CHARACTERIZATION AND DATING OF ARCHAEOLOGICAL EXCAVATED HUMAN BONE FROM JORDAN BY HIGH-RESOLUTION ³¹P AND ¹⁴C NMR AND FOURIER TRANSFORMATION INFRARED

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ABSTRACT. *Solid-State Nuclear Magnetic Resonance (SS-NMR) and Attenuated Total Reflection Fourier Transformation Infrared (ATR-FTIR) spectroscopy have excellent measurement performance for both organic and inorganic parts of bone or dental dentin. Solid-State Magic-Angle Spinning Nuclear Magnetic Resonance (SS-MAS-NMR) spectroscopy is an effective and constructive method for classifying samples, whether they are new or old. The objectives of this study include finding a new method for dating bone by SS-MAS-NMR and ATR-FTIR studies of old bone, supported by absolute dating of radioactive carbon isotopes. The specific objectives can be addressed by measuring the decomposition factor of the organic fraction in ancient bones and dentin in modern teeth, which are most similar to bones in terms of chemical composition, to arrive at a new time formula for the dating method. Eight old samples and one fresh tooth sample were taken for comparison. The method studied will be established as a new tool for characterizing ancient bone samples and detecting hydroxyl in bone minerals by SS-MAS-NMR.*

KEYWORDS. *Ancient bone; Jordan; hydroxyapatite; SS-MAS-NMR; ATR-FTIR; AMS dating; DIPSHIFT experiments; adenosine triphosphate (ATP); hydroxyproline.*

Figure 1. Bone samples from Tell al-Hosn and Yasiela sites, Jordan.

1. INTRODUCTION

A significant set of bone objects was discovered in Tell al-Hosn and Yasiela by the Yarmouk University team from 1992 to 2012, with the support of the Jordanian Ministry of Antiquities and Archaeology.

Under the direction of archaeologist Prof. Zeidoon Al-Moheisen, a significant number of bone objects were found in these sites, located in the northwestern part of Jordan. The goals of the investigations include several aspects of the novel method of dating ancient bone by SS-NMR and ATR-FTIR. The specific aims can be addressed by measuring the degradation factor of the organic part in the ancient bone to achieve a new time formula for the dating method.

SS-MAS-NMR is a useful analytical tool for studying archaeological artifacts. Equally, SS-NMR under

Magic-Angle Spinning (SS-MAS-NMR) can examine the inorganic and organic components of bone material (Klinowski 2004). Moreover, ^{13}C spectra deliver valuable information on the structure and dynamics of the collagen phase in bone or dentin teeth. Likewise, ^{31}P NMR can detect the inorganic apatite phase in bone. Both methods are quantitative (DiMasi & Gower 2014).

The analytical results of ^{31}P and ^{13}C NMR depend on time and will give new quantitative information about the inorganic and organic phases of these specimens (Ducheyne *et al.* 2017). This means that these parameters seem to be good for dating.

In this work, we will look at the MAS-NMR spectra of human bones that were buried between 2000 and about 1000 years BP. We will compare these spectra to the spectra of a modern human tooth and look for a possible link between the spectra of the teeth and the time they were buried. Modern teeth have been used for comparison with ancient bone since dental material is similar in structure to bone material. In the literature, papers detailing SS-MAS-NMR studies on teeth about their age are scarce.

New trends in dating methods for bone objects are identical and important to reveal the ancient aspects of important sites in Jordan. The SS-MAS-NMR technique is a new method for studying ancient bone; furthermore, we will use MAS-NMR not only for dating ancient bone, but we will also try to use it to study ancient bone diseases such as tumors. A collection of bone fragments has been gathered and will be ready for measuring using this novel method.

The bone samples are different and date back more than 2000 years, as concluded from a collection of coins and other artifacts found in the same bone layer. The age is consistent with that estimated by archaeological (typology) studies. Furthermore, the use of radioactive carbon isotope dating supports the work.

The method examined is to be established as a new tool to characterize ancient bone specimens to detect hydroxyl in bone minerals by SS-MAS-NMR and pioneer work (Cho *et al.* 2003; Wu *et al.* 2003).

There are a couple of examples in the literature on how SS-MAS-NMR can be used to study healthy bone specimens as well as bone implants (Cho *et al.* 2003; Reichert *et al.* 2004; Huster *et al.* 2002; Wu *et al.* 1994; Aue *et al.* 1984).

However, the application of the method to study ancient bones and the dating of those ancient samples is new. Ancient mummies have been investigated by MRI methods (Münnemann *et al.* 2007), but SS-NMR spectroscopy has not been applied so far. Also, bone analysis by CT (Computed Tomography) and X-ray is quite common (Münnemann *et al.* 2007). However, the advantages of the SS-NMR of bone are obvious; this technique can provide a quantitative assessment of the molecular structure of bone.

The hydroxyapatite compound $(Ca_{10}(PO_4)_6(OH)_2)$ is the microcrystalline arrangement of phosphorus and calcium formed by the body in a precise proportion that is chemically connected to the mineral component of human bones and other hard tissues (Habibah *et al.* 2018; Al-Sekhaneh 2016). It is a complex salt consisting of six molecules of calcium phosphate coupled with two molecules of calcium hydroxide, as well as minerals such as manganese, potassium, magnesium, copper, zinc, iron, and silicon (Zandi 2008).

The inorganic matrix of bones is responsible for their strength, permitting them to resist aggressive impact. The organic part of bone plays a significant role in the functionality of the bone matrix (Wu *et al.* 2003). The incorporation of collagen permanently alters the properties of the matrix. Collagen induces a lipid condensation effect in bone structure, giving ductility or plasticity to bone (Umar *et al.* 2013).

2. MATERIAL AND EXPERIMENTS

Specimens: eight contemporary human bone samples were gathered from Tell al-Hosn and Yasiela sites excavated by the Yarmouk University team from 1992 to 2012, then purified, disinfected, and kept at a temperature of 4°C until they were utilized for SS-NMR analysis.

NMR Experiments

The NMR experiments were carried out on DIPSHIFT (Dipolar Chemical Shift Correlation) experiments, and C–H dipolar couplings can be determined to characterize the molecular mobility of these bonds as order parameters.

Cross-polarized (CP) and directly polarized ^{31}P SS-MAS-NMR spectra for the ancient bone samples, mixed with adenosine triphosphate (ATP), can determine the hydroxyapatite (HAP) amount in the samples.

The solid-state NMR spectrometers at 600 and 750 MHz ^{1}H resonance frequencies were used to investigate a bone specimen with samples at 7 kHz in a 4 mm
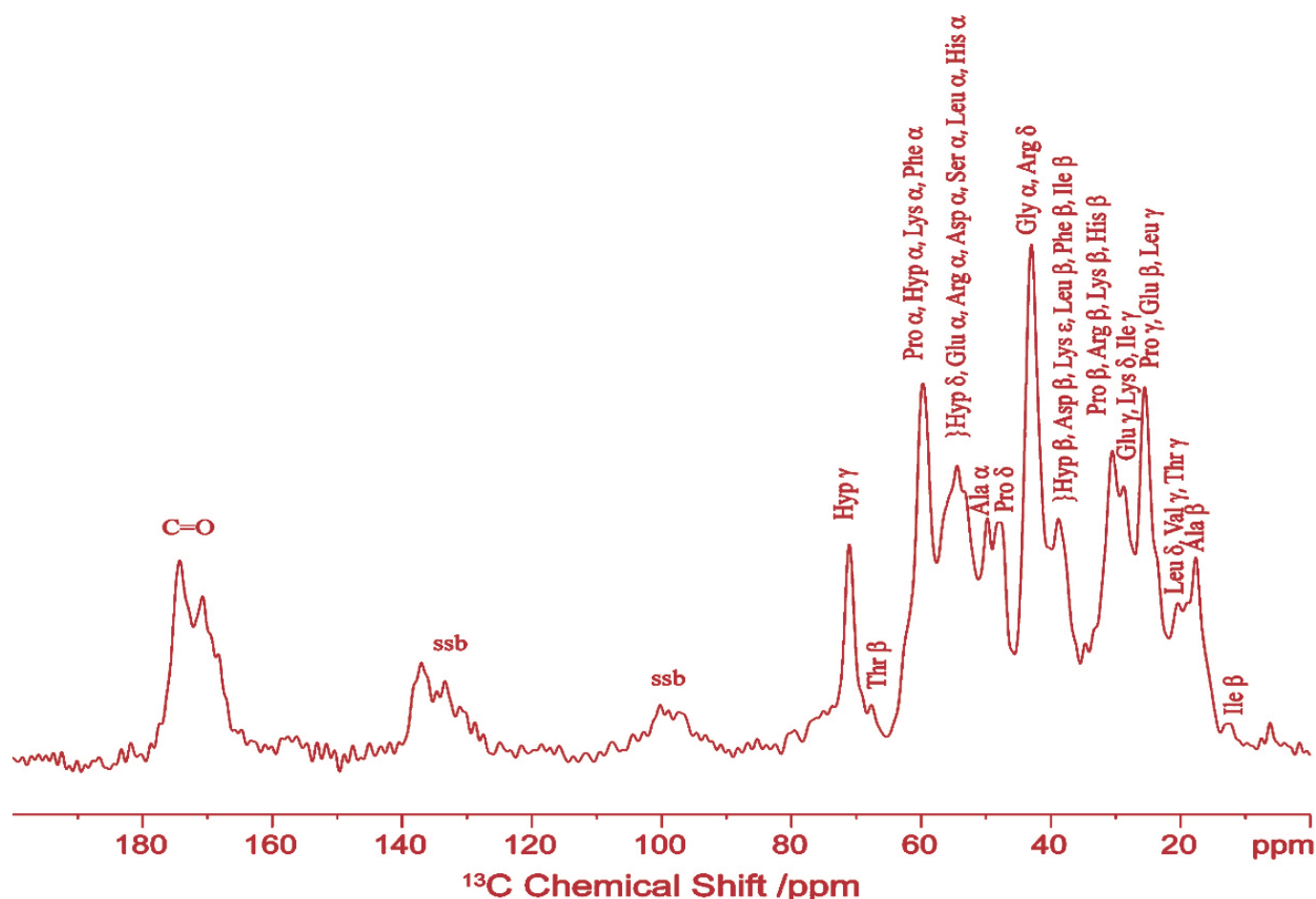
Figure 2. Typical ^{13}C CP SS-MAS-NMR spectrum of an ancient bone sample. MAS frequency: 7,000 Hz, temperature: 303 K.

zirconia (MAS) rotor utilizing single pulse stimulation with a pulse duration of 1 microsecond, equivalent to a flip angle significantly less than 90 degrees. The acquisition consisted of 500 scans with a 2-second recycle delay. The proton chemical shift has been discussed in relation to the tetramethylsilane (TMS) proton resonance.

ATR-FTIR Experiments

Eight human bone samples were gathered from Tell al-Hosn and Yasiela sites in Jordan, then purified, disinfected, and kept at a temperature of 4°C until they were utilized for ATR-FTIR analysis.

Mid-infrared spectra were taken of both the modern teeth and ancient bone samples using NMR and an ATR-FTIR spectrometer equipped with a diamond-attenuated total reflectance (ATR) attachment (4,000–400 cm^{-1}).

The light was transmitted through the samples while the spectra were recorded so that any changes in light intensity could be observed. The samples data were collected and analyzed, and ATR-FTIR spectra were generated with the use of specialized software. The ATR-FTIR band intensities and peak locations in the spectra of the ancient bone and modern tooth samples were found to be distinct.

Changes in ATR-FTIR bands were used to describe the tooth samples' crystallinity and chemical composition, and this information was subsequently given as an estimation of the samples' ages. Crystallization levels were higher in the ancient bone samples compared to the modern ones, suggesting that the ancient samples were older and more developed.

The findings of this work may be used as a benchmark for future archaeometry, and they pave the way for promising new areas of research that combine ATR-FTIR spectroscopy with other spectroscopic methods to further our understanding of ancient artifacts. This study provides conclusive evidence that ATR-FTIR spectroscopy may be used to accurately date ancient teeth discovered at archaeological sites.
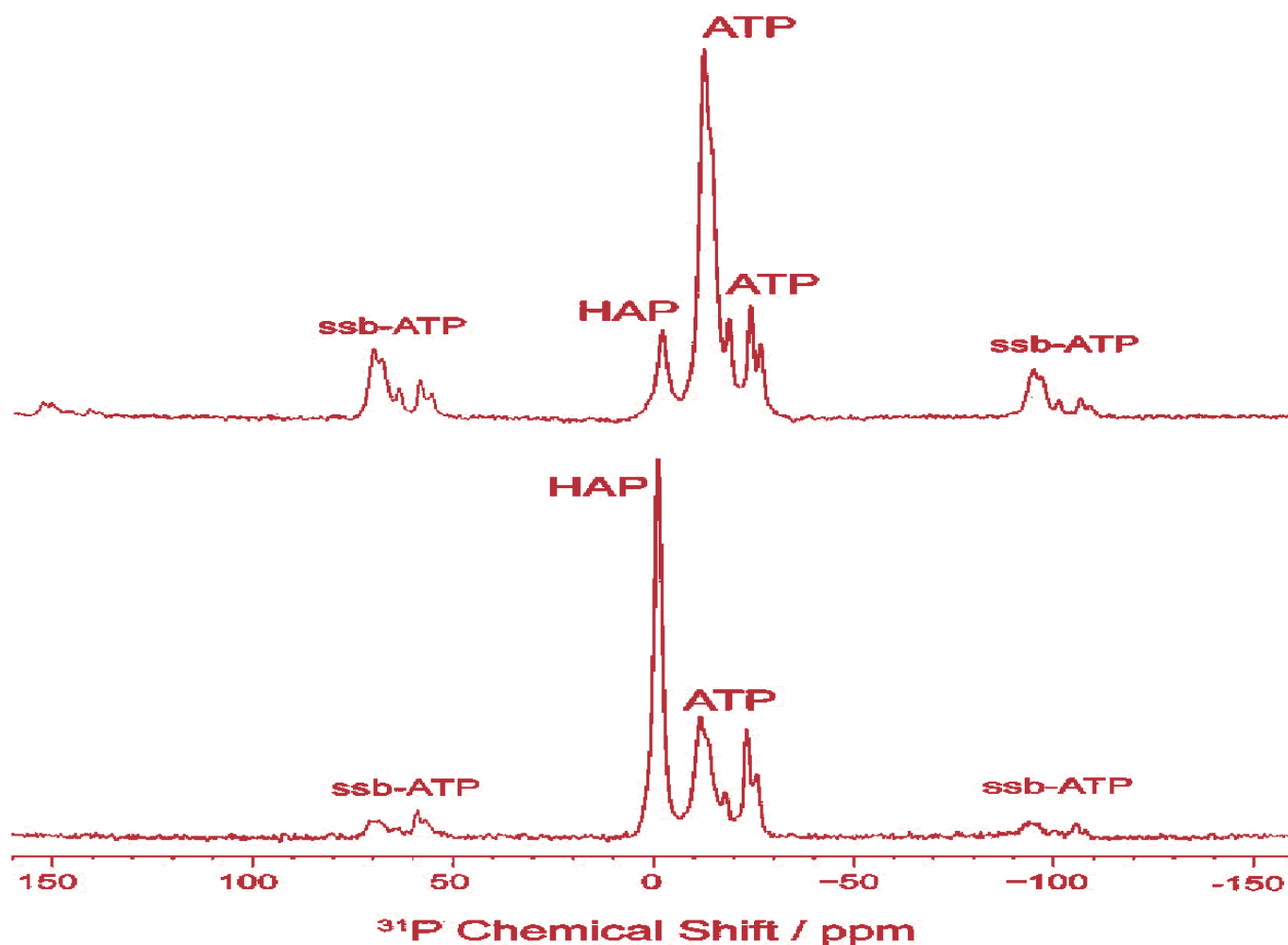
Figure 3. Example of a cross-polarized (above) and directly polarized (below) ^{31}P MAS spectra—here for sample 2a mixed with ATP. MAS frequency: 25,000 Hz, temperature: 303 K.

3. RESULTS AND DISCUSSION

We would like to determine the absolute content of the SS-NMR signals of the organic and inorganic molecules in bone. The organic components are degraded faster in bone than the inorganic components. So, by measuring the amount of ^{13}C and ^{31}P NMR signals at the same time, it is possible to figure out how old the bone is (Aue *et al.* 1984). The idea is that the organic component of the buried bone decreases with age; however, the inorganic component is expected to remain constant in the samples. We will use ATP and lysine as internal standards to determine absolute amounts of collagen and apatite, respectively (Huster *et al.* 2002; Schulz *et al.* 2007).

In preparation for this work, we have already carried out test measurements on ancient bone samples from the excavation site in Jordan. Figures 2 and 3 show typical ^{13}C MAS-NMR and ^{31}P MAS-NMR spectra of one

bone specimen, respectively. Both collagen and hydroxyapatite can be identified in these samples. We also added ATP to the bone sample as an internal standard for the quantification of the apatite content (Schulz *et al.* 2007).

These worldwide pilot studies show the feasibility of the approach, but the correlation between the age of the samples and the NMR spectra remains to be more established.

Solid-State NMR Investigations of Ancient Bone from Jordan

The Study used ^{13}C and ^{31}P MAS-NMR to investigate eight different samples of ancient bone. To this end, pieces of the ancient bones were grained and transferred into MAS rotors.

The cross-polarized ^{13}C SS-MAS-NMR spectra (Figure 2) exhibit the typical spectra of collagen as seen in
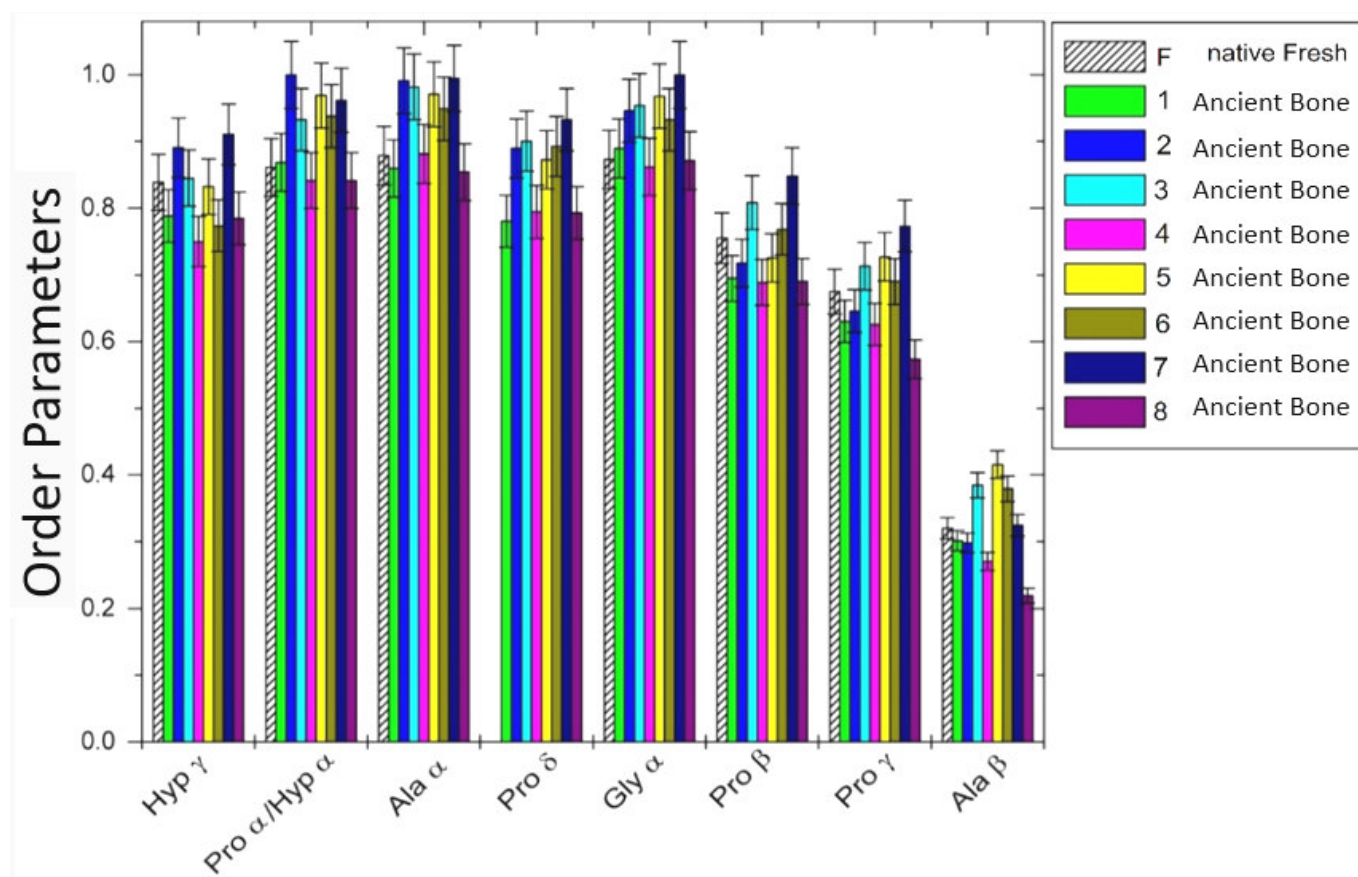
Figure 4. Comparison of the measured (C–H) order parameters or molecular mobility
of the C–H bonds in different ancient bone samples (F, 1, 2, 3, 4, 5, 6, 7, and 8).

fresh bone before. The hydroxyproline C gamma peak at 71 ppm is a unique fingerprint for collagen. There are no significant differences in the NMR spectra of the eight investigated samples.

In DIPSHIFT experiments, the C–H dipolar coupling was measured to find the ^{13}C–^{1}H order parameters. This was done to find out how the molecules in the samples move. A fully rigid C–H bond would exhibit an order parameter of 1, and an order parameter of 0 would mean a fully isotropic motion of these bonds. The order parameters (Figure 2) of the different resolved molecular groups are quite high, as is typical for collagen in bone. There is a slight tendency towards somewhat increased order parameters in the ancient samples compared to fresh bone due to the lower water content of the ancient samples. Between the different investigated samples, there is some variability but no clear trend for a specific sample.

For the ^{31}P MAS-NMR measurements, the samples were mixed with known amounts of crystalline ATP as an internal standard to obtain quantitative information about the content of inorganic matter in the samples. The spectra (Figure 3) show the typical hydroxyl apa-

tite (HAP) peak, as it can be cross-polarized in contrast to other remnants, together with the signals of ATP.

The analysis of the HAP amount exhibits huge variability between the samples and a value that is lower than for the fresh bone. This is somewhat unexpected: the ancient bones have a significantly reduced water fraction compared to fresh bone, and this should yield a higher relative HAP amount than fresh bone. However, the origin of the fresh bone is not human but from rat tibia, and therefore it may have a different density.

In Figure 4, a comparison of the measured C–H order parameters of the different samples and fresh bone of hydroxyproline (Hyp γ), proline (Pro α), hydroxyproline (Hyp α), alanine (Ala α), proline (Pro δ), glycine (Gly α), proline (Pro β), proline (Pro γ), and alanine (Ala β) is shown. It describes the secondary structure of proteins, specifically referring to the arrangement of the protein backbone.[1]

This trend is directly linked to the age of the sample, providing significant outcomes through the application

[1] The terms α, β, γ, and δ are often used to describe the secondary structure of proteins, specifically referring to the arrangement of the protein backbone.
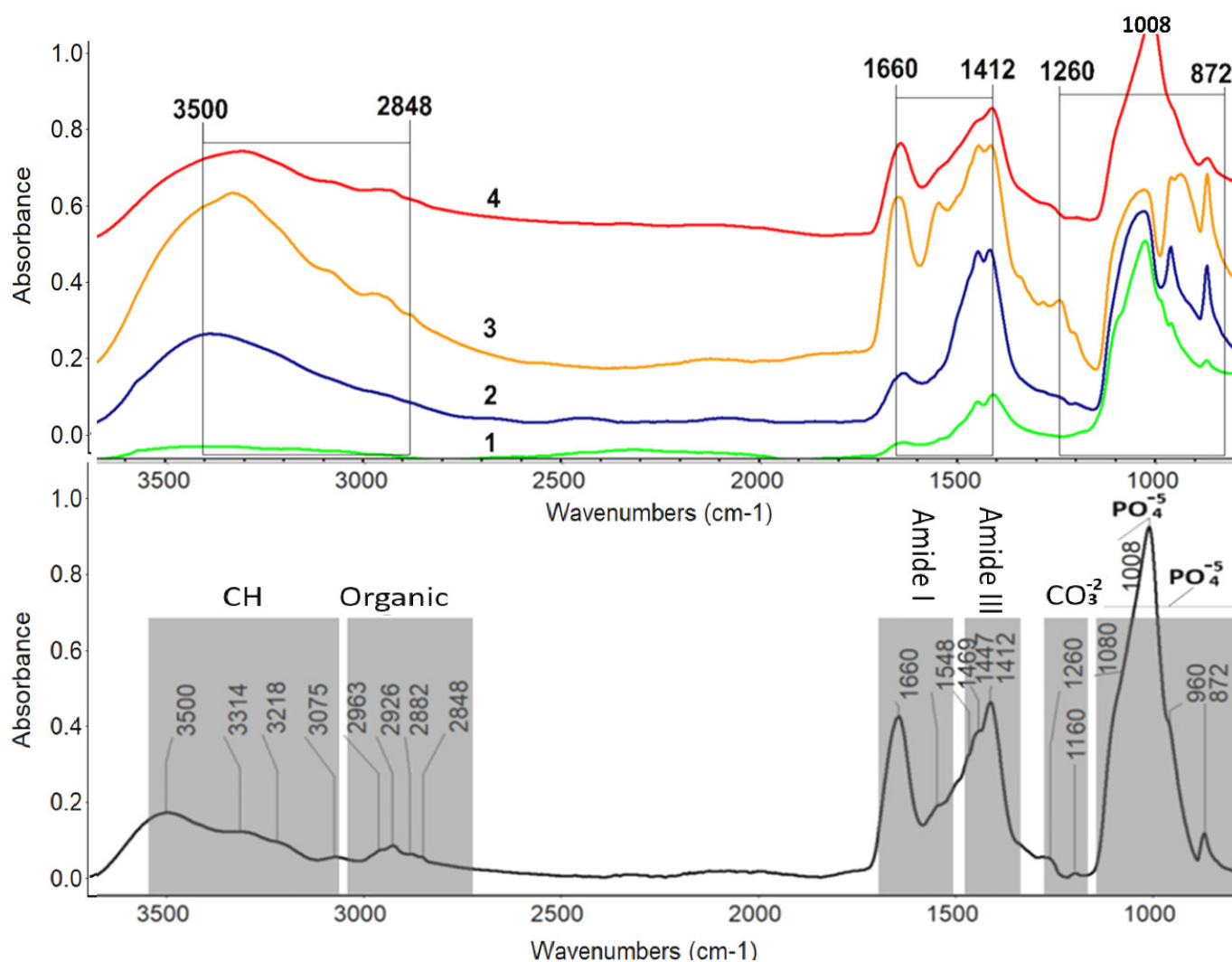
Figure 5. ATR-FTIR spectrum of bone, recorded and analyzed by identifying the characteristic peaks of organic and inorganic components of the ancient bone.

of radiocarbon dating measurements on archaeological bone. It is noteworthy that the peak values of samples 1, 7, and 15 on the y-axis indicate an inverse relationship with the age and quantity of the samples.

Initially, a standard ATR-FTIR spectrum of bone was recorded and analyzed to identify the characteristic peaks (as shown in Figure 5). The organic component of the ancient bone was represented by a band peak at 2,963, 2,926, 2,882, and 2,848 cm⁻¹ on the right. Additional peaks were observed at 1,660, 1,548, 1,469, 1,447, 1,426, 1,412, and 1,260 cm⁻¹. The inorganic (mineral) portion of the spectrum contained sub-signals at specific wavenumbers, including 1,080, 1,008, 960, and 872 cm⁻¹.

Certain peaks in the ATR-FTIR spectrum, like the amide I and amide III bands, can be used to tell the difference between the organic parts of bone. The amide I band, which is located at 1,660, 1,548, and 1,429 cm⁻¹,

is primarily associated with carbonyl stretching. The amide III bands are characterized by two vibrational modes observed at 1,260 cm⁻¹, and a weak carboxyl group stretching vibration was noted at 1,160 cm⁻¹, which corresponds to stretching between carbon-nitrogen atoms and bending of a secondary amine. These bonds are of particular interest for studies on the composition of teeth. Additionally, bending of methylene groups (CH) and stretching between carbon and hydrogen atoms (C–H) are common vibrational modes found in several peptides and can be observed at 1,412, 1,427, 2,848, 2,882, 2,926, and 2,963 cm⁻¹ (Bryan *et al.* 2007).

Apatite, which exhibits distinct phosphate and carbonate signals, makes up a significant portion of the teeth's inorganic composition. The most prominent and well-defined band at 960 cm⁻¹ corresponds to the symmetrical stretching mode of the phosphate group v1
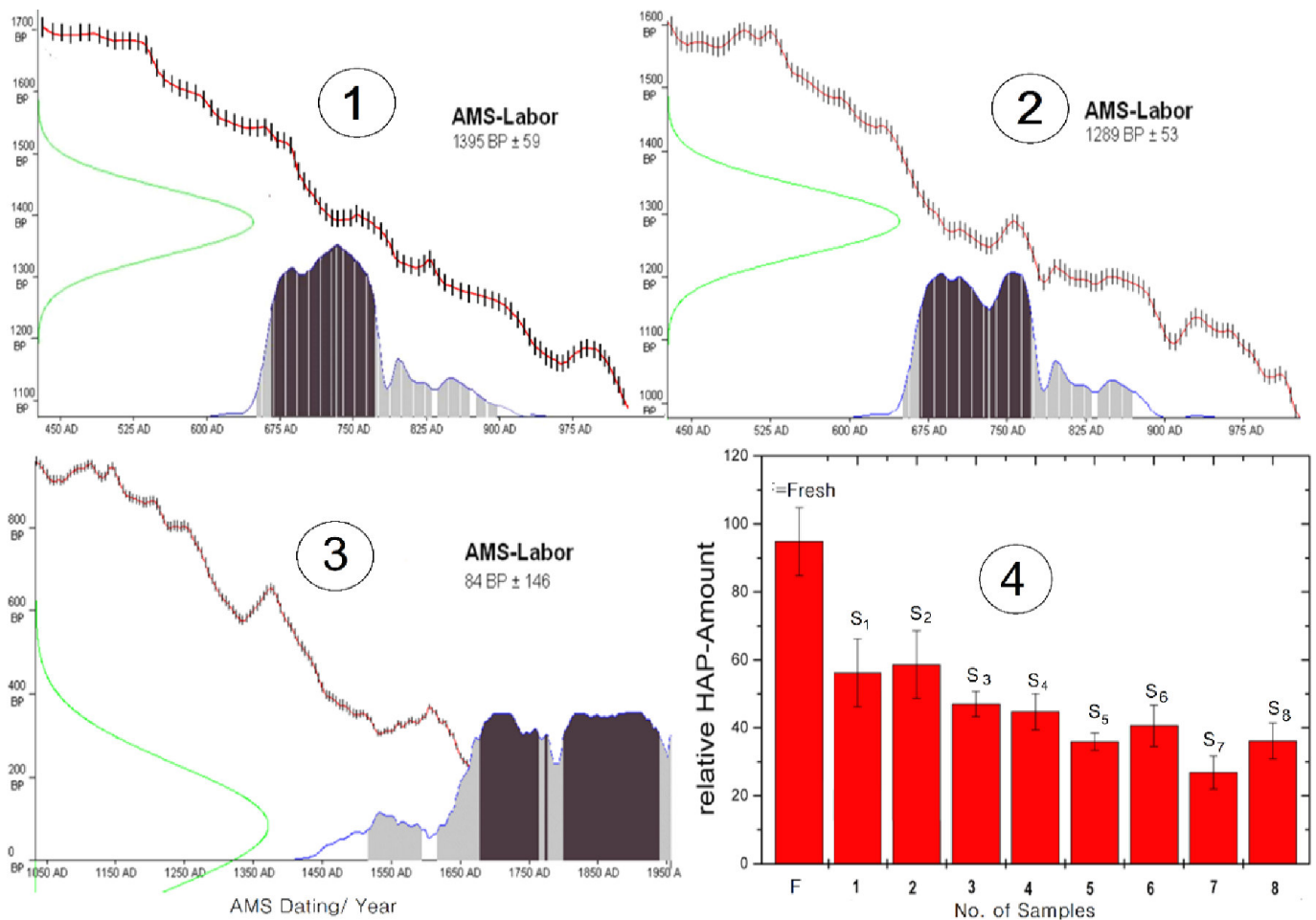
Figure 6. 1, 2, and 3 are the radioactive carbon isotope absolute dating of the samples; 4 shows
the relative amount of HAP in the different samples compared to fresh-cut bone.

(PO_4^{-3}), which is an ancient and highly abundant component of apatite (as illustrated in Figure 5). Additionally, several smaller bands can be attributed to the stretching and bending modes of phosphate groups at 872 cm^{-1} and the central big band at 1,008 cm^{-1}. Furthermore, the bands at 1,160 cm^{-1} and 1,260 cm^{-1} can be assigned to carbonate groups.

In a study, distinct spectral variations were detected at specific wavenumbers in the ATR-FTIR spectra of ancient bone, including 960, 1,050, and a triplet (2,882, 2,950, 2,962) cm^{-1}.

A notable finding was that, in all ATR-FTIR bone spectra, the ancient phosphate peak ν1 (PO_4^{-3}) vibration band observed at 963 cm^{-1} was shifted 5 cm^{-1} toward a higher wavenumber compared to modern bone samples, which was attributed to the substitution of another group instead of OH in bone, resulting in the formation of fluorapatite. This band shift provided information about the structure and crystallinity of apatite, which could potentially be used to qualitatively determine the age of bone.

The carbonate apatite band located at 1,050 cm^{-1} was attributed to exhibit intensity and broadening directly correlated with time, as depicted in Figure 3. Under conditions of burial and chemical changes, the organic component of teeth undergoes degradation at a faster rate compared to the mineral component.

The organic component, i.e., collagen, dominates the process of matrix degradation, which includes both organic and inorganic components. Furthermore, the degradation of collagen's organic part leads to a modification in the orientation of carbonate crystals; this phenomenon can be accounted for by the substitution of B-type carbonate (Serrahima 2018).

Teeth exhibit two forms of carbonate substitution: A-type (carbonate replacing phosphate) and B-type (carbonate replacing hydroxyl), with B-type being more prevalent. Additionally, the broadening of the signal could be associated with carbonate crystallinity. Previous research has suggested that the crystallinity of dental hydroxyapatite reduces with age while the carbonate content in bone increases with age (Peroos *et al.* 2006).

The absolute dating with AMS was used to back up the preliminary results from ATR-FTIR spectroscopy. This was done to compare and back up the preliminary ATR-FTIR dating of the bone samples that were studied.

4. CONCLUSION

SS-NMR and ATR-FTIR spectroscopy have excellent measurement performance for organic and inorganic components of bone. SS-NMR spectroscopy is an effective and constructive method for classifying samples, whether new or old. First, the proposed ratio (polarized ^{13}C MAS-NMR spectra shows typical collagen spectra found in fresh dental dentin. In particular, the hydroxyproline C gamma peak at 71 ppm is a unique fingerprint of collagen. It serves as an indicator of aging because it corresponds proportionally with aging and agrees well with ^{14}C dating.

Second, infrared light analysis showed that the 1-phosphate vibration in old bone had moved toward a high wavenumber (964 cm^{-1}), which backs up the NMR results even more. Third, the density of hydroxyapatite in old dentin shows a peak at 1,050 cm^{-1}, which is related to the carbonate replacement due to the long burial time. NMR and ATR-FTIR spectroscopy can be used as qualitative tools for sorting archaeological bone samples before the use of ^{14}C dating.

This instrument can save effort, time, and money and should be scheduled as a pre-order for AMS analysis, especially for large samples. NMR and infrared analysis can aid in the examination of archaeological bone samples for the highest purity and quality of organic material, thus determining the best methods for DNA analysis. In the future, the described method may be extended and applied to specific cases in ancient archaeology.

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