JOURNAL OF FORENSIC SCIENCES

J Forensic Sci, May 2011, Vol. 56, No. 3
doi: 10.1111/j.1556-4029.2010.01690.x
Available online at: onlinelibrary.wiley.com

PAPER

ANTHROPOLOGY

Sophie Beckett,[1] *Ph.D.; Keith D. Rogers,*[1] *Ph.D.; and John G. Clement,*[2] *Ph.D., B.D.S.*

Inter-Species Variation in Bone Mineral Behavior upon Heating*,†

ABSTRACT: The characterization of inter-species variation in bone mineral (b-HAP) is of relevance to forensic science and archeology, but has not previously been widely explored. Results of an investigation into unheated bone mineral and behavior of bone upon heating for 12 animal species (including human) demonstrate that b-HAP characteristics, quantitatively measured using X-ray diffraction (XRD) analysis, exhibit significant inter-species variation. Human bone was found to be significantly different to all other species in terms of b-HAP lattice parameter values from unheated and heated bone and in terms of recrystallization behavior of b-HAP upon heating bone to 600°C. The amounts of b-HAP thermal decomposition products were also significantly different for human bone heated to 1400°C compared to those obtained for most other species. Therefore, there is potential for the development of an XRD-based method of species identification, particularly one that distinguishes human from non-human bone.

KEYWORDS: forensic science, forensic anthropology, bone mineral, heated bone, species identification, X-ray diffraction

Bone mineral (b-HAP) is perhaps the most studied natural mineral and numerous characteristics of b-HAP have been investigated over many years, across a wide range of disciplines for a variety of research goals (1). The composite nature of bone consisting of inorganic, organic, and water components is therefore widely known (2). The nature of the inorganic component (bone mineral, bio-apatite, or b-HAP) as a poorly crystalline nonstoichiometric form of the mineral calcium hydroxy-apatite ($Ca_5(PO_4)_3(OH)$, HAP) is also widely accepted (3). A diagram showing the crystal lattice structure of HAP is presented in Fig. 1.

Bio-apatite is poorly crystalline and nonstoichiometric due to ion substitutions and ion vacancies within the b-HAP crystal lattice (lattice defects) and ion adsorptions on crystal surfaces (4,5). An approximation to the general formula of b-HAP is presented in Formula 1. This is inevitably far from a complete account of b-HAP composition as, for example, it does not include consideration of adsorbed ions. Nevertheless, it does clearly demonstrate that b-HAP composition and structure is heterogeneous and variable.

Inter-species variation in bone mineral has been reported by previous researchers (6–14). However, the nature and extent of such variation is an area of research that has not been extensively explored. Characterization of b-HAP with respect to inter-species variation is of particular relevance to medicine, forensic science, and archeology. For example, in medical research, such characterization can be of benefit to bone disease and aging studies and in the development of biomaterials (7,15).

Within forensic and archeological contexts, b-HAP can be the most significant surviving component of bone, and it is therefore a valuable resource. Advances in the development of analytical techniques have resulted in an increase in forensic and archeological research activity with respect to b-HAP over recent years. Previous studies have investigated b-HAP characteristics with respect to changes that occur on heating and burning bone, the effects of diagenesis, and for the purpose of human identification (16–24). The characterization of b-HAP with respect to inter-species variation has the potential to have a significant impact in enabling species identification, particularly in distinguishing human from non-human bone in cases of fragmented, burned, and pulverized bone (17,25–31).

The study of heated bone often forms part of investigations into b-HAP characteristics (9,16,32,33). The heating of bone induces changes in b-HAP such as (16,33):

- Recrystallization: increase in "crystallinity": increase in crystal size and/or decrease in microstrain (lattice disorder).
- Changes to lattice defects (such as ion substitutions and vacancies).
- Thermal decomposition: formation of additional mineral phases at the expense of b-HAP.

In the theoretical case of stoichiometric HAP ($Ca_{10}(PO_4)_6(OH)_2$), thermal decomposition would occur as a result of dehydroxylation (loss of gaseous H_2O) and the formation of β-tri-calcium phosphate ($\beta Ca_3(PO_4)_2$, β-TCP), tetra-calcium phosphate ($Ca_4(PO_4)_2O$, TTCP) and calcium oxide (CaO) at the expense of the HAP (see Reactions 1 and 2). The amount of each decomposition product would be dependent on the relative thermal stabilities of the products and the heat treatment conditions employed. The extent of decomposition would

[1]Department of Engineering and Applied Science, Cranfield Forensic Institute, Cranfield University, Shrivenham, Wiltshire SN6 8LA, U.K.
[2]Faculty of Medicine, Dentistry and Health Science, Melbourne Dental School, The University of Melbourne, 720 Swanston Street, Melbourne 3010, Vic., Australia.

†An earlier version of this paper was presented at the 60th Annual Meeting of the American Academy of Forensic Sciences, February 18–23, 2008, in Washington, DC.

*Funded by a Doctoral Training Award from the Engineering and Physical Sciences Research Council and a Ludo Frevel Crystallographic Scholarship (2008) from the International Centre for Diffraction Data.

Received 5 Jan. 2010; and in revised form 8 Mar. 2010; accepted 13 Mar. 2010.

© 2011 American Academy of Forensic Sciences

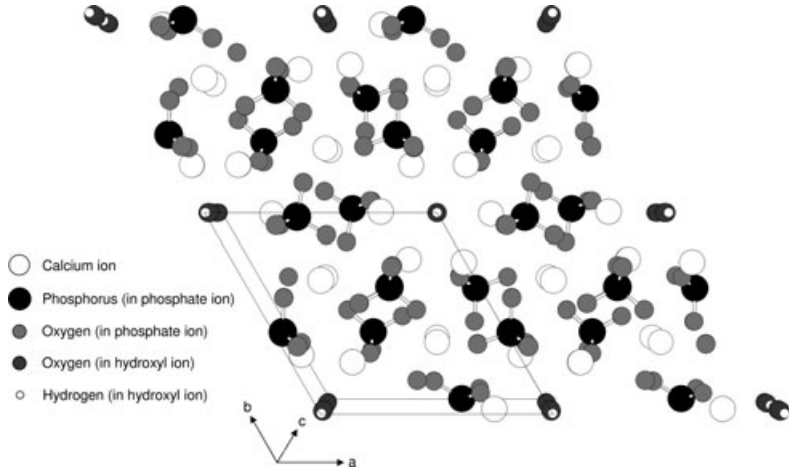

FIG. 1—*Diagram showing the crystal lattice structure of calcium hydroxy-apatite (HAP) and the HAP unit cell.*

FORMULA 1—*Approximation to the general formula of b-HAP, accounting for ion substitutions and vacancies (lattice defects) and based on the formula of HAP (using two stoichiometric $Ca_5(PO_4)_3(OH)$ formula units).*

$$\left[\left(Ca^{2+}\right)_{10-a-b}\left(Sub^{Ca^{2+}}\right)_a\left(V^{Ca^{2+}}\right)_b\right]\left[\left(PO_4{}^{3-}\right)_{6-c-d}\left(Sub^{PO_4^{3-}}\right)_c\left(V^{PO_4^{3-}}\right)_d\right]\left[\left(OH^-\right)_{2-e-f}\left(Sub^{OH^-}\right)_e\left(V^{OH^-}\right)_f\right]$$

$Sub^{Ca^{2+}}$ = calcium ion substitution (for example Mg^{2+}, Na^+, K^+, Sr^{2+});

$V^{Ca^{2+}}$ = calcium ion vacancy;

$Sub^{PO_4^{3-}}$ = phosphate ion substitution (for example CO_3^{2-}, HPO_4^{2-});

$V^{PO_4^{3-}}$ = phosphate ion vacancy;

Sub^{OH^-} = hydroxyl ion substitution (for example CO_3^{2-}, F^-, Cl^-);

V^{OH^-} = hydroxyl ion vacancy

$$\begin{array}{lll} 10 > a > 0 & 10 > b > 0 & 10 > (a+b) \\ 6 > c > 0 & 6 > d > 0 & 6 > (c+d) \\ 2 > e > 0 & 2 > f > 0 & 2 > (e+f) \end{array}$$

also depend on the heat treatment conditions as, for example, complete decomposition of HAP would not be achieved using short heat times.

$$Ca_{10}(PO_4)_6(OH)_2 \rightarrow 2\,\beta Ca_3(PO_4)_2 + Ca_4(PO_4)_2O + H_2O \quad \text{(Reaction 1)}$$

$$Ca_{10}(PO_4)_6(OH)_2 \rightarrow 3\,\beta Ca_3(PO_4)_2 + CaO + H_2O \quad \text{(Reaction 2)}$$

The behavior of nonstoichiometric HAP materials such as b-HAP upon heating is additionally influenced by the composition and structure of the material prior to heat treatment. As examples, calcium deficiency may result in the formation of less TTCP than expected for stoichiometric HAP (see Reaction 1) and specifically, the presence of magnesium ions in HAP materials is known to favor the formation of β-TCP. Thus, the study of heated bone can provide additional relevant information for investigations into b-HAP composition and structure. In particular, inter-species variation in behavior of b-HAP upon heating under standard conditions corresponds to any inter-species differences in b-HAP prior to heat treatment. The increase in the number of b-HAP characteristics available for use in the development of species identification methods and the ease of analysis of heated bone compared to unheated bone are further advantages of heated bone studies.

Unfortunately, it is difficult to distinguish between biological (both intra- and inter-species) variation and that due to experimental or environmental factors within previously published results (13,32,34,35). This is because the behavior of b-HAP upon heating is dependent on both the heat treatment conditions and the composition and structure of the unheated bone (16,36). Therefore, specific research into the extent of biological variation in bone mineral chemistry of unheated and heated bone under standardized conditions is vital to the future development of analytical tools that will incorporate measurement of b-HAP characteristics. This study aims to identify and quantify inter-species variation in the behavior of bone to standardized heat treatment at 600°C and 1400°C.

The study aim was achieved through the use of X-ray diffraction (XRD) analysis to measure bone mineral characteristics. XRD

analysis has, for many years, played a key role in structural studies of bone mineral (1,4). The characteristics measured in this study included:

- The relative coherence length of b-HAP crystals in the <0 0 ℓ> direction (b-HAP <0 0 ℓ>) for unheated bone and bone heated to 600°C. This was used as a measure of "crystallinity" of b-HAP.
- The lattice parameters of b-HAP (b-HAP "*a*" and b-HAP "*c*") in unheated and heated bone. These are measures of the b-HAP unit cell dimensions and were used as indicators of the net extent of lattice defects.
- The weight percentage of each mineral phase detected (b-HAP%, β-TCP%, TTCP%, CaO%, MgO%, and α-TCP%) in unheated and heated bone. These were used as measures of the nature and extent of the thermal decomposition of b-HAP.

Materials and Methods

Table 2 provides a list of the 12 species that were investigated in this study and the number of different individuals from each species. Names commonly used for species are used in this paper and a list of the corresponding taxonomic terms can be found in a previous publication, along with further details of the materials and methods employed in this study (37). Contemporary femoral (cortical) bone was used in all cases, was collected shortly after death, and was stored frozen. Left femur samples were used if a choice between left or right was available. Non-human bone material was obtained from several sources: local butchers, abattoirs, and pet food stores, Bristol Veterinary College, the Department for the Environment and Rural Affairs Veterinary Laboratories Agency, the Ministry of Defence, and Whipsnade Zoo. Human bone was obtained from the North London Tissue Bank.

Three bone specimens were cut from each bone sample, using water-cooled band and circular saws. Each specimen was a segment cut from an annulus of bone from the periosteal to endosteal surface. One specimen was left unheated, one was heated to 600°C, and one was heated to 1400°C. The unheated specimen was cut to height of approximately 1 mm, and the specimens to be heated were cut to a height of approximately 5 mm. Owing to the small physical size of rat femoral bone, only two specimens per individual were prepared for heat treatment. These were prepared by using a scalpel to remove both epiphyses from a femur and longitudinally sectioning the entire bone shaft into halves, each half becoming a test specimen.

Heat treatment of bone specimens was carried out using a Carbolite tube furnace (CTF 16/75; Carbolite Ltd., London, U.K.) sealed at each end with a ceramic fiber bung. Each bone specimen was placed into a separate alumina crucible and specimens were placed into the furnace at room temperature in batches. The furnace program increased the temperature at a rate of 10°C per minute and then maintained a temperature of 600 or 1400°C for 2 h, switching off at the end of this dwell time. Bone specimens were allowed to cool to room temperature before removal from the furnace.

All heated bone specimens were powdered, using an agate pestle and mortar, and sieved through a stainless steel mesh of 106 μm, in preparation for XRD analysis. Powdered specimens were mounted onto silicon (low background scattering) slides, using a thin film of petroleum jelly as an adhesive, and flattened to produce an even layer of powder with a thickness of approximately 0.5 mm and of an area slightly larger than the incident area of the X-ray beam. All unheated bone specimens were prepared for XRD analysis by mounting specimen segments onto microscope cover slips. A strip of adhesive tape was used between specimen and slide to secure each specimen.

Powder XRD analysis of all heated bone specimens was carried out using Phillips 1820 diffractometers with Cu Kα radiation. One diffraction run was carried out per bone specimen. Data were collected as stepped scans across an angular range of 10–80°/2θ (4.43–0.78 Å d-spacing). The count time at each step was 5 sec, with a 0.02°/2θ step size. XRD analysis of unheated bone specimens was carried out using a Bruker D8 X-ray diffractometer with Cu Kα radiation. One diffraction run was carried out per bone specimen. A General Area Detector Diffraction System (GADDS) and a 500-μ collimator were used to collect data over an angular range of approximately 21–55°/2θ (2.15–0.94 Å d-spacing).

The mineral phase(s) present within each specimen were identified by comparison of the data with the International Centre for Diffraction Data (ICDD) Powder Diffraction File (version PDF-2, 2004), using Crystallographica Search-Match software (version 2.1.1.1 1996–2004; Oxford Cryosystems Ltd., Oxford, U.K.).

Bruker Topas software (version 2, 2000) was used to carry out "whole pattern fitting" Rietveld refinement (38,39) of all diffraction profiles to obtain phase quantification and b-HAP lattice parameters. The structural models used for each mineral phase were derived from the ICDD Powder Diffraction Files: hydroxy-apatite (HAP) 9-432, beta-tri-calcium phosphate (β-TCP) 9-169, alpha-tri-calcium phosphate (α-TCP) 29-359, tetra-calcium phosphate (TTCP) 25-1137, CaO 37-1497, and magnesium oxide (MgO) 45-946.

Bruker Topas software (version 2, 2000) was also used to carry out profile fitting of the b-HAP 002 peaks in diffraction profiles obtained from unheated bone and bone heated to 600°C. A silicon powder standard (NBS 640c) and a corundum (Al_2O_3) standard (NIST SRM 1976) were used to correct for instrumental broadening. Values of relative coherence length of b-HAP crystals in the <0 0 ℓ> direction (b-HAP <0 0 ℓ>) were calculated using the Scherrer equation (40) applied to the 002 reflection.

One-way analysis of variance (ANOVA) and Bonferroni *post hoc* tests were carried out to identify significant differences between species for b-HAP lattice parameter and b-HAP <0 0 ℓ> values obtained from unheated bone and bone heated to 600°C and for mineral phase weight percentage values obtained from bone heated to 1400°C. It was not possible to include monkey and elephant within these tests owing to the analysis of bone from only one individual from each species. Significant differences between these species and all other species were tested using one-sample t-tests, and elephant and monkey were not tested against each other (see Fig. 4).

Results

The general characteristics of unheated bone and the general behavior of bone upon heating are first presented, followed by results relating to inter-species variation. The results obtained for human, chicken, and rat are specifically discussed as these species were found to exhibit the most notable inter-species variation.

General Characteristics of Unheated and Heated Bone

The mineral component of contemporary unheated bone, for all specimens analyzed ($n = 68$), was identified as calcium hydroxy-apatite (bio-apatite) (see Fig. 2 and Table 1). This was poorly crystalline as all values of b-HAP <0 0 ℓ> were less than 300 Å (see also the broad diffraction peaks in Fig. 2). In general, b-HAP "*a*" values (99%) were greater than the stoichiometric (defect free) value of HAP "*a*" = 9.42 Å, and all b-HAP "*c*" values were

greater than the stoichiometric value of HAP "*c*" = 6.88 Å (5) (see Table 1). Therefore, the lattice parameter value results suggest that the b-HAP was also nonstoichiometric and that b-HAP possesses a greater than stoichiometric average unit cell volume.

In general, upon heating bone to 600°C, no significant recrystallization or thermal decomposition occurred but in all cases (n = 73), a reduction of both b-HAP lattice parameter values compared to unheated bone was observed (see Tables 1 and 2).

Upon heating bone to 1400°C, significant recrystallization of b-HAP was apparent (see the narrow b-HAP diffraction peaks in Fig. 3). In general, both b-HAP lattice parameter values were reduced compared to corresponding values obtained for unheated bone. The b-HAP phase underwent thermal decomposition and β-TCP was always detected as a decomposition product. In general, TTCP, CaO, and MgO were also detected but α-TCP was rarely observed (n = 7/73).

Inter-Species Variation

The results of this study clearly demonstrate significant inter-species variation in b-HAP characteristics of unheated and heated bone (see Fig. 4) that was greater than the variation observed within species (see Tables 1–4). Between some species such as cattle and deer, no significant inter-species variation was observed for the b-HAP characteristics measured in this study. However, most species were significantly different ($p < 0.05$) to all other species in terms of at least one b-HAP characteristic.

Human

Unheated human bone possessed small b-HAP "*a*" values that were significantly different to those of all other species ($p < 0.05$, see Fig. 4). Large values of b-HAP <0 0 ℓ> were obtained although these were not significantly different to most other species ($p > 0.05$, see Table 1 and Fig. 4).

Recrystallization of b-HAP occurred on heating human bone to 600°C, in contrast to the general findings for most other species. The b-HAP <0 0 ℓ> values obtained for human bone were significantly different to those obtained for most other species ($p < 0.05$, see Table 2 and Fig. 4). Human bone heated to 600°C possessed relatively large b-HAP "*c*" values that were significantly different to those of all other species (see Table 2 and Fig. 4). A plot of b-HAP "*a*" values against b-HAP "*c*" values (see Fig. 5) for bone heated to 600°C clearly shows a separate cluster of values that distinguishes human bone from all other species investigated.

Relatively large values of b-HAP "*c*" were also obtained for human bone heated to 1400°C, and these were also significantly different to those obtained for all other species ($p < 0.01$, see Table 3 and Fig. 4). Furthermore, human bone was significantly different to most other species in terms of β-TCP and TTCP weight percentage values ($p < 0.01$, see Table 4 and Fig. 4).

Chicken

The b-HAP phase of unheated chicken bone was significantly less crystalline (along <0 0 ℓ>) compared to most other species ($p < 0.01$, see Table 1 and Fig. 4). Upon heating chicken bone to 600°C, β-TCP was detected. This thermal decomposition finding is inconsistent with the bone mineral behavior of most other species. On heating chicken bone to 1400°C, TTCP weight percentage values of <5% were obtained and these were significantly different to those of most other species which were in general >5% ($p < 0.05$, see Table 4 and Fig. 4). In general, CaO was not detected. However, α-TCP, which was not detected for most species, was detected in chicken bone specimens (see Table 4 and Fig. 4).

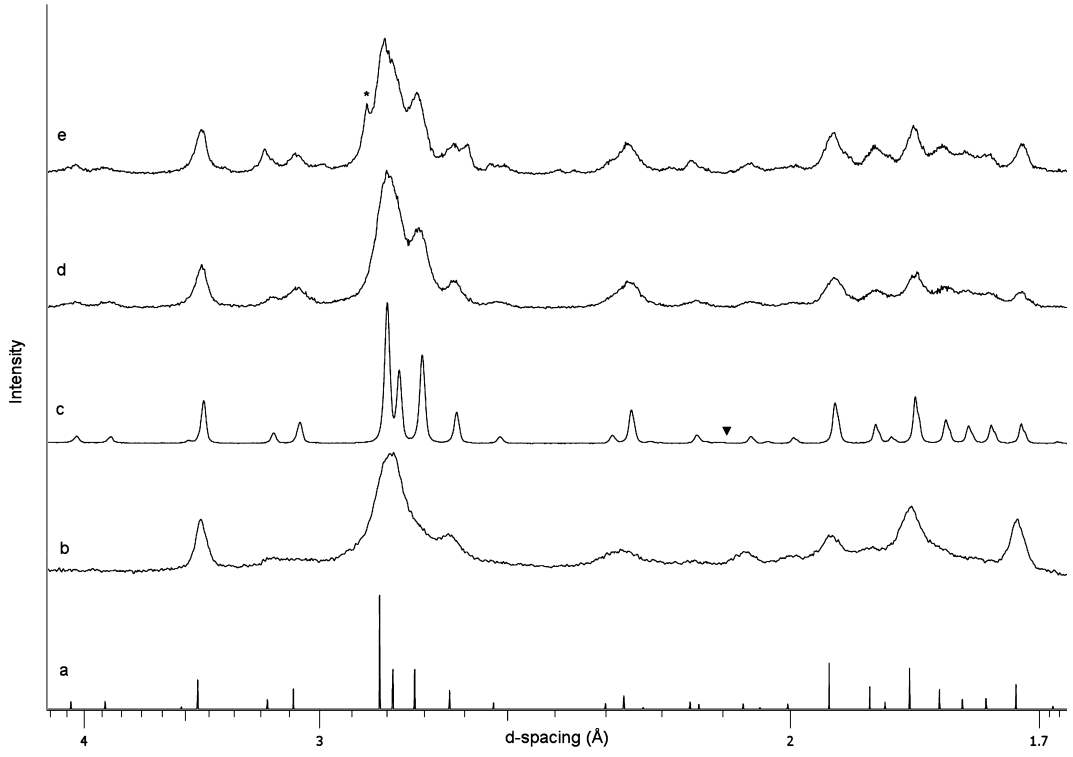

FIG. 2—*Examples of X-ray diffractograms from unheated bone (b: human) and bone heated to 600°C (c: human, d: cattle, e: rat). For clarity, diffractograms have been stacked and the expected peak positions for calcium hydroxy-apatite (from PDF 9-432) are presented (a). Most intense peaks for additional mineral phases are denoted by * (β-TCP) and ▼ (MgO).*

TABLE 1—*Mean, minimum, and maximum values for relative coherence length of bone mineral (b-HAP) crystals in the <0 0 2> direction (b-HAP <0 0 ℓ>) and b-HAP lattice parameters (b-HAP "a" and b-HAP "c") obtained from unheated bone, grouped by species (no data for rat). Values for the standard error of the mean are presented in parentheses, corresponding to the last significant figure of each mean value.*

Species	Number (Total 68)	b-HAP <0 0 ℓ> (Å)			b-HAP "*a*" (Å)			b-HAP "*c*" (Å)		
		Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
Chicken	5	194 (4)	187	210	9.482 (3)	9.475	9.493	6.895 (2)	6.888	6.901
Cattle	10	238 (4)	215	255	9.464 (4)	9.441	9.482	6.900 (1)	6.893	6.906
Deer	5	243 (5)	228	255	9.479 (4)	9.468	9.490	6.900 (2)	6.893	6.906
Dog	12	248 (4)	211	268	9.467 (4)	9.438	9.491	6.902 (2)	6.894	6.912
Elephant	1	217	–	–	9.463	–	–	6.907	–	–
Goat	8	251 (3)	238	265	9.460 (2)	9.450	9.471	6.904 (1)	6.902	6.907
Human	8	262 (5)	241	281	9.441 (4)	9.419	9.458	6.902 (1)	6.898	6.906
Monkey	1	258	–	–	9.467	–	–	6.906	–	–
Pig	7	217 (7)	175	231	9.484 (5)	9.468	9.508	6.899 (2)	6.888	6.904
Rabbit	5	237 (5)	224	251	9.471 (7)	9.452	9.489	6.898 (2)	6.892	6.906
Sheep	6	234 (4)	218	246	9.479 (2)	9.471	9.484	6.898 (4)	6.891	6.918

TABLE 2—*Mean, minimum, and maximum values for relative coherence length of bone mineral (b-HAP) crystals in the <0 0 2> direction (b-HAP <0 0 ℓ>) and b-HAP lattice parameters (b-HAP "a" and b-HAP "c") obtained from bone heated to 600°C, grouped by species. Results for the weight percentage of β-TCP and MgO detected, when detected, in bone heated to 600°C are also presented. Values for the standard error of the mean are presented in parentheses, corresponding to the last significant figure(s) of each mean value.*

Species	Number (Total 73)	b-HAP <0 0 ℓ> (Å)			b-HAP "*a*" (Å)			b-HAP "*c*" (Å)		
		Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
Chicken	5	237 (8)	206	255	9.423 (1)	9.419	9.427	6.883 (1)	6.880	6.885
Cattle	10	224 (6)	201	263	9.414 (1)	9.410	9.418	6.8842 (4)	6.883	6.886
Deer	5	247 (10)	226	283	9.414 (1)	9.411	9.417	6.884 (1)	6.882	6.887
Dog	12	236 (4)	208	260	9.413 (1)	9.407	9.421	6.888 (1)	6.884	6.890
Elephant	1	236	–	–	9.402	–	–	6.891	–	–
Goat	8	259 (16)	210	330	9.413 (1)	9.408	9.416	6.8863 (4)	6.885	6.888
Human	8	412 (64)	225	723	9.414 (1)	9.410	9.419	6.894 (1)	6.891	6.897
Monkey	1	519	–	–	9.413	–	–	6.886	–	–
Pig	7	315 (32)	209	410	9.414 (1)	9.408	9.417	6.885 (1)	6.883	6.890
Rabbit	5	255 (3)	248	261	9.413 (1)	9.411	9.416	6.886 (1)	6.885	6.889
Rat	5	232 (6)	222	250	9.428 (1)	9.423	9.430	6.8828 (3)	6.882	6.883
Sheep	6	235 (4)	217	247	9.416 (2)	9.405	9.420	6.883 (1)	6.881	6.886

The mineral phase β-TCP was detected in all chicken bone specimens (mean 6.9 [5]%, min. 5.9%, max. 8.2%), in all rat bone specimens (mean 8 [2]%, min. 3.6%, max. 13.9%), and in one (of 6) sheep bone specimen (3.1%). The mineral phase MgO was detected in one (of 8) human bone specimen (0.7%) and in one (of 1) monkey bone specimen (0.8%).

Rat

The characteristics of heated rat bone were found to be similar to those of heated chicken bone. The mineral phase β-TCP was detected in bone heated to 600°C. For bone heated to 1400°C, TTCP weight percentage values of <5% were obtained (see Table 4) and in general, α-TCP was also detected. CaO was not detected in any rat bone specimens. For all rat bone heated to 600°C, b-HAP "*a*" values were greater than 4.920 Å and significantly different to all other species, except chicken ($p < 0.05$, see Table 2 and Fig. 4).

Discussion

General Findings

The results for the typical characteristics of unheated bone and the typical behavior of bone upon heating were generally found to be in agreement with results of previously published studies (13,16,20,32,33,35,41,42). However, the general lack of recrystallization of b-HAP upon heating bone to 600°C was surprising as many studies report the observation of recrystallization at this temperature. This is likely to be due to variation between studies with respect to heating times and also, possibly, variation in the extent of removal of the organic component during sample preparation stages prior to heating. Inter-species variation and the bias within the literature toward studies of human bone for which, this study has demonstrated, b-HAP undergoes recrystallization at a lower temperature compared to most other species, may also partly explain the discrepancy.

The detection of CaO and MgO, for most species, in bone heated to 1400°C was also an unexpected result. Although both CaO and MgO are reported in the literature as mineral phases that have been detected in heated bone (13,32,43), they are not widely considered to be consistent thermal decomposition products of b-HAP (9,13,20,35,44) (see later Discussion). Again, this may partly be due to the variety of experimental conditions that have been employed by previous studies, some of which may be conditions that do not result in the formation of CaO or MgO. However, varying reports of CaO and MgO detection may simply be a reflection of the small weight percentage presence of these phases compared to the bulk decomposition products. This study therefore demonstrates the value of a quantitative whole pattern fitting analysis approach in b-HAP research to detect these low-quantity phases.

Inter-Species Variation—b-HAP Lattice Parameters

Of the measured b-HAP characteristics, b-HAP "*a*" values most clearly demonstrated inter-species variation in unheated bone and

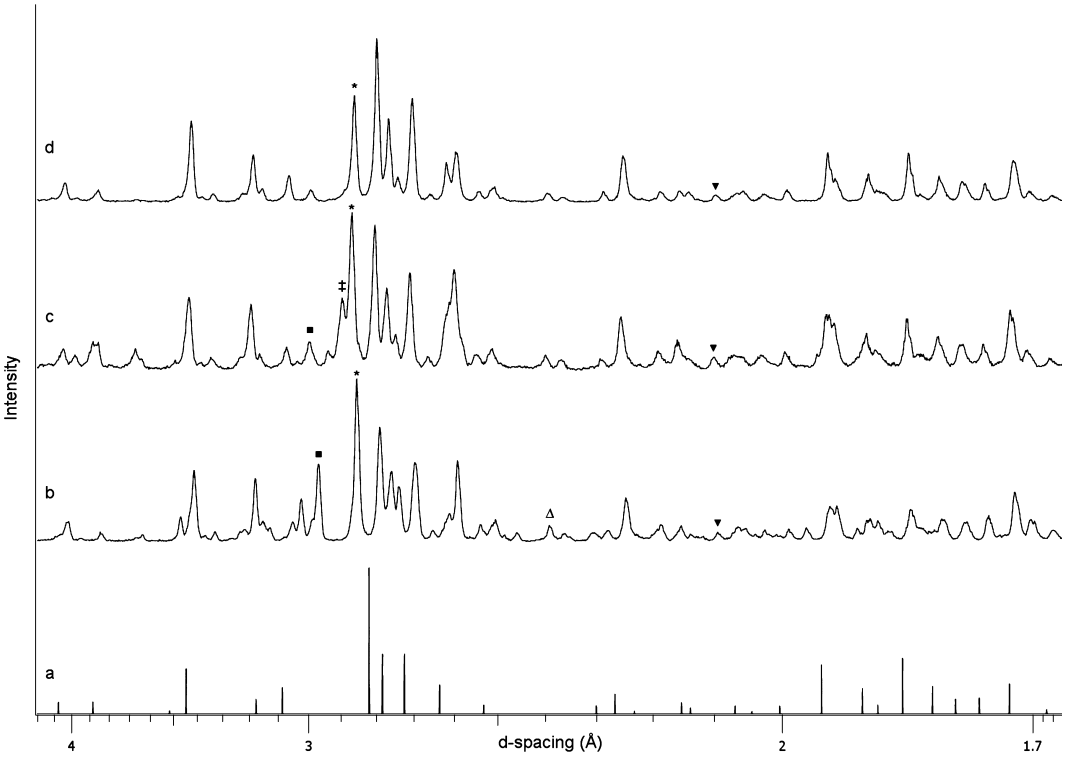

FIG. 3—*Examples of X-ray diffractograms from bone heated to 1400°C (b: human, c: rat, d: chicken). For clarity, diffractograms have been stacked and the expected peak positions for calcium hydroxy-apatite (from PDF 9-432) are presented (a). Most intense peaks for additional mineral phases are denoted by * (β-TCP), ■ (TTCP), ‡ (α-TCP), Δ (CaO), and ▼ (MgO).*

human could be distinguished from all other species on the basis of b-HAP lattice parameters obtained from unheated and heated bone. Thus, b-HAP lattice parameter values should be considered for inclusion in future development of species identification methods based on b-HAP characteristics. Furthermore, the inter-species variation observed with respect to lattice parameter values suggests that there are significant elemental composition differences between species. Future investigation into inter-species variation in elemental composition of b-HAP in conjunction with XRD analysis may enable a better understanding of the inter-species differences in b-HAP characteristics observed in this study.

Inter-Species Variation—Crystallinity

Inter-species differences in b-HAP <0 0 ℓ> values observed for unheated bone may be due, in part, to lattice defect variation between species. Certain lattice defects are known to affect b-HAP crystallinity. For example, fluorine ion substitutions cause an increase in crystallinity (45). The differences between human and most non-human bone in terms of the b-HAP <0 0 ℓ> values observed in this study have demonstrated the potential of measures of recrystallization upon heating for use as species differentiating characteristics. As recrystallization did not occur for most species upon heating to 600°C, investigation into heat temperatures between 700 and 1000°C may enable significant inter-species variation between non-human species to be identified.

Inter-Species Variation—Thermal Decomposition of b-HAP

The inter-species variation upon heating bone to 1400°C in terms of the extent of thermal decomposition of b-HAP and the quantities of the decomposition products suggests that there is significant inter-species variation in terms of b-HAP composition and structure of unheated bone. For example, the relatively high weight percentage values obtained for TTCP for human bone suggests that human bone may be less calcium deficient and/or less phosphate deficient compared to bone of most other species. It is also plausible that human bone may possess greater quantities of TTCP-stabilizing ions or smaller quantities of β-TCP-stabilizing ions compared to bone of most other species. Magnesium is known to substitute for calcium ions within the b-HAP lattice and is also known to stabilize β-TCP upon heating (42). However, less is known about the effects of other calcium-substituting ions such as sodium and potassium on the thermal decomposition of b-HAP. A combined investigation into elemental and XRD analysis, as suggested above, may enable a better understanding of the effects of b-HAP lattice defects on b-HAP thermal decomposition, especially if lattice parameters of the decomposition products are also considered. This has the potential to have a significant impact in the development of bio-materials in addition to the benefit it would have in the development of species identification methods.

While thermal decomposition of b-HAP upon heating bone to 1400°C was expected, observations of additional mineral phases in bone heated to 600°C were surprising results of this study. The detection of β-TCP in chicken and rat bone suggests that thermal decomposition of b-HAP occurred at a lower temperature compared to other species and prior to any significant recrystallization of b-HAP. It is likely that this finding is due to a combination of factors. The b-HAP of unheated chicken bone, with small b-HAP <0 0 ℓ> values, may have a relatively large degree of microstrain, and this may result in a much lower thermodynamic barrier to b-HAP decomposition than expected. Unheated chicken and rat bone may possess greater amounts of β-TCP-stabilizing ions such as magnesium ions as preliminary elemental investigations into chicken and rat bone from this study suggest (37). Furthermore,

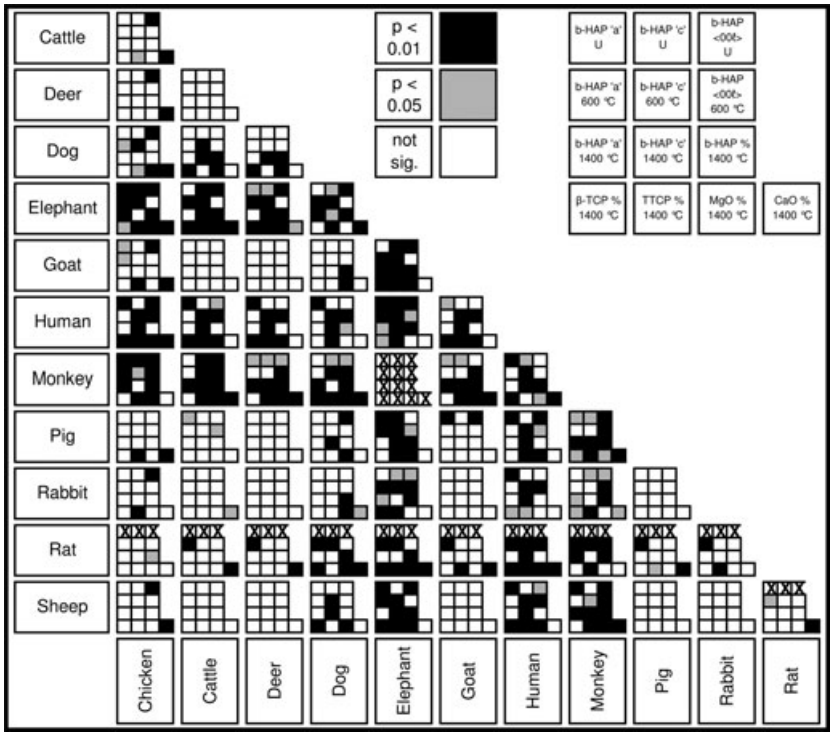

FIG. 4—*Results of ANOVA Bonferroni* post hoc *tests for significant inter-species differences for measured characteristics of unheated (U) and heated bone (600 and 1400°C). Results presented for elephant and monkey are for one-sample t-tests. An "X" within a box indicates that no test was carried out. Significant differences are indicated by gray shading for* $p < 0.05$ *and black shading for* $p < 0.01$. *Unshaded boxes indicate no significant difference (not sig.).*

TABLE 3—*Mean, minimum, and maximum values for the weight percentage of bone mineral (b-HAP) and the b-HAP lattice parameters (b-HAP "a" and b-HAP "c") obtained from bone heated to 1400°C, grouped by species. Values for the standard error of the mean are presented in parentheses, corresponding to the last significant figure(s) of each mean value.*

		b-HAP%			b-HAP "*a*" (Å)			b-HAP "*c*" (Å)		
Species	Number (Total 73)	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
Chicken	5	52 (3)	45.94	60.66	9.413 (1)	9.409	9.414	6.888 (1)	6.887	6.890
Cattle	10	40 (1)	35.06	48.82	9.416 (1)	9.413	9.419	6.8842 (3)	6.882	6.886
Deer	5	37 (1)	33.27	40.26	9.416 (1)	9.414	9.418	6.8833 (3)	6.883	6.884
Dog	12	55 (2)	43.57	72.33	9.414 (1)	9.409	9.418	6.890 (1)	6.885	6.896
Elephant	1	24.56	–	–	9.417	–	–	6.887	–	–
Goat	8	38 (2)	26.47	43.52	9.413 (1)	9.410	9.415	6.8880 (2)	6.887	6.889
Human	8	41 (3)	31.88	56.47	9.411 (2)	9.400	9.422	6.897 (1)	6.892	6.900
Monkey	1	22.22	–	–	9.407	–	–	6.889	–	–
Pig	7	44 (4)	29.66	60.04	9.4165 (3)	9.415	9.417	6.8848 (4)	6.884	6.886
Rabbit	5	38 (2)	33.42	44.95	9.413 (2)	9.408	9.417	6.886 (1)	6.883	6.890
Rat	5	34 (2)	28.55	39.37	9.416 (1)	9.412	9.419	6.888 (2)	6.885	6.894
Sheep	6	43 (4)	31.65	59.02	9.417 (1)	9.414	9.419	6.884 (1)	6.882	6.886

chicken and rat bone had greater percentage mass loss values upon heating to 600°C (37), indicative of greater loss of organic and/or water components compared to other species. This may have resulted in higher relative temperatures within the local environments of chicken and rat bone, enabling decomposition to occur at an apparently lower experimental temperature.

There are several reports within the literature of the formation of CaO in the absence of β-TCP (33). Several studies have demonstrated that b-HAP is calcium deficient compared to stoichiometric HAP, and on this basis CaO is not a phase that would be expected. However, b-HAP is also phosphate deficient owing to carbonate ion substitutions, and therefore it is plausible that the formation of CaO occurs as a result of recrystallization of b-HAP without the formation of any calcium phosphate decomposition products (see Reaction 2) (46). Although CaO was not detected in bone heated to 600°C, MgO was detected in some bone specimens heated to this temperature. Previous reports of the detection of MgO within the literature have been for bone heated to temperatures higher than 600°C and the mechanism for MgO formation in heated bone is perhaps a subject for debate and further investigation. Is MgO simply formed instead of CaO in phosphate-deficient bone that possesses high quantities of magnesium ions or is it formed through an entirely different mechanism such as oxidation of residual magnesium ions from the combustion of the organic component of bone (13)?

The formation of α-TCP occurs from the high temperature conversion of β-TCP (47). Based on evidence of previous studies, it was expected that α-TCP would be a consistent decomposition product detected in bone specimens heated to 1400°C (16). However, α-TCP was only detected in chicken and rat bone specimens. The lower conversion temperature observed for chicken and rat

TABLE 4—*Mean, minimum, and maximum values for the weight percentage of β-TCP, TTCP, CaO, and MgO detected in bone heated to 1400°C, grouped by species. Results for the weight percentage of α-TCP detected, when detected, in bone heated to 1400°C are also presented. "No." indicates the number of specimens for which the mineral phase was detected (β-TCP and MgO were detected in all specimens). Values for the standard error of the mean are presented in parentheses, corresponding to the last significant figure of each mean value.*

Species	Number (Total 73)	β-TCP%			TTCP%				CaO%				MgO%		
		Mean	Min	Max	No.	Mean	Min	Max	No.	Mean	Min	Max	Mean	Min	Max
Chicken	5	44 (3)	35.12	49.15	3	2.5 (2)	2.09	2.81	1	0.43	–	–	0.98 (3)	0.88	1.09
Cattle	10	45 (1)	40.33	50.60	10	14 (1)	8.68	18.74	10	1.04 (7)	0.57	1.32	1.03 (2)	0.89	1.14
Deer	5	48 (1)	43.33	52.05	5	13 (1)	8.40	16.41	5	1.04 (7)	0.83	1.17	0.94 (3)	0.88	1.05
Dog	12	31 (3)	13.37	41.20	12	12 (2)	4.33	20.02	12	0.98 (5)	0.72	1.26	0.53 (3)	0.36	0.77
Elephant	1	34.36	–	–	1	39.72	–	–	1	0.79	–	–	0.57	–	–
Goat	8	42 (1)	39.26	48.26	8	18 (2)	10.81	25.98	8	0.67 (6)	0.41	0.97	0.98 (3)	0.85	1.18
Human	8	26 (3)	15.33	41.58	8	32 (2)	24.67	38.26	8	0.8 (1)	0.36	1.39	0.65 (4)	0.48	0.77
Monkey	1	44.16	–	–	1	32.85	–	–	0	–	–	–	0.78	–	–
Pig	7	38 (2)	30.91	42.77	7	16 (3)	7.12	26.17	7	0.72 (5)	0.52	0.84	0.97 (5)	0.72	1.11
Rabbit	5	41 (1)	38.41	43.11	5	19 (3)	12.00	24.60	4	0.7 (1)	0.31	0.90	0.9 (1)	0.68	1.40
Rat	5	51 (3)	43.43	60.34	5	2.8 (2)	1.96	3.22	0	–	–	–	0.98 (8)	0.67	1.13
Sheep	6	46 (3)	34.80	55.55	6	9 (3)	3.97	22.15	6	0.91 (9)	0.56	1.08	1.13 (8)	0.78	1.36

The mineral phase α-TCP was detected in 3 (of 5) chicken bone specimens (mean 2.4 [7]%, min. 1.43%, max. 3.76%) and in 4 (of 5) rat bone specimens (mean 13 [3]%, min. 7.53%, max. 19.57%).

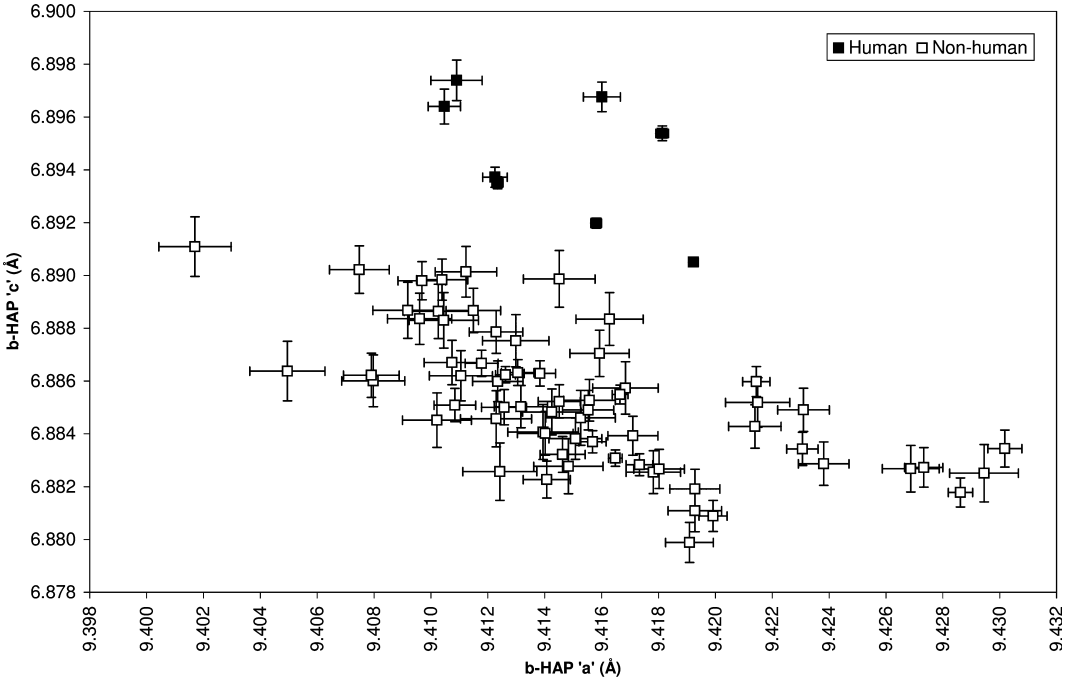

FIG. 5—*Plot of bone mineral (b-HAP) lattice parameter values* "a" *and* "c" *for bone heated to 600°C. Data include all individuals from all species investigated, labeled as either human (■) or non-human (□). The error bars indicate the magnitude of fitting errors associated with each data point.*

bone is likely to be related to the lower temperature of initial thermal decomposition that was observed for these species. Furthermore, the observed inter-species variation in β-TCP to α-TCP conversion temperature is likely to be due to inter-species variation in elemental composition of unheated b-HAP. Research into synthetic β-tri-calcium phosphates has shown that the presence of certain ion substitutions such as magnesium ions can inhibit the conversion of β-TCP into α-TCP (42,47).

Summary

The results of this study have clearly demonstrated that b-HAP characteristics of unheated and heated bone exhibit significant inter-species variation and that this is quantifiable using XRD analysis. There is potential for the development of an XRD-based method of species identification, and the potential capability for distinguishing human from non-human bone is particularly promising. Furthermore, a research approach that considers inter-species variation when investigating b-HAP characteristics contributes to a better understanding of b-HAP composition and structure which is of relevance and value to medical, forensic, and archeological research. Such studies may be further enhanced by consideration of elemental compositional information within the context of XRD data and whole pattern fitting analyses.

Acknowledgments

The authors thank M. Kirby from the Veterinary Laboratories Agency, Dr. M. Owen from University of Bristol, and

Dr. N. Martin and Dr. P. Zioupos from Cranfield University for the supply of bone tissue.

References

1. Rey C, Combes C, Drouet C, Glimcher MJ. Bone mineral: update on chemical composition and structure. Osteoporos Int 2009;20:1013–21.
2. Dorozhkin SV, Epple M. Biological and medical significance of calcium phosphates. Angew Chem Int Ed 2002;41:3130–46.
3. Wopenka B, Pasteris JD. A mineralogical perspective on the apatite in bone. Mater Sci Eng C 2005;25:131–43.
4. Elliott JC, Wilson RM, Dowker SEP. Apatite structures. Adv X-ray Anal 2002;45:172–81.
5. Young RA. Biological apatite vs hydroxyapatite at the atomic level. Clin Orthop Relat Res 1975;113:249–62.
6. Biltz RM, Pellegrino ED. The chemical anatomy of bone: I. a comparative study of bone composition in sixteen vertebrates. J Bone Joint Surg Am 1969;51(3):456–66.
7. Aerssens J, Boonen S, Lowet G, Dequeker J. Interspecies differences in bone composition, density, and quality: potential implications for *in vivo* bone research. Endocrinology 1998;139(2):663–70.
8. Danilchenko SN, Pokrovskiy VA, Bogatyrov VM, Sukhodub LF, Sulkio-Cleff B. Carbonate location in bone tissue mineral by X-ray diffraction and temperature-programmed desorption mass spectrometry. Crystal Res Technol 2005;40(7):692–7.
9. Balmain N, LeGeros JP, Bonel G. X-ray diffraction of calcined bone tissue: a reliable method for the determination of bone Ca/P molar ratio. Calcif Tissue Int 1982;34:S93–8.
10. Pellegrino ED, Biltz RM. Bone carbonate and the Ca to P molar ratio. Nature 1968;219:1261–2.
11. Bloebaum RD, Skedros JG, Vajda EG, Bachus KN, Constanz BR. Determining mineral content variations in bone using backscattered electron imaging. Bone 1997;20(5):485–90.
12. LeGeros R, Balmain N, Bonel G. Age-related changes in mineral of rat and bovine cortical bone. Calcif Tissue Int 1987;41:137–44.
13. Ravaglioli A, Krajewski A, Celotti GC, Piancastelli A, Bacchini B, Montanari L, et al. Mineral evolution of bone. Biomaterials 1996;17:617–22.
14. Rischak G, Lenart G, Pinter J. Comparative crystallographic analysis of different bones with X-ray diffractometry. Acta Biochim Biophys Acad Sci Hung 1971;6(2):157–64.
15. Joschek S, Nies B, Krotz R, Gopferich A. Chemical and physicochemical characterization of porous hydroxyapatite ceramics made of natural bone. Biomaterials 2000;21:1645–58.
16. Holden JL, Clement JG, Phakey PP. Age and temperature related changes to the ultrastructure and composition of human bone mineral. J Bone Min Res 1995;10(9):1400–7.
17. Bergslien ET, Bush M, Bush PJ. Identification of cremains using X-ray diffraction spectroscopy and a comparison to trace element analysis. Forensic Sci Int 2008;175(2):218–26.
18. Enzo S, Bazzoni M, Mazzarello V, Piga G, Bandiera P, Melis P. A study by thermal treatment and X-ray powder diffraction on burnt fragmented bones from tombs II, IV and IX belonging to the hypogeic necropolis of ''Sa Figu'' near Ittiri, Sassari (Sardinia, Italy). J Arch Sci 2007;34:1731–7.
19. Hedges REM. Bone diagenesis: an overview of processes. Archaeometry 2002;44(3):319–28.
20. Hiller JC, Thompson TJU, Evison MP, Chamberlain AT, Wess TJ. Bone mineral change during experimental heating: an X-ray scattering investigation. Biomaterials 2003;24:5091–7.
21. Piga G, Malgosa A, Thompson TJU, Enzo S. A new method of the XRD technique for the study of archaeological burned human remains. J Arch Sci 2008;35:2171–8.
22. Pijoan CM, Mansilla J, Leboreiro I, Lara VH, Bosch P. Thermal alterations in archaeological bones. Archaeometry 2007;4:713–27.
23. Stiner MC, Kuhn SL, Weiner S, Bar-Yosef O. Differential burning, recrystallization, and fragmentation of archaeological bone. J Arch Sci 1995;22:223–37.
24. Thompson TJU. Recent advances in the study of burned bone and their implications for forensic anthropology. Forensic Sci Int 2004;146S:S203–5.
25. Martiniakova M, Grosskopf B, Omelka R, Vondrakova M, Bauerova M. Differences among species in compact bone tissue microstructure of mammalian skeleton: use of a discriminant function analysis for species identification. J Forensic Sci 2006;51(6):1235–9.
26. Cattaneo C, DiMartino S, Scali S, Craig OE, Grandi M, Sokol RJ. Determining the human origin of fragments of burnt bone: a comparative study of histological, immunological and DNA techniques. Forensic Sci Int 1999;102:181–91.
27. Cuijpers AGFM. Histological identification of bone fragments in archaeology: telling humans apart from horses and cattle. Int J Osteoarchaeol 2006;16:465–80.
28. Hillier ML, Bell LS. Differentiating human bone from animal bone: a review of histological methods. J Forensic Sci 2007;52(2):249–63.
29. Owsley DW, Mires AM, Keith MS. Case involving differentiation of deer and human bone fragments. J Forensic Sci 1985;30(2):572–8.
30. van Raamsdonk LWD, von Holst C, Baeten V, Berben G, Boix A, de Jong J. New developments in the detection and identification of processed animal proteins in feeds. Animal Feed Sci Technol 2007;133:63–83.
31. Whyte TR. Distinguishing remains of human cremations from burned animal bones. J Field Archaeol 2001;28:437–48.
32. Danilchenko SM, Koropov AV, Protsenko IY, Sulkio-Cleff B, Sukhodub LF. Thermal behaviour of biogenic apatite crystals in bone: an X-ray diffraction study. Cryst Res Technol 2006;41(3):268–75.
33. Rogers KD, Daniels P. An X-ray diffraction study of the effects of heat treatment on bone mineral microstructure. Biomaterials 2002;23:2577–85.
34. Chakraborty S, Bag S, Pal S, Mukherjee AL. Structural and microstructural characterization of bioapatites and synthetic hydroxyapatite using X-ray powder diffraction and Fourier transform infrared techniques. J Appl Cryst 2006;39:385–90.
35. Ooi CY, Hamdi M, Ramesh S. Properties of hydroxyapatite produced by annealing of bovine bone. Ceram Int 2007;33:1171–7.
36. Mkukuma LD, Skakle JMS, Gibson IR, Imrie CT, Aspden RM, Huskins DWL. Effect of the proportion of organic material in bone on thermal decomposition of bone mineral: an investigation of a variety of bones from different species using thermogravimetric analysis coupled to mass spectrometry, high-temperature X-ray diffraction, and Fourier transform infrared spectroscopy. Calcif Tissue Int 2004;75:321–8.
37. Beckett S. Inter-species variation in bone mineral [dissertation]. Shrivenham, UK: Cranfield University, 2008.
38. Reid JW, Hendry JA. Rapid, accurate phase quantification of multiphase calcium phosphate materials using Reitveld refinement. J Appl Cryst 2006;39:536–43.
39. Rietveld HM. A profile refinement method for nuclear and magnetic structures. J Appl Cryst 1969;2:65–71.
40. Jenkins R, Snyder RL. Introduction to X-ray powder diffractometry. New York, NY: John Wiley & Sons, Inc., 1996.
41. Haberko K, Bucko MM, Brzezinska-Miecznik J, Haberko M, Mozgawa W, Panz T, et al. Natural hydroxyapatite—its behaviour during heat treatment. J Euro Ceram Soc 2006;26:537–42.
42. Baravelli S, Bigi A, Ripamonti A, Roveri N, Foresti E. Thermal behaviour of bone and synthetic hydroxyapatites submitted to magnesium interaction in aqueous medium. J Inorg Biochem 1984;20:1–12.
43. LeGeros RZ. Apatites in biological systems. Prog Cryst Growth Charact 1981;4:1–45.
44. Ubelaker DH. The forensic evaluation of burned skeletal remains: a synthesis. Forensic Sci Int 2009;183:1–5.
45. Posner AS, Eanes ED, Harper RA, Zipkin I. X-ray diffraction analysis of the effect of fluoride on human bone apatite. Arch Oral Biol 1963;8:549–70.
46. Raynaud S, Champion E, Bernache-Assollant D, Laval J-P. Determination of calcium/phosphorous ratio of calcium phosphate apatites using X-ray diffractometry. J Am Ceram Soc 2001;84(2):359–66.
47. Ando J. Tricalcium phosphate and its variation. Bull Chem Soc Jpn 1958;31:196–201.

Additional information and reprint requests:
Sophie Beckett, Ph.D.
Department of Engineering and Applied Science
Cranfield Forensic Institute
Cranfield University, Shrivenham
Swindon, Wiltshire SN6 8LA
U.K.
E-mail: s.beckett@cranfield.ac.uk