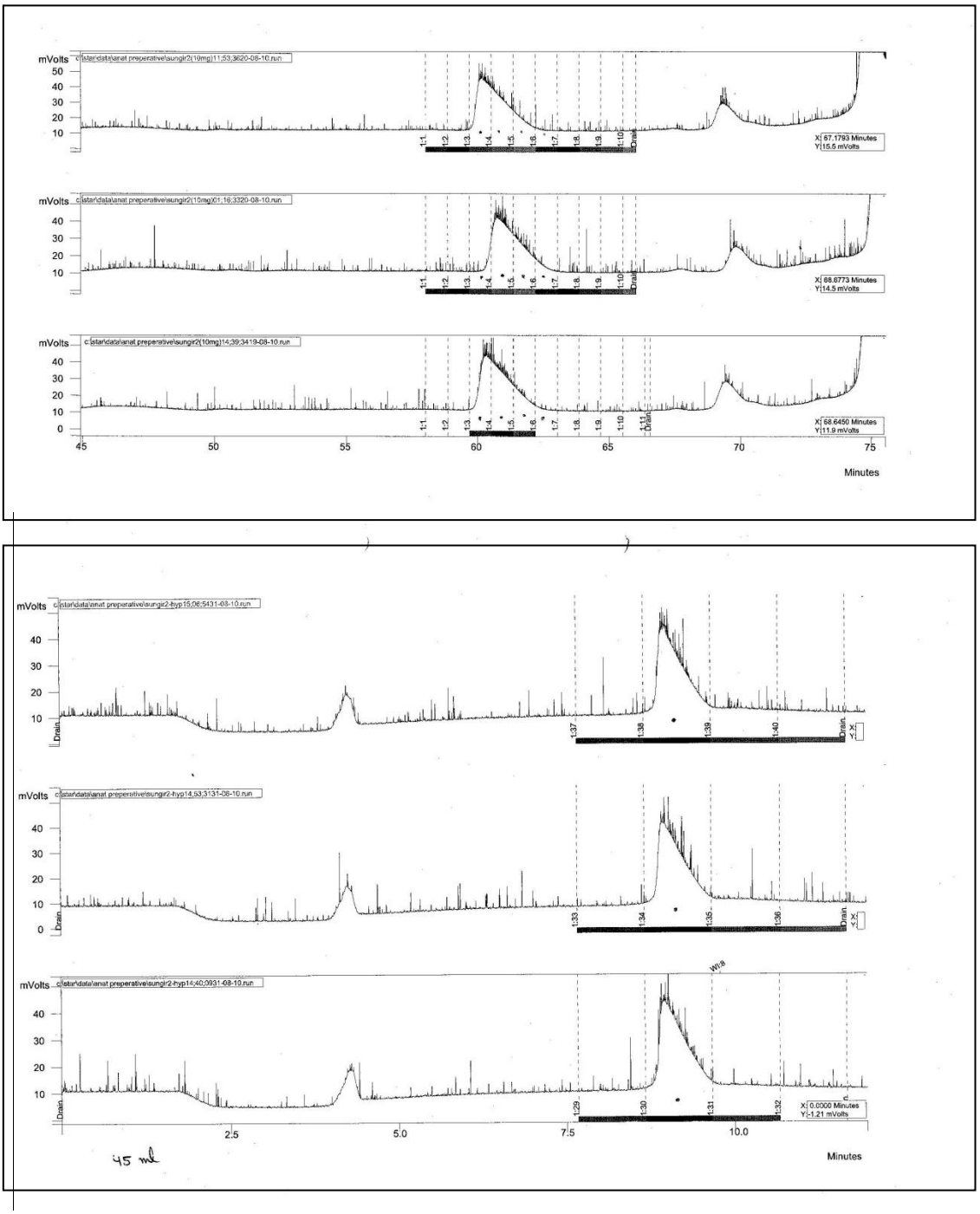
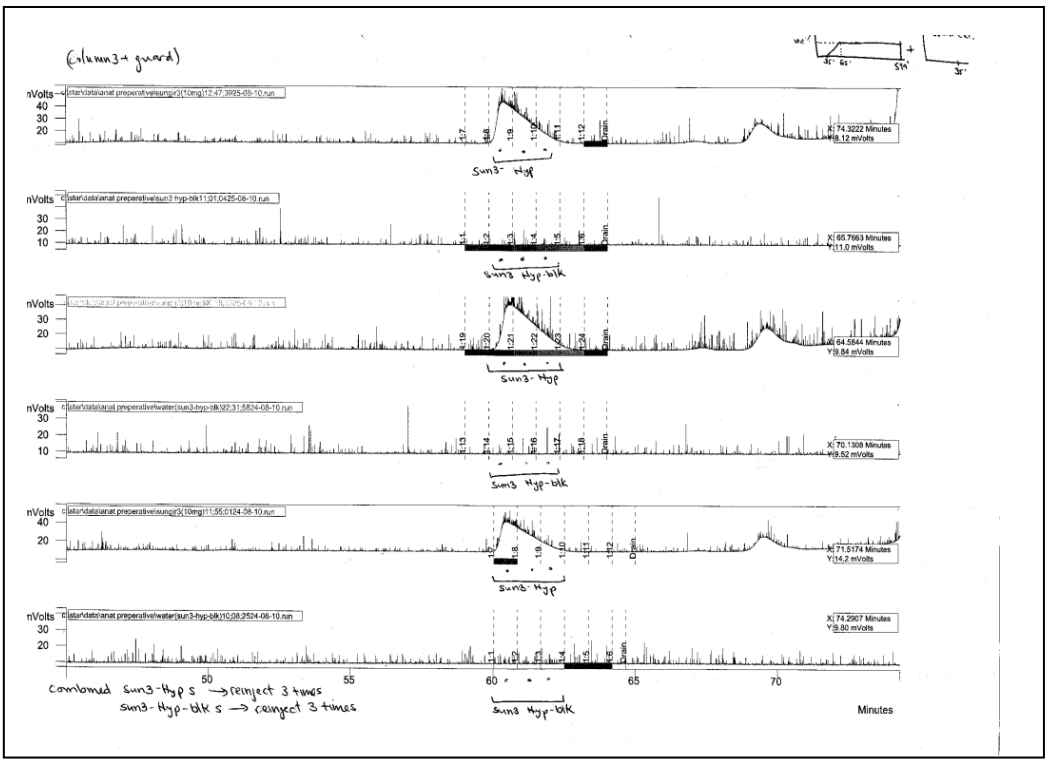


Figure D.7. Sungir 2 first injections (zoomed in) (top); Sungir 2 second injections (bottom).



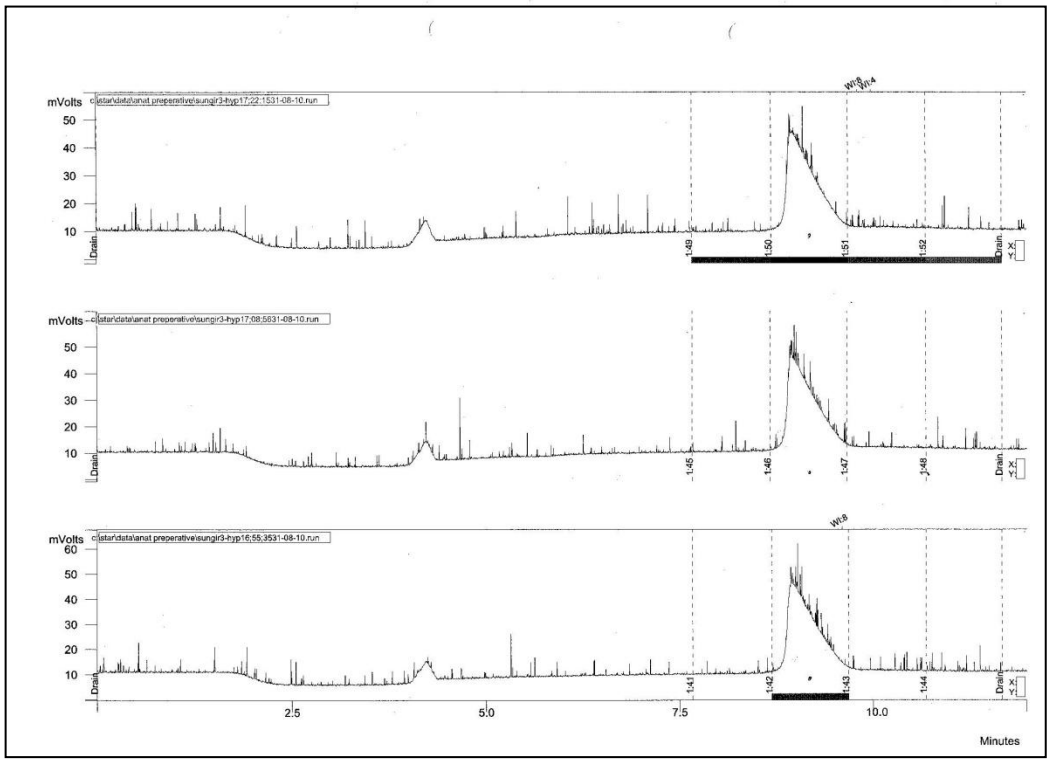


Figure D.8. Sungir 3 first injections (zoomed in; each run is followed by a water injection) (top); Sungir 3 second injections (bottom).

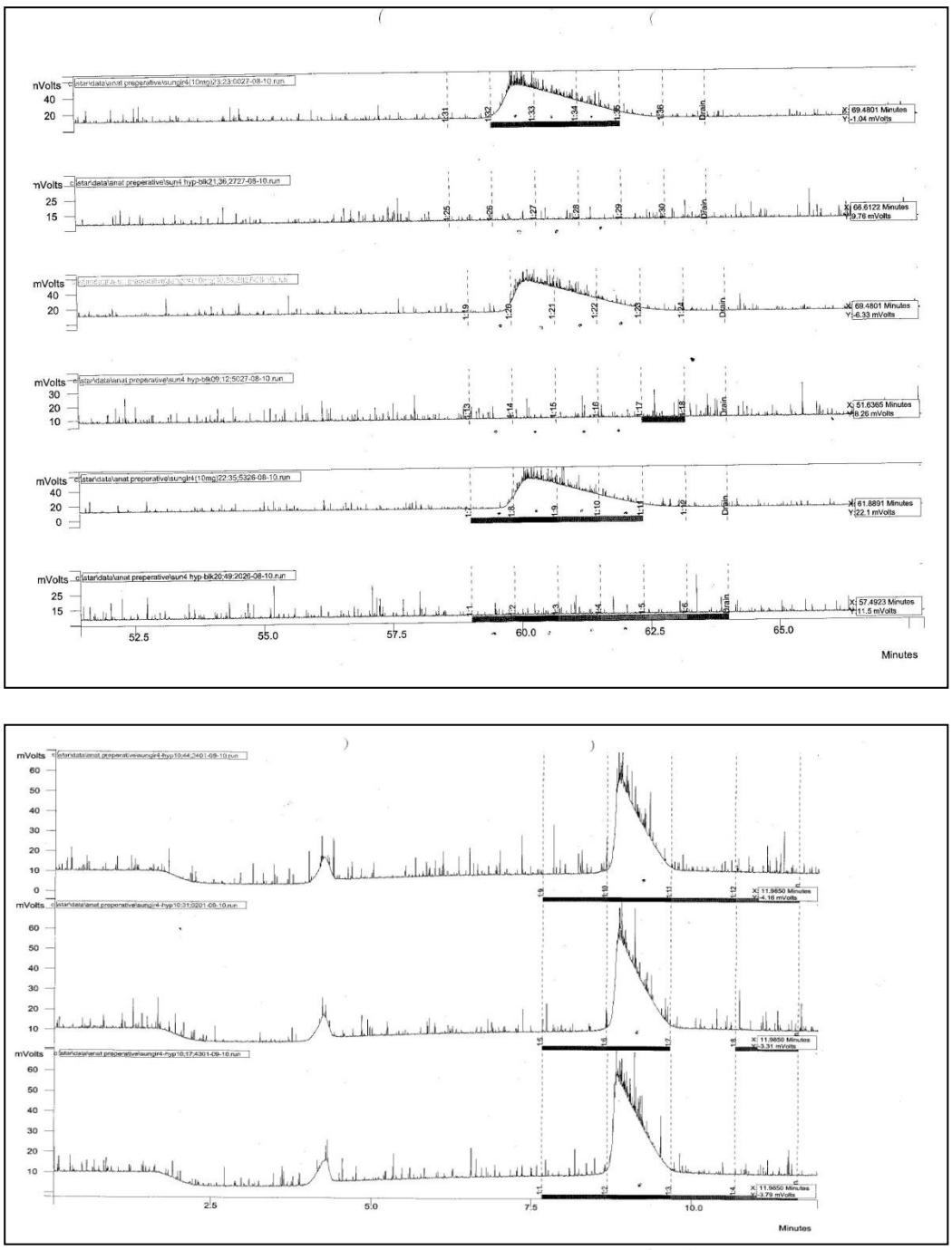


Figure D.9. Sungir mammoth first injections (zoomed in; each run is followed by a water injection) (top); Sungir mammoth second injections (bottom).

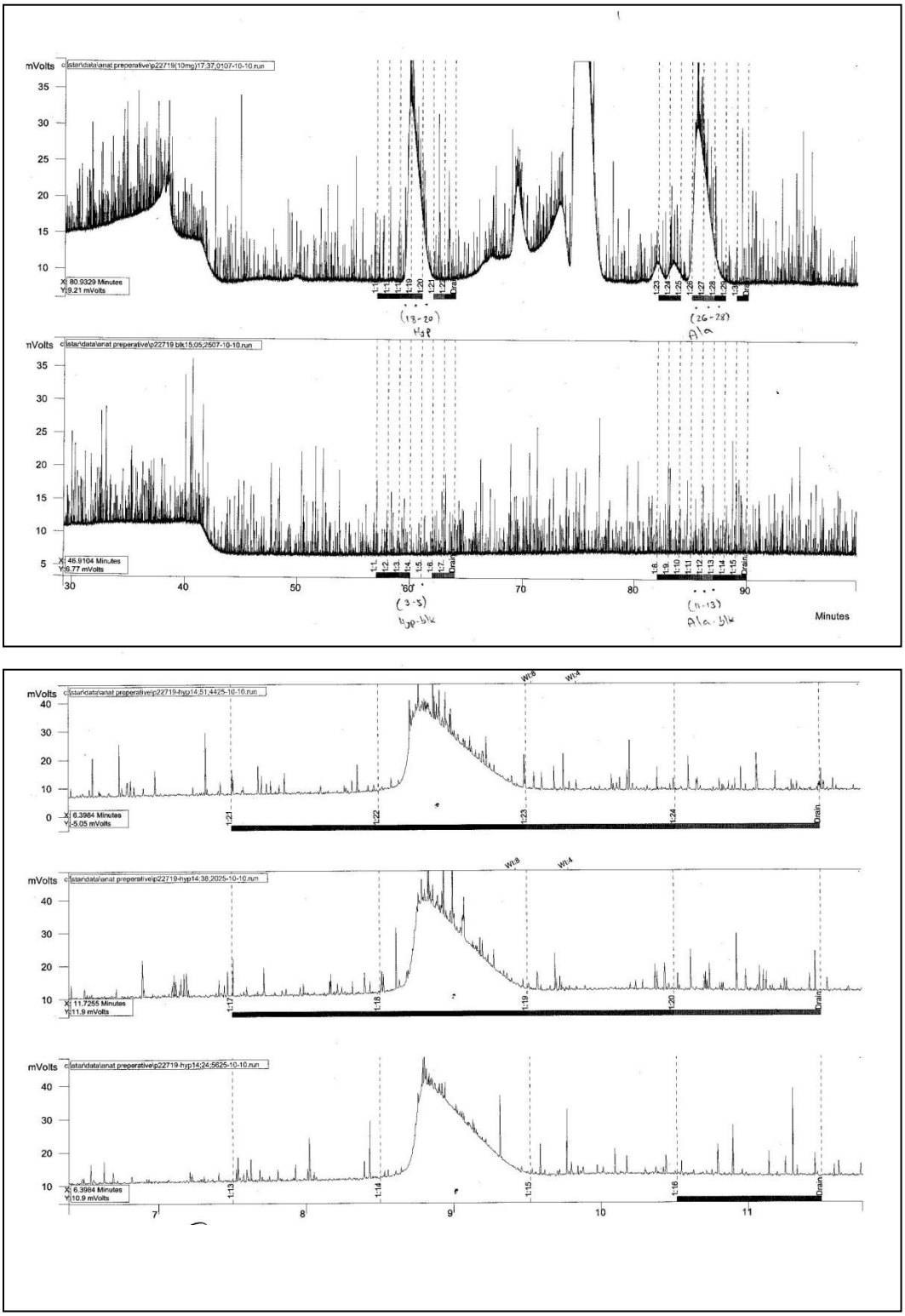


Figure D.10. La Ferrasie left tibia first injection (zoomed in; each run is followed by a water injection; one of three) (top); La Ferrasie left tibia second injections (bottom).

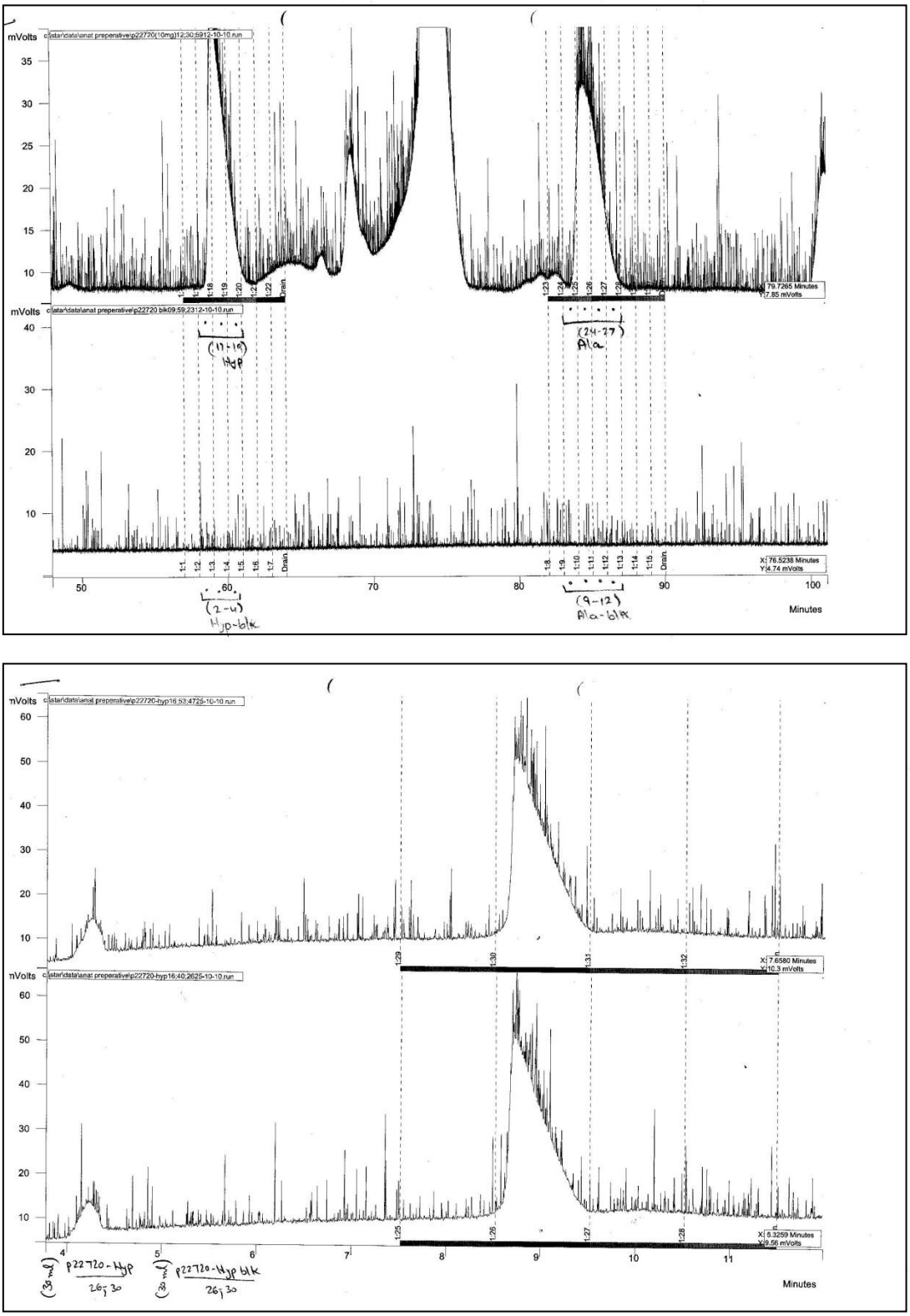


Figure D.11. La Ferrasie right tibia first injection (zoomed in; each run is followed by a water injection; one of two) (top); La Ferrasie right tibia second injections (bottom).

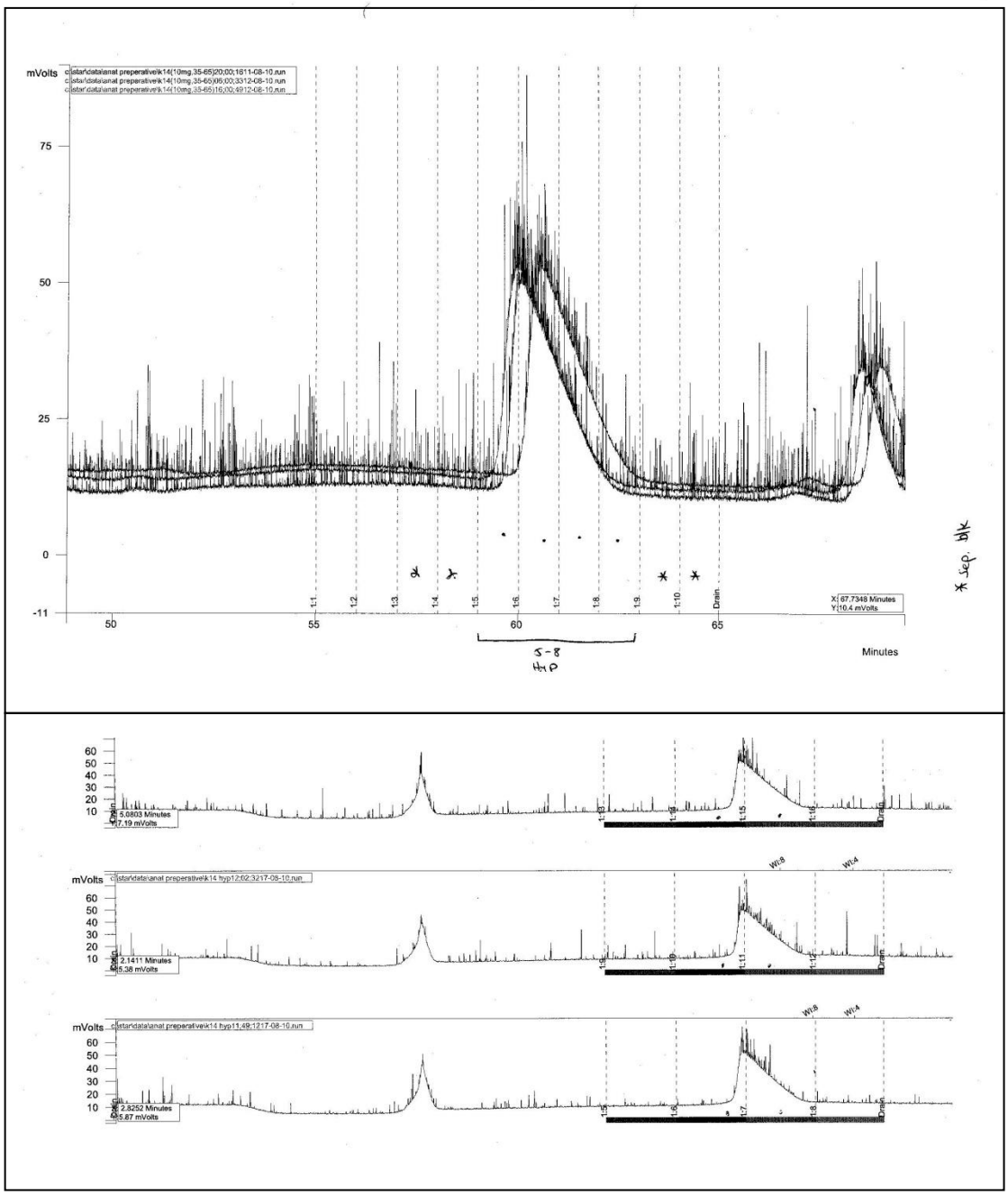


Figure D.12. Kostenki horse first injections (zoomed in; overlaid) (top); Kostenki horse second injections (bottom).

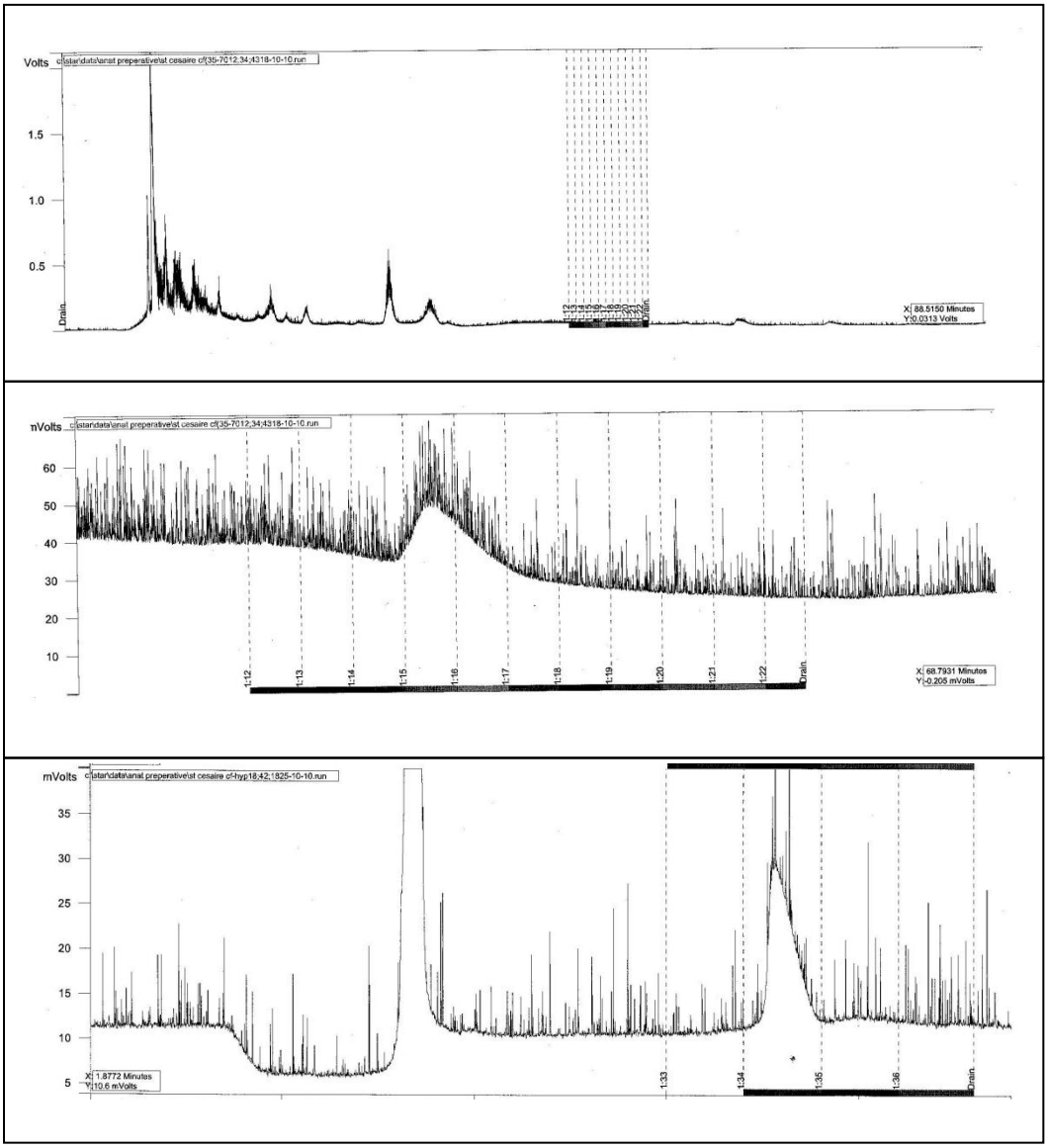
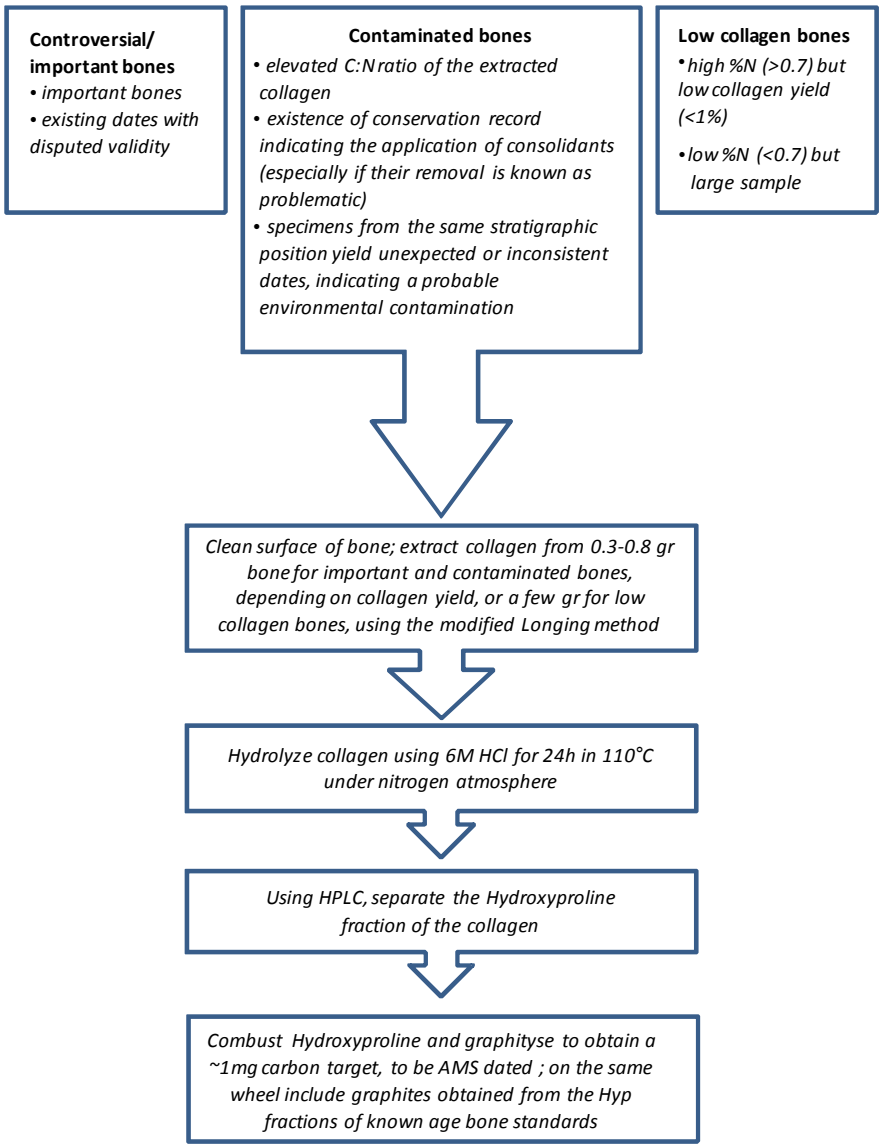


Figure D.13. St. Cesaire CF first injection (top); the same run, zoomed in (middle); St. Cesaire CF second injection (bottom). It was attempted to get a date out of this sample, but it was too small to be pressed into a graphite target. There was no Hyp found in the SF.

Appendix E: Summary of the recommended methodology for Hyp dating:



Appendix F: Papers published from the research done for this thesis

Marom, A., J. S. O. McCullagh, T. F. G. Higham, A. A. Sinitsyn and R. E. M. Hedges (2012). Single amino acid radiocarbon dating of Upper Paleolithic modern humans. Proceedings of the National Academy of Sciences of the United States of America**, 109**(18): 6878-6881.

McCullagh, J. S. O., Marom, A. and Hedges, R. E. M. (2010). Radiocarbon dating of individual amino acids from archaeological bone collagen. *Radiocarbon* **52**(2): 620-634.

Single amino acid radiocarbon dating of Upper Paleolithic modern humans

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Edited by Ofer Bar-Yosef, Harvard University, Cambridge, MA, and approved March 20, 2012 (received for review October 6, 2011)

Archaeological bones are usually dated by radiocarbon measurement of extracted collagen. However, low collagen content, contamination from the burial environment, or museum conservation work, such as addition of glues, preservatives, and fumigants to "protect" archaeological materials, have previously led to inaccurate dates. These inaccuracies in turn frustrate the development of archaeological chronologies and, in the Paleolithic, blur the dating of such key events as the dispersal of anatomically modern humans. Here we describe a method to date hydroxyproline found in collagen (~10% of collagen carbon) as a bone-specific biomarker that removes impurities, thereby improving dating accuracy and confidence. This method is applied to two important sites in Russia and allows us to report the earliest direct ages for the presence of anatomically modern humans on the Russian Plain. These dates contribute considerably to our understanding of the emergence of the Mid-Upper Paleolithic and the complex suite of burial behaviors that begin to appear during this period.

HPLC | accelerator mass spectrometry | Kostenki | Sungir

Radiocarbon dating of bone collagen routinely focuses on the extraction of bulk proteins that are then purified before radiocarbon measurement. However, the extracted bulk gelatin can be heterogeneous and include, or be cross-linked to, potential contaminants from the depositional environment, such as humic and fulvic acids, rootlets, cellulose, sediments, and other plant and animal remains including amino acids from bacteria and microorganisms (1, 2). For some samples curated in museums, additional contaminating compounds, such as glues, consolidants, and fumigants, can affect accurate dating, if not removed. In archeology, reliable chronologies are critical if an accurate picture of the human past is to be reconstructed. One area of pressing need in this respect is dating the spread of early anatomically modern humans out of Africa and into Europe and Eurasia. Direct dating of hominin fossils, as a means to assess the nature and timing of major demographic dispersals, Neanderthal extinctions, and admixture across Eurasia is usually based on radiocarbon dating and to a lesser extent optically stimulated luminescence (OSL) measurements. The radiocarbon method can be problematic, however, due to the difficulties associated with geological and museum-derived contamination, which become increasingly important as the ~50-ky dating limit of radiocarbon is approached. Evidence suggests that perhaps ~70% or more of the bone dates from the Middle and Early Upper Paleolithic are liable to be underestimates of the true age (3). The significance of this statistic should not be underestimated; its effect on our understanding of archaeological chronology has profound implications. Although the application of more rigorous ultrafiltration protocols has improved this situation recently (3), if the contaminants in bone are of high molecular mass, then they will not be removed using this technique.

Standard sample preparation protocols for dating bones generally follow an acid–base–acid (ABA) treatment, involving a decalcification step to mobilize hydroxyapatite, followed by a dilute NaOH or KOH wash that removes some humic and fulvic acids, followed by reacidification. After washing, the extracted collagen is usually gelatinized (solubilized) at pH 3 at temperatures ranging from 58 °C to 100 °C and filtered. Some laboratories apply ultrafiltration to remove low molecular mass material, retaining >30,000-Da peptides for dating (4). More elaborate approaches to dating compound-specific fractions from bone have been explored since the 1960s, most having focused on hydroxyproline (Hyp) because collagen is almost unique in nature in containing large amounts of this amino acid (5–15). One factor that makes them difficult to evaluate is that the amount of carbon derived from the laboratory protocols themselves was not reported. These approaches have not been widely adopted.

We have developed a protocol on the basis of preparative HPLC separation of amino acids hydrolyzed from bone collagen. Mixed-mode HPLC extraction of Hyp could prove very useful in dating bone with too little surviving collagen to be datable by the bulk collagen method. Analysis of bones of this type using our technique shows that it is possible to extract sufficient Hyp from a large enough sample of bone and thereby produce a radiocarbon determination where previously this had been impossible. Further application of the method to low collagen bones, as well as to highly contaminated ones, may result in reliable archaeological chronologies for parts of the world that have previously been impossible to effectively date.

We have applied the technique to a set of important anatomically modern human bones from the Early and Mid-Upper Paleolithic of Russia. These are bones that previously have proved impossible to reliably date due, it is thought, to the effects of museum conservation or to site-based organic contaminants.

Results and Discussion

Radiocarbon dating of Paleolithic bones has frequently resulted in severe underestimates of the real age, but direct dating of Neanderthal and modern human fossil remains is crucial to understanding the mechanics of the extinction of the former and the initial wide dispersal of the latter. Paleogenetic studies have shown that humans sharing haplogroup U characteristics dispersed into Europe (U5) and North Africa (U6 and M1), but dating of this diaspora is not certain (16). The Kostenki 14 (Markina Gora) human skeleton excavated near Voronezh, Russia (Fig. S1), is one of only three fossil human remains with a "complete" published mtDNA sequence (17) and it shows the five diagnostic substitutions defining haplogroup U2, present also in modern populations in Europe. Although the specimen is suspected of being Paleolithic in age, direct radiocarbon dates

Author contributions: A.M., J.S.O.M., and R.E.M.H. designed research; A.M. performed research; J.S.O.M. contributed new reagents/analytic tools; A.A.S. excavated the Kostenki site; A.M., J.S.O.M., T.F.G.H., A.A.S., and R.E.M.H. analyzed data; and A.M., J.S.O.M., and T.F.G.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1116328109/-/DCSupplemental.

Table 1. C:N atomic ratios and radiocarbon ages from Kostenki 14 (Markina Gora) skeleton (a tibia)

Fraction/treatment	Laboratory code	C:N	^{14}C age, BP, ±1 σ error	Source
Gelatin	OxA-7126	3.7	4,750 ± 40	(18)
Gelatin	GrA-9303	NP	3,730 ± 40	(18)
Gelatin	SR-7366/UCIAMS-61666	NP	13,610 ± 40	(19)
Ultrafiltered collagen	NA	4.1	Not dated	(17)
HPLC-separated Hyp fraction	OxA-X-2395-15	5.1	33,250 ± 500	This paper

Note the theoretical C:N ratio of Hyp is 5.0. NA, not available; NP, not published.

are much younger (~3.7–13.6 kaBP) (18, 19). One indication of a problem with the collagen from the bone is that the C:N ratio is higher than expected (Table 1: values outside 2.9–3.5 are considered problematic). Pure biochemically characterized collagen has a carbon to nitrogen ratio of 3.21 (20). Values higher than this indicate exogenous carbon (Table 1). We extracted bone powder from the right tibia of the skeleton and attempted a new direct date using an ultrafiltration protocol but this again resulted in high C:Ns (3.8) and the date was not attempted. We then took 40 mg of the contaminated collagen and used the HPLC protocol described above to separate the Hyp fraction. The C:N ratio of the separated Hyp was 5.1, close to the theoretical value of 5.0. The resulting 1.2 mg graphite, produced for dating by accelerator mass spectrometry (AMS), yielded an age of 33,250 ± 500 yBP (Table 1 and Table S1). This date is significantly older than all previous determinations.

There is independent evidence for the age of the burial, based on the excavated sequence at Kostenki. The burial lies under cultural layer III, but no signs of a burial pit were observed from the level of this cultural layer. A. N. Rogachev (21, 22), the excavator, rejected any possibility for the burial to be attributed to cultural layer III, which is dated to ~28.3–31.7 kaBP (19). The pit containing the body had cut through the volcanic ash horizon at the site, the Campanian Ignibrite (CI), which was clearly visible in the walls but absent from the burial fill (22, 23). The most probable context for the burial is thought to be with the "cultural layer in volcanic ash" of Aurignacian attribution (between cultural layers III and IVa). This level was unknown in 1954. The stratigraphic context and direct radiocarbon dates of material from the same cultural level therefore suggest that the age of the human must be at least 30 kaBP. Its maximum age is probably ~35 kaBP, because of the presence of the CI tephra within and below cultural layer IVa [the CI is dated to ~39.3 ka calibrated (cal) BP, which, based on the IntCal09 calibration curve, would be equivalent to ~35 kaBP] (24). The radiocarbon date therefore fits perfectly into this chrono-stratigraphic schema.

At Sungir, another key Russian Upper Paleolithic site, discovered in 1955, several spectacular burials were excavated (25). The remains of eight individuals were found, buried and ornamented with ivory spears, bracelets, brooches, numerous ivory beads, and perforated fox teeth, attesting to the technical sophistication of its inhabitants. The cultural assemblage and the red ochre covering the skeletons imply strongly that the burials are related to the wider Mid-Upper Paleolithic cultures of European modern humans (26). Sungir is the northernmost of these Upper Paleolithic sites and has a Streletskian artifact assemblage, which comprises triangular bifacial points with concave bases and therefore suggests the site is a transitional cultural phase related to the previous Early Upper Paleolithic (EUP). An Aurignacian component in the lithic assemblage supports this EUP affiliation, albeit of a more recent manifestation. Direct radiocarbon dating of three of the skeletons was attempted previously in Oxford, Arizona, and Kiel (26–28), but the results were highly inconsistent, both between the laboratories and between the different individuals dated. The results ranged between 19,160 and 27,210 yBP and this wide variability has led to problems in placing the burials into their proper context. The relatively high C:N ratio of some of the samples (Table 2) again

Table 2. C:N atomic ratios and radiocarbon ages from Sungir

	Laboratory code	C:N	^{14}C age BP, ±1σ error	Source
Sungir 1	AA-36473 (vertebra fragment)	NP	19,160 ± 270	(27)
	OxA-9036*	NP	22,930 ± 200	(26)
	KIA-27006†	3.1	27,050 ± 210	(28)
Sungir 2	AA-36474 (right side ribs)	NP	27,210 ± 710	(27)
	AA-36475 (left side ribs)	NP	26,200 ± 640	(27)
	OxA-9037*	3.5	23,830 ± 220	(26)
	OxA-15753†	3.3	25,020 ± 120	NP
	OxX-2395-6 Hyp fraction	5.0	30,100 ± 550	This paper
Sungir 3	AA-36476 (rib fragments)	NP	26,190 ± 640	(27)
	OxA-9038*	3.4	24,100 ± 240	(26)
	OxA-15751†	3.2	25,430 ± 160	NP
	OxA-15754†	3.2	24,830 ± 110	NP
	KIA- 27007†	3.5	26,000 ± 410	(28)
	OxX-2395-7 Hyp fraction	5.0	30,000 ± 550	This paper
Sungir mammoth bone	OxA-9039*	3.5	27,460 ± 310	(26)
	OxA-15752†	3.1	29,640 ± 180	NP
	OxA-15755†	3.2	29,450 ± 180	NP
	OxX-2395-8 Hyp fraction	5.1	30,100 ± 400	This paper

Note that the theoretical C:N ratio of Hyp is 5.0. NP, not published.
*Samples pretreated with a gelatinization method.
†Samples ultrafiltered before AMS dating.

indicates that carbonaceous conservation material may have been applied to the bone, which the various chemistry pretreatment methods performed on the sample were unable to remove. It should be noted, however, that C:N ratios are not greatly sensitive to small amounts of exogenous carbon contamination so could mask potential problems. Dobrovolskaya et al. (28) provided two new determinations recently from Sungir 1 and 3, which they suggested were sufficient to establish the geological age of the skeletons at 26–27 kaBP because the results overlapped at 2 SDs (Table 2).

Hyp was separated from two samples of 30 mg collagen that had previously been extracted and dated. The samples came from the Sungir 2 and 3 individuals, both buried in the same grave. Unfortunately, there was not enough material remaining to redate Sungir 1 as well. In addition, Hyp was also extracted from a 30-mg collagen sample of a mammoth bone that came from the same occupation area of the site. The Sungir 2 and 3 Hyp fractions both yielded graphites of ~0.7 mg. The Sungir mammoth yielded a graphite of 0.9 mg (Table S2). The new dates, shown in Table 2, are in close agreement and therefore consistent with a single event for the burials (Table S1). The fact that the two human dates are internally consistent provides some support for their accuracy because they are known to be contemporaneous burials and were interred together. Fig. 1 shows the calibrated ages for the Sungir samples. The new date for the Sungir burials, 30.1 ± 0.3 kaBP (between 34.1 and 35.2 ka cal BP) is appreciably older than previously assumed. Before this work, the earliest direct date for a Mid-Upper Paleolithic individual was ~29,000 yBP for the "Red Lady" of Paviland in the British Isles (29). These dates may suggest an earlier onset in the beginnings of Mid-Upper Paleolithic technocomplexes and complex ritual burial behaviors in Eastern Europe compared with Western Europe, but more dating is required.

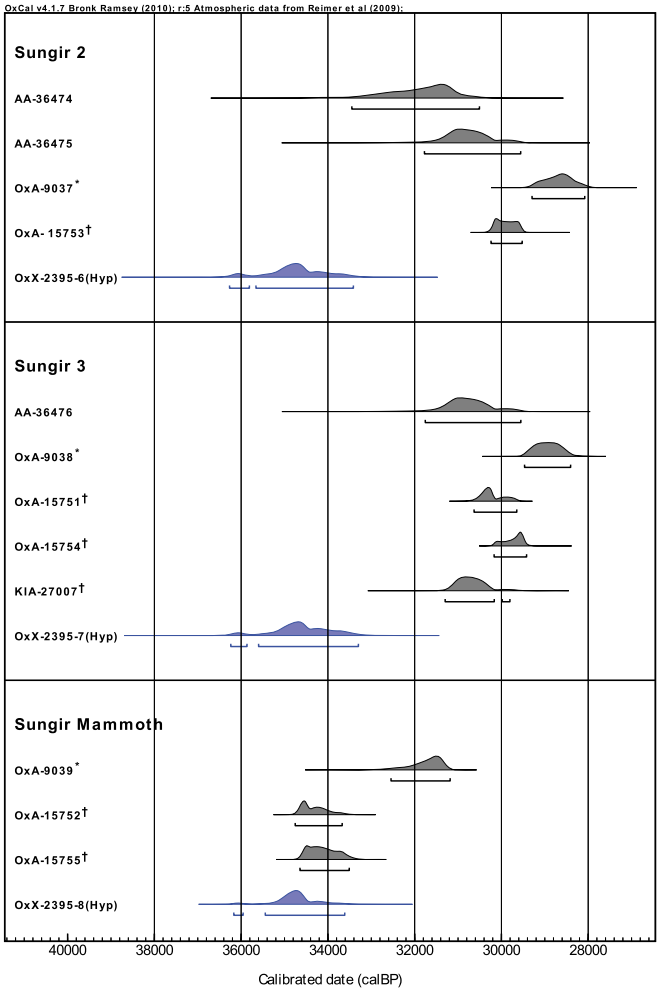

Fig. 1. Calibrated radiocarbon dates (radiocarbon likelihoods) for Sungir 2, Sungir 3, and the Sungir mammoth bones, produced using OxCal 4.1 and the INTCAL09 calibration curve (30, 31). * denotes samples pretreated with a gelatinization method; † denotes samples ultrafiltered before AMS dating. Bulk calibrated dates range between ~28 ky cal BP and 35 ky cal BP, but the three Hyp fraction dates all fall between 33.3 ky cal BP and 36.3 ky cal BP and can be combined with >95% probability to a single calibrated date range that falls between 34.1 and 35.2 ky cal BP. Interestingly, for the mammoth bone, the ultrafiltered bulk dates (OxA-15752 and OxA-15755) are very similar to the Hyp date, implying probably that this bone was not preserved and so the contaminant that made it appear more modern was effectively removed by the ultrafiltration. Sungir 2 and 3 probably had some preservation material that was not fully removed by the ultrafiltration, and only the Hyp method was able to date them accurately as implied by the fact that the three Hyp dates were the same.

The results demonstrate the potential problems that exist with direct dating of human remains using less rigorous pretreatment chemistries. Often these bones are those that are conserved in museums and collections and potentially contaminated with more modern carbon. The few human fossils dating to the Middle to Upper Paleolithic make it imperative that when direct dating is undertaken, reliable measurements can be ensured. In the case of Sungir and Kostenki the direct radiocarbon dates previously obtained are erroneous and should henceforth be set to one side by prehistorians. Our methodology provides a chemically characterizable, compound-specific molecule that eliminates the contamination that other methods cannot. Although in the majority of cases an ultrafiltration preparation is quite sufficient to decontaminate bones before AMS dating, our work shows that in some cases, where there is contamination and the samples are precious, the single amino acid method is the most optimal technique that can be applied.

Methods

The collagen is extracted by crushing ~0.5 g cleaned bone (or more, depending on its collagen yield). It is then demineralized and gelatinized. The resulting collagen is hydrolyzed and finally separated into individual amino acids, using a mixed-mode HPLC separation method incorporating weak cation exchange combined with reversed-phase components combined in the same stationary phase. The amino acid retention times were identified using standards, and spiking natural samples confirmed these retention times in archaeological samples. Detection by UV absorbance (32–34) and LC/MS analysis of selected amino acid fractions was used to confirm identification of peaks by mass. It is crucial that laboratory-derived carbonaceous material be kept as close to zero as possible. Amino acids do not require derivatization and no organic solvents are used, to avoid adding carbon to the eluate (35). We have verified, to a precision of ±30 y, that independently isolated Hyp fractions from known-age bone give the correct ^{14}C age, indistinguishable statistically from the bulk collagen age (Fig. S2). Independently isolated Hyp fractions from a ^{14}C-free bone were all >41 kaBP (Fig. S3 and Table S1). The procedure background was calculated to be 3.3 ± 1.4 μg carbon, of which 1.5 ± 0.3 μg was modern and 1.8 ± 1.1 μg was ^{14}C-free. These results demonstrate that the isolation of hydroxyproline does not add a significant background carbon to the radiocarbon measurement (*SI Methods*, Figs. S2–S4, and Tables S1, S3 and S4). The Hyp isolation procedural background has been quantified. This is an important requirement for validating the method before it can be applied to archaeological samples of unknown age. The Hyp method is dramatically different from most protocols used for dating bone and teeth today, as the date is produced from a specific, endogenous single molecule. In addition to the traditional pretreatment method, a preparative HPLC system with UV detection and an evaporator capable of removing several hundred milliliters of water-based eluent are required. The additional time required is mostly for sample hydrolysis (24 h) and separation and evaporation of the mobile phases (38 h). Different approaches have now shortened this significantly in our laboratory. After isolation of Hyp the graphitization procedure is the same as for bulk samples. The benefit of the compound-specific approach is the ability to effectively remove exogenous

contamination from a wide range of samples, providing the potential for it to play a significant role in the expansion of more accurate archaeological chronologies.

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RADIOCARBON DATING OF INDIVIDUAL AMINO ACIDS FROM ARCHAEOLOGICAL BONE COLLAGEN

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ABSTRACT. Since the development of accelerator mass spectrometry (AMS) for radiocarbon dating in the late 1970s, its ability to date small samples of bone has been of huge importance in archaeology and Quaternary paleoecology. The conventional approach to sample preparation has been to extract and gelatinize protein, which is then combusted and graphitized for analysis. However, this "bulk protein" can contain a heterogeneous mixture of non-collagenous molecules, including humic acids and other soil components that may be of a different age than the bone and therefore affect the accuracy of its ^{14}C date. Sample pretreatment methods have been an important area of development in recent years but still show inadequacies for the dating of severely contaminated bone. The idea of isolating and dating individual compounds such as single amino acids, to improve dating accuracy, has been discussed in the literature since the 1960s. Hydroxyproline, for example, makes up over 10% of bone collagen but is extremely rare in most other animal proteins, increasing the chances of its presence being endogenous to the individual being dated. Its successful isolation has therefore been considered a potential "gold standard" for dating archaeological bone; however, extracting and suitably purifying single amino acids from bone has proved a challenging task.

This paper presents a novel method for the compound-specific ^{14}C dating of individual amino acids, including hydroxyproline, from archaeological bone protein. It is based on a preparative, mixed-mode liquid chromatography separation of underivatized amino acids, entirely in aqueous solution and free of organic solvents. The method is presented here in detail including application to standard bone samples establishing its accuracy and background carbon contribution. Results from ^{14}C dating hydroxyproline and other individual amino acids, from both historical and archaeological bone, are shown to provide AMS dates that are statistically indistinguishable from those of the bulk protein.

INTRODUCTION

Bone from archaeological and geological sites is subject to chemical and environmental processes that can lead to degradation of molecular structure and the incorporation of exogenous molecules from the burial environment. It has been shown that these processes can influence subsequent radiocarbon dates (Hedges and Wallace 1978; Gillespie and Hedges 1983; Gillespie et al. 1984; Hedges and van Klinken 1992; Bronk Ramsey et al. 2004b,c). This contamination can be in the form of organic compounds in soil and sediments, in particular humic acids, and other metabolic products such as amino acids and lipids from microorganism degradation (Hedges and van Klinken 1992; van Klinken and Hedges 1997; van Klinken 1999; Bronk Ramsey et al. 2004b). Sample handling and treatment after excavation can also result in contamination from preservatives and fixatives or proteins and carbohydrate such as hair, wool, or cellulose, all potentially influencing the apparent age of the material being dated. Effective sample pretreatment prior to dating is therefore an essential part of the dating process.

Much work has been done over the last 50 yr to minimize the problem of contamination, but the complex nature of bone in particular still makes it one of the most difficult materials to date with high precision (Bronk Ramsey et al. 2004b,c). A strong focus on sample pretreatment has led to 2 alternative approaches. The first is to remove extraneous carbon-containing compounds and leave those native to the original organism for dating. This is the most common approach used by the majority of ^{14}C laboratories. Typically, bone protein (of which ~90% is type-1 collagen) is extracted

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and acid-base washed to remove contaminants before gelatinization and filtering (Longin 1971; Ambrose 1990; Lambert and Grupe 1993; Ambrose and Krigbaum 2003). A recent success has been the use of ultrafiltration to remove non-proteinaceous material. Here, the collagen is extracted and washed with an ultrafilter that has a molecular weight cut-off removing molecules below approximately 30 kD (Brown et al. 1988; Bronk Ramsey et al. 2004c; Higham et al. 2006; Brock et al. 2007). The Oxford Radiocarbon Accelerator Unit (ORAU) has used this method since 2000 to obtain more accurate accelerator mass spectrometry (AMS) dates on bone. The method is relatively cheap and straightforward, but does not guarantee to remove all contaminating carbon.

The second approach to preparing bone samples for ^{14}C dating has been to isolate chemically homogenous subunits of the extracted protein, discarding the rest of the material, including any contamination. So-called "molecular level" dating involves the separation and isolation of single compounds inherent to the sample to be dated; in the case of bone, peptides, individual amino acids, and even CO_2 from the peptide bonds between amino acids (ninhydrin method) have been attempted (Bada et al. 1984; van Klinken and Hedges 1998; Nelson 1991; Taylor et al. 1995; Stott et al. 2001, 2003; Tripp et al. 2006). These approaches inherently mitigate against contamination by removing all extraneous compounds; it is possible, however, that single amino acids found in bone may have multiple sources. Some may be derived from bacteria or other organisms in the bone's depositional environment, for example, having found their way into bone by leaching under conditions of poor preservation. Ho and coworkers first suggested isolating and dating hydroxyproline specifically to circumvent this potential problem (Ho et al. 1969; Hedges and van Klinken 1992; van Klinken and Hedges 1997).

Hydroxyproline is a major component of collagen resulting from the post-translational hydroxylation of proline residues, which are arranged in a common triad Xaa-Yaa-Gly, where Xaa and Yaa may be any amino acid but often proline in type-1 collagen (Udenfriend 1966; Vaughan 1975). This modification of the proline residue increases the stability of the collagen triple helix, but importantly for AMS dating it is found only in a very few mammalian proteins to any appreciable amount, and collagen is by far the most important. It was first isolated from fossil bone in 1981 and then AMS dated by Gillespie and Hedges (Wand 1981; Gillespie et al. 1984). Gillespie and coworkers used cation-exchange chromatography for its isolation, and results showed that the method provided dates that at the time were as accurate as conventional bulk collagen dates (Gillespie et al. 1984). Both Stafford and van Klinken went on to show that bone dates could be improved by the isolation of hydroxyproline, with van Klinken using a tripeptide approach where GlyProHyp triplets were isolated from enzymatic digests using cation-exchange chromatography; however, some self-declared problems with reliability of the method were encountered (Stafford et al. 1991; van Klinken 1991). With these notable exceptions, molecular-level dating methods are rare in the literature despite the obvious theoretical benefits over conventional techniques (Gillespie and Hedges 1983; Gillespie et al. 1984; Stafford et al. 1987, 1988, 1991; van Klinken and Mook 1990; van Klinken 1991; van Klinken et al. 1994; Tripp et al. 2006). One of the main problems has been that some methods introduce extraneous carbon into the separated fractions and sample blanks were not always reported, making it difficult to judge their success.

Each part of the process of sample preparation for ^{14}C analysis (chemical purification, combustion, and graphitization) may add small amounts of exogenous carbon to the original sample. By measuring blank (or background) samples, the laboratory is able to quantify a purification blank, a combustion blank, and a graphitization combustion blank, respectively, with the overall blank for the whole dating procedure termed the "procedure blank" (Mollenhauer and Rethemeyer 2009). The graphitization blank is consistently low at ~0.1 μg carbon, while the purification blank is usually the largest

and dependent upon the way in which samples are treated. It should be noted that these blanks will not be constant from one AMS laboratory to the next even if the same procedures were followed. They are dependent inherently on the materials used as well as the process followed; metal lines, pumps, and combustion materials will all differ slightly in this respect and contribute uniquely to an overall blank. Determining a new procedure's blank carbon contribution is an important part of method development for AMS dating, particularly at the compound-specific level.

Dating amino acids has historically relied almost exclusively on ion-exchange chromatography for the separation process, but it has been suggested that this high-performance liquid chromatography (HPLC) component is responsible for the majority of procedural blank carbon (Mollenhauer and Rethemeyer 2009). Pretreatment contamination can also come from chemical reagents; glassware; CO_2 dissolved in reagents; plastic or metal sample lines; gloves; dust particles and many others. It is possible to eliminate many of these sources of contamination, however, if reasonable precautions are taken. In this study, all reagents are analytical-grade or above and glassware is baked out at 500 °C before use. All HPLC lines were metal, where appropriate, and the system was free of organic solvents. Tin capsules were washed and cleaned and Chromosorb™ (see below) was baked at 500 °C. The ORAU's AMS background is reported to be ~0.15% (52 ka BP) and in the best conditions can be as low as 0.1% (55 ka BP), as measured by graphitizing a gas sample containing no ^{14}C (Bronk Ramsey et al. 2004b).

By its very nature, dating single amino acids has the potential to eliminate much of the molecular and isotopic heterogeneity that results from protein diagenesis and to provide more reliable dates. In this paper, we present a method for the preparative isolation and AMS dating of individual amino acids for archaeological bone proteins, introducing preparative mixed-mode chromatography to ^{14}C dating. The method is tested using well-preserved pig bone collagen obtained from Henry VIII's flagship, the *Mary Rose*, and older archaeological protein from Chalk Hill. These provide test cases to demonstrate the accuracy and precision of the procedure. ^{14}C dates for individual amino acids are shown to be statistically indistinguishable from bulk protein AMS dates and, in turn, in agreement with their historic age. The carbon contribution of the procedure is shown to be low, and on average 0.8% of the burn yields in this study.

MATERIAL AND METHODS

Materials, Reagents, and Standards

Amino acid standards were purchased from Sigma. Water was purified using a Milli-Q™ reverse osmosis system. All other solvents were HPLC-grade and purchased from Fisher and Sigma. All glassware was baked at 500 °C for 3 hr prior to use.

Archaeological and Historical Bone Standards

The *Mary Rose* was the flagship of Sir George Carew, Vice Admiral of Henry VIII. It sank off the coast of Portsmouth, UK, on 19 July 1545. During excavations prior to raising the ship in 1982, barrels of provisions were found containing beef, pork, mutton, and fish (Bradford 1982). Collagen isolated from a pig bone from a pork barrel aboard the ship has been used as a standard at Oxford Radiocarbon Accelerator Unit (ORAU) for a number of years and was adopted as a suitable protein standard for this project due principally to its known age and the fact that its bulk collagen ^{14}C date has been previously well characterized. Due to the exceptional preservation, it was hypothesized that individual amino acid dates should correspond directly with bulk collagen and historical dates, within standard errors. Over 40 dates on this material provide an average bulk collagen ^{14}C age of 321 ± 6.5 yr BP with a average precision of 23.7 yr (Bronk Ramsey et al. 2004a). In practice, the

bulk collagen dates ranged from 280 to 390 BP. There is a slight dependency on collagen yield as reported by Bronk Ramsey et al. (2004a).

Sample Preparation

Collagen was extracted using standard procedures at the Research Laboratory for Archaeology and the History of Art (RLAHA) improved from Longin (1971). Cleaned and freeze-dried bone samples were cut into small chunks approximately 1 cm^3 and left in 1M HCl for ~36 hr to solubulize and remove hydroxyapatite. The remaining solid material containing the organic fraction was washed and then sealed and heated in water at 90 °C for 24 hr to extract gelatin in solution. Freeze-drying of the extracted gelatin led to recrystallization of the crude protein ready for further analysis. For bulk AMS dating of the intact collagen, approximately 2.5 mg of extracted protein was wrapped in cleaned tin capsules for graphitization.

Hydrolysis

Hydrolysis was undertaken using approximately 50-mg aliquots of collagen with excess 6M HCl in a sealed tube in a nitrogen atmosphere at 110 °C for 24 hr. Samples were frozen and HCl removed using 1 of 2 methods. The first by rotary evaporation followed by freeze-drying and the second by using a Genevac EZ-2 (Genevac Ltd, Ipswich, UK). Milli-Q water was added to dry hydrolysates and samples ultrafiltered to remove large molecular weight compounds. The filtrate was freeze-dried once more and then made up to a concentration 8 mg/mL with Milli-Q water and used immediately for HPLC preparative separation or frozen until use (within 1 week).

Preparative Scale High-Pressure Liquid Chromatography (HPLC)

Chromatography was performed on a Varian ProStar HPLC system consisting of 2 isocratic pumps, a 410 autosampler, a 320 dual-pathlength UV detector, and a 701 fraction collector, all controlled by Star workstation PC software. The autosampler was fitted with a 1-mL syringe and 2-mL sample loop and pumps were upgraded with 25-mL/min titanium heads. Amino acid separation was performed on a Primesep A column (22 × 250 mm, particle size 5 μM; SIELC Technologies, Prospect Heights, Illinois, USA). This is a mixed-mode separation column combining reversed-phase (RP) interactions provided by the stationary phase (C_{12} alkyl groups bonded to the surface of the silica backbone) with ion-exchange interactions provided by an additional charge on the surface via ionized carboxylic acid groups.

The embedded group is negatively charged in the working pH range. The stationary phase's ability to interact is influenced by the pH of the mobile phase, as is the charge state of the amino acids in the mobile phase solution (see McCullagh et al. 2006; McCullagh 2010 for further details).

The amino acid separations were carried out using a linear gradient program. Pump A (100% water) was pumped isocratically for the first 40 min. Then, from 40 to 70 min a linear gradient from 100% A to 100% B (0.3% phosphoric acid by volume) was held until just before the end of the run when the column was re-equilibrated with mobile phase A. Throughout the run, the flow rate of the mobile phases was maintained at 6 mL/min. Amino acid standards were used to determine the elution order and the retention times of each amino acid.

For each sample, 3 injections were made and overlaid in order to comfortably obtain enough of each amino acid for AMS dating (corresponding to 0.5–1.5 mg C yield after combustion). The column's loading capacity (~15 mg/mL of hydrolysate) and injection loop size (1000 μL) limited the concentration and volume of sample that could be injected per chromatographic run. Fractions of the eluent

were collected every 30 seconds with a fraction collector and those that fell within the elution of each individual amino acid were combined. The excess water was removed by rotary evaporation, gyro-vacuum evaporation (Genevac EZ-2), lyophilization, or a combination of these. (The 3 methods were used interchangeably and as available in the laboratory. Each was tested to show they did not contribute exogenous carbon to the sample using an elemental analyzer).

Removing Mobile Phase Acidity

The use of dilute phosphoric acid in mobile phase B meant that amino acid fractions contained concentrated H_3PO_4 in the evaporate due to the fact that it is not volatile under atmospheric conditions. The resulting total sample volume was therefore significantly greater than the 30-μL maximum that was determined could be loaded onto Chromosorb (an inert silica material used as a combustion substrate at the ORAU: W/AW, Mesh Size 30–60, Phase Separations Ltd) in a tin capsule appropriate for combustion. The H_3PO_4 therefore had to be removed prior to sample preparation for AMS analysis and a number of methods were investigated to try and achieve this. The first was precipitate using barium hydroxide. This led to a gelatinous precipitate of barium phosphate from which amino acid yields were extremely low, presumably as the amino acids became incorporated into the gelatinous precipitate. This method was abandoned. A second approach used a weak ion-exchange resin (DOWEX 66) with the analyte washed through a bespoke glass column at low pressure. This was successful in removing phosphate, but it also retained some amino acids, glycine in particular, and this method was the also abandoned due to the risk of contamination and low yield of some amino acids. A second precipitation method was then tried, which involved the addition of finely powdered iron to the concentrated analyte mixture. This reacted, forming insoluble iron phosphate (solubility 1.86 × 10–12 g/L at 25 °C), which was in the form of hard, fine powder. After agitation for 1 hr with the evolution of hydrogen gas, the reaction slowed and the reaction mixture was filtered under centrifugation using a 10-kD ultrafilter. This removed excess iron as well as iron phosphate, leaving a pH 6 solution and no loss of amino acids. This was then lyophilized further to dryness and to a sufficiently small size to allow loading onto Chromosorb in baked-out tin capsules. This method was adopted for the *Mary Rose* samples, but at a later stage a chromatographic method was developed to replace this chemical precipitation procedure. This involved re-injection of the analyte/H_3PO_4 mixture for a second time onto the same HPLC column using isocratic elution conditions (100% Milli-Q water). The amino acid was retained by the column, unlike the phosphoric acid that eluted with the void volume. The amino acid fraction was then collected in the usual way via the fraction collector and water removed by EZ-2 evaporation and lyophilization (see Figure 1). This modification reduced sample handling processing time and was adopted for analysis of the Chalk Hill samples.

The preparative chromatographic method described in this section is based on an analytical method developed for the separation of underivatized amino acids from bone collagen using mixed-mode chromatography. Background to this method can be found in previous publications (McCullagh et al. 2006, 2008; McCullagh 2010).

Background Carbon and Column Bleed

Measurement of relative carbon in the mobile phases and the contribution of column bleed were made using a Thermo Scientific LC-IRMS system consisting of a Surveyor LC system connected to a Thermo Scientific LC-Isolink and a Delta Advantage mass spectrometer as detector. Isodat 2.0 (ThermoFinnigan) was used to control the HPLC-IsoLink-IRMS system. The mobile phase and any column bleed pass directly through a 6-port valve on the liquid interface into a mixing T where the inorganic oxidation reagents are mixed at a flow rate of 50 μL/min each. Quantitative oxidation takes place in a reactor held at 99 °C. The CO_2 gas is separated from the liquid phase in a 3-phase

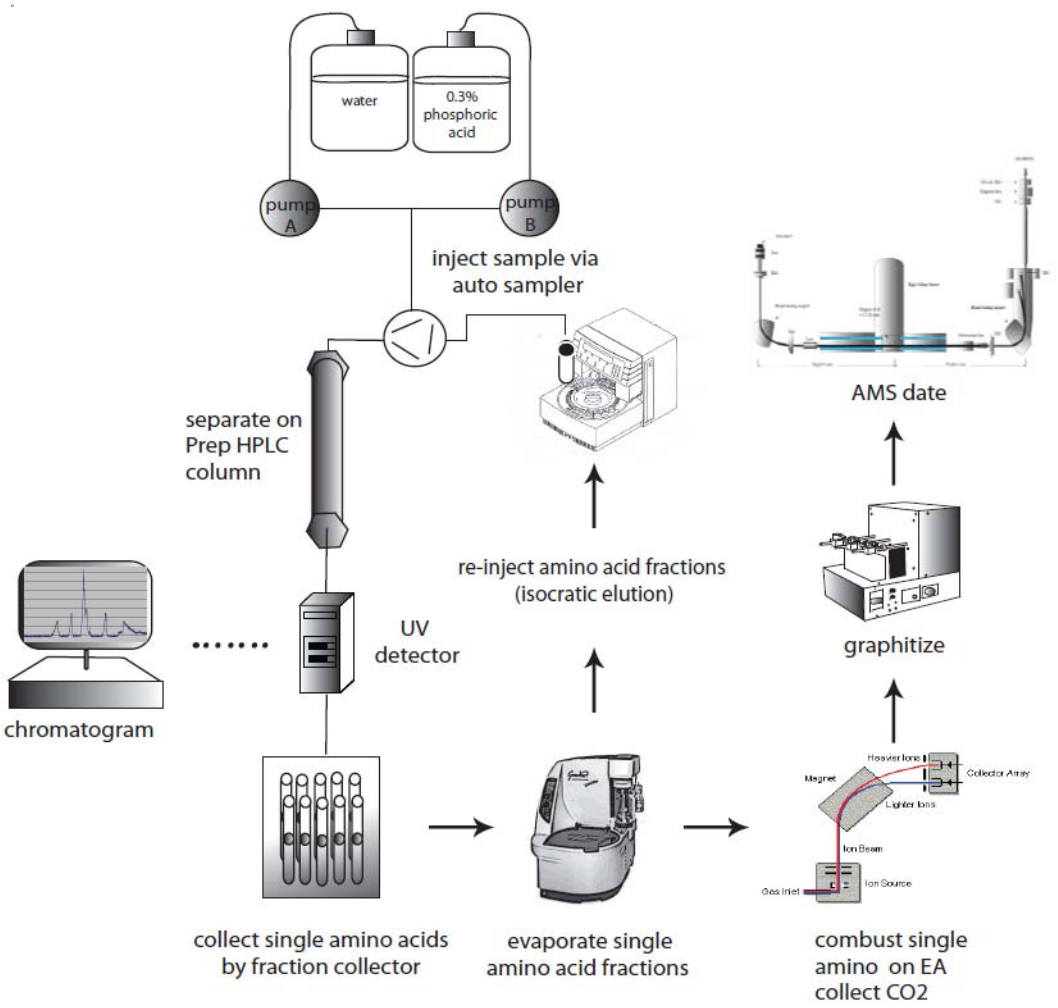

Figure 1 Flow diagram for the method to ^{14}C date individual amino acids

CO_2 separation unit using helium carrier gas. The gas is further dried using 2 Nafion driers and a helium counter flow prior to reaching the open split interface to the mass spectrometer where ionization and CO_2^+ signal intensity is measured. The phosphoric acid and magnesium persulphate, used as the IsoLink reagents, were also purchased from Sigma-Aldrich. Working solutions of 1.5M orthophosphoric acid and 100g/L of $M_2S_2O_8$ ($M^+ = Na^+, K^+, NH_4^+$) were made with Milli-Q water. Solutions were degassed under vacuum and sonicated for 1 hr before use. They were prevented from regassing *in situ* by continuous sparging with nitrogen gas. Nitrogen was found to be effective and replaced helium in the interest of economy.

AMS Analysis

^{14}C dates were measured on the AMS at the ORAU. Graphite was prepared by reduction of CO_2 over an iron catalyst in an excess H_2 atmosphere at 560 °C prior to AMS ^{14}C measurement (Dee and Bronk Ramsey 2000). Calibration and statistical analyses were performed using OxCal (Bronk Ramsey 1995, 2001) and IntCal04 calibration curve data (Reimer et al. 2004).

RESULTS AND DISCUSSION

Chromatographic Separation

A mixed-mode approach was developed to separate amino acids in bone collagen using preparative HPLC with pH gradient elution under completely inorganic conditions. Chromatographic separation was optimized by injecting a standard mixture of amino acids in a collagen-like composition (see Figure 2). When suitable elution conditions were identified, 15-mg quantities of collagen hydrolysate were injected onto the preparative column and gradient.

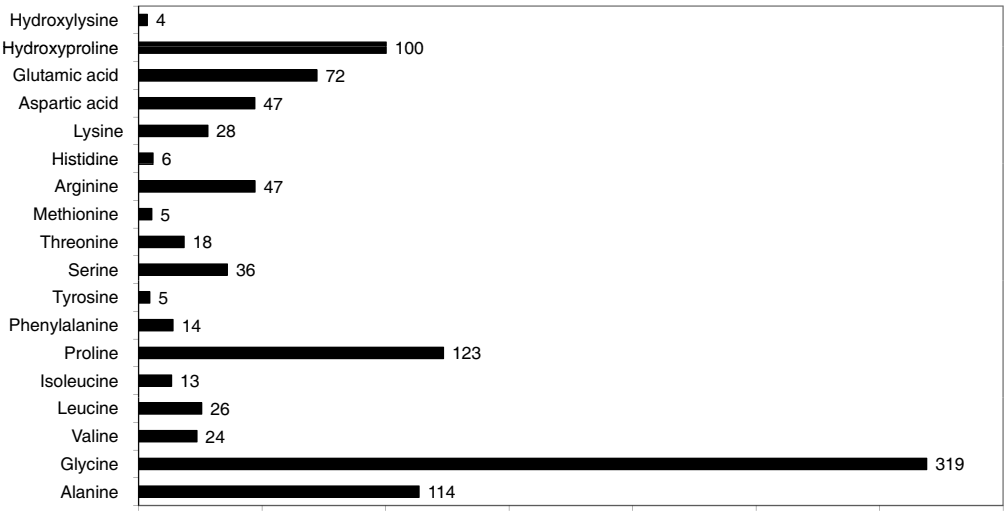

Figure 2 Amino acid composition of human bone, residues per 1000 (Eastoe et al. 1955)

Figures 3 and 4 show the location of individual and mixtures of amino acids on the chromatograms obtained from the preparative separation. These locations were identified by individually spiking the samples with each synthetic amino acid. Figure 1 shows a flow path for the method from separation to AMS measurement.

Dating Amino Acids from *Mary Rose* Pig Bone Collagen

With the use of up to 3 overlayed preparative chromatographic runs, the method was applied first to acid hydrolyzed collagen extracted from the *Mary Rose* standard. Hydroxyproline (Hyp), proline (Pro), alanine (Ala), glycine (Gly) with glutamate (Glu), and intact collagen were isolated and AMS dated (see Figure 2). AMS dates, burn yields, and standard errors are shown in Table 1.

Statistical Significance

The concordance of bulk and amino acid dates from the *Mary Rose* pig collagen in Table 1 were addressed statistically using the chi-squared (χ^2) test and Student's *t* distribution with 95% confidence. Three separate bulk collagen dates from the *Mary Rose* pig bone were AMS dated. Each of the resulting dates was shown to be internally consistent (see top of Table 2). Dates for each amino acid separated from the hydrolyzed collagen were then added in turn to the bulk values to determine that each concurred individually with the bulk date and finally together as a group without the bulk date. A "pass" indicates agreement between the dates with 95% confidence (see Table 2). Weighted

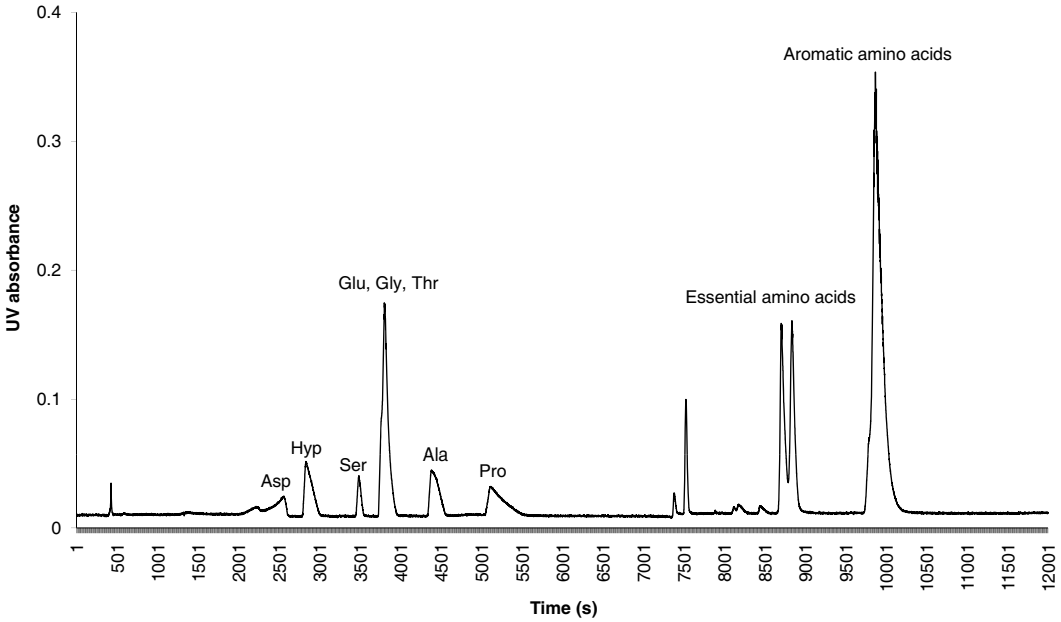

Figure 3 Separation of a standard amino acid mixture (collagen-like composition equivalent to 20 mg/mL, see Figure 2). Note: peak height and area relative to UV absorbance at 205 nm.

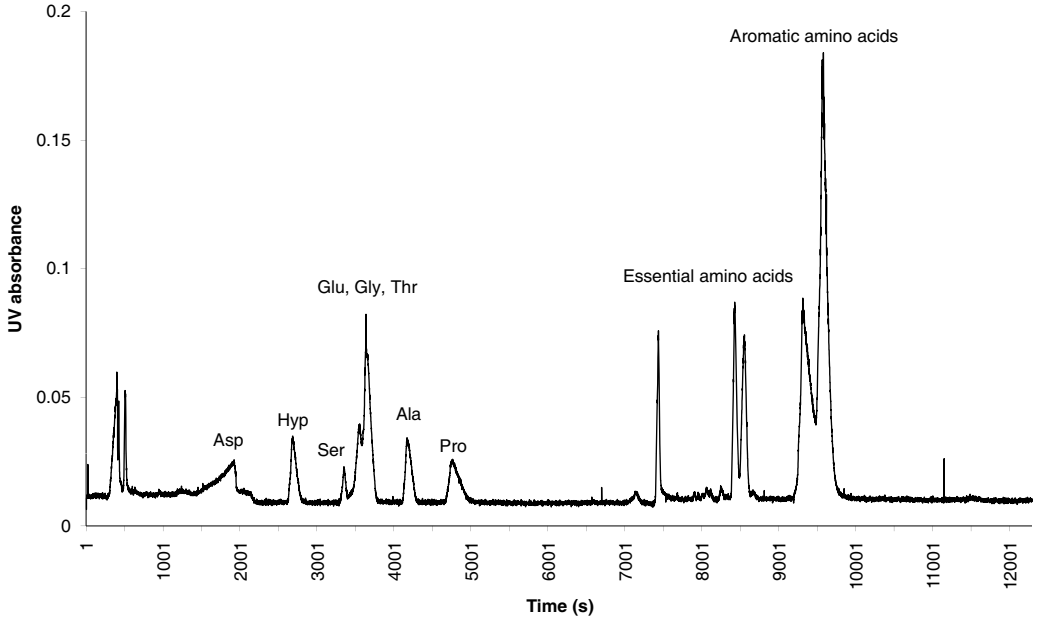

Figure 4 Separation of bone collagen hydrolysate,15 mg/mL (*Mary Rose* pig bone collagen; UV absorbance at 205 nm)

averages were obtained for the groups of measurements using the R_Combine function in OxCal (Bronk Ramsey 1995, 2001). A systematic error of 8 was included in this calculation to account for the annual fluctuation in atmospheric ^{14}C levels.

Table 1 AMS dates for individual amino acids and bulk samples from *Mary Rose* (MR) pig bone and Chalk Hill (CH) cattle bone.

Sample	AMS date (^{14}C yr BP)	Error ±	Burn yield (mg)
MR Hyp(1)	368	29	0.61
MR Hyp(2)	378	29	0.77
MR Hyp(3)	329	29	0.54
MR Gly/Glu	349	28	0.99
MR Ala	400	31	0.46
MR Pro	363	28	0.82
MR Bulk-A	339	30	0.83
MR Bulk-B	326	26	2.53
MR Bulk-C	343	27	1.95
CH Hyp	5010	36	0.61
CH Gly/Glu/Thr	4993	35	1.17
CH Ala	4995	40	0.44
CH Pro	5005	36	1
CH Bulk	4928	30	2.22

Chalk Hill

The *Mary Rose* data suggested that the new method provided both precise and accurate dates; however, with these relatively modern samples modern carbon contamination might be imperceptible and still strongly affect older samples. Application of the method to older material, with good collagen preservation, would therefore address this concern. Chalk Hill is a UK site of current archaeological interest on the western outskirts of Ramsgate near Upper Chalk Cliffs overlooking Pegwell Bay (Oswald et al. 2001). A sample of well-preserved bovine scapula was used to extract gelatinized protein, which was then subjected to bulk and compound-specific dating (results are shown in Table 1). Amino acid dates demonstrate statistical agreement with the bulk date of 4928 ± 30 yr BP. Like the *Mary Rose* samples, those of Chalk Hill are statistically indistinguishable, but the average for the Chalk Hill amino acid dates is slightly older than the average bulk date by 72 yr and the *Mary Rose* amino acids were on average 28 yr older than the bulk date.

Sample Blanks and Background Carbon

All the individual amino acid and bulk collagen dates in this study showed good statistical agreement, illustrating the efficacy of the new compound-specific approach. For any new dating method, however, it is important to investigate how much extraneous carbon (procedural blank) is introduced as part of the process. The statistically insignificant increase in age of the amino acids over the bulk values suggested some extraneous carbon was present that was ^{14}C-dead or a concomitantly larger amount that was at least on average older than 5000 yr. The addition of carbon contamination with no ^{14}C content (^{14}C-dead) contributes ~80 yr per 1% contamination irrespective of the age of the sample (Bowman 1995).

A potential source of extraneous carbon is from the material added during sample preparation. In this case, tin capsules used to contain the sample, Chromosorb used to absorb liquid samples, the liquids used in the mobile phases, and CO_2 in the air. We estimated the carbon contribution of all these using an elemental analyzer with results in Table 3. These show a very small amount of carbon comes from tin, Chromosorb, and the liquids combined (between 2 and 4 μg) as expected. All tin capsules and Chromosorb used in our experiments were cleaned and baked out at 500 °C; however,

Table 2 Results of the chi-squared tests for *Mary Rose* and Chalk Hill bulk collagen and single amino acid dates.

Sample name	Type	Date (^{14}C yr BP)	Error ±	*t* statistic	χ^2 test (Pass/Fail)
Mary Rose-A	Bulk	339	30	0.0	
Mary Rose-B	Bulk	326	26	0.1	
Mary Rose-C	Bulk	343	27	0.1	
Average (weighted)		336	18	0.2	Pass
Mary Rose-A	Bulk	339	30	0.0	
Mary Rose-B	Bulk	326	26	0.4	
Mary Rose-C	Bulk	343	27	0.0	
Mary Rose-Hyp1	Hyp1	368	29	0.7	
Average (weighted)		343	17	1.2	Pass
Mary Rose-A	Bulk	339	30	0.0	
Mary Rose-B	Bulk	326	26	0.3	
Mary Rose-C	Bulk	343	27	0.0	
Mary Rose-Hyp2	Hyp2	378	40	0.8	
Average (weighted)		341	17	1.2	Pass
Mary Rose-A	Bulk	339	30	0.0	
Mary Rose-B	Bulk	326	26	0.1	
Mary Rose-C	Bulk	343	27	0.1	
Mary Rose-Hyp3	Hyp3	329	29	0.0	
Average (weighted)		334	17	0.3	Pass
Mary Rose-A	Bulk	339	30	0.0	
Mary Rose-B	Bulk	326	26	0.2	
Mary Rose-C	Bulk	343	27	0.0	
Mary Rose-Gly/Glu	Gly/Glu	349	28	0.1	
Average (weighted)		339	16	0.4	Pass
Mary Rose-A	Bulk	339	30	0.1	
Mary Rose-B	Bulk	326	26	0.8	
Mary Rose-C	Bulk	343	27	0.0	
Mary Rose-Ala	Ala	400	31	2.7	
Average (weighted)		349	17	3.6	Pass
Mary Rose-A	Bulk	339	30	0.0	
Mary Rose-B	Bulk	326	26	0.4	
Mary Rose-C	Bulk	343	27	0.0	
Mary Rose-Pro	Pro	363	28	0.6	
Average (weighted)		342	16	1.0	Pass
Mary Rose-Hyp1	Hyp1	368	29	0.0	
Mary Rose-Hyp2	Hyp2	378	40	0.1	
Mary Rose-Hyp3	Hyp3	329	31	1.2	
Mary Rose-Gly/Glu	Gly/Glu	349	28	0.3	
Mary Rose-Ala	Ala	400	31	1.4	
Mary Rose-Pro	Pro	363	28	0.0	
Average (weighted)		363	15	3.0	Pass
Chalk Hill	Bulk	4928	30	1.3	
Chalk Hill-Hyp	Hyp	5010	36	1.8	
Average (weighted)		4962	25	3.1	Pass
Chalk Hill	Bulk	4928	30	0.8	
Chalk Hill-Gly/Glu/Thr	Gly/Glu/Thr	4993	35	1.1	
Average (weighted)		4956	25	2.0	Pass

Table 2 Results of the chi-squared tests for *Mary Rose* and Chalk Hill bulk collagen and single amino acid dates. *(Continued)*

Sample name	Type	Date (^{14}C yr BP)	Error ±	*t* statistic	χ^2 test (Pass/Fail)
Chalk Hill	Bulk	4928	30	0.6	
Chalk Hill-Ala	Ala	4995	40	1.1	
Average (weighted)		4952	26	1.8	Pass
Chalk Hill	Bulk	4928	30	1.1	
Chalk Hill-Pro	Pro	5005	36	1.6	
Average (weighted)		4960	25	2.7	Pass
Chalk Hill-Hyp	Hyp	5010	36	0.1	
Chalk Hill-Gly/Glu/Thr	Gly/Glu/Thr	4993	35	0.1	
Chalk Hill-Ala	Ala	4995	40	0.0	
Chalk Hill-Pro	Pro	5005	36	0.0	
Average (weighted)		5001	20	0.1	Pass

Table 3 Average values for 5 total procedure blanks and the effect of washing and baking tin and Chromosorb, respectively, on the sample blank.

Treatment	μg/carbon	Error	*n*
Chromosorb + tin (not cleaned or baked out)	4.7	1.6	7
Chromosorb + tin (baked and cleaned)	1.2	0.6	25
Chromosorb + tin + 30 μL water	4	2	30
Chromosorb + tin + 30 μL acid	2	1	12
Total procedure blank	8.2	5	5

these data show that if this precaution is not taken, considerably more carbon (4.7 ± 1.6 μg in our experiments) may be contributed.

Procedure blanks (including Chromosorb, tin capsules, and all sample processing up to graphitization) was tested by combining the results of three 1-mL injections of Milli-Q water, in place of the amino acid mixture, with collection of the mobile phase equivalent to an amino acid peak and subsequent sample processing was carried out in the same way as for the Chalk Hill samples. Results from the 5 procedure blanks ranged from 2 to 16 μg of carbon for the individual amino acids with an average of 8 μg of carbon. This corresponds to 0.8% of carbon in the Chalk Hill samples and 1% for the *Mary Rose* samples. This did not tell us where the carbon was coming from, but considering the relatively small size of the preparation blanks and the marginal shift to older ages for both *Mary Rose* and Chalk Hill samples, the evidence implies a small amount of ^{14}C-dead material.

Column Bleed

An LC-IsoLink system has a chemical oxidation unit that oxidizes and measures carbon content in liquid phases and enables an HPLC column to be put in-line (McCullagh et al. 2006, 2008; McCullagh 2010). This was used to measure the relative carbon content of the mobile phases and the amount of carbon coming off the preparative column used in this study ("column bleed"). Figure 5 reports their relative proportions. One drawback with this analysis is that it was not possible to compare data at the same flow rates used for the preparative separation (due to limitations of the instrumentation); however, it was possible to show that the amount of column bleed was directly proportional to the amount of acid present in the mobile phase (Figure 5).

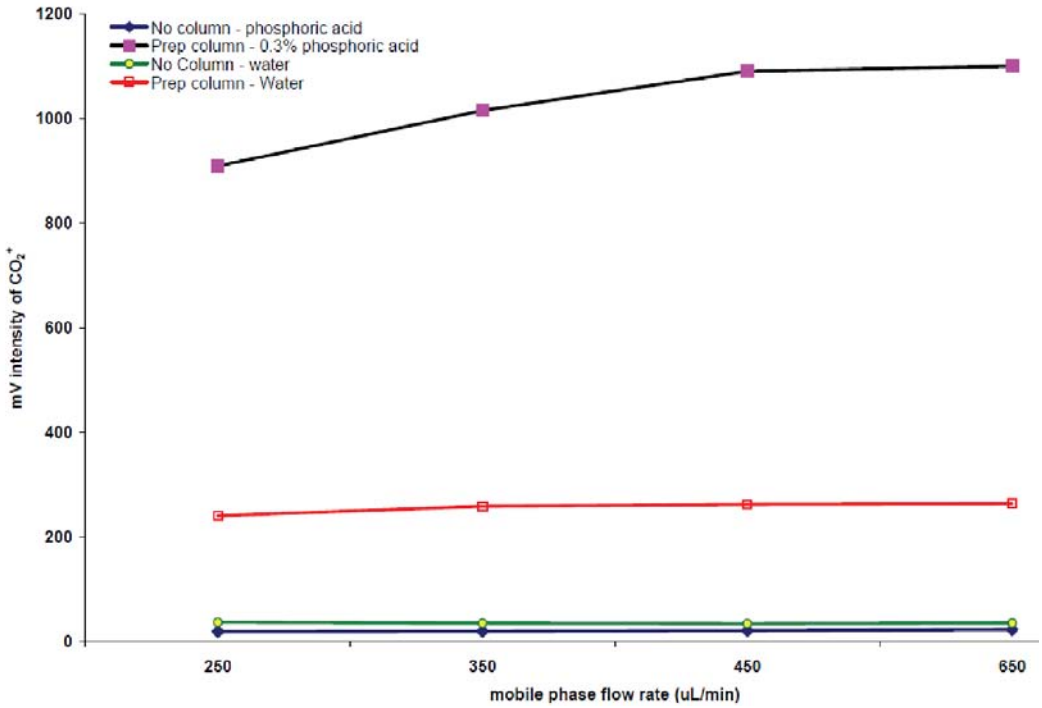

Figure 5 Data showing the relative proportion of carbon in the mobile phase eluent of the HPLC system for both 100% water and 0.3% phosphoric acid with and without the preparative column inline.

This was not unexpected but provided some important information. First, the majority of the carbon was bleeding from the preparative column. Second, more carbon comes from the column under acidic conditions than purely aqueous. Third, the small amount of carbon in the mobile phases was only 2% of the carbon coming from the column under acidic conditions. As we have found no other significant sources of carbon contamination, we conclude that the column bleed is responsible for the majority of carbon in the sample blanks.

Column bleed itself is a well-known phenomenon in chromatography and these findings are not surprising but do not tell us the ^{14}C age of this bleed. Previous studies using C18 reversed-phase columns have shown that carbon bleed is in fact the alkyl chain (ligand) supported by the silica backbone of the column, becoming detached and passing through the column in the mobile phase flow. These ligands are composed of dimethyloctadecylsilanes that would be modified with carboxylic acids groups in the case of the Primesep A column used in this study. It has also been previously demonstrated that low pH (or high temperature) leads to an increase in the hydrolysis of the siloxane bonds that attach these ligands to the stationary phase surface (Teutenberg et al. 2006; Luo and Carr 2008). This is commensurate with our findings and the evidence suggests that these alkyl chains are ^{14}C-dead, most likely having originated synthetically and ultimately from the carbon of petroleum products.

The slight (non-statistical) increase in age for both that *Mary Rose* and Chalk Hill samples is commensurate with the addition of approximately 1% dead carbon. For example, in order to obtain the ^{14}C date observed for the Chalk Hill hydroxyproline (5010), the amount of dead-carbon contamination required is 7 μg for the sample burn yield (0.6 mg) in comparison with the bulk carbon date.

This is extremely close to our average of 8 μg of carbon contamination calculated from the procedural blank without (excluding combustion blank) experiments.

The experiment of opening up a column and dating the stationary phase material itself was contemplated, but it was decided not to pursue this due to the relatively large expense and the fact that we have no direct link between the "whole" stationary phase and the column bleed observed. It is conceivable that only part of the stationary phase carbon is being eluted as column bleed and that this may have a different ^{14}C composition from that of the total carbon content of the column.

CONCLUSIONS

It is well known that conventional pretreatment chemistries for ^{14}C dating bone do not completely remove all extraneous carbon resulting from diagenesis and other types of contamination. ^{14}C dating individual amino acids from bone proteins presents a pragmatic approach to achieving more accurate dates under such circumstances, and the process of isolating individual amino acids also discards extraneous carbon by the nature of the separation processes involved.

This paper has presented a technique for underivatized amino acid separation that is based on mixed-mode chromatography, a departure from classical cation-exchange methods that have been used previously. It was demonstrated that the method could be used to preparatively isolate hydroxyproline, alanine, proline, and glutamate/glycine from hydrolyzed bone protein and was tested using *Mary Rose* samples with a known historical date. Its constituent amino acids were shown to be statistically indistinguishable from well-preserved collagen using a χ^2 test with 95% confidence limits.

The background carbon contribution was investigated using a conventional elemental analyzer to measure the procedural carbon blank and an LC-IsoLink system to identify column bleed. The blank was determined using Milli-Q water injections in place of the amino acid hydrolysates and it was estimated that on average 8 μg carbon came from the preparation process, which corresponded to on average 0.8–1% of the total carbon of an amino acid sample. It was shown that the majority of this carbon came from column bleed, which evidence suggests is ^{14}C-dead.

The future aims of this work are to set up a dating program to apply this new method to contaminated samples that would otherwise fail the selection process for conventional AMS dating due to severe contamination or poor preservation. Its success would provide the possibility of a permanent system capable of routinely dating material rejected by conventional approaches.

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