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Paleodiet characterisation of an Etrurian population of Pontecagnano (Italy) by Isotope Ratio Mass Spectrometry (IRMS) and Atomic Absorption Spectrometry (AAS)

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Human bones recovered from the archaeological site of Pontecagnano (Salerno, Italy) have been studied to reconstruct the diet of an Etrurian population. Two different areas were investigated, named Library and Sant’ Antonio, with a total of 44 tombs containing human skeletal remains, ranging in age from the 8th to the 3rd century B.C. This time span was confirmed by 14C dating obtained using Accelerator Mass Spectrometry (AMS) on one bone sample from each site.

Atomic Absorption Spectrometry (AAS) was used to extract information about the concentration of Sr, Zn, Ca elements in the bone inorganic fraction, whilst stable isotope ratio measurements (IRMS) were carried out on bone collagen to obtain the δ13C and δ15N. A reliable technique has been used to extract and separate the inorganic and organic fractions of the bone remains.

Both IRMS and AAS results suggest a mixed diet including C3 plant food and herbivore animals, consistent with archaeological indications.

Keywords: Ancient populations; Atomic absorption spectrometry (AAS); Carbon-13; Carbon-14; Isotope ratio mass spectrometry (IRMS); Natural isotope variations; Nitrogen-15; Paleodiet

1. Introduction

Dietary investigation plays a major role in answering questions concerning human biological and cultural past, addressed by archaeologists, anthropologists, etc. [1].

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Chemical and isotopic techniques are used on inorganic and organic bone fractions to reconstruct the diet of ancient populations, giving additional information about lifestyle and alimentary habits [2, 3].

Due to their long preservation time, bones are extensively used as a source of information in archaeological research. They are made of inorganic (≈80%) and organic (≈20%) fractions. The inorganic part is essentially calcium hydroxyapatite $\text{Ca}_10(\text{PO}_4)_6(\text{OH})_2$ with additional substances such as carbonate, fluoride, sodium, and magnesium ions [4]. It is well known that by metabolic pathway, calcium ions can be substituted by other ions with similar metabolic behaviour such as strontium [5] and zinc ions, during the lifetime of an individual. For this reason Sr and Zn in bone inorganic phase reflect their proportion in human diet [6]. On the other hand, concentrations of Sr and Zn in the food chain can vary because of their different behaviours in the food chain: Sr, found at a relatively high level in plants, decreases in the series herbivore $\rightarrow$ omnivore $\rightarrow$ carnivore whereas Zn increases over the same series; Zn occurs in relatively high levels in meat; it is also found at high levels in nuts and mollusces. Consequently, the amounts of Sr and Zn in the inorganic part of bones, expressed as the ratios to the Ca concentration (Sr/Ca and Zn/Ca), can be directly used to gain information about the diet [7, 8]. Only Sr, Zn and Ca were used for paleodietary reconstruction in complete agreement with the study of Parker and Toots [9]. They affirm that Sr, Zn and Ca were incorporated into bone apatite matrix during the life, and are thus representative of the bone composition, whereas many elements as Mn, Al, K etc., which are the major components of the soil, may give analyses that largely reflect soil contamination rather than the bone itself.

The organic fraction of bones is composed of protein (mainly collagen) and lipids. The mineral matrix protects collagen from denaturation, thus allowing the recovery of large collagen peptides from fossil bones over centuries or millennia [10]. As for the mineral part of bone (Sr, Zn and Ca), isotopic ratios $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ of collagen can be used as dietary indicators since they reflect the isotopic composition of the food that people used to eat [11]. In fact, along the food chain isotopic ratios of carbon ($^{13}\text{C}/^{12}\text{C}$) and nitrogen ($^{15}\text{N}/^{14}\text{N}$) go through different fractionation processes that make the isotopic signal of each trophic level different from the others. The analysis of carbon and nitrogen stable isotopes in bone collagen can reveal the position of an individual within the food chain, the types of vegetables consumed and whether the diet was terrestrial or marine [12, 13].

Here, IRMS and AAS are combined to study the diet of the Etrurian population discovered in the settlement of Pontecagnano (Salerno, Southern Italy). Moreover, by using AMS, a chronological characterisation of two representative remains has been given.

2. Materials and methods

2.1 Site description

Pontecagnano’s necropolis [14, 15] is one of the most important funerary complexes left by Etruscan culture in South Italy. Archaeological excavation of those areas led to the recovery of many individuals. Two sets of remains were analysed: the first one belongs to the area named Library located in the western necropolis while the second one belongs to the site named Sant’Antonio located in the eastern necropolis (figure 1).

The excavation of Library site started in 1980 and lasted one year. Out of 70 tombs found in this area, 35 contained bone remains, dated around the 8th century B.C. from archaeological studies (L. Cerchiai, private communication). Together with human bone remains, several pottery samples, personal ornament and animal (herbivores) remains were recovered. Archaeological studies indicate that 9 samples from Sant’Antonio excavation site can be dated...
around the 3rd century B.C. In this area some herbivore animal skeletals, probably horses, were found, besides many objects of common use. Due to the limited number of findings recovered in Sant’Antonio, it is suggested that this area could be an extra urban settlement.

### 2.2 Sample preparation

From all the samples collected, only bones without observable pathological characteristics were selected and analysed.

All of the bone fragments were cleaned by abrasion to remove any surface material that could have been contaminated in different ways. After cleaning, the abraded fragments were dried at 100 °C for 20 hours to remove surface hydration water before being pulverised.

Starting from this point, different procedures to extract the inorganic and organic bone fractions were used.

*Extraction of the inorganic fraction* [8]: a bone amount of 10 g was treated with 25.0 ml HCl 6.0 M until complete dissolution. The resulting solution was filtered and then analysed by AAS. The Sr/Ca × 1000 and Zn/Ca × 1000 ratios for all treated samples were determined.

*Extraction of the organic fraction* [16]: bone samples, of mass between 1–2 g, were pulverised and immersed in an acid bath (HCl 0.6 M) for ~1 h at 0 °C to remove the inorganic
component without altering the organic one. This cycle was repeated at least 3 times, depending on the bone preservation state. In each step, the organic fraction was separated from the inorganic one by centrifugation. The residue was then neutralised with water, dried and pyrolysed in a quartz tube under a nitrogen stream flow at 600 °C.

2.3 Analysis

Atomic Absorption Spectrometry: AAS analyses were performed with a Perkin Elmer Analyst 100. For strontium analysis a wavelength of 460.7 nm was used. No further treatments were needed on the samples before the measurements. For zinc analysis a wavelength of 213.9 nm was used and the samples were further diluted at 1:20. The analysis for calcium was performed with much higher dilution (1:20000) and with the use of a wavelength of 422.7 nm. Potassium salt was added (2000 mg/ml) to all the samples in order to offset ionisation and interference from phosphate. The analysis was performed using calibration curves obtained by standard solutions, purchased from the Aldrich Chemical Company.

Isotope Ratio Mass Spectrometry: A ThermoFinnigan Delta plus mass spectrometer coupled with an elemental analyser (ThermoFinnigan CHNS EA 1112) via CONFLO II interface was used for IRMS analyses. Solid samples were combusted by the EA and the resulting CO2 and N2 entered (via CONFLO) the mass spectrometer ion source to measure the ratios $^{45}\delta^{13}R(=^{45}CO_2/^{44}CO_2)$, $^{46}\delta^{15}R(=^{46}CO_2/^{44}CO_2)$ and $^{29}\delta^{13}R(=^{29}N_2/^{28}N_2)$ (then converted to $^{13}\delta^{13}C(=^{13}C/^{12}C)$ and $^{15}\delta^{15}N(=^{15}N/^{14}N)$). Finally, the isotopic ratios for carbon and nitrogen are expressed in δ (‰) notation:

$$\delta^{13}C = \left(\frac{^{13}R_{sample}}{^{13}R_{reference}} - 1\right) \times 1000$$

and

$$\delta^{15}N = \left(\frac{^{15}R_{sample}}{^{15}R_{reference}} - 1\right) \times 1000.$$

The measured $\delta^{13}C$ and $\delta^{15}N$ are reported against the international standards for carbon and nitrogen isotopic analyses: PDB (Pee Dee Belemnite) and AIR (atmospheric nitrogen), respectively. In order to do that, two primary standards were used, calibrated against PDB and AIR (pure CO2 and N2 cylinders from Messer, namely ISO-TOP), with $\delta^{13}C_{VPDB} = -25.4 \pm 0.2$‰ and $\delta^{15}N_{AIR} = -13.2 \pm 0.2$‰.

3. Results and discussion

3.1 Atomic absorption spectrometry

According to a consolidated procedure [7] the Sr/Ca × 1000 ratio of human bones, indicated as $R_{bone}$, is compared to the Sr/Ca × 1000 ratio of fauna herbivores, indicated as $R_{herbivore}$, which were found in the same archaeological site: $R_{bone}/R_{herbivore}$ ratios close to 1 indicate a vegetable-based diet. The zinc values are reported as Zn/Ca × 1000 ratio and indicated as $R_{Zn/Ca}$. The information one can gain from Zn concentrations in bone is quite difficult to interpret as no carnivore animal remains were found. In addition to this, high levels of Zn are found not only in meat, but also in milk, molluscs and nuts.

In table 1 the average values of $R_{bone}$, $R_{bone}/R_{herbivore}$ and $R_{Zn/Ca}$ both for the human and the animal remains analysed, are reported.

Both samples from Library and S. Antonio sites show a $R_{bone}/R_{herbivore}$ ratio close to 1, giving support to a vegetable-based diet [2]. This last indication is also supported by the observed values of $R_{Zn/Ca}$ for human bones which are the same (as indicated by a t-test at
Table 1. Ratios of Sr and Zn concentration to Ca concentration for all investigated samples.

<table>
<thead>
<tr>
<th></th>
<th>Human remains of Library site</th>
<th>Human remains of Sant’Antonio site</th>
<th>Herbivore remains of Library site</th>
<th>Herbivore remains of Sant’Antonio site</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_{\text{Sr}/\text{Ca}}$</td>
<td>0.18 ± 0.08</td>
<td>0.14 ± 0.03</td>
<td>0.24 ± 0.03</td>
<td>0.18 ± 0.04</td>
</tr>
<tr>
<td>$R_{\text{bone}/\text{R_{herbivore}}}$</td>
<td>0.75 ± 0.08</td>
<td>0.78 ± 0.04</td>
<td>0.20 ± 0.03</td>
<td>0.20 ± 0.05</td>
</tr>
<tr>
<td>$R_{\text{Zn}/\text{Ca}}$</td>
<td>0.29 ± 0.09</td>
<td>0.21 ± 0.05</td>
<td>0.20 ± 0.03</td>
<td>0.20 ± 0.05</td>
</tr>
</tbody>
</table>

the significance level of 1 %) as those of the herbivore remains (as indicated by a t-test at the significance level of 1 %).

3.2 Contamination from soil

In contrast to modern bone, excavated bone has been exposed to an environment in the soil that might lead to contamination. Permeation of elements from the soil through ion exchange or other mechanisms may occur. To verify the preservation state of bone and check if the concentration of metals determined were at physiological values, soil samples adhering to the skeletal remains were analysed. Soil dissolution and analysis were performed according to the procedures described in literature [17].

In table 2 the metal concentration has been reported according to the convention used for soil chemical analysis. Values collected for soil are consistent with those existing in literature [17]. The amount of metals in bones is significantly different from the one in soil. This result suggests that Sr, Zn and Ca of the bones are at physiological concentrations [18].

3.3 Isotope Ratio Mass Spectrometry

Figure 2 shows the isotopic composition for the three groups of samples (mean $\delta^{13}$C and $\delta^{15}$N± standard deviation: Sant’Antonio ($-19.7 \pm 0.4$‰, $9.2 \pm 1.0$‰), Library ($-19.5 \pm 0.9$‰, $9.5 \pm 1.6$‰) and herbivore animals ($-22.8 \pm 1.0$‰, $7.8 \pm 1.7$‰).

It is evident that there is no significant difference between Sant’Antonio and Library samples, both in $\delta^{13}$C and $\delta^{15}$N values as indicated by a t-test at the significance level of 1 %. Animal samples have a $\delta^{13}$C significantly lower than that of Sant’Antonio and Library samples (t-test, 1 %). On the contrary, the $\delta^{15}$N of animals is not significantly different from that of Sant’Antonio and Library samples (t-test, 1 %).

Isotope measurement results show that for all the investigated samples a large amount of the assumed proteins came from C 3 terrestrial-based resources [19]. Assuming a mean $\delta^{13}$C value for C 3 plants of $-26.5$‰, due to isotopic fractionation associated with biochemical reactions (+5 ‰), pure C 3 feeders should have a $\delta^{13}$C value around $-21.5$‰. The isotopic composition of human bone collagen for all samples (Sant’Antonio and Library together,

Table 2. Concentration of Ca (g/kg), Zn (mg/kg) and Sr (mg/kg) in reference soil (as indicated in the workbook of the Agrarian Politics Ministry–Italian Society of Soil Science [17]), in soil adhering to bones and in analysed bones.

<table>
<thead>
<tr>
<th></th>
<th>Reference concentration values for soil</th>
<th>Soil values</th>
<th>Bone values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>10–250</td>
<td>115 ± 3</td>
<td>270 ± 7</td>
</tr>
<tr>
<td>Zinc</td>
<td>10–150</td>
<td>41 ± 1</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>Stronzium</td>
<td>8–200</td>
<td>170 ± 2</td>
<td>46 ± 1</td>
</tr>
</tbody>
</table>
Figure 2. Isotopic composition for $^{13}$C and $^{15}$N of the group of samples analysed: S. Antonio ($\Delta$), Library (●) and Herbivore animals (×).

$\delta^{13}$C = $-19.6 \pm 0.7$‰, $\delta^{15}$N = $9.4 \pm 1.3$‰ suggests a mixed diet including C$_3$ plant food and, probably, herbivore animals. Isotopic results are consistent with atomic absorption results.

3.4 $^{14}$C dating

Although the findings from the two studied sites were archaeologically correlated with time spans ranging in age from the 8$^{\text{th}}$ to the 3$^{\text{rd}}$ century B.C., no date attribution based on quantitative chemical-physical analytic methods had been performed so far. One of the methods available to the research group of the present study is the radiocarbon dating using AMS.

To chronologically characterise the remains, two of the skeletal bones, tomb 8057 from Library and tomb 3857 from Sant’Antonio were treated in the preparation laboratory of the Environmental Science Department in Caserta [20]. The few milligrams of graphite obtained at the end of the treatment process was used to produce cathodes that were inserted in the sputtering source of the Bochum (D) University Accelerator Mass Spectrometry (AMS) system [21]. The radiocarbon ages of the samples were converted to calendar ages using the CALIB 4.4 software [22]. In table 3 the radiocarbon age and calibrated age for the two investigated samples are showed.

These results confirm the historical indication about the site and the remains found.

Table 3. Radiocarbon age and calibrated age, obtained using the software CALIB 4.4, for the two investigated samples.

<table>
<thead>
<tr>
<th>Laboratory code</th>
<th>Sample</th>
<th>Radiocarbon age</th>
<th>Calibrated age (1 sigma)</th>
<th>Calibrated age (2 sigma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSA261</td>
<td>Tomb 3857</td>
<td>2582 ± 101</td>
<td>BC 832–755 (33.4 %)</td>
<td>BC 901–BC 409 (100 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BC 723–538 (64.5 %)</td>
<td>BC 411–AD 26 (99.6 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BC 530–521 (2.1 %)</td>
<td></td>
</tr>
<tr>
<td>DSA217</td>
<td>Tomb 8057</td>
<td>2199 ± 98</td>
<td>BC 385–165 (97.7 %)</td>
<td>BC 128–121 (2.3 %)</td>
</tr>
</tbody>
</table>


4. Conclusion

On the basis of combined AAS and IRMS analyses it has been shown that the main dietary elements consumed by populations in Pontecagnano came from a vegetable diet, with particular use of C3 plant food. These results, together with date values obtained through AMS analysis, are in agreement with archaeological indications.

The two key objectives of the present research were (a) the comparison of the two employed techniques (AAS and IRMS) in order to verify their validity and complementarity for paleodiet reconstructions; (b) the development of a reliable protocol for sample preparation procedure to determine the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios and metal amount preserved in archaeological bones.

A major future challenge will be the improvement of the capability to isotopically characterise the studied site through the correlation between the sample isotopic signals and the site characteristics and to extend the metal analysis to different bone markers (i.e.: Ba, Fe) which are representative of the bone composition.

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