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# Boiled versus unboiled: a study on Neolithic and contemporary human bones

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ABSTRACT

Bones treated at low temperature do not present major modifications although, macroscopically, boiled bones may be distinguished from unboiled ones as they are smoother, lighter and more transparent. Such observations should correspond to textural modifications at a nanometric level and should depend on boiling time. In this study, contemporary human bones, boiled during various time intervals, were characterized using scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDS), X-ray diffraction (XRD) and nitrogen adsorption (BET). The results were used to estimate the boiling time in four archaeological samples (Neolithic bones from Malalmuerzo cave, Spain). It is concluded, comparing Neolithic bones to contemporary ones and from the textural characterization at a nanometrical level, that two of the archaeological bones were boiled and that they were boiled for less than 6 hours.



## 1. Introduction

Information on paleodiets, paleoclimates, or behavior may be inferred from the chemistry of archaeological bones (Hurlbut, 2000). As frequently reported, buried bone is altered by time, diagenetic factors, or temperature (Malgosa et al., 2008; Munro et al., 2007; Lozano et al., 2003, 2002; Botella López et al., 2000; White and Hannus, 1983). Structural and textural bone modifications due to heat exposure have to be correctly understood because they are related to such human habits as boiling and grilling meat. In this sense, the understanding of bone modifications at 100 °C in water, *i. e.* a boiling process, is crucial. Indeed, bone burnt at high temperature may be distinguished through macroscopical as well as microscopical observations. For instance, it becomes black. Instead, bone treated at a temperature lower than 100 °C is difficult to distinguish from bone untreated or from bone which has suffered taphonomic processes.

Bone is a mineralized connective tissue mainly composed of biological apatite (a natural mineral also present in teeth) whose crystal sizes range in length from 2 nm for the smallest particles, to 110 nm for the largest ones (Barrère et al., 2006), and collagen (a natural polymer, also found in skin and tendons); it is a composite material (Munro et al., 2007; Wahl and Czernuszka, 2006; White and Hannus, 1983). The type of bone is defined depending on the orientation of collagen fibers (Weiner and Wagner, 1998; Bigi et al., 1997).

Collagen, which is a scleroprotein rich in amino acid glycine, is converted into gelatin, when boiled in water. If collagen is inmersed in a warm liquid, it forms a sol within the liquid that increases in viscosity and solidifies to form a gel as it cools. In contrast, biological hydroxyapatite is not dissolved but it can either undergo ion exchanges or be degraded by the $CO_2$, $HCO_3$ and $H^+$ ions produced by the collagen desintegration (White and Hannus, 1983).

Boiled bone has not been thoroughly studied since conventional characterization techniques have not detected any significant difference between unboiled and boiled materials (Ríos-Díaz et al., 2008; Malgosa et al., 2008; Outram, 2001). It is only by a careful study of collagen through transmission electron microscopy that it has been possible to sort the butchered and unboiled bone from the boiled one, in bovine cases (Koon et al., 2010). This work revealed that, by boiling, mineralized collagen accumulates only minor damage as the mineral matrix seems to stabilize the collagen. It has

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also been shown that when bone is boiled, protein is lost and crystallinity increases, as does porosity (Roberts et al., 2002). These two conclusions seem contradictory as, on the one hand, it is said that crystallinity is lost and, on the other, that collagen is stabilized, which means that bone composition and structure are maintained. It is clear that a rigorous characterization and a mechanism that explains bone behavior treated at 100 °C in presence of water is required. Furthermore, a factor not often considered and which is crucial for archaeologists are the subsequent diagenetic alterations of boiled and unboiled bones with time.

The purpose of the present study is to obtain a correlation between textural properties (at a nanometrical level) and boiling time, from the study of contemporary materials; then, to compare those results with the corresponding features of Neolithic bones in order to determine if the archaeological materials were really boiled and, finally, to estimate the boiling time.

The chosen characterization methods, commonly used in chemistry of materials, are complementary. They should provide a description of boiled bone that should lead to understand the modifications of bone structure and texture at low temperature, if any. Of course, this study is descriptive as we will, only, indicate if the bone has been boiled or not, not why.

## 2. Materials and methods

In this work, the contemporary samples are human bones boiled during various time periods. The chosen Neolithic human bones were found in the Malalmuerzo cave, located in the Granada zone (Jiménez Brobeil, 1990; Ramos Muñoz, 1988–1989), (see Map). We have chosen two apparently unboiled and two apparently boiled Neolithic samples. The choice was based on macroscopic criteria, *i.e.* on changes in color and surface texture (Botella López et al., 2000; Hurlbut, 2000; White, 1992).

The bones are characterized through gas adsorption technique, X-ray diffraction and scanning electron microscopy coupled with energy dispersive X-ray spectroscopy. With gas adsorption the specific surface and the pore size distribution in the range 1–30 nm can be determined (Robinson et al., 2003; Cardoso et al., 2007), X-ray diffraction identifies crystalline compounds (in bone mainly hydroxyapatite) and estimates the degree of crystallinity (Malgosa et al., 2008), scanning electron microscopy reveals the surface texture (Weiner and Wagner, 1998), and energy dispersive X-ray spectroscopy provides the local elemental composition. These techniques are complementary and sensitive to bone microstructure (Smith et al., 2008; Robinson et al., 2003).

### 2.1. Contemporary samples

Fresh samples were obtained from the same human female tibia, 65 years old. Approval from the research ethics committee of the Facultad de Medicina, Universidad de Granada, and family consent for the donation of organs for autopsy and procedures included in this research were obtained. After autopsy, four slices of the central part of the defleshed bone, *ca.* 2 cm, were cut. These pieces were defleshed with bisturi and boiled in water for 2, 4 or 6 h. A sample was not treated for comparison purposes. The samples were boiled in stainless steel pots and covered. The pots were shut to minimize water loss during boiling. To summarize, the bone tissue was at 100 °C (temperature of boiling water) in contact with water for 2, 4 or 6 h. The covered pots were allowed to cool to room temperature for 24 h. The bones were, then, air-dried for 72 h and weighted.

The contemporary bones were defleshed with a bisturi before boiling. Indeed, temperature in water would not be significantly different and the cleanliness of the experiment is improved. Furthermore, as bones were degreased the resulting samples would be similar. The boiling time periods were chosen in order to reproduce cooking times. It is known that stews are cooked in water for *ca.* 2 h, for instance “rabo de toro”, but tendons require 4 h. Tendon may be eaten or used, as Troyans did, to construct bows. The sample treated for 6 h has no reference in everyday life. Still, the corpse of the German Emperor Lothar I was boiled for 6 h to deflesh it (Bada et al., 1989).

### 2.2. Sample degreasing

To prevent putrefaction, the boiled contemporary materials were all frozen for two days at −14 °C. They were defrosted at ambient temperature for 2 h and immersed in undissolved trichoroethylene for 11 days to degrease them and eliminate all organic material. The bones were heated at 37 °C for one day, in an oven, to dehydrate them before cutting a small piece (*ca.* 1 g) of the central part. Those pieces were milled to obtain a fine powder appropriate for the physical characterization techniques.

Unfortunately the degreasing process has not been normalized and several procedures may be used. For instance bones may be kept in ammonia solution for less than one week followed by bleaching with hydrogen peroxide solution and cleaning with commercial detergent (Hussain et al., 2007). Our procedure is close to the method reported by Dayal et al. (2009) which has been shown to be very effective.

### 2.3. Neolithic samples

The second group of samples corresponds to Neolithic bones from Malalmuerzo cave (in Alfacar, close to Granada, Spain), bag 617 deposited in the Anthropology Department of the Granada University. From a macroscopical criterion (Botella López et al., 2000), *i. e.* smoothness, weight, and transparency, two of them, numbers 278 and 282, were not thermally treated; instead, the numbers 471 and 2013, were boiled. The bones were cut and milled as the fresh ones. Of course, the degreasing process was not required due to the old age of those bones.

### 2.4. Characterization techniques

#### 2.4.1. Powder X-ray diffraction (XRD)

X-ray diffraction is a conventional analytical method that identifies the crystalline compounds present in the sample, since each compound has a unique diffraction pattern. The compounds may be identified by comparing the experimental pattern with those reported in the cards of the JCPDS (Joint Committee on Powder Diffraction Standards). Only if the compound is crystalline, with a crystallite size larger than *ca.* 3 nm, and if the content is higher than *ca.* 3%, peaks are observed; otherwise, a broad and undefined enhancement of the background line is registered. Therefore, in bone, the sharp peaks present in the diffractogram correspond to crystalline hydroxyapatite, and the broad background line is due to collagen as collagen is not crystalline.

X-ray diffraction patterns were obtained with a Bruker D8 Advance diffractometer coupled to a copper anode X-ray tube. K$\alpha$ radiation was selected, with a diffracted beam monochromator. The patterns were processed with the software Xpowder (http://www.XPowder.com).

#### 2.4.2. Scanning electron microscopy/energy dispersive X-ray spectroscopy (SEM/EDS)

In scanning electron microscopy, an electron beam is focused on the sample in a vacuum environment. The information is local and corresponds to a selected fraction of the bone. From the micrograph, it is possible to determine the morphology of the various compounds that constitute bone but it is not possible to identify the

compounds unless their morphology is known. Note that in SEM micrographs, the observed objects are in the micron range, whereas in XRD the patterns are due to the atomic structure of the full sample, *i.e*, to the ordered arrangement of atoms in space and the results correspond to the bulk of the sample.

In the microscope, the elemental identification provided by the EDS probe is very useful. When the sample is homogeneous, one EDS analysis is representative; instead, if the sample is inhomogeneous an EDS analysis of each zone has to be performed. Elements with an atomic weight lower than fluorine are not detected and carbon peak is due to the sample as well as to the layer sputtered on the sample to guarantee conductivity.

For SEM/EDS studies, a Hitachi S-510 scanning electron microscope was used. The bone splinters were sputtered with carbon to avoid charge problems. The samples were studied at 2000×, 5000× and 10,000× magnifications. The elemental analysis of some selected zones was made with a Rontec Energy dispersive X-ray probe (EDS) instrument.

#### 2.4.3. Nitrogen adsorption

Adsorption is the adhesion of atoms, ions, molecules of gas or liquids to a surface. This process creates a film of the adsorbate (the molecules or atoms being accumulated) on the surface of the adsorbent. Adsorption is a consequence of surface energy. For pore structure analysis, the nitrogen adsorption–desorption isotherms were determined. In principle, nitrogen isotherms of Types II and IV are amenable to the Brunauer–Emmett–Teller (BET) analysis provided that pores of molecular dimensions are absent and that the BET plot is obtained over an appropriate range of the isotherm (Sing, 2001). All samples were characterized by $N_2$ adsorption at 77 K by applying the BET equation to the $N_2$ adsorption isotherm, after a sample treatment *in vacuo* at 80 °C. Still, the porosity obtained through BJH equation corresponded only to the interparticular voids and therefore will not be presented.

## 3. Results

### 3.1. X-ray diffraction

The X-ray diffraction patterns of the contemporary samples are compared in Fig. 1. The peaks are due to crystalline material, they all correspond to the well known reflections of crystalline hydroxyapatite, $3Ca_3(PO_4)_3.Ca(OH)_2$ (Gutowska et al., 2005). The broad and undefined peak from 15 to 60°(2θ) superimposed on the background line corresponds to an amorphous material which in fresh bone is collagen (Gutowska et al., 2005; Rogers and Daniels, 2002). All patterns are similar. The hydroxyapatite peaks maintain their shape and their size showing that, as expected, the hydroxyapatite crystallinity is not altered by the boiling process. In Fig. 1, the peak positions do not shift (2θ), thus apatite lattice parameters are not altered, and the hydroxyapatite is not exchanged: lattice parameters, and hence peak positions, should vary depending on the size of the ions incorporated into the exchange positions of the crystalline lattice. The ratio between peaks and the broad line due to amorphous material remains constant showing that the percentage of organic compound is not altered, independently of boiling time. This fact does not mean that collagen remains stable; it only suggests that the amount of non-crystalline material is not modified.

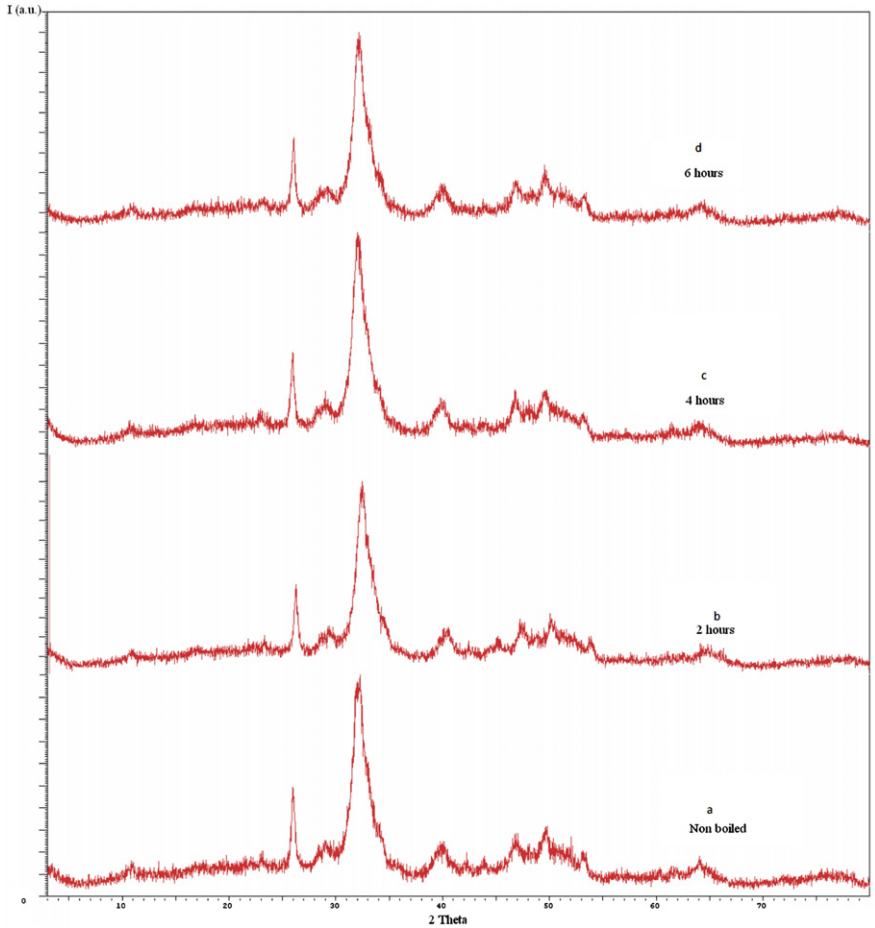


**Fig. 1.** X-ray diffraction of contemporary bone: a) unboiled bone; b) boiled for 2 h; c) boiled for 4 h; and d) boiled for 6 h.

Diffractograms of the two assumed unboiled Neolithic samples, Fig. 2, present sharp and well defined hydroxyapatite peaks, narrower than those of contemporary samples: in the zone $30 < 2\theta < 35^{\circ}$ the peaks are resolved. Hence, the hydroxyapatite crystals are larger than those present in the contemporary materials, indeed, the broader the X-ray diffraction peaks are, the smaller the corresponding crystallite dimensions. Furthermore, if sample 278 is compared to sample 282 or to contemporary bones, an extra peak is found at $2\theta = 29.52^{\circ}$(interplanar distance $d = 3.02$ Å), that may be attributed to calcite whose main peak is at $d = 3.03$ Å. In Neolithic materials, the ratio of crystalline hydroxyapatite (peaks) to amorphous material (background) is systematically slightly higher than in contemporary fresh bone; then, the amount of amorphous material (organic) has diminished.

The diffractograms of Neolithic bone assumed to be boiled during the Neolithic are similar to the unboiled materials. The differences are, indeed, in the range of variations between the two unboiled materials. No calcite seems to be present. Again, within error range, peaks are not shifted and, therefore, no exchange of hydroxyapatite with other ions is present.

### 3.2. Scanning electron microscopy (SEM)/energy dispersive X-ray spectroscopy (EDS)

#### 3.2.1. Contemporary samples

Fig. 3 shows the micrographs of the contemporary samples and Fig. 4 the corresponding analyses by EDS of the most representative areas. As expected, the unboiled bone presents the hierarchical structure, typical of cortical bone morphology (Weiner and Wagner, 1998; Weiner and Traub, 1992). The arrangement of lamellae can be observed. The amount of calcium and phosphorus determined by EDS in the zone labeled as A in the microscopy image, molar ratio $Ca/P = 1.097$, Table 1, is in agreement with the previous X-ray diffraction result which identified hydroxyapatite with chemical formula$3Ca_3(PO_4)_3.Ca(OH)_2$ whose molar ratio is $Ca/P = 1.11$ (Gutowska et al., 2005). Again, carbon peak is due to collagen and the carbon layer sputtered on top.

The sample boiled for 2 h reproduces the features of the fresh material. The pores are the same size (*ca.* 4 μm) and the platelets are typically 3–20 μm. The EDS analysis ($Ca/P = 0.813$, 1.068; in positions A or A1, respectively) reproduces the results obtained for the previous sample, and shows the heterogeneity of the surface composition.

The sample boiled for 4 h shows a smoother surface. The macropore mouths are no more apparent and a layered morphology appears. The elemental analysis value ($Ca/P = 1.16$) obtained by EDS is again similar to the previous ones.

After a boiling time of 6 h, the surface turns out to be definitely different. A surface coverd by drop-like interconnected particles appears, the particles are >0.5 μm as it is commonly observed in a solidified gelatin solution. The composition is no more the typical composition of hydroxyapatite, the ratio $Ca/P$ has increased almost twice, it is now $Ca/P = 2.61$ or 1.61. Phosphates, then, are replaced by ionic species whose elements are invisible in our experimental

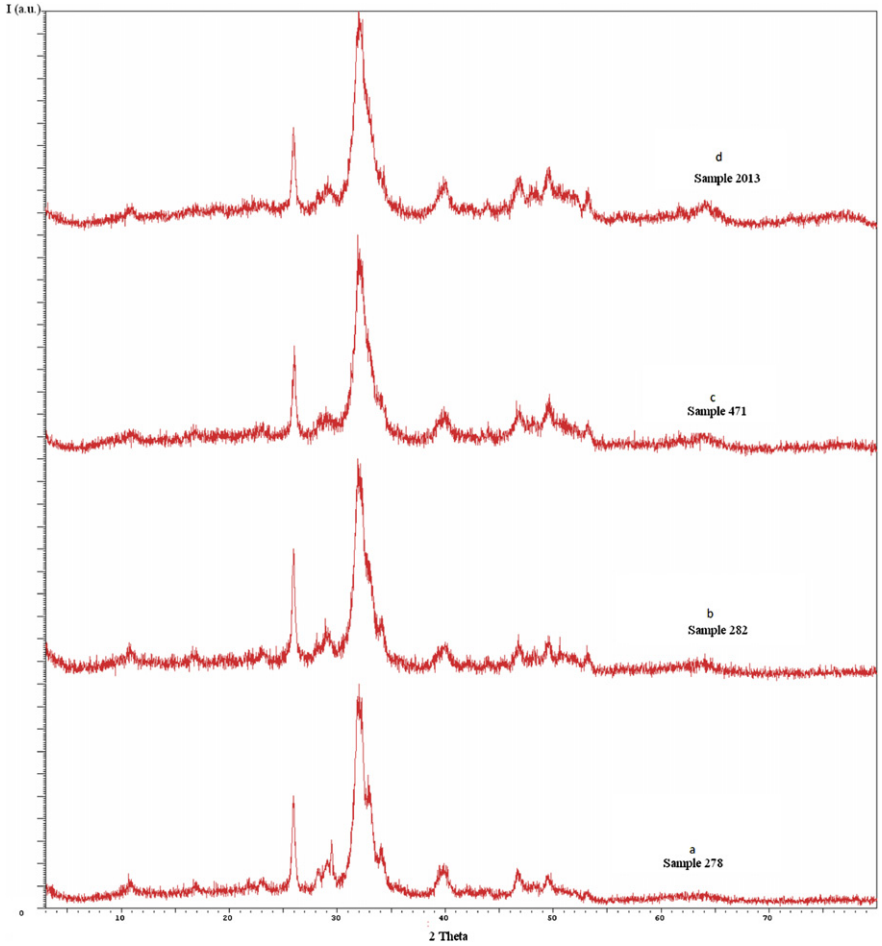


**Fig. 2.** X-ray diffraction of Neolithic bones: a) 278 unboiled sample; b) 282 unboiled sample; c) 471 boiled sample; and d) 2013 boiled sample.

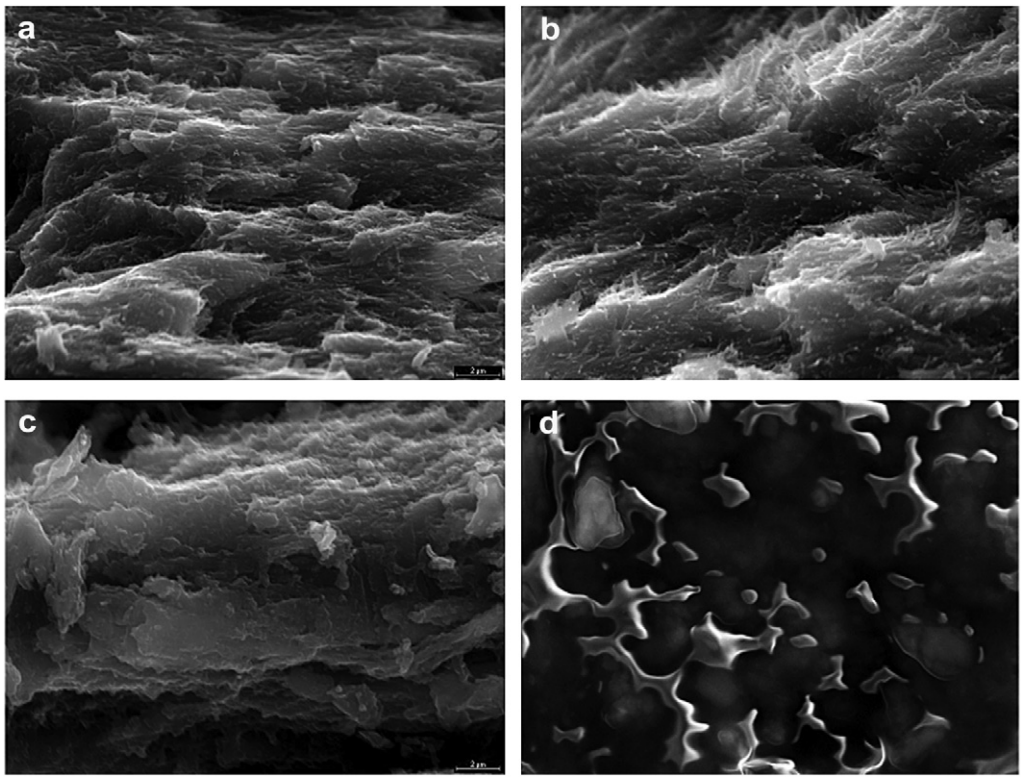


**Fig. 3.** SEM of contemporary bone (magnification: 5000×): a) unboiled bone; b) boiled for 2 h; c) boiled for 4 h; and d) boiled for 6 h.

conditions, most probably carbonate or hydroxyl ions. This result confirms the observation of White and Hannus (1983) who proposed that apatite may be degraded by the $CO_2$, $HCO_3$ and $H^+$ ions produced by collagen disintegration. Processes such as prolonged boiling produce bones similar to those that have been chemically deproteinated (Collins et al., 2002; Roberts et al., 2002).

This result seems to contradict the corresponding X-ray diffraction pattern discussed previously where no shifted peaks were observed. As EDS is a surface local analysis and X-ray diffraction patterns correspond to the bulk of the sample, it has to be concluded that such an exchange only affects some zones of the bone surface.

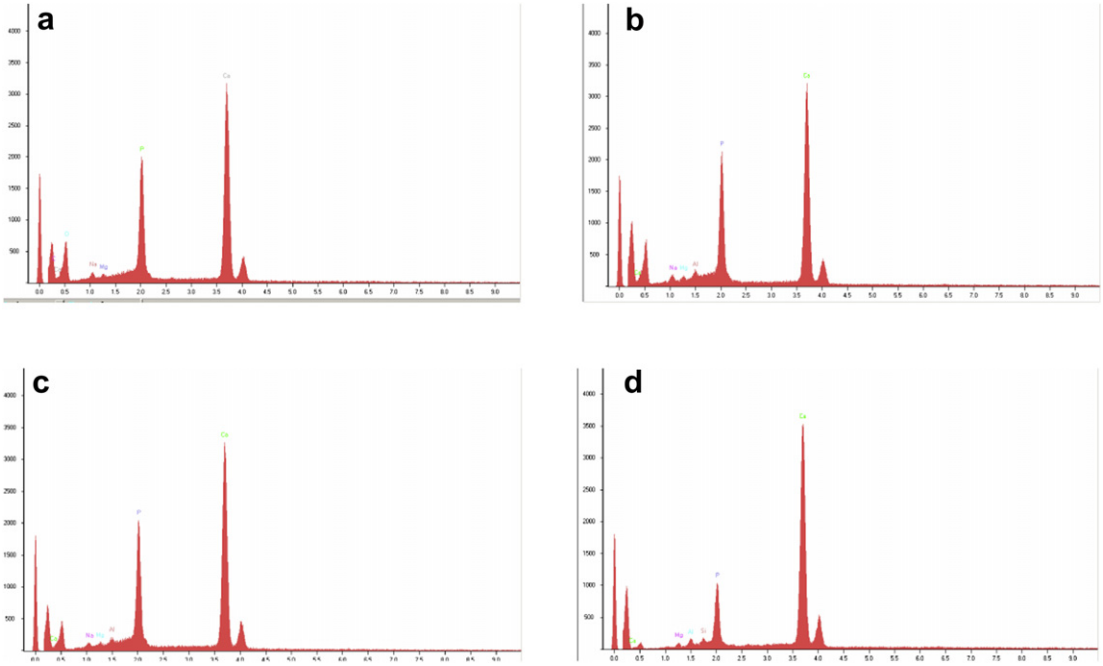


**Fig. 4.** EDS of contemporary bone: a) unboiled bone; b) boiled for 2 h; c) boiled for 4 h; and d) boiled for 6 h. The position of the beam for the analysis is shown in Figs. 3 and 5 by A.

**Table 1**
Weight percentage of calcium, phosphorus, and molar ratio Ca/*P*, obtained by EDS, in contemporary bone samples.

| Sample | Spot | Ca (wt%) | Error | P (wt%) | Error | Ca/*P* (molar) |
|---|---|---|---|---|---|---|
| Unboiled | A | 27.84 | 8.6 | 19.51 | 13.2 | 1.097 |
| Boiled for 2 h | A | 26.91 | 8.4 | 25.58 | 13.0 | 0.813 |
| | A2 | 27.63 | 8.5 | 19.90 | 13.1 | 1.068 |
| | A3 | 28.77 | 8.2 | 19.83 | 11.1 | 1.118 |
| | A4 | 33.33 | 8.3 | 20.06 | 12.9 | 1.211 |
| Boiled for 4 h | A | 30.85 | 8.5 | 20.44 | 13.0 | 1.166 |
| Boiled for 6 h | A | 40.66 | 8.0 | 12.00 | 13.2 | 2.61 |
| | A2 | 40.65 | 8.1 | 19.40 | 13.2 | 1.61 |

Note that all samples present small amounts of magnesium and sodium, those elements have to be attributed to the use of non distilled water. Of course, this observation is valid for contemporary samples boiled in tap water and not for the Neolithic materials.

Last but not least, non boiled sample was homogeneous, but sample boiled for 2 h presented a zone with some reticular solidified material, Fig. 5, whose punctual analysis by EDS reproduces the results already reported for this sample (position A3, Ca/*P* = 1.118). Hence, those membrane-like formations must be constituted by elements undetected by EDS, for example an organic material, probably gelatin formed from heated collagen or by some spongy tissue. The last hypothesis may be discarded as the sample comes from the same bone as the others.

### 3.2.2. Neolithic samples

Although sample 278 looks similar to the non boiled contemporary bone, the contrast is variable; some zones appear more brilliant than others. Such contrast effect can be related to the material density. The EDS analyses show that in the brighter zone the Ca/*P* molar ratio is as high as 3.98 and that a high amount of Si and Al is present. Instead, in the darker zones, where bone lamellae are found, the Ca/*P* molar ratio is the expected one (Table 2). Thus, some alumino-silicates are present as patches on the surface; some voids may be filled by calcite, quartz, hematite and zeolite (Downing and Park, 1998). This observation is in agreement with the X-ray diffraction patterns where calcite was identified, but the alumino-silicate must be a non-crystalline material as no other diffraction peaks were found.

In sample 283, which is another unboiled Neolithic sample, bone lamellae are less defined and a gruyère-like morphology, with holes smaller than 1 μ, is the main feature. Such unusual texture may be attributed to the large group of organisms, as fungi, algae, or bacteria that can destroy archaeological bone remains. Particularly, fungi and algae create small holes, cavities, and channel-like structures (Ortner, 2003). The corresponding spectra do not present the characteristic peaks of Si, Al and Ca of the environmental alumino-silicate, as in the previous sample, but the molar Ca/*P* ratios are all higher than the hydroxyapatite theoretical value. Thus, this Neolithic sample is highly calcified.

**Table 2**
Weight percentage of calcium, phosphorus, and molar ratio Ca/*P*, obtained by EDS, in Neolithic bone samples. The position of the beam corresponding to analaysis EDS A2 in sample 283 is not shown as it corresponds to a lower magnification in a very bright zone.

| Sample | Spot | Ca (wt%) | Error | P (wt%) | Error | Ca/*P* (molar) |
|---|---|---|---|---|---|---|
| 278 | A | 29.19 | 8.8 | 18.96 | 12.7 | 1.02 |
| | A2 | 20.06 | 9.3 | 3.88 | 20.3 | 3.98 |
| | A3 | 34.46 | 8.2 | 20.57 | 12.3 | 1.29 |
| 283 | A | 51.27 | 8.0 | 12.93 | 12.1 | 3.06 |
| | A2 | 35.37 | 8.3 | 16.48 | 12.3 | 1.65 |
| | A3 | 31.21 | 8.4 | 18.49 | 12.1 | 1.30 |
| | A4 | 45.46 | 8.0 | 13.12 | 12.6 | 2.67 |
| 471 | A | 39.62 | 8.0 | 18.11 | 12.7 | 1.69 |
| 2013 | A | 36.72 | 8.2 | 20.16 | 13.1 | 1.40 |
| | A2 | 31.83 | 8.1 | 12.30 | 12.2 | 2.00 |
| | A3 | 45.15 | 8.1 | 16.07 | 11.4 | 2.17 |
| | A4 | 48.86 | 8.1 | 16.27 | 12.9 | 2.32 |

The Neolithic samples, 2013 and 472, Fig. 6c and d, are rather similar to the boiled contemporary samples for 2 or 4 h. Compare Fig. 6c, sample 472, to Fig. 3c, both present similar smoothed lamellae, thus the Neolithic material seems to have been treated for *ca*. 4 h. The morphology observed in the micrograph of sample 2013, Fig. 6d, is in between the morphology of the contemporary samples boiled for 2 and 4 h, Fig. 3b and c. The Neolithic bones assumed to be boiled are as inhomogeneous as the unboiled Neolithic samples. Note that the values of the Ca/*P* molar ratio may oscillate between 1.29 and 3.98 (the unreproduced value 1.02 seems to be an artifact). Still, a common feature is detected, all Ca/*P* values are higher than 1.29, such value is much higher than 1.11, which is the value determined in the contemporary materials (Fig. 7, Table 2).

### 3.3. Nitrogen adsorption

A similar isotherm was obtained for unboiled or boiled contemporary bones, Fig. 8. The shape corresponds to a non porous solid and the adsorption is only due to interstitial space between the powdered bone particles. The access to the smallest pores, *i.e.* microporosity due to the lacunae and/or the canaliculi, is blocked.

BET equation was applied to $N_2$ adsorption isotherms at −196 °C from which a BET surface area, S(BET), was obtained, considering as 0.162 $nm^2$ the molecular area of $N_2$ at that temperature (Rodríguez-Reinoso and Linares-Solano, 1989). The corresponding specific surface areas, *i.e.* the area of surface per gram of material, were all comprised between 2 and 7 $m^2/g$ and the differences between samples are not significant.

In Neolithic materials, the specific surface area of boiled bones is as low as the specific surface area of contemporary bones. Instead, the specific surface area of the non boiled materials turns out to be significantly higher (20 $m^2/g$); the differences between isotherms are clear, Fig. 9. Therefore, pores are accessible to nitrogen molecules, which means that the organic fluids and molecules filling the bones have been eliminated.

## 4. Discussion

Contemporary bones boiled for 0, 2, 4 or 6 h, *i.e.* free of the usual diagenesis effects present in ancient bones, show the same structural and compositional features if characterized by gas adsorption, EDS, or X-ray diffraction. Instead, SEM study revealed that the morphology evolves as the surface is progressively smoothed. To summarize, all contemporary samples are composed by hydroxyapatite, $3Ca_3(PO_4)_3.Ca(OH)_2$, with a Ca/*P* molar ratio of 1.11, and some non-crystalline compound that must correspond to collagen, gelatin, or dried organics. Bone surface is progressively smoothed with boiling time until non-crystalline crusts appear on the surface.

Such results are in agreement with previous studies which state that X-ray diffraction cannot differentiate boiled from unboiled bone (Ríos-Díaz et al., 2008; Malgosa et al., 2008). The measured specific surface areas reproduce the values reported by Robinson et al. (2003), although the degreasing and the washing methods were different. Porosity may vary with diagenetic or pathological factors, or with thermal treatment (Smith et al., 2008; Cardoso et al., 2007; Kolmas et al., 2007; Wang and Ni, 2003; Roberts et al., 2002). Furthermore, micro and macroporosity play an important role in the physicochemical dissolution process of

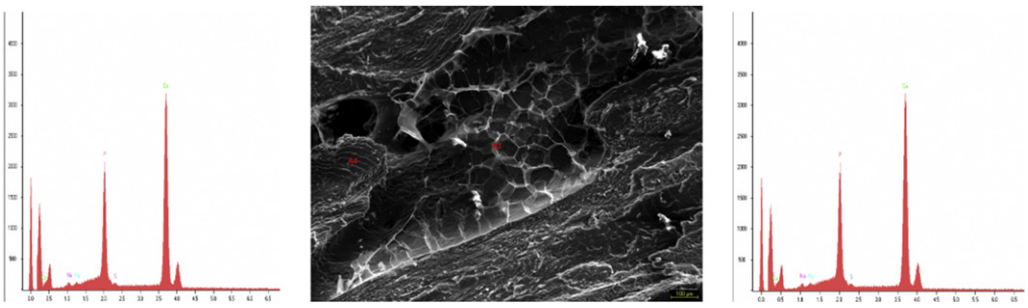

**Fig. 5.** SEM and EDS analyses showing the heterogeneity of the sample boiled for 2 h. Two zones are analyzed, one corresponding to the solid phase and the other to the reticular formation, spots A3 and A4 shown in the image.

calcium phosphates. The larger the exposed surface to the environment, the faster the biomaterial dissolves, simply because larger quantities of exchanges can take place (Barrère et al., 2006).

Since these cavities (or pores) in cortical bone are filled with fluids the reported porosity measurements in non-treated bones are very disparate. It is not surprising that, if the measurement is made by NMR, the obtained pore size distribution presents much smaller pores than by histomorphometry (Wang and Ni, 2003). Sample preparation and the characterization technique are, indeed, crucial as the content of water and grease may modify the experimental values. Reported specific surface area values vary from 2 to 100 $m^2/g$ (Figueiredo et al., 2010; Smith et al., 2008).

The smoothing of bone surface, observed by SEM, in this study, as well as by the macroscopic reports of anthropologists (Botella López et al., 2000), may be explained considering that the collagen fibril is modified by boiling time as revealed by transmission electron microscopy (TEM) (Koon et al., 2010). Indeed, with boiling, collagen turns out to be beaded or dumbbell shaped instead of fibrillar. Furthermore, it is suggested that the presence of a mineral matrix stabilizes the collagen enabling the damage to accumulate, but preventing it from causing immediate gelatinization.

In our work, the morphology determined by SEM may be attributed, then, to a progressive diffusion of the lightest modified collagen fibrils, first, and afterwards, to the heaviest species, all resulting from the damaged collagen observed by TEM. These residues are uniformly adsorbed on the full surface and on the pore mouths blocking nitrogen access and softening rugosity.

The features of the Neolithic bones divide them in two groups, one whose main characteristics are surface inhomogeneity and the other a smooth homogeneous surface. They correspond to the assumed unboiled and boiled bones respectively. The surface of the assumed unboiled Neolithic bones turned out to be destroyed by algae or funghi; the surface was very inhomogeneous and it was covered by calcium carbonate. Instead, the assumed boiled Neolithic bones were not as degraded, no holes or calcium carbonate were observed. Therefore, it seems that boiling process reduces diagenesis. Furthermore, in the zones free of diagenesis, the assumed boiled Neolithic bones reproduce the surface morphology of the contemporary bone boiled for 4 h and they are definitely different from the contemporary bone boiled for 6 h, which would mean that those Neolithic bones were boiled, and, furthermore, that they were boiled between 2 and 4 h.

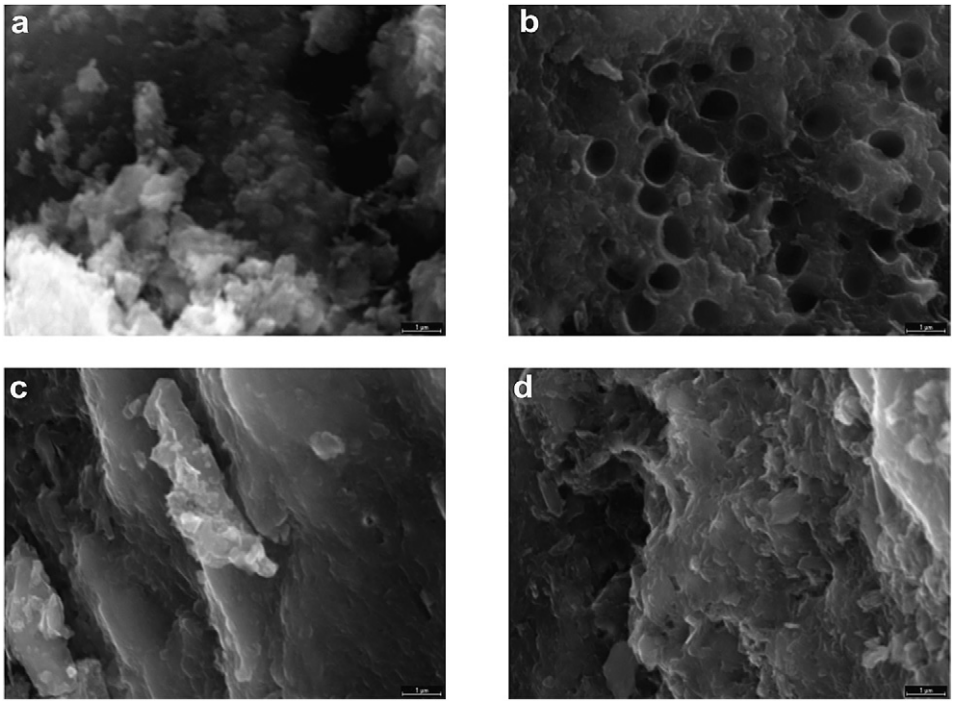


**Fig. 6.** SEM of Neolithic bone (magnification: 10,000×): a) unboiled bone (sample 278); b) unboiled (283); c) boiled bone (471); and d) boiled bone (2013).

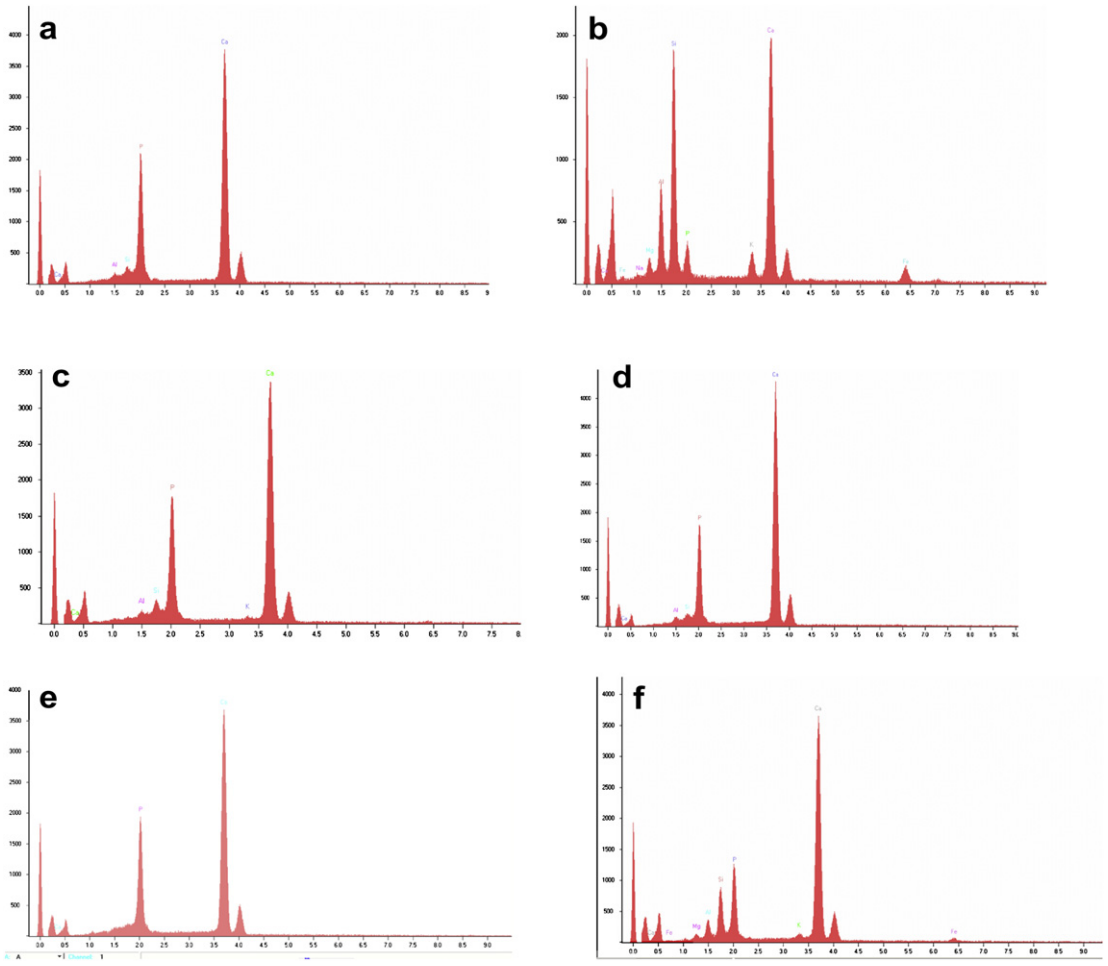


**Fig 7.** EDS of the Neolithic bones: a) sample 278, clear contrast zone, corresponding to spot A2 in Fig. 6a; b) sample 278, dark contrast zone, corresponding to spot A3 in Fig. 6a; c) sample 283, corresponding to spot A3 in Fig. 6b; d) sample 471, corresponding to spot in A in Fig. 6c; e) sample 2013, corresponding to spot A in Fig. 6d; f) sample 2013, corresponding to spot A2 in Fig. 6d.

As expected, crystalline hydroxyapatite was identified in the X-ray diffraction patterns of Neolithic bones, but through EDS analyses it was shown that the corresponding Ca/*P* molar ratio (*ca.* 1.3) was higher than in the contemporary samples, *i.e.*, the composition is closer to a more stable hydroxyapatite such as $Ca_5(PO_4)_3(OH)$, with Ca/*P* = 1.66, than to the less calcified hydroxyapatite $3Ca_3(PO_4)_3.Ca(OH)_2$, Table 2, Fig. 7. Roberts et al. (2002) argue that boiling, in disrupting the mineral–organic

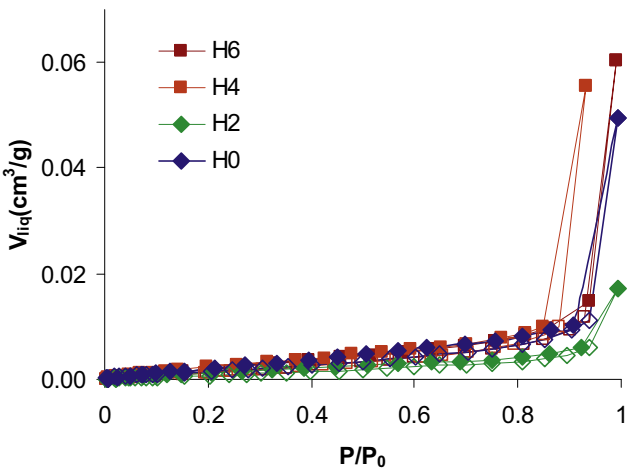


**Fig. 8.** Adsorption isotherms of contemporary samples (H0 = unboiled bone, H2 = boiled for 2 h, H4 = boiled for 4 h, and H6 = boiled for 6 h). Adsorption curve corresponds to filled symbols and desorption to empty symbols.

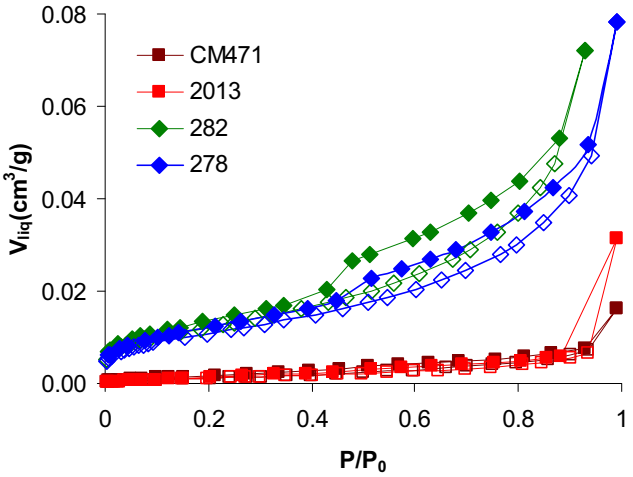


**Fig. 9.** Adsorption isotherms of Neolithic samples (278 and 282 = unboiled bone, 471 and 2013 = boiled bone). Adsorption curve corresponds to filled symbols and desorption to empty symbols.


**Map.** Map Location of the Malalmuerzo cave in Andalucia and map of the cave.

interface and in increasing porosity, might have an important influence on bone survival, but this will be entirely dependent on the duration of cooking. Therefore, increased boiling times could mirror diagenetic effects observed in archaeological bone.

Such variability has to be attributed to contaminants as well as to the solubility of bone in certain conditions. Indeed, bone mineral is much more soluble than synthetic hydroxyapatite. A re-crystallization window of hydroxyapatite between pH 7.6 and 8.1 has been determined, it defines the conditions under which bone crystals dissolve and re-precipitate as a more insoluble form of carbonated hydroxyapatite (Berna et al., 2004). It seems, then, that the Malalmuerzo Neolithic materials have re-precipitated. The observed development of bentonic clays around concretions during pedogenesis has already been reported (Downing and Park, 1998). Such observation confirms the mechanism proposed previously as hydroxyapatite re-crystallizes without filling fluids.

## 5. Conclusion

Bone is a composite material with a hierarchical structure. To explain the present results, a mechanism consisting on the progressive diffusion of degraded collagen is proposed. Residues cover the bone surface and smooth it. Boiling only modifies the organic component of bone and the effect is observable at micron range as a softening and smoothing of the surface which is clearly observed in a 4 h boiled human bone by scanning electron microscopy.

The comparison between boiled and unboiled Neolithic bones confirms the previous proposition and shows that boiling can stabilize the structure against diagenesis because porosity is closed and becomes inaccessible to degrading agents. Using the results obtained with boiled contemporary bones as reference, boiling time in Neolithic bones can be estimated. Two of the Neolithic samples studied in this work were boiled for 2–4 h, the other two were unboiled.

Thus, we have found that the macroscopic criteria to distinguish a boiled bone from an unboiled one are coincident with the microscopic results: the differences are only morphological. Contemporary bones had the advantage to be diagenesis free. In this sense, the comparison with Neolithic bones suggests that diagenesis manifestation, in our case, is circunscribed to fungi attack.

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