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EVALUATION OF POSSIBLE CONTAMINATION SOURCES IN THE ^{14}C ANALYSIS OF BONE SAMPLES BY FTIR SPECTROSCOPY

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ABSTRACT. In the sample preparation laboratory of CEDAD (CEnter for DAting and Diagnostics) of the University of Lecce, a protocol for the quality control of bone samples based on infrared spectroscopy has been set up. The protocol has been recently developed as a check-in test with the aim to identify the presence of collagen in the samples, assess its preservation status, and determine whether the submitted bone samples are suitable for accelerator mass spectrometry (AMS) radiocarbon measurements or not. We discuss in this paper the use of infrared-based techniques to identify the presence of "contaminants" such as restoration and consolidation materials, humic acids, and soil carbonates, which, if not removed by the sample processing chemistry, can be sources of exogenous carbon and can thus influence the accuracy of the ^{14}C determinations.

Bone samples recovered in well-defined and previously dated archaeological contexts were intentionally contaminated, submitted to the standard method for collagen extraction and purification, and then characterized by means of Fourier transform infrared (FTIR) spectroscopy analyses performed in attenuated total reflection (ATR) mode before being combusted, converted to graphite, and measured by AMS. The study shows that the ATR-FTIR technique is an extremely powerful method for the identification of both the collagen and its contaminants and can supply important information during the selection and processing of samples to be submitted for ^{14}C dating.

INTRODUCTION

Bones are one of the materials most commonly found in archaeological contexts and are an ideal material for isotopic study, used for both radiocarbon dating and stable isotope analysis (Collins et al. 2002). Bone is a composite material formed by an organic fraction (20–30 wt%), mainly collagen; an inorganic phase (60–70 wt%); and water (~10 wt%) (Reiche et al. 2002). The bone mineral phase consists of non-stoichiometric, poorly crystalline hydroxyl/apatite in which the basic apatite structure, $Ca_{10}(PO_4)_6(OH)_2$, is modified by substitution of phosphate with the carbonate groups. Several investigations dedicated to the study of the structure of both the mineral and the organic phase, as well as to the diagenesis of this kind of sample, suggested the introduction of different parameters to be used as indicators of the preservation status of bones, such as the C/N ratio, $\delta^{13}C$ and $\delta^{15}N$ ratios, and amino acid composition as summarized by Hedges (2002) and van Klinken (1999). Furthermore, the relationship between the different indicators and the burial and environmental conditions have been extensively studied as well (Hedges and van Klinken 1992; Shahack-Gross et al. 1997; Nielsen-Marsh and Hedges 2000; Berna et al. 2004; Trueman et al. 2004).

Quality indicators of the bone preservation status based on the evaluation of characteristic features of the infrared spectra have been also suggested, such as the infrared splitting factor (IRSF) (Weiner and Bar-Yosef 1990) as an index of the crystallinity of the bone mineral phase, the C/P ratio as related to the apatite carbonate content (Wright and Schwarcz 1996), and the evaluation of the characteristic infrared absorption bands of the bone organic phase (Stiner et al. 1995, 2001; Yizhaq et al. 2005).

At CEDAD (CEnter for DAting and Diagnostics), about 40% of the specimens submitted every year for ^{14}C dating are osteological samples, and systematic quality control protocol has been recently set

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up based on the Fourier transform infrared spectroscopy (FTIR) analysis in attenuated total reflection (ATR) mode of the bone organic phase (Gianfrate et al. 2007). We showed that the advantages of ATR-FTIR methods—namely the high absolute sensitivity of the technique in the IR fingerprint region where the characteristic IR absorption bands of collagen are present—can result in the development of an extremely powerful tool for the assessment of the preservation status of the collagen extracted from bone samples submitted for ^{14}C AMS dating. Furthermore, another fundamental advantage of ATR-FTIR methods is that they are sensitive not only to the presence of collagen but also to that of several carbon compounds, which, being sources of exogenous carbon, can act as contaminants in the ^{14}C analysis if not removed by proper sample processing procedures. We thus investigated the possibility of using ATR-FTIR methods as a check for the presence of contaminants in the collagen extracted from bones and to assess the effectiveness of the standard sample processing chemistry usually applied during the processing of bone samples.

For this purpose, a bone sample taken from a well-defined archaeological context (a Neolithic necropolis in southern Italy) previously extensively ^{14}C dated was split into different fragments that were then intentionally contaminated with consolidation or restoration substances, calcite and humic acids, before being processed by standard methods for the extraction of collagen. The extracted collagen was then characterized by ATR-FTIR before being submitted for ^{14}C AMS dating.

MATERIALS AND METHODS

At CEDAD, osteological samples submitted for ^{14}C AMS dating are first mechanically cleaned, crushed to powder, demineralized by 1% HCl at room temperature; gelatinized in hot (85 °C), acidified (HCl) water at pH = 3, and finally filtered using a 0.45-μm pore silver filter for the extraction of collagen (Longin 1971; D'Elia et al. 2004; Quarta et al. 2004). The purified collagen gelatin is then combusted to CO_2 in sealed quartz tubes, reduced to graphite at 600 °C by using H_2 as the reducing medium and iron powder as the catalyst, and finally pressed in the aluminium target holders of the accelerator mass spectrometers for the determinations of the carbon isotopic ratios. Thus, the filtered gelatin is the fraction of the sample retaining the original isotopic information that is finally submitted for isotopic analysis, and it represents the ideal material when any evaluation of the presence of contaminants and/or of the effectiveness of the sample preparation should be carried out. Nevertheless, the amount of dried gelatin is usually limited to a few milligrams (mg) and thus any control protocol of its quality should be based on an analytical technique requiring much smaller amount of material, in the microgram (μg) range. In this respect, the ATR-FTIR method is ideal since it combines the advantages of the FTIR-based methods (i.e. high sensitivity to collagen functional groups) and the reduction of the required sample mass to a few μg (Derrick et al. 1999). The only disadvantages are those related to a restriction of the investigated spectral region that, at least in our experimental setup, is limited by the IR absorptions characteristic of the ATR diamond crystal and of the ZnSe optics in the region between 4000 and 600 cm^{-1}. All the ATR-FTIR measurements were carried out with a Spectrum-One (PerkinElmer) spectrometer equipped with an ATR accessory. The internal reflection element was a 3-reflection diamond microprism. For each spectrum, 16 interferograms with a resolution of 4 cm^{-1} were collected and averaged.

IR ANALYSIS RESULTS

Figure 1 shows the characteristic FTIR spectrum collected as a reference, in attenuated total reflection (ATR) mode, for the collagen extracted from modern animal bones. In fact, no differences are to be expected in the IR spectra of collagen extracted from animal and human bones. The characteristic "fingerprint" absorption bands of collagen are clearly visible and are indicated in the figure:

Amide I, Amide II, Amino acid proline, and Amide III (Warren et al. 1969; Susi et al. 1971; Doyle et al. 1975; Steiner et al. 1995; Friess and Lee 1996). A bone sample (LTL126A) collected from a Neolithic burial in Carpignano Salentino (Lecce, Italy), and already the subject of an extensive ^{14}C dating campaign, was used for the intentional contamination experiment (Quarta et al. 2006). Different portions of ~2.5 g each were obtained from the sample: the first one was used as a (blank) reference while the others were intentionally contaminated by using a waterproof pen ink; calcite; humic acids; and Paraloid 72™, an acrylic resin (chemically an ethyl methacrylate copolymer) widely used by archaeologists and conservators as a consolidant of bone samples (Johnson 1994).

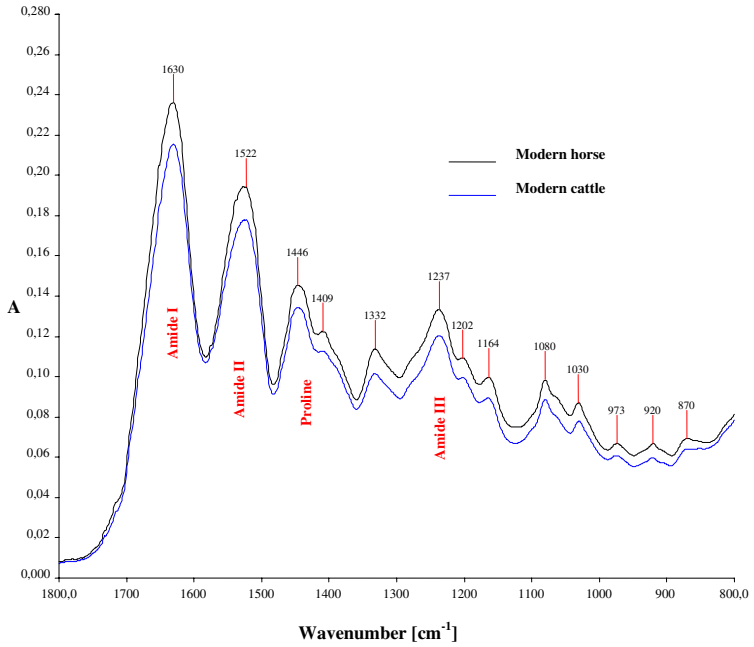

Figure 1 FTIR spectra of collagen extracted from modern bones by standard sample treatment with the identification of the characteristic IR bands of collagen.

The first step of the experiment was to quantitatively estimate the collagen preservation status in the original uncontaminated (blank) reference sample and to measure its carbon isotopic composition by AMS. The crystallinity of the bone mineral phase was estimated through the infrared splitting factor (IRSF) as defined by Weiner and Bar-Yosef (1990) by performing IR transmission analyses on KBr pellets on the bone powder with a Thermo-Nicolet Nexus FTIR spectrometer (Figure 2). The measured IRSF value of 3.1 can be considered an indication of good preservation of the mineral phase (Weiner and Bar-Yosef 1990). Figure 3a shows the ATR-FTIR spectrum obtained on purified gelatin extracted from the sample. The good preservation status of the collagen is testified by comparison with the ATR-FTIR spectrum measured for the modern samples (Figure 1): intense, sharp collagen-related peaks are clearly distinguishable. The sample was then AMS ^{14}C dated to 5665 ± 50 BP.

For the intentional contamination experiment, Paraloid 72™, a commercial waterproof marker (Lumocolor® permanent marker by Staedtler®, Germany), soil calcite obtained from local geological sediments (^{14}C age >50 kyr as measured by ^{14}C AMS), and humic acids extracted from an "archaeological" charcoal (^{14}C content = 24.5 ± 0.5 pMC) were used. Each portion of the sample was exposed to only 1 contaminant.

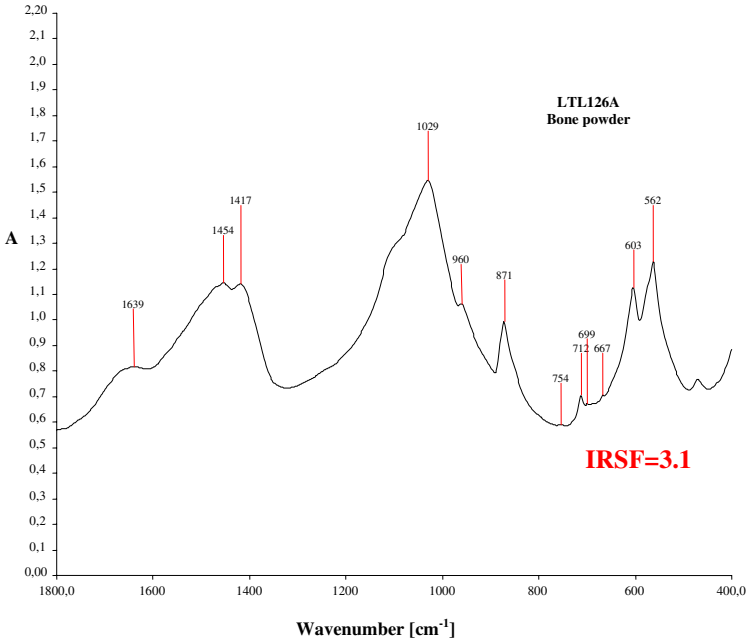

Figure 2 Transmission mode FTIR spectrum of LTL126A bone powder

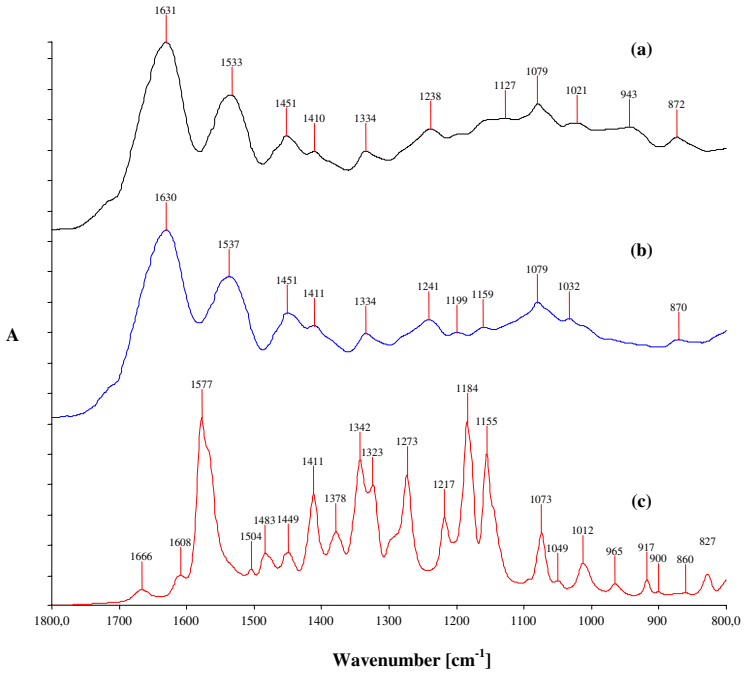

Figure 3 ATR-FTIR spectra of the collagen gelatin extracted from the (a) uncontaminated sample and (b) the sample contaminated by the marker pen. The FTIR spectrum of the contaminant is also shown (c).

Contamination with the waterproof pen was performed by completely covering the samples with the ink at room temperature for 8 hr. For Paraloid 72™, the samples were completely immersed in the contaminant for 8 hr at room temperature. For the contamination with calcite and humic acids, the samples were immersed in a water solution of the contaminant for 48 hr at 60 °C, 100 °C, and 200 °C.

The contamination protocol was designed in order to reproduce, in accelerated time, the worst possible environmental conditions for each kind of contaminant. For this reason, for Paraloid 72™ and the pen, the contamination was carried out at room temperature for 1 day, while for the humic acids and calcite the experiment was designed in order to evaluate also the influence of the temperature on contaminant uptake and in the loss and degradation of collagen (Roberts et al. 2002; Lozano et al. 2003). The duration of the contamination with humic acids was set to 48 hr since it has been found by others that the uptake of humic substances is a fast process taking place in a few hours (van Klinken and Hedges 1995). Ambient humidity and pH play an important role in hydration and denaturation of collagen, as humid and highly alkaline burial environments alter the organic component of bones (Fernández-Jalvo et al. 2002), so the contaminations with calcite and humics were made in alkaline water solutions.

Once contaminated, bone fragments were dried and submitted to the standard treatment, the aforementioned procedure for the extraction of the bone collagen gelatin, which was then characterized by ATR-FTIR. The IR spectra obtained for the gelatin extracted from the samples are shown in Figures 3, 4, 5, and 6, together with the IR spectrum of the reference (blank) sample. The IR spectrum of the corresponding contaminant is also shown for comparison in each figure.

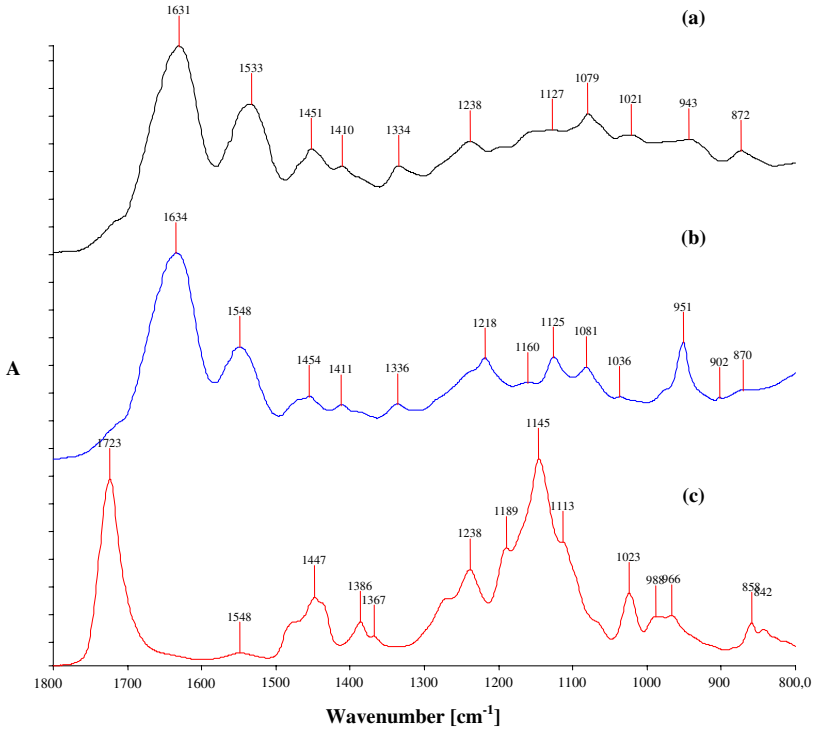

Figure 4 ATR-FTIR spectra of the collagen gelatin extracted from an (a) uncontaminated sample and (b) a sample contaminated by Paraloid. The FTIR spectrum of the contaminant is also shown (c).

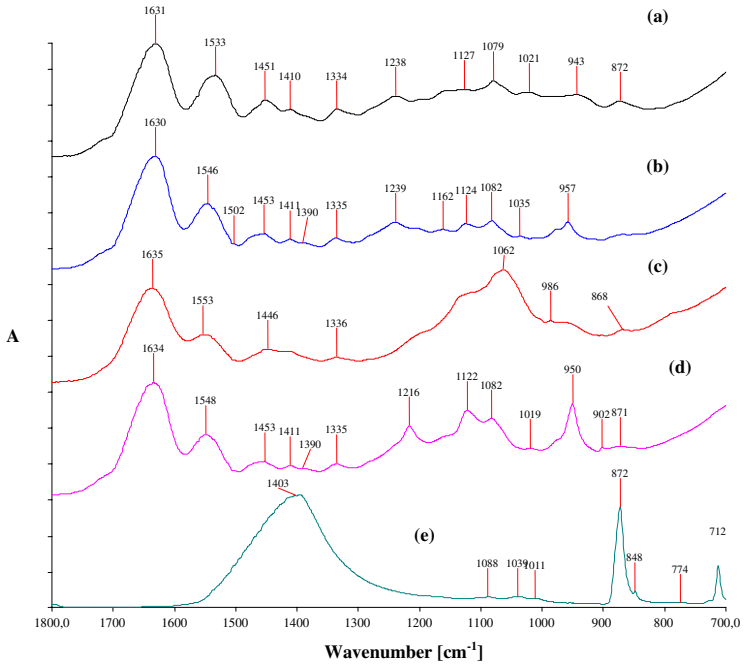

Figure 5 ATR-FTIR spectra of the collagen gelatin extracted from an (a) uncontaminated sample and a sample contaminated by calcite at different temperatures: 60 °C (b), 100 °C (c), and 200 °C (d). The FTIR spectrum of the contaminant is also shown (e).

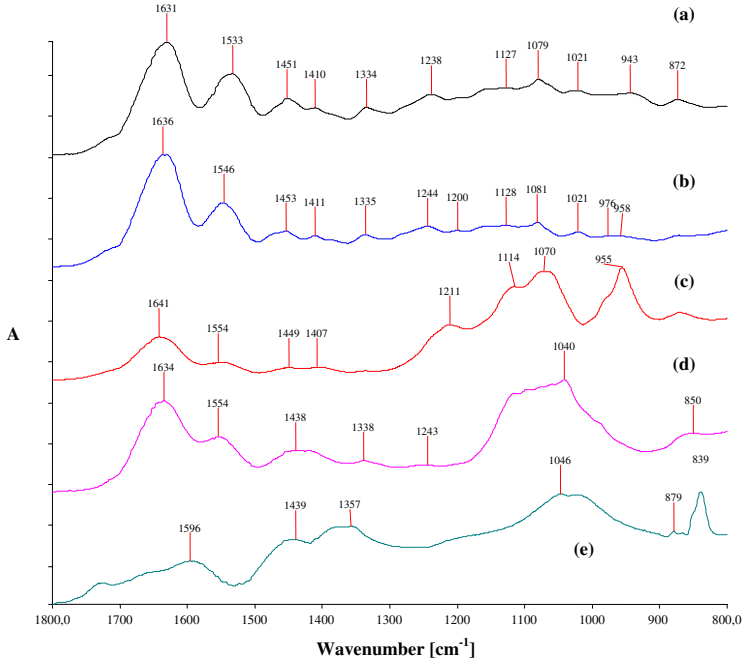

Figure 6 ATR-FTIR spectra of the collagen gelatin extracted from an (a) uncontaminated sample and a sample contaminated by humic acids at different temperatures: 60 °C (b), 100 °C (c), and 200 °C (d). The FTIR spectrum of the contaminant is also shown (e).

The ATR-FTIR spectrum (Figure 3b) of the collagen extracted from the sample contaminated with the pen does not show any of the IR bands characteristic of the contaminant itself (Figure 3c), while all the fingerprint bands characteristic of collagen are sharp and intense (Figure 3a). In this case, the IR analysis shows that the standard sample processing is effective for contamination removal. This is also the case of the sample contaminated with Paraloid (Figure 4b), although in the spectrum of the gelatin extracted from the contaminated Sample 4, intense and sharp peaks appear at 1218, 1160, 951, and 902 cm^{-1}, the origins of which have not been determined. Although an effect of the contaminant in the appearance of these bands cannot be excluded *a priori*, the absence of corresponding bands in the spectrum of the contaminant, and the fact that these peaks were also found in previous investigations (Gianfrate et al. 2007) for uncontaminated bones, seems to relate their presence to the fragmentation or degradation of the collagen structure, which happens during the sample preparation rather than at the introduction of exogenous compounds.

In the spectrum of calcite (Figure 5e), the characteristic peaks at 872 and 712 cm^{-1} are clearly distinguishable, while that at 1415 cm^{-1} (also characteristic of calcite) is covered by the large band probably originating from soil substances. Also, in this case the IR analysis indicates an effective removal of the contamination: the peak at 712 cm^{-1} disappears in the spectrum of the purified gelatin and the ratio of the bands at 1415 and 872 cm^{-1} with the proline peak is the same as the reference (blank) sample. With increasing temperatures, the spectrum of the contaminated collagen sample—which is similar to the blank at 60 °C and 100 °C—shows an increasingly large band at 1062 cm^{-1}, in which some shoulders can be identified as corresponding to the 4 bands of unknown origin already found in the spectrum of the sample contaminated with Paraloid. The bands become clearly visible in the spectrum of the sample treated at 200 °C.

The IR spectra of the collagen gelatin extracted from the bone samples contaminated with humic acids are shown in Figure 6, together with the IR spectrum of the humic acids used as contaminants with the characteristic large band at 1046 cm^{-1}. In this case, IR analysis of the collagen gelatin extracted from the contaminated samples shows that the removal of humics becomes less and less effective as the exposure temperature increases from 60 to 200 °C. In particular, while no signs of the humic acids band at 1046 cm^{-1} can be seen in the IR spectrum of the samples exposed at 60 °C, this becomes completely evident for the sample exposed at 200 °C, when degradation of collagen is also evident from the shape and height of collagen characteristic bands, which become less sharp and intense. This seems to point, as already observed by others (van Klinken and Hedges 1995), to incomplete removal of humic acids through the standard Longin method.

Furthermore, also in the case of humic contaminants, the 100 °C spectrum shows an increasingly large band at 1070 cm^{-1} corresponding, within the spectral resolution of the analysis, to the band at 1062 cm^{-1} seen in the case of calcite contamination. In this situation, some shoulders corresponding to unassigned peaks at 1218, 1160, 951, and 902 cm^{-1} are present and, while not related to any particular contaminant, seem to be associated, as mentioned before, to the degradation of the complex collagen macromolecule.

Radiocarbon Measurements

The ^{14}C content in all the samples (except the sample contaminated by calcite at 100 °C whose preparation failed) was measured by AMS at CEDAD, by following standard procedures for combustion, reduction to graphite, and measurement of the carbon isotopic ratios (Calcagnile et al. 2005).

The ^{14}C concentration expressed as conventional ^{14}C yr is given, for all the samples, in Figure 7 and summarized in Table 1, where also the $\delta^{13}C$ terms measured with the AMS system are listed.

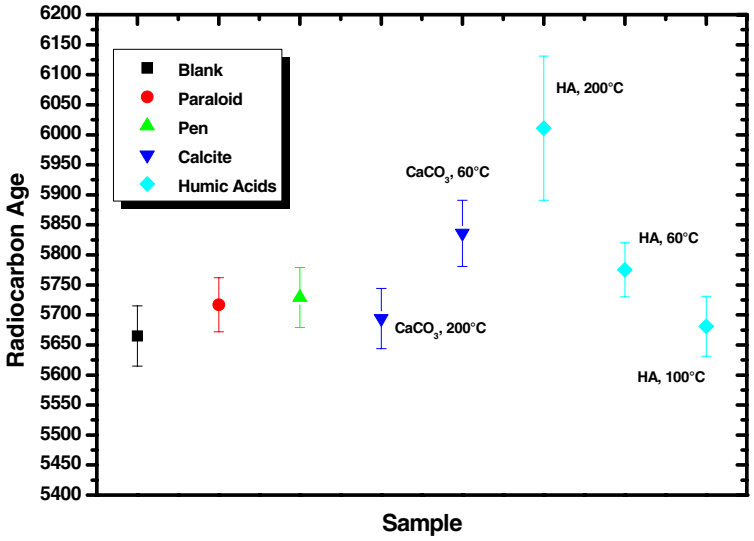

Figure 7 Uncalibrated ^{14}C dates (1-σ uncertainty) measured for the blank and the intentionally contaminated samples.

Table 1 Uncalibrated ^{14}C dates and $\delta^{13}C$ values (1-σ uncertainty) measured for the blank and the intentionally contaminated samples. The $\delta^{13}C$ term was measured by accelerator mass spectrometry.

Lab code	Contamination protocol	^{14}C age (yr BP)	$\delta^{13}C$ (‰)
LTL126A	(Blank) reference sample	5665 ± 50	–19.9 ± 0.3
LTL126A(a)	Paraloid	5717 ± 45	–17.2 ± 0.1
LTL126A(b)	Marker	5729 ± 50	–18.7 ± 0.1
LTL126A(c)	Calcite, 60 °C	5836 ± 55	–18.9 ± 0.1
LTL126A(e)	Calcite, 200 °C	5694 ± 50	–21.3 ± 0.1
LTL126A(f)	Humic acids, 60 °C	5775 ± 45	–19.1 ± 0.1
LTL126A(g)	Humic acids, 100 °C	5681 ± 50	–25.7 ± 0.5
LTL126A(h)	Humic acids, 200 °C	6011 ± 120	–25.7 ± 0.2

The ^{14}C ages of all the samples contaminated with Paraloid, pen, and calcite, are in agreement within 1 σ with the age of the (blank) reference sample, confirming, as already pointed out by the IR analyses, the absence of sources of exogenous carbon, and thus the effective removal of the contamination. Also significant is that samples where the presence of the 4 "unexplained" bands were observed do not show any anomalous ^{14}C age.

This is also the case for the samples contaminated with humics at 60 °C and 100 °C, while for the samples contaminated at 200 °C a more significant variation of the ^{14}C age seems to be present—although still within the 2-σ range when compared with the blank uncontaminated sample.

CONCLUSION

A systematic quality control procedure based on ATR-FTIR analysis has been set up at CEDAD, University of Lecce, for assessment of the presence and the preservation status of the collagen extracted from osteological samples submitted for ^{14}C AMS dating. The possibility of using the same experimental approach in order to identify the presence of sources of exogenous carbon was

explored by intentionally contaminating samples with different kind of "contaminants"; thus, the effects of Paraloid 72, waterproof pen, calcite, and humic acids were investigated. Our results show that the ATR-FTIR technique can supply fundamental information not only with respect to the preservation status of collagen at the molecular level but also to identifying the presence of sources of exogenous carbon not removed by sample processing chemistry. Future investigations will be addressed towards defining the detection limits of the technique (i.e. the smallest amount of a given contaminants whose presence can be identified) and to a better understanding of the mechanisms of collagen degradation and denaturation.

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