Progressive Dehydration in Decomposing Bone: A Potential Tool for Forensic Anthropology

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Abstract

The aim of this pilot study was to determine whether collagen and/or water content of bone vary during soft tissue putrefaction by thermogravimetric analysis with a view to eventually developing a possible forensic application to determine postmortem interval. Porcine bone decomposed in a shallow burial showed an approximate difference in average mass loss of 15 +/- 8% when heated between 22 and 100˚C, compared to 14 +/- 3% for porcine bone decomposed in a surface deposition, equating to water loss. Mass loss showed peaks at 0, 250-500 and 1200-1500 cumulative cooling degree days' (CCDD) deposition for the experimental porcine bone. Should these measurements prove consistent in future studies on a wider variety of porcine and eventually human skeletal elements, they may have potential to be corroborated with other data when determining post-mortem interval, especially with disarticulated bones. A downward trend in mass loss was apparent within shallow burial and surface deposition scenarios (inclusive of freeze dried controls) for the thermolysis of collagen (and other proteins) between 220 and 650˚C during thermogravimetric analysis. This was inconsistent within the timeframe examined (0-1450 cumulative cooling degree days), and so demonstrates less potential as an indicator of post-mortem interval during soft tissue putrefaction.

Introduction

During thermogravimetric analysis, mass loss at a particular temperature over a given period of time illustrates the thermal decomposition of specific molecules and is thus indicative of the chemical composition of the substance (Heal, 2002). Jong Jin Lim's 1975 paper 'Thermogravimetric Analysis of Human Femur Bone' (Journal of Biological Physics) is arguably the first use of thermogravimetric analysis that is applicable to forensic anthropology. Lim thermally decomposed a 2 mm^3 sample of 20 year old human femur bone in air in the temperature range 25˚C to 1000˚C, for which the differential primary loss curve yielded four distinct peaks. Drawing on previous data from tendon collagen, and known values for water, Lim identified the first mass loss peak between 25˚C and

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200˚C as dissociation of water from collagen, the second significant mass loss peak occurred between 200˚C and 400˚C and was identified as resulting from the thermal decomposition of collagen and other proteins, and the third peak at 597˚C as residual organic components (non-collagenous proteins) associated with collagen. The fourth peak was identified by Lim as being linked with water and other losses associated with the thermally mediated transformation of hexagonal α-tricalcium phosphate crystals to rhombohedral β-tricalcium phosphate crystals between 700˚C and 780˚C. Raja et al (2009) obtained comparable results when using TGA to estimate pig bone age (at death). They dried 2 mm thick bone slices from pigs aged 3 months to 7 years that had been subjected to shallow burial at 50˚C for 2.5 hours to remove moisture. Thermogravimetric analysis of the samples was then carried out. Raja et al concluded from their results that total mass loss during thermogravimetric analysis decreased with post mortem age up to 23 months, demonstrating potential forensic application of TGA in this context. They found that, for the purposes of storage of forensic bone specimens, freeze dried ground; as opposed to oven dried ground samples (again determined by experimentation on porcine bone) provided the most consistent results, with the only variation in mass loss being consistent with age at death (Ibid). Drying does however, negate measurement of mass loss due to water desorption.

With regards to time elapsed since deposition of remains (bone) in the archaeological context, rwork by Tomassetti et al on 23 samples of fossil human bone obtained from the El Geili necropolis in Egypt showed that chemometric processing of data obtained from TGA (again stepwise increments between ambient and 1000˚C at 10˚C min^{-1}) accurately facilitated distinction between (relatively) more recent, and older, more mineralized samples according to % collagen / % carbonate ratios (Tomassetti et al, 2013). More recent archaeological samples showed greater mass loss attributed to the thermal decomposition of collagen, ergo more collagen was evidently present in comparison to the older samples. With regards to locus of deposition, Onishi et al's thermogravimetric mass spectrometry study of 45kg pigs decomposed over a period of 1 to 5 years in a range of deposition scenarios (clothed/unclothed, surface/burial, car purged with carbon monoxide – 'suicide') determined that significant differences in mass loss (again during TGA at stepwise increments between ambient and 1000˚C at 10˚C min^{-1} in an inert argon gas atmosphere) could not only be attributed to age at death, but also to locus of deposition. Unclothed surface deposited pigs' bone showed less water loss (220˚C step) than its buried counterpart for example (Onishi et al, 2008).

Previous studies have therefore indicated that TGA has some validity in determining age at death of porcine bone from collagen levels, and that water desorption can be similarly quantified. Lim's study indicated that comparable data can be elucidated from human bone (Lim, 1975). Further to this,

there is a general consensus in the literature that dehydration affects the mechanical characteristics of bone. A literature review by Bandini et al (2013) showed a consensus that soft tissue removal modifies water content of bone, leading to drying or hydrated conditions. Dall 'Ara et al (2007) likewise found a 10% increase in Vickers microhardness of trabeculae from wet to dry bone that had been dehydrated by exposure to a concentration gradient of 70% to absolute ethanol. Drying was found to increase the elastic modulus and hardness of bone, and to decrease the toughness and the strain to fracture (De-Macedo-Soares-Silva et al, 2005; Rho and Pharr, 1999; Townsend et al, 1975; Amprino, 1958). Currey (1988) showed that rewetting dried bone restored the mechanical properties to values similar to those of fresh specimens.

The purpose of the TGA experiment under discussion here from a forensic anthropological perspective was thus threefold. Primarily, any changes in water and/or collagen content could be correlated to time and temperature in the immediate postmortem interval (soft tissue putrefaction period), for use as a potential indicator of time passed during that interval. Secondly any such changes in hydration could potentially be correlated to changes in the mechanical characteristics of bone observed during the course of putrefaction, such as changes in micro-crack characteristics, or Vickers hardness, for example, which may themselves have potential uses for corroborative forensic testing in this context. Finally, it was used to test if TGA can discriminate between porcine bone samples decomposed in surface and burial deposition scenarios (Raja et al, 2009). Any such differential in the rate of dehydration of bone during soft tissue putrefaction could also have potential with regards to developing a forensic field application.

Methods and Materials

Materials

Porcine bone samples were obtained from adolescent male pigs killed within the previous 24 hours at abattoir. Bone was exposed to a series of scenario to simulate surface deposition and shallow burial. In both scenarios Porcine weight bearing long bones: femora, tibiae, and fibulae were deposited intact under a porous layer of 30 µm micro-mesh membrane before being buried, to facilitate decomposition via microbial action, and water perfusion whilst preventing more severe taphonomic damage to the samples.

Surface Deposition Scenario: The bones were placed on the ground covered in a 50mm deep layer of leaf litter, and for a period of six months within a privately owned enclosed area adjacent to woodland. Temperature readings were taken from the closest weather station, Ebbw Vale via http://www.degreedays.net/ : IWALESEB2: Ebbw Vale, Ebbw Vale, Wales (3.22W, 51.80N)

(1mi/2km). This is within 3.5 miles of the experimental site and shares the same temperature range and weather pattern. Cumulative Cooling Degree-days were calculated for comparison. Cooling degree days are a measure of how much (in degrees), and for how long (in days), the outside air temperature was *above* a certain level. For this study it is indicative of all days where temperature is above 4˚C and thus within the temperature range of active microbial decomposition, and enzymolysis. Where D_d is the daily (cooling) degree-days for one day, $Ø_b$ is the base temperature and $Ø_{0,j}$ is the outdoor temperature in hour j, the value is calculated as follows. The subscript denotes that only positive values are taken.

$D_d = \sum_{j=1}^{24} (Ø_b - Ø_{0,j})_{\left((Ø_b - Ø_{0,j}) > 0\right)} / 24$ (Buyukalaca et al 2001).

At 28 day intervals (t0-t140 days) the bone samples (n=5) were carefully retrieved and frozen at -20°C. Typically this was for a period of one to four months prior to analysis. As access to the TGA laboratory was limited this was to ensure all experimental samples would be available during the allotted time slot.

Shallow Burial Scenario: The bones were buried at a depth of 300mm below 25 litres of loam soil of known pH in an aerated trough (1000mm (l) by 200mm (w) by 330mm (d)), for a period of 140 days . A predator cage (a rabbit run was utilised) was erected over the samples within the privately owned enclosed area adjacent to woodland, to prevent scavenging by local wildlife. Cooling Degree-days were calculated for comparison. Samples (n=5) were again retrieved at 28 day intervals (t0-t140 days) and frozen and stored as above.

Comparative Human Bone Samples

Fresh (Perimortem Analogue) Sample

A head and neck of femur of a 60-70 year old female was retrieved from the operating theatre subsequent to a hip replacement operation, after the patient had consented for this sample to be donated for research purposes and with ethical approval. As the tissue was non-viable, it was exempt from the UK Human Tissues Act, 2004. The sample was then frozen, machined, defrosted and thermogravimetrically analysed.

Dry (Postmortem Analogue) Sample

A complete femur, part of a teaching skeleton, was sourced from a reputable antiques dealer, legally entitled to trade this item. It was certified as over 100 years old and is exempt from the UK Human

Tissues Act, 2004. The femur was natural dry bone and had not been treated with any preservative agent. The sample was then frozen, machined, defrosted and thermogravimetrically analysed.

The porcine long bones, and comparative human bone samples, were frozen for a period of not less than 48 hours at -20°C (48 hours to two weeks for the human bone samples). The purpose of this was dual fold, firstly to facilitate easier machine cutting of samples, and secondly to negate the effects of friction generated heat when cutting, to minimise thermal damage (Evans, 1969). The bones were then machined into 10mm thick longitudinal and transverse section samples with a Draper 100 W band saw at low speed. Cortical bone from these samples was then cut into smaller sections of approximately 5mm by 2mm with a Dremel 3001 powered hand saw. Half of the samples were freeze dried in a Thermo© Heto PowerDry model PL3000 freeze dryer at -50˚C at an atmospheric pressure of 2 Pa, for 7 days. This was both to provide a baseline for measurement of water desorption and thus act as a control. The other half of the samples were left untreated. All samples were then ground to a rough powder with a pestle and mortar to give an amorphous consistency prior to TGA. Thermal decomposition of the bone samples was performed by thermogravimetric analysis on a Stanton Redcroft (STA-780 series).

Ground bone samples (15-20 mg) were placed in a ceramic crucible and analysed under constant flowing helium (2 bar, 20 ml/min) to provide an inert atmosphere, from ambient (room) temperature (22°C) to 800°C at 10°C min^{-1} with a view to examining the degree of water desorption and thermal decomposition of collagen as summarised next.

Results

Mass loss due to thermal decomposition in the ranges of 22˚C to 100˚C (suspected free water desorption), 100˚C to 220˚C (suspected bound water desorption), and 220˚C to 650˚C (thermal decomposition of collagen and other organic matrix components) have been summarised according to the duration of putrefaction, measured in cumulating cooling degree days, for surface and burial scenarios for experimental porcine bone samples. The same measurements were recorded for the comparative human bone samples.

Porcine bone allowed to decompose in a shallow burial showed an approximate difference in average mass loss of 10 +/- 4% when heated between 22 and 100˚C during TGA, compared to 8.75 +/- 3% for porcine bone allowed to decompose in a surface deposition. Porcine bone allowed to decompose in a shallow burial showed an approximate difference in average mass loss of 15 +/- 8% when heated between 22 and 100˚C during TGA, compared to 14 +/- 3% for porcine bone

decomposed in a surface deposition. The range of difference was within 11% mass loss for all parameters. (It was within 2% for the human perimortem analogue and postmortem comparatives). Percentage mass loss shows peaks at 0, 250-500, and 1200-1500 cumulative degree days' (decomposition) for both deposition scenarios. Porcine bone allowed to decompose in a shallow burial showed an approximate difference in average mass loss of 4.46 with a confidence interval of +/- 0.5% when heated between 100 and 220˚C, compared to 4.9 with a confidence interval of +/- 2% for porcine bone decomposed in a surface deposition, after being subjected to freeze drying (see table 1 and figure 1).

Porcine bone allowed to decompose in a shallow burial showed an approximate variation in average mass loss of 3.9 +/- 3.4% when heated between 100 and 220˚C, compared to 4.5 with a confidence interval of +/-1.3% for porcine bone decomposed in a surface deposition. The range of difference was within 5% mass loss for both deposition scenarios. Freeze drying appears to have had minimal effect on adsorbed water evaporated during thermogravimetric analysis in this temperature range. Percentage mass loss appears relatively consistent across deposition scenarios over time. Porcine bone decomposed in both deposition scenarios showed an average mass loss of 29.6% when heated between 220 and 650˚C (where thermolysis of collagen and other proteins occurs) after being subjected to freeze drying. Porcine bone decomposed in a shallow burial showed an average mass loss of 26.25% when heated between 220 and 650˚C, and 23.74% for porcine bone decomposed in a surface deposition. The range of difference was within 6% mass loss for both deposition scenarios. A downward trend in % mass loss was apparent over time, but it was inconsistent compared with mass loss due to water evaporation (see figure 1 and table 1).

A hierarchical, supervised, nearest-neighbour fuzzy cluster analysis was utilised to determine if the percentage mass loss from the bone samples within the specified temperature ranges showed any interdependence with the periodicity of soft tissue putrefaction, deposition scenario, the use or omission of freeze drying, and if the human comparative samples shared any commonality with the experimental porcine bone samples in these respects. In a fuzzy cluster analysis, every object belongs to every cluster with a membership weight between 0 (does not belong) and 1 (absolutely belongs); the clusters are treated as fuzzy sets (University of Minnesota, 2017). The NCSS™ software utilised for this statistical test also computed the probability with which each point must belong to each cluster, the sum of which must equal 1 (Ibid). Fuzzy (probabilistic) clustering can be interpreted as exclusive clustering by assigning each object to the cluster in which its membership weight or probability is highest (Ibid).

With the maximum number of potential clusters set to four, four distinct clusters of bone samples became apparent. This remained consistent for when the maximum number of potential clusters was set to five, six and seven. With the maximum number of potential clusters set to seven, accounting for each experimental parameter, four distinct clusters (according to ascribed weighting) with two outliers became apparent (see figure 2). The first cluster was occupied by the T28 (250 CCDD) porcine bone sample from the burial deposition scenario, and the second cluster was occupied by the T56 (551 CCDD) sample from the same scenario. These constituted the two outliers (see figure 2). The third discernible cluster was occupied primarily by porcine bone samples from the latter part of the soft tissue putrefaction period ranging from 551 to 1450 CCDD from both deposition scenarios. Two of the freeze dried samples, including the human postmortem sample were included. The subsequent cluster contained primarily surface deposition samples ranging in decomposition periodicity from 250 CCDD to 1450 CCDD, the freeze dried T0 sample, and the 1450 CCDD buried porcine bone sample. The next cluster contained predominately freeze dried bone samples with one exception, the human perimortem sample (head of femur donated from a hip transplant). The final cluster contained the T0 (untreated perimortem analogue) porcine bone sample, the untreated human postmortem bone sample (from the femur of a 100+ year old teaching skeleton) and three freeze dried porcine bone samples ranging in decomposition periodicity from 551 to 1450 CCDD from both deposition scenarios.

With regards to the positioning of clusters relative to axes in a fuzzy cluster analysis such as this, the mean vector is coincident with the axis along which the sum of the squared projections of the vectors is at maximum because the mean of a group of unit vectors is the eigenvector corresponding to the maximum eigenvalue (Hammah and Curran, 2000). For this experiment, this means that the position of any cluster along a particular axis is indicative of the influence of the parameter to which that axis refers. Referring to figure 2, it can be seen that clustering of points identifying bone samples predominates between the axes pertaining to the two temperature ranges where water is desorbed. The results thus suggest that water content changes as decomposition progresses as samples from the earlier (0-551 CCDD) part and latter part (551 CCDD+) of the soft tissue putrefaction period tended to be in the same clusters, albeit with some outliers such as the T0 sample in the final cluster (Tomassetti et al, 2013). There may be variation in water content within samples from different locations in one bone, between bones, and between animals. These are factors that would need to be addressed in any unscaled version of this experiment. As was to be expected, freeze drying appears to have had a demonstrable effect on the water content of the bone. There did not appear to be any consistent clustering according to deposition scenario.

Pearson tests of potential linear correlation between each of the temperature parameters utilised in this experiment were conducted at 95% confidence (Puth et al, 2014). The results suggested some limited negative correlation between the amount of water desorbed between 22-100˚C and that desorbed between 100-220˚C (Pearson correlation -0.44, p = 0.03, see fig. 9.1). No significant correlation was demonstrated between the amount of water desorbed between 22-100˚C and the thermal decomposition of collagen between 220-650˚C (Pearson correlation -0.32, p = 0.18, see fig. 9.2). Some positive correlation was evident between water desorbed between 100-220˚C and the thermal decomposition of collagen (Pearson correlation 0.13, p = 0.52, see figures 3.1 to 3.3).

Discussion

To apply Pineri's water-collagen binding interaction hierarchy, water loss in the 22 and 100˚C temperature range (less was lost in the freeze dried control samples as was to be expected, but also indicated that even with 7 days freeze drying, water remained adsorbed within the bone) could be posited as having mainly weaker hydrogen bonds, such as those between water molecules, equating to the third regime of double hydrogen bonding of the water molecules between the collagen triple helix and between collagen microfibrils (the second level of organisation) which was found by Pineri et al to be responsible for a large increase in the rigidity modulus of the collagen; and the final regime posited as free water between collagen microfibrils (Pineri et al, 1978). Water loss in the 100 and 220˚C temperature range could be posited as having stronger hydrogen bonds, such as those between water molecules and collagen, equating to Pineri's first regime is that of triple hydrogen bonding inside the collagen triple helix molecule, associated with hydroxyproline at distinct sites. The second is that of double hydrogen bonding between water molecules at available sites within the collagen triple helix molecules, resulting in a small increase in the rigidity modulus of collagen (Pineri et al, 1978). Porcine bone decomposed in a shallow burial showed an average mass loss of 29.6% when heated between 220 and 650˚C (where thermolysis of collagen and other proteins occurs), and for porcine bone decomposed in a surface deposition, after being subjected to freeze drying. The clustering between 100-220˚C could be considered in conjunction with the original principle component analysis and in accordance with Pineri's water-collagen binding interaction hierarchy, again suggesting there may be more water between collagen microfibrils at these times during decomposition and that water adsorption to collagen via hydrogen bonding is stronger at these times (Pineri et al, 1978; Dubey and Tomar, 2013).

Conclusions

The peaks of water loss between 22 and 100 ˚C noted during thermogravimetric analysis of the current samples, suggests there may be more water between collagen microfibrils at 250 and 1450 cumulative cooling degree days decomposition, which may be of potential use when determining the postmortem interval. There appears to be sufficient difference between water loss as % of total mass in this temperature range (at least 5%) to distinguish between surface and shallow burial deposition scenarios, in agreement with Onishi et al (2008). With regards to further research, an initial expanded study on all bones of whole porcine carcasses in a variety of deposition scenarios would be an appropriate first step, to determine if the % mass loss as water in the 22-100˚C temperature range during thermogravimetric analysis is universally applicable to porcine bone during soft tissue decomposition. That experiment could then be repeated at a dedicated taphonomic research facility with donated human cadavers to fully determine potential validity of said difference in the forensic context. % mass loss attributed to water desorption between 100-220˚C appears relatively consistent i.e. little change, for surface and shallow burial deposition scenarios (inclusive of freeze dried controls) when measured against increase in cumulative degree days' deposition (decomposition) for the timeframe examined (0-1450 cumulative cooling degree days), and so does not appear to have any value as a potential forensic indicator of corpse deposition time within the soft tissue putrefaction period. A downward trend in % mass loss is apparent within shallow burial and surface deposition scenarios (inclusive of freeze dried controls) for the thermolysis of collagen (and other proteins) between 220 and 650˚C during thermogravimetric analysis when measured against increase in cumulative degree days' deposition (decomposition), but it was inconsistent within the timeframe examined (0-1450 cumulative cooling degree days), and would therefore lack validity as a forensic indicator of corpse deposition time within the soft tissue putrefaction period. However it should be reiterated that this does not detract from its potential as a forensic indicator for longer term corpse deposition time, as outlined by Tomassetti et al (2013) and indicated by the 6.8% greater loss of mass in this temperature range shown by the 100+ year old postmortem human bone sample (teaching skeleton) as compared to the perimortem analogue human bone sample (head of femur sourced from a hip replacement procedure).

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Table 1: Summary of Bone Mass Loss According To Temperature Range during TGA

Temperature	Water Desorption 22˚C to 100˚C		Water Desorption 100˚C to 220˚C		Thermal Decomp. Collagen and other Proteins 220˚C to 650˚C	
Parameter: T time in days; FD Freeze Dried	Mass Lost Mg	% Mass Lost	Mass Lost Mg	% Mass Lost	Mass Loss Mg	% Mass Loss
T0	2.31	16.53	0.68	4.86	3.94	28.20
T0 FD	2.08	11.44	0.86	4.73	5.00	27.52
T28 Burial FD	0.98	6.55	0.62	4.14	4.27	28.54
T28 Burial	2.32	14.81	0.08	0.5	5.29	33.78
T28 Surface FD	1.04	5.36	1.4	7.22	8.71	44.96
T28 Surface	2.84	16.74	0.69	4.06	4.39	25.88
T56 Burial FD	2.33	13.77	0.83	5.12	4.73	29.21
T56 Burial	3.67	23.22	0.66	4.17	3.86	24.43
T56 Surface FD	1.73	9.81	0.87	4.94	5.46	31.02
T56 Surface	1.93	12.70	0.89	5.85	3.34	21.98
T84 Burial FD	1.24	6.93	1.09	6.09	4.81	26.88
T84 Burial	1.54	9.75	0.91	5.76	3.63	22.98
T84 Surface FD	1.26	7.66	0.74	4.50	4.98	30.29
T84 Surface	1.72	10.51	0.90	5.50	3.31	20.23
T112 Burial FD	1.67	10.42	0.80	4.99	5.98	37.32
T112 Burial	1.9	11.68	0.91	5.59	3.79	23.30
T112 Surface FD	1.22	7.59	0.65	4.04	3.99	24.82
T112 Surface	2.26	15.50	0.45	3.08	3.37	23.11
T140 Burial FD	1.76	11.36	0.63	4.06	4.29	27.69
T140 Burial	2.88	15.91	0.74	4.08	4.49	24.80
T140 Surface FD	1.66	10.57	0.55	3.50	3.06	19.49
T140 Surface	2.02	12.60	0.70	4.29	3.70	23.08
Human Peri FD	0.95	5.55	0.87	5.09	5.16	30.19
Human Peri	1.33	8.48	0.82	5.23	5.15	32.86
Human Post FD	1.39	7.97	1.07	6.13	4.17	23.91
Human Post	1.58	10.08	0.71	4.53	4.08	26.03
Days Deposition	T0	T28	T56	T84	T112	T140
Cumulative Cooling Degree Days	0	250	551	879	1219	1450

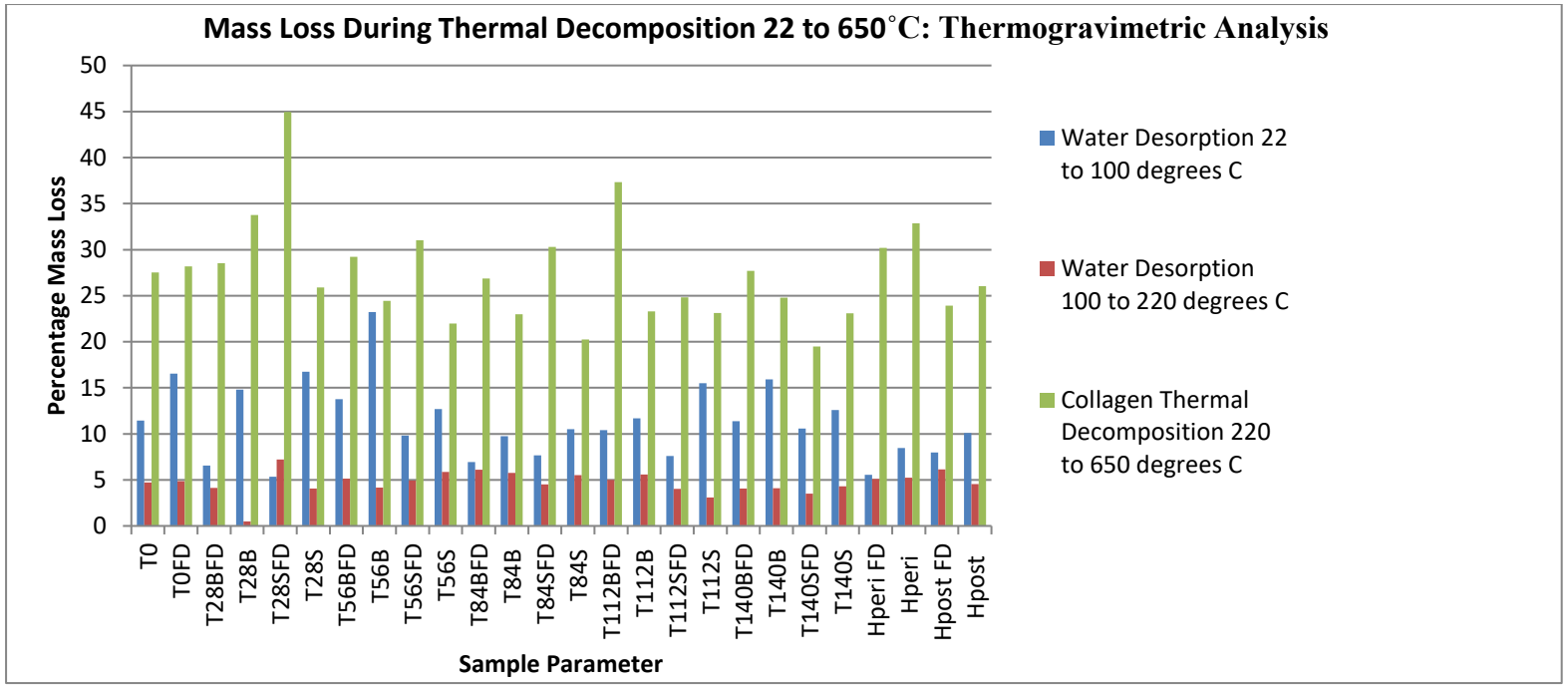

Figure 1: **Percentage of total mass loss during thermal decomposition 22 to 650ºC**

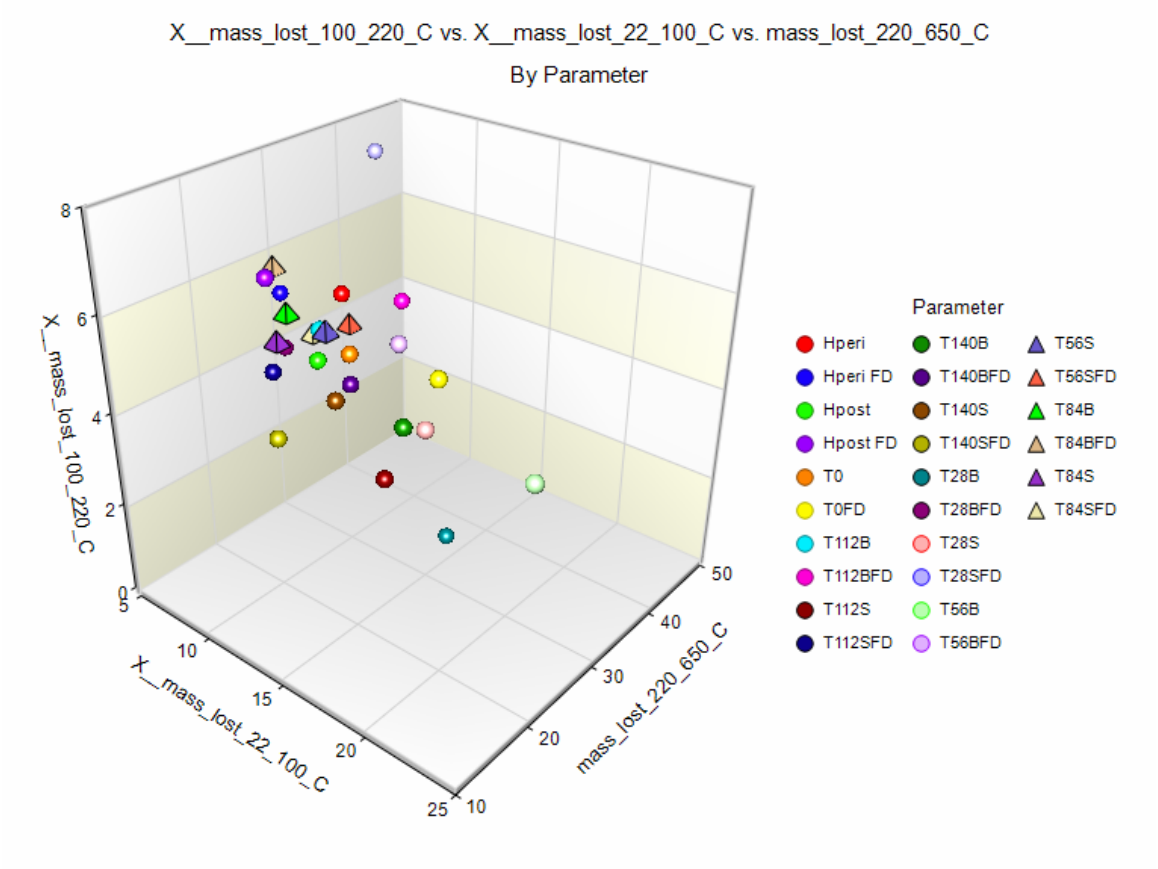

Figure 2: Cluster Analysis of TGA Results: 3D Scatter Plot Generated from the NCSS Report for the Maximum of 7

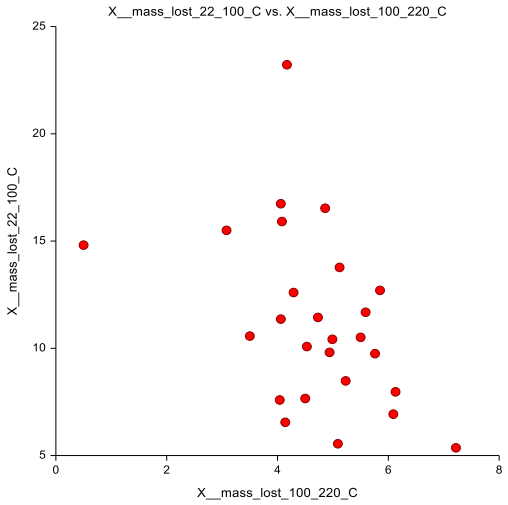

Figure 3.1: Pearson's correlation between exprimental porcine bone samples subjected to 22-100˚C and 100-220˚C temperature ranges.

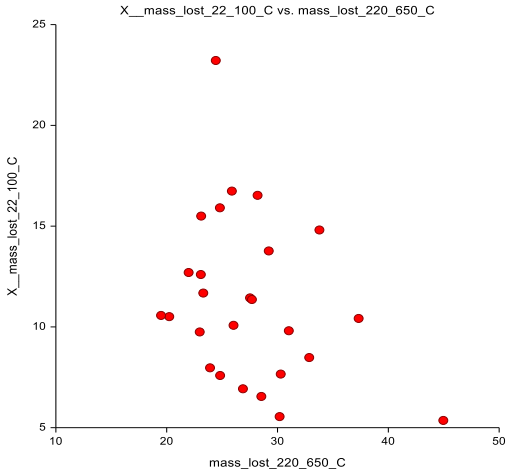

Figure 3.2: Pearson's correlation between exprimental porcine bone samples subjected to 100-220˚C and 220-650˚C temperature ranges.

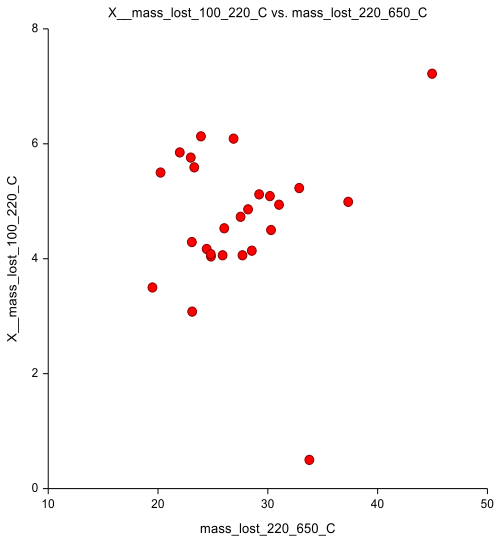

Figure 3.3: Pearson's correlation between exprimental porcine bone samples subjected to 100-220˚C and 220-650˚C temperature ranges.