

# Ashing of Bone: Errors Due to Loss of CO<sub>2</sub> and Its Correction

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## Research article

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# Abstract

**Background:** Ashing is widely used to determine weight fraction of water-free bone that is mineral, but no standard procedure exists and the range of techniques used spans a range of temperatures and times over which the amount of weight loss is variable. We show that variability is largely due to progressive loss of  $\text{CO}_2$  from  $\text{CO}_3^{2-}$  ions in the apatite crystal lattice, beginning at  $600^\circ\text{C}$ , typically used for ashing. We test the effect of varying temperature, time and weight of sample and develop a reliable method, using small samples.

**Methods:** Replicate samples of bovine cortical bone were tested at  $500^\circ\text{C}$ ,  $600^\circ\text{C}$  and  $700^\circ\text{C}$  for times ranging up to 24h. We also tested samples of multiple humans at what we concluded to be the optimal conditions

**Results:** Varying conditions of ashing resulted in variations in apparent ash weight % by up to 7%. Samples between 5 and 20 mg heated to  $600^\circ\text{C}$  for 1 h gave results agreeing with generally accepted values, but with much smaller variability. Ash wt% values for multiple human bone samples differed by up to 4.8% but replicate data for individuals agree to  $\pm 1$  wt%.

**Conclusions:** A satisfactory method is given for ash weight determination using small samples, and yielding highly reproducible data. If accepted widely, ash weight values between laboratories could be used to study variations due to diet, age, drug treatment and disease.

## Introduction

### Nature of the problem

Bone is composed of about equal volumes of organic matter and mineral, together with about 10% of water [1]. It is of importance in the study of bone to know the weight fraction of mineral in the material. In the study of the effects of aging, disease, diet, and drug treatment on bone, the mineral fraction should be a significant variable. The classic method for determination of the mineral fraction of bone is to heat a weighed sample of bone to a high temperature ( $\geq 600^\circ\text{C}$ ) in contact with air, and then to reweigh it. The ratio of the second weight to the first is the ash content, usually given as a weight percent (wt%). Previous ashing studies (Table S1) have used widely varying pre-treatment, heating temperature, duration of ashing, and size of sample. Although analyses of control samples (e.g., wild-type experimental animals) should give closely similar values, we see large variability in results. For example, five studies of rat femora give values ranging from 47 to 64 wt%. Within single studies, the range is always much smaller, showing that use of the same method in one lab is capable of giving reproducible results. Why are there such large variations between laboratories in such a simple method?

We have discovered one cause for the erratic variations observed in ash weighing, namely, loss of  $\text{CO}_2$  during heating of bone mineral. We have shown how this problem can be dealt with experimentally, thereby developing a more reliable method for the measurement of ash weight percent.

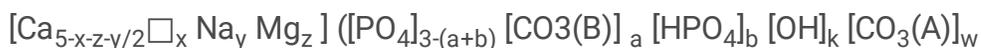
This allows us to determine variations in ash weight of only a few percent which could be due to changes in the life history of the organism such as age, disease, diet and drug treatment.

### Other methods of determination

While it would be useful to compare the results of these measurements with other standard techniques, it appears that there are essentially no other acceptable methods to determine the mineral content of a sample of bone. Infra-red analysis (FTIR) of the ratio of  $\text{PO}_4$  to amide[2,3] must make assumptions about the composition of the mineral phase. Likewise, the use of back-scattered electron intensity to measure the Ca fraction in a polished section of bone can determine local point-to-point variations in the apparent mineral content but cannot be converted to total mineral content since the composition of the mineral phase is not determined [2,4]. Bulk chemical analysis (e.g., by ICP/MS) has some promise. For example, one could remove all the protein from a bone sample by treatment with an oxidant such as bleach[5] and then chemically determine its bulk composition; then, from the Ca content of the total bone sample it would be possible to determine the weight percent of mineral in the sample, assuming that the organic component contained no Ca. However, this would be a tedious procedure if it is even possible. Altogether, ashing of bone continues to be an attractive, technically simple procedure if its precision could be improved.

### Composition of bone mineral; volatile constituents

To understand the origin of variations of ash weight on heating at high temperatures, we must consider the chemical composition of the mineral component of bone. This mineral is a carbonate apatite whose formula can be written as



where  $\square$  = a vacancy at one of the two Ca sites in the lattice and A, B represent the two sites where carbonate ions,  $\text{CO}_3^{2-}$ , can reside; also,  $x = (a + b)/2$  and  $k + w \leq 1$ . One of the components of apatite, namely  $\text{CO}_3^{2-}$  is partially lost during heating. Above some critical temperature, the reaction  $\text{CO}_3^{2-} = \text{CO}_2 + [\text{O}]^{2-}$  occurs; an atom of oxygen [O] is left in the crystal in some stable configuration, e.g. as CaO when  $\text{CaCO}_3$  is heated. Carbonate makes up approximately 5 wt% of the inorganic component of bone [6]. Therefore, the weight of the sample during heating would be expected to be lowered by up to ~ 3% during heating due to loss of  $\text{CO}_2$ . As we shall see, actual weight losses are even greater than this. The progressive loss of  $\text{CO}_3^{2-}$  during heating can be seen in a series of FTIR patterns of heated samples (Fig. 1). Both the  $\nu_2$  and  $\nu_3$  (split) peaks begin to decrease in height after the samples are heated to 600°C, but the peaks are still visible at 700°C, showing that the loss of  $\text{CO}_3^{2-}$  continues with increasing temperature.

Another volatile component,  $\text{OH}^-$ , could be lost during heating, but in fact experiments have shown that this does not occur up to temperatures well above the range used for ashing (~900°C) [7].

## Goal of study

The goal of this current study is to explore the effect on ash weight percent (wt%) of variation in the principal variables in ashing: a) sample size; b) sample preparation prior to ashing; c) temperatures used in ashing; and d) duration of ashing. These studies were carried out on homogenized samples of a single piece of animal bone. We attempted to develop a standard method of ashing that would yield reproducible and comparable values for all bone samples. We end by applying the method to samples of human bone to test whether we can identify significant differences in ash content.

## Methods

### Samples

Two types of samples were employed in the survey: bovine bone and a set of human bones to test for our ability to detect intra- and inter-sample variability.

#### *Bovine bone:*

We used a single homogeneous sample of femoral cortical bovine bone as a standard reference material, to eliminate biological variability as much as possible. Part of the cortical bone from the femur of a bovine (*Bos taurus*) was obtained from a butcher. The part selected for analysis was from the interior of the cortical region where there is little variation on the mm scale in the average composition; this will be made evident in the homogeneity of results. The total sample weighed approximately 80 grams. No animal care ethical approval was required for this sample.

#### *Human bone:*

Samples of cortical bone were extracted from femoral diaphyses of six individuals obtained from Science Care Inc, (Phoenix AZ). Ethics approval for use of these samples was obtained from HiREB (Hamilton Integrated Research Ethics Board) as a human tissue research study, approval 2016-2346-T. The samples were received in a fresh-frozen state. They represent females ranging in age from 57 to 85 years. None of the individuals sampled had experienced antemortem femoral fractures. Samples were obtained using a hand saw and were approximately 3 cm x 2 cm, each weighing 10-20 grams.

One additional human sample was obtained of fragments of cortical bone from the femur of a healthy, 19-year-old male; the sample was obtained with ethical approval as a by-product of restorative surgery [9].

The human sample collection is summarized in Table 1.

**Table 1:** Human samples used in this study (all female except 19y old)

Sample no.	Age
1553L	70
1493	57
1640L	73
161569	94
172202	85
171519	76
19-year-old	19

## Sample Preparations

### *Preliminary treatment*

Each sample of bone (bovine and human) was treated with formalin for 48 hours. This was required, as the laboratory in which these studies were carried out did not have biohazard approval for analysis of unfixed tissue. While treatment with formalin affects cross-linking of collagen and other structural proteins, it is not expected to affect the wt% of organic matter in the bone after removal of all traces of the fixative. The samples were then subjected to the following sequential procedures: 1) dried for 24 hours at room temperature; 2) residual fat and tissue from the surface of the bone was removed using a scalpel; 3) defatted using a 1:3 mixture of methanol and chloroform, stirring with a stir bar for 1 hour in the fume hood; 4) rinsed twice with methanol for 10-15 minutes; 5) placed in an oven at 60°C for 30 minutes; 6) broken into 5-125 mg fragments by cooling in liquid nitrogen and crushing in a steel mortar and pestle; 7) the pieces were stored in the freezer at -80°C until ashing. Single fragments of appropriate weight were used in the heating runs.

### *Pre-ashing drying*

Before ashing, all samples were placed in ceramic crucibles and heated in a warming oven held at 60°C, for either 30 min or 24h. The purpose of this procedure was to eliminate loosely bound water (liquid, adsorbed on surfaces or interstitial).

### *Ashing*

A muffle furnace (Lindberg/Blue M Box Furnace, Model 30620-004; Thermo-Fisher, Waltham, MA) was preheated to the temperature to be used for the incineration. The furnace is equipped with an electronic temperature gauge attached to a thermocouple in the wall of the furnace. Weighed, preheated samples were placed in ceramic crucibles, introduced into the furnace, and heated for various times and temperatures. Four samples were used in each heating run, consisting of similar sized bone fragments with aggregate weights that varied by up to a factor of two. The principal variables investigated were

temperature (T), time (duration) of heating (t) and sample mass (M). After ashing, all samples were immediately placed in a desiccator and allowed to cool for 24 hours before reweighing.

Ash weights were always calculated as the difference between weights after preheating and after high-temperature heating, as is done in some other studies of ashing [10,11].

### *Thermogravimetric (TGA) analysis*

In order to establish the thermal landscape over which mass loss would be occurring, it was useful to also make measurements by TGA. This technique has been used previously to determine ash weight [12]. Samples of bovine bone weighing 20 to 35 mg were analyzed using a Netzsch apparatus (Waldkraiburg, Germany). Two samples were first preheated overnight at 60°C, and then heated in the TGA from 25 to 800 °C in air at a rate of 10°Cmin<sup>-1</sup>.

### *Data Analysis*

Statistical analysis of the data used GraphPad Prism Software 8.3 (GraphPad Software Inc., San Diego, CA, USA). One-way ANOVAs were performed to determine significance of results obtained for ashing samples with 3 or more data sets. For multiple comparisons, one-way ANOVA with Tukey's multiple comparison tests were performed to determine significance of the results. Unpaired t-tests were performed to determine significance of results obtained for ashing samples with two or more data sets. Results are presented as box and whisker plots with mean and median. Probability values of <0.05 were considered statistically significant.

## **Results**

### **TGA**

Mass loss occurs over three distinct temperature ranges (Fig. 2).

First, when heated from 25 to 180°C, the sample loses about 8 wt%, due to loss of H<sub>2</sub>O from various sites including collagen water of hydration, and water trapped in voids in mineral. From about 300 to 550°C, the sample loses about 27 wt% by the burning of collagen and other organic components. The minimum in the derivative curve at ~560°C suggests that burning of organic matters has ended. Between 550 and 900°C there is an additional loss of about 4.1 wt% presumably due to decomposition of CO<sub>3</sub><sup>2-</sup> in apatite, liberating CO<sub>2</sub>. The pattern shown here is very similar to that shown by Greiner *et al.* [13]. We can generally recognize three distinct stages in weight loss, separated by points at which the rate of mass loss goes through minima (red vertical lines on Fig. 2).

### **Constancy of furnace temperature**

When a sample is inserted into the furnace, the temperature immediately falls below the nominal temperature to which the furnace was set, which was displayed on the thermometer before insertion.

After closing the furnace door, T slowly returns to the nominal temperature (Fig. 3).

## **Preheating**

Preheating was carried out at 60°C for 30 min or 24 h. The average weight loss of liquid and adsorbed water for samples ranging in weight from 10 to 100 mg was  $7.8 \pm 0.8$  wt% for both preheating times.

## **Testing effects of variation in conditions of ashing**

Samples were ashed at 500, 600 and 700°C. Samples of both larger weights, between 50 and 100 mg, and smaller weights between 5 and 20 mg were tested. Ashing times of one hour and 24 hours were used. Preheating at 60°C was also tested for 30 minutes and 24 hours.

The data obtained using these various methods are given in Supplement 2, and are summarized in Figs. 4-7.

From Fig. 4, we immediately see the nature of the problem of obtaining a single ash weight from a bone sample. As the temperature of ashing increases, the weight of the residue gradually decreases, lowering from  $69.4 \pm 1.1$  to  $64.3 \pm 1.1$  wt%. This is a 7.3% increase in weight loss. Which of these weights is the “correct” ash weight?

The heating experiment was repeated using sample weights of 5 to 20 mg to test the reproducibility of the method using smaller samples. The results are shown in Fig. 5.

We again see a drop in ash weight of 7.1% as the ashing temperature rises from 500 to 700°C. Some of this loss could be due to continued burning of organic material which appears to end at ~560°C.

The time of heating the sample would presumably also have some effect on ash weight; to test this we increased both the preheating and ashing periods to 24h (Table S2.3). The longer preheating and ashing times resulted in an average wt% ash of  $66.2 \pm 0.55$  while preheating for 30 min and ashing for 1 hour had given a significantly larger ash wt% of  $68.0 \pm 0.9$  (Table S2.1).

## **Standard method of ashing**

Based on the prior results, it was apparent that the ash weight result would depend critically on the time and temperature of heating. We attempted to develop a standard method for determining the ash weight of bone which would achieve the following: a) minimize dispersion of the data (reproducibility); b) minimize the amount of sample needed for analysis; and c) minimize time needed for the analyses. Based on our experimental runs, we selected four possible sets of conditions (Tables S2.4 and S2.5). Fig. 6 displays the results of the experiments with larger weights of bone, while Fig. 7 displays the results with smaller weights of bone.

In principle, either the 600°C or 700°C ash weights could be used as the “reference method” for determination of ash weight. For reasons presented in the “Discussion,” we have selected the smaller

weights of samples and heating at 600°C as the standard. The complete method would be as follows:

Sample weight: 5 - 20 mg

Preheat treatment: 60°C for 24h, followed immediately by

Ashing: at 600°C for 1 h

The average obtained for our standard bovine sample using these conditions was  $70.5 \pm 1.1$  wt%; individual percent ash weights ranging from 68-73% (Table S2.5, Fig. 7).

## Human samples

In order to test the efficacy of this method, it was applied to a series of distinct human bone samples from older women and one young man. The data are summarized in Table S2.6 and Fig. 8.

**Table 2:** p-values for statistically significant samples shown in Fig. 8

Samples Compared	P- value
161569 vs. all other samples	<0.0001
172202 vs. Bovine bone	0.0003
171519 vs. 1553	0.0261
1553 vs. 1493	0.0051
1553 vs. 1640	0.0065
1553 vs. Bovine bone	<0.0001
19 yr vs. Bovine bone	0.0242

## Discussion

### Analyses of bovine bone

Almost all studies of ashing of bone samples in the literature report data obtained by heating samples at 600°C or higher temperatures. The TGA results reported here and in other studies [13] show that starting at 600°C, bone samples lose additional mass beyond that lost from burning of organic material. As we showed earlier, this is mainly due to loss of CO<sub>2</sub> from the breakdown of CO<sub>3</sub><sup>2-</sup> molecules in the apatite structure.

In spite of this uncertain degree of loss of CO<sub>2</sub>, it is advisable to carry out the ashing at  $T \geq 600^\circ\text{C}$  to assure the complete loss of organic matter since, up to the point at which CO<sub>3</sub><sup>2-</sup> decomposition begins, the TGA curve shows a continuous decrease in bone mass due to combustion of residual organic matter (including previously pyrolyzed and less readily flammable carbonaceous substances). Greiner *et al.* found that at up to 600°C, bone retains a slightly greyish colour but after heating at 700°C it appears

white. We presume that the greyish colour signified the continued presence of traces of organic matter that are eventually burned away, leading to further mass loss.

It would seem therefore that we should have selected a higher temperature than 600°C as the ideal temperature to be used in a standard procedure. Although the dispersion of ash wt% values is negligibly different for ashing at 700°C than at 600°C (coefficient of variation = 1.4% vs 1.6%), the average ash wt% is significantly less (by 3.2 wt%) for samples heated at the higher temperature, presumably due to additional loss of CO<sub>2</sub>. Since we would like to obtain an ash wt% value as close as possible to the actual mass of inorganic matter in bone, we have selected 600°C as the standard temperature for ashing. As well, in order to minimize loss of CO<sub>2</sub>, we have used a relatively short heating time of one hour. Heating for 24h at 600°C resulted in additional 1.8 wt% loss in ash weight (Table S2.1 and S2.3), due to additional slow loss of CO<sub>2</sub>.

Comparisons of the ashing of larger and smaller sample weights show that using a sample as small as 10 to 20 mg did not compromise the precision or accuracy of the measurement. The ash wt% data were identical for the two weight sizes when heated to either 600°C or 700°C, and the standard deviations were indistinguishable (Figs. 6 and 7, Tables S2.4 and S2.5).

Significant fluctuations in temperature of the furnace after insertion of the sample are inevitable for most furnace designs (where the door to the furnace must be completely open to insert the sample). The effect is reproducible for a given oven temperature and could be taken as another standardized variable. However, variable types of furnaces in use in research laboratories could introduce some offset in the ash wt% measured.

We only used formalin-fixed, defatted cortical bone, and did not investigate effects of differing methods of defatting or using fresh bone. We also did not study trabecular bone, which, from previous studies cited in Table S1, may lead to greater uncertainties in the ash weight percent.

### **Analyses of human bone**

With the exception of one obvious outlier (161569), which happened to be the oldest individual analyzed, the average ash weight of the human bone samples is  $67.3 \pm 1.7$  wt%; this is close to published values [14]. Individual humans differ from one another by up to 4.2 wt%, while the average error in the quadruplicate analyses (mean of the s.d.'s for each analysis) is 0.9 wt%. This suggests that relatively small differences (~1 wt%) between individuals could be resolved.

### **Influence of internal burning**

When bone is heated in the presence of air, the organic material (largely collagen) ignites and begins to burn from the outside of the piece toward the center. As the collagen burns the internal temperature could rise higher than the ambient temperature of the furnace, leading to more complete thermal degradation of the bone, especially in larger pieces of bone. The largest samples analyzed here (100 mg) appear to have

been too small to produce such effects, as seen by comparing data for larger and smaller samples (Tables S2.4 and S2.5).

## Conclusions

The goal of this study was to develop a high-precision method of ashing of bone. During heating above  $\sim 500^{\circ}\text{C}$ , the mineral content of bone begins to decompose due to the release of  $\text{CO}_2$  from  $\text{CO}_3^{2-}$  molecules in the apatite crystals. This process is gradual and there is no fixed temperature or time of treatment at which the process is complete within the range of accessible heating temperatures. The loss of  $\text{CO}_2$  results in a decrease in the mineral mass so that the apparent ash weight after a certain duration of high-temperature heating will depend on the temperature and duration of heating. This will complicate measurement of ash wt% as an indicator of the fraction of bone mass that is mineral. We have shown that it is possible to find a single set of values of pre-treatment, ashing temperature and heating time that, when used together, generates a reproducible value of ash weight which could be accepted as the “standard” for comparison between laboratories or in a series of analyses in one laboratory and which achieves a precision of about 1wt%. When used on a set of human bones of varying medical history and age, each sample gives a well-defined value which is highly reproducible, but we see differences of up to 4.2 wt% between multiple samples. This would not be possible using other ashing techniques and the results would not be comparable between laboratories.

Hopefully, use of this method would allow scientists to characterize the effects on ash wt% of variation in treatment or in phenotype of experimental animals, or where human subjects were being compared to investigate the effects of age, disease, and diet.

## Declarations

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### Availability of data and materials

Not applicable

### Authors' contributions

The analyses were carried out by AT. Human samples donated by CQ. Writing by HPS, followed by editing by AT and CQ.

### **Ethics approval and consent to participate**

Not applicable

### **Consent for publication**

Not applicable

### **Competing interests**

The authors declare that they have no competing interests

### **Ethical approval**

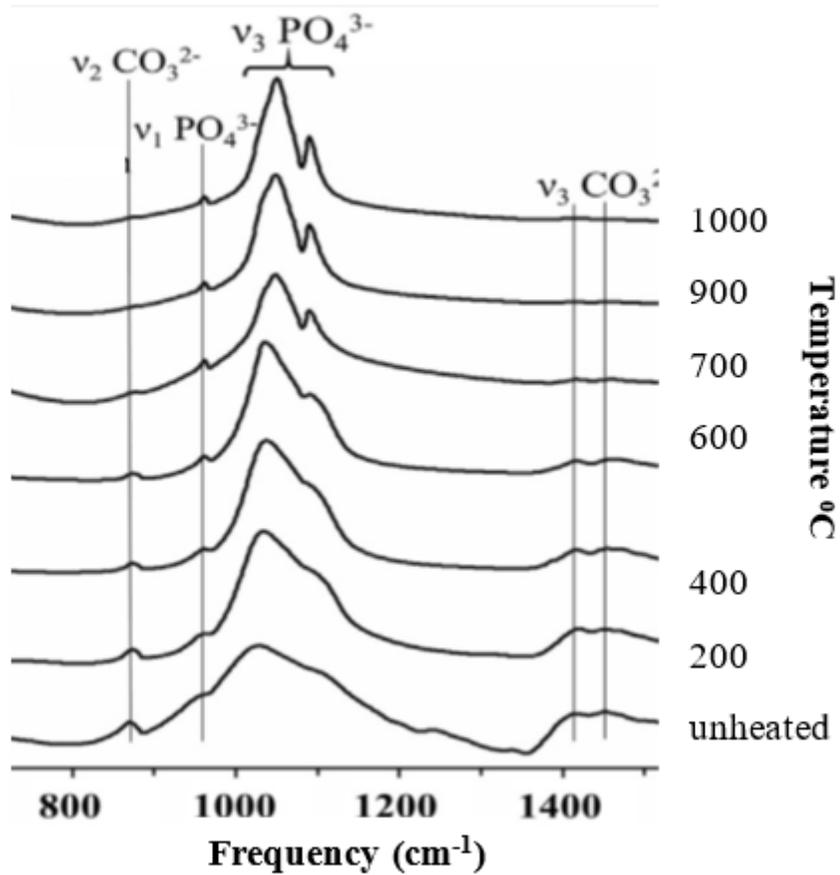
Ethics approval for use of human samples was obtained from HiREB (Hamilton Integrated Research Ethics Board) as a human tissue research study, approval 2016-2346-T

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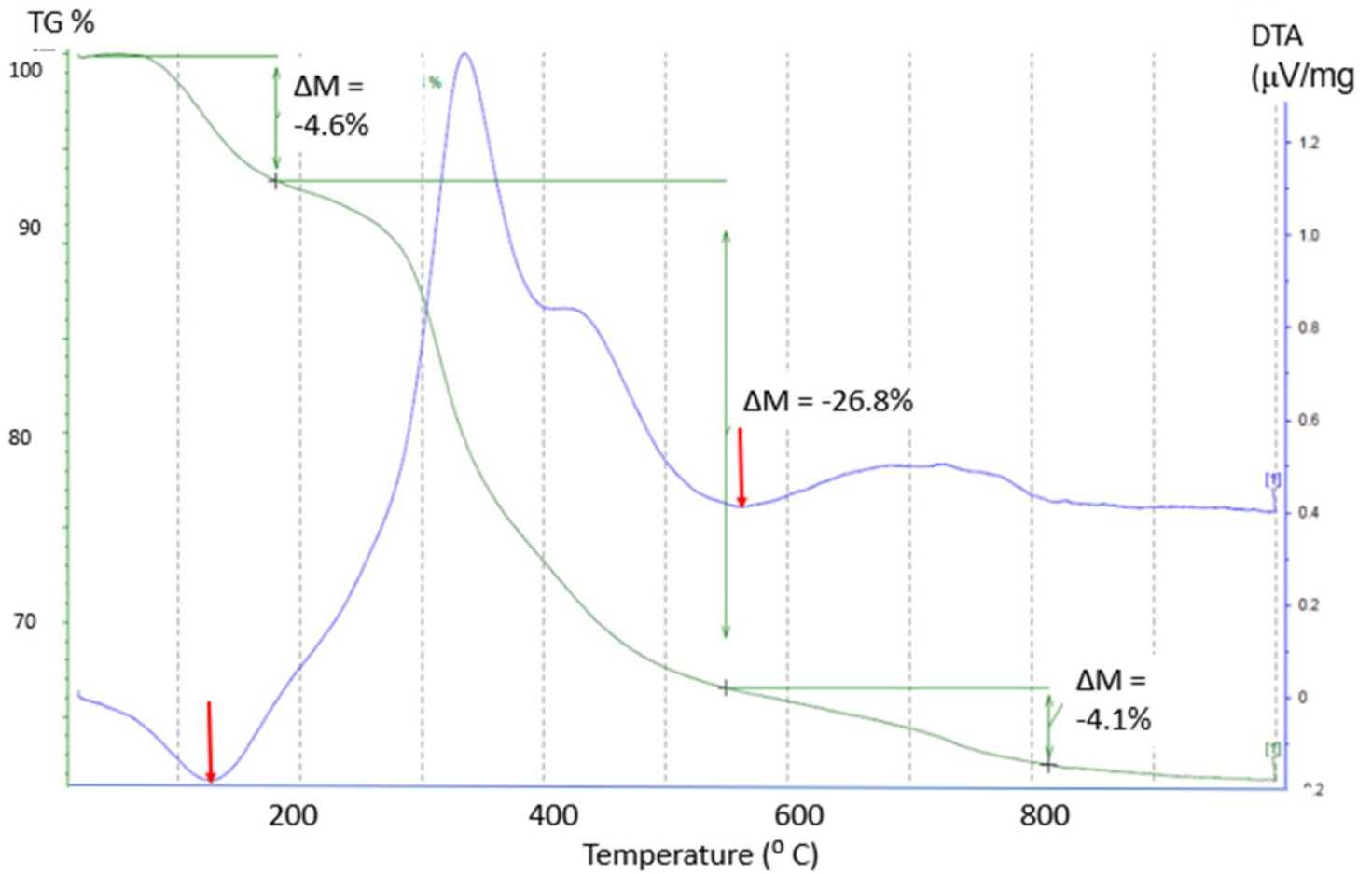
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## Figures



**Figure 1**

Variation in intensity of FTIR absorption peaks of  $\text{CO}_3^{2-}$  ion with heating. Each sample was heated at the specified temperature for 150 minutes. Temperatures in  $^{\circ}\text{C}$ ;  $\nu_i$  = vibrational mode. From Greiner et al. [10] with permission.



**Figure 2**

TGA record for sample of bovine bone. Green curve: weight loss; blue curve: derivative of green curve with respect to temperature. Red arrows: minima in derivative curve representing changes in mode of mass loss.

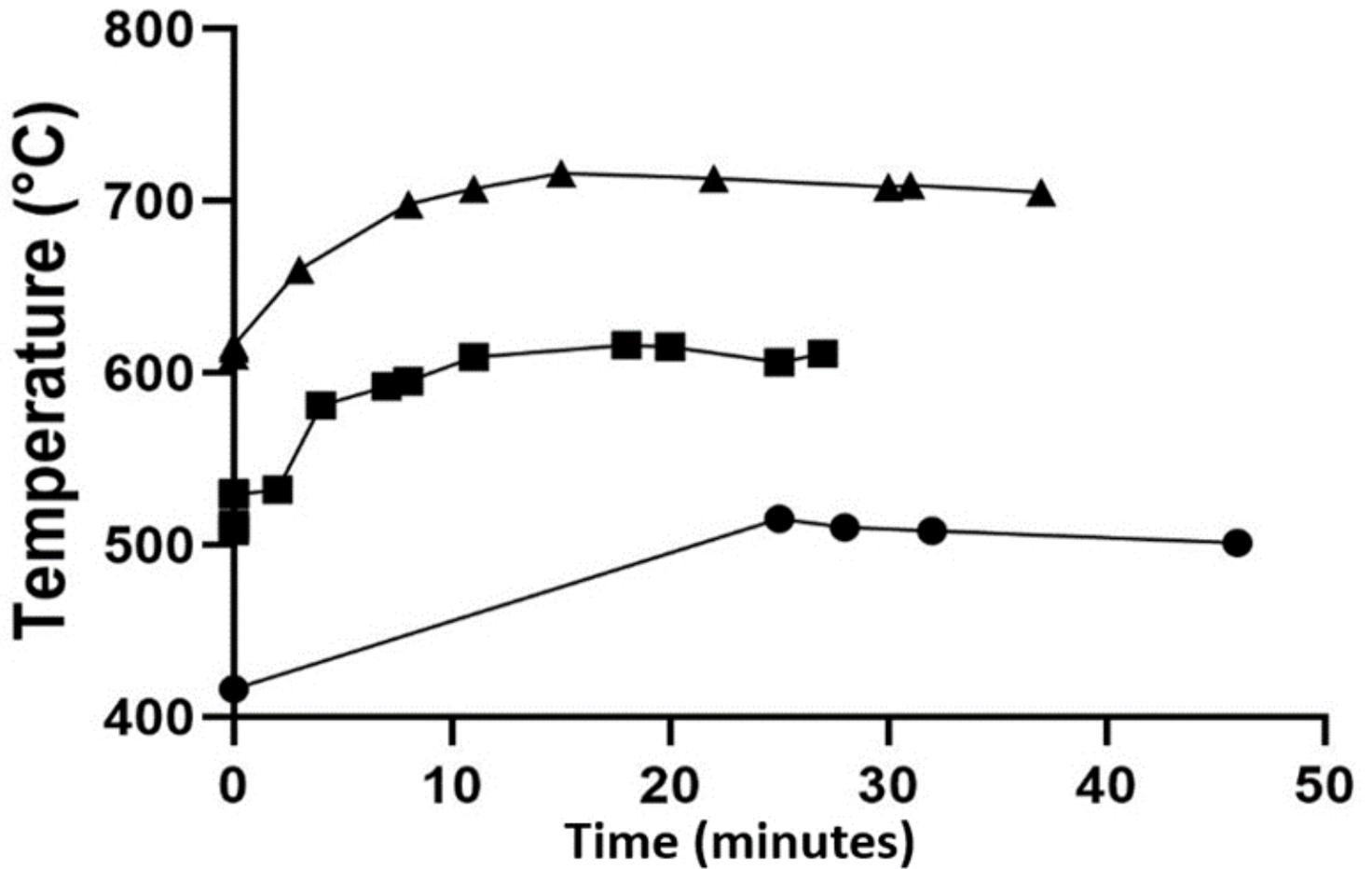


Figure 3

Variation in temperature after insertion of the sample into the furnace. The nominal temperatures were: circles: 500°C, squares: 600°C; triangles: 700°C. For each set of data, the temperature just prior to the insertion of the sample was the nominal temperature.

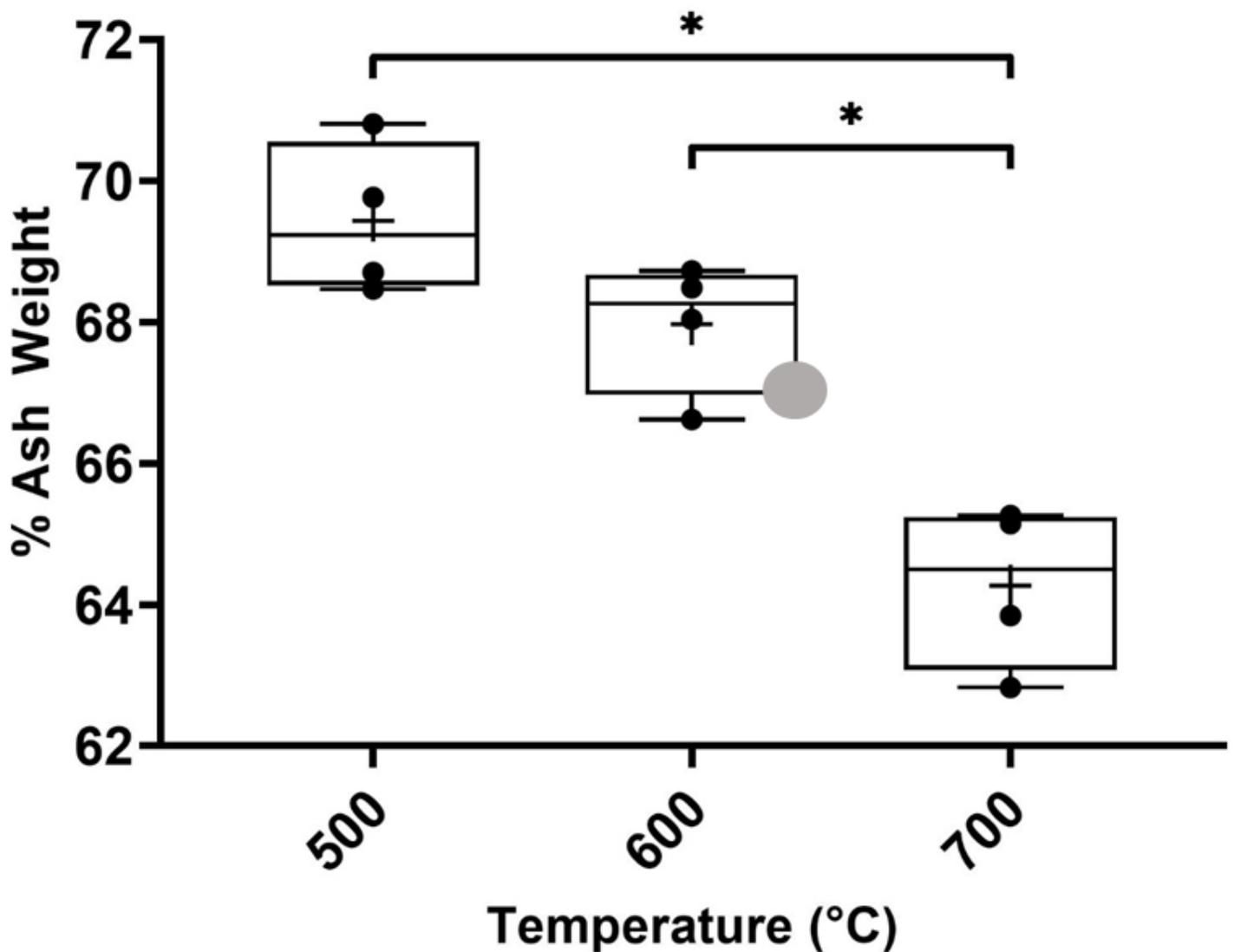


Figure 4

Variation in ash weight percent with temperature of ashing for samples of bovine bone weighing between 40 and 100 mg. All samples were preheated at 60°C for 30 minutes before ashing for 1 hour. The midline of each box marks the median while + marks the mean. The grey circle is the value obtained at 600°C after heating samples of the same size for 24h (data from Table S2.1); the radius of the circle is 1 s.d. of the quadruplicate samples. In this and all subsequent figures the mean is shown by a +, the median by the midline of the rectangular box. Mean ash weight at 500°C was significantly higher than at 700°C ( $p=0.0002$ ). Mean ash weight at 600°C was also significantly higher than at 700°C ( $p=0.0021$ ).

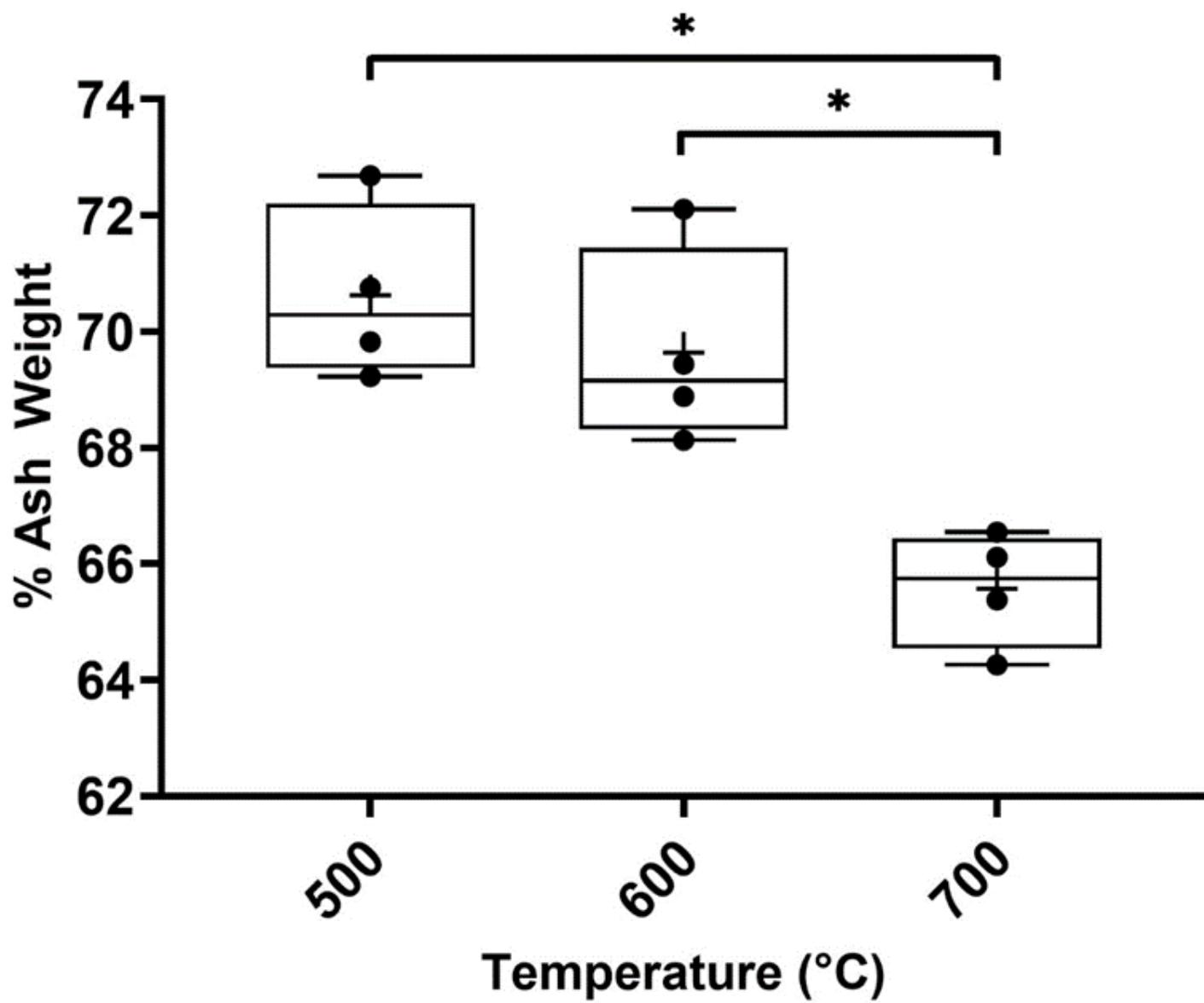


Figure 5

Percent ash weight of bovine bone samples weighing 5-20 mg, preheated at 60°C for 30 minutes and ashed for 1 hour at various temperatures (data from Table S2.2). Mean ash weight at 500°C was significantly higher than 700°C ( $p= 0.0021$ ). Mean ash weight at 600°C was also significantly higher than 700°C ( $p= 0.0081$ ).

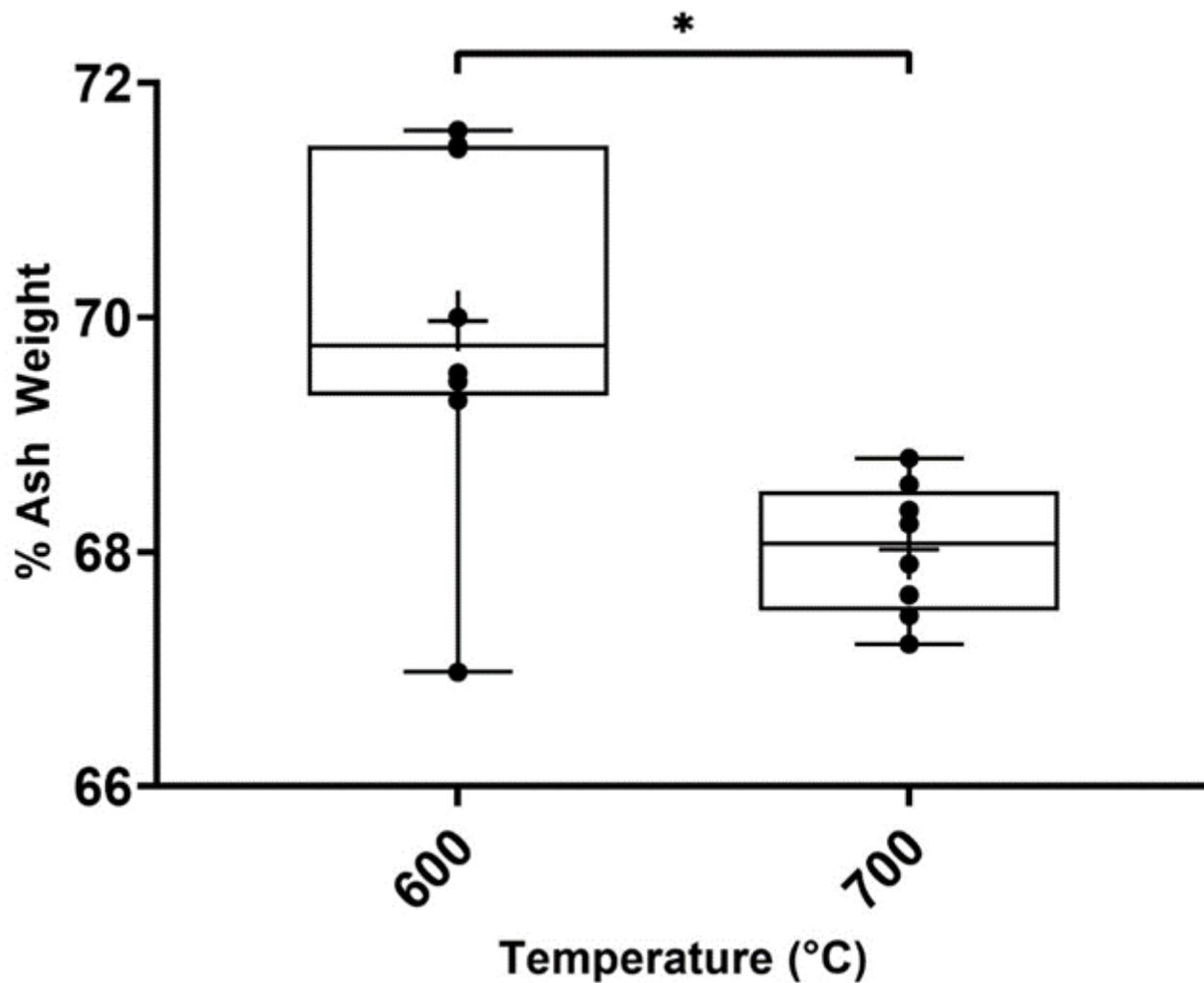


Figure 6

Ash weights of samples of bovine bone of 50-100 mg preheated at 600°C for 24 h and ashed at 600 or 700°C for 1 hour (data from Table S2.4). Mean ash weight at 600°C was significantly higher than 700°C ( $p= 0.0007$ ).

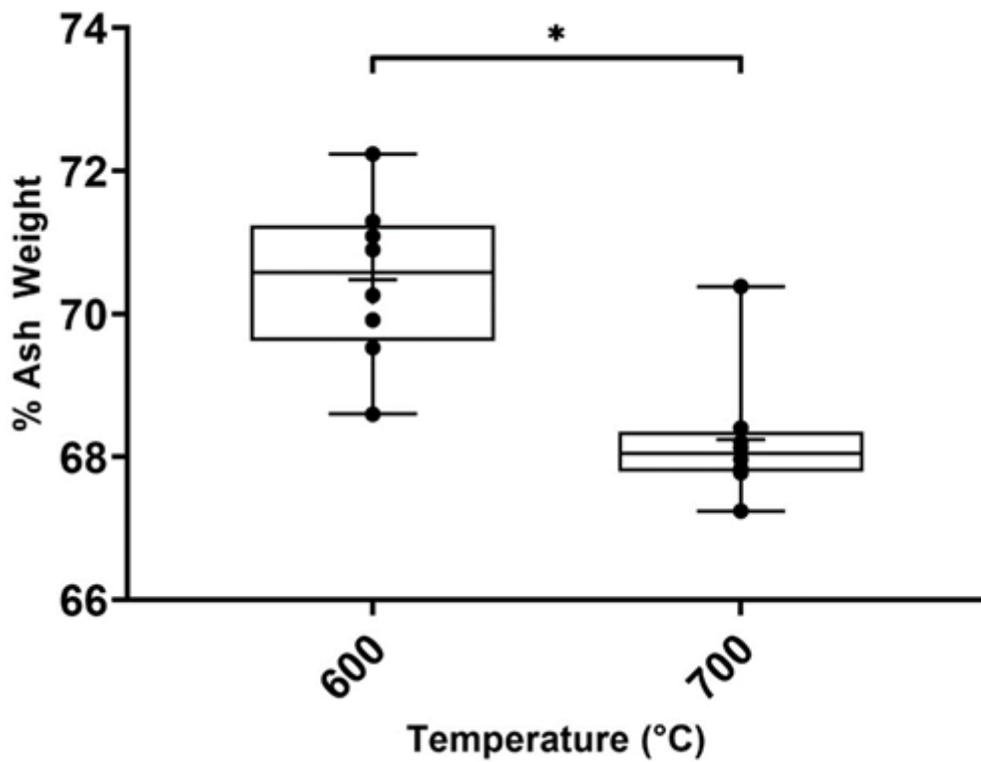


Figure 7

Ash weights of samples of bovine bone of 5– 20 mg, preheated at 60°C for 24 h, and ashed at 600 or 700°C for 1 hour (data from Table S2.5). Mean ash weight at 600°C was significantly higher than 700°C ( $p= 0.0007$ ).

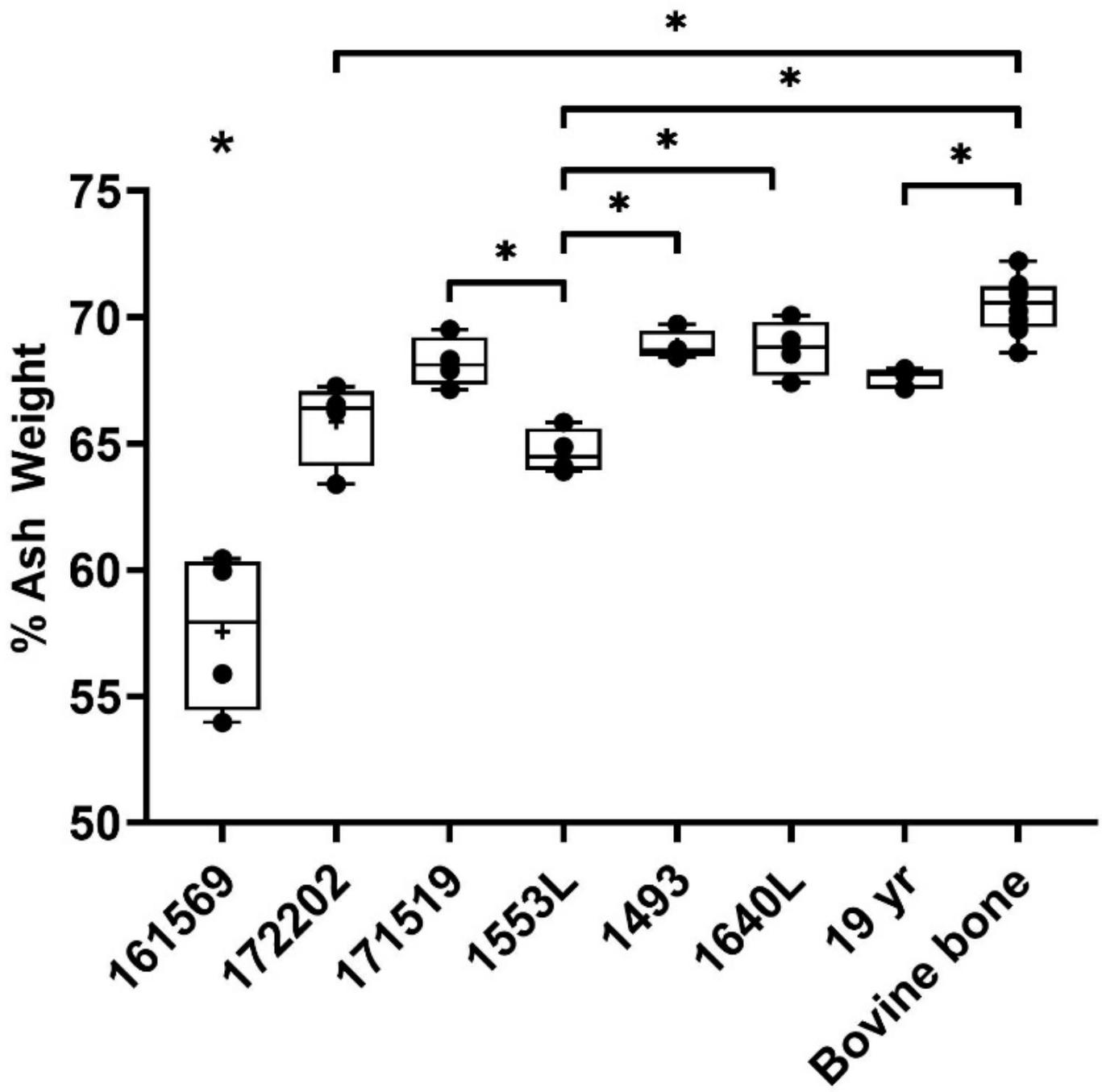


Figure 8

Percent ash weight of human and bovine bone samples of 5-20 mg ashed for one hour at 600°C. Samples were pre-heated at 60°C for 24 hours (data from Table S2.6). Table 2 summarizes the p-values of statistically significant samples.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SuppTable1RevisedOct42021.docx](#)
- [SuppTable2RevisedAug192021.docx](#)