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COMPARISON BETWEEN THE PROPERTIES OF "ACCELERATED-AGED" BONES AND ARCHAEOLOGICAL BONES

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ABSTRACT

This study focuses on the changes in the properties of bones that resulted from "heat-ageing" at different temperatures and long term of exposure compared to archaeological samples. It also aims to prepare aged samples similar to archaeological samples for the experimental studies in the conservation of bone artifacts. FTIR, XRD, UV spectrophotometry, dual-energy X-ray absorptiometry, polarizing and SEM microscopes were used as analytical techniques. The results revealed that "heat ageing" technique used at different temperatures (200°C and 300°C) and times (from 1 hour to 13 hours) affected the properties of change in colour, loss of bone density, destruction of the surface morphology, increasing the crystallinity index which, was similar with the archaeological sample after 8 and 12 hours of exposure. The study concluded that "heat ageing" at 300°C after 8 hours can give properties close or similar to archaeological samples.

KEYWORDS: Archaeological bone, accelerated ageing, FTIR, XRD, Spectrophotometer, DEXA, microscopes

INTRODUCTION

Bone consists of two structural components: inorganic mineral, calcium hydroxyapatite Ca_{10} $(PO_4)_6$ $(OH)_2$, and organic material which is made up of collagen, non-collageneous protein, lipids, mucopolysaccharides and other carbohydrates (Goffer 1980; Child, 1995). The mineral and organic phases of bone differ in their response to taphonomic processes. Deterioration of archaeological bones begins immediately between death and final burial. Diagenesis of bones is a combination of physical, chemical and biological processes that alter bone. The characteristics of the soil environment also play an important role in the deterioration or preservation of bone (Cook and Heizer 1953; Ezra and Cook 1957; White and Hannus 1983; Price *et al.* 1992; Child 1995; Tuross and Dillehay 1995; Wilson and Pollard 2002; Collins *et al.* 2002).

"Accelerated-aging" is now vital in experimental studies in order to understand and predict the long-term behavior of materials. "Heat ageing" was used for different purposes such as to gain information regarding the actual decay rates; to generate samples of the actual decay products; and to provide unambiguous analytical data (Kiszely 1973; Bigi *et al.* 1997; Rogers and Daniels 2002; Hedges 2002; Koon *et al.* 2003; Hiller *et al.* 2003; Fantner *et al.* 2004; Pijoan *et al.* 2007). Most of the previous studies focused on short -term exposure (40 minutes) (Fanter et al, 2004), or one and two hours (Rogers and Daniels, 2002) that had been used during heat ageing.

In the last decades, many authors have studied "accelerated aged" and archaeological bone artifacts and animals and human remains using different approaches. Investigations and analyses detected: (1) the changes in the organic matrix (Ortner *et al.* 1972; Fantner *et al.* 2004); (2) mineralogical changes to bone by a series of diagenetic reactions (Wilson and Pollard 2002); (3) isotopic signature of bone, molecular, biochemical structure (Hedges 2002); (4) the reaction of bone to the environmental conditions at the moment of excavation and after long-term exposure after discovering in archaeological sites or after exhibition in museums; and (5) the response to the methods and materials used in the preservation and stabilization (McGowan and LaRoche 1996). Fourier Transform Infrared Spectroscopy (FTIR) was one of analytical techniques that had been previously used to gather information on the composition and crystallinity of the bone mineral (Pleshko *et al.* 1991; Pegnier *et al.* 1994; Schiegl *et al.* 2003; Reiche *et al.* 2003; Fook and Guastaldi 2005; Petra *et al.* 2005; Fuchs *et al.* 2008; and Nagy *et al.* 2008).

X-ray diffraction has been used to determine components and crystallinity of bone and other associated materials (Robles 2002; Meneghini *et al.* 2003; Reiche *et al.* 2003; Fantner *et al.* 2004). Change of colour by spectrophotometer became a useful tool for the measurement of colour change especially in experimental studies (Abdel-Maksoud and Marcinkowska 2000). Bone mineral density and content were measured by dual-energy X-ray absorptiometry (DEXA), which had always been used to measure bone loss (Kreutzer 1992; Broughton *et al.* 2007). Light microscopes or a scanning electron microscope are considered now very good tools to investigate the histology and any abnormal changes in the surface morphology (Wyckoff and Doberenz 1965; McConnel *et al.* 1971; Banks 1980; Stiner *et al.* 1995; Stutz 2002; Farlow and Argast 2006; Chinsamy and Hurum 2006; Odriozola and Pérez 2007; da Cruz *et al.* 2007). Analyses and investigations mentioned above were

very effective in revealing the state of both accelerated and archaeological bone properties.

This study aims to comprehend the changes between the properties of "accelerated-aged" and archaeological bones, in order to assess the conditions of burial of archaeological bones, and to know how to prepare "accelerated-aged" bones similar or close in their properties with the properties obtained from archaeological samples.

MATERIALS AND METHODS

Preparation of modern bone

Bone specimens were prepared from long bones (radius) of sheep. Ages of bones prepared were approximately two years. Bone specimens were boiled in water cautiously cleaned from fat or impurities by using scalpels without either scratching the surface or affecting the surface morphology, and the marrow materials were removed by using dental tools. Prepared bone samples were then left for one week to dry at the room temperature (from 22°C to 25°C)

Archaeological samples

Archaeological long bones of sheep were collected from El-Turra excavation, situated at a distance of 9 kilometers to the north and north-west of El-Ramtha, north of Jordan. This site dates back to different periods (Roman, Byzantine, Umayyad, Abbasid and Mamluk periods) (Mittmann 1979). The chosen bone samples date back to Roman and early Byzantine times.

Heat ageing technique

The prepared bone samples were placed to oven (Heraeus D.63450 Hanau, Type: VT 6130M). Bone samples were divided into two groups, one exposed to 200°C and the other exposed to 300°C. The ageing time was between 1 and 13 hours. After every one hour of "heat ageing" at temperatures used, enough samples were taken for the analysis and investigation in order to follow the changes in properties selected and to determine the suitable temperature and time for preparing samples similar to archaeological samples.

INVESTIGATION METHODS

Fourier Transform Infrared Spectroscopy (FTIR)

Samples from compact bone combined with spongy bone were prepared in accordance with Greene *et al.* (2004): A small quantity of matter (each sample 1 – 3 mg) was collected by carefully scraping the outer sample surface with a steel file. The sample was thin ground to a fine powder with an agate mortar and pestle. FTIR grade potassium bromide (97-99 mg) was ground to a fine powder in a separate agate mortar and pestle. The two powders (100 mg total) were then combined and mixed with a spatula. An additional 100 mg of KBr was ground into a fine powder, and then used to obtain background spectria. The sample was transferred into a sample cup to overflowing, and a cover slip was dragged across the top of the cup to remove excess powder and smoothed the sample surface in order to maintain uniform distribution of particle size. Each sample then mixed with KBr and placed in a DRIFT cell. Absorbance infrared spectra were obtained between 4000 cm^{-1} and 400 cm^{-1} wavenumber. This method of analysis gives information on the composition and crystallinity of the bone mineral, and at the same time gives an indication on the behavior of the protein materials in bone. Spectra were assigned for modern, archaeological and aged samples

after 4, 8 and 12 hours with each group of ageing (200°C and 300°C).

X-ray diffraction

X-ray powder diffraction data were collected on x-ray diffractometer 6000 (Shimadzu, Japan) using Cu Kα radiation from a tube operated at 45 kv and 35 mA. All the samples studied were measured from 0° to 65° 2θ to obtain a general diffraction pattern.

Crystallinity index of modern, archaeological and aged samples was determined using x-ray diffraction (XRD) by the basis of the full width of half maximum (FWHM) of the apatite diffraction 002 (Armelagos *et al*. 1989; Hedges *et al.* 1995; Fratzl *et al.* 1996; Rinnerthaler *et al.* 1999; Zizak *et al.* 2000; Reiche *et al.* 2002; Zizak *et al.* 2003; Danilchenko *et al.* 2004).

UV-Spectrophotometer

The colour coordinates L*a* and b* were determined for each sample. The colour difference (ΔE) between unexposed and heat exposed samples were calculated using CIE 1976 L*a*b* formula (Abdel-Maksoud and Marcinkowska 1999; Abdel-Maksoud and Marcinkowska 2000; Kohara *et al.* 2001; Yatagai *et al.* 2001): $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$, where ΔL^* is the change in lightness, from lighter (+) to darker (-),Δa^* is the change in shade from red (+) to green (-), and Δb^* is the change in shade from yellow (+) to blue (-).

The measurement was made using Macbeth color eye 7000 (U.S.A.) UV-Spectrophotometer.

Dual-energy X-ray absorptiometry (DEXA)

Bone mineral density (BMD) and bone mineral content (BMC of archaeological and modern bone before and after heat ageing at different temperatures and times) were determined by DEXA Norland XR-46 Version 3.6.9. The measurement was in accordance with Dirrigl *et al.* (2004); Carlson and Pickering (2004); Ives and Brickley (2005); but with minor changes. Bone was examined under radiation sources.

The pattern of radiation absorption enabled a measurement of bone mineral content and density. Norland XR-46 (DEXA) measures BMC for each pixel of the scanned image, and then calculates BMD by dividing BMC by the bone area represented by ROI (skeletal elements and portions represented by regions of interest). For aged bone samples, each bone sample was measured two times (before ageing and after ageing at specific temperature and time). Bone density for all aged and archaeological samples was measured at mid shift of bone.

Light microscope

Thin sections were prepared in accordance with Hanson and Cain (2007). Transverse sections were cut using a diamond-tipped saw, then ground by hand to a thickness of approximately 50 mm and polished. All thin sections were examined and studied using transmitted and polarizing light microscopy.

Each section was viewed at several magnifications and digitally photographed. The experimental sections were assessed for evidence of burning and all archaeological sections were assessed for both diagenetic change and evidence of burning.

Scanning electron microscope (SEM)

A scanning electron microscope FEI (Netherlands) Model Quanta 200 Supplement with W/EDAX was used to observe the surface morphology.

RESULTS AND DISCUSSION

Fourier Transform Infrared Spectroscopy (FTIR)

The results are explained as follow:

Organic compound phase (Collagen): It is clear (Fig. 1) that the band at 3337.21 cm^{-1} in the modern sample (control) assigned to a broad band represents (OH) hydroxyl stretching due to intermolecular hydrogen bonding of the hydroxyl group. This band includes multiple bands due to multiple N-H groups (it is primary amides. In the solid state and in the presence of hydrogen bonding, these bands are shifted to about 3350 cm^{-1} and 3200 cm^{-1}) in the molecule, which represent a symmetric N-H stretching. In the archaeological sample, this band shifted to a higher position (3420.14 cm-1). At 200°C, the position of this band increased after 4 hours and decreased after 8 and 12 hours. At 300°C the position of this band increased more than the modern sample. The band at 2928.38 cm^{-1} was assigned to a symmetric C-H (residual alkane group that are found in a very large number of compounds. The C-H stretching vibrations occur in the region 2975-2840 cm^{-1}) stretching of aliphatic groups, and was found in all the samples that were studied. The position of this band was very close to the band of the modern sample, either at lower position (in the case of archaeological sample, at 200°C after 8 and 12 hours, and at 300°C with all ageing times), or at higher position than the modern sample (in the case of "heat ageing" at 200°C after 4 hours ageing). The bands between 3420.12 cm^{-1} and 2924.52 cm^{-1} in the samples are protein characteristics, and the increase or decrease of these bands may give an indication on the expansion or contraction of the protein areas (e.g, the position in the archaeological sample was 3420.14 cm^{-1} and it ranged from 3335.28 to 3381.57 cm^{-1} with heat ageing at 200°C).

Collagen exhibits a series of absorptions from 1659.45 cm^{-1} to 1240.97 cm^{-1}. Band at 1653.66 cm^{-1} (C=O stretching) in the modern sample is assigned to amide I and the position of this band increased with "heat-ageing" at 200°C after 4 and 8 hours (1659.45 cm^{-1}) but decreased after 12 hours (1654.62 cm^{-1}). C=O stretching vibration (amide I) disappeared with "heat ageing" at 300 °C after 8 and 12 hours. Increasing or decreasing of C=O is dependent on the physical state of the sample. In the solid phase , the frequency of the vibration is slightly decreased. The presence of hydrogen bonding is an important contributing factor to this decrease in frequency.

The similarity of the position of this band was noticed in the archaeological and aged samples. The bands at 1541.81 cm^{-1} (NH, CN stretching) in the modern sample are assigned to amide II, which increases with "heat ageing" at 200°C after 8 hours, and at 300°C after 8 and 12 hours. The position of the band decreased in the archaeological sample. The band at 1507.1 cm^{-1} in the modern sample is also assigned to amide II. Amide II disappeared in the archaeological sample, and with "heat ageing" at 200°C and 300°C after all ageing times. The band at 1240.97 cm^{-1} is assigned to amide III which involves C-N stretching and N-H bending. The observed wavenumber of these peaks depends on the secondary structure of the protein (e.g., α-helix, β-sheet, β-turn, random coil). This band appeared only with the modern sample and disappeared with the other aged samples.

The results revealed that most of the positions and intensities of bands of amide I in the "heat aged" samples at 200°C at all ageing times increased more than the

modern sample. Amide I of aged samples at 300°C after 8 and 12 hours disappeared. The changes and disappearance in the FTIR spectra were due to the state of hydration in the proteinaceous materials. Collins *et al.* (2002) reported that at high temperatures, the rate of collagen loss will be accelerated. According to Götherstrom *et al.* (2002), the collagen was influenced by the presence or absence of water.

Centeno *et al.* (2004) classified the changes that resulted from FTIR spectra into two groups: the first group resulted from the influence of the hydrogen bonding of water on hydration sites such as C=O and N-H, resulting in the intensities and positions of the corresponding IR bonds (IR spectra look quite complex because the bond vibrations create absorption bands.

The intensity of an absorption band depends on the change in the dipole moment of the bond and the number of the specific bonds present). The second group was due to changes in the second structure of the protein. Upon removal of water molecules from the vicinity of the peptide linkage, a shift of the amide I band to higher wavenumbers was expected as a result of the combined effect of an increase in the force constant of the C=O bond (bond strength and a decrease in the reduced mass of the C-O vibration in view of the decoupling with O-H. The amide II at 1507.1 cm^{-1}disappeared at 200 °C and at 300 °C during all ageing time.

According to Centeno *et al.* (2004), this may attributed to the force constant of the C-N decreases "stiffness" of the H-N-CO angle due to a higher "mobility" of H through a decrease in the hydrogen bonding in addition to a decrease in the hydrogen bonding and decrease of strength of the C-N bond.

Inorganic phase: FTIR gives accurate information on the mineral matrix ratio and crystallinity. These properties are very important. Crystallinity reflects the mineral crystallite size and degree of crystal lattice order. For the inorganic part of the studied bone samples, band at 1455.99 cm-1 in the modern sample is assigned to carbonate (-CO_3) type A. The position of this band decreased or increased in the samples studied. The band at 1418.39 cm^{-1} in the modern sample is assigned to carbonate (type B), COO^- and –CO_3. This band was stable with heat ageing technique, and only decreased with heat ageing at 200°C after 8 hours.

The band at 871.667 cm-1 relates to carbonate (-CO_3). The strongest bands appeared at 1028.84 cm^{-1} (plus or minus) and are assigned to (PO^{3-}_4) symmetric stretch, which are mainly from hydroxyapatite. The band at 603.61 cm^{-1} and the band at 562.148 cm^{-1} (plus or minus) are assigned to (PO_4^{3-}) (antisymmetric phosphate).

The carbonate/phosphate ratio (C/P) was calculated using the peaks at around 1418.39 cm^{-1} (CO_3^{2-}) and 1028.84 cm^{-1} (PO_4^{3-}) (Koon *et al.* 2003). The carbonate ratio was higher than the phosphate ratio in the modern (1.378), archaeological (1.370), and aged samples at 200°C (1.337, 1.337 and 1.376) and 300°C (1.376, 1.376, 1.379).

The data obtained from FTIR stated that "the accelerated heat ageing" especially at 300°C after 8 and 12 hours of exposure led to degradation of collagen (loss of amide I, amide II and amide III) which, was similar to collagen degradation obtained from the archaeological sample. There was similarity in the ratio of carbonate/phosphate between aged samples (at 300°C after 8 and 12 hours of exposure) and the archaeological sample).

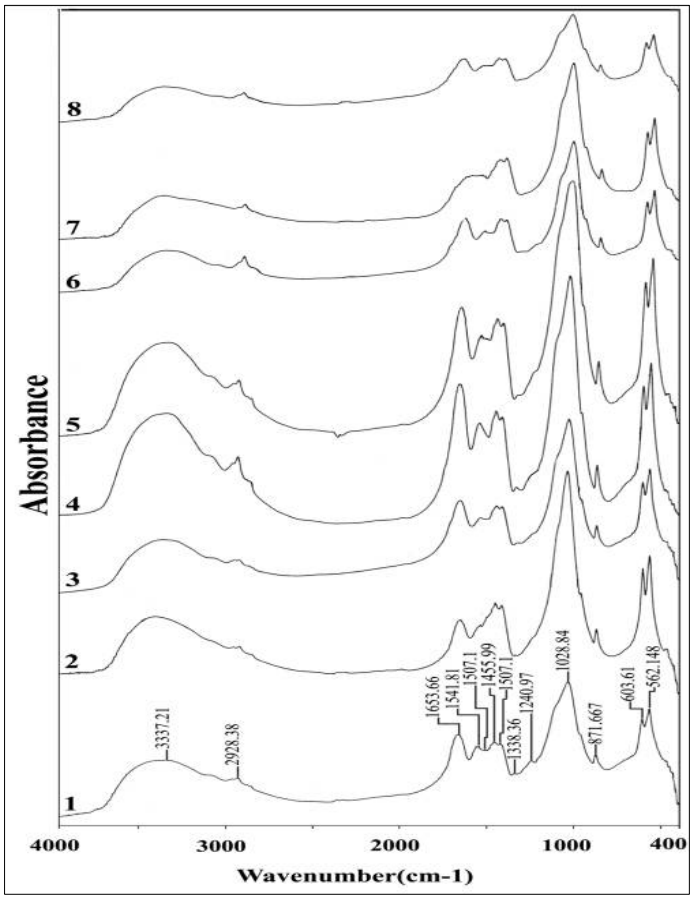


Fig. 1: FTIR spectra of the modern, archaeological and "heat-aged" samples: (1) Modern sample, (2) Archaeological sample, (3) "Heat-aged" sample at 200°C after 4 hours, (4) "Heat-aged" sample at 200°C after 8 hours, (5) "Heat-aged" sample at 200°C after 12 hours, (6) "Heat-aged" sample at 300°C after 4 hours, (7) "Heat-aged" sample at 300°C after 8 hours, (8) "Heat-aged" sample at 300°C after 12 hours

X-ray diffraction

The main aim of using XRD in this study is to measure the crystallinity index of bone. Sillen (1989) explained that bone mineral is generally described as having poor "crystallinity". He also defined crystallinity as a term, which connotes both large size and the absence of structural defects, qualities which tend to be found together.

In this technique the crystallinity index measurement by the width at half-maximum of the 002 reflection (Fig. 2) was used. This method of measurement is considered as the most commonly used. All samples examined generally present characteristic X-ray diffraction patterns typical of poorly crystalline hydroxyapatite, since results showed that there is little difference between the modern sample and aged samples. The crystallinity index of the modern sample was 0.30 mm. The crystallinity of the peak (002) reflection that was obtained after 4, 8 and 12 hours of "heat ageing" at 200°C and 300°C were 0.15, 0.12, 0.11 cm (at 200°C) and were 0.14, 0.12 and 0.10 cm (at 300°C respectively). It was noticed that the highest crystallinity index of the peak (002) diffraction was obtained after 4 hours with heat ageing at 200°C and 300°C. The increase in the crystallinity index after 8-12 hours was very small compared to the aged samples after 4 hours. The crystallinity of the peak 002 of archaeological sam-

ple was 0.11 cm. It was clear that the crystallinity was equal between the archaeological sample and "heat ageing" at 200°C after 12 hours, and approximately at 300°C after 12 hours.

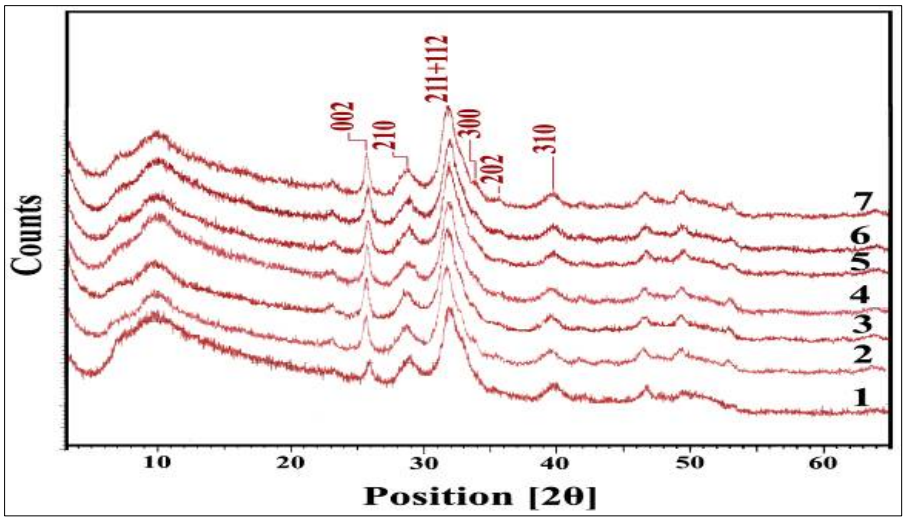
For the analysis of bone by X-ray diffraction, the following observations were noticed:

- Very small shift of the peak (002) position to lower 2θ for all aged samples compared to the modern sample. This may be due to the ageing of bone which led to the loss of water at 200°C and partially or totally decomposing the organic matter at 300°C.

- These results agreed to a far extent with Rogers and Daniels (2002); Fanter *et al*. (2004) and Pijoan *et al.* (2007). They reported that temperature up to 200°C did not result in a change in the fraction of organic material, which was lost above this temperature.

- The relative intensity of peaks diffraction (e.g. 002, 211, 202) of the "heat-aged" samples at 200°C and 300°C increased little more than the modern sample. This might have been attributed to the loss of water and collagen, where the apatite became more crystalline in the course of "heat aged" samples. The relative intensity of archaeological samples decreased compared to the modern and "heat-aged" samples.

Fig. 2: XRD diagrams of modern and "heat aged" bones at different temperatures: (1) Modern sample, (2) 200°C after 4 hours, (3) 200°C after 8 hours, (4)) 200°C after 12 hours, (5) 300°C after 4 hours, (6) 300°C after 8 hours, (7) 300°C after 12 hours



X-ray diffraction was also used to determine compounds of the archaeological sample and the burial soil sample.In the archaeological bone sample (Fig. 3A), calcium carbonate was found with hydroxyapatite. Visual assessment by critical eye observation, the porosity of the archaeological sample increased compared to the modern sample. According to Reiche *et al.* (2003), this might have been caused by the degradation of the organic matter and its leaching induced an increase in bone porosity, which allowed the pore water contained to dissolve chemical species and to penetrate more deeply into the bone structure and to be in contact with a large available surface of apatite crystals. The analysis of the burial soil of the archaeological bone by XRD showed that the main components of soil are calcite as well as quartz, which indicates

that the burial soil is calcareous soil, rich in calcium carbonate. The author supposes that the burial soil of studied bone may have fluctuated water content. Reiche *et al*. (2003) also reported that the water content fluctuation enables the trapping of sediment particles in bone like quartz and calcium carbonate precipitates. These particles at the surface of the bone pores could act as diffusion barriers that hamper water penetration and therefore may limit bone deterioration to a certain extent.

The results obtained proved that the "accelerated heat ageing" at 200°C and 300°C after 12 hours of exposure gave crystallinity similar with the crystallinity obtained from the archaeological sample. Calcium carbonate was the major compound in the archaeological bone sample and in the burial soil sample.

UV-Spectrophotometer

"Heat ageing" cycles changed the color of bone samples. The changes in color of unexposed and exposed and archaeological samples at different temperatures were shown in Table 1. Based on the results, the "heat ageing" cycle changed the color of bone samples. The following observations were noticed:

- The L scale measures lightness, and varies from 0 (black) to 100 (perfect white). Lightness (L – value) decreases as the ageing time increases. The lightness was reduced by 61% at 200°C and 89% at 300°C after 13 hours of exposure, which means that the color became darker. A reduction of 30% was measured in the archaeological sample in comparsion to the modern sample (control).

- The a-scale measures red-green; +a means more red and –a means green. For the a – value, the color of the archaeological sample and the color of "heat-aged" samples were red. The red color of "heat-aged" samples increased with the increase of ageing time indicating that the color of the samples changed towards red during the ageing process. Regarding to the temperatures used, the a – value of samples aged at 200°C with different ageing times was higher than the aged samples at 300°C. The a – value increased in the archaeological sample.

- The b-scale measures yellow-blue, +b means more yellow and –b means more blue. The yellow color (b – value) of aged samples at 200°C increased more than the modern sample. The yellow color of the aged samples at 300°C decreased in comparison to the modern sample. The b – value increased in the archaeological samples.

- The color difference (ΔE*) between the unexposed and the heat exposed samples increased with time of exposure. ΔE* of the archaeological sample increased in comparison to the modern sample. A highly larger discoloration was associated with "heat-ageing" technique. The color of the aged samples at 300°C became progressively darker and the colour difference increased more markedly than the aged samples at 200 °C.

- All samples at 200°C showed a gradual darkening of their color to dark brown; at 300°C, there was a striking, transient shift in the color of all samples from brown to black. Walker *et al.* (2008) explained that this is presumably the pyrolytic (charring) temperature at which of the non-carbon elements of the organic components of bone disassociate, leaving only carbon.

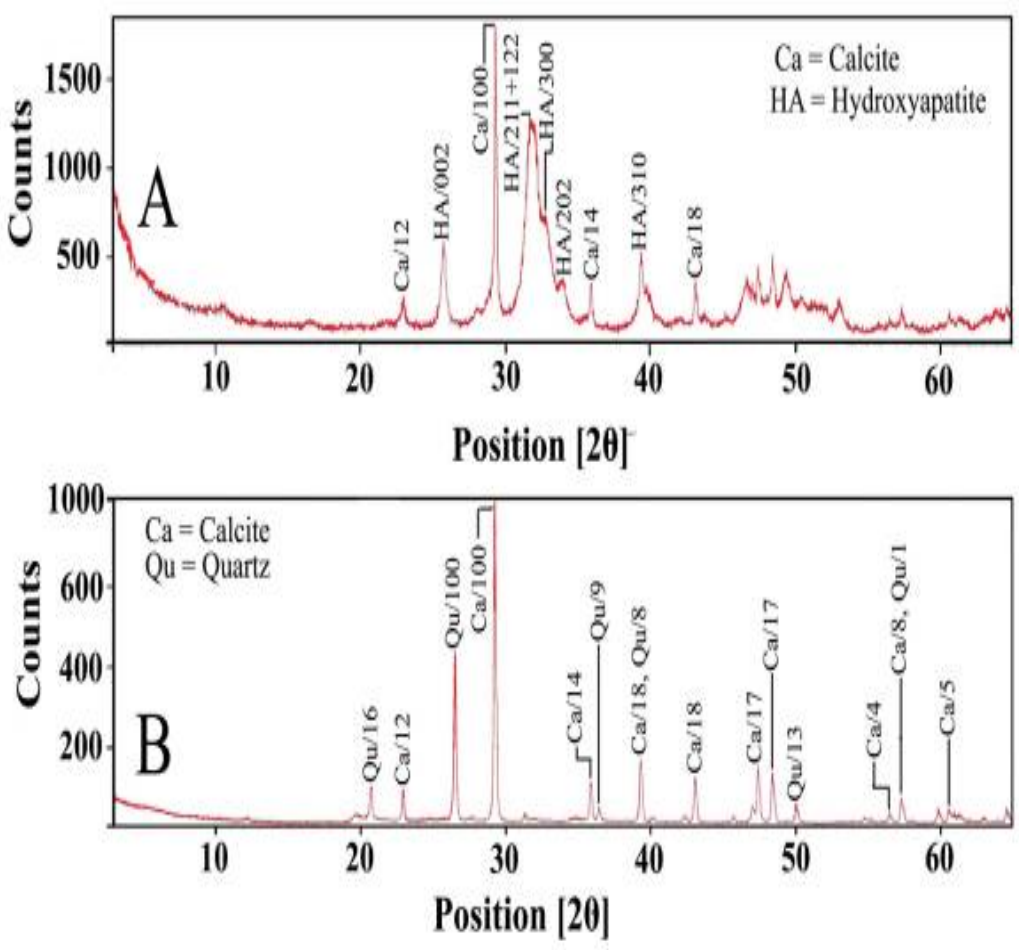


Fig. 3: XRD diagrams of archaeological and soil samples: (A) Archaeological sample, (B) Soil sample

- The results showed that the higher change in color values and the total color values was obtained after 1 hour of exposure to "heat-ageing". This may due to that heat caused initial shock to bone and provide energy to break the chemical bonds of the different chemical compounds of bone.

- The change of color of the studied samples is attributed to the energy (dependent on temperature and time of ageing) and air in particular oxygen which, is a vital ingredient of the chemical reactions occurring in bone as previously mentioned by Walker *et al.* (2008) and Arroyo *et al.* (2008).

Shipman *et al.* (1984) burned sheep and goat bones, and found that the color changed from white at 20°C, to pale yellow at 285°C, and the color became a pale bluish grey at 940°C. Pijoan *et al.* (2007) argued that the reddish brown color may represent bone that decomposed in the presence of flesh, as well as the presence of minerals in the soil, where the presence of iron salts infiltrate the bones, dyeing them red or dark brown due to the presence of manganese. In the present work, the dark brown color was obtained from heat ageing at 300°C after 13 hours, although Shipman *et al.* [64] stated that these colors could be obtained at temperatures from 525 – 645°C after short time.

Table 1: Colour changes of modern, aged and archaeological bones

Temperature (hours)	"Heat-ageing" at 200°C				"Heat-ageing" at 300°C			
	Color values			Total color difference	Color values			Total color difference
	L	a	b	ΔE	L	a	b	ΔE
Control	89.7	-1.5	11.2	0.0	90.3	-1.2	10.8	0.0
1	78.2	3.0	31.1	23.4	43.1	0.6	4.6	47.6
2	75.7	5.0	29.2	23.7	35.4	0.9	4.0	55.4
3	69.8	6.4	28.0	27.2	34.3	1.3	3.5	56.5
4	65.8	7.3	27.8	30.4	33.1	2.5	3.1	57.8
5	63.4	8.5	27.5	32.4	31.1	3.2	2.6	59.9
6	54.5	10.3	27.0	40.3	27.6	4.7	2.3	63.5
7	53.5	10.5	26.2	41.0	25.2	5.2	2.0	66.0
8	52.5	10.9	25.2	41.6	24.3	5.7	1.8	67.0
9	50.4	11.5	24.7	43.5	21.4	6.1	1.5	70.0
10	44.2	12.3	23.5	49.1	18.5	6.8	1.2	72.9
11	41.7	12.8	20.8	51.0	15.7	7.3	1.0	75.7
12	38.6	13.2	20.2	54.0	13.4	7.9	0.8	78.1
13	35.3	14.0	16.3	56.8	10.1	8.4	0.5	81.4
	Archaeological sample							
	L	**a**	**b**	**ΔE**				
Sample 1	61.5	15.1	36.0	41.4				

The data obtained showed that "the accelerated heat ageing" led to the darkness of bone samples. L-value of "accelerated heat aged" samples after 5 hours of exposure at 200°C gave lightness close to the lightness obtained from the archaeological sample. The reduction of "heated aged" samples at 300°C after 1 hour was higher than the reduction obtained from the archaeological sample. a-value and b-value of "heat aged" samples at 200°C and 300°C increased with increasing ageing time. a-value of "heat aged" sample at 200°C after 13 hours of exposure was approximately similar to a-value obtained from the archaeological sample. Changes in the total color difference of "heat aged" samples at 300°C were higher than changes obtained from "heat aged" samples at 200°C and the archaeological sample.

Dual-energy X-ray absorptiomtry (DEXA)

The main aim of this measurement is to take an account on the behavior of bone with "accelerated heat-ageing" technique at different temperatures. It was clear from the data obtained (Table 2) that "heat-ageing" lead to bone loss. Bone mineral density (BMD) and bone mineral content (BMC) decreased after ageing compared to the modern sample before ageing. The results revealed that the bone mineral density of the archaeological sample reduced in comparison to the

other studied samples. The result of the bone mineral content of the archaeological sample was very close to the results of bone mineral contents of some aged samples especially at 300°C.

The results also showed that there was a reduction in the dimensions of the aged samples. It was found by measurement of bone mineral content and bone mineral density as well as area that the values of some samples with long time of exposure were lower than other samples exposed to shorter time at the same temperature. This means that the accuracy and precision of this method is varied depending on the scan site.

These results were confirmed by the study of Hellström (2007), which concluded that DEXA gives many advantages for measuring mineral density and mineral content of bone, although the accuracy of DEXA is about 5-8% and the precision is approximately 1-2%. The measurements are affected by the size and shape of the bone, hence larger bones always appear more density compared to smaller ones.

The variation of accuracy and precision according to scan site is probably the result of variation of the thickness of soft tissue that covers the centre of the bone being studied. According to Dirrigil *et al.* (2004), the variation in the BMD and BMC measured by DEXA might be due to the disadvantages of DEXA measurement. These disadvantages include inability to distinguish flat, mineral rich from thick, mineral poor bone ambiguity between elements of varying shape and dependency on bone orientation during scanning. The precision of DEXA measurement is dependent also on the position of the animal and region of interest (ROIs) as defined (Butler and chatters 1994). These results were also confirmed by Symmons (2004), who said that where measurements areas are large or linear, bone thickness is likely to vary and becomes problematic.

Dirrigl *et al.* (2004) found that the vertebrate BMD varies intra – and interspecifically with age, sex, nutrition and genetics. Ives and Brickley (2005) explained that with increased age an imbalance in the process of bone remodeling results in loss of bone. The structural changes caused by age – related bone loss differ between cortical and trabecular bone. Cortical bone loss in the long bone can be identified through the increasing width of the medullary cavity indicating a thinning of the cortical walls.

Age the trabecular bone in the vertebrae suffer a depletion in the number of vertical plates with subsequent thickening of the remaining vertical struts, although not enough to compensate for the original loss of bone. Horizontal trabeculae are thinned and connective struts in the trabecular framework are frequently broken resulting in 'free-end's. Excessive resorption can also perforate the trabeculae removing the existing bone surface and preventing the osteoblasts from laying down new bone.

It was found from the data obtained that the "accelerated heat ageing" led to reduce bone mineral density and bone mineral content. Bone mineral density of the archaeological sample was higher than bone mineral density of "heat aged samples" at temperatures used during all ageing times. Bone mineral content of the archaeological sample was close to bone mineral content of "heat aged" samples especially at 300°C.

Table 2: Measurement of bone mineral density (BMD) and bone mineral content (BMC) of Aged and archaeological samples

Ageing time (hours)	BMD g/cm²	BMC g	Area cm²	Length Cm	Width cm
	Archaeological sample				
Arch	0.3375	1.418	4.200	1.90	2.80
Samples at 200 °C before and after accelerated-ageing at different temperatures					
Before (3 hours)	0.6185	2.320	3.751	2.40	2.10
After (3 hours)	0.6179	2.132	3.450	2.20	2.40
Before (6 hours)	1.021	6.228	6.101	3.60	2.20
After (6 hours)	0.9696	5.050	5.209	2.80	2.30
Before (9 hours)	0.7686	4.420	5.750	3.50	2.30
After (9 hours)	0.7580	3.534	4.661	2.60	2.70
Before (12 hours)	0.7474	2.501	3.347	1.60	2.60
After (12 hours)	0.7473	1.835	2.456	1.20	2.50
Samples at 200 °C before and after "accelerated-ageing" at different temperatures					
Before (3 hours)	0.6811	1.871	2.747	1.70	2.00
After (3 hours)	0.5993	1.585	2.644	1.40	2.40
Before (6 hours)	0.9781	5.389	5.510	3.30	2.10
After (6 hours)	0.8988	3.896	4.335	2.30	2.60
Before (9 hours)	0.5731	1.592	2.778	1.40	2.40
After (9 hours)	0.5650	1.491	2.639	1.30	2.40
Before (12 hours)	0.8011	2.165	2.703	1.20	2.90
After (12 hours)	0.7047	1.510	2.143	0.90	2.40
	Archaeological samples				
Sample 1	0.3375	1.418	4.200	1.90	2.80

Light microscope

The use of light microscope became a vital tool for histological examination either under transmitted or polarized light. It also gives many details about the changes in the structure of bone. The interpretation for histology and structure of bones studied was established after Hammersen 1980; Halluche and Fauger 1986, Hedges *et al.* 1995.

The results revealed that the structure of the modern bone sample demonstrated the subdivision into smaller fiber bundles by strands of loose connective tissue (Fig. 4A). The modern sample showed high fraction of trabecular surface covered by osteoid seams, high osteoid-osteoblast interface, and high bone-osteoclast interface with appearance of tunneling resorption (Fig. 4B). Under Crossed Nichols of polarized light microscope (Bone appears dark and bright with different colours) (Fig. 4B) shows yellow 1st interference color in association with the grey 1st order interference color (which were either related to thick-

ness variation or to mineralogical variation). It also illustrated two phases of apatite, one amorphous and isotropic (cellophane or amorphous uncrystalline apatite), and the other completely crystalline of longitudinal internal structure. It was also clear from the modern sample (Fig. 4C) that the birefringence of lamellar bone typically appeared dark and bright when observed under crossed Nichols of the polarizing microscope. In the case of Haversian systems (also so-called osteons), which is the basic structural unit of adult compact lamellar bone, birefringence appeared as a "maltese cross" (Guarino *et al.* 2006).

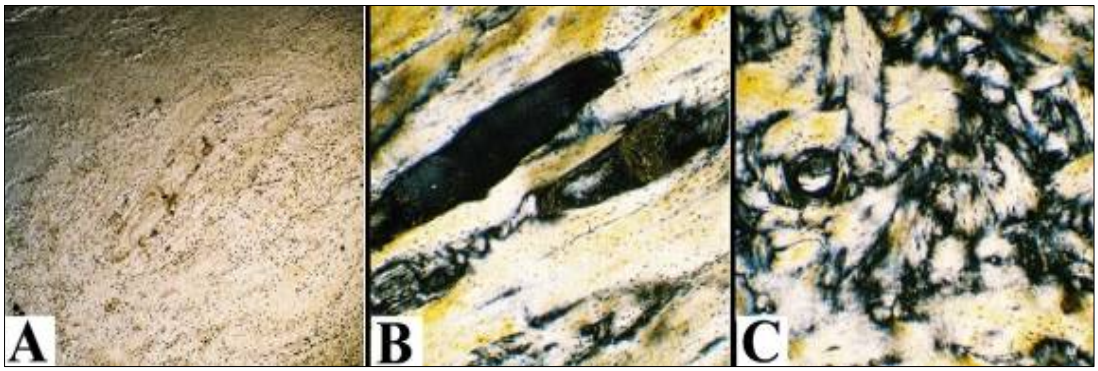


Fig. 4: Modern sample (control): (A) A cross-section of modern bone sample observed under transmitted light, magnification 40. (B) Crossed Nichols under polarized light, magnification 100. (C) Crossed Nichols under polarized light, magnification 40

Archaeological sample showed numerous Haversian systems, each of which contained several osseous lamellae concentrically arranged around a circular opening (An Haversian central canal running longitudinally in the center of an osteon of mature compact bone. It contains blood and lymphatic vessels and nerves). It also showed the alteration of the surface of bone to the black or grey altered surface, and the surface appeared coarse (Fig. 5A). Under crossed Nichols of the polarizing microscope, the birefringence was completely absent, since the osteon in the center and osteons in the surrounding were completely dark. It also showed alteration by calcite within the white longitudinal fibrous matrix. The sample showed a widespread crystal formation showing confluence within the mineralized bone and osteoid (Fig. 5B). Relics of the yellow and white minerals within the grey 1st order were evident, but the Haversian systems were not recognized. The color of the specimen was white-blue color. This result was confirmed by Hanson and Cain (2007) who reported that the archaeological bone surface of some intensely heated bone surface did turn white and shades of light blue. They also said that in thin section these bones displayed white edges and a lack of histological structure with wide cracks at the edges. They confirmed that these seemed to be a reliable indicator of very intense heating rather than specifically high temperature. The changes in colour and surface textures do not necessary come from the effect of heat at high temperature. Nicholson (1993) argued that surface texture and colour changes can occur on unburned bones because of alteration by weathering and fossilization. The grey-blue colour may be due to heating and digenetic changes – such as modification by pathologies and destruction by microbial action (Hanson and Cain 2007).

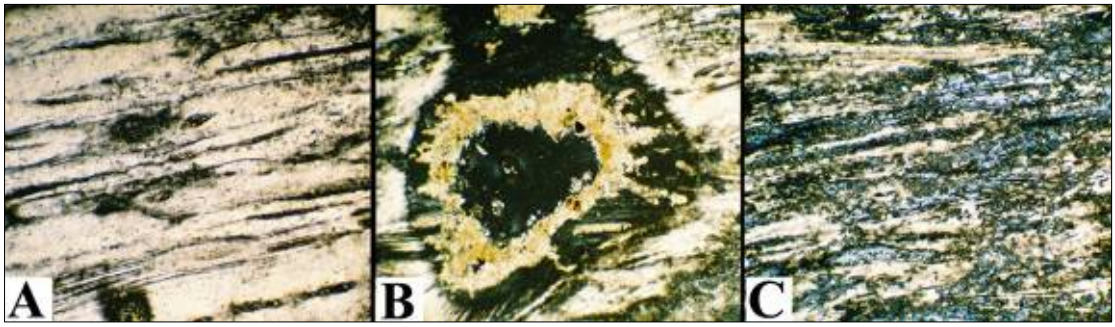


Fig. 5: Archaeological sample: (A) Cross Nichols light microscopy, magnification 100. (B) Cross cutting under polarizing microscopy, magnification 100, (C) Cross Nichols light microscopy, magnification 100

"Accelerated-aged" samples at 200°C after an exposure of 5 hours showed numerous Haversian systems as is the case of the archaeological sample, but the color was darker and the surface was smooth (Fig. 6A). The lamellar bone typically appears dark and bright as observed under crossed Nichols of the polarizing microscope. Birefringence appeared as a "maltese cross". All the osteons of the microscope view field appeared completely dark, and the darkness of the bone was due to ageing process (Fig 6B). It also showed a pseudo-oolitic structure. After 9 hours of exposure, the sample showed the appearance of more apatite of grey 1st order and the darkness of color was also noticed. This photo revealed the osteocytes as dark dots wedged in between the osseous lamellae and aligned into concentrical circular lines. Remnants of former Haversian systems formed the interstitial lamellae filling the spaces between the osteons (Fig. 6C). It also illustrated (Fig. 6D) that the Haversian systems became completely black, and could not be recognized, in addition to the spread of black color through the bone surface, with only a few bright areas. After 13 hours of exposure at the same temperature increase in the black color was noted, the Haversian systems could not be recognized, and the sample showed the alteration of the longitudinal structure into disrupted grey order structure (Fig. 6E). The sample also showed close up details, with most of bone turning black. Much deformation in morphology and structure was noticed in the sample (Fig. 6F).

For "accelerated-aged" samples at 300 °C after 5 hours of exposure, the accumulation of carbon inside the Haversian system and lacunae of bone were noted, which indicate the decay of organic materials in bone (Fig. 7A). Hanson and Cain (2007) confirmed these results as the deposition of carbon in the spaces throughout the matrix, which was a very good indicator of burning process. It was also noticed that the carbon accumulation began at the edges of the samples, accentuating the lamellate structure, and continued inwards as burning intensity increased, depositing carbon throughout the entire structure and sometimes rendering the section opaque. Very thin cracks were noticed, probably due to post-depositional burning. The sample (Fig. 7B) showed turning the color to yellow – red and dark red or brownish. The color of the sample was non-homogeneous, and very small holes with white color in different places were noticed with some deterioration of morphology and structure as a result of temperature.

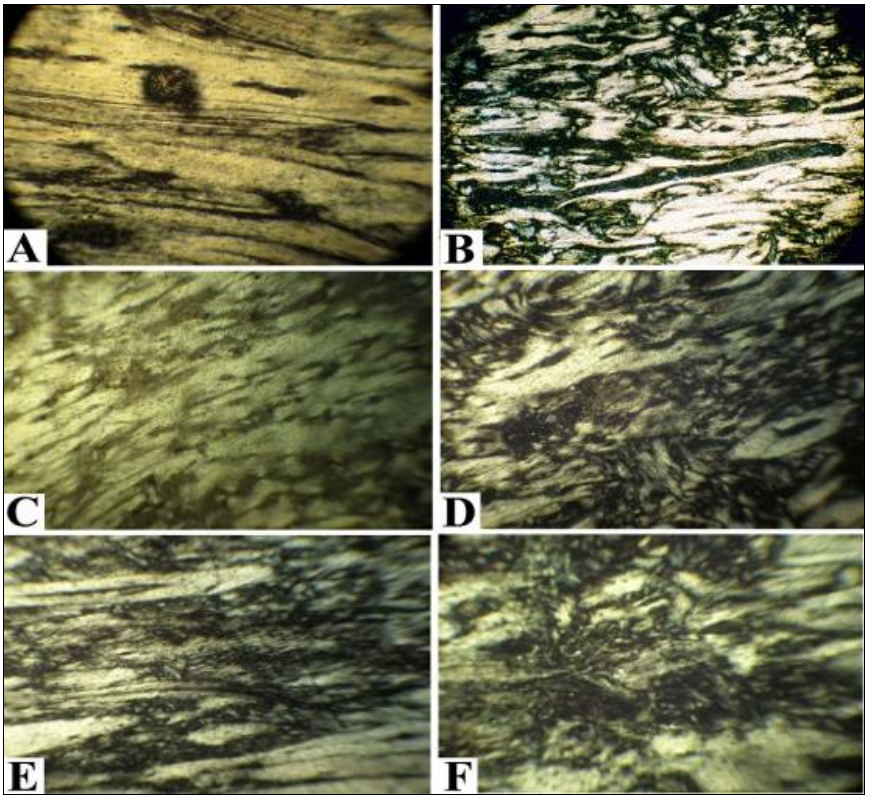


Fig. 6: "Accelerated-aged" samples at 200 °C with different exposure times: (A, B) After 5 hours, magnification 40, 40, (C, D) After 9 hours, magnification 40, 100, (E, F) After 13 hours, magnification 40, 100

"Accelerated-aged" bone after an exposure of 9 hours at 300°C (Fig. 7C) showed that the color changed from pale brown to dark brown in some parts of the photo. Schiegl *et al.* (1996) reported that the colour of the burned bones in thin section varied from pale brown to dark brown and deep black. The white holes were larger than the previous exposure time.

The sample also showed (Fig. 7D) more darkness in color. The lamellate and the Haversian system was white in color. The edges ranged from dark brown to black colour. After 13 hours of exposure, bone sample (Fig. 7E) illustrated advanced state of histological deterioration. The colour ranged from red to dark brown and became black in most of the sample. Shipman *et al.* (1984) found that some surfaces changed and colour occurred while using heated bone, where the first indication of burning appeared between 185°C and 285°C. The same sample (Fig. 7F) showed changes in the surface texture (big erosion in Haversian canal which became wide and the surface became coarse) and color. The colour ranged between dark brown and black and the spreading of white colour was noticed. Carbon deposition still occured in some areas of the bone; entirely calcined, while white color and loss of structure were noticed as previously mentioned by Hanson and Cain (2007).

Hanson and Cain (2007) said when burned, the bone surface first darkens (typically around 400 – 500°C after short time of exposure), then carbonizes turning black (typically around 400 – 500°C after short time of exposure), and once the organic components are burned, the bone

becomes grey to grey-blue in colour (at 600 – 900°C). The present study differs than the results given by Hanson and Cain.

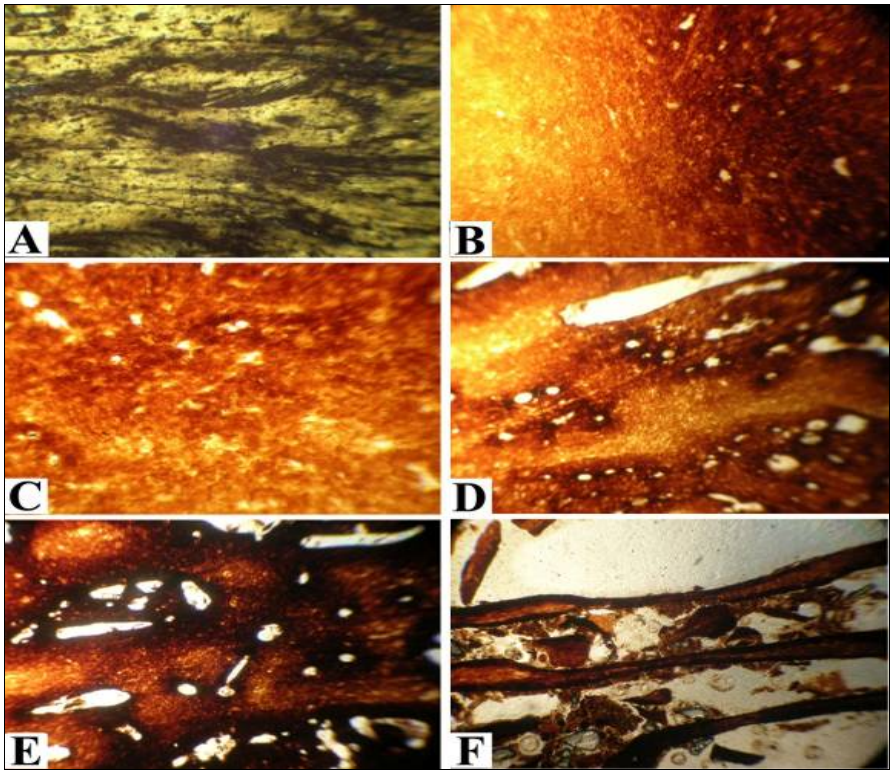


Fig. 7: "Accelerated-aged" samples at 300 °C with different exposure times: (A, B) After 5 hours, magnification 200, 200, (C, D) After 9 hours, magnification 40, 40, (E, F) After 13 hours, magnification 40, 100

It was clear from this study that the darkness in color and carbonization (turning black) depended not only on the temperature used but also on the duration of exposure at temperatures used (200°C and 300°C). The color changed to black at 200°C after 9 and 13 hours of exposure. It was noticed that the long duration of exposure especially at 300°C gave high degradation and the histological structure could not be recognized. Wegweiser (2006) reported that at temperatures from 105°C – 300°C, water was desorbed and evaporated from the surface of the bone. During dehydration, bubbles appeared in the bone external laminae and the bone crashed. However, the cortical bone structure still remained visible. The lacunae expanded and became tightly packed, and the surface was scabrous as temperature rose up to 285°C. Upon reaching temperatures of 285°C to 600°C, the bone surface turned glassy and flushed while an increase in material hardness and brittleness was evident.

The long time of exposure gave high degradation in the histology and structure of bone. This degradation may be equal to the degradation resulting from temperatures higher than 300°C. These results were confirmed by Stiner *et al.* (1995) who found that at temperature below 650°C, the changes occurred in the crystals of heated bone which were probably similar to those occurring at low temperatures, but above this temperature, a solid state of recrystallization occurs.

The results obtained showed that "Accelerated heat aged" samples at 300°C after 9 hours of exposure gave degradation in the histological structure higher than "heat aged" samples at 200°C after 12 hours. The histological structure of the archaeological sample suffers from degradation more than "heat aged" samples at temperatures used.

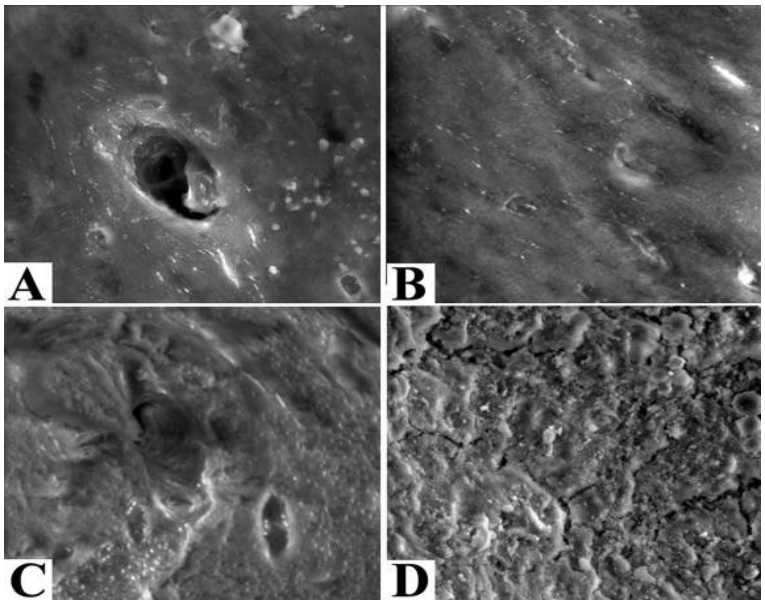


Fig. 8: Scanning electron micrographs illustrate modern sample (A) Magnification 1200, (B) Magnification 2000, and archaeological sample (C) Magnification 1200, (D) Magnification 2000

Scanning electron microscope

The investigation of the surface morphology of bone using scanning electron microscope gave more details about the state of the surface. In the modern bone (Fig. 8A), the Haversian system was clearly seen, which disposed in concentric laminae or sheets around an osteonal canal or Haversian canal. It was noticed that the peripheral limits of the osteonal bone were marked by a reversal line or cement line. Osteon in its simplest form is a long cylinder with a hollow cavity, where the osteonal canal is in its center. Modern bone (Fig. 8B) also showed a very smooth surface, and that the distribution of the Haversian systems was in many parts of bone. For the archaeological sample (Fig. 8C), the results showed Haversian systems in many places of the surface.

The Haversian systems are not arranged as in modern bone. The surface was coarse and some pittings were noticed. The equality of the flattening surface was absent. It was clear that archaeological sample suffered from deterioration (Fig. 8D), and some cracks and some soil crystals were noticed.

For "heat-aged" samples at 200°C after 5 hours of exposure (Fig. 9A), the Haversian systems were noticed but with little destruction. The surface was coarse and the equality in the surface flattening could be recognized in most of the micrograph. After 10 hours of exposure at the same temperature (Fig. 9B), the destruction increased and the Haversian systems could not be identified. The surface flattening was absent in most of the surface.

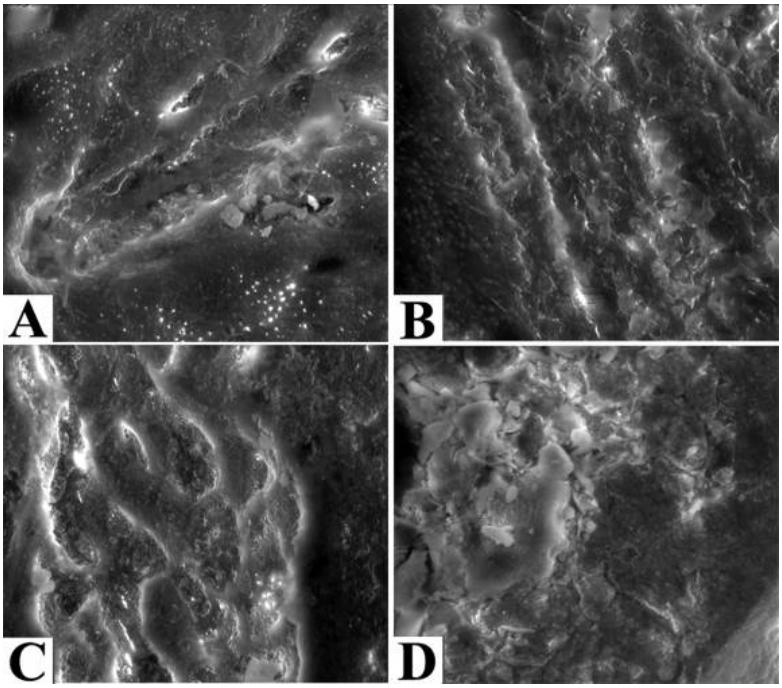


Fig. 9: Scanning electron micrographs illustrate "accelerated-heat-aged" samples at different temperatures and exposure times, Magnifications 2000 (A) At 200 °C after exposure 5 hours, (B) At 200 °C after exposure 10 hours, (C) At 300 °C after exposure 5 hours, (D) At 300 °C after exposure 10 hours

For "heat-aged" samples at 300°C after 5 hours of exposure (Fig. 9C), many types of erosions and many zigzags were noticed on the surface. After 10 hours of exposure at the same temperature (Fig. 9D) deformations and cracks were noticed. The equality in the surface flattening was absent.

It was clear from the data obtained that both "heat aged" samples and the archaeological sample suffer from the degradation of the surface morphology, but the surface morphology of the archaeological bone degraded more than "heat aged" samples.

CONCLUSIONS

"Heat-ageing" affects the collagen especially amide II and amide III, but it did not give any significant change in the carbonate and phosphate distribution, since the carbonate ratio was more than the phosphate ratio. "Heat-aged" samples were similar to the archaeological sample regarding crystallinity of the inorganic phase. By XRD analysis: the relative intensity of "heat-aged" bones increased and the archaeological sample decreased compared to the modern sample, but the crystallinity index measurement by the width at half-maximum of the 002 reflection of aged samples was similar to the archaeological samples. Archaeological bone had been buried into calcareous soil; and contamination with calcite was a problem. In cases were soil is considered alkaline, archaeological bones are preserved.

"Accelerated-heat-aged" bones gave variation in the degradation of the histological structure of aged bones. These variations ranged between accumulation of carbon, where the histological structure was still recognized, and the heated bone with different colors which ranged from pale yellow, red, brown, dark brown and

black at 300°C, especially after 9 and 13 hours of exposure.

Entirely calcined bones, white color and loss of structure were also noticed. Most of the properties obtained from "heat-ageing" as an individual factor after 8 hours of exposure especially at 300°C were close or similar with the same properties obtained from archaeological samples. This means that the ageing technique deteriorated the bone, but at the same time the properties were still recognized. Ageing technique succeeded in preparing aged samples that can be used in experimental studies for the evaluation of conservation methods and materials.

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