CHARACTERIZATION OF DIFFERENT CHEMICAL PROCEDURES FOR ^{14}C DATING OF BURIED, CREMATED, AND MODERN BONE SAMPLES AT CIRCE

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ABSTRACT. Bone chemical treatment for radiocarbon dating has drawn the attention of different laboratories because dates of bones and charcoals found in the same layer often disagree. Excluding diet-related reservoir effects, this observation is likely due to a nonoptimized procedure of contaminant removal from the extracted collagen. In this study, systematic work on the bone chemical treatment was performed with the aim to investigate the effect of each known procedure (i.e. AAA, GEL, and ULTR) on the collagen used for ^{14}C dating. Isolation and purification of lipids from animal tissues were performed to estimate eventual offsets induced by the applied methods, by comparing the ^{14}C ages of lipids with those of collagen. Moreover, cremated bones were treated for the first time at CIRCE. Measured ^{14}C isotopic ratios on these samples were used to evaluate the accuracy of the applied procedure by comparing against the results for charcoals found in the same archaeological context as the bones.

INTRODUCTION

Bones represent a significant repository of archaeological information and, within an archaeological context, bones acquire increasing importance especially when they are the only available material at a given site. Human and animal bones contain carbon in both organic and inorganic form, with collagen being the commonly analyzed fraction for radiocarbon dating.

The known protocols used to extract and purify the collagen are (i) low concentration acid digestion plus base/acid attack (AAA) to remove humic acids (dark colored acids comprising moderately high-molecular weight polymers of indefinite structure) that can contaminate the bone sample (e.g. Arslanov and Svezhentsev 1993); (ii) gelatinization (GEL) where the AAA-extracted collagen is dissolved in pH 3 solution (Longin 1971; Stafford et al. 1987); and (iii) ultrafiltration (ULTR) (Brown et al. 1988; Bronk Ramsey et al. 2004; Brock et al. 2007; Hüls et al. 2007) to remove low-molecular weight contaminants from the gelatin solution. Since the 3 procedures (AAA-GEL-ULTR) can be performed in sequence, this study applies a unique procedure (from AAA to ULTR), sampling and measuring the produced fractions during each treatment. The aim is to test the effect of each protocol on the extraction and purification of collagen.

Lipids, characterized by very fast turnover times, preserve an isotopic ratio $^{14}C/^{12}C$ equal to the atmospheric CO_2 in the last 1–2 yr of life of an individual. Meanwhile, collagen is characterized by slower turnover time compared to lipids and produces an isotopic ratio that is the weighted average of a number of years before the death of the individual (Wild et al. 2000).

The first part of this study assesses the presence or absence of systematic offsets introduced during the collagen extraction from modern bones via a comparison between collagen fractions (AAA, GEL, ULTR) with 1 non-collagen fraction (lipids). In the absence of procedure-induced contaminations, for short-lived animals (few months of life) no differences are expected between these fractions, in terms of ^{14}C dating.

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The 3 collagen fractions (AAA, GEL, ULTR) were extracted also from the standard VIRI E sample (mammoth) and compared with the lipids fraction. Bone is a connective tissue largely composed of organic proteins, collagen, and the inorganic mineral hydroxyapatite, which includes calcium phosphate, calcium carbonate, calcium fluoride, calcium hydroxide, and citrate ($[Ca_3(P)_4)_2]_3Ca(OH)_2$), which together combine to provide the structural support in the body (Smith et al. 1983). The substitution mechanisms, which occur in the hydroxyapatite of bones during burial, include intercrystalline exchange and recrystallization with the addition of new ions into the crystal structure. The hydroxyapatite can therefore contain environmental carbonates and, for this reason, this fraction is not used for ^{14}C dating of buried bones. Instead, during the funeral ritual when the bone is heated at high temperature (>600 °C), the inorganic mineral component survives the cremation process while the organic component, mainly collagen, does not. During cremation, changes in the color of the bone are observable depending on the exposure temperature (Shipman et al. 1984). Mays (1998) studied the changes in crystal structure as a function of temperature, conducted by heating modern bone samples and observing X-ray diffraction patterns, with results showing a gradual increase in the size of the crystals, indicated by the rising and narrowing of the peaks, up to ~525 °C.

The cremation tests of Van Strydonck et al. (2005) highlighted that, also as a function of exposure time and not only of the temperature, an increase of the crystallinity and a compaction of the bone as well as a loss of CO_2 take place. This occurs at lower temperatures due to the combustion of the organic fraction and at higher temperatures due to the decomposition of the bioapatite. The recrystallization of the bone matrix forms a barrier that protects the remaining structural carbonate. Therefore, compaction of the bone and the very low concentration of structural carbonate after cremation prevent reactive agents in the environment from arriving at the reactive part of the bone and causing intercrystalline exchange introducing carbonate ions.

Recently, further tests by Van Strydonck et al. (2010) and Hüls et al. (2010) showed that during the cremation process there is not only a decrease of carbon content in the apatite but also a carbon substitution. The isotope results indicate an effective carbon exchange between bone apatite carbonate and CO_2 in the combustion gases during the cremation, which varies with CO_2 concentration and duration. Archaeological cremated bone apatite may thus contain a significant amount of carbon originating from the burning fuel, and its ^{14}C dates may thus suffer from an old-wood effect. Thus far, however, there are few publications studying the fuel in prehistoric pyres. If relatively young trees were used as firewood for the pyre, the old-wood effect would be comparable to the reservoir age of the calcined bones; thus, a possible carbon exchange will not be recognizable in the ^{14}C date. More tests are needed to understand fully the dynamics of the cremation process.

In order to assess the feasibility of dating cremated bones from an Italian site (see below), we analyzed cremated bones and compared the results with those for buried bones and charcoals. Archaeological bone samples from a large cemetery in Campania (Italy), where inhumations were found together with several cremations, were used to compare the fractions (AAA-GEL-ULTR) extracted by the 3 protocols. Moreover, the possibility of using cremated bones for ^{14}C dating allowed us to treat these samples for the first time at CIRCE and to verify the accuracy of the applied procedures for cremated and buried bones by comparing against charcoals used for the funeral rituals found in the same archaeological context.

METHODS AND MATERIALS

Lipid Extraction

The extraction of lipids was carried out using the Folch protocol (Folch et al. 1957). The bone powder is treated with a chloroform-methanol mixture (2:1) to a final volume of ~25 times the mass of the analyzed sample (1 g in 25 mL of solvent mixture). Extraction is achieved by ultrasonication for 30 min (repeated for 3 times on the bone powder with a new solvent each time). This step produces apolar substances in the solution (including lipids). After recovery of the solvent by centrifugation, 20% of the total volume of a 0.9% NaCl solution is added (5 mL for 25 mL). After shaking for a few seconds, a biphasic system is generated: an upper, more polar phase with gangliosides or small organic polar molecules and the lower phase containing lipids. After centrifugation and removal of the upper phase, the lower chloroform phase with lipids is evaporated under vacuum at high temperature (45 °C).

Collagen Extraction

Before chemical treatment, the bone sample is scraped clean in order to remove external contaminants like roots or soil, and then crushed by drilling. After mechanical cleaning, collagen extraction is conducted following the main chemical pretreatments during which the 3 fractions (AAA, GEL, ULTR) are extracted:

1. About 1 g of bone powder is treated with 0.6N HCl at room temperature (20 °C) for 2 hr followed by a second HCl attack overnight and a final HCl attack for 1 hr. The sequence of attacks is performed in order to increase the yield apatite (carbonate and phosphates) removal (i.e. an access of protons is supplied to the solution 3 times).
2. The acid-insoluble component is then treated with 0.1M NaOH for a short time in order to remove base-soluble contaminants such as humic acids (Minami et al. 2004), followed by HCl to remove dissolved atmospheric CO_2 from the sample. Several rinses with deionized water are done after each step. Before the solution becomes neutral, after the second rinse with water a part of the collagen is kept and stored in an oven overnight (AAA).
3. The remaining collagen, neutralized by water in the last rinse, is heated to 70 °C in a pH 3 solution for 20 hr, gelatinizing the collagen. The gelatin solution is then filtered using a 60–90 μm polyethylene Eezi-filter™ (cleaned in Milli-Q™ water for 30 min by ultrasonication). A part of this gelatin is kept and freeze-dried, obtaining the GEL fraction.
4. The residual filtered gelatin is put into a special polyethersulfone (PES) ultrafilter (Vivaspin™ 15–30,000 MWCO), previously cleaned by several centrifugations and ultrasonication with ultrapure water (Brock et al. 2007; Hüls et al. 2007), and centrifuged until only higher-molecular weight (>30 kD) proteins remain, removing contaminants of low molecular weight. This gelatin is freeze-dried, obtaining the ULTR fraction.

Cremated Bones Treatment

The treatment of cremated bone samples was carried out for the first time at CIRCE following the Lanting et al. (2001) method that is currently in use at the ORAU laboratory in Oxford. After cleaning and crushing 2–3 g of cremated bone, the bone powder is attacked with 1.5% sodium hypochlorite solution at pH 3 for 48 hr at room temperature (20 °C). In this way, the organic material is removed. Then, 1M acetic acid is added to the bone over 24 hr at 20 °C to remove calcite and adsorbed carbonates. Finally, the bone is washed in pH 3 water, freeze-dried, and submitted to acid digestion. By using special Pyrex® vessels, the CO_2 is produced by reaction between the bone and 85% phosphoric acid.

Graphitization and Measurement

The CO_2 sample, produced by combustion (Passariello et al. 2007) or by acid digestion, is purified into a steel cryogenic line through H_2O and CO_2 spiral traps and transferred to a sealed, pretreated Pyrex tube with Zn and TiH_2 powder where graphitization takes place at 565 °C for 8 hr (Marzaioli et al. 2008). Finally, the graphite is pressed in an aluminum cathode and measured at CIRCE using a NEC 3MV accelerator mass spectrometer (Terrasi et al. 2008). All measured ^{14}C data were background-subtracted by means of processed Aesar graphite (i.e. a blank graphite processed in the preparation line). The values of all measurements are expressed as pMC or ^{14}C ages calculated according to Stuiver and Polach (1977) and calibrated by using the OxCal v 4.1.3 program (Bronk Ramsey 2009) and the IntCal09 calibration curve (Reimer et al. 2009).

STUDY CASES

Modern Bones

To verify the presence or absence of systematic offsets that could occur during the extraction of collagen in the different collagen fractions, the 3 fractions (AAA, GEL, ULTR) and the lipids were extracted and compared in 2 chicken leg bone samples.

In the first part of our study, we used a leg bone coming from an industrially reared chicken (breading and death in 2010). This bone was split into 2 halves: both halves were treated separately for AAA-GEL-ULTR extraction and one half was used to extract lipids before extraction of the collagen fractions.

In the second part of this study, a leg bone from a chicken raised on an organic farm (breading and death in 2010) was used in order to avoid possible interferences arising from the industrial feeding. Also in this case, the bone was divided into 2 parts. Both halves were used to extract the 3 collagen fractions, but one half was subdivided into 2 parts obtaining 2 lipids fractions and 2 AAA-GEL-ULTR fractions after the lipid extraction. Chickens bred for consumption are short-lived animals (a few months of life); therefore, there should be no difference between the lipids and collagen in terms of ^{14}C dating. We thus aim to compare the ^{14}C ages of lipids and collagen coming from each step and to verify whether the presence of lipids in bones affects the ^{14}C ages obtained by dating collagen extracts. In addition, the lipids fraction was extracted also from a 2010 extra-virgin olive oil sample from EC 692/2003_ DOP (Designations of Origin for Agricultural Products and Foodstuffs) and used as a control in order to test if contamination was introduced during the extraction by comparison with a bulk fraction of the same oil.

VIRI E (Mammoth)

To check our procedures and results and to verify the quality assurance of our laboratory, we successfully participated in the VIRI intercomparison exercise (Scott et al. 2010). In this study, the VIRI E sample was used to characterize the different chemical procedures for ^{14}C dating. Two reasons led us to this choice: 1) the bone is a certified sample and 2) the mammoth bone is a very old sample, but well preserved, so we can better assess eventual differences among the 3 collagen fractions, especially in the ULTR fraction. In fact, particularly for old bones, the ultrafiltration method should provide improved removal of contaminants for bone dating (Bronk Ramsey et al. 2004).

In the third part of our study, the mammoth bone was pulverized. One part of the powder was used to extract the 3 fractions (AAA-GEL-ULTR) and the other part was used to extract lipids before the extraction of the collagen fractions and used for comparison with the 3 fractions.

Archaeological Samples: Nuovo Mattatoio Necropolis in Parisi, Italy

The third study case concerns a big necropolis, Nuovo Mattatoio, occupying a 3500-m^2 area between Capua and Santa Maria Capua Vetere, Italy. During excavations in 2005, a total of 459 tombs were found (362 inhumations and 97 cremations) attributable to the Early Iron Age. These inhumations and cremations coexist and are distributed equally without demarcations, frequently overlapping. The burials are covered by local white limestone pebbles and dug into a layer of red silt, corresponding to the plane of use of the cemetery, which lies just 50 cm below present ground level. The covers of the graves were badly damaged by agricultural work (Colombo and Stanislao 2011). The prevailing orientation of the pits is NW-SE, although many burials are N-S or NE-SW. In some cases, the adoption of a different orientation emphasizes the aggregation between neighboring graves.

The first archaeological studies (Colombo and Stanislao 2011) date the necropolis between 1000–800 BC and a more recent period, identifying 2 contiguous phases. Among all these tombs, only a few were found with materials (bones or charcoal) suitable for ^{14}C dating. Specifically, 7 cremations (all dated in this work), only one of which contained charcoal (Tomb 56_*vitis vinifera*) (many cremations were empty or only had ashes) and very few inhumations with bones (many empty or with only funeral goods), were found geographically arranged in the same area of the necropolis. Several years ago, this necropolis was looted: this explains the almost total lack of materials useful for ^{14}C dating. For these reasons, the choice of which graves to date was forced. Nevertheless, the materials found and used for this study were sufficient to obtain an initial chronology for the site.

The possibility to treat and measure both buried and cremated bones, belonging to the same archaeological context, constituted an opportunity to check and compare the different protocols of extraction and purification of collagen also on archaeological samples and to verify the accuracy of the treatment applied on cremated bones by comparison with charcoals found in the same archaeological context. We used 4 buried samples from which the 3 fractions were extracted and 6 cremated bone samples, together with 2 charcoal samples, one of which belonged to the same dated cremation tomb. The charcoal samples were chemically treated using the AAA protocol (Mook and Streurman 1983).

RESULTS AND DISCUSSION

Modern Bone Samples

^{14}C results on 2 chicken leg bone samples, one from an industrially reared chicken and one from an organic chicken, are shown in Table 1, together with the results of an olive oil sample and the lipid fraction coming from the same oil. The oil is used as a control in order to test contamination introduced during the extraction by comparison with the bulk fraction of the same oil.

Some fractions were not large enough to yield high-precision results and are thus not included in Table 1. The results from bulk oil and 2 lipid fractions were obtained in 2 different replicates from the same oil sample. The pMC values of the oil (Table 1) suggest that, with respect to the result of bulk oil, the oil lipids appear to be more enriched in ^{14}C in both extractions. We could exclude fossil-like contamination coming from the lab atmosphere or from solvents because, in this case, we should observe more depleted values, although there is a possibility, albeit remote, that the solvents are modern. Therefore, most likely the latter possibility and/or a fractionation during the lipid extraction induces this enrichment observable in both extractions. The variability of this fractionation could also be the reason for the discrepancy between the 2 results. More detailed studies will be performed to characterize this phenomenon and to understand the origin of this enrichment, also by means of isotope ratio mass spectrometry (IRMS).

Table 1 ^{14}C results of the AAA, GEL, and ULTR fractions on 2 chicken legs, one coming from an "industrial" chicken and one from an organic chicken (indicated by *), together with the results of an oil sample and the lipids fraction coming from the same oil, used as control. The 1° part and 2° part terms refer to the 2 lipid extractions and to the 3 collagen fractions, obtained after the lipid extraction, coming from half of the organic chicken leg bone (nl = after lipid extraction).

CIRCE code	Sample name	pMC	Error
DSH2009	Oil	104.88	0.44
DSH2010	Oil lipids	106.29	0.30
DSH1990	Oil lipids	107.93	0.68
DSH1982	Chicken_AAA	104.52	0.24
DSH1986	Chicken_GEL	105.44	0.39
DSH1988	Chicken_ULTR	104.82	0.51
DSH1981	Chicken_nl_AAA	105.26	0.37
DSH1987	Chicken_nl_GEL	105.14	0.47
DSH1985	Chicken_Lipids	107.42	0.42
DSH2026	*Chicken 1_AAA	105.51	0.33
DSH2087	*Chicken 1_nl_AAA_1° part	104.39	0.31
DSH2039	*Chicken1_nl_GEL_1° part	105.56	0.43
DSH2044	*Chicken1_nl_ULTR_1° part	104.73	0.26
DSH2032	*Chicken 1_Lipids_1° part	105.83	0.32
DSH2035	*Chicken 1_nl_AAA_2° part	107.44	0.50
DSH2046	*Chicken1_nl_ULTR_2° part	104.39	0.24
DSH2034	*Chicken 1_Lipids_2° part	107.55	0.43

The results from chicken samples are better highlighted in Figure 1. In the upper panel, corresponding to the "industrial" chicken, the results of the 3 fractions (circles) extracted from one half of the chicken are shown, together with the results of the lipids (diamonds) and of the fractions extracted from lipid residues from the other half of the chicken leg. The horizontal black line represents the pMC value corresponding to 2010 (104.5 ± 0.1 pMC; Marzaioli et al. 2011), i.e. the date of death of the chicken, with the observed atmospheric variability. Also in this case, the trend of lipids is the same as the oil (Table 1). The results of the AAA, GEL, and ULTR fractions, from both halves, all agree within 1σ and it thus seems that the extraction of lipids does not interfere with the procedure of extraction of the 3 fractions obtained from collagen. Also, for the organic chicken (Figure 1, lower panel), we note the same trend of lipids in both extractions, coming from one half of the chicken leg, similar to the oil and to non-organic chicken results. All fraction results, apart from Chicken 1_NL_AAA_2, are in agreement and represent the expected age.

VIRI E (Mammoth)

For modern bone samples, we have shown that the AAA fraction sometimes fluctuates, often deviating from the other 2 fractions (GEL and ULTR). This is seen also in the results of the VIRI E sample (Table 2). Although we had a very old but well-preserved mammoth bone, we were able to extract lipids enriched in ^{14}C as for the other samples. From Table 2, it is evident how the AAA fraction fluctuates and deviates from the other 2 fractions, especially in the lipids sample. On the other hand, the GEL and ULTR fractions are in agreement in both cases (in the sample with lipids and in that after lipid extraction). From these first results on modern and mammoth samples, we thus deduce that it is not possible to use lipids to compare the applied treatments and estimate offsets

induced by the treatment stages. Moreover, it appears that there is no difference between the GEL and ULTR fractions for the young sample nor the mammoth VIRI sample.

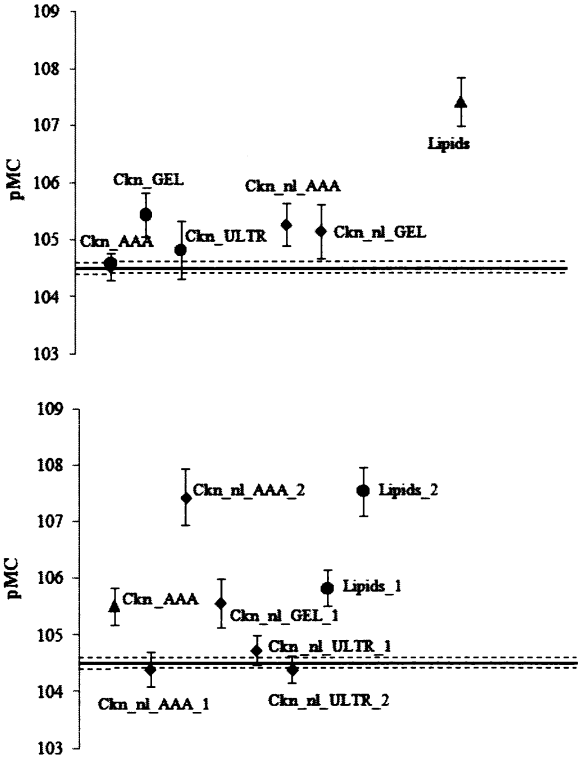

Figure 1 pMC values of AAA, GEL, and ULTR fractions (upper) extracted from one half of an "industrial" chicken leg bone and from one half of an organic chicken (lower), together with the results of the lipids and of fractions extracted without lipids (nl) from the other half of the bone. The horizontal black line represents the pMC value corresponding to the date of chicken death with the observed atmospheric variability (104.5 ± 0.1; Marzaioli et al. 2011). Some fractions were not large enough to yield high-precision results and are thus not included in the figure.

Table 2 ^{14}C ages and pMC values obtained on the mammoth sample (VIRI E) coming from lipids and from the AAA, GEL, and ULTR fractions on the bulk sample and on the same sample after the lipid extraction (nl = after lipid extraction).

CIRCE code	Sample name	pMC	Error	^{14}C age (BP)	Error
DSH2488	Mammoth_AAA	1.43	0.09	34,140	480–510
DSH2511	Mammoth_GEL	0.71	0.09	39,800	939–1064
DSH2506	Mammoth_ULTR	0.77	0.09	39,100	856–958
DSH2492	Mammoth_nl_AAA	0.74	0.08	39,380	844–944
DSH2512	Mammoth_nl_GEL	0.74	0.09	39,380	895–1007
DSH2508	Mammoth_nl_ULTR	0.81	0.09	38,700	849–949
DSH2538	Mammoth_Lipids	8.34	0.22	19,960	207–212

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Archaeological Samples: Buried Bones

^{14}C ages for 4 buried bone samples are shown in Table 3, together with the ^{14}C ages of the cremated bones and charcoals. For the 4 buried samples (T7, T94, T8, T24), the 3 fractions were extracted and measured. As seen in the table, the results from the T7 and T94 tombs highlight a case in which there is no difference among the 3 fractions (AAA-GEL-ULTR) within 1σ or 2σ. Conversely, a very big difference between the AAA fraction and the others (GEL and ULTR) is evident in the results of the T8 and T24 tombs. Therefore, when the sample suffers more contamination, the ^{14}C ages derived from the GEL and ULTR fractions are much more reliable than that of AAA, which often fluctuates, as observed in the previous results.

Table 3 ^{14}C ages and pMC values of all samples from Santa Maria Capua Vetere (Campania, Italy), together with the calibrated ages obtained using OxCal v 4.1.3 program (Bronk Ramsey 2009) and the IntCal09 (Reimer et al. 2009) calibration curve. Note that sample names with * represent the weighted average between the GEL and ULTR fractions extracted on buried bone.

CIRCE lab code	Sample name	Material	pMC (error)	Age (BP) ±1σ	Calibrated age (BC, 1σ)	Calibrated age (BC, 2σ)
DSH2440	T81	Cremated bone	70.42 (0.47)	2820 ± 50	1043–906	1125–837
DSH2441	T35	Cremated bone	72.58 (0.27)	2570 ± 30	801–675	810–566
DSH2445	T1	Cremated bone	72.59 (0.41)	2570 ± 50	809–595	827–541
DSH2446	T14	Cremated bone	70.51 (0.34)	2810 ± 40	1006–913	1056–841
DSH2199	T56	Cremated bone	72.39 (0.55)	2600 ± 60	833–593	900–538
DSH2521	T56_charcoal	Charcoal	72.40 (0.29)	2610 ± 30	811–777	836–601
DSH2311	T65_charcoal	Charcoal	70.94 (0.30)	2760 ± 30	928–842	997–828
DSH2200	T74	Charcoal	71.29 (0.55)	2720 ± 60	916–811	1006–796
DSH2359	T7_AAA	Buried bone	70.44 (0.18)	2800 ± 20	979–917	1006–904
DSH2565	T7_GEL	Buried bone	71.94 (0.52)	2650 ± 60	896–787	974–592
DSH2564	T7_ULTR	Buried bone	70.49 (0.43)	2800 ± 50	1016–896	1112–832
	*T7			2740 ± 40	914–836	976–811
DSH2364	T94_AAA	Buried bone	70.98 (0.18)	2750 ± 20	915–845	970–832
DSH2377	T94_GEL	Buried bone	71.94 (0.29)	2650 ± 40	841–792	900–782
DSH2378	T94_ULTR	Buried bone	71.41 (0.22)	2700 ± 20	893–814	897–811
	*T94			2690 ± 20	889–809	896–806
DSH2357	T8_AAA	Buried bone	61.40 (0.29)	3920 ± 40	2472–2346	2562–2290
DSH2369	T8_GEL	Buried bone	70.13 (0.20)	2850 ± 20	1048–976	1112–930
DSH2368	T8_ULTR	Buried bone	71.11 (0.39)	2740 ± 40	917–834	976–810
	*T8			2830 ± 20	1008–935	1041–918
DSH2563	T24_AAA	Buried bone	73.16 (0.28)	2510 ± 30	769–553	789–538
DSH2561	T24_ULTR	Buried bone	69.84 (0.27)	2880 ± 30	1115–1014	1193–941

Cremated Bones

Figure 2 presents a multiplot of ^{14}C ages on the cremated samples shown together with some charcoals found in the same site and the 4 uncremated bones (T7, T94, T24, T8), here shown as a weighted average between the GEL and ULTR fractions (with the exception of T24 where only the GEL fraction is present). In particular, by comparing the ^{14}C ages of bone and charcoal samples belonging to the same tomb (Tomb 56) the accuracy of the applied procedure can be evaluated. As the figure shows, the results are in agreement.

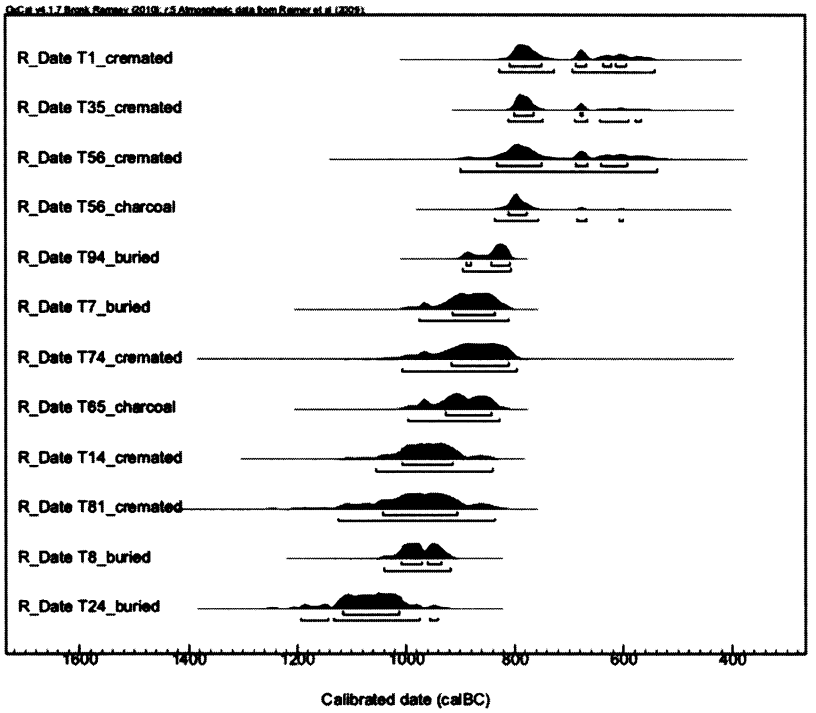

Figure 2 Multiplot of calibrated results on buried and cremated bone samples, together with some charcoals found in Santa Maria Capua Vetere. Calibration was obtained using OxCal v 4.1.3 (Bronk Ramsey 2009) and the IntCal09 (Reimer et al. 2009) calibration curve.

CONCLUSIONS

This work on the characterization of the different chemical procedures applied on several types of bone samples brought us to several conclusions. The first is that the lipid extraction induces an enrichment in ^{14}C (probably due to fractionation) that, however, does not influence the 3 AAA-GEL-ULTR fractions after the lipid extraction. Therefore, for short-lived animals, it is not possible to use the lipid fraction to estimate eventual offsets induced by the applied methods comparing the measurements of lipids with those of collagen obtained by the other 3 fractions. We are working to understand the cause of this fractionation also by using IRMS analysis. Moreover, it seems that there is no difference between the GEL and ULTR fractions for relatively old samples, nor for the very old but well-preserved VIRI E sample (Hüls et al. 2009). In Figure 3, we calculated R as the ratio of the values of pMC between the GEL and ULTR fractions for the bone samples where both fractions are present. The average (1.00) and the standard error (0.01) show that all values are, on average, in agreement with a value of 1, within 2σ.

Concerning the AAA fraction, when the sample suffers more contamination the results derived from the GEL and ULTR fractions are much more reliable than those for AAA, which frequently fluctuates as seen in the study cases. Finally, treatment on the cremated bones, applied for the first time at CIRCE, allowed us to verify the accuracy of the procedure. Also, comparison was made with charcoals coming from coeval trees used as firewood (^{14}C dates should thus not suffer from an old-wood effect) and uncremated bones found in the same archaeological context, as cited in Naysmith et al. (2007).

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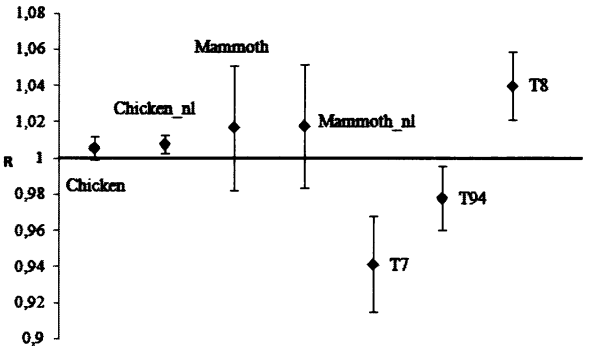

Figure 3 *R* as the ratio of the values of pMC between the GEL and ULTR fractions for the bone samples where both fractions are present. All values are in agreement with a value of 1, within 2σ.

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