RADIOCARBON DATING OF INDIVIDUAL AMINO ACIDS FROM ARCHAEOLOGICAL BONE COLLAGEN

James S O McCullagh[1] • Anat Marom[2] • Robert E M Hedges[2]

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

[1]Chemistry Research Laboratory, Mansfield Road, Oxford OX13TA, United Kingdom. Corresponding author. Email: james.mccullagh@chem.ox.ac.uk.
[2]Research Laboratory for Archaeology and the History of Art (RLAHA), Dyson Perrins Building, South Parks Road, Oxford OX1 3QY, United Kingdom.

Proceedings of the 20th International Radiocarbon Conference, edited by A J T Jull
RADIOCARBON, Vol 52, Nr 2–3, 2010, p 620–634

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

https://doi.org/10.1017/S0033822200045653 Published online by Cambridge University Press

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

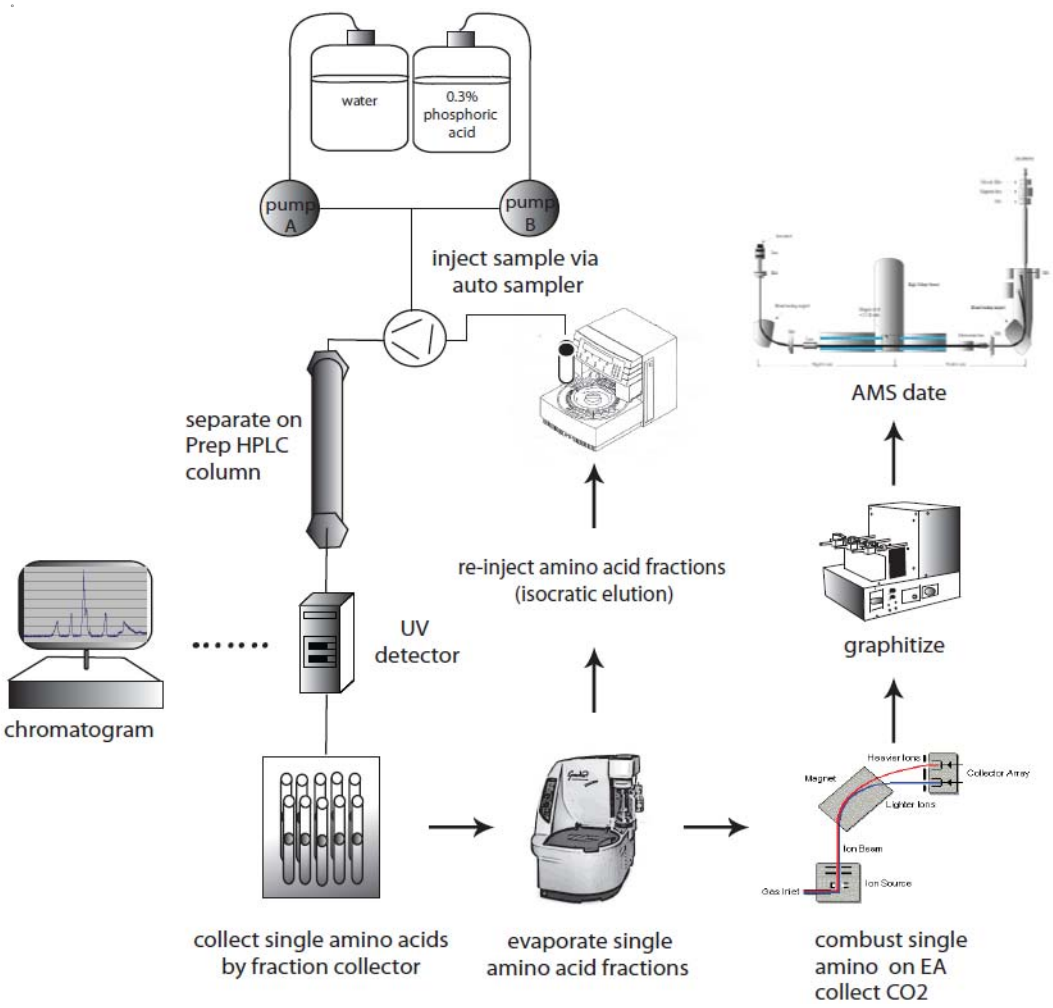

Figure 1 Flow diagram for the method to ^{14}C date individual amino acids

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).

https://doi.org/10.1017/S0033822200045653 Published online by Cambridge University Press

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.

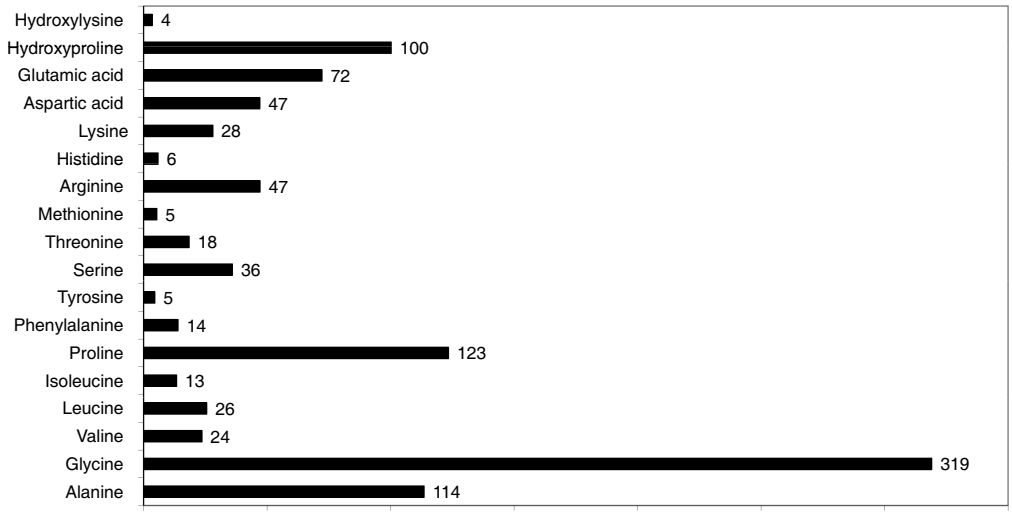

Figure 2 Amino acid composition of human bone, residues per 1000 (Eastoe et al. 1955)

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

https://doi.org/10.1017/S0033822200045653 Published online by Cambridge University Press

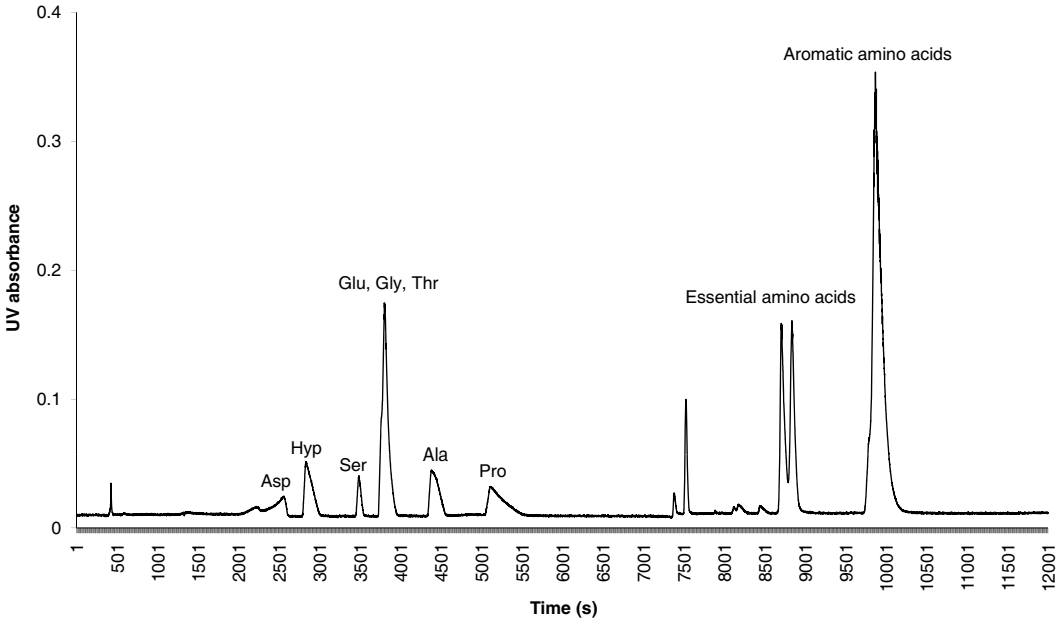

Figure 3 Separation of a standard amino acid mixture (collagen-like composition equivalent to 20 mg/mL, see Figure 2). Note: peak height and area relative to UV absorbance at 205 nm.

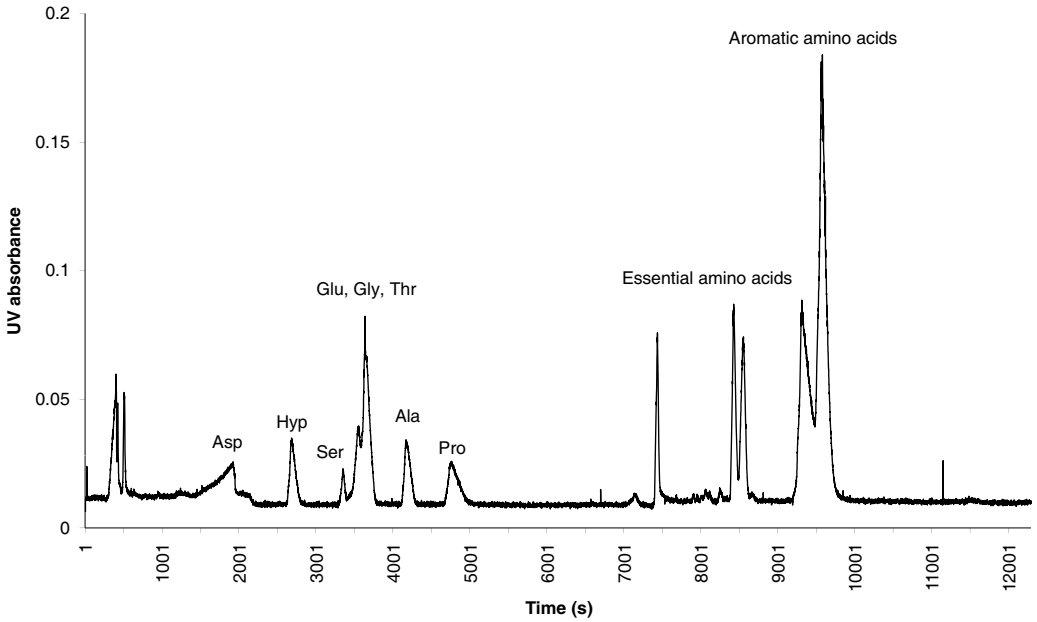

Figure 4 Separation of bone collagen hydrolysate,15 mg/mL (*Mary Rose* pig bone collagen; UV absorbance at 205 nm)

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.

https://doi.org/10.1017/S0033822200045653 Published online by Cambridge University Press

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,

https://doi.org/10.1017/S0033822200045653 Published online by Cambridge University Press

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

https://doi.org/10.1017/S0033822200045653 Published online by Cambridge University Press

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).

https://doi.org/10.1017/S0033822200045653 Published online by Cambridge University Press

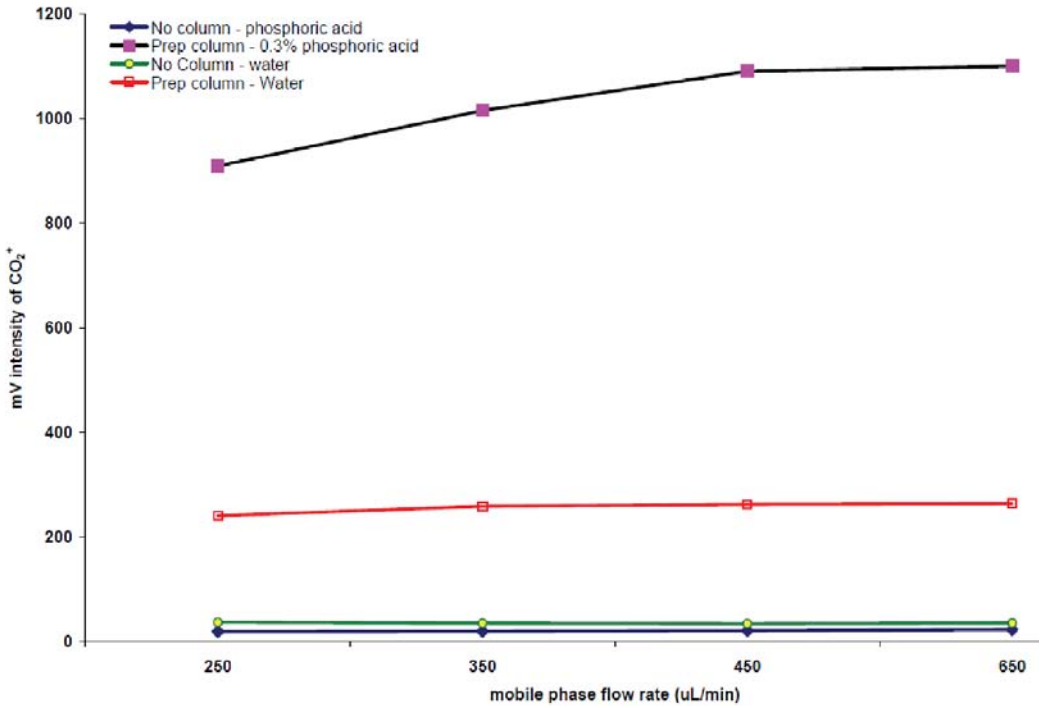

Figure 5 Data showing the relative proportion of carbon in the mobile phase eluent of the HPLC system for both 100% water and 0.3% phosphoric acid with and without the preparative column inline.

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.

https://doi.org/10.1017/S0033822200045653 Published online by Cambridge University Press

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.

ACKNOWLEDGMENTS

We are especially grateful to Angela Bowles for her work on combustion and graphitization of amino acid fractions for this project and to all the people at the ORAU who helped with this project and to Peter Ditchfield for EA analysis. Additional thanks go to Christopher Bronk Ramsey, Tom Higham, and Michael Dee at the ORAU who provided invaluable support including provision of the *Mary Rose* standards and statistical help. Gratitude also goes to Julie Hamilton at the RLAHA for providing access to the Chalk Hill samples and to Dr Wen-Wu Li for ideas which helped improve the chromatographic method.

REFERENCES

Ambrose SH. 1990. Preparation and characterization of bone and tooth collagen for isotopic analysis. *Journal of Archaeological Science* 17(4):431–51.

Ambrose SH, Krigbaum J. 2003. Bone chemistry and bioarchaeology. *Journal of Anthropological Archaeology* 22(3):193–9.

Bada JL, Gillespie R, Gowlett JAJ, Hedges REM. 1984. Accelerator mass spectrometry radiocarbon ages of amino acid extracts from Californian palaeoindian skeletons. *Nature* 312(5993):442–4.

Bowman S. 1995. *Radiocarbon Dating*. London: British Museum Press. 64 p.

Bradford E. 1982. *The Story of the Mary Rose*. Over Wallop: BAS printers. 207 p.

Brock F, Bronk Ramsey C, Higham T. 2007. Quality assurance of ultrafiltered bone dating. *Radiocarbon* 49(2):187–92.

Bronk Ramsey C. 1995. Radiocarbon calibration and analysis of stratigraphy: the OxCal program. *Radiocarbon* 37(2):425–30.

Bronk Ramsey C. 2001. Development of the radiocarbon calibration program. *Radiocarbon* 43(2A):355–63.

Bronk Ramsey C, Ditchfield P, Humm M. 2004a. Using a gas ion source for radiocarbon AMS and GC-AMS. *Radiocarbon* 46(1):25–32.

Bronk Ramsey C, Higham T, Leach P. 2004b. Towards high-precision AMS: progress and limitations. *Radiocarbon* 46(1):17–24.

Bronk Ramsey C, Higham T, Bowles A, Hedges R. 2004c. Improvements to the pretreatment of bone at Oxford. *Radiocarbon* 46(1):155–63.

Brown TA, Nelson DE, Vogel JS, Southon JR. 1988. Improved collagen extraction by modified Longin method. *Radiocarbon* 30(2):171–7.

Dee M, Bronk Ramsey C. 2000 Refinement of graphite target production at ORAU. *Nuclear Instruments and Methods in Physics Research B* 172(1–4):449–53.

Eastoe JE. 1955. The amino acid composition of mammalian collagen and gelatin. *Biochemical Journal* 61(4):589–600.

Gillespie R, Hedges REM. 1983. Sample chemistry for the Oxford high energy mass spectrometer. *Radiocarbon* 25(2):771–4.

Gillespie R, Hedges REM, Wand JO. 1984. Radiocarbon dating of bone by accelerator mass spectrometry. *Journal of Archaeological Science* 11(2):165–70.

Hedges REM, van Klinken GJ. 1992. A review of current approaches in the pretreatment of bone for radiocarbon dating by AMS. *Radiocarbon* 34(3):279–91.

Hedges REM, Wallace CJA. 1978. The survival of biochemical information in archaeological bone. *Journal of Archaeological Science* 5(4):377–86.

Ho TY, Marcus LF, Berger R. 1969. Radiocarbon dating of petroleum-impregnated bone from tar pits at Rancho La Brea, California. *Science* 164(883):1051–2.

Higham TFG, Jacobi RM, Bronk Ramsey C. 2006. AMS radiocarbon dating of ancient bone using ultrafiltration. *Radiocarbon* 48(2):179–95.

Lambert B, Grupe G. 1993. *Prehistoric Human Bone: Archaeology at the Molecular Level*. Berlin: Springer-Verlag.

Longin R. 1971. New method of collagen extraction for radiocarbon dating. *Nature* 230(5291):241–2.

Luo H, Carr PW. 2008. Silica-based, acid-stable stationary phases for high performance liquid chromatography. *Analytical and Bioanalytical Chemistry* 391(3): 919–23.

McCullagh JSO, Juchelka D, Hedges REM. 2006. Analysis of amino acid ^{13}C abundance from human and faunal bone collagen using liquid chromatography/isotope ratio mass spectrometry. *Rapid Communications in Mass Spectrometry* 20(18):2761–8.

McCullagh JSO, Gaye-Siessegger J, Focken J. 2008. Determination of underivatized amino acid $\delta^{13}C$ by liquid chromatography/isotope ratio mass spectrometry for nutritional studies: the effect of dietary non-essential amino acid profile on the isotopic signature of individual amino acids in fish. *Rapid Communications in Mass Spectrometry* 22(12):1817–22.

McCullagh JSO. 2010. Mixed-mode chromatography/isotope ratio mass spectrometry. *Rapid Communications in Mass Spectrometry* 24(5):483–94.

Mollenhauer G, Rethemeyer J. 2009. Compound-specific radiocarbon analysis—analytical challenges and applications. *IOP Conference Series: Earth and Environmental Science* 5(1):012006, doi:10.1088/1755-1307/5/1/012006.

Nelson DE. 1991. A new method for carbon isotopic analysis of protein. *Science* 251(4993):552–4.

Oswald A, Dyer C, Barber M. 2001. *The Creation of Monuments: Neolithic Causewayed Enclosures in the British Isles*. Swindon: English Heritage. 175 p.

Reimer PJ, Baillie MGL, Bard E, Bayliss A, Beck JW, Bertrand CJH, Blackwell PG, Buck CE, Burr GS, Cutler KB, Damon PE, Edwards RL, Fairbanks RG, Friedrich M, Guilderson TP, Hogg AG, Hughen KA, Kromer B, McCormac G, Manning S, Bronk Ramsey C, Reimer RW, Remmele S, Southon JR, Stuiver M, Talamo S, Taylor FW, van der Plicht J, Weyhenmeyer CE. 2004. IntCal04 terrestrial radiocarbon age calibration, 0–26 cal kyr BP. *Radiocarbon* 46(3):1029–58.

Stafford TW Jr, Jull AJT, Brendel K, Duhamel RC, Donahue D. 1987. Study of bone radiocarbon dating accuracy at the University of Arizona NSF Accelerator Facility for Radioisotope Analysis. *Radiocarbon* 29(1):24–44.

Stafford TW Jr, Bendel K, Duhamel RC. 1988. Radiocarbon ^{13}C and ^{15}N analysis of fossil bone: removal of humates with XAD-2 resin. *Geochimica and Cosmochimica Acta* 52(9):2257–67.

Stafford TW Jr, Hare PE, Currie L, Jull AJT, Donahue DJ. 1991. Accelerator radiocarbon dating at the molecular level. *Journal of Archaeological Science* 18(1):35–72.

Stott AW, Berstan R, Evershed RP, Hedges REM, Bronk Ramsey C, Humm MJ. 2001. Radiocarbon dating of single compounds isolated from pottery cooking vessel residues. *Radiocarbon* 43(2A):191–7.

Stott AW, Berstan R, Evershed RP, Bronk-Ramsey C, Hedges REM, Humm MJ. 2003. Direct dating of archaeological pottery by compound-specific ^{14}C analysis of preserved lipids. *Analytical Chemistry* 75(19): 5037–45.

Taylor RE, Hare PE, Pior C, Kirner D, Wan L, Burky R.

1995. Radiocarbon dating of biochemically characterized hair. *Radiocarbon* 37(2):319–30.

Teutenberg T, Holzhauser MT, Kiffmeyer TK. 2006. Evaluation of column bleed by using an ultraviolet and a charged aerosol detector coupled to a high-temperature liquid chromatographic system. *Journal of Chromatography A* 1119:197–201.

Tripp JA, McCullagh JSO, Hedges REM. 2006. Preparative separation of underivatized amino acids for compound-specific stable isotope analysis and radiocarbon dating of hydrolyzed bone collagen. *Journal of Separation Science* 29(1):41–8

Udenfriend S. 1966. Formation of hydroxyproline in collagen. *Science* 152(3727):1335–40.

van Klinken GJ. 1991. Dating and dietary reconstruction by isotopic analysis of amino acids in fossil bone collagen - with special reference to the Caribbean [PhD dissertation]. University of Groningen, Netherlands.

van Klinken GJ. 1999. Bone collagen quality indicators for palaeodietary and radiocarbon measurements. *Journal of Archaeological Science* 26(6):687–95.

van Klinken GJ, Hedges REM. 1992. Experiments on ^{14}C dating of contaminated bone using peptides resulting from enzymatic cleavage of collagen. *Radiocarbon* 34(3):292–5.

van Klinken GJ, Hedges REM. 1997. Chemistry strategies for organic radiocarbon samples. *Radiocarbon* 40(1):51–6.

van Klinken GJ, Mook WG. 1990. Preparative high performance liquid chromatographic separation of individual amino acids derived from fossil bone collagen. *Radiocarbon* 32(2):155–64.

van Klinken GJ, Bowles AD, Hedges REM. 1994. Radiocarbon dating of peptides isolated from contaminated fossil bone-collagen by collagenase digestion and reversed-phase chromatography. *Geochimica et Cosmochimica Acta* 58(11):2543–51.

Vaughan J. 1975. *The Physiology of Bone*. Oxford: Clarendon Press. 324 p.

Wand JO. 1981. Microsample preparation for radiocarbon dating [unpublished PhD dissertation]. Oxford University.