A Study of Bone Chemistry in Forensic Applications

by

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Certificate of authorship and originality

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

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Sophil Raja

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Table of Contents

List of Figures

List of Tables

Abbreviations

AFC = Advanced Flow Control

AFM = Atomic Force Microscopy

BMD = Bone Mineral Density

BSE = Back-Scattered Electron

C = Clay

Cont = Control (Loam Defleshed)

DSC = Differential Scanning Calorimetry

DNA = Deoxyribonucleic Acid

DTA = Differential Thermal Analysis

DTG = Derivative Thermogravimetry

DRIFT = Diffuse Reflectance Infrared Spectroscopy

DXA = Dual-energy X-ray Absorptiometry

EGA = Evolved Gas Analysis

EVA = Evaluation

ESEM = Environmental Scanning Electron Microscopy

FTIR = Fourier Transform Infrared

GC = Gas Chromatography

GE = Gel Electrophoresis

GSE = Gaseous Secondary Electron

HAP or HA = Hydroxyapatite

HTXRD = High Temperature X-ray Diffraction

ICDD-JCPD = International Centre for Diffraction Data - Joint Committee of Powder Diffraction

IR = Infrared

LA = Loam Acidic

LB = Loam Basic

LBO = Loam Boiled

LD = Loam Dry

LDG = Loam Degreased

LR = Loam Refrigerator

LW = Loam Wet

MS = Mass Spectrometry

NHMRC = National Health and Medical Research Council

NIST = National Institute of Standards and Technology

PCA = Principal Component Analysis

PCR = Principal Component Regression

PLS = Partial Least Squares

PMI = Post-Mortem Interval

Py = Pyrolysis

Py-GC-MS = Pyrolysis Gas Chromatography-Mass Spectrometry

S = Silt

SA = Sand

SD = Standard Deviation

SDT = Simultaneous Differential Techniques

SE = Secondary Electron

SEM = Scanning Electron Microscopy

SPSS = Statistical Package for the Social Sciences

TA = Thermal Analysis

TG = Thermogravimetric Analysis

UTS = University of Technology, Sydney

UV = Ultraviolet

UWA = University of Western Australia

XRAS = X-ray Absorption Spectroscopy

XRD = X-ray Diffraction

Abstract

The primary aim was to develop a method for accurately estimating the post-burial time of bones. Bones were buried in diverse soil environments for 18 months and subsequently examined using various analytical techniques. Pig rib bones were used as an analogue for human bones. The burial environments varied in factors including soil type, soil pH, moisture content and temperature. Environmental Scanning Electron Microscopy (ESEM) allowed the classification of bone samples into two categories of young and old based on differences in surface morphology. X-ray Diffraction (XRD) results showed no changes in crystallinity for a post-burial period of 18 months, making this technique unsuitable for post-burial time estimation. Thermogravimetric analysis (TG) showed an overall increasing trend in mass loss in all the bone samples up to a post-burial time of 8 months. Bones buried in an acidic soil environment showed a decreasing trend in mass loss with increasing burial time, indicating that an acidic environment is the most destructive environment. Pyrolysis Gas Chromatography-Mass Spectrometry (Py-GC-MS) was identified as being the most useful and accurate technique for estimating the post-burial time of recovered bone samples. The data showed a direct correlation between the actual and predicted post-burial time of bones for all the pre-treatment procedures studied except for boiling. The pyrograms collected for the different post-burial times demonstrated the process of diagenesis and highlighted the identifiable compounds most susceptible to degradation, as well as the identifiable compounds which persist after longer periods of burial. Comparison of the different burial environments also demonstrated that it is possible to estimate the post-burial period of bones without knowledge of the burial environment, however, information about the burial environment allows for a more accurate estimation of the post-burial time.

Chapter 1

Introduction

1.1 Background and Significance

Gaining an understanding of the ageing of bone fragments or decomposed skeletal remains provides a challenge for forensic practitioners. Bones are complex in structure and are sensitive to environmental factors. Extensive studies of the visual and physical properties of bones have been undertaken; however, there have been few investigations into the changes to the chemical structure of bones in a forensic context.

In the context of crime investigations, the role of forensic scientists involves the identification and characterisation of various forms of evidence. The different forms of forensic evidence including bone, hair, fabric, blood and deoxyribonucleic acid (DNA), all play a vital role in the investigation process. One of the primary forms of forensic evidence found at death scene investigations are skeletal remains. These skeletal remains can provide useful information about the crime and the victim in terms of height, sex, physique, ancestry. Therefore, the study of skeletal remains is critical for forensic scientists to understand the underlying post-mortem alterations that occur in bone once a crime has taken place.

Forensic taphonomy involves the use of analytical and experimental techniques to estimate the post-mortem interval (PMI). Due to the shorter time frame, forensic taphonomy deals with changes to soft tissue and bone, particularly the rate of decomposition and factors affecting this rate. The process of decomposition can help estimate PMI as it can provide useful information about the history of skeletonised remains and soft tissue (Micozzi, 1991). The PMI is defined as the length of time between death and the discovery of the remains. Post-burial time (PBT) refers to the period of time between deposition in a burial site and the recovery of bones. PBT differs from PMI in that it represents the time in which skeletal remains have been buried as opposed to the entire period since death. Taphonomic approaches such as entomological, geological and botanical can also be employed to estimate PBT in forensic

investigations. In many forensic cases, the PBT will be the same as the PMI (Knight and Lauder, 1969) and therefore, the estimation by a forensic anthropologist will simply represent the PMI.

Bone is a complex biological material which can undergo a variety of pre- and post-depositional processes, causing physical and chemical changes, resulting in either destroying or adding to the forensic value (Millard, 2001). As bones contain both inorganic and organic components, there is potential to gain an insight into the post-mortem decomposition processes occurring using techniques that are sensitive to changes in these components. An investigation of these decomposition processes may aid in the determination of the PMI, which is crucial in any suspicious death investigation.

The forensic parameters essential in determining the cause, manner and time since death begin to degrade as soon as death occurs. It becomes increasingly difficult to determine the PMI as soft tissue decomposition progresses (Anderson and VanLaerhoven, 1996). In criminal investigations, when the identity of the person is unknown, a PMI determination is essential for establishing the timeframe, which limits the fields of suspects and possible missing persons.

Forensic investigations involve studying the events that occurred in the ante-, peri-, or post-mortem period surrounding death. The PMI can span a period of days, weeks, months or years. Contrary to the conventional period of time in taphonomy that spans hundreds to thousands of years, the estimation of PMI in a forensic context needs to be much more precise.

Currently, estimating the time elapsed since death remains one of the most elusive determinants present at a crime scene. There is no simple technique for calculating this time interval, which significantly hinders forensic investigations. In the early post-mortem period, when flesh is still present on a cadaver, it is possible to determine time since death based on

the progression of rigor, livor and algor mortis (Di Maio ad Di Maio, 2001). However, these stages of decomposition are not useful for estimating time since death in the extended post-mortem period. Similarly, forensic entomology uses the presence of insects and their progression at a crime scene to estimate PMI within hours, days and weeks after death (Goff, 2000). In addition, a reconstruction of events that occurred after death can be carried out using anthropological methods. The main biological parameters such as age, sex, stature and ancestral origin are used in an initial assessment of PMI. These parameters only allow broad categorisations which limit the applicability of these parameters in a forensic context. Overall, several studies have investigated the estimation of time since death based on the stages of decomposition of soft tissue (Coe, 1993; DiMaio and DiMaio, 2001), however, the stages of decomposition following skeletonisation are not well documented. As a result, the use of these approaches is limited in forensic investigations where skeletal remains are discovered and so, the application of other approaches and techniques is required. Therefore, research into this area is required to assist forensic investigators in accurately estimating PMI.

The chemistry of archaeological bones has been widely studied (Ubelaker, 2001; Ambrose and Krigbaum, 2003), however, the structures of lesser aged bones (less than 75 years) such as those that are encountered in a forensic context, have not been as extensively studied. Even though the chemistry associated with the decomposition and destruction of skeletal tissue is complex, it is still important to study this bone chemistry in order to relate variations to different stages of decomposition. The extent of changes in the organic and inorganic content of bones as a result of decomposition could be related to different lengths of burial, which could aid in the estimation of PMI. In a recent study, it has been demonstrated that changes to relatively young bones can be detected using techniques that examine the decomposition of the organic phase of bone (Onishi *et al.*, 2008). This study further demonstrates the need for

research investigating the potential of using changes in the organic and inorganic content of bone to estimate PMI.

Estimating the PMI of bones based on the degree of bone degradation is a difficult task since bone decomposition is affected by environmental factors. Each burial environment is comprised of unique factors that affect the type and extent of bone decomposition, which directly impacts PMI estimation. To eliminate errors, estimates need to be based on the environment that the body was discovered in and the condition of the remains. Under controlled conditions, decomposition follows a predictable pattern but the rate at which a body passes through the decomposition stages is variable.

1.2 Human Decomposition

The decomposition of skeletal remains is a lengthy process with the first signs appearing as cracks along the bone's surface and can eventually lead to the complete loss of shape and structural integrity of the bones. This process can take anywhere from 6 years up to 30 years and is significantly dependent on the bone's micro-environment (Ross and Cunningham, 2011). Any changes in the micro-environment can alter the rate at which bone decomposes.

Historically, human decomposition has been categorised into three stages; fresh, decomposed and dry. The process of decomposition follows certain predictable stages, but the rate of decomposition is highly variable and it is therefore difficult to define each stage and its duration. However, by observing the condition of the body including the state of decay, an *approximation* of time since death can be calculated (DiMaio and DiMaio, 2001).

A body is considered "fresh" during the first 24 to 48 hours after death (Galloway *et al.*, 1989). During this stage of soft tissue decomposition, the processes of rigor mortis, livor mortis and algor mortis occur. Following decomposition, a build-up of lactic acid in the tissues leads to

muscle stiffening, which is known as rigor mortis (Janaway, 1996). Livor mortis involves blood settling in the lowest points of the body, creating visible red areas on the skin (Baden and Hennessee, 1989). The process of algor mortis refers to the lowering of the body temperature after death (Perper, 1993).

The process of autolysis begins immediately after death. Autolysis is a process involving the extensive self-digestion of cells and results in a complete loss of cellular integrity and widespread necrosis (Love and Marks, 2003). The onset of putrefaction, a gradual process involving the loss of organic material followed by a relative enrichment in mineral substances, generally commences 48 to 72 hours after death (Janaway, 1996), following autolysis. During this stage of decomposition, internal bacteria begin to break down the surrounding tissue (Coe, 1993). Distinct colour changes, skin slippage and bloating are observed and this is known as the "decomposed" stage (Galloway *et al.*, 1989).

As putrefaction begins to cease, initial skeletonisation begins (Love and Marks, 2003). The final stage ("dry" stage) is characterised by little or no soft tissue remaining and bone exposure over the entire body (Galloway *et al.*, 1989; Rodriguez and Bass, 1983). This stage can persist for months or years. Throughout burial, estimation of PMI can be quite difficult because burial factors affect bone degradation processes. In particular, once a body has reached the "dry" stage, determination of PMI becomes difficult because bone degradation processes are dependent upon the burial environment. Many burial factors which include soil type, acidity, moisture content, temperature and weather conditions as well as animal and insect scavenging can affect bone deterioration.

There is still much to do to understand the processes involved in bone deterioration, in order to gain information about dating bones (Millard, 2001). Bones can be found in varying environmental conditions and therefore, it is important to establish reliable, reproducible and

accurate methods for determining the PMI of bones. Additionally, the identification of characteristics such as species, age, sex and ancestry of the specimen is equally important to reach an accurate conclusion in any criminal investigation.

1.3 Bone

1.3.1 Definition

Bone is a composite of an organic and an inorganic phase with a highly hierarchical structure (Robinson, 1979). The main building blocks of bone are long collagen fibrils of approximately 100-nanometre diameter that contain nanometre-sized carbonated apatite crystals (Weiner and Wagner, 1998). One of the major challenges for forensic examiners is the ageing and characterisation of bone fragments or decomposed skeletal remains.

The adult human skeleton normally contains 206 bones. These vary in size ranging from the femora, which can be greater than 450 mm in length to the almost microscopic ossicles of the inner ear. This great variation in size complemented by variation in shape makes identification of individual bones relatively straightforward. There are however some bones that are more difficult to identify than others. Identification of bones of the hands, feet, rib cage and vertebral column require closer examination than the rest (Brown, 2007). This is true both within the human species and other mammals.

1.3.2 Chemistry

Bone is a complex, dynamic tissue, composed of approximately 65 per cent mineral (mainly hydroxyapatite) and 35 per cent organic (collagen and other noncollagenous protein, bone cells, and water) components (Samuel *et al.*, 2009). The major bone protein is collagen, a long

chain structural protein which coils in a left-handed helix. Three molecules of collagen supercoil in a right-handed manner to form a tropocollagen triple helix. Tropocollagen molecules combine into fibrils with spaces between the molecules, where crystals of bone mineral form. The mineral component is also present between the fibrils of mature bone. Bone mineral is a carbonated-hydroxyapatite (HA) that can accommodate a large number of trace elements by surface and lattice substitutions. The carbonated-hydroxyapatite is also mineralogically termed *dahllite*. The crystals are very small with dimensions typically 2-5 x 40-50 x 20-25 nanometre. The significance of their small size is that it provides bone mineral with a very large and chemically reactive surface area of 85-170 m^2/g (Millard, 2001).

1.3.3 Classification

There are several ways of classifying bones including categorisations based on structure and morphology of the bone. The structure, chemical composition and isotopic data provide information on diet. The morphological structure forms the basis for species identification (Millard, 2001). Each method of classification of bone carries information which can be used in the forensic context.

1.3.3.1 Structure

Classification based on structure divides bones into two principal types. This classification is based particularly on the denseness of the bone. The difference lies in how tightly the tissue is packed together. Bones are classified as:

- compact or cortical; or
- cancellous or trabecular bone (Samuel *et al.*, 2009).

Adult human skeletal mass consists of 80% compact bone and 20% cancellous bone. Compact bone is hard and dense and found in the shafts of bones, surrounding the marrow cavity with closely packed osteons or haversian systems (Figure 1-1). The Haversian systems are packed tightly together to form what appears to be a solid mass. The osteonic canals contain blood vessels, which run parallel to the long axis of the bone (Samuel *et al.*, 2009). These blood vessels interconnect, by way of perforating canals, with vessels on the surface of the bone.

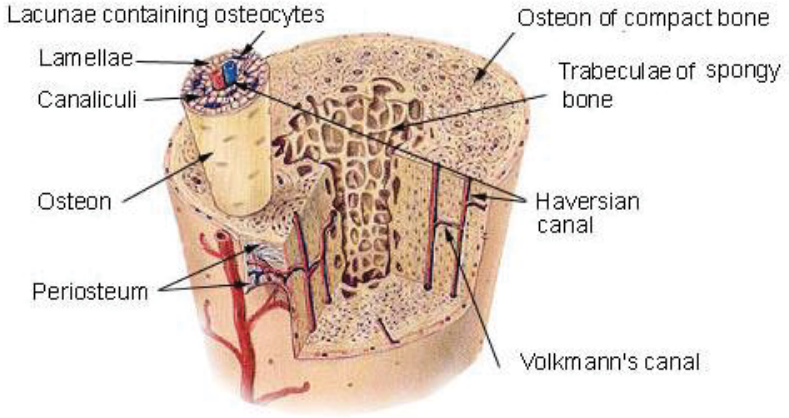

Figure 1-1. Compact and cancellous bone (SEER Training Modules, 2007)

Cancellous bone is spongy, consisting of fine interlacing trabeculae which contain marrow. The examples include vertebrae, flat bones and the ends of long bones (Figure 1-1). Cancellous bone is lighter and less dense than compact bone. This type of bone consists of trabeculae and bars of bone adjacent to small, irregular cavities which contain red bone marrow. The trabeculae are organized to provide maximum strength. The trabeculae follow the lines of stress and can realign, if the direction of stress changes (Samuel *et al.*, 2009).

1.3.3.2 Morphology

Every bone has specific surface markings and characteristics that make it identifiable. There are holes, depressions, smooth facets, lines, projections and other markings that represent passageways for vessels and nerves, points of articulation with other bones or points of attachment for tendons and ligaments (Gray, 2000). The bones of the body come in a variety of shapes, contours and sizes. Based on their morphology alone, the bones are classified into four principal types. These are long, short, flat and irregular bones.

a. Long bones: Long bones (Figure 1-2) are longer than they are wide. They consist of a long shaft with two bulky ends or extremities. The long bone is primarily a compact bone, which may have a large amount of spongy bone at the ends or extremities. Examples are the femur, tibia, fibula, humerus, radius and ulna (Gray, 2000).

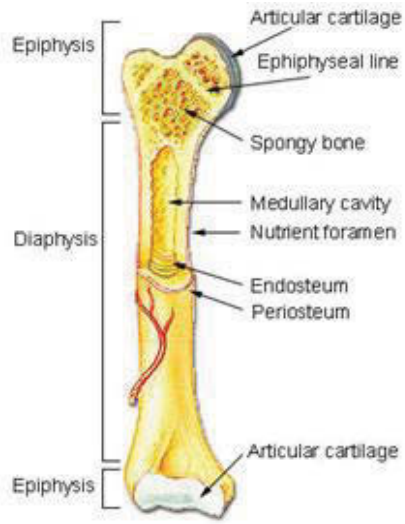

Figure 1-2. Long Bone (SEER Training Modules, 2007)

b. Short bones: Short bones are roughly cube-shaped bones, with both the vertical and horizontal dimensions approximately the same. They consist primarily of spongy bone, which is

covered by a thin layer of compact bone (Gray, 2000). They include the carpals (wrist) and talus bone (ankle).

c. Flat bones: Flat bones (Figure 1-3) are thin, flattened and usually curved bones (Gray, 2000). They consist of thin plates of periosteum-covered (fibrous sheath containing blood vessels and nerves) compact bone on the outside and endosteum-covered (thin layer of connective tissue lining the walls of the bone marrow cavities) spongy bone within. These bones have no shafts or epiphyses (end of a long bone that is originally separated from the main bone by a layer of cartilage). They contain bone marrow between their trabeculae, but no marrow cavity is present (Marieb, 2004).

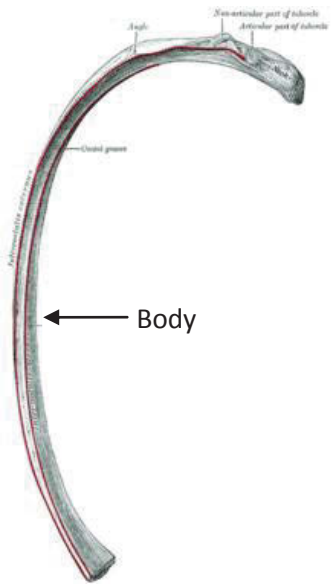

Figure 1-3. Rib (flat) bone (Gray, 2000)

d. Irregular bones: Bones that are not in any of the above three categories are classified as irregular bones. This type of bone is mainly spongy, that is covered with a thin layer of compact bone (Gray, 2000). The vertebrae and some of the bones of the skull belong to the category of irregular bones.

1.3.4 Bone Diagenesis

Diagenesis is an important phenomenon associated with post-mortem changes in bone. It is a natural process that affects the organic and inorganic components of bone exposed to environmental conditions. Interaction with the environment initiates an extremely complex series of processes including the exchange of natural bone components, deposition in voids, adsorption onto the bone surface and leaching from the bone (Vass, 2001). These post-mortem processes are known as diagenesis (Millard, 2001). Diagenesis takes into account all of the chemical changes and microbial attacks on bone. It also encompasses the various processes that are responsible for the deterioration of bone.

Three general pathways of bone diagenesis have been identified that include the chemical deterioration of the organic phase (collagen), chemical deterioration of the inorganic phase (hydroxyapatite (HA)) and the microbiological attack of the composite (Collins *et al.*, 2002). The major diagenetic change in the organic phase of bone is collagen loss and decay (Millard, 2001). The dissolution of collagen depends on time, temperature, water content and environmental pH. It has been demonstrated that higher temperatures accelerate rate of collagen loss and extremes of pH can cause collagen swelling and accelerated hydrolysis (Collins *et al.*, 2002). Collagen loss leads to an increase in porosity and therefore HA, with its affinity for amino acids, permits charged species of endogenous and exogenous origin to take up residence (Hedges, 2002). Deterioration of the inorganic phase leads to chemical changes that affect crystallinity. The mechanisms of chemical change, such as the uptake of F^- or CO_3^{2-} ions may cause recrystallization where HA is dissolved and re-precipitated allowing for the incorporation of exogenous material (Hedges, 2002). The third pathway of bone diagenesis, the microbiological attack of the composite, is the most common mechanism of bone deterioration. The dissolution of the mineral phase caused by low pH permits access to collagen by extracellular microbial enzymes (Collins *et al.*, 2002). During this phase, most bone

collagen is lost and porosity is increased (Hedges, 2002). All three processes can add vital information to the bone regarding the burial environment, which in turn can aid in the estimation of the PMI of recovered skeletal remains.

There are some diagenetic processes which are partially understood e.g., uranium uptake, and exchange of carbonate ions. However, evidence shows that hydrolysis is crucial to the process of diagenesis as water acts as an agent of transport (Millard, 2001). It has been shown that it is important to take into account the porosity of a bone, which plays a key role in understanding water movements in bone (Hedges and Millard, 1995).

The presence of microbes in the environment is primarily responsible for the loss in the organic component of bone. Approximately 20% of weight loss and a substantial increase in porosity (approximately 50%) are linked to collagen decay (Hedges, 2002). Diagenesis of the inorganic content of bone can be better understood, in terms of its loss, gain and the internal changes it goes through. Most of the research so far has focussed on the uptake and exchange of matter. Excavated bone can consist of a large number of elements that are not substantially present in living bone e.g. uranium and fluorine. Secondary minerals e.g. calcite and pyrite can form in voids in archaeological bone, and sometimes, they replace bone mineral itself. Elements and ions like calcium and phosphate, which are already present in bone may also be obtained from, or exchanged because of their presence in the burial environment (Millard, 2001).

Internal changes to the inorganic phase are expressed by changes in crystallinity. Crystallinity is the reordering of material in the bone, which can be monitored by a 'crystallinity index' based on X-ray diffraction or infra-red spectra (Millard, 2001).

1.3.4.1 Factors Affecting Bone Diagenesis

In a burial environment, several different factors can inhibit bacterial growth. This in turn delays decomposition. Other burial factors including insect and animal activity can affect the stages involved in bone decomposition by accelerating the entire process. The most common factors that influence decomposition rates are the type of soil, soil pH, moisture and temperature. The effect of the type of burial is also significant in terms of differences being observed in remains left on the surface and those that have been buried. The combination of these factors in a burial environment makes the rate of decomposition unpredictable and varied.

1. **Surface vs Buried**

The decomposition of bone is significantly dependent on whether skeletal remains are deposited on the surface or buried. Insect and animal scavenging is less evident in buried remains and temperatures below the ground are typically lower than above the surface. These factors tend to slow down decomposition (Janaway, 1996). Buried remains are generally more protected from weathering activities and insect and animal activity (Ross and Cunningham, 2011). It is interesting to note that the depth of burial also affects decomposition rates – a deeper burial provides better preservation of remains. This is because temperatures are more stable and the bone is sheltered from the effect of surface factors (Mant, 1987). Also, soil becomes more depleted at greater burial depths, which reduces biological activity to bacterial activity.

Interpretation of the sequence of events surrounding primary and secondary dispositions using bone colour was performed by Huculak and Rogers (2009). Comparison was made between bones buried and then exposed on the ground surface and bones exposed then buried. The

results demonstrated five main surface colours due to soil, sun, haemolysis, decomposition, and fungi. The presence of fungi on buried bones indicated prior surface exposure. Interestingly, the results also emphasised the importance of conducting bone surface analysis since the cross-sections of buried bones were identical to buried then exposed bones. Cross-sections of the bone samples could, however, help verify remains that have been exposed then buried. The effect of the presence of decomposing tissue on bone colour was also studied, showing that decomposing tissue leads to minimal colour staining.

2. Soil Type

Decomposing remains are directly affected by the surrounding environment, and, in the case of buried remains, the soil represents the matrix. All soils are made up of two components – the organic and inorganic components. The inorganic soil fraction (also known as the mineral fraction) can be divided into three major soil fractions based on size – clay, silt and sand. Each fraction has different physical properties including particle sizes ranging from small clay particles to large and irregular sand particles, resulting in different effects on the soil in terms of drainage and charge.

Clay particles are the smallest particles in soil. They have colloidal properties and are the most chemically active inorganic component of soils. They carry a negative charge and therefore increase the water-holding and cation exchange capacities of the soil. Clay soils are sticky (plasticity) when wet. Silt particles are intermediate in size and possess characteristics between those of sand and clay particles. Silt particles can show some plasticity due to clay film coatings on the silt particles. This leads to a greater absorptive capacity of silty soils for water and cations compared to sandy soils. Sand grains are irregular in size and shape and their presence in soil leads to a loose soil, allowing rapid water and air movement. Overall, sand and silt particles generally promote good soil drainage and air movement while clay

particles absorb water and have an overall negative charge which means they have a tendency to hold onto cations (Tan, 1994).

The mineral fraction of soils can vary greatly as it depends on the composition of the rocks and sediments from which it was formed. The elements most commonly found in the rocks and sediments are oxygen (O), aluminium (Al), silicon (Si), iron (Fe), calcium (Ca), magnesium (Mg), sodium (Na) and potassium (K) and therefore, the minerals in soil are also made up of these elements. Most of the minerals are silicates or oxides, which is typical in many Australian soils. However, carbonate rich soils can also lead to a significant presence of carbonates and sulphates in the soil.

Geochemical changes, including concentration of solutes and redox potential, have been suggested as affecting many of the detailed differences in bone diagenesis (Quattropani *et al.* 1999). Studies investigating the effects of soil type on decomposition have been previously conducted (Mant, 1987; Janaway, 1996). Generally, different soil types cause differences in the micro-environment which leads to a difference in the type and quantity of mould growth on bone samples. Even though fungi are present in all soils, it is the soil type that determines what kind of fungi predominate (Brady and Weil, 1999).

Soil particle size affects water permeability and air exchange within a soil environment and is therefore a pertinent factor in studying decomposition rates. It has been shown that clay soils retain more moisture because of their small particle size compared to sandy soils. As a result, clay soils generally slow down decomposition (Bethell and Carver, 1987).

A study monitoring the effect of bone weathering patterns on PMI estimation showed that the variation in the micro-environment which was partially caused by soil composition introduced variability in bone weathering rates. The investigation, which used metatarsal and femur bones

in Southern Ontario, demonstrated that the water-holding capacity of sand is low whereas the water-holding capacity of clay is high. Therefore, the drainage rate of sand and clay is high and low, respectively. Soils containing a higher clay content lead to a more moist environment (Janjua and Rogers, 2008).

Coarse-textured soils such as sand with a low moisture content generally promote desiccation, which can inhibit decomposition (Fiedler and Graw, 2003; Santarsiero *et al.*, 2000). This is because the large pore content of coarse-textured soils enables the relatively rapid movement of gases and moisture through the soil matrix. Another reason for the promotion of desiccation by coarse-textured soils with low moisture contents is because coarse-textured soils retard hydrolytic enzymes associated with the cycling of carbon and nutrients (Skujins and McLaren, 1967). Eventually, this can result in the natural preservation of a cadaver for thousands of years (Micozzi, 1991).

Fine-textured soils such as clay have been shown to retard bone decomposition due to low rates of gas diffusivity or wet conditions not being able to meet aerobic microbial demand (Carter, 2007). Anaerobic microorganisms lead to a slower decomposition rate compared to aerobic microorganisms. Where reducing conditions are present, anaerobic microorganisms dominate decomposition (Swift *et al.*, 1998).

3. Soil pH

Data investigating the effect of soil composition and chemistry on bone decomposition confirm that pH plays a major role in bone diagenesis (Nafte, 2000; Nielsen-Marsh *et al.*, 2007). Even though several different burial environments and bones with burial ages ranging from pre-modern to the Mesolithic times were studied, it was still possible to categorise all the samples into four main diagenetic types – basic vs acidic soils; human vs animal remains. The study by

Nielsen-Marsh *et al.* (2007) demonstrated that only the decomposition of the mineral component of bones is affected by soil chemistry (acidic soils). When observing bone samples buried in neutral or basic soil environments, the controlling factor in terms of bone decomposition appears to be microbial activity (Nielsen-Marsh *et al.*, 2007).

Highly acidic soils appear to be the most influential in accelerating decomposition processes within bones. Correlations between bone deterioration and soil acidity have been shown to be significant. According to Gordon and Buikstra (1981), soil pH has the largest influence on bone preservation, with preservation generally effective in soils above pH 5.3. Soils with a highly acidic pH will decompose bone more rapidly due to the dissolution of the inorganic matrix of bone (Nafte, 2000). The inorganic component of bone, hydroxyapatite, is insoluble in water (Morse *et al.*, 1983), but in the presence of an acidic environment hydroxyapatite decomposes into soluble salts of calcium and phosphorus. In contrast, basic and neutral soils can lead to the preservation of buried skeletal remains for centuries (Nielsen-Marsh *et al.*, 2007).

Low soil pH enhances the growth of fungi in the burial environment, which enhances degradation of buried bones (Tortora *et al.*, 1994). Acidic soils also enhance plant activity by increasing rates of nutrient uptake in roots, which may lead to increased rate of skeletal decomposition (Degaetano *et al.*, 1992).

Alkaline soils are usually found in arid and semiarid areas where the amount of rainfall is too low to leach the base forming cations (such as calcium, magnesium, potassium and sodium). Low rainfall and poor drainage leads to the accumulation of sodium in many soils. The sodium in saline soils is found as a soluble salt, usually as sodium chloride or sodium sulfate. Salts are usually formed from natural weathering of rocks and minerals or are transferred to the soil by rainfall or irrigation. The effect of the presence of salts in a burial environment on bone decomposition was investigated (Abdel-Maksoud and Abdel-Hady, 2011). During the

excavation of the Egyptian-Polish mission in 2008, different archaeological remains including crocodile skeletons were discovered in Hawara, Fayoum, Egypt. Surface modifications and colour changes were observed in the crocodile skeletons as a result of salt crystallization, erosion and pitting caused by the burial environment. The effects of other burial factors including soil composition and soil pH were also studied in terms of their effects on bone crystallinity, collagen degradation, bone histology and surface morphology. It was noted that both soluble (sodium chloride) and insoluble (calcium sulphate) salts play a vital role in the degradation of bones.

4. Moisture Content

The moisture content of the soil surrounding skeletal remains also affects the rate of bone decomposition. According to Swift *et al.* (1979), soil moisture can have a significant effect on decomposition rates. Generally, a moist environment enhances bone decomposition while a dry environment delays the process (Smith, 1983). Extremely dry environments promote desiccation (Galloway, 1997; Galloway *et al.*, 1989) whereas extremely wet environments promote waterlogging and adipocere formation (Forbes *et al.*, 2005a). Both of these processes slow cadaver decomposition because soil moisture can affect the metabolism of micro-organisms responsible for decomposition. Additionally, oxygen dissolved in groundwater can act as an oxidant in decomposition reactions (Dent *et al.*, 2004). In addition, Mant (1987) demonstrated that well-drained, dry soils favour mummification i.e. the dehydration or desiccation of tissue. Both very dry and very wet environments inhibit microbiological attack (Hedges, 2002).

Under field conditions, variations in the moisture content of soil are one of the most significant factors influencing microbial activity (Lund and Goksoyr, 1980). Similarly, changes in microbial biomass have been shown to be linked to the wet-dry cycle of the environments studied

(Jenkinson and Ladd, 1981) with significant increases in microbial activity being observed after rewetting. Thus, decay rates may exhibit seasonal fluctuations in some regions, which is dependent on the soil texture. Bioavailablility of moisture is determined partly by the power with which water is held between soil particles (Tibbet and Carter, 2008). As a result, soil texture affects the moisture content of soils with fine textured soils retaining more moisture than sandy or silty soils (Krogman and Iscan, 1986).

The significance of moisture content in the decomposition process was also investigated by Aturaliya and Lukasewyz (1999). The study used rat carcasses and it was observed that the most significant impact in moisture loss or retention was a result of the environment (including materials) that was in direct contact with the decomposing body. Materials that allowed water to collect on the carcass prevented mummification, irrespective of whether the body was placed on the surface or buried. In contrast, materials that absorbed moisture and then allowed for evaporation accelerated water loss (Aturaliya and Lukasewyz, 1999), which led to faster desiccation of the tissue.

The effect of soil moisture on the weight and overall condition of bone samples in terms of the estimation of the post-mortem interval of bones was studied (Jaggers and Rogers, 2009). Changes in pig bone samples were observed over two time intervals (2 and 5 months) using two soil environments (one drier than the other). Weight loss was observed in both sets of bone samples; however, greater weight loss was seen in the bones buried in the environment with the higher moisture content. This confirms results of previous research that a moist environment accelerates bone decomposition. Interestingly, no changes occurred in colour, texture and condition of the bone samples buried in both burial environments. This suggests that the burial period used was not long enough for the observation of visible evidence of such changes.

5. Temperature

Temperature also plays an influential role in the process of bone decomposition (Gill-King, 1997; Mann *et al.*, 1990). According to Mount and Paetzold (2002), temperature has an important influence on biological, chemical, and physical processes occurring in soil. Research shows that temperature can affect decomposition and associated microbial activity in soil (Tibbet and Carter, 2008). An increase in soil temperature leads to an increase in biological activity and chemical reaction rates and can therefore be linked to an increase in the rate of decomposition of buried cadavers (Vass *et al.*, 1992).

Tuross *et al.* (1989) stated that multiyear climatic cycles affect macroscopic weathering rates and weathering progresses at varying velocities intra and inter skeleton, even within one macro environment. In their study, they found that bone strontium levels and HA crystal sizes increased with time in two exposed wildebeest skeletons. Human subfossil bone recovered archaeologically also showed evidence of diagenetic change that resulted in crystal growth and elevated strontium levels.

Studies have shown that most bacterial activity occurs at an optimum temperature of approximately 37 °C (Polson *et al.*, 1985; Chamberlain and Parker Pearson, 2001). Similarly, putrefaction, the decomposition of organic matter by microorganisms, occurs optimally between temperatures of 21 and 38 °C. The rate of putrefaction is significantly slowed at temperatures below 10 °C or above 40 °C (Mant, 1987; Polson *et al.*, 1985). Therefore, the temperature of a burial environment significantly affects the rate of decomposition, in particular the survival of collagen in bones (Hedges, 2002).

Generally, warmer conditions promote decay as they favour bacterial activity, and colder climates delay the process of decomposition (Smith, 1983). It was observed that an increase in

temperature due to heat retained by straw and pine needles covering decomposing matter accelerated the rate of decomposition (Mant, 1987). Since soil temperatures are generally lower than the optimum temperature, bacterial activity can be hindered, which ultimately affects the extent of decomposition and preservation that occurs (Forbes *et al.*, 2005a). A cold environment acts in a preservative manner and often discourages both insect and scavenger activity (Janaway, 1996).

Interestingly, it has also been shown that extremely high temperatures can inhibit bacterial growth and proliferation (Micozzi, 1997). Following the same principle, freezing can significantly delay the decomposition process (Micozzi, 1991). Therefore, it is expected that cold soil temperatures will slow bone decomposition since most microbial activity is inhibited by cold and freezing soil conditions.

6. Insect and Animal activity

Insect activity, including ants, blowflies, beetles and cockroaches, has been shown to increase decomposition rates (Gonzales *et al.*, 1954). Animal scavengers can also affect decomposition rates by dismembering parts of a body during decomposition as well as scattering the remains. Animal scavengers include large carnivores that are normally responsible for gnawing at the face, neck and abdominal areas as well as disarticulating limbs from the torso (Willey and Snyder, 1989). Rodents typically gnaw at the long bones, in particular the epiphyses, rather than the diaphysis (Haglund *et al.*, 1989; Klippel and Synstelien, 2007).

1.3.5 A Suitable Analogue for Human Bone

A suitable analogue for human bones was required as access to human remains for research purposes is limited due to ethical implications. A study by Robinson *et al.* (2003) explored the differences between the same bone type from various animals e.g. ribs from cow, deer, sheep,

wild boar; different bones from the same animal e.g. ribs, scapula, radius, humerus; and bones of immature and adult animals. The compact regions of the bones were used for analysis. Their results showed that the differences in porosity between the same bones from skeletally mature cow, red deer, sheep and sub-adult wild boar were relatively minor and less in scale than the differences between the different bones of various mammalian taxa. However, it is still important to remember that variations can exist, even between the same bones of the same animal, e.g. rib bones vary in size and mass considerably for the one specimen. The heterogeneous nature of such bone samples needs to be taken into account when interpreting data for research purposes. Robinson *et al.* also found that in general, long bone shafts were less porous than the ribs and scapula and, therefore, any investigations using changes in porosity as a means of measuring decay should avoid comparing results from different skeletal elements. Their findings also revealed that the porosity was greatest in the juvenile specimens tested when compared with their adult counterparts.

Different bone specific features including bone macrostructure, microstructure and composition, and remodelling have been compared to determine similarities and differences between animal models and the human clinical situation (Pearce *et al.*, 2007). This review demonstrated that an understanding of the similarities and differences in bone architecture is required when choosing a suitable model for research. However, it is important to remember that there is no species that exhibits exactly the same characteristics and functions as the human bone. Research confirms that no single animal model is appropriate for all purposes, but this does not mean that a model is therefore inappropriate for all purposes (Hazzard *et al.*, 1992). Depending on the purpose of the research, certain model types would be suitable. Pearce *et al.* (2007) found that the most commonly used animal for implant research purposes is the rabbit, however, this species shows the least similarities to human bone. Choosing the right species based on the size of the bones and the ease of handling alone, the dog and the

sheep/goat appeared to be the most suitable models. Generally, pig bones showed many similarities to human bone (Pearce *et al.*, 2007).

1.3.5.1 Factors Used for Ideal Animal Model Selection

When choosing the ideal animal model for a specific research purpose, there are many factors that need to be considered including both physiological and pathological factors (Schimandle and Boden, 1994). Ideal animal model selection factors include:

- cost
- availability
- acceptability to society
- ease of handling
- interanimal uniformity
- biological characteristics analogous to humans
- an existing database of biological information for the species (Schimandle and Boden , 1994)

1.3.5.2 Comparison of Different Species

Rat

The rat is usually the preferred species for medical research purposes, however, the significant differences between human and rat bones, particularly, differences in size, indicate that the rat model is not suitable for bone research. In general, small bones are not as well preserved as large bones (Von Endt and Ortner, 1984). Biochemical analyses on seven vertebrates (human, dog, pig, cow, sheep, chicken and rat) that are commonly used in bone research showed that rat bone was most different to human bone (Aerssens, 1998).

Dog

A common animal species used for musculoskeletal and dental research is the dog and there have been numerous studies performed which compare human and dog bones (Aerssens, 1998; Schimandle and Boden, 1994). However, since dogs are considered to be companion animals, their use in medical research has decreased as a result of an increasing recognition of ethical issues.

Human and dog bones were found to be the most similar in terms of bone composition (Aerssens *et al.*, 1998). Based on density, dog and pig bones were the most similar to human bones. Dog bone has been shown to have significantly higher mineral density when compared with human bone but the organic content and composition of both human and dog bones is relatively similar (Wang *et al.*, 1998). Using these results, it was concluded that the dog bone most closely represented the characteristics of human bone. These results agree with the research conducted by Gong *et al.* (1964) in that human and dog cortical and cancellous bones were shown to be similar in terms of the water, organic, volatile inorganic and ash fractions.

Sheep/Goat

Even though most literature demonstrates the suitability of dog bone as an ideal model for human bone, the use of sheep/goat bones for research purposes has increased over the last decade. This has occurred as a result of the ethical issues associated with using companion animals for such purposes.

Sheep bones are very similar to human bones macroscopically; however, comparison of the histology shows that the bone structure of the two species is very different. The density of sheep bones is significantly higher than that of human bones (Nafei *et al.*, 2000).

The mineral composition of human and sheep bones has been compared and it was reported that sheep bones do not show any significant differences to human bones in this respect (Ravaglioli *et al.*, 1996). When using sheep bone for medical research, it is vital to use sheep of similar ages as it has been shown that the trabecular bone of skeletally immature sheep is weaker, less dense and more porous than that of skeletally mature sheep. Like most other vertebrate species, the younger sheep bones also contain more collagen than the older bones (Nafei *et al.*, 2000).

Tiny dissimilarities in the apparent and ash density between goat and human bones were evident; however, these dissimilarities were not as major as the differences found between anatomic sites of the same species (Liebschner, 2004).

Rabbit

For the purposes of medical research, rabbits are commonly used for musculoskeletal studies as they are easy to handle (Neyt *et al.*, 1998). Rabbit and human bones are very different in terms of the size and shape of the bones as well as the differences in loading. Also, rabbit long bones and human long bones have different microstructures (Wang *et al.*, 1998).

Pig

Pig (porcine) bones are used regularly for research purposes as the bone anatomy, morphology, healing and remodelling capabilities of the pig are considered to be closely representative of human bone (Thorwarth *et al.*, 2005). According to Raab *et al.* (1991), the femoral cross-sectional diameter and area of both human and pig bones is very similar. Also, Mosekilde *et al.* (1987) showed that pig and human bones have a similar lamellar bone structure.

Comparison of the bone composition of various species found that even though dog bones represent human bones most closely, porcine bones also show similarities to human bone in terms of bone mineral density and bone mineral concentration (Aerssens *et al.*, 1998).

Pigs have been shown to have a similar rate of bone regeneration to humans when compared to dogs (Laiblin and Jaeschke, 1979). Investigation of the effects of fluoride on cortical bone remodelling in growing pigs demonstrated that pigs have a similar cortical bone mineralisation rate to human (Kragstrup *et al.*, 1989). In addition, pig rib bone samples generated the most reproducible results when compared to cattle and sheep bones (Howes *et al.*, 2012).

1.3.5.3 Summary

Of the species discussed, dog bones can be seen to be the most similar to human bones in terms of macrostructure, microstructure and bone composition. However, ethical implications associated with using companion animals for research purposes means that using dog bones for research purposes is restricted in Australia (National Health and Medical Research Council (NHMRC) Guidelines on the care of dogs used for scientific purposes, 2009). The second most similar animal model for human bone is porcine bone. Pig bones closely represent human bones with regards to bone structure and properties. They are easily available, relatively inexpensive and their use does not have significant ethical implications.

1.3.6 The Effect of Pre-treatment Procedures on Bone

Pre-treatment procedures have been shown to have an effect on the macro- and micro-structure of bones (Connelley *et al.*, 2010; Nicholson, 1996; Mant, 1987). Pre-treatment procedures were used to represent different conditions skeletal remains can be found in prior to burial, for example, with or without flesh, with or without fats and oils, etc. Determining the effect of pre-treatment procedures on bone is essential in providing accurate and meaningful

results when bones are analysed. Investigation of the differences in bones that are fleshed and defleshed; defleshed and degreased; and defleshed and boiled is important to be able to accurately estimate PMI.

1.3.6.1 Fleshed versus Defleshed Bones

The need for research into this area was highlighted by Connelley *et al.* (2010) as it was indicated that fleshed bones may decompose in a different manner to defleshed bones. The study was based on an accelerated ageing method using heat to simulate decomposition of buried bones. Results showed that the texture of the bone surfaces in both defleshed and fleshed bones became more porous and grooved as 'burial time' increased. It was also observed that the fleshed bone samples had less prominent ridges than the defleshed bone samples, which indicated that the flesh on the bones acted in a preserving manner and reduced surface degradation caused by the elements. In addition, it was found that fleshed bones were brighter in colour and retained more surface lipids compared to the defleshed bones. After a year of burial, it was seen that bones buried with flesh intact were stronger and harder, which suggested that less degradation of the mineral component had occurred in these bones as opposed to defleshed bones (Connelley *et al.*, 2010).

The extent of differences between defleshed and fleshed fish bones has also been assessed (Nicholson, 1996). The defleshed bones appeared to decompose at a faster rate in all of the types of fish bones studied except for one case. This was explained by Nicholson to have occurred as microorganisms in the soil had limited access to bones which had flesh covering them. These results were confirmed by Mant (1987) who studied buried human corpses in 1987. A study by Hanson and Cain (2007) also agreed on the insulating nature of flesh in protecting fleshed parts of bones from significant burning damage compared to defleshed bones.

A study investigating the characterization of chopping weapon wounds using fleshed pig hind limbs to determine the effects of wounds inflicted using two axes and two hatchets has been reported (Lynn and Fairgrieve, 2009a; Lynn and Fairgrieve, 2009b). These results were compared with results of defleshed humeri and femora. No great differences were observed between the fleshed and defleshed specimens. Findings in a similar study suggest that the defleshed bones exhibited characteristics more indicative of a higher energy impact because of the absence of flesh. In addition, it was also shown that fleshed bones will depict the opposite, showing characteristics more indicative of a lower energy impact (Lynn and Fairgrieve, 2009b).

1.3.6.2 Degreased Bones

Modern fresh bone contains a large amount of surface lipids and it is important to remove the lipids in order to produce meaningful and reproducible results (Robinson *et al.*, 2003). On the other hand, the surface lipids may be a vital part of the diagenetic process and, therefore, important in predicting PMI. Therefore, it is important to compare the effects of degreased bones with non-degreased bones. The study showed that the pre-treatment procedure of degreasing can significantly affect the porosity of bones. Similarly, an evaluation of the taphonomic changes to blunt force trauma demonstrated that the taphonomic effects varied between fresh and degreased bone as well as cortical and cancellous bone (Calce and Rogers, 2007). In a study examining the internal structure of bones, Hanson and Cain (2007) demonstrated that degreased bone burns more rapidly when compared to greasy bone.

Research by Fenton *et al.* (2003) proposed a "fast and safe non-bleaching method for forensic skeletal preparation" that includes a method for defleshing and degreasing bone samples using readily available ingredients. This technique has been shown to be the least destructive towards bone DNA compared to the other two cleaning processes studied (boiling and bleach). The human bones showed much lower yields of DNA when they were cleaned using bleach.

The detergent/carbonate method allowed the largest segments of DNA to be amplified (Fenton *et al.*, 2003), which suggests that this cleaning process may have a less degradative effect on bone DNA (Rennick and Fenton, 2005).

1.3.6.3 Boiled Bones

Boiling of bones has been observed to significantly accelerate diagenesis. Other methods of cooking involving lower temperatures were not seen to have the same effect on the bones examined (Nicholson, 1996). In a similar study conducted in 2002, Roberts *et al.* confirmed these results. It was observed that bones subjected to boiling mirror diagenetic effects that are usually seen in archaeological bone, such as loss of collagen as well as increased crystallinity and porosity. Therefore, it is suggested that boiling may be used as an analogue for bone diagenesis and that the process of boiling may cause accelerated bone diagenesis by 'loosening' the bone structure. Roberts *et al.* (2002) went on to propose that once a boiled bone is buried in soil, changes to the mineral component of the bone and decomposition caused by microbial activity might more readily occur.

1.3.7 Estimation of Post-Mortem Interval using Analytical Techniques

A number of studies have been conducted to date bone material and have reported difficulties in achieving accurate estimations (Onishi *et al.*, 2008; Villanueva, 1976; Nielsen-Marsh *et al.*, 2000; Mkukuma *et al.*, 2004). Archaeological bone remains have been the subject of many research projects and certain methods have been developed for dating bones. However, in a forensic context, it is crucial to be able to date relatively young bones and more research is required in this area.

Ross and Cunningham (2011) highlight the importance of understanding the decomposition mechanisms that occur in different burial environments for the accurate estimation of the length of burial of discovered bones. They also emphasise the need to accurately estimate the time since burial of bones in both an archaeological and forensic context. Their study focussed on bone decomposition in a tropical environment (Isla de Coiba, Panama) and observations made during the excavation as well as analysis of the burial environments were used to create a timeline to estimate the time since burial. The timeline was based on visual observations of the bones' surfaces and consists of five stages with an accuracy of ± 10 years. Ross and Cunningham (2011) went on to conclude that further research is necessary to identify "geographic-specific stages" which would assist in estimating the PMI of bones in different micro-environments.

1.3.7.1 Microscopy

Scanning electron microscopy (SEM) has been used in several studies for estimating time since death (Onishi, 2008; Chatterji and Jeffery, 1968; Abdel-Fattah and Nour, 1993; Fantner *et al.*, 2004; Ooi *et al.*, 2007). In a study using human compact bones, SEM was employed to estimate PMI of skeletal remains. The bones were fractured longitudinally and the fractured surface was used for analysis. They discovered that the first noticeable signs of degradation in compact bones, buried in soil, were visible after 2.5 years of burial. Apart from the 2.5 year bone sample, no morphological changes were observed in the bone samples with PMIs less than 4 years. For the bone samples with PMIs ranging from 5 to 10 years, a labyrinth-like structure formed by small tubules or vacuoles of 0.5-1 μm in diameter was observed in the fractured surface of the bones. No morphological changes were revealed in bones left in the open air. In bones left in soil, vacuoles of 5-10 μm were found in the peripheral zone of the bones with a PMI of approximately 5 years and in bones of 6 years or more, this change extended to the mid-zone. In bones left in the sea for 4-5 years, these same vacuoles were found in the outer

peripheral zone of the bones. This study reinforces the need to pay special attention to the varying environmental conditions of the resting place of bones when estimating the time since death of skeletal remains (Yoshino *et al.*, 1991).

An SEM evaluation of the structural changes in paleopathology and diagenesis using backscattered electron (BSE) imaging was conducted (Bell, 1990). The results obtained suggested that macroscopic and X-ray interpretations of archaeological bone, both normal and pathological, can be misinterpreted due to the diagenetic change occurring. This study suggests that the variation of density within diagenetically altered bone, illustrated by BSE imaging, may result from the wide ranging elemental composition of the remineralized archaeological bone and from its stage of diagenesis. Further experimental investigation into the progress of diagenetic alterations should provide a valuable insight into this process in normal and pathological bones.

Changes in the properties of bone due to heat-induced degradation of the organic matrix were demonstrated by Fantner *et al.* (2004). They compared the microscopic fracture behavior (SEM), the topography of the surfaces (atomic force microscopy (AFM)), the condition of bone constituents (x-ray diffraction (XRD), thermogravimetric analysis (TG) and gel electrophoresis (GE)), and the macromechanical properties of healthy bovine trabecular bone with trabecular bone that has a heat-degraded organic matrix. Their results showed that heat treatment changes the microfracture behavior of trabecular bone in a number of ways. Baking most effectively weakens the mechanical strength of bone, creating the most brittle material. Boiled bone was observed to be stronger than baked bone but weaker than untreated bone. Boiled bone was also more elastic than untreated bone, which was in turn more elastic than baked bone (Fantner *et al.*, 2004).

Transmitted light microscopy has been employed to examine the internal structure of bones for the purpose of distinguishing burned from unburned bone (Hanson and Cain, 2007). The effect of diagenesis on heat-induced histological changes in bone was explored to determine if histological evidence of burning preserved through fossilisation and diagenetic processes. This study confirmed the insulating effect of flesh in protecting fleshed parts of bones from significant burning damage. It also demonstrated that degreased bone burns more rapidly when compared to greasy bone. Two studies were performed by Holden *et al.* (1995) using SEM to observe heat-treated and incinerated human bone. In their study on heat-treated bones, they found that at high magnifications, heat-induced effects made it possible to define the temperature attained in the bone tissue to within 200 °C and to estimate the age at death of a fire victim as being young (1-22 years), adult (22-60 years) or old (≥ 60 years). Similarly, in their study of incinerated human bones, they were able to make an estimate of the age of the fire victim and the temperature and duration of the fire (Holden *et al.*, 1995). These studies demonstrated the use of different microscopy techniques in determining certain factors including age and temperature of a fire and, therefore, suggest that further investigation into these techniques might enable the estimation of the PMI of bones. It might also allow the observation of the effects of heat-treatment of bones on the estimation of the PMI of bones.

The thermal effects induced by the electron beam interaction with the surface of the bone tissue and the surface damage was analysed as this could aid in the analysis of bone tissue (Holmes *et al.*, 2000). This could result in erroneous analytical interpretations of the mineral content and elemental proportions of the bone. The results of this study showed that as magnifications and probe currents increased, the surface temperature also significantly increased. This study suggests that thermal effects were minimal at lower accelerating voltages, lower probe currents and lower magnifications, but surface damage can still occur during the analysis of bone tissue.

1.3.7.2 Thermogravimetric Analysis

Thermal methods lend themselves to the examination of both the inorganic and organic components of bone (Brown, 2001). Villanueva *et al.*(1976) argued that differential thermal analysis (DTA) and TG could be used show the mineralisation process in post-mortem bones, not only statistically, but also dynamically by recording how the loss of weight evolves, how long it takes and the physicochemical processes involved. Their data showed a correlation between DTA and TG data and they also obtained patterns with high significance for the extreme series. DTA has not been widely used as a tool for dating bone remains in a forensic context except by Villanueva *et al.* (1976). However, they were unable to draw any definitive conclusions and, therefore, it was recognised as worthwhile to pursue this technique to determine its validity in calculating the PMIs of bones in a forensic context.

The thermal behaviour of HA prepared from bovine tibia was observed using both DTA and TG (Abdel-Fattah and Nour, 1993). The research also used the supplementary techniques of XRD and SEM. They stated that the importance of HA lies on the compatibility as a bone substitute and therefore, complete characterization of such potential graft material needs to be carried out to determine its biocompatibility. This study showed that the existing structure of the HA persisted up to and above 1000 °C. When heated between 700 °C and 1000 °C, the samples developed a highly crystalline structure as proved by their diffraction patterns and surface topography analysis. The results of this study demonstrate the potential of using DTA and TG to analyse bone samples as these techniques focus on the changes occurring in different bone samples upon heating, which may be useful in dating bone samples.

Similarly, the properties of porous HA bioceramic produced by heat treatment of bovine bone were evaluated between 400 °C and 1200 °C (Ooi *et al.*, 2007). The annealed body was characterized by TG-DTA. The TG-DTA thermogram of the bovine bone indicated the presence

of organic compounds, which upon annealing above 600 °C were completely removed from the matrices. Bovine bone annealed between 800 °C and 1000 °C revealed the characteristics of a natural bone with the interconnecting pore network being retained in the structure. This study demonstrates that TG techniques can be used to characterise the subtle differences in bones based on the organic content of the bones. However, the need to be careful in the use of TG methods for characterising materials based on the subtle morphological differences between the samples has been emphasised (Liu *et al.*, 2004). A study by Onishi *et al.* (2008) also confirmed that environmental conditions significantly affect TG by conducting a study of samples deposited on soil and buried in soil. They observed that the buried samples followed a sequence of PMI with the total percentage mass loss.

Bones have been analysed from ovariectomized (ovary removed surgically) rats using TG and DTA (Okamoto *et al.*, 1998). The results suggested that a kinetic parameter calculated from TG data may be a useful method for assessing experimentally induced osteoporosis and drug effects. TG used for calcified tissues provided parameters related to water, protein and carbonate content (Aoki *et al.*, 1977; Aoba *et al.*, 1978). DTA employed in combination with TG for heat energy changes in bones (Aoki *et al.*, 1977; Fages *et al.*, 1994), calcified tissues (Aoki *et al.*, 1977; Aoba *et al.*, 1978) and purified proteins (Steim, 1965) may be useful in studying osteoporosis and the effects of drugs upon it because of the changes in organic and inorganic substances. This study demonstrates that DTA and TG may be effective techniques for measuring the changes in mass loss and therefore, in the determination of the PMI of bones.

A study investigating the potential application of differential scanning calorimetry (DSC) to archaeological bone collagen deterioration reported a correlation between the maximum temperature of thermal transition (T_{max}) and age in young bone samples (Nielsen-Marsh *et al.*, 2000). This correlation improved when thermal age, a parameter that integrates thermal history with the temperature dependence of collagen gelatinisation, was included in the

analysis. T_{max} displayed little variation in thermally older bones. This was possibly due to the small sample size and limited number of sites and therefore, this study recommended further research using larger sample sizes and a wider range of archaeological sites. They also observed that the methods of sample preparation have a significant effect on the ability to produce reproducible and reliable thermal data from the collagen. They tested three main methods of collagen extraction and the method found be the most reliable (10%, w/v ethylenediamine tetraacetic acid demineralisation of bone shards) was slow, which reduced the overall utility of DSC for archaeology.

The decomposition of the organic and inorganic components of bone has been studied using TG (Mkukuma *et al.*, 2004). Thermogravimetric analysis-mass spectrometry (TG-MS) showed changes in mass and identified gases evolved when the samples were heated. The samples were classified as having a high or low proportion of organic material by heating them to 600 °C. At higher temperatures, the mineral phase of the bones decomposed. Fourier transform infrared (FTIR) spectroscopy of bones heated to different temperatures showed that loss of carbon dioxide (as a result of decomposition of carbonate ions) was accompanied by the appearance of hydroxide ions. This study demonstrated that TG-MS can be used to observe differences in the organic content and mineral phases of bones respectively, and this may be useful in comparing bone samples of different ages.

The importance of the organic matrix in affecting the fracture mechanics of bone as well as how TG techniques can be used to study these fracture mechanics have been clearly emphasized. It was observed that TG techniques may be used in determining the PMI of bones using the differences in the organic matrices (Fantner *et al.*, 2004).

Studies of biomaterials by Budrugeac *et al.* (2004) and Tonin *et al.* (2004) found TG to be a reliable technique for characterisation. Budrugeac *et al.* carried out identification of collagen-

based materials while Tonin *et al.* performed thermoanalytical characterisation of modified keratin fibres. The chemical and physical properties of healthy and pathologic bones were also studied using TG, derivative thermogravimetry (DTG) and DSC analysis (Utech *et al.*, 2005). It was discovered that in all the healthy bones the TG curve presents a mass loss of approximately 35% and the DTG curve shows three peaks due to three effects of mass loss – the first peak is due to desorption of water; while the second and third peaks are due to decomposition of organic substances in bone. The pathologic bones presented different curves to the healthy bones. This study concluded that TG can be used as a qualitative method to measure the severity of diseases in bones.

A recent study used TG as the technique to characterise bones due to the sensitivity of thermal methods to morphological states (Onishi *et al.*, 2008). In this preliminary study, TG-MS was applied to the characterisation of bone fragments derived from the compact bone of pig rib specimens in a forensic context. The bone samples were heated to 1000 °C in an argon atmosphere and TG-MS curves were created using the mass loss data collected. Both the organic and inorganic phases decomposed, producing a variety of organic fragments and carbon dioxide. The organic phase is predominantly composed of collagen and pyrolysis of this phase resulted in the observation of ion fragments up to 110 amu. Ion fragments were selected and monitored and changes in these ions were related to the decomposition of both the collagen phase and inorganic carbonated hydroxyapatite phase. This study concluded that observing changes in particular decomposition fragments using TG-MS shows promise as an approach for determining the PMI of bones but without further studies, conclusions could not be drawn with certainty.

Although differences between young and old bones are detectable, there is an interest in determining differences between younger bones for forensic purposes. Since TG techniques are not commonly employed for investigations related to forensic purposes, an attempt has

been made to explore the potential of this technique in relation to forensic applications. In previous research carried out by the author (Raja *et al.*, 2009), this technique has proven to be useful in estimating the PMI of bone samples up to 7 years (Figure 1-4).

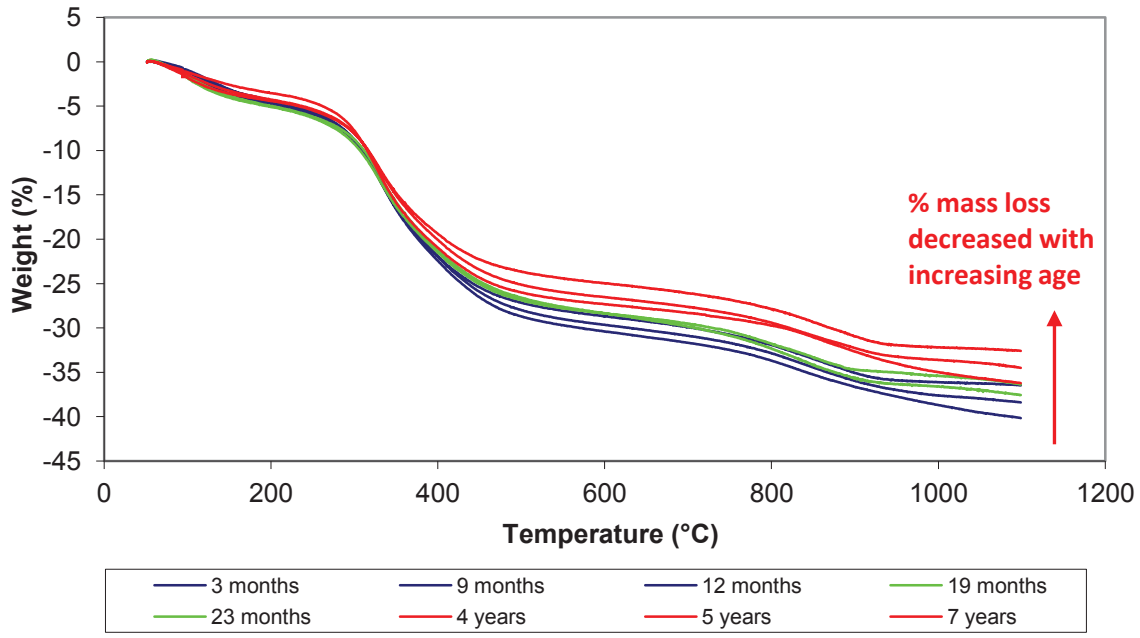

Figure 1-4. TG curves of different post-mortem ages

TG data from the Raja *et al.* (2009) study shows mass losses at particular temperatures that may be correlated with the organic and inorganic phases present in bone. A step in the temperature range 50–220 °C is associated with water loss, while a second step appearing up to 600 °C represents the decomposition of organic components. A third step in the range 650–850 °C is associated with the release of carbon dioxide from the carbonated hydroxyapatite if the decomposition is carried out in an air atmosphere, but is also associated with the further decomposition of the products of pyrolysis produced in the second step if the decomposition is carried out in an inert atmosphere (Onishi *et al.*, 2008).

Figure 1-5 illustrates the TG curves obtained for a specimen of bone with a 3 month PMI in both air and nitrogen atmospheres (Raja *et al.*, 2009). The data has been plotted as a function

of '% mass gain' based on the final mass rather than the more traditional mass loss as water content can be variable resulting in significant variation in percentage mass losses based on initial mass. It is typical also to plot the data as the derivative of mass loss (DTG) to help identify the number of decomposition processes (Figure 1-6) (Raja *et al.*, 2009). For the 3 month sample shown in Figure 1-6, for both the air and nitrogen atmospheres, four mass loss steps are observed. A water loss step is observed below 220 °C. There are two steps associated with the thermal decomposition of organic matter at 220–420 °C and 420–600 °C. The first stage of organic decomposition involves the breakdown of polymeric material into solid and volatile degraded substances and the second stage involves the further breakdown of the solid degraded substances. A small step, step 3 is observed above 600 °C and is associated with carbonate decomposition in an air atmosphere, but also includes further pyrolysis of the organic matter in the inert (nitrogen) atmosphere. The mass loss steps are fairly consistent for the decomposition of the air atmosphere samples up to 60 months PMI. The pyrolysis in an inert atmosphere, however, was observed to be more complex resulting in an increase in the number of mass loss steps observed.

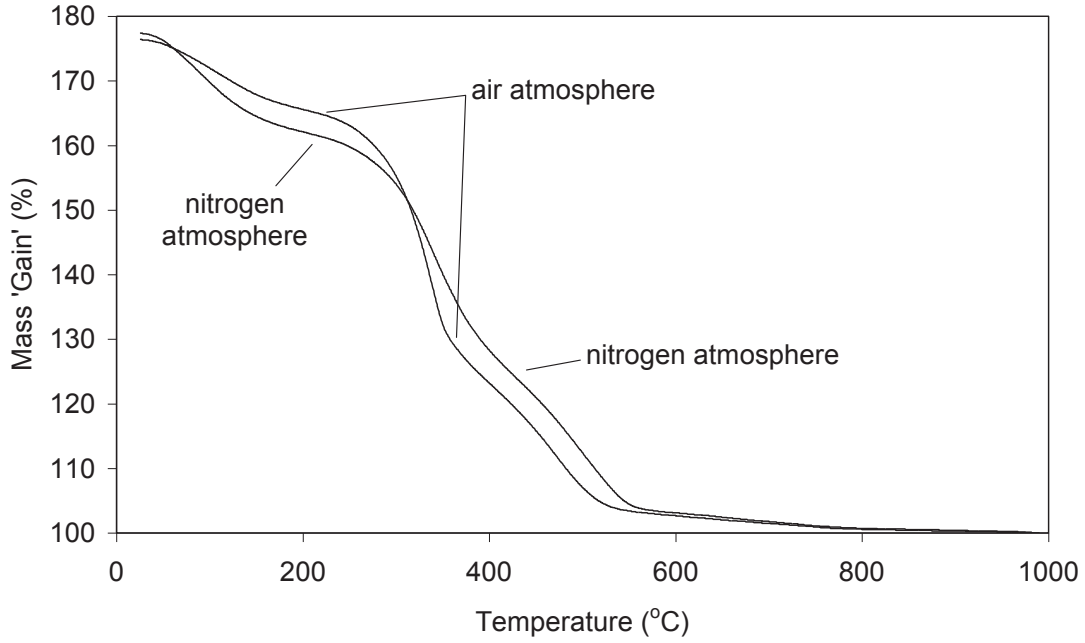

Figure 1-5. TG curves for a bone sample with a 3 month post-mortem age in both an air and a nitrogen atmosphere

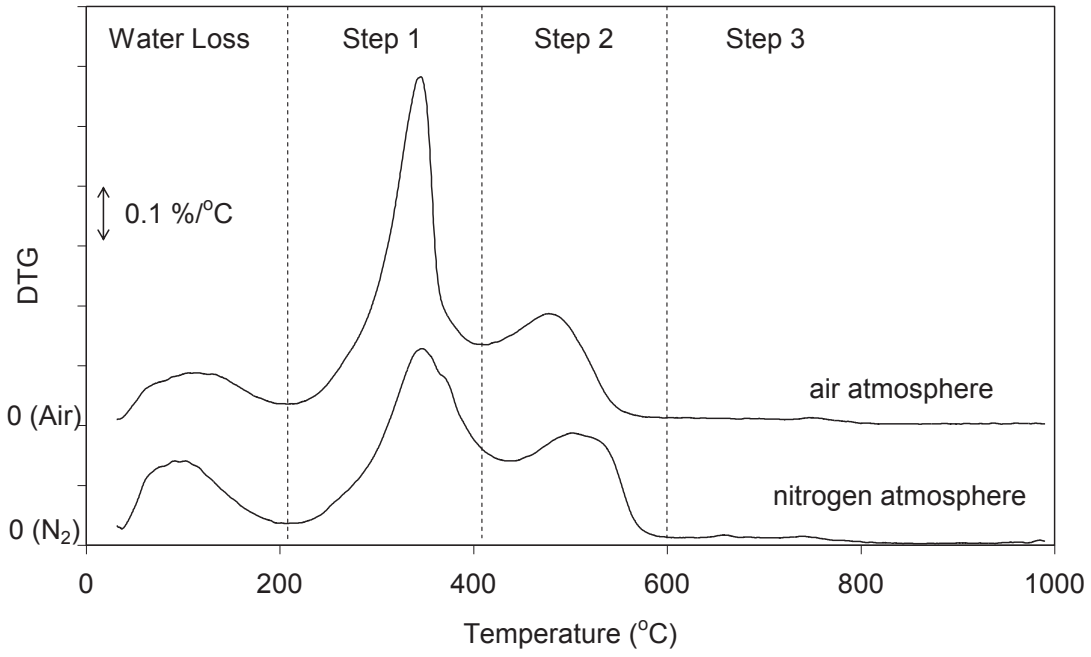

Figure 1-6. DTG curves of a bone sample with a 3 month post-mortem age in both an air and a nitrogen atmosphere

1.3.7.3 Pyrolysis Gas Chromatography-Mass Spectrometry

Pyrolysis gas chromatography – mass spectrometry (Py-GC-MS) is established as an effective technique for the characterisation of complex organic molecules. This technique has the added attraction for forensic practitioners of being a cost-effective method that provides good discrimination, involves minimal sample preparation and is able to detect small quantities of material. Py-GC-MS has been found to be an effective technique to study a majority of organic molecules; however, interpretation of the data is more complex compared to other analytical techniques. It is recognised as a useful tool in particular areas of forensic analysis, including automotive paints (Zieba-Palus, 2008), tire trace analysis (Sarkissian, 2004; Sarkissian, 2007), adhesives (Huttunen, 2007; Bakowski, 1985), lubricants (Campbell, 2007) and fingerprints (Richmond-Aylor, 2007). However, literature is limited for the Py-GC-MS analysis of bone and thus other studies of protein materials using Py-GC-MS have been sourced. One study that used GC-MS to examine animal bone pyrolysis tar produced in Mongolia found that the major decomposition products were nitriles, pyridines, pyrroles and amides (Purevsuren *et al.*, 2004a). Other aromatic compounds were only present as minor components of the tar. The use of a solvent (toluene) may have produced the small amounts of benzene, xylenes and propyl benzenes as impurities in the samples, but these could have also been produced during pyrolysis. The absence of significant aromatic compounds in the bone tar was confirmed by the probe-mass spectra. The elemental composition of the tar was C: 73.3%, H: 10.1%, N: 11.3%, O: 5.3%. Using size-exclusion chromatograms, it was found that smaller molecular mass material was present compared to coal tars.

Flash pyrolysis with GC and MS was used to assess the quality of protein preservation in archaeological remains in which some have yielded DNA sequences. Several samples yielded abundant pyrolysis products assigned to 2,5-diketopiperazines; the relative amounts of these products provide a good estimation of the amount of peptide hydrolysis and DNA

preservation. They concluded that this technique is a rapid and effective method for assessing fossils for the possibility of DNA preservation (Poinar and Stankiewicz, 1999).

The hypothesis that proteins may be sterically protected from enzymatic attack via intimate associations with refractory, macromolecular organic matter was tested using Py-GC-MS. Detrital samples were analysed and the results of this technique supported their hypothesis (Nguyen *et al.*, 2003). This study demonstrated that Py-GC-MS is effective in analysing organic matter samples and therefore may be useful in the analysis of the organic components of different bone samples.

Dating of osteological material from archaeological sources is possible through the use of dynamic TG and pyrolysis gas chromatography methods. Collagen in vertebrates reduces over time when bones are buried for a long time as they are subject to fossilization. It is also demonstrated that collagen undergoes thermal and thermooxidative degradation at high temperatures. The amount of the products released is dependent on the age of the samples (Figure 1-7) (Mognonov *et al.*, 2002).

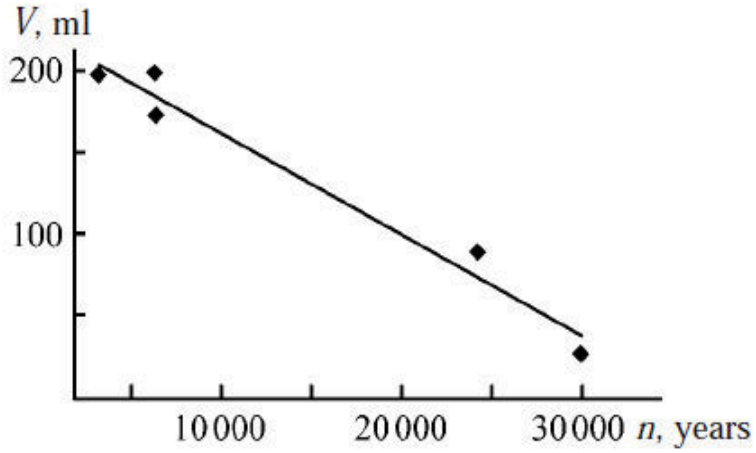

Figure 1-7. Volume of carbon dioxide liberated in pyrolysis of osteological material of different ages (Mognonov et al., 2002)

A previous study conducted by the current author, (Raja *et al.*, 2010a), investigated the potential of Py-GC-MS as a tool for the estimation of PMIs of bones. Pig bone specimens prepared under controlled burial conditions in soil were studied and the PMIs ranged from 3 to 48 months. Notable differences were observed in the data produced for younger bone specimens (< 1 year) compared to specimens of greater PMI (> 1 year). Py-GC-MS also illustrated a relationship of particular peak ratios (19/20 min; 22/23 min) with the PMI of bones. It was demonstrated that the ratios of both pairs of peaks in the pyrograms increased as the bone age increased. This relationship between the peak ratios and the PMI of bones proved the potential of this technique in estimating the time since death and is therefore, further investigated in the present study.

Recently, a study by Lodowska *et al.* (2012) examined the pyrolytic profile of lypophilized and deep-frozen compact bone as an indirect means of analysing the organic compositions of the bones. Derivatives of benzene, pyridine, pyrrole, phenol, sulfur compounds, nitriles, saturated and unsaturated aliphatic hydrocarbons, and fatty acids were observed in the pyrograms. Derivatives of pyrrole and nitriles originating from proteins were the most abundant in the pyrolyzates, with the predominant product being pyrrolo[1,2-α]piperazine-3,6-dione. This is derived from collagen and was used to differentiate the lypophilized graft from the deep-frozen one. Also, the deep-frozen implants resulted in a higher percentage of long-chain fatty acids. The predominant fatty acids in the bone samples were found to be oleic and palmitic acid.

1.3.7.4 X-ray Diffraction

The decomposition of bone involves the degradation of complex organic molecules and a proportional increase in the inorganic matrix. The main inorganic component of bones is a calcium phosphate mineral whose structure closely resembles HA (Smith *et al.*, 1983). XRD is a

powerful technique used in the qualitative and quantitative analyses of crystalline compounds. In terms of bone analysis, XRD can be used to study the crystalline structure of inorganic bone matter.

X-ray procedures (Onishi, 2008; Mkukuma *et al.*, 2004; Abdel-Fattah and Nour, 1993; Fantner *et al.*, 2004, Ooi *et al.*, 2007; Bigi *et al.*, 1997) have been studied for the purpose of PMI estimation but their use in forensic investigations is limited. XRD has been carried out on the inorganic phases in rat cortical and trabecular bones (Bigi *et al.*, 1997). It was observed that several significant differences existed even though both inorganic phases consisted of poorly crystalline B carbonated apatite. The results of trabecular bone apatite showed reduced crystallite sizes, Ca/P molar ratio and carbonate content. Trabecular bone also demonstrated a greater extent of thermal conversion into β-tricalcium phosphate than cortical bone apatite. Therefore, the authors concluded that cortical and trabecular bones differ in the extent of collagen post-translational modifications and also in the structure and chemistry of their mineral phases. This study demonstrated that XRD is a useful technique in observing the inorganic content of bones and therefore may be useful in research related to the dating of bone remains.

The analysis of bone samples of different origins has been performed using various analytical techniques including XRD (Peters *et al.*, 2000). It was demonstrated that size and morphology of the bone mineral particles were independent of the nature of the bone sample, but that significant differences existed in the overall composition of the bone samples and in the carbonate content of the mineral phase. It was noted that the biological resorption of living bone is closely related to morphology and composition of mineral phase. As a result, the necessity of further studies to relate carbonate content and the mineral content of bones to bone history such as age and function was emphasised.

Identification of burnt remains (cremains) has been conducted using XRD, the results of which were compared to the results of trace element analysis (Bergslien *et al.*, 2008). The results showed that XRD can be used to differentiate cremains from the vast majority of common filler materials. XRD has the advantage that it identifies minerals by their crystalline structures rather than their elemental composition, which takes into account any ambiguity caused by natural variation in human trace element loads. In addition, lattice parameters of the inorganic bioapatite phase of animal and human cremains were compared using powder XRD (Piga *et al.*, 2013). The results showed that the differences between the *a*-axis and *c*-axis of the monoclinic and hexagonal lattice were unimportant. In terms of lattice parameters, it was observed that the variability of human specimens was completely overlapped by the non-human specimens' variability. Therefore, it was not possible to distinguish human bones from animal bones using only lattice parameters.

Similarly, XRD has been used to date and characterise recent and fossil bones (Bartsiokas and Middleton, 1992). The results indicated that a progressive increase in bone crystallinity occurs with time during fossilisation, which means that the older the bone, the sharper is its XRD pattern (Figure 1-8). Therefore, crystallinity indices from XRD patterns of bones of the same animal taxon may provide a means of determining the relative ages of bones if important factors such as ante-mortem age, sampling location in the bone and the depositional environment are also taken into consideration, which is relevant in the present study.

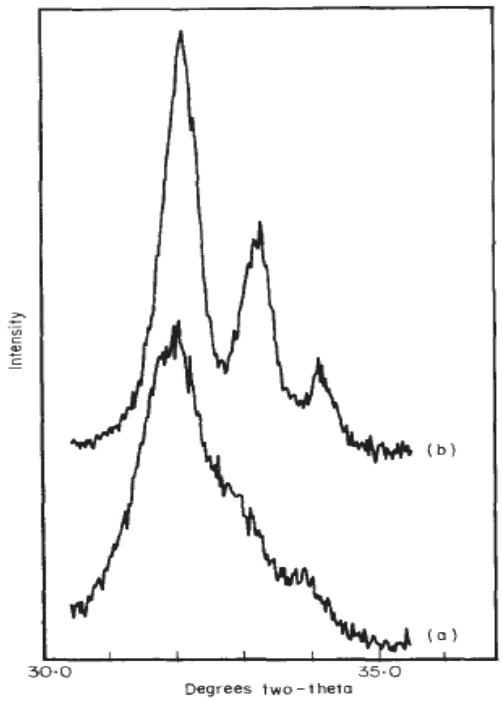

Figure 1-8. XRD diffraction patterns of young (a) and old (b) bone samples (Bartsiokas and Middleton, 1992)

Prieto-Castelló *et al.* (2007) agree that the degree of crystallinity of HA increases as the bone starts to lose organic material in its post-mortem degradation process. This results in a greater crystal size and hence, increasingly narrower XRD peaks, which was evident in the results. The study showed the potential of the use of biochemical (sulphur, potassium, urea) and XRD analyses to establish the PMI. Significant relationships between sulphur, potassium and urea content and PMI and between XRD peaks and PMI exist and therefore, the joint use of XRD and biochemical analyses could be a promising method for use in dating of bone remains. By contrast, a study performed using XRD to analyse the crystallinity of ancient bone and dentine produced results, which showed that the change in crystallinity index was not correlated with the age of the samples (Reiche *et al.*, 2002). It also showed that the local apatite crystal size was not related to crystallinity index in ancient bone or dentine samples. Therefore, it is important to investigate the function of these techniques in establishing the PMI.

The mineral phases and crystallinity in bone were studied using XRD (Miller *et al.*, 2001). The results showed that the width of an XRD line decreases as the bone matures. By combining XRD linewidths and IR data, it was shown that the peak height ratio of 603/563 cm^{-1} is directly related to crystallinity. Also, the results revealed that acid phosphate content and crystallinity are inversely related, that is, high acid phosphate content and low crystallinity are features of young bone.

Jackes *et al.* (2001) compared the XRD results of modern bone samples and two samples of archaeological bone from Moita and Arruda (Figure 1-9). These results confirm the results of a previous study by Bartsiokas and Middleton (1992) in that major differences are observed between modern and archaeological bone mineral. The XRD pattern peaks are more intense in the archaeological samples compared to the modern bone samples. The peaks become sharper with increasing crystal size (Kyle, 1986). Therefore, the broader peaks of modern bone indicate small crystals while the sharper peaks of the Mesolithic bones indicate larger crystals. Nielsen-Marsh & Hedges (1999) suggest that the increase in crystal size occurs as a result of alteration in bioapatite. This involves the removal of collagen as well as the inclusion of carbonate in the lattice. The diffraction pattern of hydroxyapatite (Coreno *et al.*, 1998) is shown in Figure 1-10.

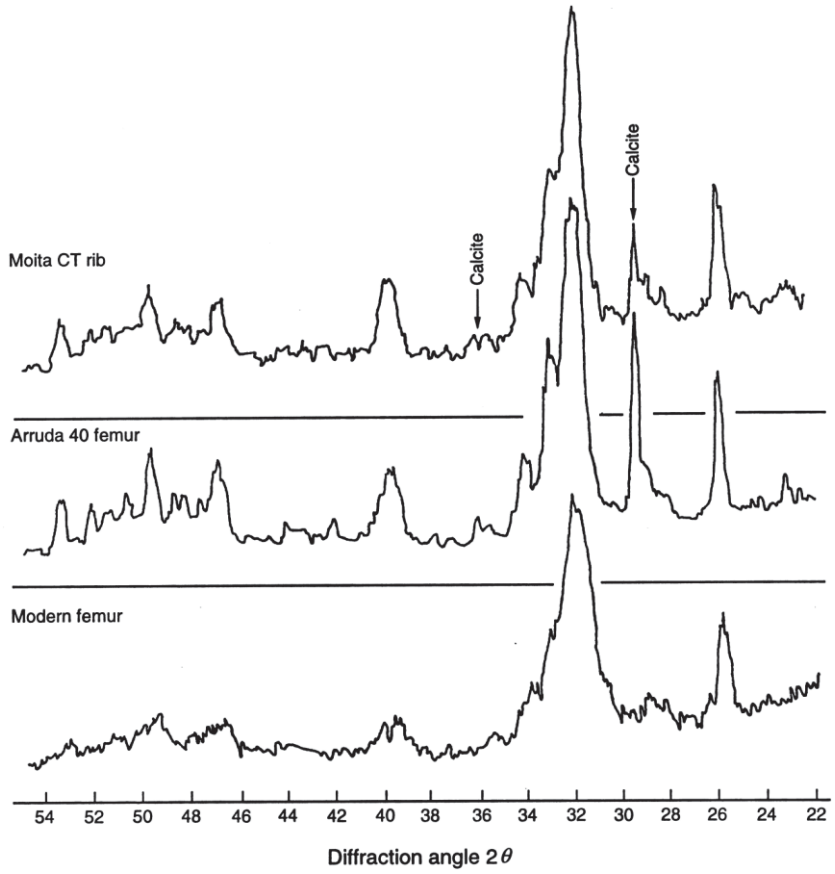

Figure 1-9. XRD diffraction patterns of modern bone and Mesolithic bone samples (Jackes et al., 2001)

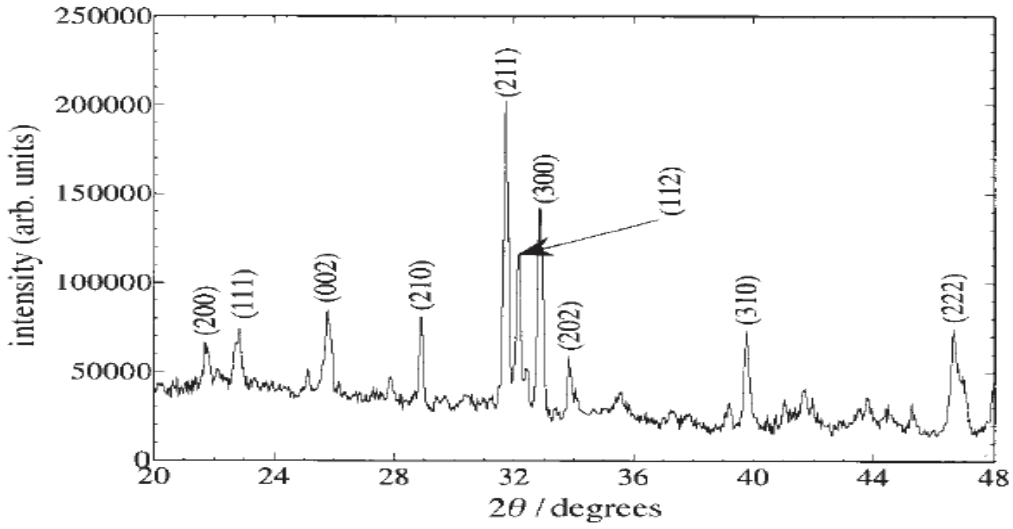

Figure 1-10. X-ray diffraction pattern of hydroxyapatite (Coreno et al., 1998)

The properties of porous HA bioceramic produced by heat treatment of bovine bone were evaluated between 400-1200 °C (Ooi *et al.*, 2007). The bovine bone was characterized by XRD and these results showed that the annealing process enhanced the crystallinity of HA phase in the bone matrix particularly when heated to temperatures above 700 °C. There was no secondary phase formation in bones annealed between 600-1000 °C. However, decomposition of HA to β-tricalcium phosphate was observed for samples heat-treated at 1100-1200 °C.

The decomposition of the organic and inorganic components of bone was studied using high temperature X-ray diffraction (HTXRD) (Mkukuma *et al.*, 2004). HTXRD showed that the main solids produced by decomposition of mineral (in air or argon at 800-1000 °C) were β-tricalcium phosphate and HA, in deer antler, and CaO and HA, in whale tympanic bulla. In carbon dioxide, the decomposition was retarded, which suggested that the changes observed in air and argon were a result of the loss of carbonate ions from the mineral. This study demonstrates that XRD can be used to observe differences in the organic content and mineral phases of bones respectively, and this may be useful in comparing bone samples of different ages.

1.3.7.5 Other Analytical Techniques

A number of researchers have acknowledged the difficulty in dating skeletal remains and have approached this problem from histological (Tirelli, 1910; Tirelli, 1912; Dell'Erba and Caretto, 1957; Aznar, 1945; Aznar and Maestre, 1945), morphological (Lopez-Gomez, 1967; Mueller, 1953), and chemical (Lopez-Gomez, 1967; Tirelli, 1910; Tirelli, 1912; Aznar, 1945; Aznar and Maestre, 1945; Dell'Erba, 1958; Taylor, 1965; Strehlow and Kneip, 1969) points of view. In their book on Legal Medicine, Lazzaretti and Orfila (1878) stated that since the 19th century it has been difficult to establish the time since death using bone remains in a legal context. This is particularly important when attempting to determine the timeline of events in forensic cases involving missing persons. Villanueva *et al.* (1976) also recognised the difficulty in dating bone

material for legal medicine. They investigated the problem using a systematic study of the most relevant organic and inorganic compounds in a set of 34 bone samples.

In 1969, Bass performed a complete test involving morphological, histological, and physicochemical aspects in the identification of human skeletal material. Knight and Lauder's preliminary study (1967) attempted differentiation based solely on morphological and physical characteristics. Knight (1968) and Knight and Lauder (1967, 1969) used different approaches which included observing soft tissue and bone texture over different PMIs. Dating methods based on morphological characteristics proved problematic since soft tissues do not generally survive the decomposition process.

Knight and Lauder then attempted to use a variety of chemical tests to differentiate modern and ancient bones (Knight, 1968, 1971; Knight and Lauder, 1967, 1969). The chemical studies involved analysing total and amine nitrogen content using fluorescence, amino acid identification, and benzidine reactions. These techniques proved to be useful in differentiating modern and ancient bones, with the results being supported by Jarvis (1997) who investigated the nitrogen levels in long bones buried for a period of 26 to 90 years. In 2001, the chemical composition of decomposing remains was studied in order to develop usable biomarkers for determining PMI. Bodies were monitored over a decomposition period of four years. Tissue samples were collected for analysis for specific biomarkers such as amino acids and neurotransmitters (Vass *et al.*, 2001), which were then correlated with PMI. However, it was demonstrated that environmental conditions were a large source of uncertainty in estimating PMI using the approach of chemical testing. This led to the conclusion that the chemical tests could not be relied upon without taking the surrounding burial conditions into consideration (Knight, 1968; Pollard, 1996). Also, the benzidine tests rely on the presence of blood, which can be easily be destroyed by certain environmental conditions.

Recently, the decompositional changes to buried human hair, buried porcine cartilage and bone were investigated using macroscopic, microscopic, molecular, chemical and microbiological analyses. A correlation between the decompositional changes and time was attempted in an effort to develop a new method for PMI estimation. Changes were observed in bone over time; however, these were only minimal. These included a change in colour of the cortical surface and the cracking and flaking of the cortical surface. Changes in colour and gradual loss of bone marrow were also observed. The results indicated that the decompositional changes to cartilage could be used to estimate PMI, however, the degradation of hair and bone was too variable to be of use in this context (Rogers, 2010).

Immunological techniques focussed on the detection of residual serological activity of bone protein, changes in the fat content of bone, histological bone sections and colorimetry techniques to estimate post-mortem bone age (Knight, 1968; Knight, 1967, 1969). These results were unreliable and therefore, cannot be used for the accurate estimation of PMI.

The use of radiocarbon dating methods has been widely studied for dating bone material in an archaeological context. This method has recently been used to date skeletal remains in a forensic context (Ubelaker, 2001) and demonstrated that the collagen fraction of recent bone does not produce accurate results. The method of radiocarbon dating is a useful method for distinguishing between modern and ancient bone, however, it is not reliable enough for use in a forensic context.

Investigation of the viability of measuring the equilibrium between two naturally occurring isotopes, ^{210}Po and ^{210}Pb, as a method of estimating the PMI was conducted by Swift (1998). This study demonstrated significant variations in isotopic concentrations in individual bone types such as ribs, vertebrae, femur and skull bones. Also, there were significant differences between trabecular and cortical bones in terms of lead isotope concentrations. Limitations of

this technique are the effects of diagenesis as well as time and cost, however, Swift suggested that a large study be undertaken to determine if this technique could provide a suitable method for the estimation of the PMI.

Infrared spectroscopy, in particular, FTIR, is a commonly used technique for bone analysis. FTIR is a form of vibrational spectroscopy that can provide a broad range of information on both organic and inorganic samples based on change in the dipole moment. Most research using FTIR for bone dating has been in an archaeological context. Sillen and Parkington (1996) compared the crystallinity values of archaeological bone samples (up to 20,000 years old) with modern references. They found that the crystallinity index increased gradually from about 2.8 to 3.5 as the crystal structure became more and more ordered. Similarly, Wright and Schwarcz (1996) found that the crystallinity of ancient bone increases with decreasing carbonate content. Very *et al.* (1997) looked at the quality of bone samples in those who had died a violent death with no known pathology and found that the organic context of the bones was changed the most significantly.

Research using FTIR has shown that in modern bone (Trueman *et al.*, 2008), the crystallinity index increases from about 2.50 to 3.25. Trueman *et al.* (2004) looked at the diagenesis of bones weathered on the ground's surface for a period of up to 40 years. They found that the crystallinity index did not change for up to 2 years, but the amide:phosphate ratio decreased from 0.8 to 0.2. Since the bones were left exposed, the diagenesis of the samples was severely accelerated and may not be an accurate representation (Trueman *et al.*, 2004).

Thompson *et al.* (2009) support the notion that higher crystallinity index values indicate a higher degree of order within the crystal lattice. They showed that the subtle change in the crystallinity index can be accelerated by burning and/or environmental conditions such as the weathering of bones which are partially or fully exposed. In 2011, Thompson *et al.* confirmed

that crystallinity index increases with burning intensity using modern burned and unburned faunal bone. The influence of internal and external variables on bone crystallinity changes was studied using Fourier Transform Infrared Spectroscopy - Attenuated Total Reflectance (FTIR-ATR). It was demonstrated that external variables (such as duration and temperature of the burning process) have a more significant influence on crystallinity index values than internal variables (such as location of sample site).

In a recent study conducted by Thompson *et al.* (2013), five new spectral indices of heat-induced crystallinity change were identified using FTIR spectra of burnt bone samples and a new statistical approach. The burning temperature of more than a hundred bone samples burned between 100 °C and 1100 °C was predicted using a statistical classification model based on the newly identified spectral indices. Using four spectral indices including the crystallinity index, a correct classification rate of 97.2% was achieved, which was significantly higher than the classification rate obtained using crystallinity index alone (66.7%).

FTIR has also been used to study the effects of environmental conditions on the colour and organic content of cremated bone (Walker and Miller, 2008). They used the potassium bromide (KBr) pellet method and discovered that neither of the peaks of intact collagen persisted in samples cremated at temperatures greater than 600 °C. Similarly, Surovell and Stiner (2001) used FTIR to monitor the effects of different sample preparation methods on the IR spectroscopic measures of bone mineral crystallinity. A standard KBr pellet method of bone preparation is proposed based on the fact that intensive grinding can make it difficult to distinguish variation in actual bone crystallinity from that introduced by sample preparation. Grinding using a ball mill is recommended in the standard method.

FTIR spectroscopy has been used to analyse pathological and non-pathological human skeletal remains (Nagy *et al.*, 2008). The KBr pellet method was used which included mortar and pestle

grinding. The modern bone samples exhibited more intense organic bands than the archaeological ones (Figure 1-11). A method based on the crystallinity index and carbonate-phosphate index was proposed to distinguish between archaeological and forensic anthropological skeletal remains. Also, francolite has been observed in archaeological bone samples and therefore, the appearance of francolite crystals can be used in the distinction of ancient and recent human bone samples.

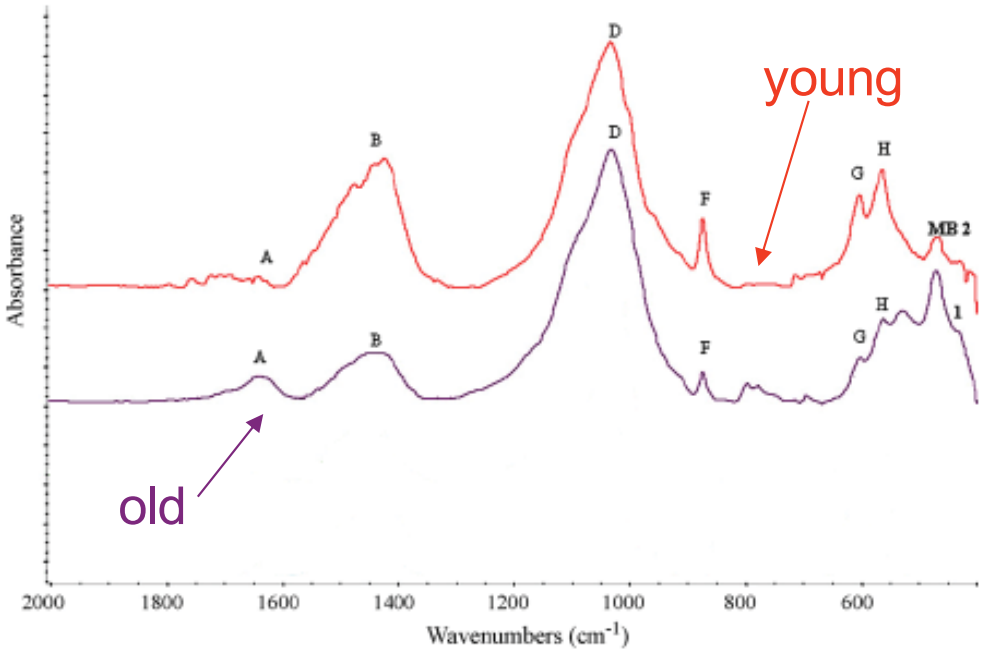

Figure 1-11. FTIR spectra of a modern (young) and archaeological (old) bone sample (Nagy et al., 2008)

Unlike the above-mentioned studies using FTIR, a study performed by Greene *et al.* (2004) did not use the KBr pellet method. Instead, they used diffuse reflectance infrared spectroscopy (DRIFT) to identify potential diagenesis and crystallinity changes in teeth. This study demonstrates that DRIFT is a convenient alternative to pellet-based transmission FTIR spectroscopy in testing diagenetic changes in hard tissue i.e. teeth and bones for archaeological investigations. The DRIFT approach aided in the characterisation of functional groups, estimation of carbonate content and the evaluation of the extent of fluoridation.

Sample preparation involving mortar and pestle grinding (only 1-3 mg required) in the DRIFT process enabled duplicate analyses of each sample, between which negligible sample-to-sample variation was observed. Therefore, DRIFT proved to be a method with high sample throughput in which errors in sample preparation or loading could be easily corrected and therefore demonstrates enormous potential in future diagenetic research.

The most common preparation method for bone samples being studied using infrared spectroscopy is chemical pre-treatment followed by grinding; however, it has been demonstrated that chemical pre-treatments can affect the interactions within bones. As a result, a recent study on bones using a non-destructive technique that requires little to no sample preparation was conducted (Gu *et al.*, 2013). The technique employed, photoacoustic Fourier transform infrared spectroscopy (PA-FTIR), detects absorbance spectrum from a sample at controllable sampling depths. Human cortical bone was studied using the photoacoustic mode, which produced similar spectra to the traditional transmission mode. It was observed, however, that the photoacoustic mode was more sensitive to the amide III and v_2 carbonate bands. Overall, it was demonstrated that chemical pre-treatment and transmission techniques affect the phosphate ion geometry and the presence of water affects the OH band, amide I and mineral bands. Interestingly, it was also observed that the longitudinal sections of bones appear to have more organic matrix exposed. The results of this research demonstrate the potential of using PA-FTIR to study bone samples without requiring sample preparation. This technique could be useful for the analysis of recovered skeletal remains in terms of achieving an accurate estimation of the post-mortem interval as interference from sample preparation methods is eliminated.

The technique of Raman spectroscopy was used by McLaughlin and Lednev (2011) to study trends in the chemical composition of turkey bones buried for intervals between 12 and 62 days. Results of the study showed that chemical changes in bones due to soil bacteria are

dependent on time with the visual comparisons of buried bones demonstrating a consistent decrease in the organic Raman bands with increasing length of burial. The spectroscopic trend observed most clearly was the broadening and diminishing amide I band. Additionally, it was noticed that peak area integration produced more correlative results with burial duration than peak height.

1.3.8 The Effect of Storage Conditions on Bone

The consequences of freezing bone samples before preparation have been studied (Robinson *et al.*, 2003). Freezing is considered to be the most convenient method of storage; however, it may alter the microstructure of the bone samples by the formation of ice-crystals. This study showed that preparation techniques, particularly freezing, can significantly affect the porosity of bones. Similarly, a study on taphonomic changes to blunt force trauma evaluated seven different taphonomic changes (Calce and Rogers, 2007). The results demonstrated that freezing and thawing and exposure to rain and snow altered pre-existing trauma. The study by Robinson *et al.* (2003) went on to conclude that any comparative measurements of bone porosity should avoid bones which have been frozen or been prepared by heating or maceration.

Experiments comparing frozen and freeze-dried particulate bone allografts (bone transplant) were conducted (Malinin and Temple, 2007). The biological behaviour of freeze-dried and frozen particulate bone allografts are assumed to be similar, however, the results of this study found that freeze-dried particulate allografts induced new bone formation and healing of the bone defects much faster than the frozen allografts. The effects of thawing frozen calcaneal (heel) bone specimens on the bone mineral density (BMD) using dual energy X-ray absorptiometry (DXA) were also examined (Trudel *et al.*, 2005). The specimens were stored at -13 °C as autolytic changes at this temperature are negligible. Their results showed that the

BMD properties in frozen or thawed states were equivalent. This means that investigators can confidently use frozen sample BMD data as a surrogate for thawed state BMD data. This will save time and eliminate the need to thaw samples before conducting BMD measurements which is crucial in musculoskeletal research. Frozen storage of bones for up to 122 days does not affect bone density (Reeves, 2002). Interestingly, Wahnert *et al.* (2009) showed that measurement of bone density is affected by storage temperature and therefore should be standardised to thawed or frozen specimens.

The technique of microscopy (light and SEM) was used to investigate frozen human bone to determine whether previously frozen bone could be identified and whether freezing alters the structural integrity enough to prevent histological ageing (Tersigni, 2007). Bone segments were stored at 0 °C for 21 days and then allowed to thaw before analysis. Even though noticeable microstructural changes due to freezing were observed using SEM analysis, these changes did not alter the structural integrity. Therefore, the results of this study suggest that freezing does not alter the process of histomorphological analysis. Statistically, no significant changes were observed in the size of the Haversian canals and lacunae due to the freezing process.

Enzymes in bone tissue degrade the osteoinductive growth factors present in demineralised bone. It has been demonstrated that storage of rat limbs at low temperatures (4 °C) before harvesting preserves the osteoinductive potential of such factors. In contrast, storage of bone samples at room temperature for more than 24 hours results in a biologically inactive bone matrix. This has been suggested to be a result of biodegradation (Yazdi *et al.*, 1991).

No difference was observed in bone ash of refrigerated and frozen samples at the three time periods studied (72, 80 and 92 weeks). However, a difference in the bone breaking strength in refrigerated and frozen samples was seen for the time period of 72 weeks (Park *et al.*, 2003).

Storage by freezing as well as several thawing and refreezing sequences did not change the stiffness of bones over a period of 100 days (Linde and Sorensen, 1993).

The measurement of bone marrow adipose content was examined for three animal species (equine, bovine and canine) at three storage temperatures (-20 °C, 4 °C and ambient temperature). It was concluded that the percentage of fat in bone marrow did not change after 30-60 days in the bovine and equine samples (Lamoureux *et al.*, 2011).

Research investigating the effects of freezing on bone samples has produced differing results for different researchers. This highlights the need for further research to be able to understand the effects of storage conditions on bone samples. Limited research on the effects of storage conditions on bone decomposition also highlights the need for more studies to be performed in this area.

1.3.9 Aims and Objectives

The present study investigates the effects of varying environments on bones using different analytical techniques. The primary aim was to identify methods of determining the PMI of skeletal remains discovered in different burial environments.

It has been identified that more research and detailed information is required on bone pre-treatment procedures and burial conditions to understand the biological and chemical processes that occur in the bone after burial. Differences have been observed in fleshed, defleshed, degreased and boiled bones and have therefore been investigated in the present study. Burial environments varying in soil types, soil pH, moisture content and temperature have also been examined in the present study as they have been observed to have an effect on bone decomposition.

It has also been highlighted that more research is needed to understand the effects of storage conditions on bones. There is limited information on how storage conditions affect bone decomposition and therefore a comparison of fresh bone and bones stored for different periods of time in a freezer was performed.

Bone is a complex material consisting of organic and inorganic components and it is important to study changes in these two components to understand how decomposition occurs. Thermal methods of analysis were the focus of this study with changes in the organic content of bones over time being studied using the techniques of TG and Py-GC-MS. The complementary technique of XRD was employed to study variations in the mineral content of bones. In addition, ESEM analysis was used to observe changes in morphology as the burial times of bones increased. Additionally, the technique of FTIR was employed by a colleague (Howes *et al.*, 2012) on the bone samples prepared in the present study.

It should be noted that the results of the present study will be discussed in terms of PBT of bones rather than PMI as the exact time of death of the specimens from which the bone samples were obtained is not known.

1.3.9.1 Primary Aim

The primary aim of the present study was to develop a method that can lead to an accurate estimation of the post-burial time of bones by burying bones in diverse burial environments and subsequently examining the buried bones using various analytical techniques.

Therefore, to achieve the primary aim several objectives were established, which included bone specimen preparation, burial of bones in different burial environments, bone analysis and bone storage.

1.3.9.2 Objectives

i) The first objective involved investigation of the effects of the slicing, drying and powdering processes during bone specimen preparation to establish the most accurate and reliable method of bone sample preparation.

ii) The second objective of the study was to observe the diagenetic processes occurring in differing burial environments including varied soil types (loam, silt, sand, clay); pH (acidic, basic, neutral); moisture content (wet, dry); and temperature (cold, room-temperature).

iii) The third objective comprised of studying the effect of the presence of flesh and/or fats and oils in bones on the diagenetic processes occurring using the pre-treatment methods of defleshing and/or degreasing.

iv) The fourth objective included observing the differences between bones subjected to accelerated diagenesis and normal diagenesis using the pre-treatment method of boiling (accelerated diagenesis).

v) The fifth objective of the study was to identify the potential of PMI determination through the use of ESEM, TG-DTA, Py-GC-MS and XRD to monitor diagenetic changes in bones in differing burial environments.

vi) The sixth objective entailed the study of the effects of storage conditions on bone samples through comparison of fresh bone samples with bones stored in the freezer for different time periods.

Chapter 2

Materials

2.1 Introduction

For this thesis, porcine bone samples were used as a representative for human bone sample. According to Thorwarth *et al.* (2005), pig bones can be considered to be a suitable analogue for human bone research in terms of human anatomy, morphology, healing and remodelling. The main difference is that pig bones have a denser trabecular network than human bones. However, the lamellar bone structure of both pig and human bones are very similar (Mosekilde *et al.*, 1993). In addition, the ease and availability of obtaining the samples for research as well as the ethical considerations for developmental research were important factors to consider.

2.2 Optimisation of Bone Sample Preparation Procedure

A standard procedure, which was reproducible and reliable, was established for preparing the bone samples for analysis. An experiment was conducted using bone specimens from pigs (*Sus scrofa*) of PMIs 3 to 20 months. These decomposed bone samples were provided by the Centre for Forensic Science at the University of Western Australia and were the result of a decomposition study carried out under controlled conditions. All these samples were flat rib bones from female pigs, sourced from the same farm and fed with an identical diet. Their weights ranged between 40-45 kg. The carcasses were buried in soil with a pH of 5 at around 60 cm below the surface for a designated amount of time. The average ambient temperature was 25 °C. After exhumation, the bones were stored in sealed plastic bags at 4 °C prior to analysis.

The bones were mechanically sectioned using a Buehler IsoMet low speed diamond saw. This is a precision sectioning saw designed for cutting various types of materials with minimal deformation and has a built-in micrometer for precise sectioning. The cortical bone was chosen for analysis as the results of a previous study demonstrated that cortical bone produces reproducible results when compared to cancellous bone (Onishi *et al.*, 2008; Raja *et al.*, 2009). The cortical bone was separated from the cancellous bone using a scalpel. Once separated, the cortical bone was prepared using four different methods to determine which method of bone preparation produced the most reliable and accurate results.

It was recognised that there could be two main potential sources of error that could occur during bone sample preparation and these needed to be eliminated. One of the errors could be introduced as a result of the incomplete removal of water from the bone sample. The second source of error could be the decomposition of the bone sample during the grinding process due to heat or mechanical pressure. Therefore, the following four methods were chosen and compared to establish a standard bone sample preparation procedure:

1. Oven-dried in a vacuum oven at 50 °C for 2.5 h in lump form (OD-L);
2. Oven-dried in a vacuum oven at 50 °C for 2.5 h and ground using a ring mill (Rocklab) for 30-45 s (OD-R-P);
3. Freeze-dried (Christ Alpha 2-4 LD Plus) at -90 °C and a vacuum of 0.0010 mbar for 24 h then hand ground in an agate mortar and pestle (FD-H-P);
4. Freeze-dried at -90 °C and a vacuum of 0.0010 mbar for 24 h then ground using a ring mill for 30-45 s (FD-R-P) (Raja *et al.*, 2010b).

Based on this study, it was concluded that the method of freeze-drying and manual grinding using a mortar and pestle (#3 above) was the most appropriate and reliable method for

preparing the bone samples after recovering them from their burial environment (results outlined in *Section 4.2.4.2).*

2.3 Burial Conditions Study

Various burial conditions for the bones were selected for a comparison of their effects on bone structure. The study included variations to factors including soil type, soil pH, moisture content, temperature and bone pre-treatment methods for a burial period of 0 to 18 months.

2.3.1 Bone Pre-treatment Procedures and Burial Environments

Fresh pig rib bones were buried at a depth of 5 cm in different types of soils (Table 2-1) in sealed 41 litre polyethylene containers. It is important to note that the sets of pig rib bones used in the current study had a relatively large mass range (2.9 g – 18.7 g) with the average mass of bones in each set being in the range of 8.0 – 12.3 g. Therefore, the variations in the masses of the rib bones compared in the study as well as the heterogeneity of the samples as a result, need to be considered during interpretation of the observed data.

The different types of soils used for this project are listed in Table 2-1. This table also shows the details of the different bone pre-treatments used for three of the sets of bones. All but two sets of bones were stored at room temperature, which ranged between 20 °C and 23 °C. The remaining two sets of bones were stored at a colder temperature of 4 °C (in a refrigerator). The rib bones were recovered at specific time intervals of 1, 2, 4, 6, 8, 10, 12, 15 and 18 months post-burial.

Table 2-1. Burial conditions of bone samples

Sample Name	Burial Conditions	Sample ID
Reference (Control) – Loam Defleshed (1 & 2)	Defleshed bones buried in loam soil, pH 7	Cont
Loam Fleshed (1 & 2)	Fleshed bones buried in loam soil, pH 7	LF
Loam Acidic (1 & 2)	Defleshed bones buried in loam soil, pH 5-6	LA
Loam Basic (1 & 2)	Defleshed bones buried in loam soil, pH 9-10	LB
Loam Wet (1 & 2)	Defleshed bones in wet loam soil pH 7	LW
Loam Dry (1 & 2)	Defleshed bones in dry loam soil pH 7	LD
Silt (1 & 2)	Defleshed bones in silt	S
Sand (1 & 2)	Defleshed bones in sand	SA
Clay (1 & 2)	Defleshed bones in clay	C
Loam Degreased (1 & 2)	Defleshed + degreased bones in loam soil, pH 7	LDG
Loam Boiled (1 & 2)	Defleshed + boiled bones in loam soil pH 7	LBO
Loam Refrigerator (1 & 2)	Defleshed bones in loam soil, stored at 4 °C, pH =7	LR

2.3.1.1 Bone Pre-treatments – Defleshed, Fleshed, Degreased, Boiled Bones

Different pre-treatments of bones were carried out before burial to examine their effects on the bones.

Defleshed and Fleshed Bones

Two sets of bones were buried without removing any flesh to observe the differences between defleshed and fleshed bones. The defleshed bones represented the reference (control) samples and were prepared using the defleshing method described in Fenton *et al.* (2003). The bones were placed in a beaker with a solution of detergent (5 g of "Surf-Sunshine Fresh") and sodium carbonate (4 g) and left in a water bath at 50 °C for 24 hours. The fleshed bones consisted of porcine rib bones with flesh completely covering the bones.

Defleshed, Degreased and Boiled Bones

The aim of the different pre-treatment methods used was to compare bones that had been defleshed, degreased and boiled prior to burial. The defleshed bones were the reference (control) samples, as previously described. In addition, two sets of bones were degreased prior to burial using the degreasing method (Fenton *et al.*, 2003), which utilised ammonia solution. Another two sets of bones were boiled in a water bath at 100 °C for 3 hours prior to burial. To be able to observe decomposition processes that occur in bone alone, it was important to eliminate factors that would otherwise affect these processes. Factors including flesh and surface lipids were taken into account and the presence or absence of these was related to the differences in the results. The pre-treatment of boiling was also studied as boiling of the bones has been shown to simulate bone diagenesis (Roberts *et al.*, 2002). Therefore, in the present study, three bone pre-treatment methods were compared.

2.3.1.2 Burial Environments – pH, Moisture Content, Soil Type, Temperature

The effect of different soil compositions on bone decomposition was observed by comparing the reference (control) soil environment with soil compositions that varied in soil type, soil pH, moisture content and temperature. Duplicates of each of the burial environments were created.

Reference (Control) Soil Environment

The 'reference' soil environment *(hereinafter referred to as the 'control' soil environment)* consisted of defleshed bones buried in neutral loam soil at room temperature. This was chosen as the control environment to be able to understand the effects of different burial factors on bones including bone pre-treatments, soil type, soil pH, moisture content and temperature. The loam soil, Brunnings Organic Garden Soil, was purchased from Bunnings Warehouse, Sydney and was chosen as it represents the common type of top soil used in gardens in Sydney.

Soil Type

Soil texture refers to the relative proportion of sand, silt, and clay particles in a soil. In terms of size, sand particles are the largest (0.05-2 mm), followed by silt particles (0.02-0.05 mm) with clay particles (<0.002 mm) being the smallest. Mineralogically, sand is predominantly composed of quartz, silt is made up of quartz and feldspar and clay consists of phyllosilicate minerals.

In the present study, bone samples were buried in different soil types for comparison with burial in a loam soil environment (control). Brickies Sand was used for the silty soil

environment, Washed Sydney Sand was used for the sandy soil environment and Renderer's Clay was used for the clay soil environment. These 'substrates' were purchased from Maroubra Building Supplies, Matraville, New South Wales (NSW).

Soil pH

For comparison with the neutral loam soil (control) environment, burial environments with differing pHs were created. For the acidic soil environment, Brunnings Azalea and Camellia Potting Mix was used and purchased from Bunnings Warehouse. Weekly monitoring of the pH was conducted and a pH of 5-6 was maintained by adding powdered sulfur as needed. The basic soil environment was loam soil with Richgro Garden Lime added, until a pH of 9-10 was achieved. This soil was also maintained at a pH of 9-10 for the duration of the study. The pH of all three burial environments was regularly tested using a Manutec Soil pH Test Kit, also purchased from Bunnings Warehouse.

Moisture Content

Bone samples were buried in wet and dry loam soil environments for comparison with the loam as supplied (control) burial environment. The 'wet soil' burial environment consisted of loam soil with distilled water added until saturation was visibly evident. The samples were visually monitored weekly and additional distilled water was added if the saturated samples became too dry. The 'dry soil' burial environment was produced by drying the soil using an oven at 50 °C for 3 hours.

Very low condensation levels were observed inside the lids of all the containers except for the Loam Wet 1 and 2 burial environments, where higher condensation levels were seen.

Temperature

Bone samples were buried in loam soil and stored in a refrigerator at a temperature of 4 °C (digitally controlled) to simulate burial in a cold environment. These were compared with bone samples buried at room temperature (control), which was digitally monitored.

All Samples

The bone samples were all buried approximately 5 cm below the surface (Figure 2-1) for an assigned time period. All the bone samples were kept buried in sealed containers and were only opened when bones were to be recovered. This meant that the burial environment remained relatively undisturbed for the duration of the study. The bones were recovered in a random manner and stored in sealed plastic zip-lock bags.

Figure 2-1. Burial of bones approximately 5cm below the soil surface (before and after placement of top soil)

The average ambient temperature was between 20-23 °C and the loam soil was neutral in pH, unless otherwise stated (Table 2-1). Macroscopically, there was noticeable microbial activity caused by bacteria or fungi on some of the bone samples and this is described in detail in the visual observations sections in Chapters 4 (Sections 4.2.1, 4.3.1, 4.4.1 and 4.5.1) and 6 (6.2.1, 6.3.1, 6.4.1 and 6.5.1).

2.3.1.3 Bone Samples

Porcine rib bone samples used were purchased from Glenmore Meat Co. Pty Ltd, Glebe, NSW. The carcasses handled by this company are transported from the abattoir daily and stored at -28 °C. The pig ribs used for the present study were purchased and immediately transported to the laboratory. The defleshing process was performed on the ribs as soon as the samples were brought to the laboratory. All porcine bone specimens were flat rib bones from butchered pigs weighing approximately 68-72 kg. Flat bones consist of thin plates of periosteum-covered compact bone on the outside and endosteum-covered spongy bone on the inside. These bones are not cylindrical and so have no shafts or epiphyses. They contain bone marrow between their trabeculae, but there is no marrow cavity present (Abdel-Fattah and Nour, 1993). The ribs were defleshed using a fast and safe method of skeletal preparation for forensic purposes (Fenton *et al.*, 2003), using a solution of detergent and sodium carbonate, before they were buried.

Once buried, the bones were recovered at different time intervals and stored in sealed and labelled plastic zip-lock bags. Firstly, photographs of the bone samples in each of the zip-lock bags were taken using a Canon Digital IXUS 500 camera. The bone samples were then removed from the bags. The bone samples that had soil and plant material still adhering were cleaned using a scalpel blade. Measurements such as length, diameter and mass of each bone were recorded. The diameter of the bone samples was the measurement of the diameter of

the 'body' section of the bone (Figure 1-3). Physical descriptions including peeling, cracking, colour and the presence of any odour were recorded for each bone at each post-burial time. After the initial inspection of the bones was completed, the bones were stored in the freezer at a temperature of -7 °C to -10 °C until required for analysis.

2.3.1.4 Bone Sample Preparation – Burial Conditions Study

Each rib bone specimen was sectioned using the Buehler IsoMet low speed diamond saw. Three slices of 1 mm widths and six slices of 2 mm widths were cut from each bone sample. 'Fractured' samples were also prepared by cutting the bone sample halfway using the diamond saw and then breaking the bone sample in half longitudinally by manually applying pressure. All the slices (1 mm and 2 mm) of each bone sample were then placed in labelled glass petri dishes. These were freeze-dried overnight (24 hours) using a Christ Alpha 2-4 LD Plus freeze dryer.

After freeze-drying, all the slices were longitudinally cut in half using a scalpel. Based on previous research, it was determined that it was important to retain the cortical section of the bone samples for TG due to the reproducibility of the results rather than the bone marrow section of the bone (Onishi *et al.*, 2008; Raja *et al.*, 2009). Therefore, the fibrous inner portion of the slices of bone (bone marrow) was removed using a scalpel blade. The hard cortical bone sections of the 1 mm slices were hand-ground using a mortar and pestle and the remaining cortical bone sections of the 2 mm slices were mechanically-ground, using a ring mill. The hand-ground samples resulted in a coarse powder containing particles of several different sizes, whereas the mechanically-ground samples resulted in a very fine powder. A previous study by Onishi *et al.* (2008) compared the shard and powdered bone samples to determine the optimum sample particle size for TG, in order to reduce sample preparation time. The sample preparation study performed as part of this research project

concluded that hand-ground powdered samples produce the most reproducible TG results. After cutting the sections and powdering, the bone samples were placed in sealed and labelled glass scintillation vials and stored in the freezer at -7 °C to -10 °C until required for analysis.

2.3.1.5 Bone Sample Preparation – Storage Conditions Study

To study the effect of storage conditions, two fresh porcine rib bone samples (same as bone samples in Section 2.3.1.3) were cut, freeze-dried and hand-ground in the same manner as described in Section 2.3.1.4. These were prepared to determine whether storage conditions of the samples prior to analysis had any effect on the results of the samples. The two bone samples were stored in the freezer at a temperature between -7 °C and -10 °C and analysed at post-burial times of 1, 2, 4, 6, 8, 10, 12, 15 and 18 months, using analytical techniques including TG, Py-GC-MS, XRD and ESEM analysis. The results for each post-burial time (length of time stored in the freezer) were compared to the results of the bones prior to storage in the freezer. For each analytical technique, the results at each post-burial time were compared to determine the extent of the effect of storage on bone samples.

Chapter 3

Methods

3.1 Introduction

In the present study, various experimental techniques were used to observe the changes that occur in bone after burial. The techniques were employed to study the changes in the organic as well as the inorganic content of the bone. Firstly, to study changes in the macro-porosity and micro-porosity of the bone samples over time, ESEM analysis was used. To investigate and monitor the changes in the organic content of the bones, the thermal analysis technique of TG-DTA was used. Py-GC-MS was used to identify the compounds that are present in bones and to observe changes in these compounds over time. Finally, XRD analysis was used to monitor the variation in the inorganic component of the bones.

3.2 Scanning Electron Microscopy

SEM has a central role in structural characterisation for material scientists due to its high magnification, high resolution and increased depth of field (Aoba *et al.*, 1978). Bombarding the surface of a material with a beam of electrons and detecting those that are emitted or backscattered allows microscopists to image samples at resolutions as low as 10 nanometres, providing intricate details of the material's structure. However, the requirements of SEM, such as a high vacuum and the need for a thin coating of electrically conducting material if an insulator is being analysed, mean that certain types of materials have proven difficult to image. The high vacuum requirements in the chamber suggest that lengthy specimen preparation techniques are required to remove the water before imaging. This raises the risk of artefacts being introduced (McDonald, 1998).

The problems of a high vacuum can be overcome with the use of an ESEM, which permits the imaging of wet systems with no prior specimen preparation. Because the sample environment can be dynamically altered, hydration and dehydration processes can be

followed as they occur in the sample chamber. The electrons emitted or backscattered from the surface of the sample can be detected by three types of detectors. The presence of secondary electrons is measured by both the secondary electron (SE) and the gaseous secondary electron (GSE) detectors. Detection of backscattered electrons is performed using a backscatter electron (BSE) detector. Using a thermal emission source or a field emission cathode, a SEM generates a beam of incident electrons in an electron column above the sample chamber. Elastic and inelastic scattering activities within the surface of the sample result in the incident electrons causing electrons to be emitted from the sample. Secondary electrons are lower-energy electrons resulting from inelastic scattering while backscattered electrons are high-energy electrons that are ejected by an elastic collision of an incident electron with a sample atom's nucleus. ESEM provides a technology for imaging hydrated or dehydrated biological samples, with minimum manipulation and without the necessity for conductive coatings (McDonald, 1998).

The ESEM instrument generally has 3 main modes of operation:

1) high resolution conventional SEM in high vacuum mode using SE or BSE with a resolution of 2nm at 30kV and magnifications of 10x to 500,000x.

2) low vacuum mode using GSE or BSE with a resolution of 3nm at 30kV at pressures of 0-1.5 Torr using gas (nitrogen or water vapour) in the microscope chamber for charge dissipation. One of the aims of using gas in the microscope chamber is to discharge any accumulated surface charge, thereby making this mode suitable for any solid including non-conducting and dry specimens. In addition, there is no need for coating specimens.

3) ESEM or "wet" mode using GSE with a resolution of 3nm at 30kV at pressures of 0-10 Torr and temperatures of ±20 °C from ambient (when a Peltier cooling stage is fitted) using water vapour in the microscope chamber. Adjusting the pressure

of the water vapour and the temperature of the cooling stage to saturation conditions (100% relative humidity) allows a wet sample to remain hydrated whilst inside the chamber. This is particularly useful for biological specimens (McDonald, 1998).

3.2.1 Experimental Details

In this study, ESEM was used to view the differences in surface morphology in the bone samples. The instrument used to perform the microscopy analysis was the tungsten filament electron microscope, Philips XL 30 Environmental Scanning Electron Microscope. This is a high resolution SEM allowing magnifications up to 500,000x on conventional coated/conducting samples, but it also has the capability for high resolution SEM of uncoated and even hydrated "wet" samples. This instrument's magnification in 'wet mode' ranges from 20X – 50,000X; the relative humidity range is 0-100%; and the temperature range is from -30 °C to 30 °C.

The 'fractured' bone samples were placed directly onto the mounting stage using double-sided conducting stickers. Eight samples were mounted on to the stage and analysed in one session. The ESEM was operated using wet mode with a low vacuum pressure of about 0.8-1 Torr. For viewing purposes, a working distance of 10-13 mm was used with a spot filter of 5.0 at an accelerating voltage of 20.0 kV. An accelerating voltage of 20.0 kV was employed as a compromise between excessive charging at higher accelerating voltages (up to 30.0 kV) and poor resolution at lower accelerating voltages.

Two detectors, GSE and BSE were used to acquire images at magnifications of 50X, 100X, 200X, 400X and 800X. The technique of scanning electron microscopy was chosen over optical microscopy as SEMs have a large depth of field and a high resolution while optical microscopes have a small depth of field and a low resolution. The micrographs (Figures 3-1 and 3-2) present the differences in images produced using the two detectors, GSE and BSE

used to record the images. At a magnification of 100X, the GSE detector produced clear images of *all* the surface features present in the bone samples, while the images produced using the BSE detector focussed mainly on the pores.

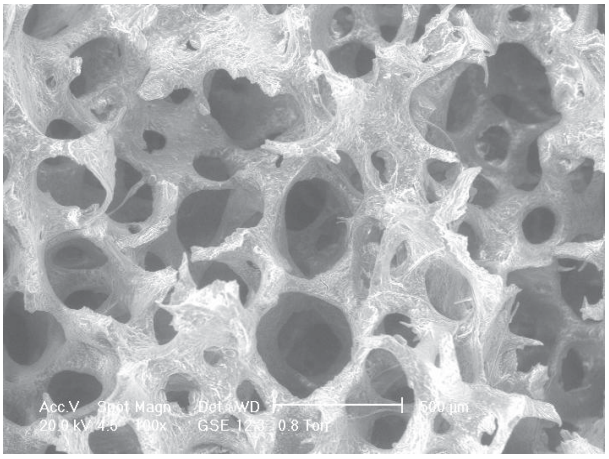

Figure 3-1. GSE detector image of a bone sample

At a magnification higher than 100X, it was observed that the depth of field and surface topography of the samples was more detailed using the BSE detector when compared to the GSE detector. For this reason, the BSE detector was used for ESEM analysis of the bone samples in the current study.

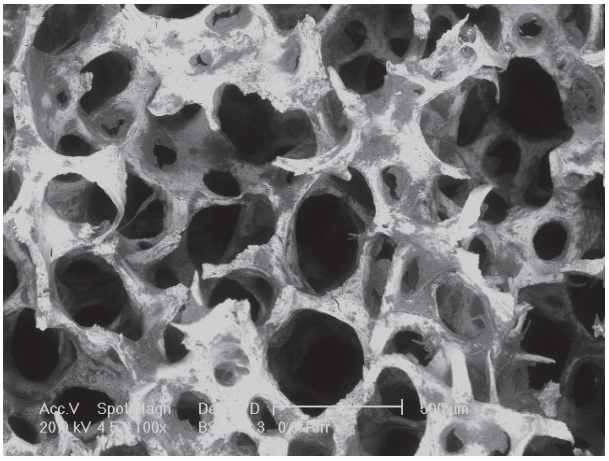

Figure 3-2. BSE detector image of a bone sample

The measurement feature of the microscope was used to determine the number of pores and the pore sizes of each bone sample (recorded on the images captured). Such measurements are useful in the analysis of the morphology of the samples, and therefore allow comparison between the samples. ESEM analysis was performed on all 24 samples at each post-burial time and the images were compared to observe if there were any visible changes in the porosity of the bone samples.

3.3 Thermal Analysis

Thermal methods of analysis are used in a range of scientific investigations such as polymers, pharmaceuticals and materials science. Thermal analysis is defined as a group of techniques in which a property of the sample is monitored as a function of temperature over a period of time. When the mass of a sample is monitored, the technique is known as thermogravimetric analysis (TG). The temperature of the sample is programmed in a specified atmosphere, either at a fixed temperature gradient or at a constant temperature, or a combination of both (Brown, 2001).

Thermal analysis techniques are not commonly employed in forensic investigations and, therefore, an attempt has been made in the present study to explore the use of these techniques. As bones contain both inorganic and organic components, there is potential to gain an insight into the post-mortem decomposition processes occurring using techniques that are sensitive to changes in such components. Thermal analysis techniques are sensitive to changes in organic compounds and can therefore be used to study the decomposition of the organic phase of bone.

In the present study, TG was employed to monitor the changes in the mass of bone samples recovered from various burial conditions and with post-burial times that would be useful in a forensic context.

3.3.1 Thermogravimetric Analysis (TG)

TG is a technique in which the mass of the sample is measured and monitored as a function of time or temperature while the temperature of the furnace at the sample crucible, in a specified atmosphere, is programmed (Hill, 1991) using a thermobalance (also known as a thermogravimetric analyser) (Haines, 1995). The thermobalance is used for weighing a sample continuously, while it is being heated or cooled. In order to enhance the steps in the thermogravimetric curve, the derivative thermogravimetric (DTG) trace is frequently produced. This produces the rate of change in mass with time, dm/dt, or rate of change in temperature with time, dT/dt.

As with most thermal analysis systems, the thermobalance has four major parts (Haines, 1995).

1. electrobalance and its controller;
2. furnace and temperature sensors;
3. programmer or computer; and
4. data acquisition device.

The balance used in many of the commercial apparati is a modified electronic microbalance. The balance operates on a null-balance principle, in which the amount of current applied to return the balance to the null position is proportional to the weight loss or gain.

The furnace used is generally comprised of non-inductively wound electrical resistance heaters. The furnace temperature is measured by a thermocouple. This temperature is compared with the temperature set in the programme by the operator. The sample temperature is measured by a thermocouple placed close to, but not in contact with, the sample. Platinum/Platinum-Rhodium (Type R) thermocouples are used and since the thermocouples are in close proximity to the sample, they are able to monitor any changes in the sample and any products formed. The high ΔT sensitivity of these thermocouples (0.001 °C) enables the detection of minor endothermic and exothermic changes when compared to a reference (Haines, 1995).

TG is most frequently performed on solids. The requisite for the samples is that they should ideally be small, powdered and spread evenly in the crucible (Haines, 1995). Liu *et al.* (2004) demonstrated the effect of sampling conditions on the decomposition of electrolytic manganese dioxide using TG, demonstrating that both sample size and environment type (open pan vs closed pan) affected the decomposition mechanism. Therefore, it is important to maintain the sample conditions to obtain reproducible and reliable results.

It is important to calibrate the thermobalance in conditions that reproduce those conditions used experimentally. The temperature measured by a thermocouple, which is not in contact with the sample, may be subject to a thermal lag of several degrees. The lag with vary with temperature unless the experiment is carried out isothermally on a very small sample. In addition, this lag will vary with the reactions proceeding, with the furnace atmosphere, the heating rate and the geometry of the system (Haines, 1995).

The need to surround the sample in an inert or a reactive atmosphere and to control the evolution of gases from the sample generally means that TG is conducted in a flowing gas stream. Flow rate can disturb the balance mechanism and, therefore, a constant flow rate of

about 10-30 cm^3/min is often used. The flow of gas can also contribute to the transfer of heat and assist the transfer of products to any external gas analysis system.

TG can also be coupled with mass spectrometry (TG-MS). During a TG experiment, decomposition results in the volatilisation of products into the flowing purge gas. The evolved gases are sampled by and analysed using a mass spectrometer and this is known as an evolved gas analysis (EGA). Mass spectrometry is a technique used to study the masses of atoms, molecules or fragments of molecules. To obtain a mass spectrum, volatilised gaseous molecules are ionized and these ions are accelerated by an electric field and then separated according to their mass-to-charge ratio, m/z. Ionization involves the loss of one electron, leaving the resulting cation or molecular ion, M^+, with one unpaired electron, from which the formula mass may be determined. The molecular ion of the evolved gases is recorded over the same temperature gradient as the TG. At the same time, the molecular ion is fragmented into smaller mass ions (m/z values), which are characteristic of certain molecules evolving. This instrument can be programmed to select particular ions, which are then monitored throughout each run (Harris, 2003).

Differential thermal analysis (DTA) is a technique that monitors the difference in temperature between the sample and a reference material against time or temperature while the temperature of the sample, in a specified atmosphere, is programmed. The DTA curve is generally a plot of the difference in temperature (ΔT) as the ordinate against the temperature (T), or occasionally time (t), as the abscissa (Haines, 1995).

3.3.1.1 Experimental Details

TG was performed using the TA Instruments SDT 2960. The temperature range of this instrument is ambient to 1500 °C, the heating rate can vary from 0.1 to 150 °C/min and the sample mass range that can be analysed is 1 - 100 mg. It has an ability to detect and measure

minute mass changes as low as 0.1 µg. The instrument is calibrated using temperature calibration standards including indium, tin, lead, zinc, aluminium, silver and gold.

The TA Instruments SDT 2960 can be used to perform both TG and differential thermal analysis (DTA) at the same time. The amount and rate of weight change in a sample is monitored in a controlled atmosphere and is recorded either as a function of increasing temperature, or isothermally, as a function of time. DTA can be used to characterize material as well as detect phase changes due to decomposition, oxidation or dehydration. This aids in the identification and assessment of the percent weight change and correlation of chemical structure (Haines, 1995).

The manually-ground bone samples (2 – 3 mg) prepared using the method outlined in Section 2.3.1.4 were placed in a platinum crucible and a second empty platinum crucible was used as the reference. Similarly, for the analysis of the bone samples prepared using the four different sample preparation procedures in order to establish a standard sample preparation procedure, 3 – 5 mg of the bone samples were analysed. The balance was tared prior to the addition of each sample to correct for any potential variations that can occur in mass measurements. Mass of each bone sample was recorded, after the sample was placed in the crucible. Analysis was carried out on each sample in an air atmosphere at a flow rate of 20 mL/min. The bone samples were heated from ambient temperature up to 1000 °C at a heating rate of 10 °C/min.

3.4 Pyrolysis Gas Chromatography-Mass Spectrometry

Pyrolysis is the thermal fragmentation of a substance in an inert atmosphere. The pyrolytic process results in molecular fragments characteristic of the composition of the original macromolecular material. Pyrolysis generally occurs at high temperatures (> 400 °C) and

under pressure. It is carried out in an inert environment in order to promote the thermal fragmentation of the molecular species present while minimizing oxidative processes which predominantly yield carbon dioxide and water as products. In reality, it is practically impossible to achieve a completely oxygen-free atmosphere as there is always a small amount of oxygen present in any pyrolysis system. This means that as part of the pyrolytic process, a small amount of oxidation also occurs (Challinor, 1989).

Since the early 1970s, forensic scientists have characterized a range of crime scene evidence using analytical pyrolysis processes. The pyrolysis products may be detected and identified by coupling the pyrolysis unit to a gas chromatograph or a mass spectrometer or a combination of both instruments as in Py-GC-MS. With the pyrolyser connected to the GC-MS, analysis of trace amounts of sample is possible as the GC-MS enables high-sensitivity detection. The data collected by Py-GC-MS enables the identification and comparison of analytes using library searches of peaks present in the chromatograms (Challinor, 2001). Py-GC-MS is a relatively fast and cost-effective analytical technique; however, data analysis using Py-GC-MS results can be complex and time-consuming. Generally, Py-GC-MS provides efficient separation, involves minimal sample manipulation and has the ability to detect very small quantities of samples of interest (May *et al.*, 1977).

Despite these advantages, Py-GC-MS is a technique which is often under-utilised for examining biological materials including bone. Initially, poor reproducibility of the pyrolysis method for such materials was a major reason for the limited use of this technique; however, research and experience have shown that this is not the case, when modern instrumentation and correct analytical techniques are employed (Challinor, 2001). The variables in the pyrolysis process are temperature-rise-time, pyrolysis temperature, sample mass, the dimensions of the pyrolysis chamber, carrier gas type and flow rate. These factors influence

the primary and secondary pyrolysis products and the introduction of catalytic effects (May *et al.*, 1977).

Py-GC-MS techniques have progressed considerably in terms of requiring little to no sample preparation and sample loading. There has also been a move away from packed chromatography columns to the more versatile silica capillary columns. Silica capillary columns give improved results in terms of better peak shape and the efficiency of separation of the compounds. These columns are also able to separate a wider range of compounds of greater polarity (Challinor, 1989).

Py-GC-MS differs from conventional GC-MS in two main aspects: the type of sample being analysed and the method by which the sample is introduced to the GC-MS. In Py-GC-MS, a few micrograms of a sample can be analysed directly once it has been prepared appropriately and reproducibly otherwise variability is introduced. There is no need to use lengthy sample preparation procedures to make a solution that is suitable for injection. Before analysis, the sample can be extracted with an organic solvent to remove any free, unbound components that would interfere with the analytical data recorded. Samples are then released into a quartz chamber in a pyrolysis unit, which is at a pre-set temperature (up to 800 °C) and which consists of an oxygen free environment. This can also be followed by the use of a temperature gradient, in which the temperature is changing at a constant rate. When the sample is heated, the cleavage of chemical bonds within the macromolecular structures of interest produces a set of low molecular weight chemical components. The composition of such components is indicative of specific types of macromolecules present. This mixture of compounds is then swept onto the analytical column of the GC and the GC-MS analysis proceeds. The compounds are separated using the column in the GC and analysed using the mass spectrometer based on their mass-to-charge ratio.

In this study, the technique of Py-GC-MS has been used to analyse bone samples buried in the different environments and recovered after various time periods. This was performed to identify and monitor the progress of the decomposition reactions with burial time based on the analysis of pyrolysis fragments. The fresh bone samples stored in the freezer were also analysed to determine the effect of storing bone samples in the freezer in terms of preserving the integrity of the samples.

3.4.1 Experimental Details

The instrument used to perform the Py-GC-MS was a pyrolyser (PYR-4A) linked to the Shimadzu GC-17A, which was connected to the Shimadzu GCMS-QP5050A. This instrument has high sensitivity and can achieve a high vacuum in less than 5 minutes due to a 150 L/sec turbomolecular pump and rotary pump, which allows for high throughput. Using Advanced Flow Control (AFC), the Shimadzu GC-17A is able to produce fast and reproducible results.

The column used was a J & W Scientific DB-5MS column, 30 m in length with an internal diameter of 0.25 mm. It has previously been demonstrated that this column can be used for successful separation of the constituents of bone tar (Purevsuren *et al.*, 2004a). The temperature range of the column was -60 to 350 °C. The column pressure used was 100 kPa and the temperature of the heaters above the furnace was preset to 250 °C. The pyrolysis furnace (Shimadzu Furnace Pyrolyser-4a Ver.2) was mounted on the split injector of the GC-MS and its temperature was preset manually to 450 °C. An explanation of why this temperature was chosen is presented in Section 4.2.5.1.

A few micrograms of the hand-powdered bone samples were placed in a platinum bucket using a magnifying glass and high precision grade tweezers. The platinum bucket was cleaned between each sample by heating it using a Bunsen burner. Once the bone samples had been

introduced to the pyrolysis furnace, transfer of the pyrolysis products to the GC column was assumed to be instantaneous. Separation of these products was carried out in the GC oven, where they were heated from 40 °C to 300 °C at a temperature gradient of 10 °C/min before travelling to the mass spectrometer. Each run took 35 minutes. Prior to analysis, the GC oven was cleaned by heating the instrument to 310 °C for 10 min to remove any contaminants remaining on the column. Blank samples were also run at the beginning of each session to check for the presence of contaminants on the column. Auto-tuning was also performed prior to analysis to calibrate the mass spectrometer. The reproducibility of the results was investigated by comparing the results of two different bones buried in the same burial conditions. Analysis of the pyrograms was performed using the Shimadzu Class-5000 software and the peaks in the final pyrograms were compared to the NIST62 library database in order to determine the compounds each peak represented.

To identify trends that existed between the pyrograms of bones with different post-burial times, statistical analysis was performed. Using the 'Statistical Package for the Social Sciences' (SPSS) software, areas of the pyrograms that could be considered to be significantly changing with post-burial time were identified. Linear regression analysis in the form of partial least squares (PLS) analysis using Infometrix Pirouette 4.0 software was employed after baseline correction using a linear fit model was performed.

3.4.1.1 Background to Statistical Analysis

In Py-GC-MS analysis, large quantities of multivariate data were generated and therefore, a suitable method of examining and evaluating this data needed to be applied.

Statistical Analysis using SPSS Software

To identify trends that existed between the pyrograms of bones with different post-burial times, statistical analysis using SPSS software was performed. SPSS software enables researchers to uncover patterns and associations from all types of data. Using the linear regression analysis feature in the software (Munoz *et al.*, 2001; Madea and Rodig, 2006; Singh *et al.*, 2006), the retention times and absolute intensities of all the bone samples buried in the different environments were analysed and, hence, significant areas of the pyrograms were identified. The Pearson correlation coefficient was calculated and t-tests were performed on the data. The Pearson correlation coefficient is a measure of the strength of the association between two variables and the t-test is used to establish if the correlation coefficient is significantly different from zero, and, hence that there is evidence of an association between the two variables. All the retention times resulting in a Pearson correlation coefficient between -0.5 and -1 or between 0.5 and 1 and with a t-test significance value less than 0.05 are considered to be significant from a statistical perspective, and were therefore selected for further statistical analysis.

Statistical Analysis using Pirouette Software – Pre-processing Data

Pre-processing is a column-oriented operation and, hence, results of this operation are specific to a set of samples. Many multivariate algorithms used in statistical analysis compute results based on variance patterns in the independent variables. This is why the process of pre-processing is necessary for most sets of results (Gemperline, 2006).

Mean-centering pre-processing is recommended for most data types as it simply shifts the origin, but does so without altering relative inter-sample relationships. This is important when attempting to identify trends between samples. It is easier to visualise relationships between samples by placing the origin of the plot at the center of the data set. Therefore, data points are often centered around the mean (Gemperline, 2006).

A mean is computed for each variable via the following formula:

$\bar{x}_j = \frac{1}{n}\sum_i^n x_{ij}$ (Gemperline, 2006)

The mean is then subtracted from each data value to produce a mean-centered matrix:

$x_{ij(mc)} = x_{ij} - \bar{x}_j$ (Gemperline, 2006)

Figure 3-3 shows the effect of mean-centering pre-processing on a set of data.

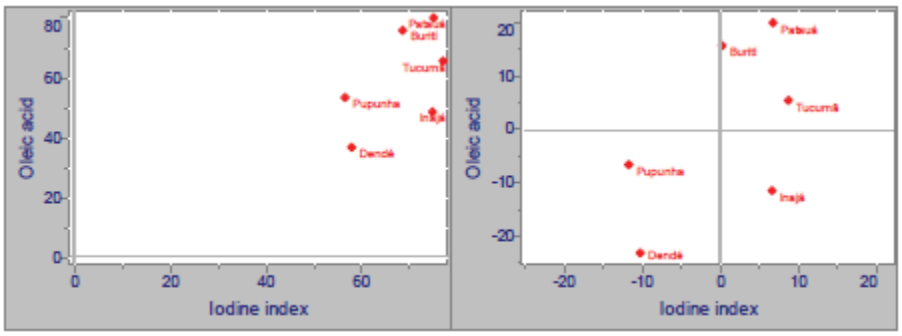

Figure 3-3. Graph of raw data (left) and mean-centered data (right) (Gemperline, 2006)

Statistical Analysis using Pirouette Software – Transformation of Data

Data contained in pyrograms require transformation prior to analysis to lessen the effects of random variations such as noise. Using Infometrix Pirouette 4.0 software, there are many possible transformations that can be performed including baseline correction, 1st and 2nd derivatives, align, log 10 and smooth (Gemperline, 2006). The two transformation methods of baseline correction and derivatives are most useful in analysis as they aid in reducing background noise as well as highlighting and separating peaks of narrow features.

Baseline Correction Transform corrects offsets by subtracting a profile, which can be a set of data or derived from a curve fit. Pirouette includes linear, quadratic and cubic curve fits,

which are based on first, second and third degree polynomials, respectively. These baseline correction curves are presented in Figure 3-4 (Gemperline, 2006).

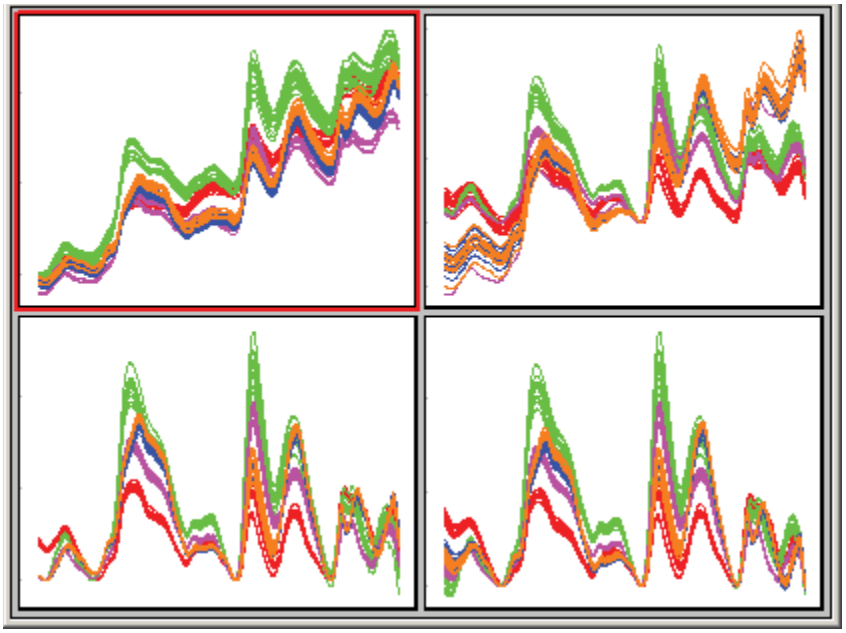

Figure 3-4. Baseline correction transform (clockwise starting from top left) - raw data, linear fit, quadratic fit, cubic fit (Gemperline, 2006)

Derivative Transforms (1st and 2nd) are based on a Savitzky-Golay polynomial filter (Savitzky and Golay, 1964), in which a convolution is applied to independent variables, with each consecutive derivative reducing the signal magnitude. The effects of this method of transform are shown in Figure 3-5 (Gemperline, 2006).

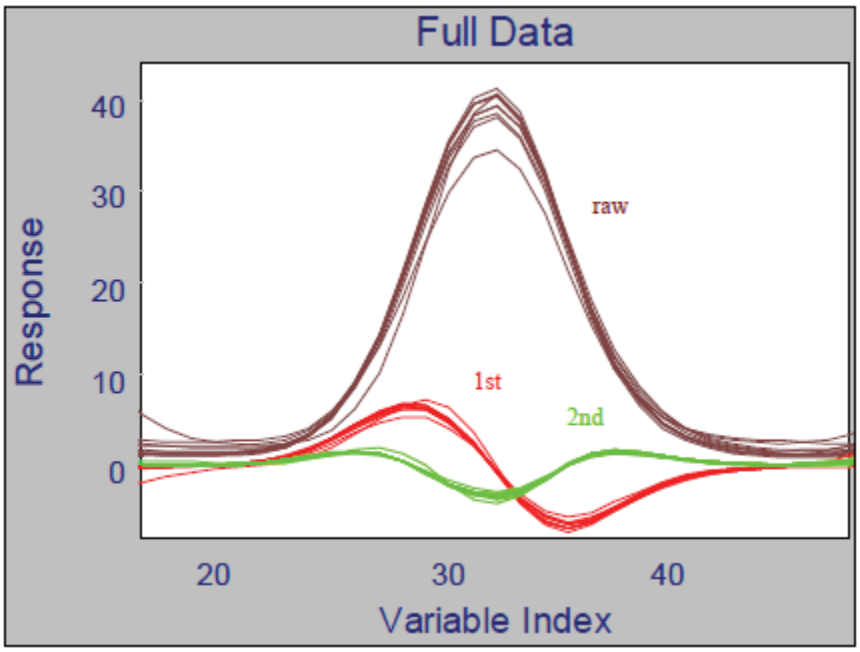

Figure 3-5. 1st and 2nd derivative transform applied to raw data (Gemperline, 2006)

Statistical Analysis using Pirouette Software – Exploratory Analysis

The aim of exploratory analysis is to reduce significant amounts of complex data sets to the point that any correlation that exists between samples and/or independent variables can be easily identified. Principal Component Analysis (PCA) is a type of exploratory analysis commonly applied (Pozo *et al.*, 2012). It is a method of graphically representing inter-sample and inter-variable relationships as well as reducing the dimensionality of complex data. PCA provides information on combinations of independent variables which account for the maximum amount of variation between data. Using PCA, it is possible to recognise the presence of natural clustering in the data, outlier samples and noise. A PCA model that is created can be used as a point of reference for future samples (Gemperline, 2006).

Statistical Analysis using Pirouette Software – Regression Methods

The ultimate goal of multivariate analyses is to develop a model that is able to predict a characteristic and the characteristic can be either categorical or continuous. Regression

methods are used to model and predict continuous properties. A relationship between a quantitative sample property, the dependent variable, and one or more independent variables can be established by regression methods. Multivariate methods offer improved precision, more sophisticated outlier detection and in the case of factor based algorithms, the possibility of compensating for interferences (Gemperline, 2006).

The statistics software, Infometrix Pirouette 4.0, employed in this study, can be used to apply two different multivariate factor-based regression methods – principal component regression (PCR) and PLS regression. Both these types of regression analysis result in a lower dimension representation of the independent variable block. The results are often similar, however, PLS may generate a model using one less factor than PCR and the process of PLS regression analysis normally does not take as long as PCR using Pirouette software. Therefore, PLS is usually the preferred choice of regression analysis (Gemperline, 2006).

PLS regression includes many of the features of PCA and PCR analysis, including finding relationships between samples and variables based on factors, similar to PCA's principal components. These factors, however, correlate X and Y block data, and therefore result in more reliable and economical models than PCR (Gemperline, 2006).

3.4.1.2 Application of Statistical Analysis

After comparisons of various methods of transformation of data as well as the two methods of linear regression analysis mentioned earlier, the most appropriate and useful method of statistical analysis was chosen. Linear regression analysis in the form of PLS analysis using Infometrix Pirouette 4.0 software was employed, after baseline correction using a linear fit model was performed.

Pre-processing Data

The data selected based on correlation coefficients and significance values calculated using SPSS software was subjected to mean-center pre-processing. This method of pre-processing data is recommended for most data types when using SPSS software as it includes an adjustable intercept in multivariate models (Gemperline, 2006).

Transformation of Data – 1st derivative vs linear baseline correction vs cubic baseline correction

Once pre-processed, the data were corrected for baseline variation using three different methods. The results were compared to determine the best method of transformation to be performed on bone pyrolysis data, without compromising the integrity of the data. The methods of 1st derivative, linear and cubic baseline corrections were used. The results are shown in Figures 3-6, 3-7 and 3-8 respectively. These graphs present comparison between the actual (Measured Y) and the predicted (Pred Cal) post-burial time values. Based on the results of all the samples, the linear baseline correction method was chosen. This was because it resulted in the most accurate correlations between the true post-burial time of the bone samples and the post-burial time of the samples predicted by the statistical software. Therefore, for all the bone samples, the data was transformed using linear baseline correction prior to any regression analysis being performed.

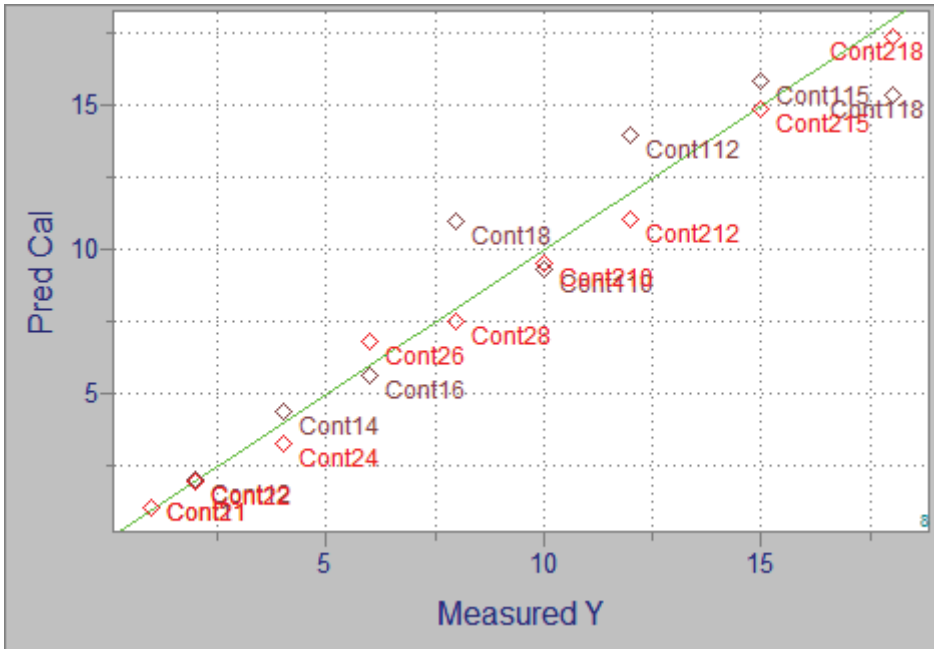

Figure 3-6. PLS regression analysis of the loam defleshed (control) samples using 1st derivative baseline correction

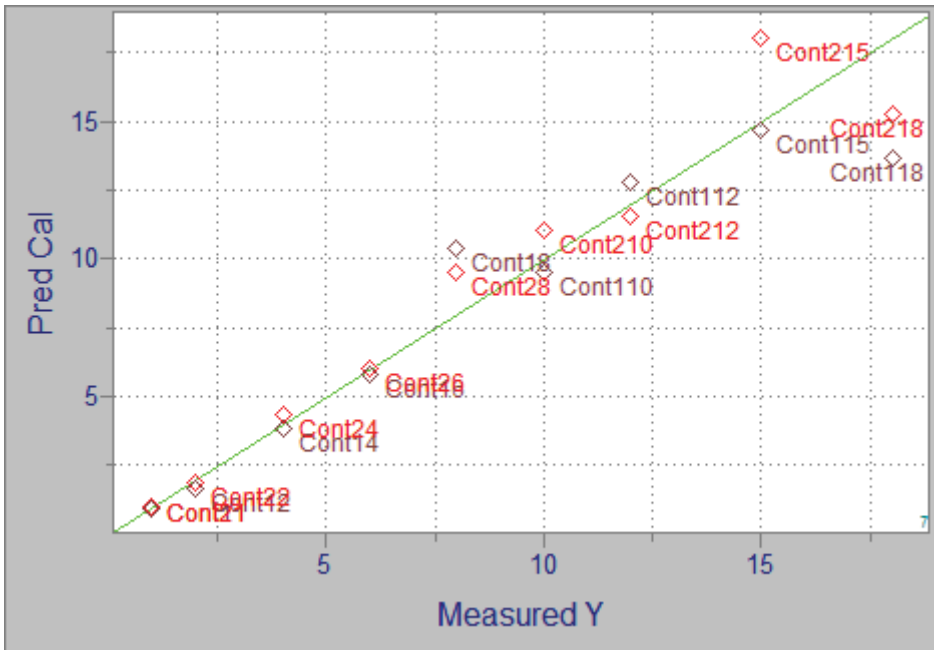

Figure 3-7. PLS regression analysis of the loam defleshed (control) samples using linear baseline correction

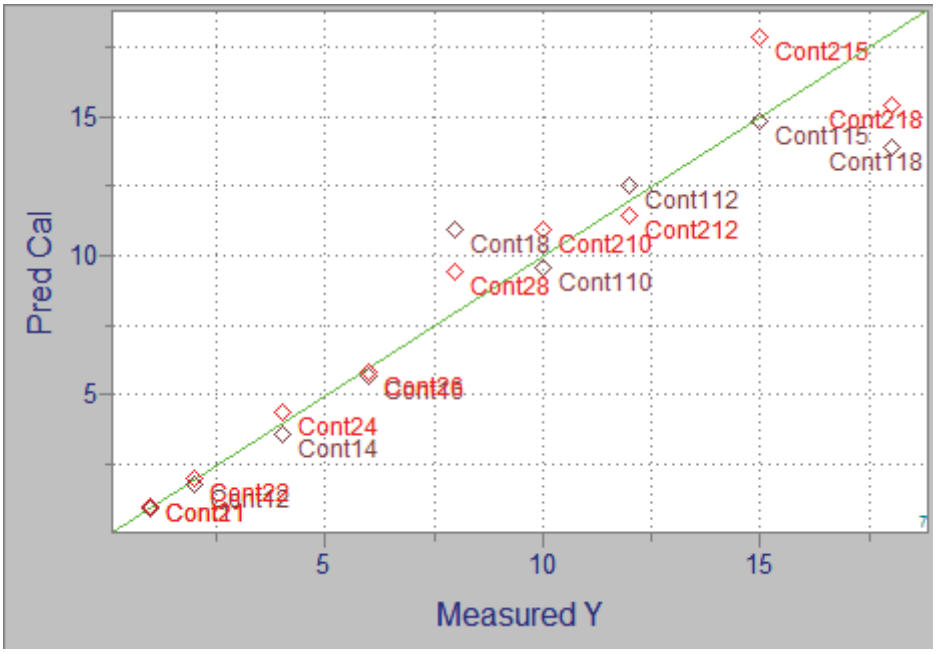

Figure 3-8. PLS regression analysis of the loam defleshed (control) samples using cubic baseline correction

PCR vs PLS

Linear regression analysis was used to analyse the retention times, absolute intensities and post-burial times of all the bone samples buried in the different environments. Using the loam defleshed samples, PCR (Figure 3-9) and PLS (Figure 3-10) regression analysis were compared to determine the more suitable method of statistical analysis for the comparison of bone data. The figures show the correlation between the actual time since burial of bones (Measured Y) and the predicted post-burial time of bones calculated by the Pirouette software (Pred Cal).

PLS regression analysis was a more accurate predictor of the post-burial time of bones based on the differences in the absolute intensity values, when compared to PCR analysis, and was therefore selected for all further analysis. PLS is a faster method of analysis compared to PCR and results in a smaller number of factors upon which the prediction of the post-burial time of the bones is based. Therefore, for all the bone samples, PLS regression analysis was performed in conjunction with linear baseline correction. The accuracy of the predicted post-burial time compared to the true post-burial time was calculated using the standard deviation as a percentage of true post-burial time.

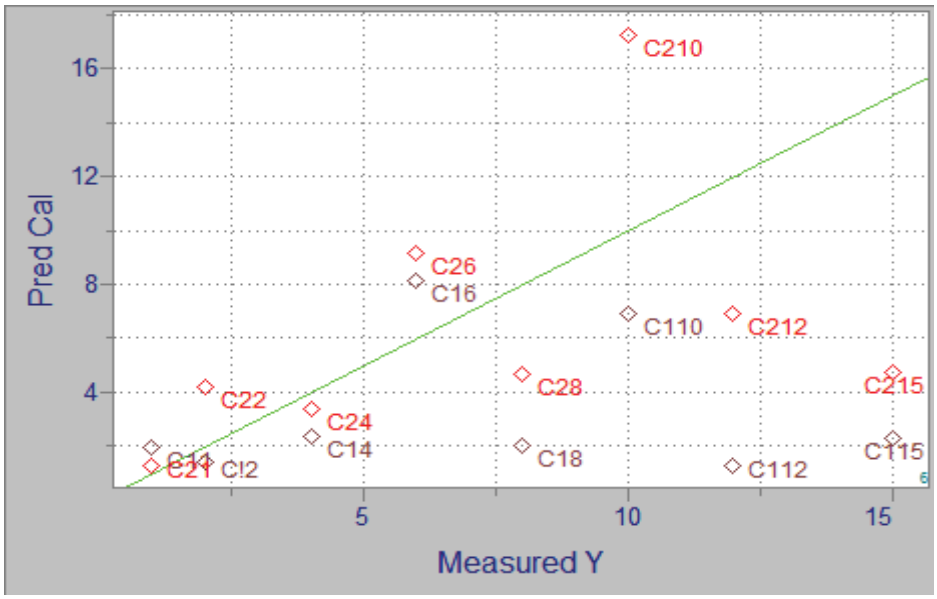

Figure 3-9. PCR analysis of the loam defleshed (control) samples

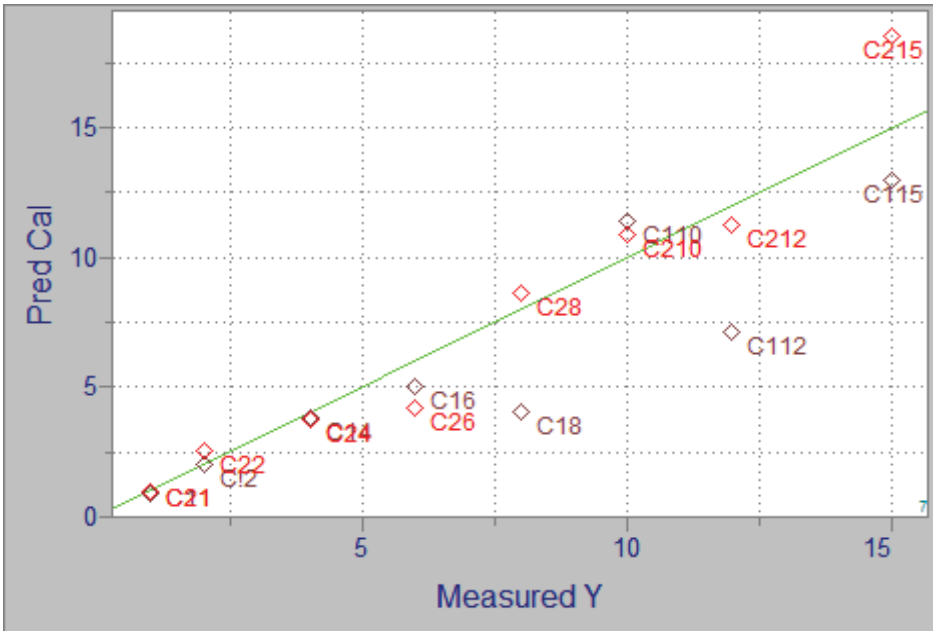

Figure 3-10. PLS regression analysis of the loam defleshed (control) samples

3.5 X-ray Diffraction

XRD is typically applied to crystalline phase analysis in heterogeneous samples and to the identification of phases present (Jenkins and Snyder, 1996). It is a commonly used technique in chemistry and materials science to characterise crystal structure because sample preparation is relatively easy. A disadvantage of this technique is that analysis times can be long compared to other techniques (hours compared to minutes). It can be used to perform both qualitative and quantitative phase analysis as well as crystal structure analysis. Changes in the positions of the diffraction peaks or the peak widths provide information on the crystal size, purity and texture of the samples being analysed (Santiago Rivera, 2006).

In XRD, the pattern produced by the diffraction of X-rays through the closely spaced lattice of atoms in a crystal is recorded (Santiago Rivera, 2006). All crystals are made up of repeating planes of atoms that form a lattice. X-rays, when focussed on a crystal, excite the electrons in

each atom of the crystal. The excited electrons vibrate with the same frequency as that of the incoming radiation and therefore become secondary sources of X-rays. The electrons transfer the radiation in all directions at the same frequency as the incoming radiation. This is known as coherent scattering. These secondary X-rays are also known as diffracted X-rays and form interference patterns, which can be classified as constructive (addition of waves) or destructive (cancelling out of waves). The interference pattern reveals valuable information about the structure of the crystal, as it is dependent on the chemical composition, the distance between the atomic layers and the angle that the X-rays diffract away from the atoms (Bergslien *et al.*, 2008).

The diffraction pattern created by constructive interference is recorded through the use of a beam detector, which measures the intensity of the diffracted light as the X-ray tube and the detector are rotated around the sample.

Bragg's law, $n\lambda = 2d \sin\theta$, outlines the relationship between the angle at which diffraction peaks occur (θ), usually measured as degrees two theta ($°2\theta$) experimentally, and the spacing between the atoms of a crystalline lattice (d-spacing). X-ray diffraction patterns are normally expressed using $°2\theta$ along the x-xis and counts or counts per second along the y-axis (Bergslien *et al.*, 2008).

Each crystalline structure and the diffraction pattern created by it is unique and hence crystalline samples of unknown composition can be identified by comparing the positions and intensities of the diffraction peaks against a library of known crystalline materials. These diffraction peaks broaden when disorder occurs in the crystalline structure, which is useful in identifying changes occurring in samples (Bergslien *et al.*, 2008).

3.5.1 Experimental Details

XRD analysis was carried out on the bone samples recovered from burial. Firstly, XRD analysis was performed on fresh bone samples (same as bone samples in Section 2.3.1.3) to determine the best instrument and method parameters. Results of the preliminary analysis of the fresh bone samples showed that it was necessary to scan more slowly between certain °2θ regions to ensure diffraction peaks are not overlooked as some substances do not diffract as strongly as others. Table 3-1 shows how the 5-90 °2θ range was analysed.

Table 3-1. XRD scan range

°2θ Range	Scan Rate
5 – 13.7	Fast scan (3 s / 0.02 °2θ)
13.7 – 30	Slow scan (13 s / 0.02 °2θ)
30 – 43	Fast scan (3 s / 0.02 °2θ)
43 – 56.3	Slow scan (13 s / 0.02 °2θ)
56.3 – 60.9	Fast scan (3 s / 0.02 °2θ)
60.9 – 66	Slow scan (13 s / 0.02 °2θ)
66 – 70	Fast scan (3 s / 0.02 °2θ)
70 – 79.1	Slow scan (13 s / 0.02 °2θ)
79.1 – 90	Fast scan (3 s / 0.02 °2θ)

Analysis of each powdered sample took approximately 9 hours. The samples analysed at each post-burial time using XRD were Loam Defleshed (Control) 1 and 2, LF1, LA1, LB1, LW1, LD1, S1, SA1, C1, LDG1, LBO1, LR1, Fresh 1 and 2. Samples Fresh 1 and 2 were analysed at each post-burial time to monitor the effects of storing the samples in the freezer prior to analysis. The reproducibility of the results was investigated by comparing the results of samples that

were subjected to the same burial environments – Loam Defleshed (Control) 1 and 2 and Fresh 1 and 2 bone samples.

The Siemens D5000 X-ray diffractometer was used for the analysis of the inorganic content of bones. XRD is a technique used for characterisation purposes and involves examination of crystal structures as well as the qualitative and quantitative phase identification and analysis. The Siemens D5000 diffractometer allows for automatic analysis of up to 40 samples. The Siemens D5000 diffractometer has a large diameter goniometer (600 mm), low divergence collimator, and Soller slits. This instrument is useful for both powder and bulk materials. Data collection is performed under computer control using the "Diffrac Plus" measurement software and "EVA" evaluation software. Qualitative phase identification was performed by using search/match software and a CD-ROM archive of the International Centre for Diffraction Data - Joint Committee of Powder Diffraction (ICDD-JCPD) tables (Santiago Rivera, 2006). For samples containing mixed materials, the detection limit of this instrument is approximately 2% (Santiago Rivera, 2006).

The sample holders used for the analysis consisted of a 2 mm thick plastic plate with a 20 mm circular well in the centre, with a raised circular edge acting as a wall to hold the powdered sample in place. Samples analysed using XRD needed to be finely powdered and homogeneous for accurate identification – particle sizes of less than 50 microns were required. If the particle size is too big, it leads to crystal statistics effects, which cause incorrectly measured intensities. These effects can also influence the shape of the peaks, making them jagged which affects peak intensities. Similarly, if the particle size is too small, this can cause damage to the crystal structure or alteration of phases, making it difficult to identify or quantify the actual phases present in the sample. Achieving a fine powder is also important to avoid preferred orientation effects. Once finely powdered using a ring mill,

approximately 20 g of each sample was compacted into the circular sections of the sample holders.

All of the plastic sample holders containing the samples were then loaded directly into the horizontal stage and analysed using the theta-theta Siemens D5000 diffractometer equipped with a Cu-tube. The system has a Kristallo-Flex 710D X-ray generator using monochromatic Cu $K\alpha_1$ radiation (λ = 1.5406 Å) selected with a graphite incident-beam monochromator. Each sample was analysed at 40 kV and 30 mA from 5 2θ to 90 2θ in scanning mode with a step size of 0.02° and 3 seconds (fast scan regions) or 13 seconds (slow scan regions) counting time per step using the Bragg–Brentano geometry. Qualitative phase identification was performed using "Diffrac Plus" software and the ICDD-JCPD database (Santiago Rivera, 2006).

Chapter 4

The Effect of Pre-Treatment Procedures on Bone Structure

4.1 Introduction

The aim of the present study was to establish a method that can ultimately enable the estimation of the length of time for which a bone has been buried. To achieve this, it was critical to observe the decomposition of bones once they were buried. Observing the changes that occur in bone during bone diagenesis involves understanding factors that may influence the processes involved. In bones, these factors include the presence of flesh and surface lipids and the presence/absence of both of these was investigated in the study. The boiling of bones is also considered to mirror the processes that occur in bone diagenesis (Roberts *et al.*, 2002) and, therefore, a burial environment created by boiling bones prior to burial reproduces accelerated bone decomposition. In the present study, bones were subjected to the different pre-treatments and visual observations were recorded. They were also examined using ESEM, TG, Py-GC-MS and XRD analysis to determine if these pre-treatments have an effect on bone diagenesis.

4.2 Defleshed Bones

4.2.1 Visual Observations

In this study, bones were buried in various burial conditions and subjected to different pre-treatments. Upon recovery of each bone, measurements including length, diameter and mass were recorded. Physical descriptions including peeling, cracking, colour and the presence of any odour were recorded for each bone at each post-burial time. The physical characteristics of the defleshed bone samples are reported in Table 4-1.

Table 4-1. Physical characteristics of the defleshed bone samples analysed at each post-burial time (n = 18)

Bone Pre-treatment	Average Length (cm)	Standard Deviation (±)	Average Diameter (cm)	Standard Deviation (±)	Average Mass (g)	Standard Deviation (±)
Defleshed (Control)	8.2	0.49	1.5	0.46	6.5	1.9

For all the defleshed (control) samples, which were buried in a loam soil environment, visual comparison demonstrated that the colour did not change from the initial light brown colour. Tiny cracks and holes started appearing after 2 months of burial and were evident in all the samples up to and including 18 months of burial. No odour was present in any of the bone samples. In terms of microbial activity, white fungus was present on only one of the Loam Defleshed 1 (Control 1) samples (8-months). By contrast, white fungus was present on all the Loam Defleshed 2 (Control 2) samples with post-burial times of 6 months and greater. After 4 months of burial, a layer of white, jelly-like mucus covered the entire surface of soil in the Loam Defleshed 2 (Control 2) samples and could be the result of a greater extent of microbial activity in the Loam Defleshed 2 (Control 2) samples. Also, for both sets of defleshed samples, cockroaches were present in the containers after and including 15 months of burial. Since the containers were tightly sealed, the only explanation could be that cockroach eggs were initially present in the soil.

Once the bones were recovered (after 1 month), sectioned and dried, photographs were taken of the samples. These photographs were examined and compared to determine if it was possible to differentiate between the different conditions using the naked eye. Differences in physical characteristics such as colour were recorded and a summary of the descriptions of the bones that can be used to identify the burial conditions of a bone sample and hence, differentiate it from other bone samples, are presented.

4.2.2 Defleshed (buried) and Defleshed (not buried) Bones

Defleshed bones that have been buried and not buried can be easily differentiated as visible colour differences exist between the bones. As evident in Figure 4-1, bones that were not buried are predominantly white and red in colour, while defleshed bones buried in loam soil and recovered after 1 month are predominantly brown in colour. Similarly, defleshed bones recovered after 2 months of burial and up to 18 months of burial, had a similar appearance. Therefore, it can easily be determined if a bone has been buried for a month or longer.

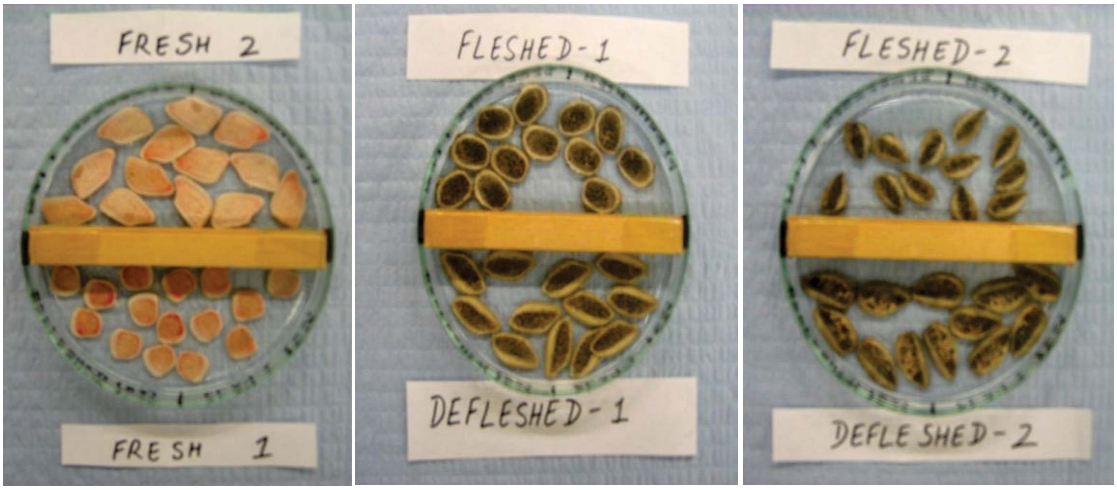

Figure 4-1. Photographs of fresh, fleshed and defleshed bone samples

4.2.3 Environmental Scanning Electron Microscopy

A bone's interaction with its burial environment and the changes that occur in the bone as a result, can be monitored via changes in the organic and inorganic content of bones. This is performed using the techniques of TG, Py-GC-MS and XRD analyses. To complement these techniques, the technique of ESEM was employed to observe any changes in the morphology of the bone samples being investigated. This refers to the presence of striations, vacuoles and pores. It also refers to the condition of the bone's surface, in terms of being rough or smooth.

The technique of ESEM was used in conjunction with the BSE detector to analyse the longitudinally fractured bone samples recovered from the various burial conditions at the different post-burial times.

4.2.3.1 Reproducibility

To test the reproducibility of this technique, two sets of bones buried in the same conditions were imaged at each post-burial time and then compared. The images of the defleshed samples produced using ESEM analysis and a BSE detector at the various post-burial times are presented in Figures 4-2 to 4-11. A total of six images were taken for each bone sample and the images presented in Figures 4-2 to 4-11 are representative of those images. Both defleshed samples, 1 and 2, produced relatively similar images at each of the post-burial times investigated, in terms of the surface type and the presence of pores and striations. Generally, the results of this technique can be considered to show good reproducibility.

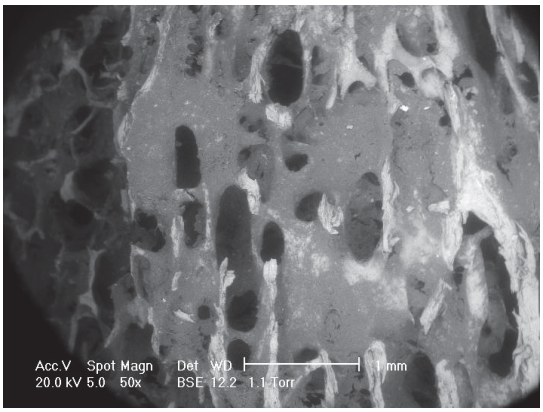

Figure 4-2. Loam Defleshed 1 - 1 month

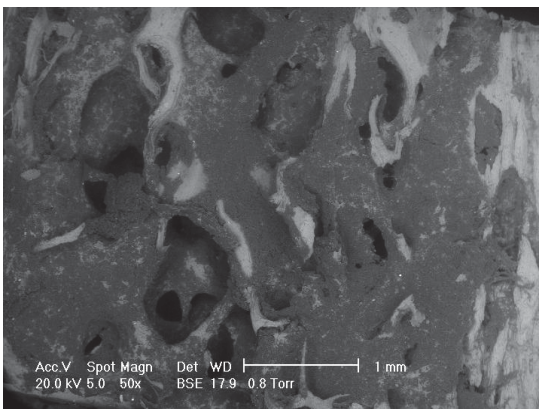

Figure 4-3. Loam Defleshed 2 - 1 month

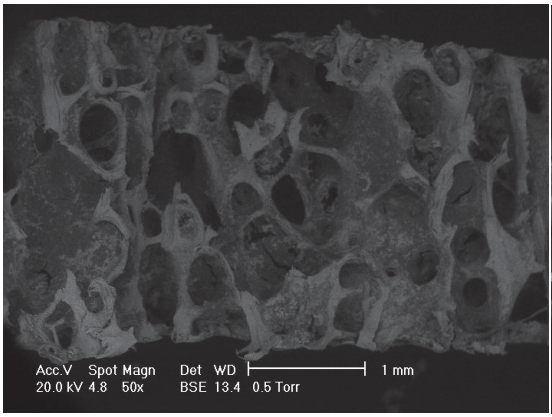

Figure 4-4. Loam Defleshed 1 - 2 months

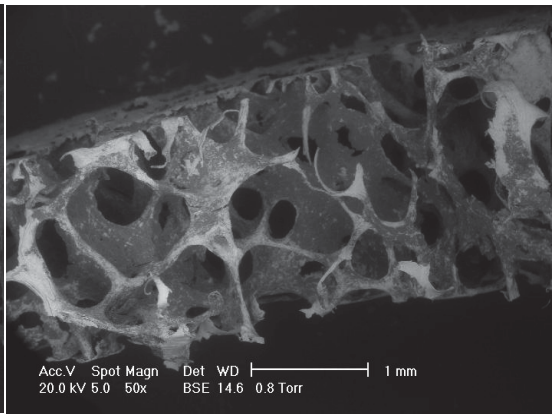

Figure 4-5. Loam Defleshed 2 - 2 months

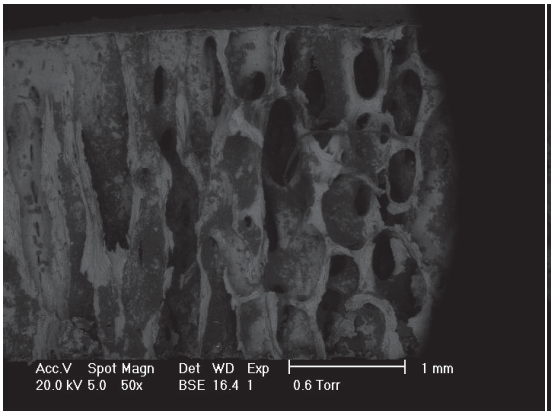

Figure 4-6. Loam Defleshed 1 - 6 months

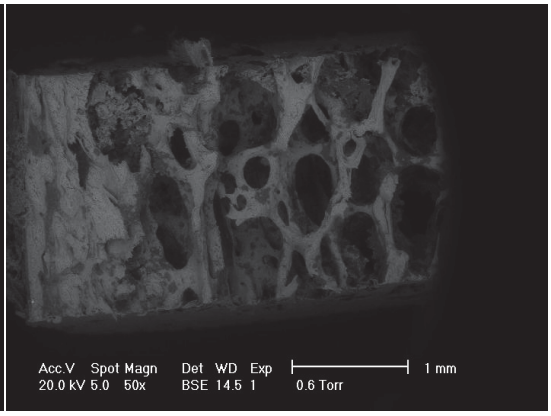

Figure 4-7. Loam Defleshed 2 - 6 months

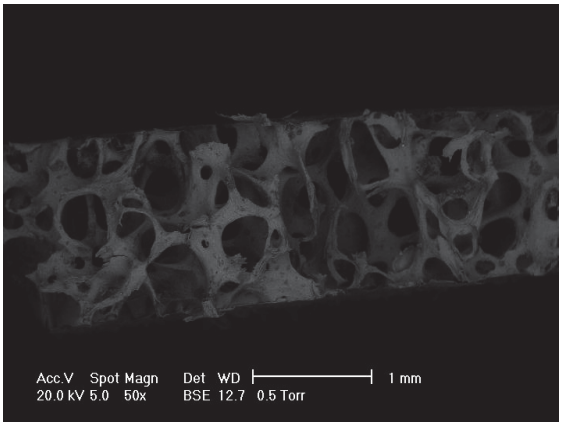

Figure 4-8. Loam Defleshed 1 - 12 months

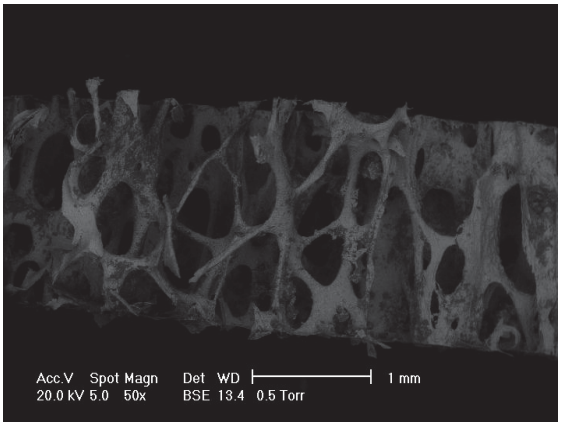

Figure 4-9. Loam Defleshed 2 - 12 months

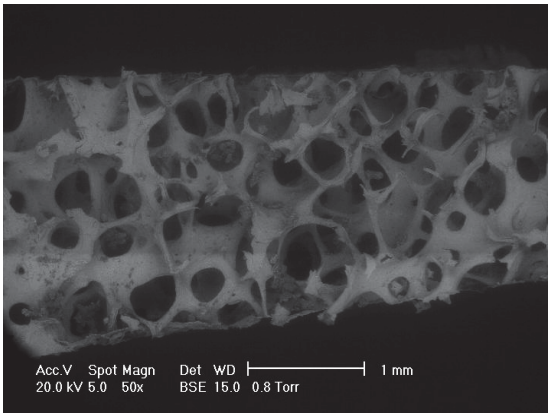

Figure 4-10. Loam Defleshed 1 - 18 months

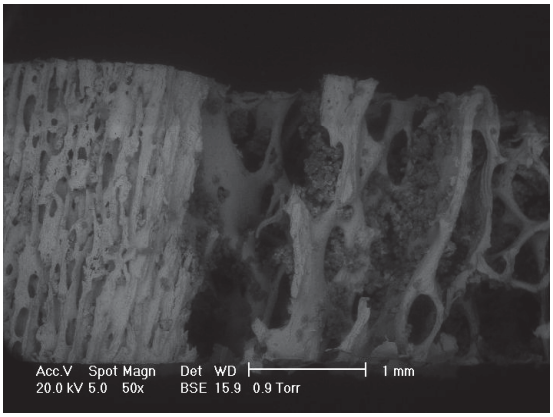

Figure 4-11. Loam Defleshed 2 - 18 months

4.2.3.2 Changes in Morphology

Using ESEM, it was observed that the bone samples were composed of different surface types at a magnification of x200 (defleshed bones shown in Figures 4-12 and 4-13 and fleshed bones shown in Figures 4-14 and 4-15). The two main reasons for the different surface types are the direction of the collagen fibres and how closely these layers of fibres are packed together.

The descriptions of the varying surface types of the defleshed samples and the approximate numbers of pores visible in one image frame are presented in Table 4-2. It is interesting to observe that the youngest samples of post-burial times up to and including 4 months had smooth surfaces (Figures 4-12 and 4-14). Older bone samples (6 months and older) varied in appearance as some demonstrated rough surfaces (Figure 4-13) while some showed smooth surfaces (Figure 4-15). All the samples had circular or relatively circular pores and consisted of tightly packed layers (Figures 4-16 and 4-17). All the samples except for the 2 and 4 month bone samples showed evidence of striations, whether they were longitudinal (Figures 4-18 and 4-19) or haphazard (Figures 4-20 and 4-21). It is also interesting to note that only the older bone samples (8 months and older) had random shallow grooves present in the

samples (Figures 4-22 and 4-23). These grooves were absent from all the younger bone samples. Therefore, the distinguishing features of the bone samples with different post-burial times were the surfaces and the presence of shallow grooves in the surfaces. Similar observational differences were evident in the bone samples recovered from the other burial conditions investigated in this study, as evident in the images below.

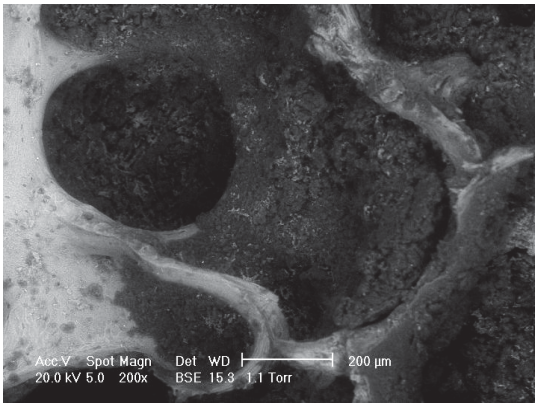

Figure 4-12. Defleshed: 1 month burial

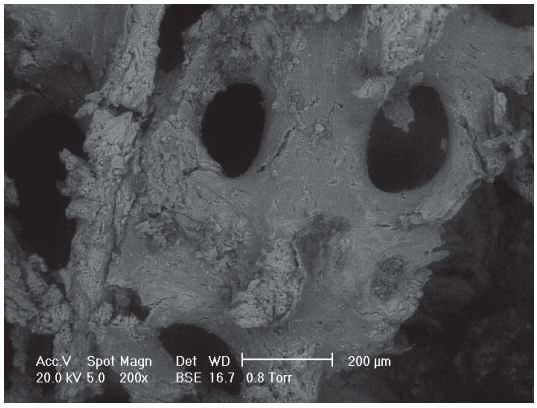

Figure 4-13. Defleshed: 18 months burial

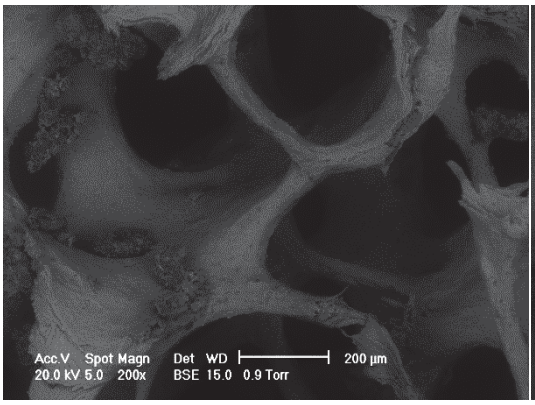

Figure 4-14. Fleshed: 1 month burial

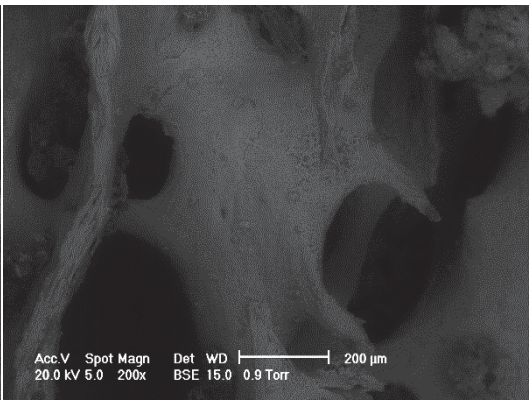

Figure 4-15. Fleshed: 18 months burial

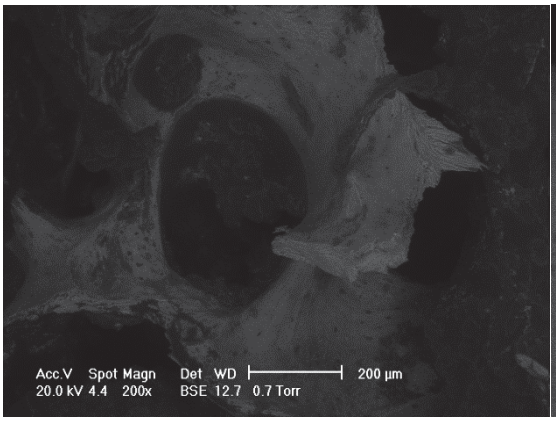

Figure 4-16. Wet: 1 month burial

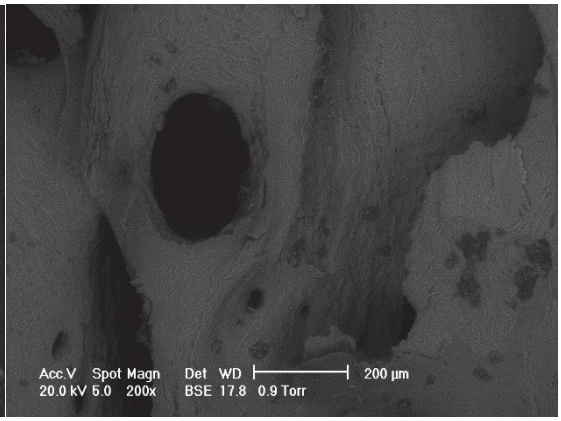

Figure 4-17. Dry: 18 months burial

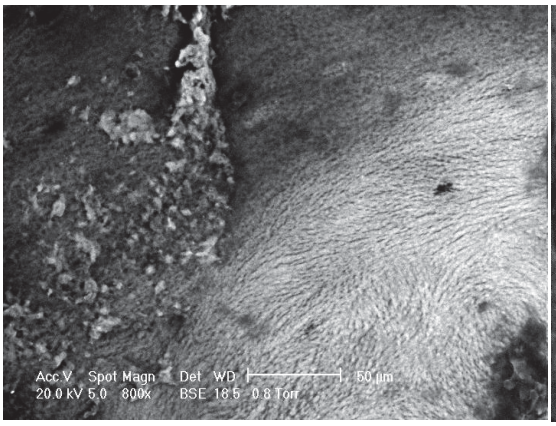

Figure 4-18. Boiled: 1 month burial

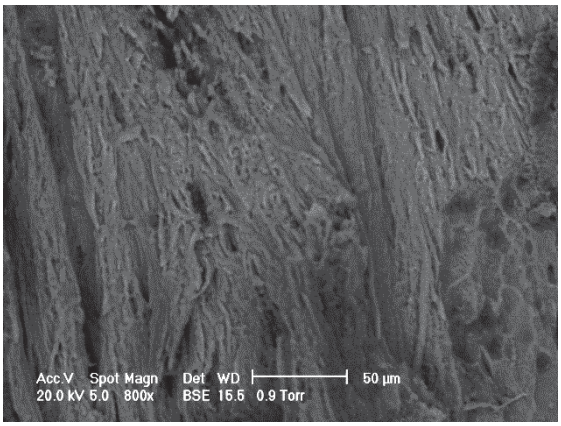

Figure 4-19. Degreased: 10 months burial

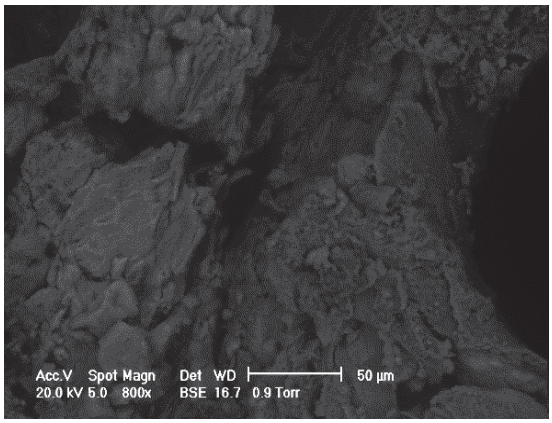

Figure 4-20. Sand: 6 months burial

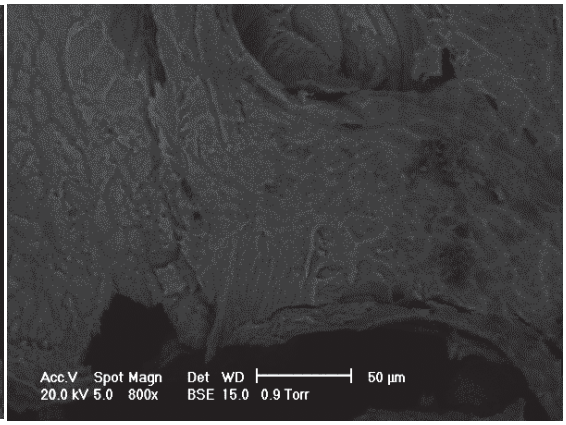

Figure 4-21. Clay: 12 months burial

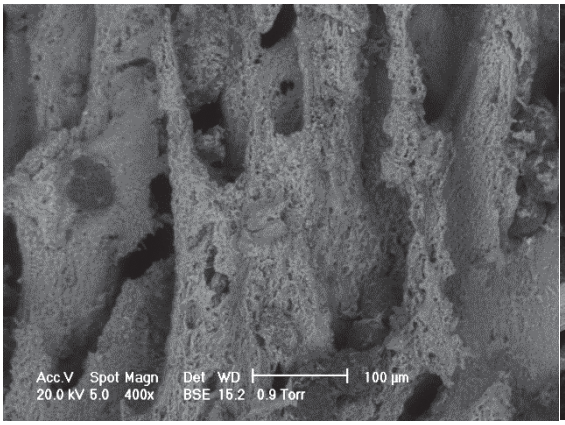

Figure 4-22: Cold: 8 months burial

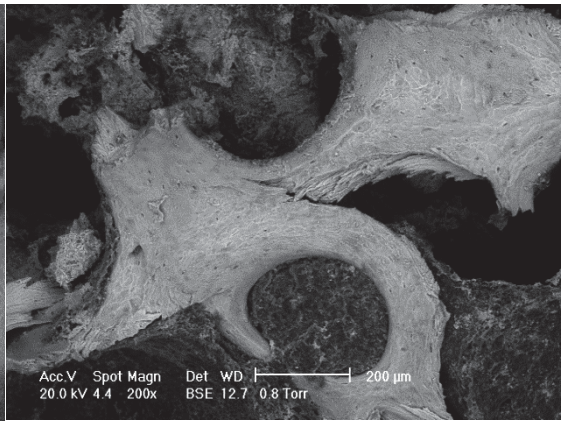

Figure 4-23. Room-temp.: 15 months burial

Table 4-2. Surface descriptions of loam defleshed (control) samples at different post-burial times (n = 18)

Post-burial time (months)	Description (x200 magnification)
1	Tightly packed layers, smooth appearance with visible haphazard striations, presence of many circular pores
2	Tightly packed layer of extremely thin straw-like fibres, relatively smooth appearance, presence of relatively circular pores
4	Tightly packed layers, relatively smooth appearance, presence of relatively circular pores
6	Tightly packed layers, slightly rough appearance, clearly visible longitudinal striations, presence of circular pores
8	Tightly packed layers, relatively smooth appearance with visible haphazard striations, presence of random grooves & circular pores
10	Relatively tightly packed layers, slightly rough appearance, presence of haphazard striations, shallow grooves and relatively circular pores
12	Moderately tightly packed layers, slightly rough appearance, presence of cracks, haphazard striations, shallow grooves and circular pores
15	Tightly packed layers, relatively rough appearance, few patches of haphazard striations, presence of random grooves & circular pores
18	Tightly packed layers, relatively smooth appearance, presence of haphazard striations, shallow grooves and tiny relatively circular pores

The relationship between post-burial time and the number of visible pores (measured using ESEM software and the BSE detector at x200) for the defleshed samples can be seen in Figure 4-24. The graph shows that as the post-burial time of the defleshed samples increased, the number of visible pores also increased; however, there is some scatter in the data.

It is interesting to note that the number of visible pores more than doubles after 6 months of burial in both the defleshed samples. This trend is visible in all the bone samples recovered from the various burial conditions investigated in this study.

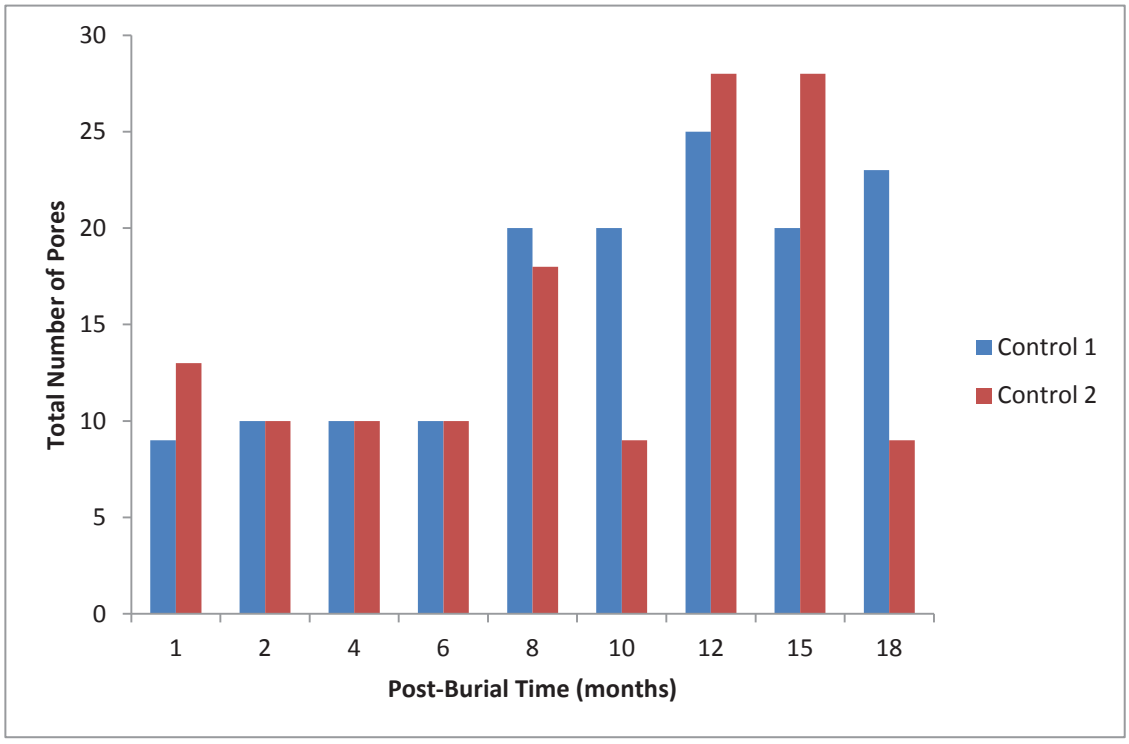

Figure 4-24. Total number of visible pores vs post-burial time for Loam Defleshed (Control) 1 and 2 bones

4.2.4 Thermogravimetric Analysis

TG was one of the techniques explored to examine the differences between the bones buried for different periods of time. Previous research (Raja *et al.*, 2009) has utilised this technique for samples up to 7 years of burial. In the present study, bone samples buried for shorter periods of time but under controlled laboratory conditions were investigated and analysed using TG. TG was chosen for analysis because diagenesis over time is expected to result in mass change in the bone samples. It was also used to identify changes in the decomposition mechanisms occurring in the different burial environments examined. The TG results were examined for the presence of trends that would help in estimating the burial time of the bones.

4.2.4.1 Cortical versus Cancellous Bone

As part of this study, TG was used to analyse the bone samples recovered after the different burial periods to investigate the potential of being able to estimate the length of burial of bones using the results. Previous research has demonstrated that the TG results obtained using cortical bone were much more reproducible than the results obtained using cancellous bone (Onishi *et al.*, 2008; Raja *et al.*, 2009). Therefore, for the purpose of this study, cortical bone was used for analytical purposes although cancellous bone was also examined for comparison and confirmatory purposes. The TG results obtained agree with the results of previous research in that cortical bone analysis using TG shows less variation than cancellous bone analysis. This is evident as the standard deviations in the results of the cortical bone are typically lower than the standard deviations in the results of the cancellous bone and are presented in Table 4-3.

Table 4-3. Comparison of TG results (mass loss as %) of the cortical and cancellous sections of the loam defleshed (control) bones

Month	Loam Defleshed (cortical) Average	St. Dev. (±)	Loam Defleshed (cancellous) Average	St. Dev. (±)	ΔSD (SD_{canc}-SD_{cort})
1	35.3	2.8	45.9	6.0	Lower
2	35.5	2.7	40.8	0.9	Higher
4	29.8	1.3	32.8	2.6	Lower
6	30.5	0.0	32.1	2.9	Lower
8	32.3	1.6	40.7	9.4	Much lower
10	32.4	5.6	41.2	5.9	≈
12	30.1	5.1	43.6	4.5	Higher
15	38.2	4.4	38.5	7.9	Lower
18	33.6	0.6	34.4	2.1	Lower
	Average SD	2.7	**Average SD**	4.7	
	SD Average SD	2.0	**SD Average SD**	2.8	

4.2.4.2 Optimisation of Sample Preparation Method

The most appropriate method of bone sample preparation was determined using TG (Raja *et al.*, 2010b). The four sample preparation methods that were tested and compared were oven-dried (OD), freeze-dried (FD), ring mill ground (R-P), hand ground (H-P) and lumps (L). The TG results reveal that the method used to prepare the bone samples has a significant effect on the results obtained. The results of the 3 month sample prepared by the four different sampling methods and heated in an air atmosphere are shown in Figure 4-25. The significant differences observed in the mass loss data can be attributed to the method of sample preparation. The water loss region (room temperature to 200 °C) is grouped into oven-dried and freeze-dried specimens, where the freeze drying process is observed to have removed less of the total water. Oven drying removed more water and this was not significantly affected by the state of the sample (lump or powder).

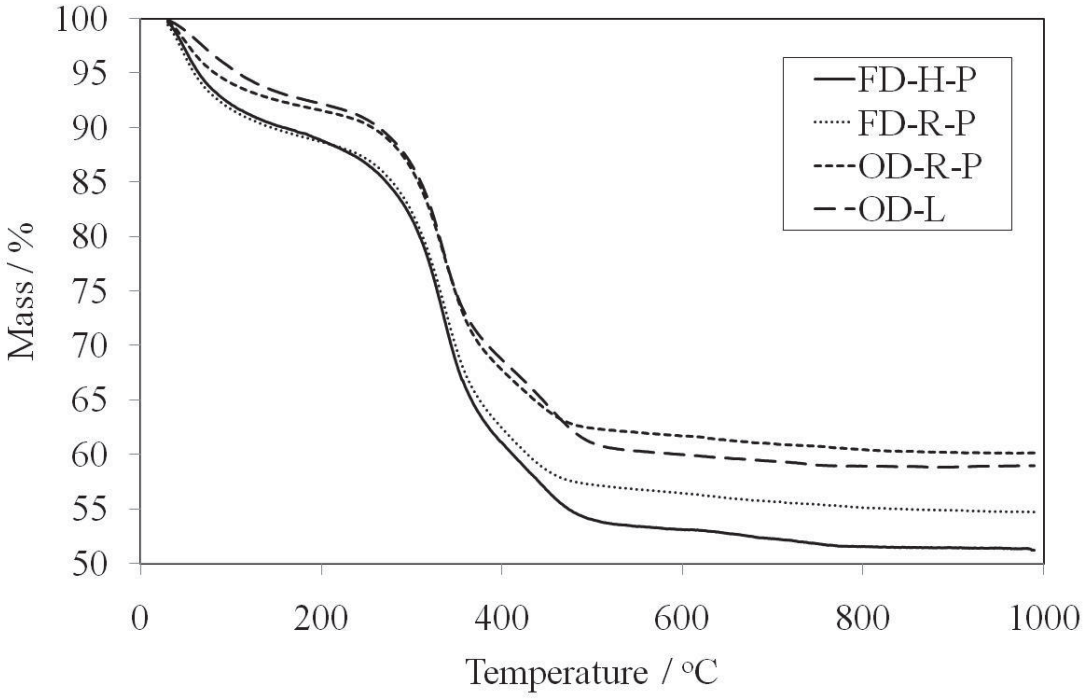

Figure 4-25. 3 month sample prepared by the four different sampling methods

Between the temperatures of 200 and 600 °C, decomposition of the organic content of bone occurs. Significant differences are evident in this region when comparing the different methods of sample preparation. The differences are based on how the bone specimens were ground. Oven-dried lumps show a greater mass loss than the ring ground oven-dried powder. Similarly, the hand-ground freeze-dried specimens show a greater mass loss than the ring-ground freeze-dried specimens. These observations indicate that the energy and heat created as a result of mechanical grinding cause degradation and volatilisation of some of the organic components of bone.

The water content of the specimens is observed to significantly vary with sample preparation method, which makes water content an unreliable method for analysing the ageing of bones. It was difficult to control the amount of water present in each bone sample. In order to minimise error in the results caused by the differing water content in the samples, changes in the water content (Figure 4-26) and the organic content (Figure 4-27) of the bones were plotted separately and in terms of the residue mass, rather than mass loss. These are plotted as a function of the burial time. The results obtained for the mass loss associated with organic matter as a function of post-burial time also show a significant degree of variability. However, the hand-ground freeze dried bone samples show a consistent reduction in organic content with increasing post-burial time. An important factor responsible for this correlation could be the minimal amount of damage endured by the samples during the preparation stage.

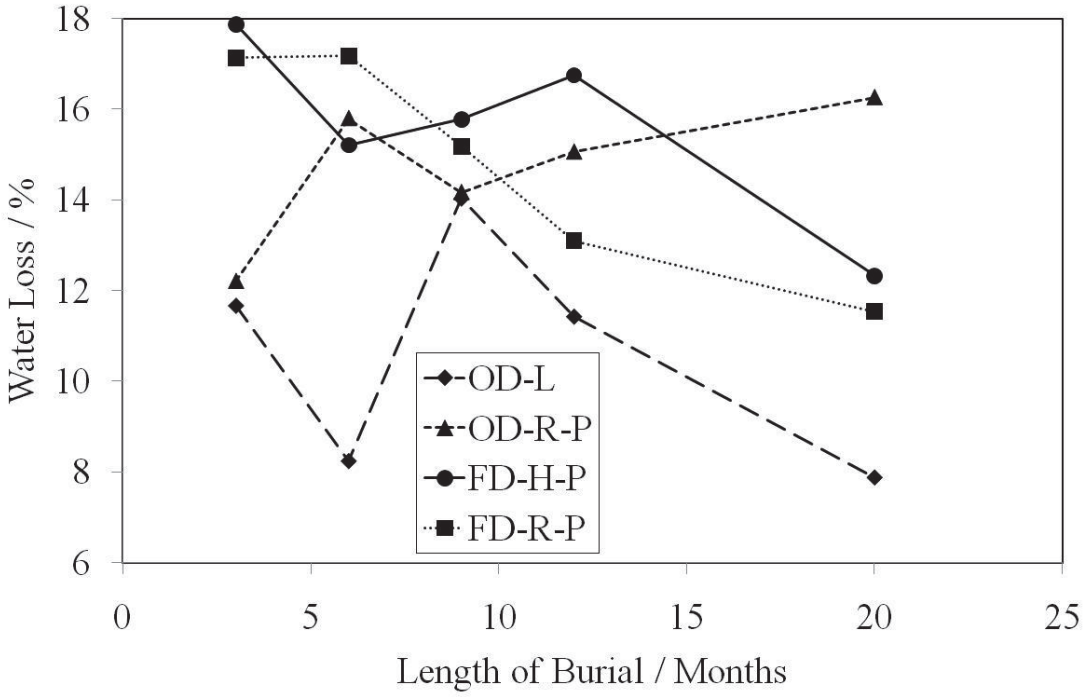

Figure 4-26. Water loss as a percentage of final mass versus post-burial time

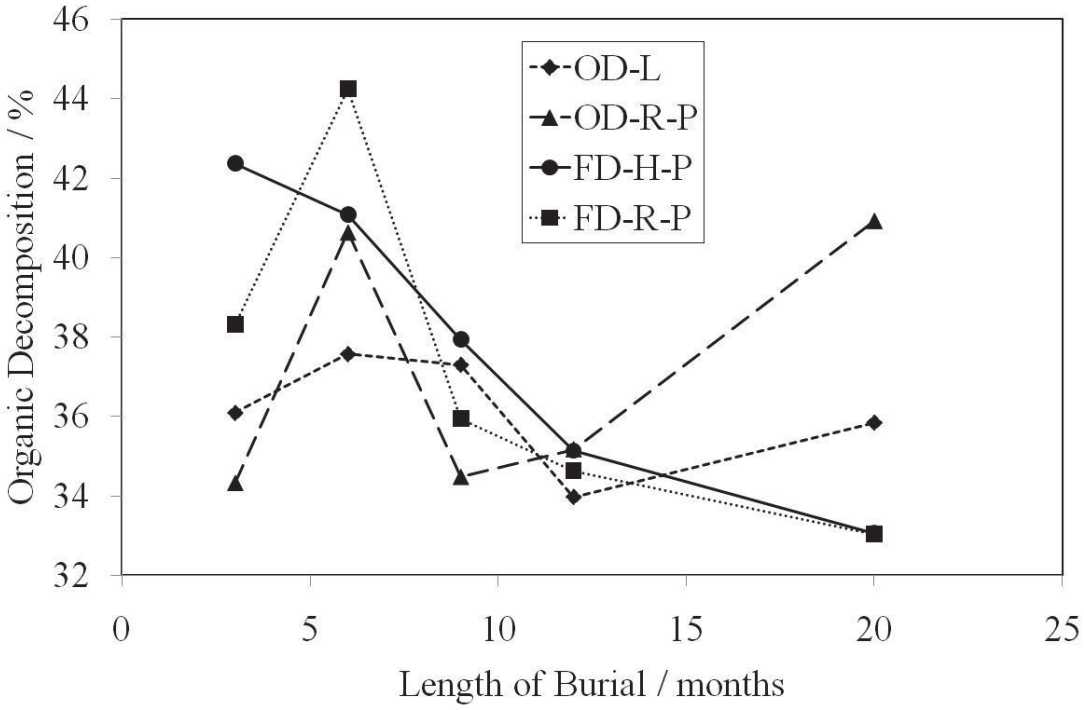

Figure 4-27. Organic mass loss as a percentage of final mass versus post-burial time

Figure 4-28 shows the residue as a percentage of the initial mass versus post-burial time. The results of the residue appear to be quite varied, except for the results of the hand-ground freeze dried samples. This can be attributed to the fact that any variation in mass loss due to water content is small in comparison to the change in mass loss due to the organic content. As a consequence, the residue mass shows a relationship with the post-burial time when the samples are freeze-dried and hand-ground. Therefore, the sample preparation procedure selected for the burial conditions study was freeze-drying followed by manual grinding using a mortar and a pestle (Raja *et al.*, 2010b).

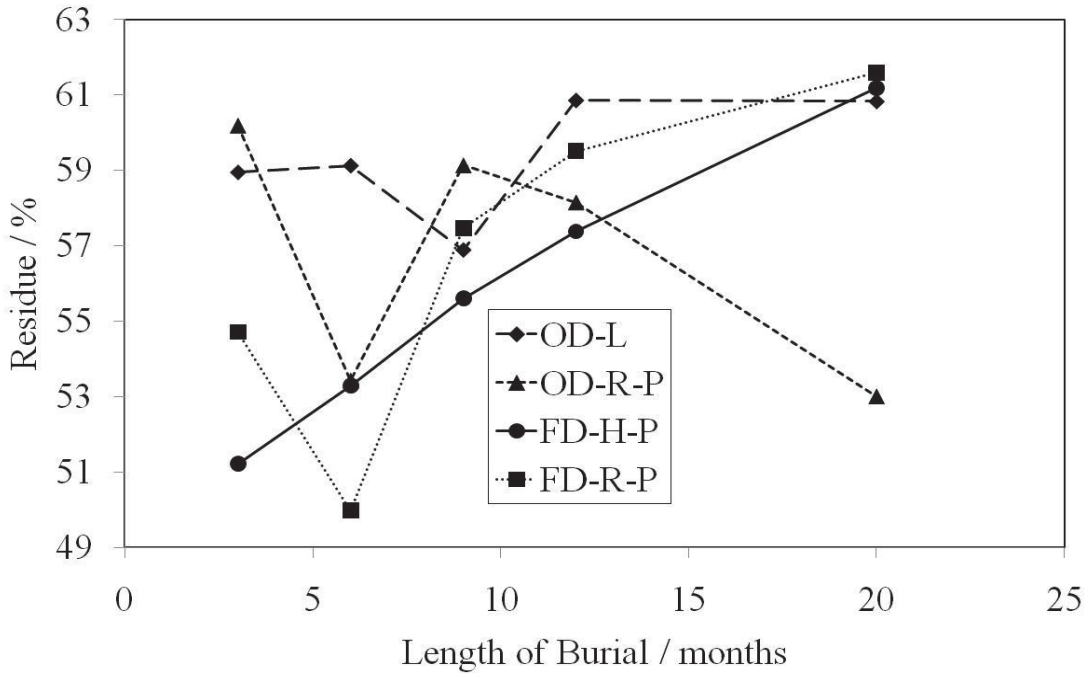

Figure 4-28. Residue as a percentage of initial mass versus post-burial time

4.2.4.3 Mass Loss Steps in Bone

Previous research has shown that the results in an air atmosphere are more consistent than the results obtained when using an inert atmosphere and therefore the samples in this study were analysed using an air atmosphere rather than a nitrogen atmosphere (Raja *et al.*, 2009). The progress of the mass loss that occurs as bone is heated from room temperature to 1000 °C is presented (as a percentage of the initial mass) for loam defleshed samples 1 and 2 at the different post-burial periods in Figures 4-29 and 4-30. These samples were heated in an air environment. The bone samples with shorter burial periods are shown in black while the bone samples with longer burial periods are shown in red and blue. As can be seen in these figures, no trend is observed with respect to the percentage of mass remaining and the burial time. Also, the results of the two loam defleshed samples do not appear to be reproducible.

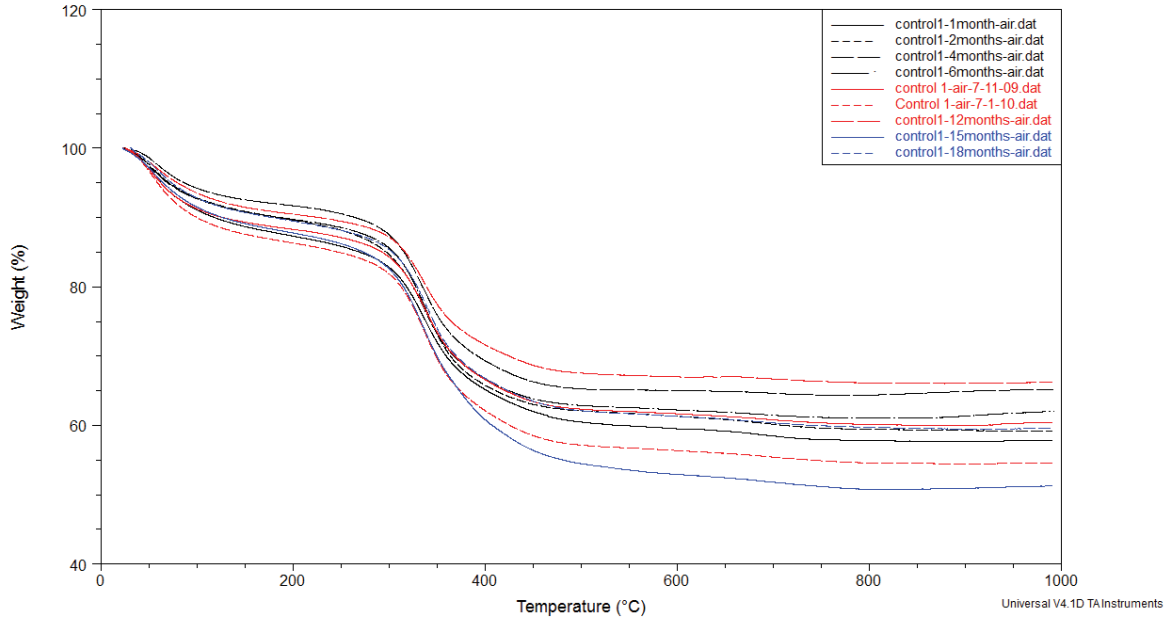

Figure 4-29. TG curves of Loam Defleshed (Control) 1 at the different post-burial times

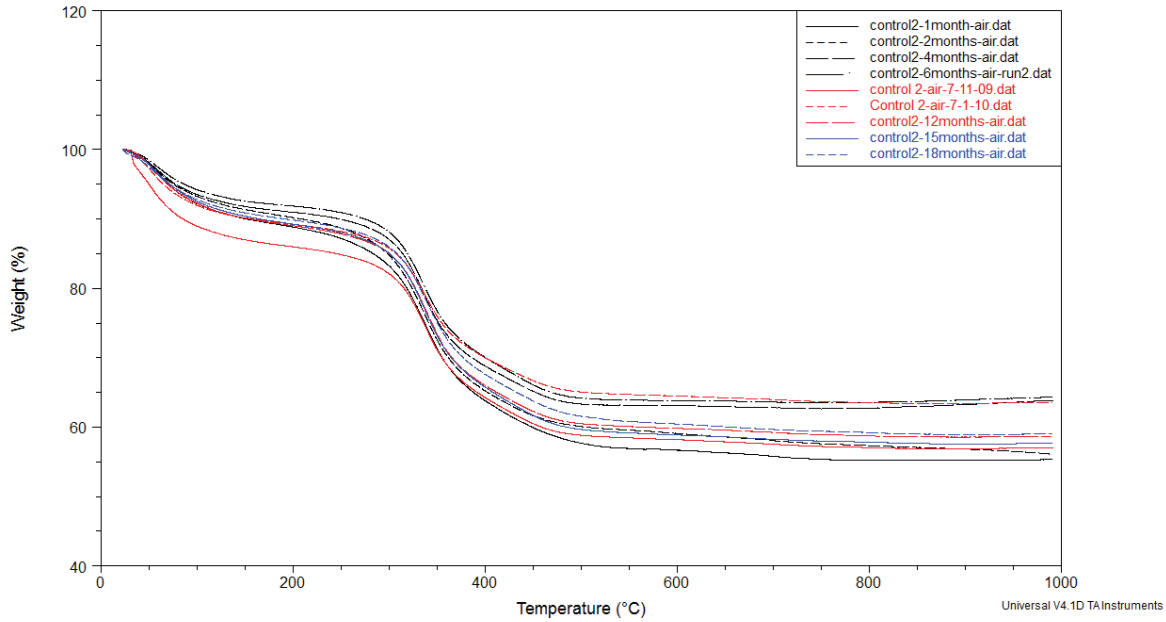

Figure 4-30. TG curves of Loam Defleshed (Control) 2 at the different post-burial times

The total mass loss of each sample can be divided into four mass loss steps as can be seen in Figure 4-31 (Raja *et al.*, 2009).

1. The first mass loss step is associated with water loss and occurs below 220 ^{o}C ('Water Loss').

2. The step corresponding to the thermal decomposition of organic matter occurs between 220-420 ^{o}C ('Step 1').

3. Mass loss associated with the loss of the organic component of bone occurs between 420-600 ^{o}C ('Step 2').

4. The final step is associated with carbonate decomposition in an air atmosphere and occurs between 600-1000 °C ('Step 3'). In an inert (N_2) atmosphere, the final step also involves further pyrolysis of the organic matter in bone.

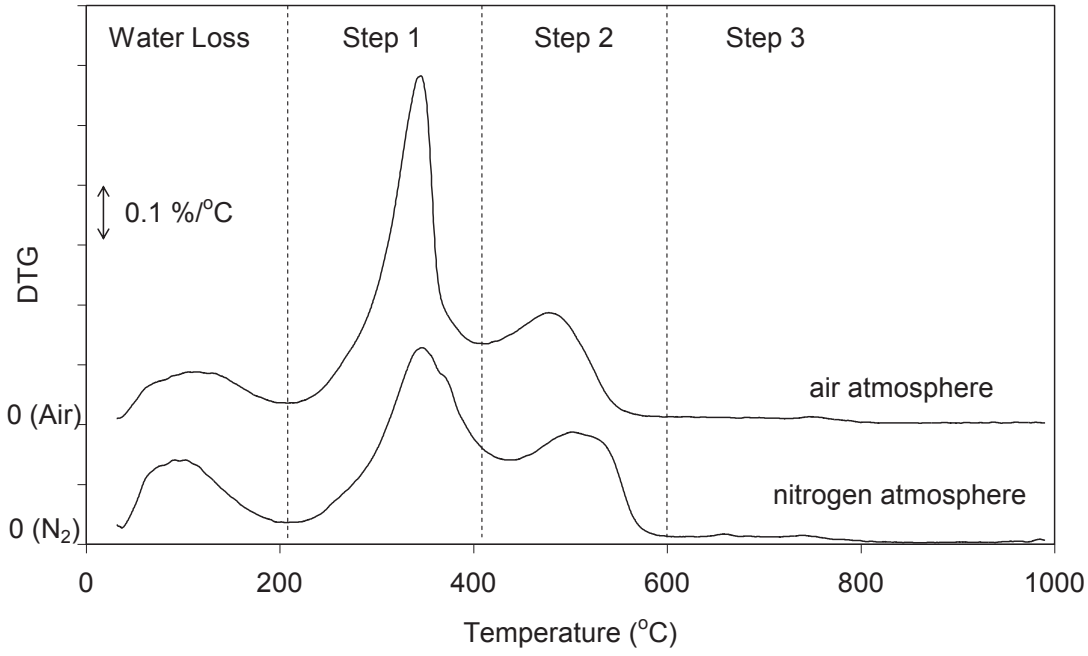

Figure 4-31. Mass loss steps for bone

The first mass loss step i.e. water loss, is excluded from the results for comparison purposes as it has been demonstrated in previous studies that it is difficult to be certain that all water has been removed from each bone sample (Raja *et al.*, 2009) and, therefore, using the water loss step as a method of estimating the post-burial time would not be suitable. Therefore, this step is not included in the calculation of the total mass loss when comparing the TG results of the bone samples in this study.

For loam defleshed samples 1 and 2, the three mass loss steps associated with the organic and inorganic decomposition of bone are presented in Figures 4-32 to 4-34 as a function of post-burial time. The three graphs showed that mass loss occurring in Step 1 is the highest and mass loss occurring as a result of Step 3 is the lowest. It is noteworthy that the variation in mass loss due to the thermal decomposition of the organic component of bone is much higher than the variation observed due to the inorganic content of bone.

Figure 4-32 shows no trend in mass loss Step 1 with the exception of a decrease in total mass loss between 2 and 4 months of burial in both loam defleshed samples. In mass loss Step 2 (Figure 4-33) and mass loss Step 3 (Figure 4-34), a similar trend is observed for both loam defleshed samples between the post-burial period of 1 and 8 months. For samples with longer burial periods, both mass loss Steps 2 and 3 do not show similar trends.

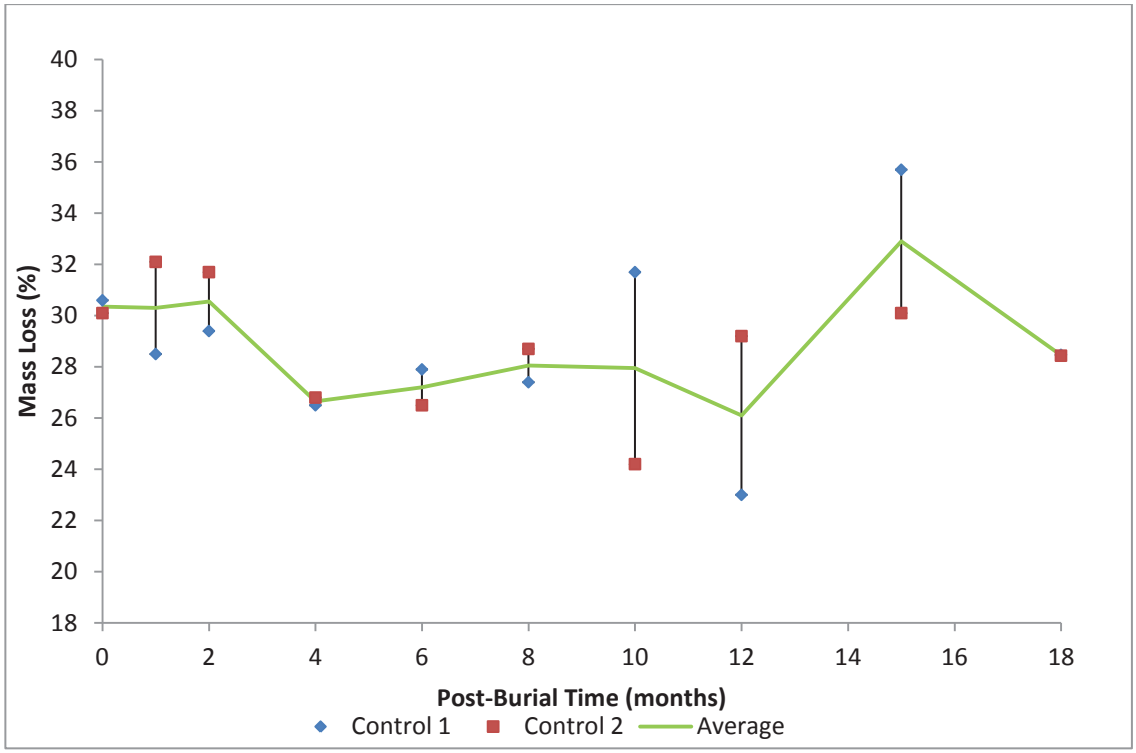

Figure 4-32. Loam Defleshed (Control) 1 and 2 - Mass Loss Step 1

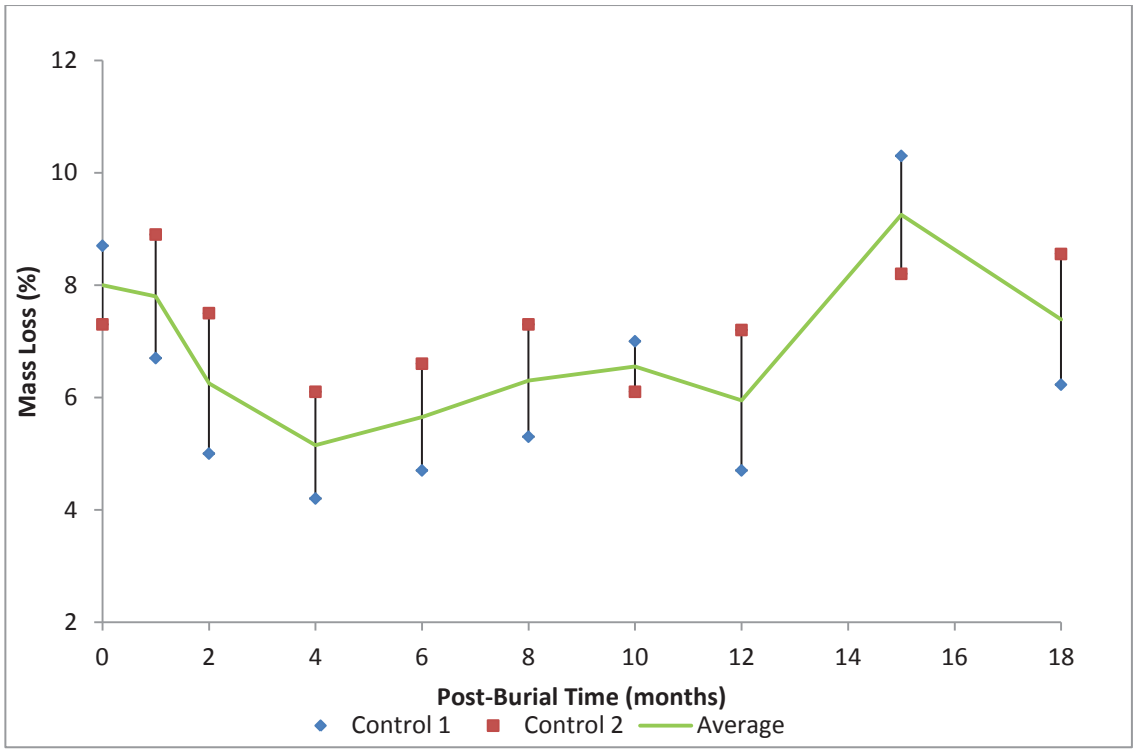

Figure 4-33. Loam Defleshed (Control) 1 and 2 - Mass Loss Step 2

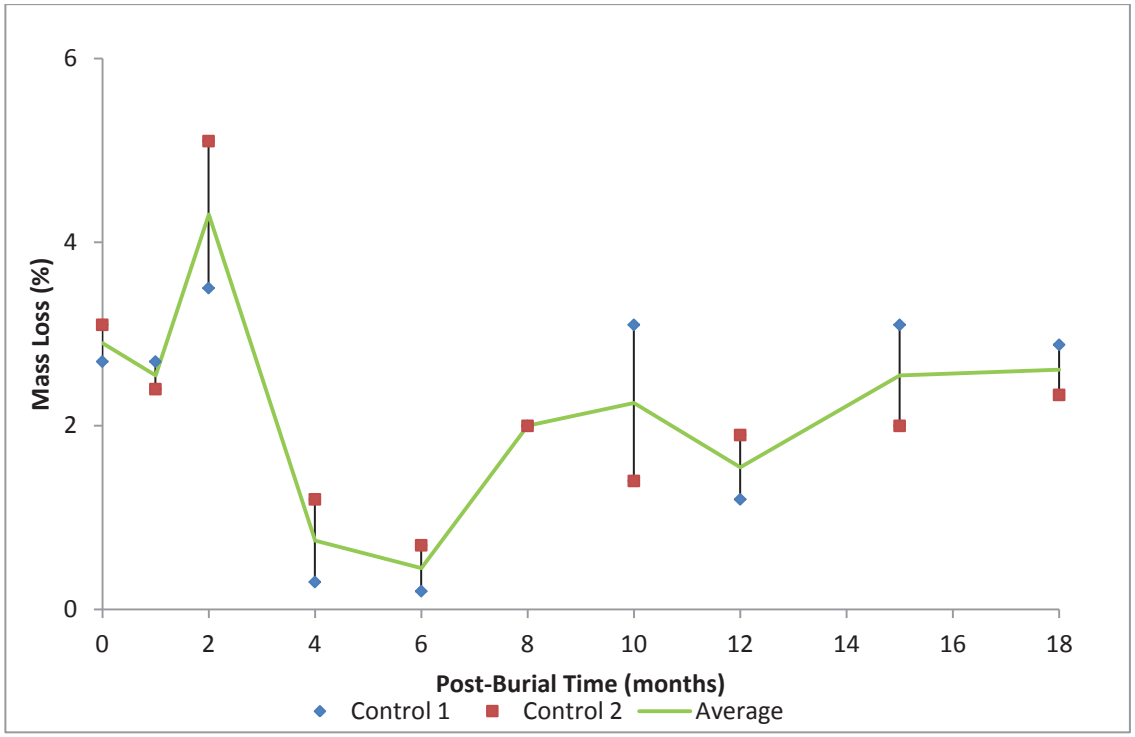

Figure 4-34. Loam Defleshed (Control) 1 and 2 - Mass Loss Step 3

4.2.4.4 Reproducibility

Two sets of bones were buried in the same burial conditions to assess the reproducibility of the results. The results of the defleshed samples buried in loam soil for the different lengths of burial from pre-burial to 18 months post-burial are presented in Figures 4-35 to 4-44. The shapes of the curves are similar for both the loam defleshed samples after all the different burial times, except for the 6 and 15 month samples. However, the total percentage mass loss differs for the two loam defleshed samples after each burial period, except for the bone samples buried for 18 months, indicating that the samples being analysed are heterogeneous.

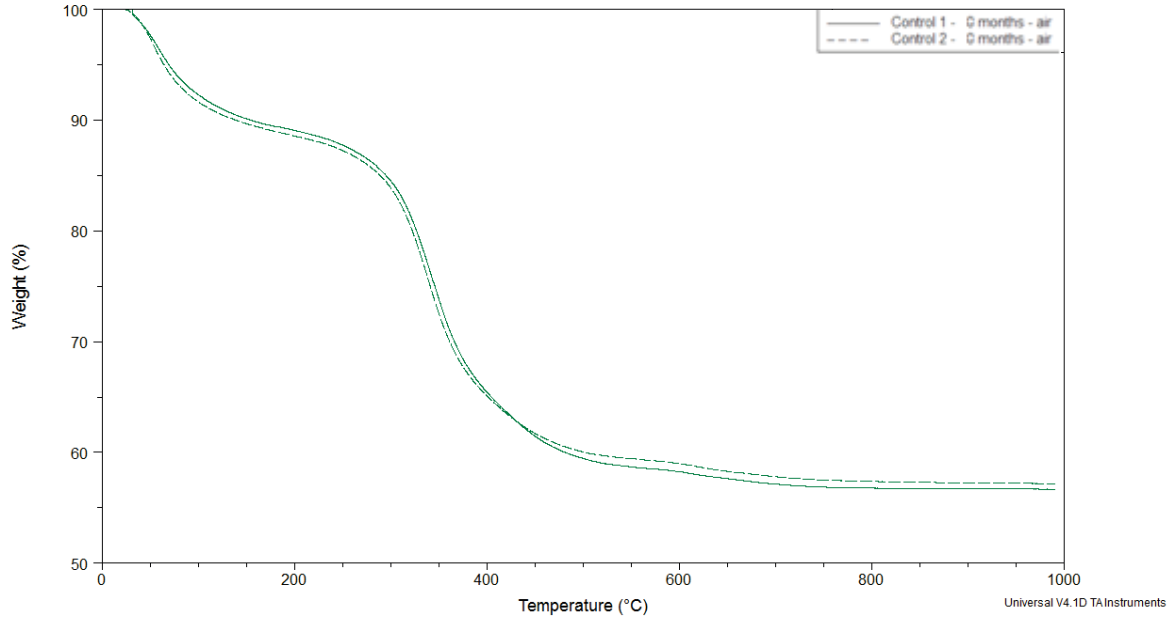

Figure 4-35. TG curves of Loam Defleshed (Control) 1 and 2 prior to burial

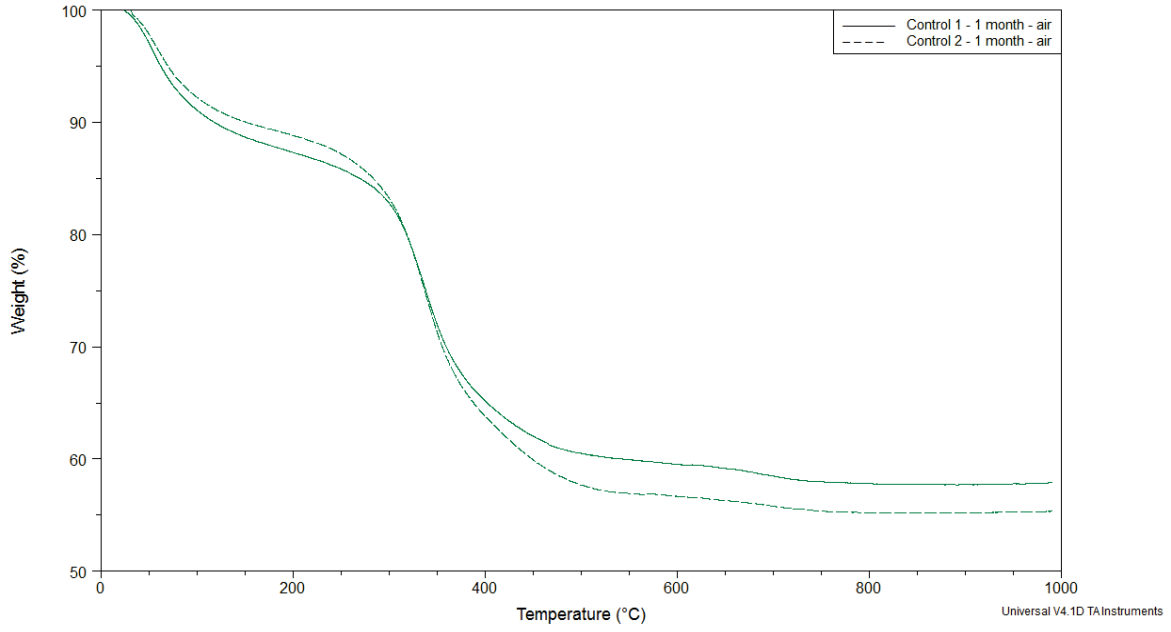

Figure 4-36. TG curves of Loam Defleshed (Control) 1 and 2 after 1 month of burial

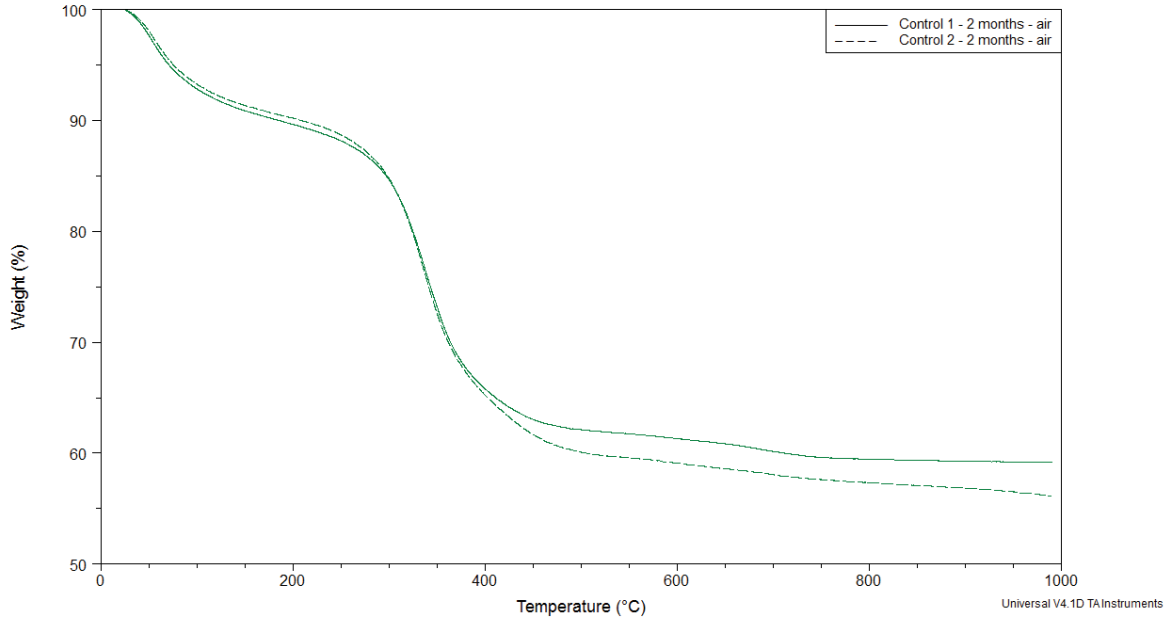

Figure 4-37. TG curves of Loam Defleshed (Control) 1 and 2 after 2 months of burial

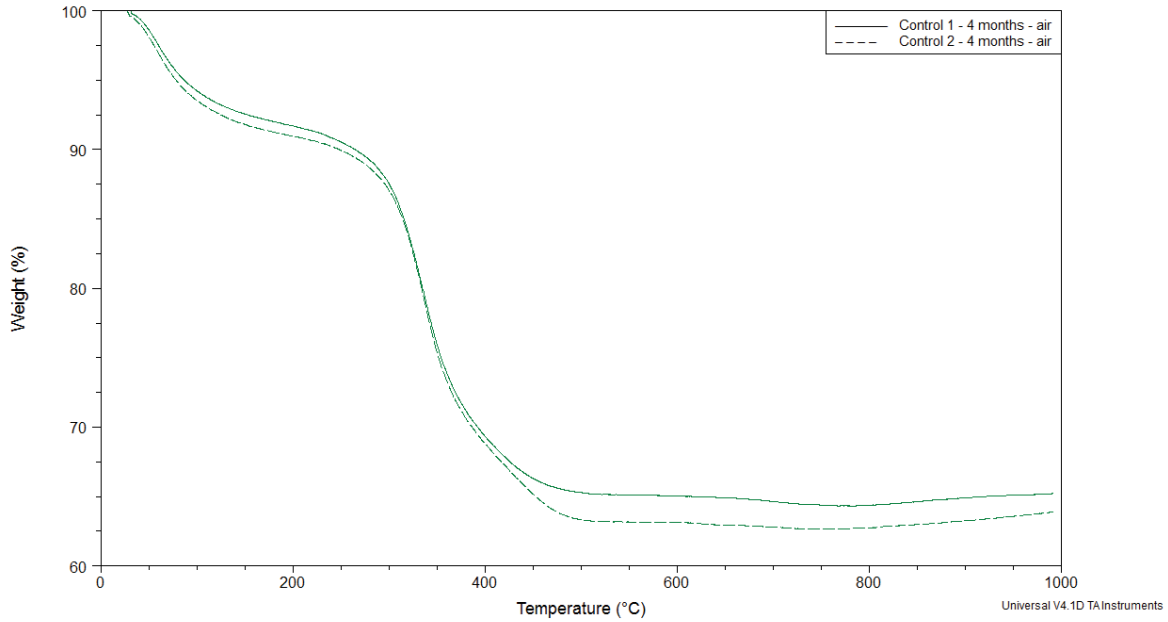

Figure 4-38. TG curves of Loam Defleshed (Control) 1 and 2 after 4 months of burial

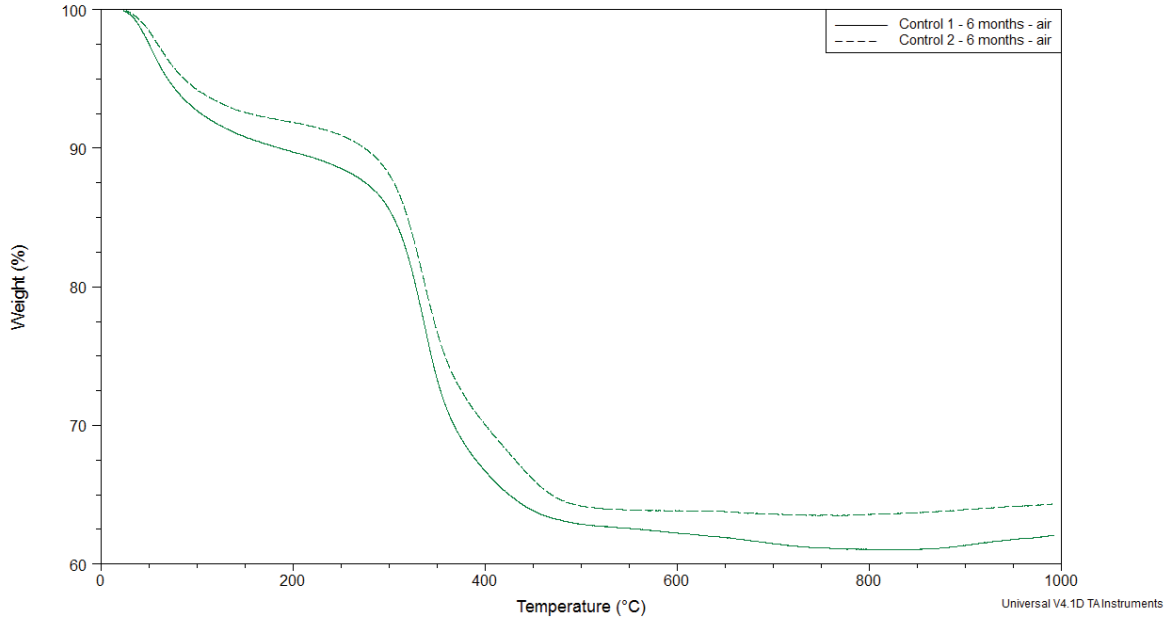

Figure 4-39. TG curves of Loam Defleshed (Control) 1 and 2 after 6 months of burial

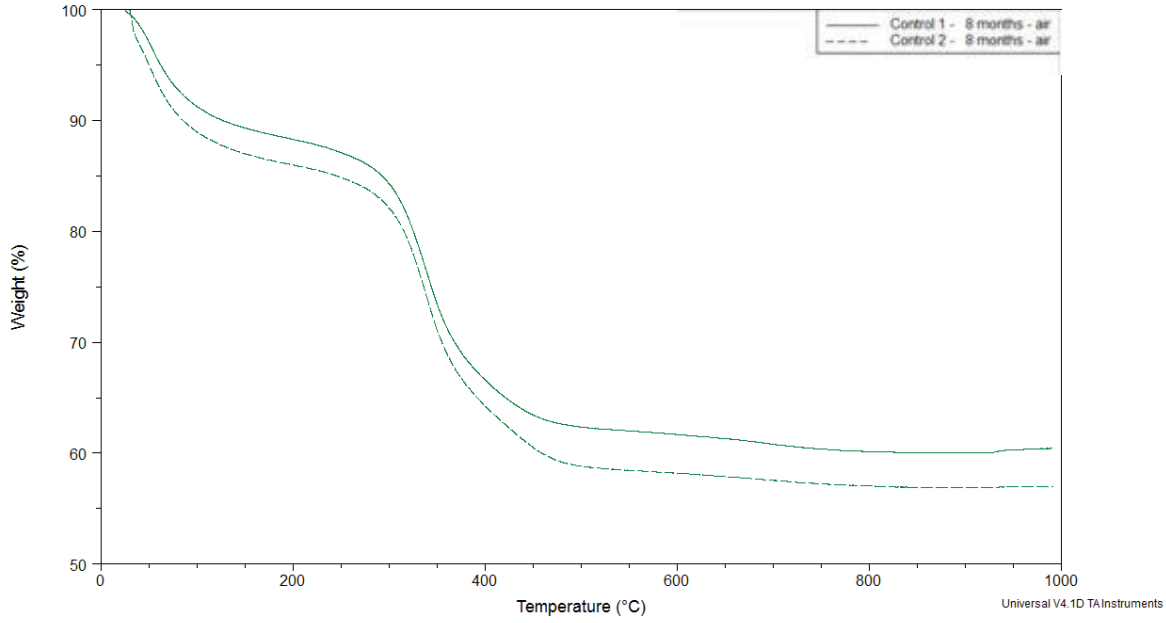

Figure 4-40. TG curves of Loam Defleshed (Control) 1 and 2 after 8 months of burial

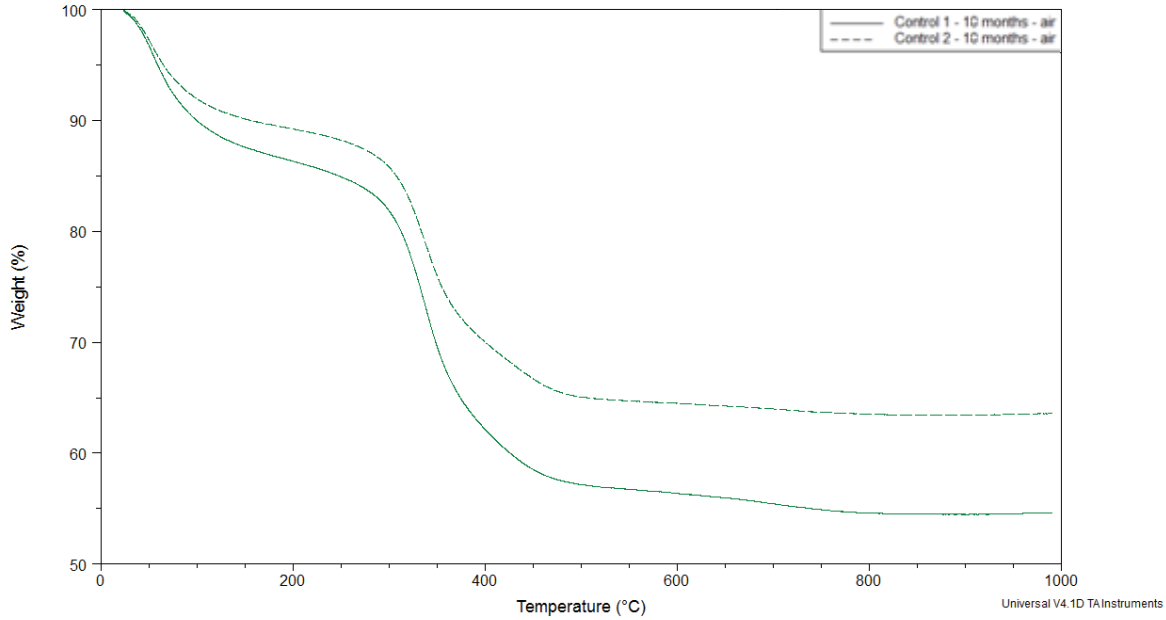

Figure 4-41. TG curves of Loam Defleshed (Control) 1 and 2 after 10 months of burial

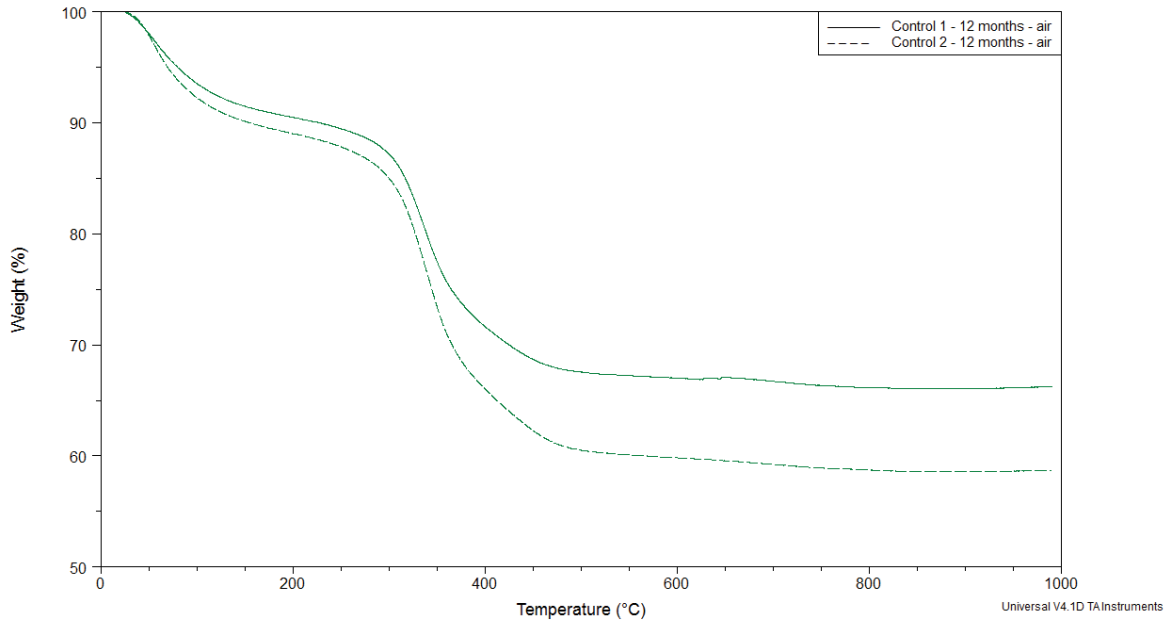

Figure 4-42. TG curves of Loam Defleshed (Control) 1 and 2 after 12 months of burial

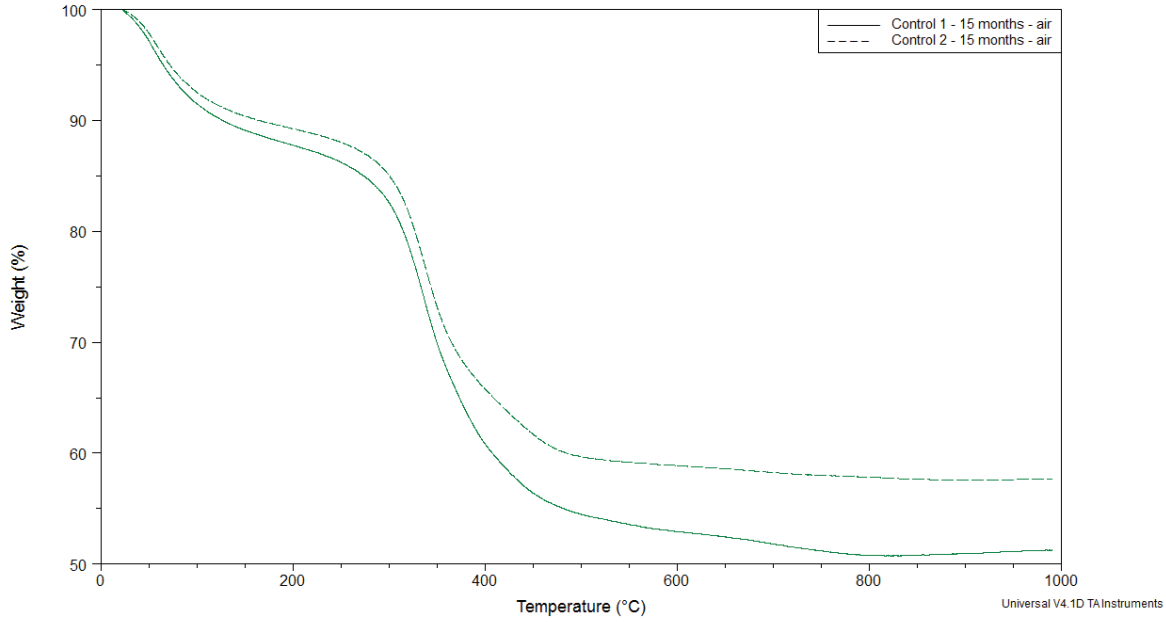

Figure 4-43. TG curves of Loam Defleshed (Control) 1 and 2 after 15 months of burial

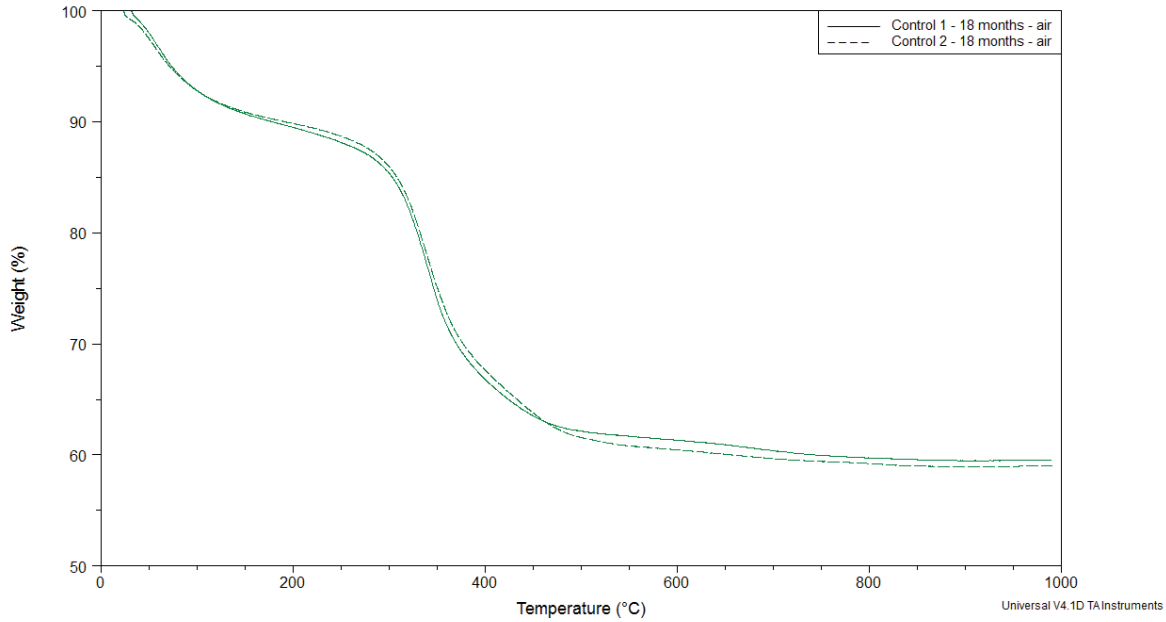

Figure 4-44. TG curves of Loam Defleshed (Control) 1 and 2 after 18 months of burial

To further assess the reproducibility of the results, the DTG curves of the defleshed bone samples buried in loam soil were compared. The DTG curves of the bone samples for the different lengths of burial from pre-burial to 18 months post-burial are shown in Figures 4-45 to 4-54. The lack of reproducibility in the DTG curves confirms the heterogeneity of the bone samples being analysed in the study.

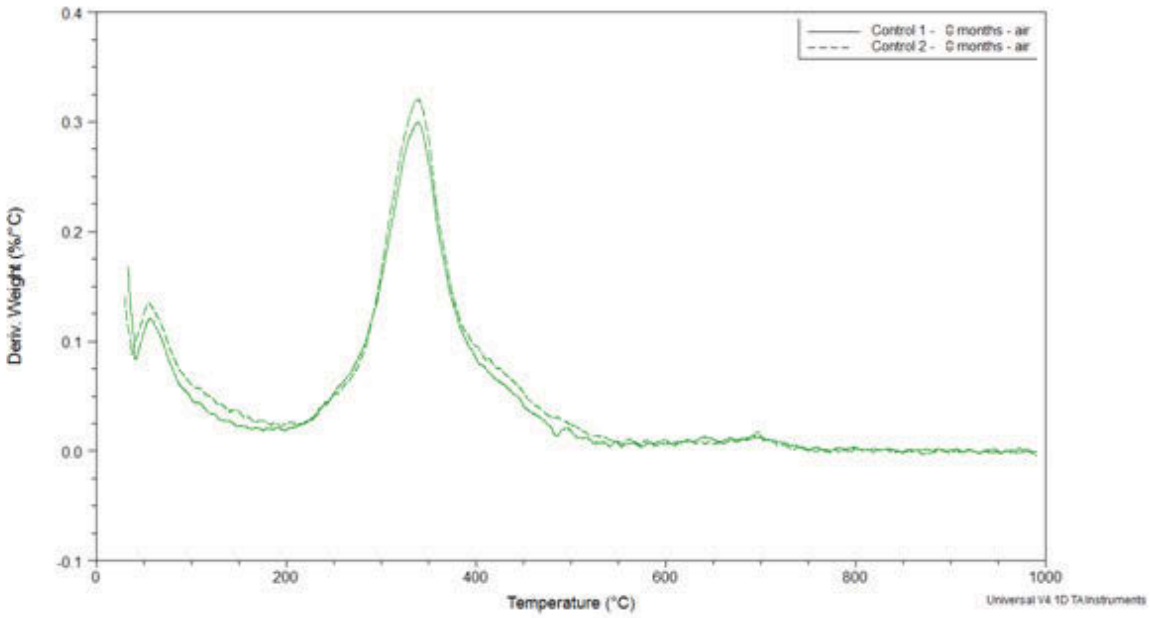

Figure 4-45. DTG curves of Loam Defleshed (Control) 1 and 2 prior to burial

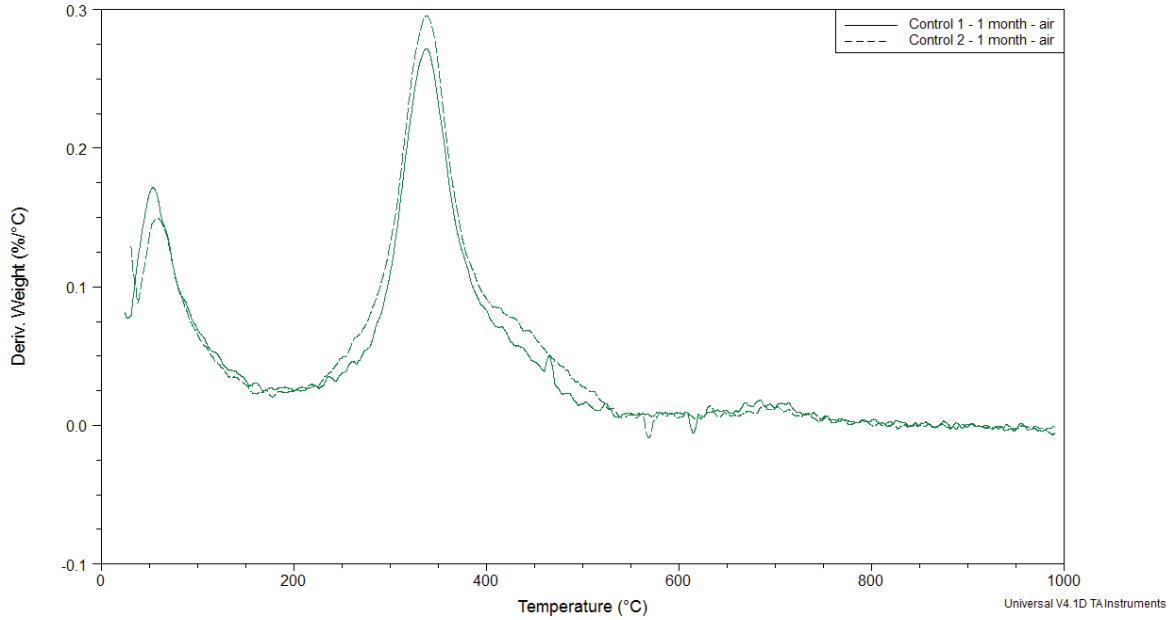

Figure 4-46. DTG curves of Loam Defleshed (Control) 1 and 2 after 1 month of burial

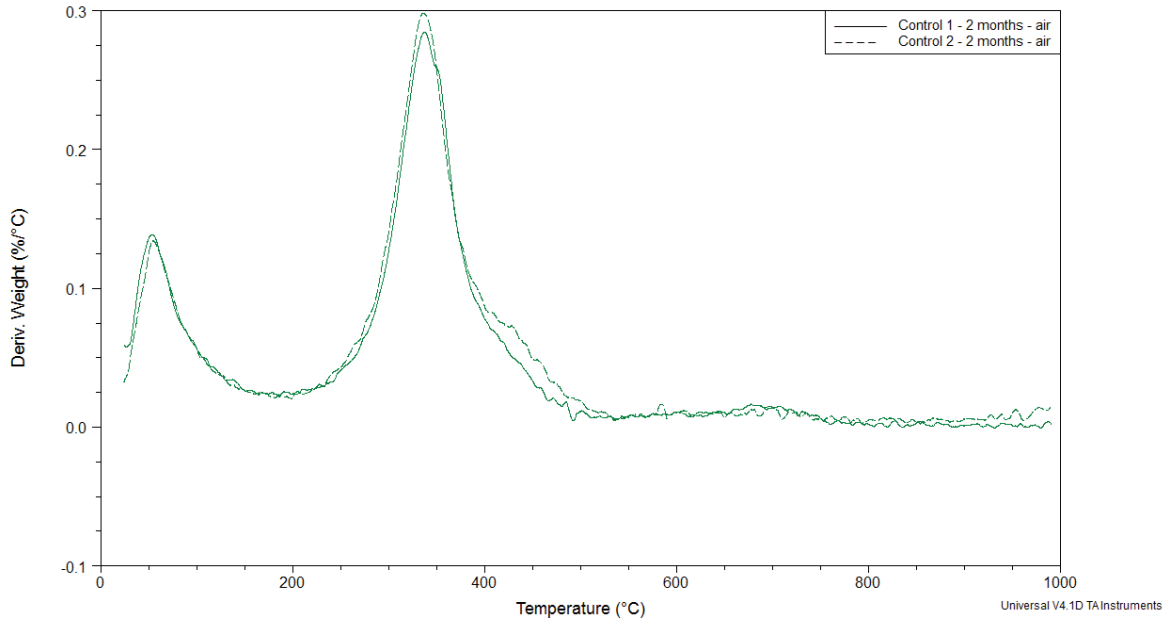

Figure 4-47. DTG curves of Loam Defleshed (Control) 1 and 2 after 2 months of burial

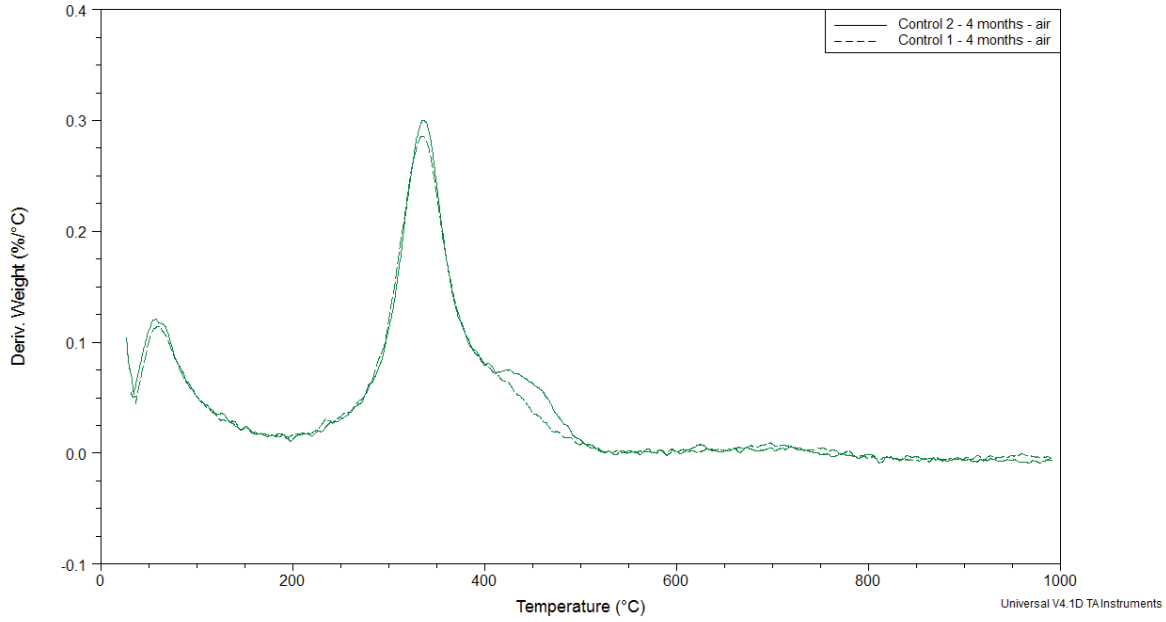

Figure 4-48. DTG curves of Loam Defleshed (Control) 1 and 2 after 4 months of burial

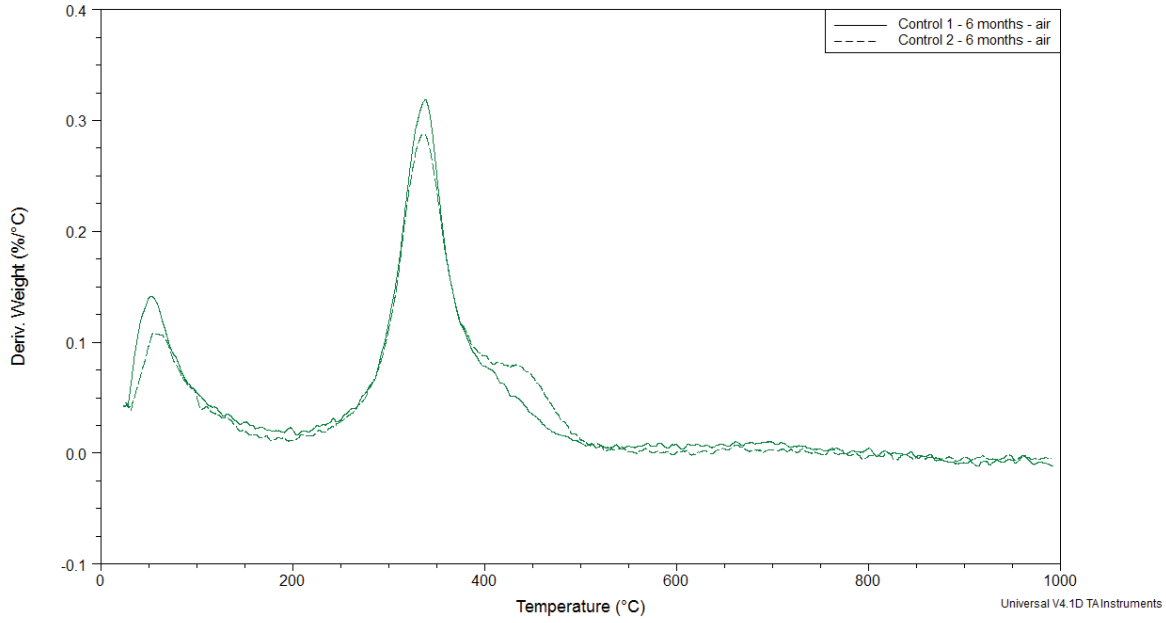

Figure 4-49. DTG curves of Loam Defleshed (Control) 1 and 2 after 6 months of burial

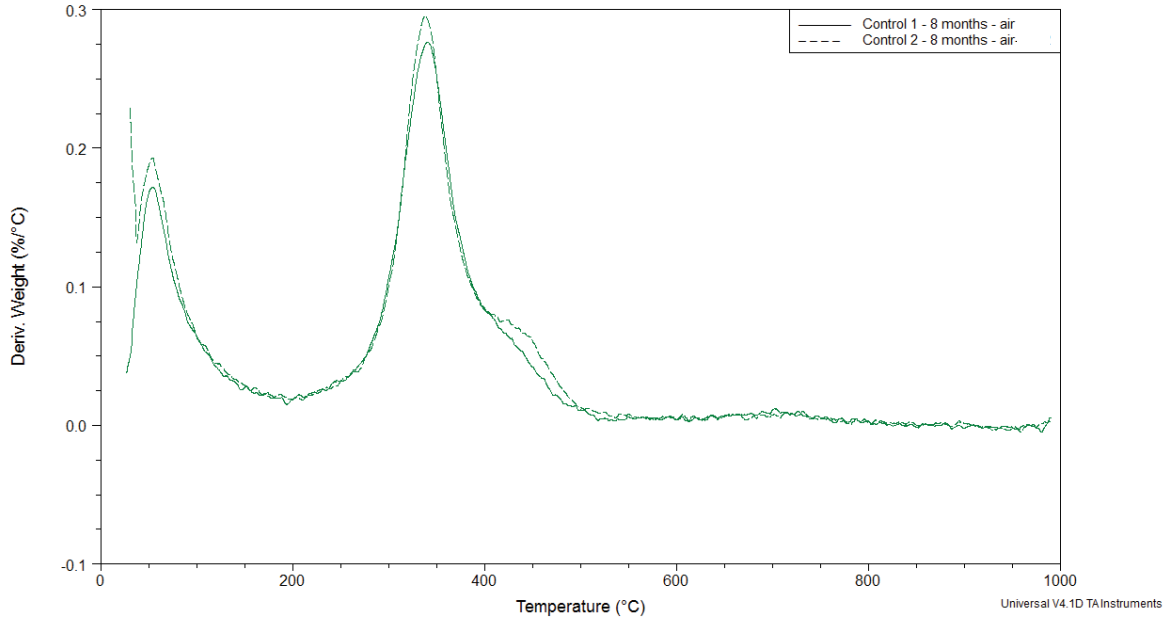

Figure 4-50. DTG curves of Loam Defleshed (Control) 1 and 2 after 8 months of burial

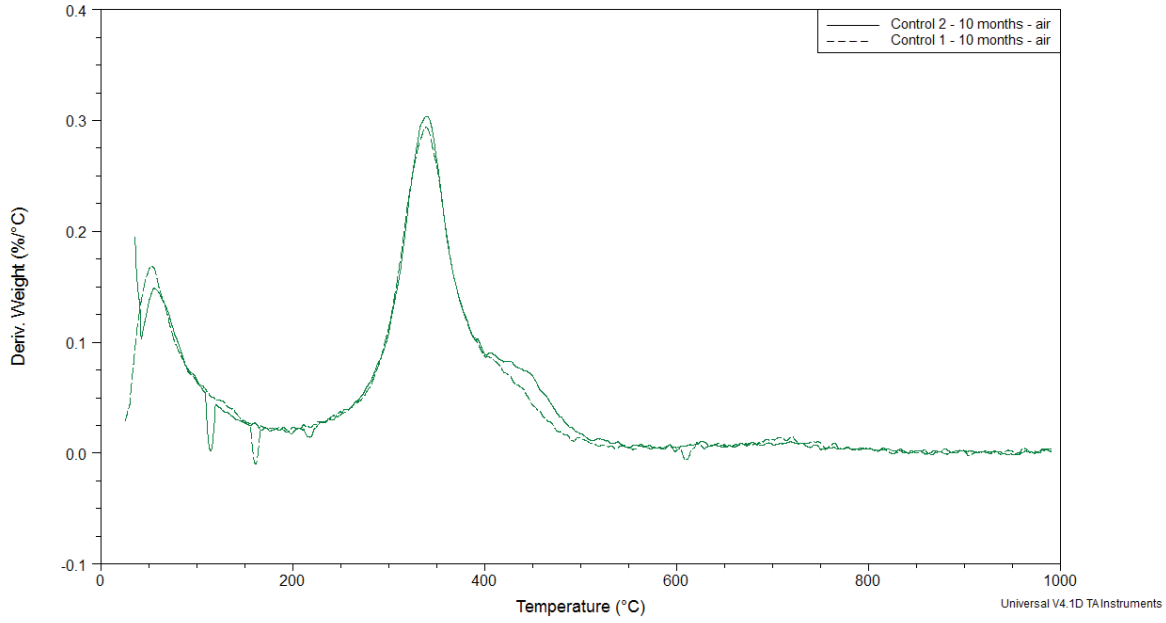

Figure 4-51. DTG curves of Loam Defleshed (Control) 1 and 2 after 10 months of burial

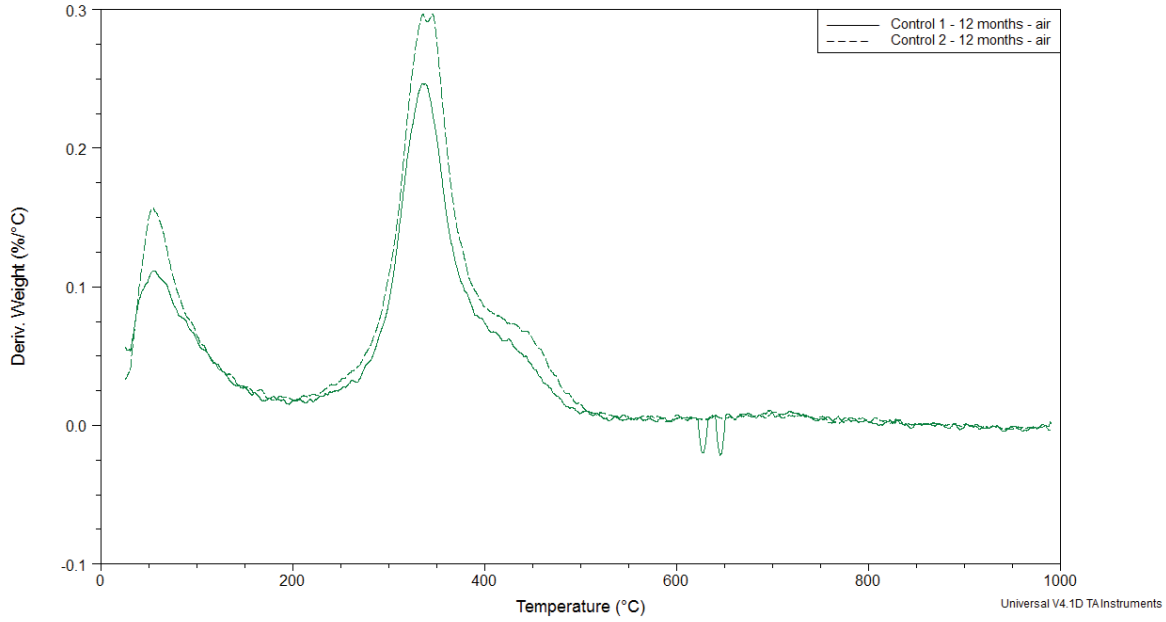

Figure 4-52. DTG curves of Loam Defleshed (Control) 1 and 2 after 12 months of burial

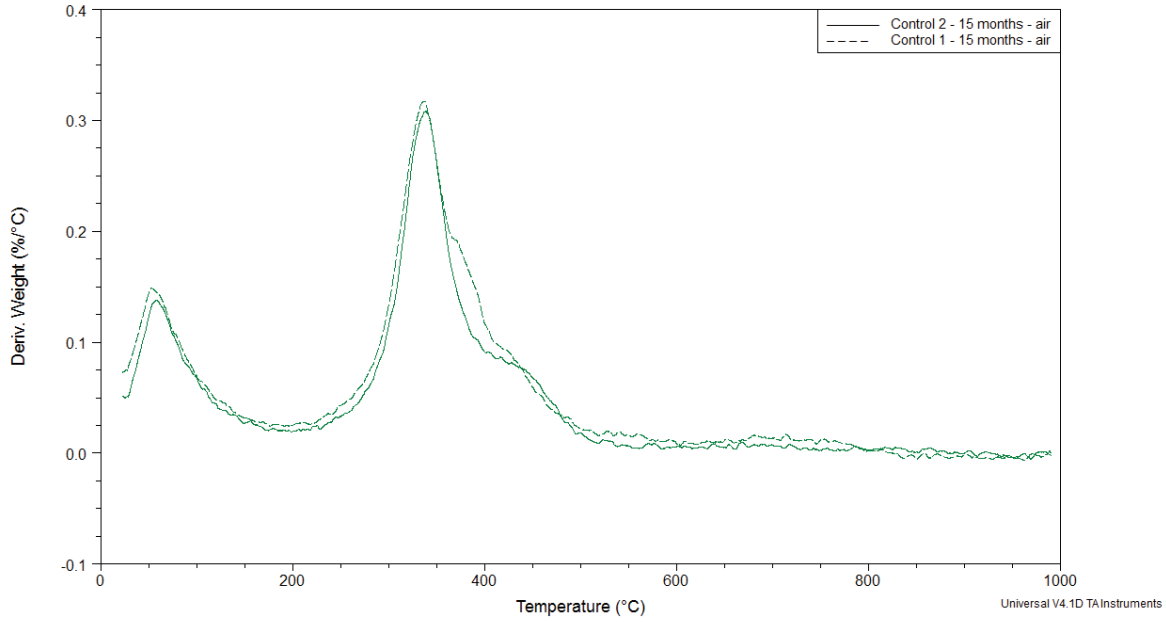

Figure 4-53. DTG curves of Loam Defleshed (Control) 1 and 2 after 15 months of burial

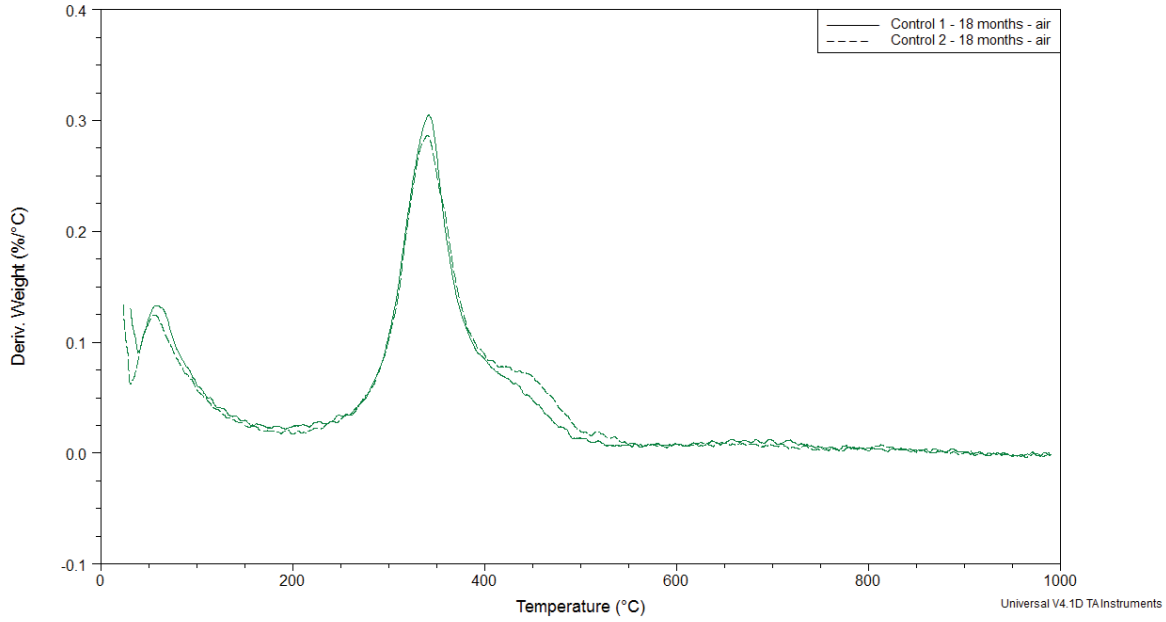

Figure 4-54. DTG curves of Loam Defleshed (Control) 1 and 2 after 18 months of burial

To visualise the differences between the results of the two loam defleshed samples more clearly, the total percentage mass loss (excluding the water loss step) was calculated and plotted as a function of the post-burial time. The results of the total mass loss as a function of the post-burial time are shown in Figure 4-55. The calculated averages and standard deviations for the results of the loam defleshed samples are presented in Table 4-4. It was observed that the total percentage mass losses were very similar for the samples of post-burial times from 1 month up to 8 months, with the values ranging from 35.3 ± 2.8 % to 32.3 ± 1.6 %. There is a certain degree of variation within the results of the samples with post-burial times greater than 10 months (38.2 ± 4.4 % to 32.4 ± 5.6 %). However, the results of the 18-month sample did not show any significant variation (33.6 ± 0.6 %). A possible explanation for the variation in the bone samples buried for a longer period could be that, as

the burial time increases, the bone's interaction with the surrounding soil, also increases. This would suggest that the extent of degradation would be more in bone samples buried for longer, and therefore more variation could exist in the rates of degradation. Assuming this to be true, the greater variation in the degradation processes may provide an explanation for the variation in the results of the samples buried for a longer period.

Based on the reproducibility results, it is important to remember that another factor responsible for the variations observed in the mass losses of the examined bone samples could be the heterogeneous nature of the bone samples. An additional factor that needs to be taken into account during interpretation of mass loss data is that 2-3 mg of each bone sample was used. The error in the absolute mass measured by the instrument was 0.1 to 0.3 mg, which possibly led to errors in the mass loss measurements being 10 % or greater (3-4 % in mass loss measurements of 30-40 %). Therefore, the variations in the mass losses observed could also be due to the heterogeneity of the samples as well as the initial mass of the samples being close to the limit of resolution of the TG instrument.

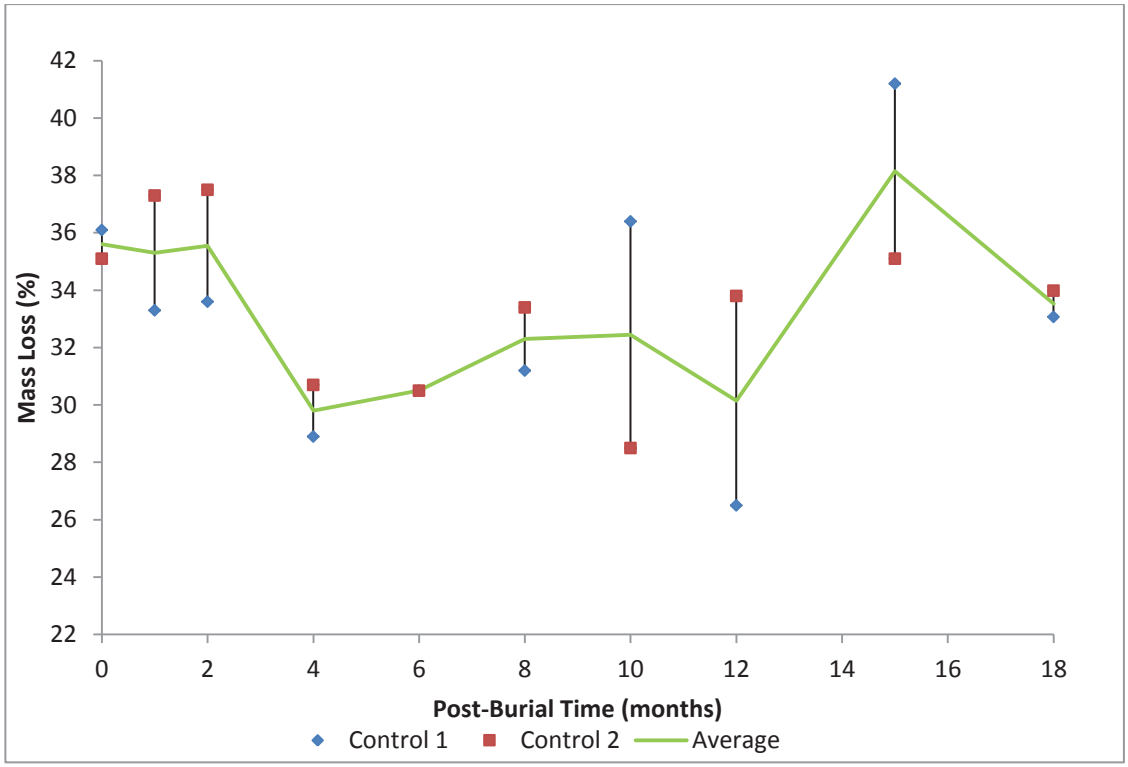

Figure 4-55. Comparison of TG results of Loam Defleshed 1 and 2 bone samples

Table 4-4. Total mass loss ± standard deviation (%) for loam defleshed bones

Post-burial time (months)	Loam Defleshed (Control) (%)
0	35.6 ± 0.7
1	35.3 ± 2.8
2	35.5 ± 2.7
4	29.8 ± 1.3
6	30.5 ± 0.0
8	32.3 ± 1.6
10	32.4 ± 5.6
12	30.1 ± 5.1
15	38.2 ± 4.4
18	33.6 ± 0.6

4.2.5 Pyrolysis Gas Chromatography-Mass Spectrometry

The technique of Py-GC-MS was investigated as a method of estimating the post-burial time of buried bones once they are discovered. Py-GC-MS is a new technique for such a purpose and was used because it is expected that diagenesis over time would lead to changes in the pyrolysis products of the bone samples. The Py-GC-MS results were examined for the presence of trends that might aid in estimating the post-burial time of bones.

4.2.5.1 Optimisation of Experimental Parameters

Optimisation of the method was performed by varying chromatographic analysis parameters and the pyrolysis temperature. This enabled the determination of the optimum analysis time required for all the compounds to elute and the pyrolysis temperature necessary for the optimum separation of the compounds eluting.

Analysis Time

Previous research by the current author has shown that the optimum oven program for an efficient run time for the Py-GC-MS of bone samples is 35 minutes (Raja *et al.*, 2010a).This involves heating of the oven from 40 °C to 300 °C at a temperature gradient of 10 °C/min. This GC oven temperature program was selected as it provided good separation of the peaks of interest in a reasonable timeframe. All compounds observable eluted in this time period and the degradation of the column was reduced by minimising the length of time at which the GC oven was kept at a high temperature.

In the current study, this result of the optimum oven program was tested and confirmed. The study examined pig bone specimens prepared under controlled burial conditions in soil with the post-mortem ages ranging from 3 to 48 months. Notable differences were observed in the data produced for younger bone specimens (<1 year) compared to specimens of greater

post-mortem age (>1 year). A run time of 35 min was shown to be enough for all the pyrolysis products to elute (Figure 4-56). Therefore, 35 min was chosen as the run time for the analysis of all the bone samples.

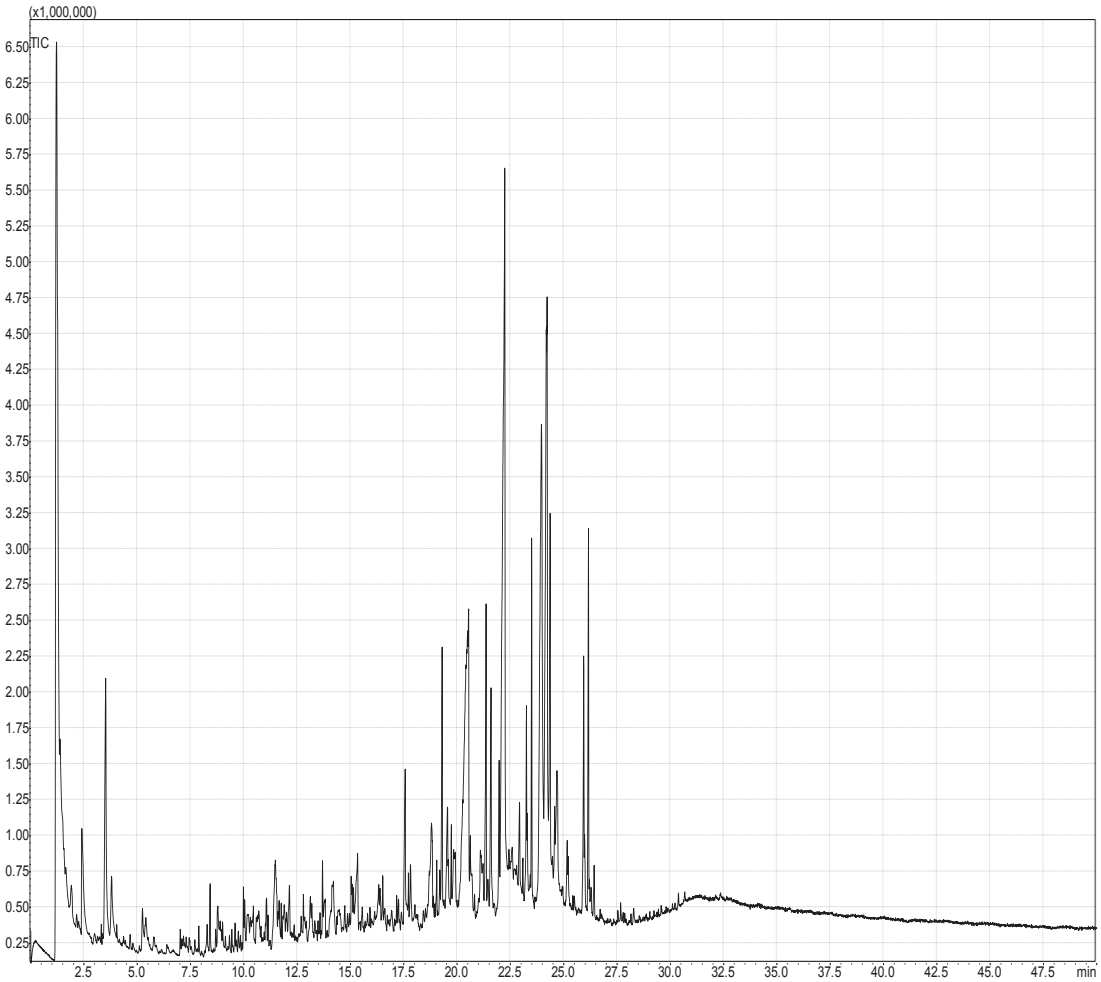

Figure 4-56. Pyrogram of bone sample showing optimum analysis time of 35 min (no peaks visible after 35 min)

Pyrolysis Temperature

Optimisation of the pyrolysis temperature was performed based on comparison of different pyrolysis temperatures utilised in previous research (Purevsuren *et al.,* 2004a; Purevsuren *et al.,* 2004b). The results of a bone sample pyrolysed at the temperatures of 300 °C, 400 °C,

450 °C, 500 °C and 600 °C are shown in Figure 4-57. This comparison was performed on five different bone samples and similar results were produced for each of the samples.

The optimum temperature of 450 °C was established and is highlighted in Figure 4-57. The temperature of 450 °C was selected as the optimum pyrolysis temperature because it provided a balance between the number of compounds eluting and the separation of the compounds. The pyrogram obtained at 450 °C showed more peaks than the pyrograms obtained at temperatures of 300 °C and 400 °C. Even though there were more peaks present in the pyrograms of 500 °C and 600 °C, the peaks in the 450 °C pyrogram were better resolved.

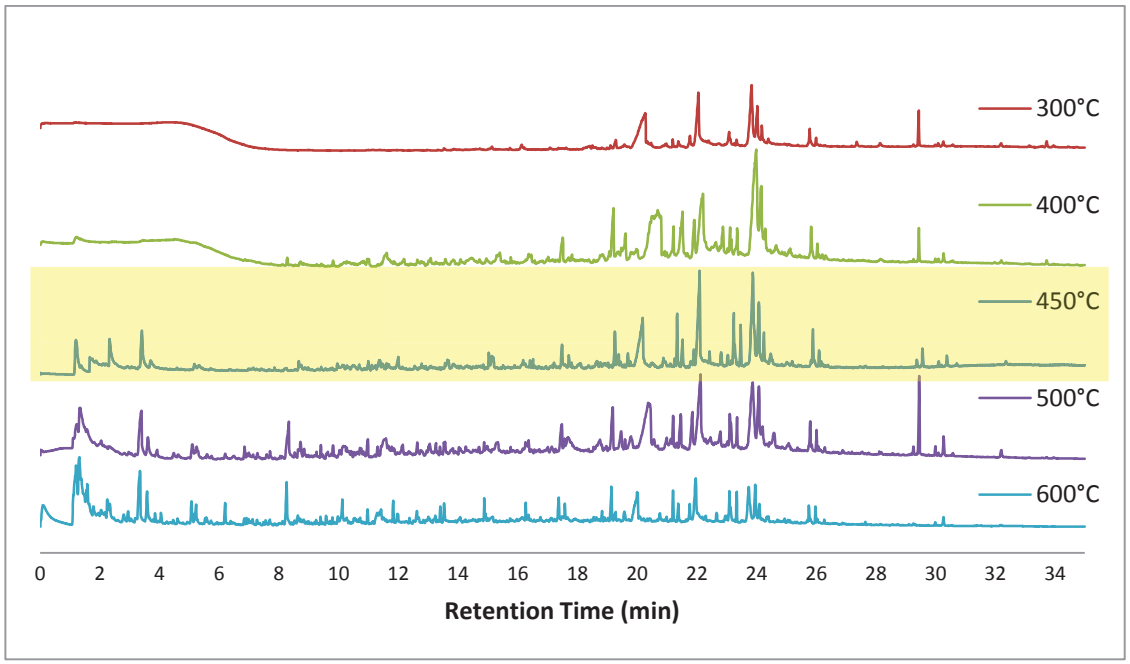

Figure 4-57. Py-GC-MS of bone at different pyrolysis temperatures

Blank Runs

At each post-burial time, a blank sample was run prior to each analysis session to check for the presence of contaminants on the column. The results of the blank samples are shown in Figure 4-58. The chromatogram shows that after a retention time of 27 minutes, column

degradation begins and, therefore, the region from 27 to 35 minutes was not included in analysis of the results.

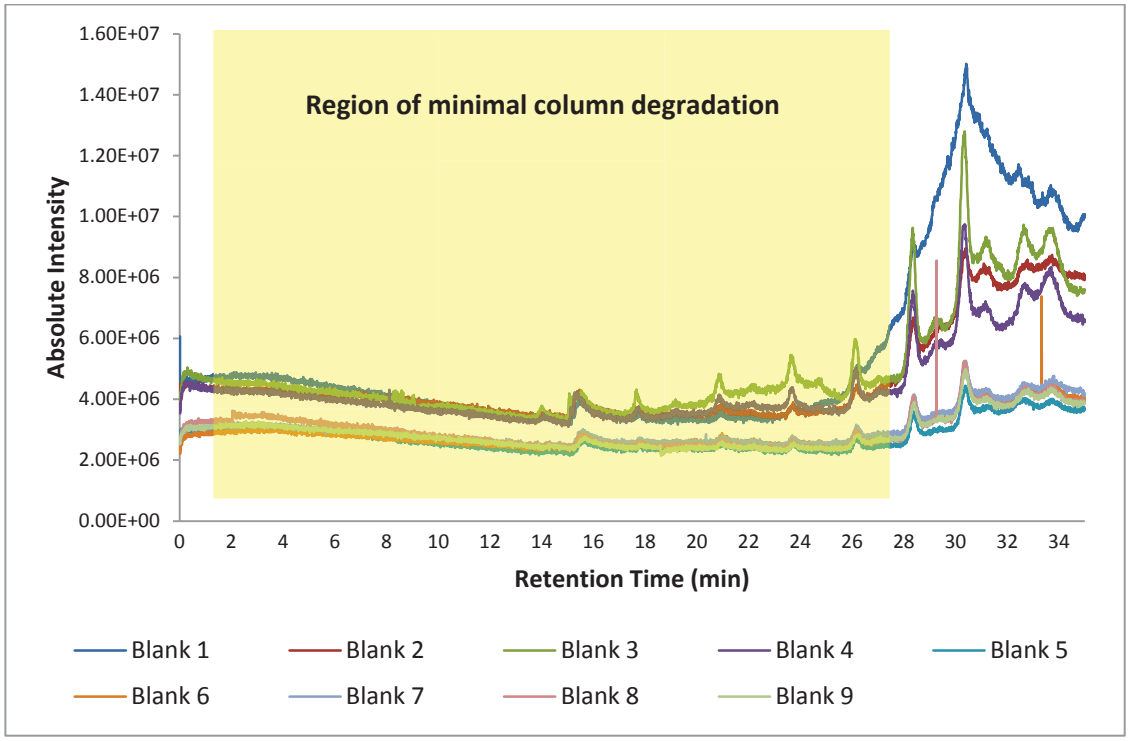

Figure 4-58. Blank runs performed on the Py-GC-MS prior to analysis at each post-burial time

The blanks performed at each post-burial time showed that no contaminants were present on the column that could potentially affect the results obtained. Figure 4-59 shows the results of the blank sample and Loam Defleshed (Control) 1 at a post-burial time of 1 month. This figure highlights that there were no compounds existing or 'sticking' to the column prior to analysis. It also demonstrates that the intensity of the peaks of the bone samples analysed were significantly higher than any peaks that arose in the blank runs. The blank runs represented a relatively flat baseline and any peaks that are observed in the blank runs are predominantly observed after a retention time of 27 minutes. Therefore, to minimise the potential for column contaminants affecting the results, the region after a retention time of

27 minutes was not included in the analysis. This figure is representative of all the different bone samples at each of the post-burial times.

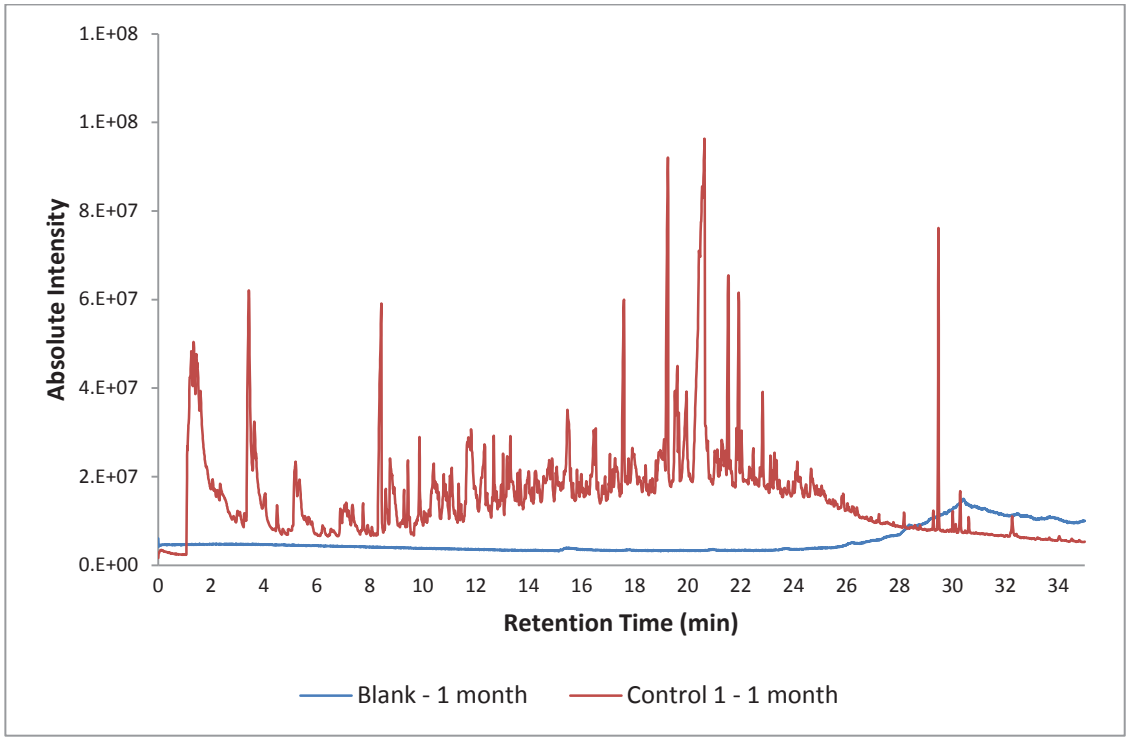

Figure 4-59. Py-GC-MS results of blank and Loam Defleshed (Control) 1 at post-burial time of 1 month

4.2.5.2 Reproducibility

Two bones with the same post-burial times and buried in the same conditions were compared to determine the reproducibility of the results of this technique. The results of the loam defleshed samples (1 and 2) for a post-burial time of 2 months are shown in Figure 4-60. The region of interest ranges from 0 – 27 minutes as any peaks eluting after a retention time of 27 minutes can be attributed to the degradation products of the column. As can be seen in the figure, the results of the loam defleshed (control) samples at a post-burial time of 2 months are reproducible, with the retention times and intensities of most of the peaks being

almost identical. Similarly, the results of all the bone samples buried in the various environments at all the post-burial times are also reproducible and are presented in Appendix 1.

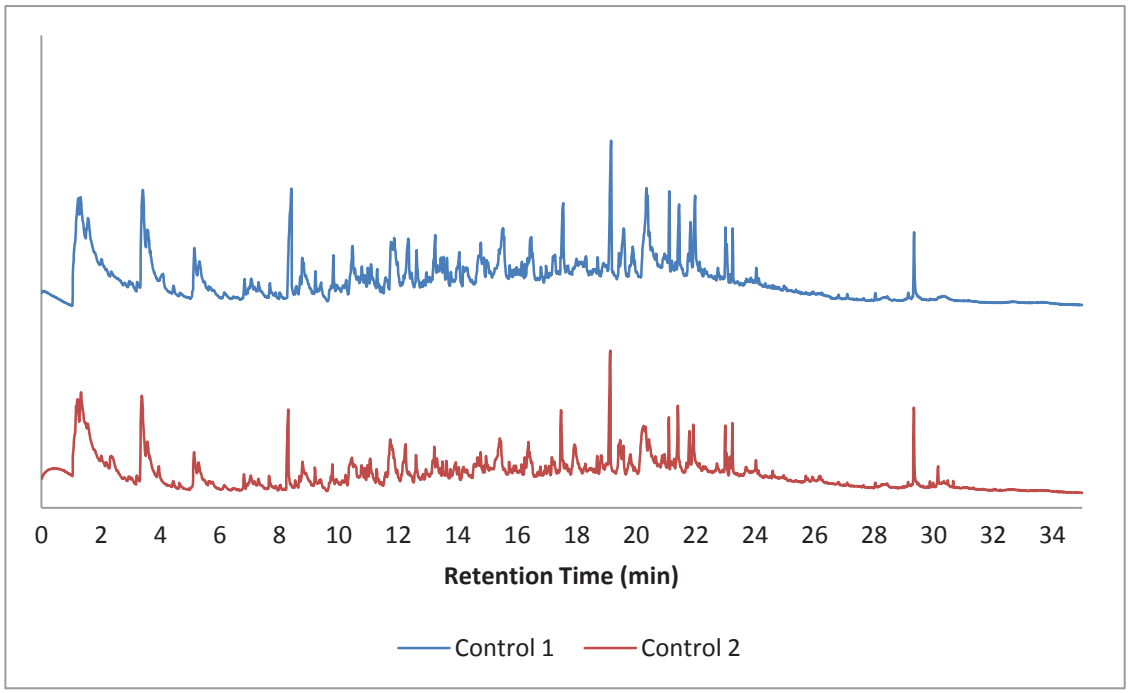

Figure 4-60. Py-GC-MS results of Loam Defleshed (Control) 1 and 2 after 2 months of burial

4.2.5.3 Visual Analysis

Bone is a complex biological material and, therefore, pyrolysis of a bone sample results in a complicated pyrogram consisting of several peaks. It is interesting to note that even on visual inspection it is possible to differentiate between bone samples up to the post-burial times of 6 months, as is evident in Figure 4-61. However, after the post-burial time of 6 months, it becomes increasingly difficult to discriminate between samples of different post-burial times using visual inspection. Therefore, statistical analysis was employed to investigate the trends that existed within the data.

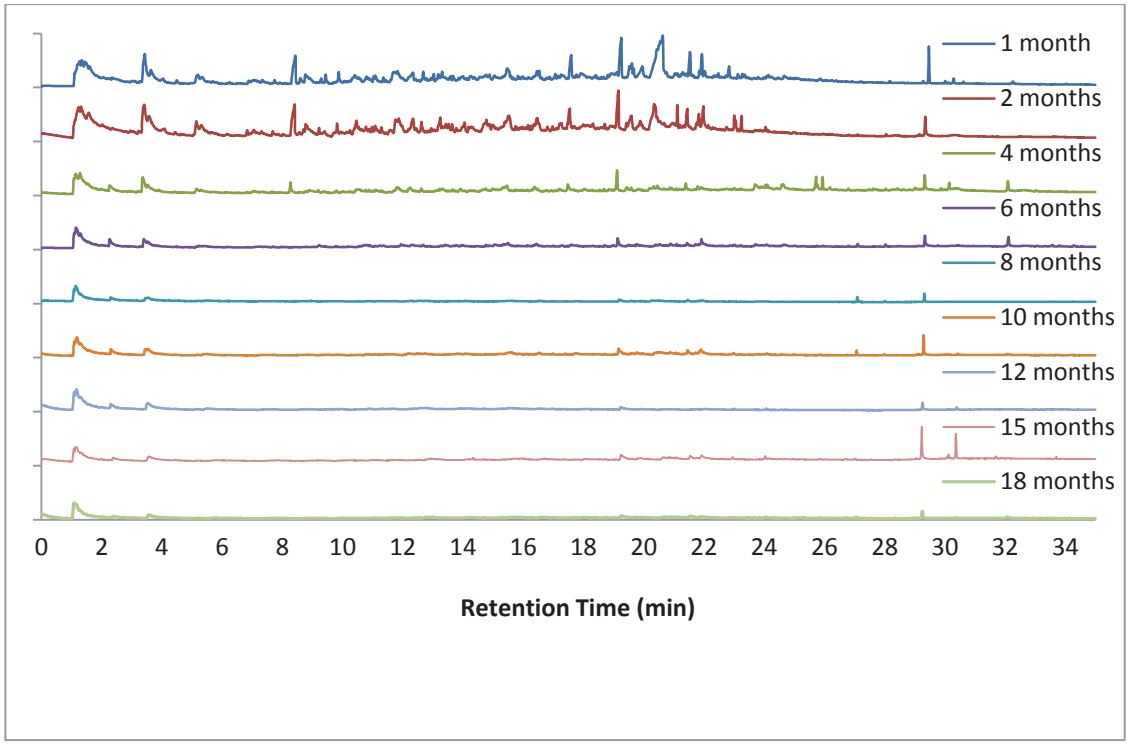

Figure 4-61. Py-GC-MS results of Loam Defleshed 1 up to post-burial time of 18 months

4.2.5.4 Linear Regression Analysis

An overall representation of the reproducibility of the results of Loam Defleshed (Control) 1 and 2 for the different post-burial times can be seen in Figure 4-62. The graph shows that the results of the younger samples (up to 8 months) are reproducible. Interestingly, results of the older samples are slightly less reproducible compared to the younger bone samples. The same trend in reproducibility has been observed in the bone samples buried in all the different burial environments.

Figure 4-62. Comparison of the Py-GC-MS results of Loam Defleshed (Control) 1 and 2

The results of the chosen method of PLS regression analysis using mean-center pre-processing and linear baseline correction on the loam defleshed (control) samples are presented in Figure 4-63. Statistical analysis of the Py-GC-MS results of the loam defleshed samples clearly show that the post-burial times of samples, up to and including 6 months, can be predicted accurately up to ± 0.5 months (R^2 = 0.99). Bones with post-burial times of 10 and 12 months can also be reliably predicted up to ± 1.0 month. For the 8, 15 and 18 months, slight variation of the predicted post-burial time value from the true post-burial time value is evident. It can be said that upon visual inspection, good correlations are observed between the actual and predicted post-burial times for all the loam defleshed samples up to post-burial times of 18 months. Therefore, using Py-GC-MS and PLS analysis, the post-burial times of defleshed bone samples buried in loam soil can be predicted relatively accurately up to 18 ± 4.0 months.

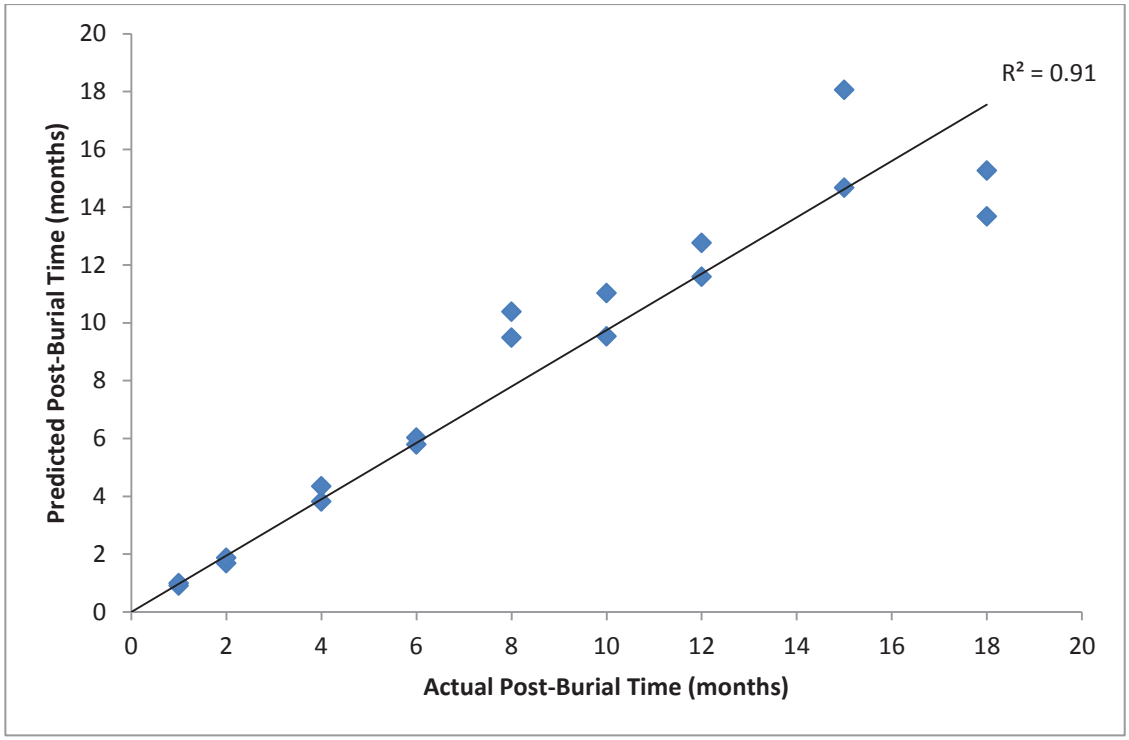

Figure 4-63. Correlation of predicted post-burial time with actual post-burial time for loam defleshed samples

Using the statistical analysis results, the retention times that were most significant in discriminating between samples of different post-burial times were determined. This was carried out by investigating the effect of each retention time on the differentiation of samples, which was recorded as a "loading" value. The retention times with high "loading" values were chosen. The compounds eluting at these retention times were identified by comparing mass spectra of the target compounds with compounds present in the NIST library. A list of these retention times and compounds is presented in Table 4-5. From the table, it can be seen that there are some compounds that are only present in the 1st month samples such as N-methyl-2-propyn-1-amine, while some compounds are present for a longer period of time such as phenoxy-phenol, which exists in samples up to a post-burial time of 12

months. Other compounds are present in different time periods during the burial e.g. hexadecanenitrile which is present during 6 to 12 months of burial.

Table 4-5. Significant retention times and corresponding compounds in loam defleshed samples at different post-burial times

		Burial Time (months)			
Retention Time (min)	**Compound Name**	**1**	**6**	**12**	**18**
8.4250	N-methyl-2-propyn-1-amine	Y	N	N	N
17.600	2,6-dihydroxy-benzoic acid	Y	Y	N	N
19.142	phenoxy-phenol	Y	Y	Y	N
20.292	trans-1,1,3,4-tetramethyl-cyclopentane	Y	N	N	Y
20.850	1-pentadecanol	N	N	N	Y
20.867	1-heptadecanol	N	N	Y	N
21.242	1-nonadecene	Y	Y	N	N
21.267	Hexadecanenitrile	N	Y	Y	N
21.442	4-amino-6-methoxy-2-(trifluoromethyl)-pyrimidine	Y	N	N	N
21.925	hexadecanoic acid	N	Y	N	Y
23.133	1,3,5-trimethyl-2-octadecyl-cyclohexane	Y	Y	Y	N
23.242	2-methyl-hexadecanal	N	Y	N	N
25.900	(Z)-9-octadecenamide	N	Y	N	N

The results of the previous Py-GC-MS bone studies by the current author used peak ratios to estimate the post-burial time of bones (Raja *et al.*, 2010a). Two different peak ratios were used – phenoxy-phenol / trans-1,1,3,4-tetramethyl-cyclopentane and octadecanoic acid / hexadecenenitrile. Both these peak ratios were chosen after visual inspection of the results and showed great promise in accurately determining the post-burial time of bones. Table 4-5 includes the peaks of phenoxy phenol and trans-1,1,3,4-tetramethylcyclopentane as compounds that are significant in discriminating between samples of different post-burial times. This confirms the results of the previous study, in which the peak ratios of these two compounds showed a distinct trend with post-burial time. On the other hand, octadecanoic

acid and hexadecenenitrile are not shown to be significantly changing with post-burial time in the results of the defleshed bone samples buried in neutral loam soil. It is noteworthy that the bone samples analysed in the previous study were buried in acidic loam soil and in the results of the acidic environment samples of the current study (Table 6-9), octadecanoic acid can be seen to be one of the compounds to be significantly changing with post-burial time as well as phenoxy phenol and trans-1,1,3,4-tetramethylcyclopentane.

4.2.6 X-ray Diffraction

As a bone interacts with its burial environment, the processes of degradation commence. These include a series of biochemical and structural changes, which eventually result in a decrease in complex organic molecules and a proportional increase in the inorganic matrix. As a result, it is expected that the degree of crystallinity of the hydroxyapatite in bones increases with increasing post-burial time. This results in narrower XRD peaks, which indicates a greater crystal size (Prieto-Castello *et al.*, 2007).

Previous research has demonstrated the potential of XRD analysis in monitoring changes in crystallinity, and hence, in dating osseous remains. However, these previous studies focussed on much older, fossilized (millions of years old) bone samples. Bartsiokas and Middleton (1992) showed that the estimation of the post-burial time of bone samples using the crystallinity indices from XRD patterns was possible for bones up to about 1 million years old.

Similarly, Prieto-Castello *et al.* (2007) demonstrated the use of this technique for samples with post-burial times up to 100 years. Their study showed that the most accurate discrimination between bone samples of different post-burial times was achieved when two post-burial time groups were established (PMI < 10 years and PMI > 10 years). The results of the study also showed the cortical section of the bone to be more efficient in this

discrimination compared to the cancellous section of the bone. They hypothesised that the cortical zone is less prone to the processes of degradation as it consists of a more homogeneous structure.

Therefore, to monitor diagenesis in terms of changes in the inorganic phase of the bone samples, XRD analysis was employed. The cortical section of the bone was used for analysis.

4.2.6.1 Reproducibility

To test the reproducibility of this technique, two sets of samples buried in the same conditions were analysed and compared. Since XRD analysis on the bone samples was a time-consuming process, only the duplicates of the loam defleshed (control) samples were analysed using this technique, rather than the duplicates of all the samples. Results of Loam Defleshed 1 and 2 at the post-burial times of 1, 6, 12 and 18 months are shown in Figures 4-64 to 4-67. The diffraction patterns of both the samples were very similar at each of the post-burial times shown in the figures mentioned above. The diffraction patterns of the loam defleshed samples at the other post-burial times are presented in Appendix 2. Both the loam defleshed samples showed similar XRD results at each of the investigated post-burial times. Therefore, the technique of XRD can be said to be reproducible for bone samples analysed up to and including 18 months of burial.

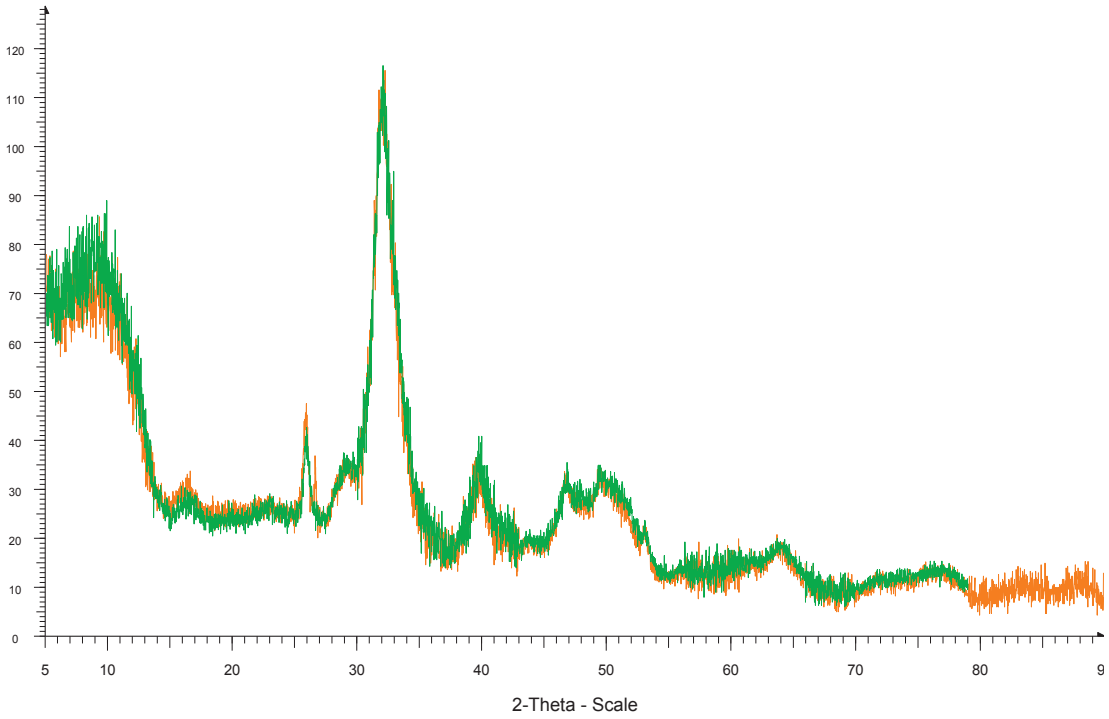

Figure 4-64. XRD results of Loam Defleshed 1 and 2 (orange and green) after 1 month of burial

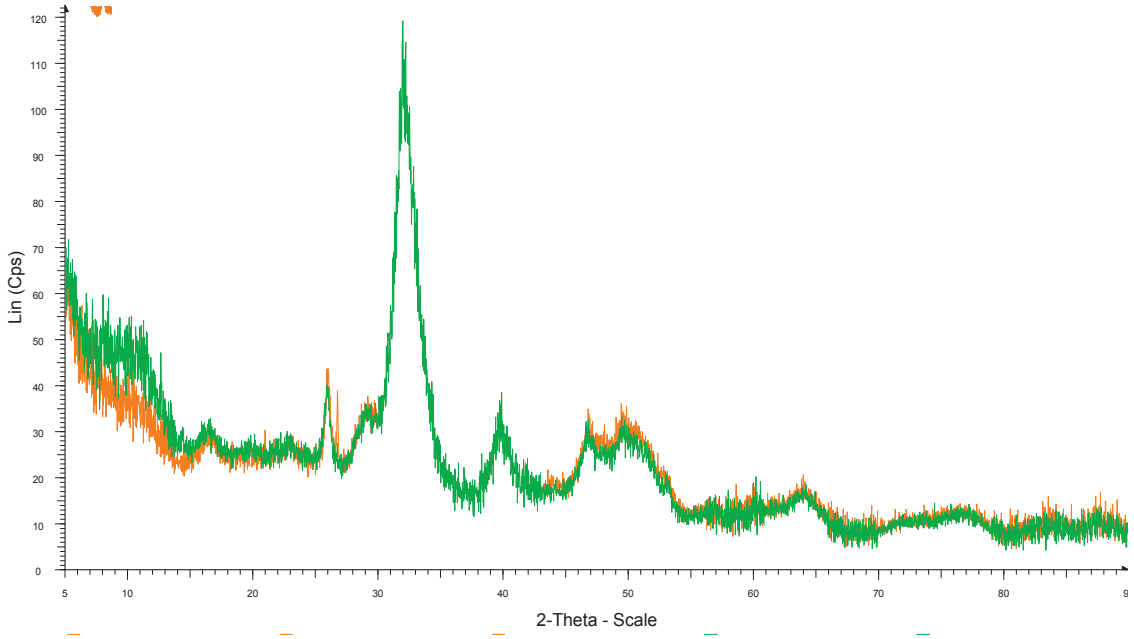

Figure 4-65. XRD results of Loam Defleshed 1 and 2 (orange and green) after 6 months of burial

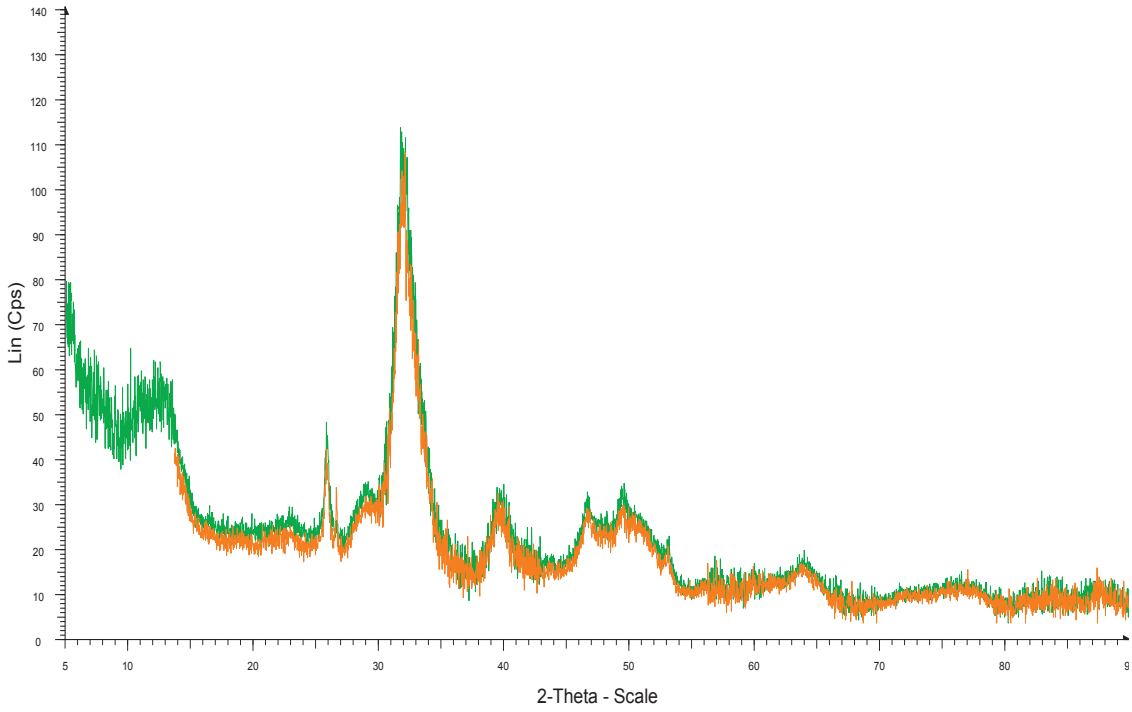

Figure 4-66. XRD results of Loam Defleshed 1 and 2 (orange and green) after 12 months of burial

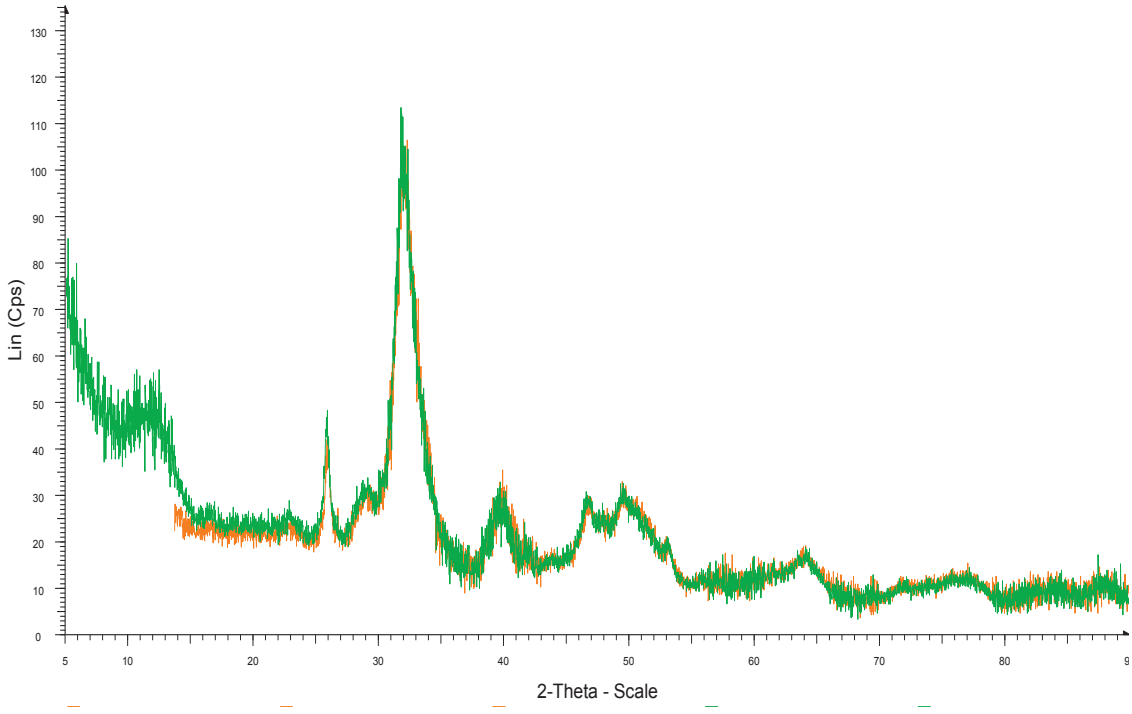

Figure 4-67. XRD results of Loam Defleshed 1 and 2 (orange and green) after 18 months of burial

4.2.6.2 Analysis

The technique of XRD is used for the analysis of the inorganic content of bones. However, the inorganic content of bones takes years to break down (Bartsiokas and Middleton, 1992; Kyle, 1986), and therefore, bone samples with post-burial times of less than 2 years are all expected to result in similar diffraction patterns. This is confirmed by the results of the current study, as the diffraction patterns of both the loam defleshed samples show very similar patterns for all post-burial times up to and including 18 months (Figures 4-68 and 4-69). Bone samples recovered from the other burial conditions investigated in the study also resulted in similar diffraction patterns for the different post-burial times. These diffraction patterns can be found in Appendix 3.

The inorganic component of bone consists of carbonated-hydroxyapatite, and the characteristic peaks of this compound can be seen using XRD analysis. The diffraction patterns of all the bone samples investigated, regardless of the burial condition or the bone pre-treatment, showed many characteristic peaks corresponding to HA. The x-ray diffraction patterns of Loam Defleshed 1 and 2 at all the post-burial times are shown in Figures 4-68 and 4-69, respectively. The primary characteristic apatite peak (Li *et al.*, 1993) consisting of an overlap of the diffraction bands of three crystalline spacings (211), (112) and (300) can be seen in the diffraction patterns of all the loam defleshed samples at 32 °2θ. Other characteristic HA peaks presented in Table 4-6 can also be seen in the diffraction patterns of all the loam defleshed samples. This confirms the results of previous research (Bartsiokas and Middleton, 1992; Kyle, 1986) that differences can only be seen when fossilised bones are compared with young bones. Relatively young bones (18 months or younger) do not show differences in XRD results, when compared with each other.

All the peaks mentioned in Table 4-6 can also be found in the diffraction patterns of all the bone samples investigated, which are presented in Appendix 3. This indicates that the different burial conditions and the different bone pre-treatment methods do not have an effect on the inorganic content of young bones (post-burial times of 18 months and less). These results also confirm the results of previous research which demonstrated that differences in the inorganic content of bones can only be seen if fossilized bones are compared with modern bones (Bartsiokas and Middleton, 1992; Kyle, 1986). It is important to note the broadness of the peaks which is consistent with carbonate hydroxyapatite of low crystallinity.

Table 4-6. Characteristic HA crystalline spacings

Degrees (2θ)	Crystalline Spacing
26	(002)
29	(210)
31	(211)
32	(112)
33	(300)
34	(202)
40	(310)
47	(222)

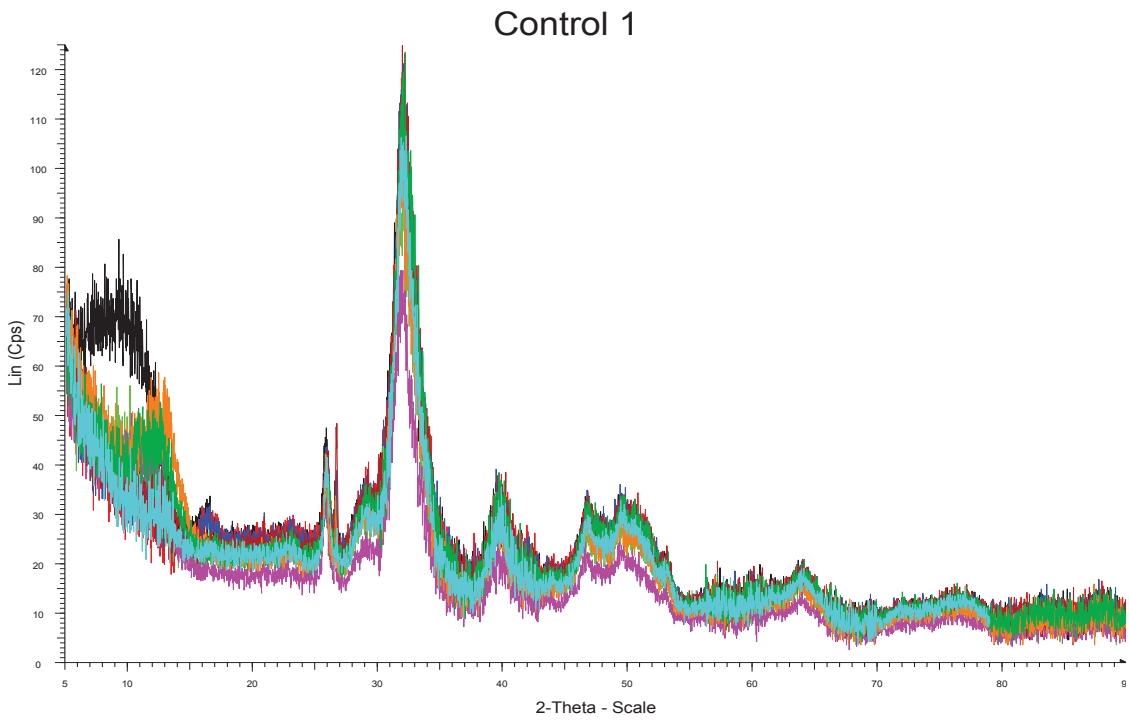

Figure 4-68. XRD results – Loam Defleshed (Control) 1 at all post-burial times

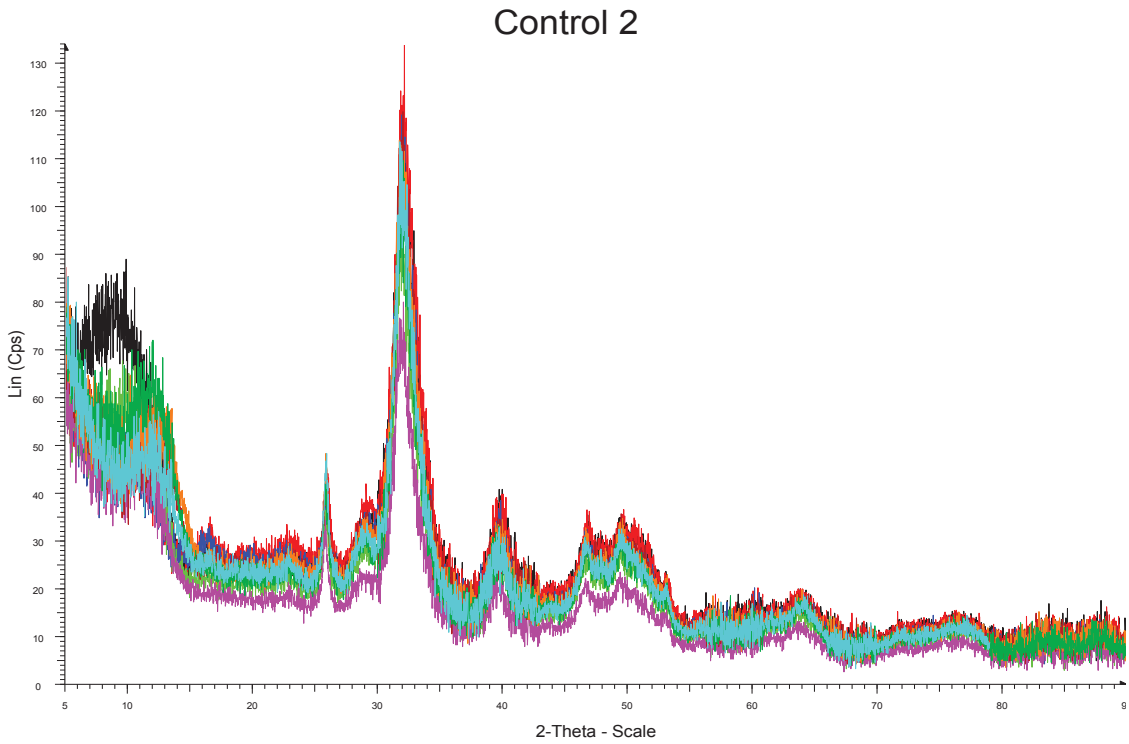

Figure 4-69. XRD results – Loam Defleshed (Control) 2 at all post-burial times

To estimate the sharpness of the peaks in the XRD patterns, a similar method to the one employed by Bartsiokas and Middleton (1992) was used. The intensity of the diffraction band of the crystalline spacing (300) of hydroxyapatite was used to calculate the crystallinity index, I:

$$I = (a)/(b) \qquad \text{Equation 4.1}$$

where 'a' and 'b' are estimates of the local and general background in the region, respectively. Two different crystallinity indices were calculated for each XRD pattern using an XRD peak at a lower degree (2θ) value and one at a higher degree (2θ) value. Crystallinity index 1 was calculated using the (300) and the (310) crystalline spacing peaks (Figure 4-70) and crystallinity index 2 was based on the (002) and the (300) crystalline spacing peaks (Figure 4-71).

Modern and archaeological bones (up to 2.8 millions of years) have previously been demonstrated to result in crystallinity indices of 0.0 to 7.2 with an error of 0.1 (Bartsiokas and Middleton, 1992). These results show that older bone samples produce higher crystallinity indices, i.e. sharper XRD patterns.

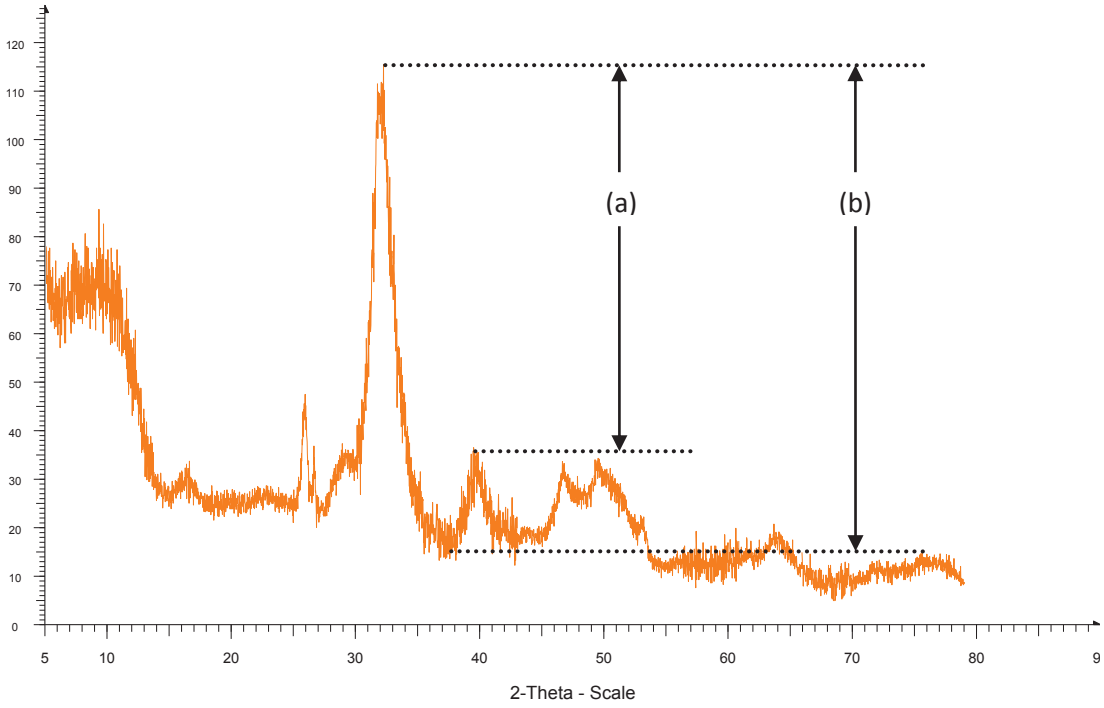

Figure 4-70. Calculation of crystallinity index 1 (a = (310) and b = (300) crystalline spacing)

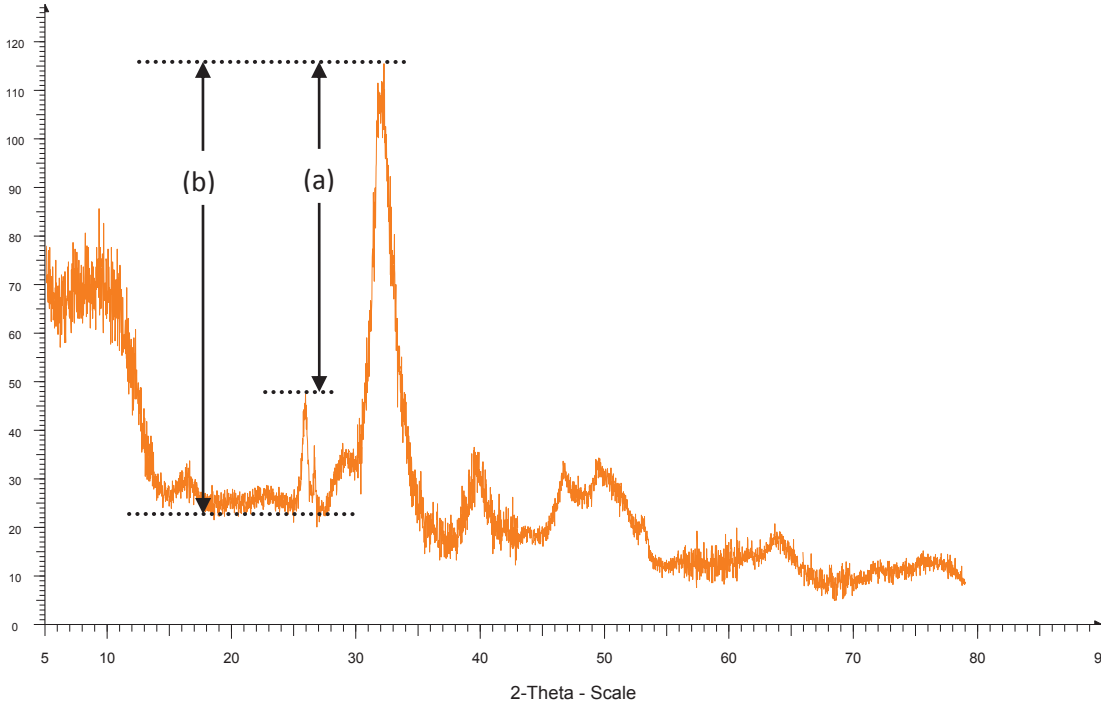

Figure 4-71. Calculation of crystallinity index 2 (a = (002) and b = (300) crystalline spacing)

Table 4-7. Crystallinity Indices of Loam Defleshed 1 and 2 bone samples using XRD results

	Crystallinity Index 1		Crystallinity Index 2	
Post-burial time (months)	**Loam Defleshed 1**	**Loam Defleshed 2**	**Loam Defleshed 1**	**Loam Defleshed 2**
1	0.79	0.76	0.72	0.78
6	0.76	0.79	0.74	0.76
12	0.80	0.80	0.73	0.70
18	0.76	0.78	0.74	0.69

The results of the calculations of crystallinity indices 1 and 2 for Loam Defleshed 1 and 2 bone samples are presented in Table 4-7. For both Loam Defleshed 1 and 2, crystallinity index 1 ranges from 0.76 to 0.80, and crystallinity index 2 ranges from 0.69 to 0.78. These crystallinity indices are very similar and, therefore, show that the inorganic component of the bone samples has not changed significantly in the 18 months of burial.

4.3 Fleshed Bones

4.3.1 Visual Observations

For the fleshed samples, the colour of the bones changed from a brown-orange colour to a light brown colour after 6 months of burial. No cracks or holes were evident in any of the fleshed 2 samples, however, in the fleshed 1 samples, tiny holes were evident in the bones with post-burial times of 10 and 12 months. No odour was present in any of the bone samples. Also, for both sets of samples, white fungus was present on the bones recovered after 1 and 2 months of burial. For the fleshed 2 samples, white fungus was also present on the 4-month samples. Like the defleshed samples, both sets of the fleshed samples contained cockroaches in the containers after and including 15 months of burial. The images of the

fleshed bone samples are presented in Figure 4-1 and the physical characteristics of fleshed bone samples are reported in Table 4-8.

Table 4-8. Physical characteristics of defleshed and fleshed bone samples analysed at each post-burial time (n = 36)

Bone Pre-treatment	Average Length (cm)	Standard Deviation (±)	Average Diameter (cm)	Standard Deviation (±)	Average Mass (g)	Standard Deviation (±)
Defleshed (Control)	8.2	0.49	1.5	0.46	6.5	1.9
Fleshed	7.6	0.86	1.5	0.34	7.2	3.6

4.3.2 Thermogravimetric Analysis

4.3.2.1 Reproducibility

The TG results of the total mass loss as a function of the post-burial time of the fleshed bones buried in loam soil is shown in Figure 4-72. The averages and standard deviations of these samples for the various post-burial times are presented in Table 4-9. The mass losses are reproducible for all the bone samples, except for the ones buried for 2, 4, 12 and 15 months. The change in mass loss ranged between 29.8 ± 0.1 % and 35.2 ± 0.5 %. One of the objectives of the study was to compare the defleshed (control) samples with the fleshed samples. Interestingly, it was found that the variation within the results of the fleshed bones was lower when compared to the defleshed bones. It is important to note that the fleshed samples used in this study had the entire bone completely covered with flesh. More reproducible results were obtained using the fleshed bones, when compared to the defleshed bones. This can be attributed to the fact that the flesh covering the bone may act as a protective barrier and restrict the movement of substances into and out of the bone, which might slow down the degradation processes that would have occurred otherwise.

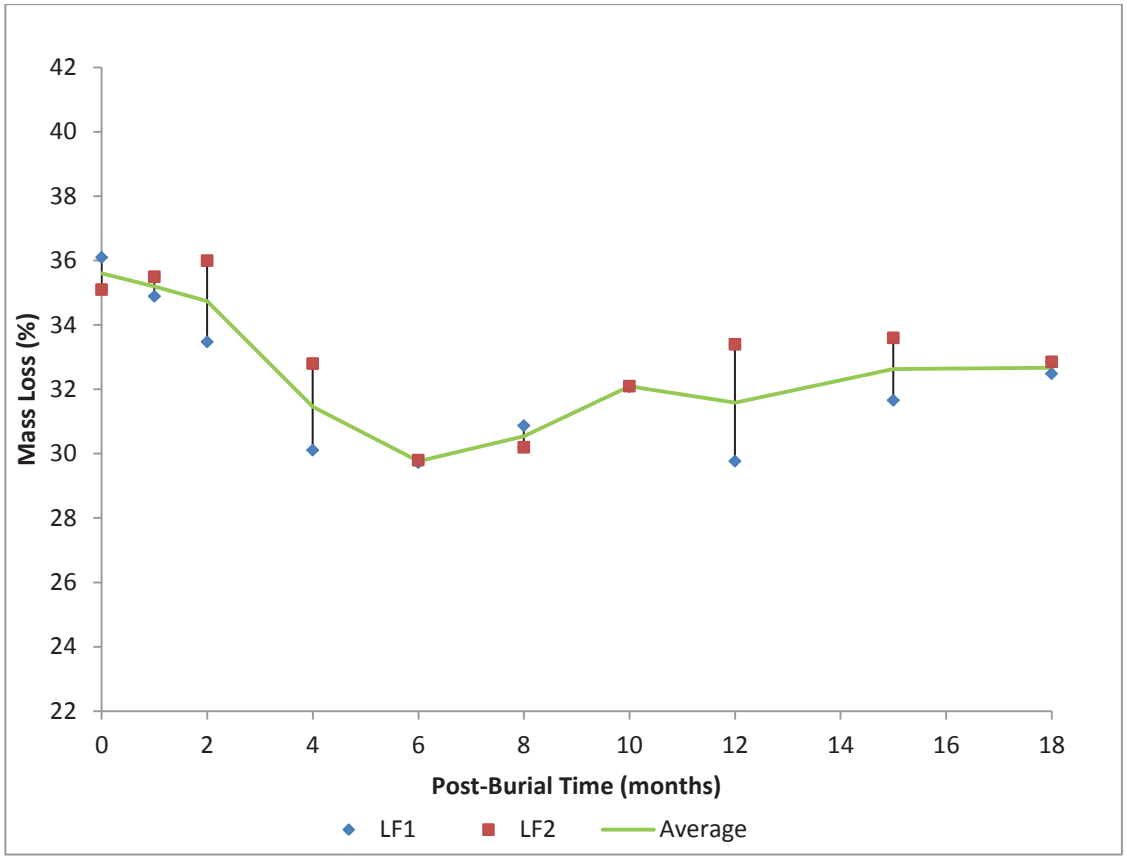

Figure 4-72. Comparison of TG results of Loam Fleshed 1 and 2 bone samples

Table 4-9. Total mass loss ± standard deviation (%) at each post-burial time for defleshed and fleshed bones (n = 40)

Post-burial time (months)	Loam Defleshed (%)	Loam Fleshed (%)
0	35.6 ± 0.7	35.6 ± 0.7
1	35.3 ± 2.8	35.2 ± 0.5
2	35.5 ± 2.7	34.7 ± 1.8
4	29.8 ± 1.3	31. 4 ± 1.9
6	30.5 ± 0.0	29.8 ± 0.1
8	32.3 ± 1.6	30.6 ± 0.5
10	32.4 ± 5.6	32.1 ± 0.0
12	30.1 ± 5.1	31.6 ± 2.6
15	38.2 ± 4.4	32.7 ± 1.4
18	33.6 ± 0.6	32.7 ± 0.3

4.3.3 Pyrolysis Gas Chromatography-Mass Spectrometry

For comparison with the defleshed bone samples, fleshed bone samples were also prepared and analysed using Py-GC-MS. Results of the PLS regression analysis using mean-center pre-processing and linear baseline correction on the fleshed bone samples buried in loam soil are presented in Figure 4-73.

Statistical analysis of the fleshed bone samples clearly shows that samples with post-burial times up to 18 months can be accurately predicted, with only minimal discrepancies present in the results of bone samples buried for 4 and 18 months. The coefficient of determination of the actual post-burial time compared with the predicted post-burial time was $R^2 = 0.98$, $p < 0.05$. Therefore, it can be said that a good correlation is observed and using Py-GC-MS and PLS analysis, the post-burial times of fleshed bone samples buried in loam soil can be estimated relatively accurately up to 18 ± 1.4 months.

It is interesting to note that the coefficient of determination of the fleshed bone samples ($R^2 = 0.98$) is higher than that of the defleshed bone samples ($R^2 = 0.91$). In a study conducted by Nicholson (1996), the differences between bones buried with flesh intact versus defleshed were evaluated. The defleshed bones degraded more rapidly than fleshed bones in all the samples (except for one type of fish). An explanation for this result suggested by Nicholson was that micro-organisms had easier access to bones when there was no flesh covering them. This reduction in the amount of micro-organism activity in bones with flesh might explain the higher coefficient of determination observed for fleshed bones as variations introduced as a result of micro-organism activity would be limited in fleshed bones.

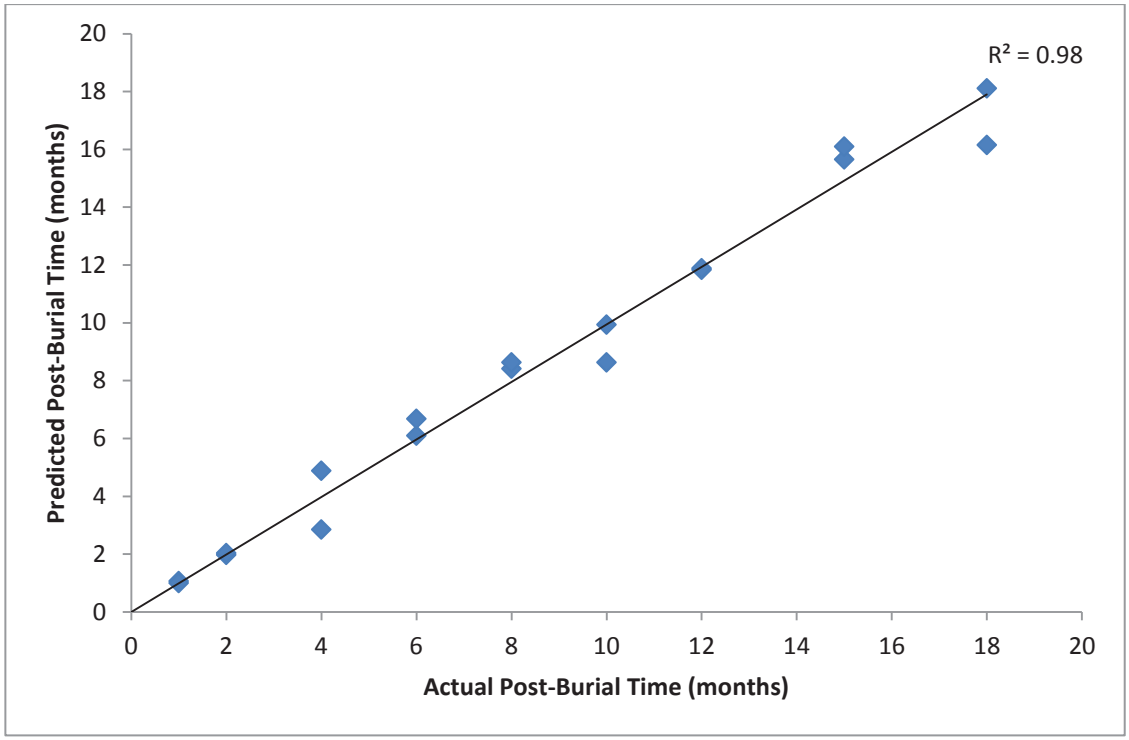

Figure 4-73. Correlation of predicted post-burial time with actual post-burial time for fleshed bone samples 1 and 2

The most influential compounds for discriminating between samples of different post-burial times were determined. These compounds and their corresponding retention times are recorded in Table 4-10. From the table, it can be seen that there are some compounds that are only present in the 1st month samples such as hexadecanal, while some compounds are present for the entire 18 months of burial, such as phenoxy-phenol. In addition, there are other compounds that are present either in the first 6 months such as butoxy-benzene or in the last 6 months of burial such as trans-1,1,3,4-tetramethylcyclopentane. There are also some compounds such as 2,6-dihydroxy-benzoic acid that are present only for the first 12 months of burial. This change in the structural composition of the bone collagen has the potential to provide markers for post-burial time estimation.

Table 4-10. Significant retention times and corresponding compounds in fleshed bone samples at different post-burial times

		Burial Time (months)			
Retention Time (min)	**Compound Name**	**1**	**6**	**12**	**18**
8.3920	2-cyclohexen-1-one	Y	Y	N	N
17.600	2,6-dihydroxy-benzoic acid	Y	Y	Y	N
19.142	phenoxy-phenol	Y	Y	Y	Y
20.292	trans-1,1,3,4-tetramethylcyclopentane	N	N	Y	Y
20.583	(Z)-2-undecene	Y	N	N	N
21.092	butoxy-benzene	Y	Y	N	N
21.233	Hexadecanal	Y	N	N	N
21.925	hexadecanoic acid	N	Y	N	N
22.100	octadecanoic acid	N	N	Y	N
23.250	Indole	Y	N	N	N

4.4 Degreased Bones

4.4.1 Visual Observations

The colour of bone samples that were degreased prior to burial was initially white but after 6-8 months of burial, it changed to pale brown-yellow. Tiny holes started appearing on the bones after 4 months of burial and persisted till the end of the study. No odour was evident in any of the bone samples. For both sets of samples, white fungus was present in the 2-month samples. For the Loam Degreased 2 bone samples, the white fungus was not evident after the first 4 months of burial, however, in the Loam Degreased 1 bone samples, white-purple fungus could be seen after 15 months of burial until the end of the study. The physical characteristics of the bone samples are reported in Table 4-11.

Table 4-11. Physical characteristics of defleshed and degreased bone samples analysed at each post-burial time (n = 36)

Bone Pre-treatment	Average Length (cm)	Standard Deviation (±)	Average Diameter (cm)	Standard Deviation (±)	Average Mass (g)	Standard Deviation (±)
Defleshed (Control)	8.2	0.49	1.5	0.46	6.5	1.9
Degreased	9.5	0.92	1.5	0.29	11.1	2.4

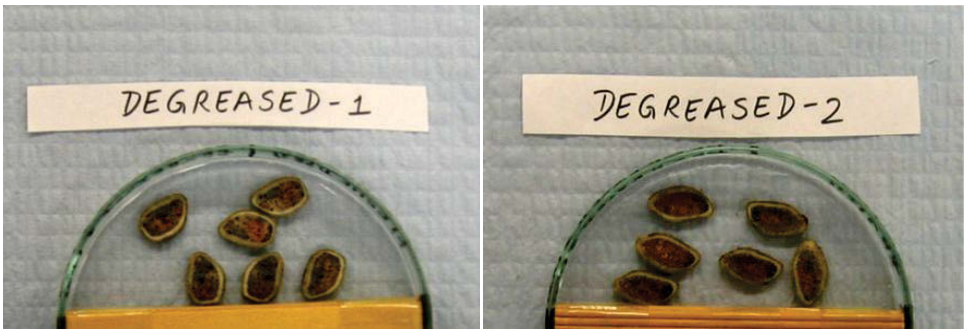

Figure 4-74. Photographs of degreased bone samples after burial

Images of bone samples that were defleshed and degreased are shown in Figure 4-74. It was observed that the bones which were defleshed and degreased prior to burial were completely white, while the bones that were defleshed only and defleshed and boiled prior to burial were white-brown-grey in colour. After one month of burial, the degreased bones were orange and black in the cancellous section and white in the cortical section. This was the same for all the degreased bones at the different post-burial times. Therefore, it is possible to identify the bones that have been degreased prior to burial compared to bones that were defleshed only and defleshed and boiled prior to burial.

4.4.2 Thermogravimetric Analysis

4.4.2.1 Reproducibility

Figure 4-75 shows the TG results of the bone samples that were degreased before being buried in loam soil. Overall, the mass loss averages for the degreased bone samples ranged from 29.0 ± 1.2 % to 36.6 ± 2.2 %. Interestingly, the mass loss results of both the degreased bones are similar for the samples buried for 1 and 18 months. Variations are present in the results of the remaining degreased bones and indicate that degreasing prior to burial may have an effect on the processes of degradation and the rates at which these processes occur once a bone has been buried. The total mass loss at each post-burial time for degreased bones is presented in Table 4-12.

Table 4-12. Total mass loss ± standard deviation (%) at each post-burial time for defleshed and degreased bones (n = 40)

Post-burial time (months)	Loam Defleshed (%)	Loam Degreased (%)
0	35.6 ± 0.7	35.6 ± 0.7
1	35.3 ± 2.8	36.6 ± 0.4
2	35.5 ± 2.7	31.5 ± 3.1
4	29.8 ± 1.3	30.4 ± 1.2
6	30.5 ± 0.0	29.0 ± 1.2
8	32.3 ± 1.6	34.2 ± 1.8
10	32.4 ± 5.6	36.6 ± 2.2
12	30.1 ± 5.1	32.5 ± 0.6
15	38.2 ± 4.4	33.1 ± 1.5
18	33.6 ± 0.6	34.1 ± 0.1

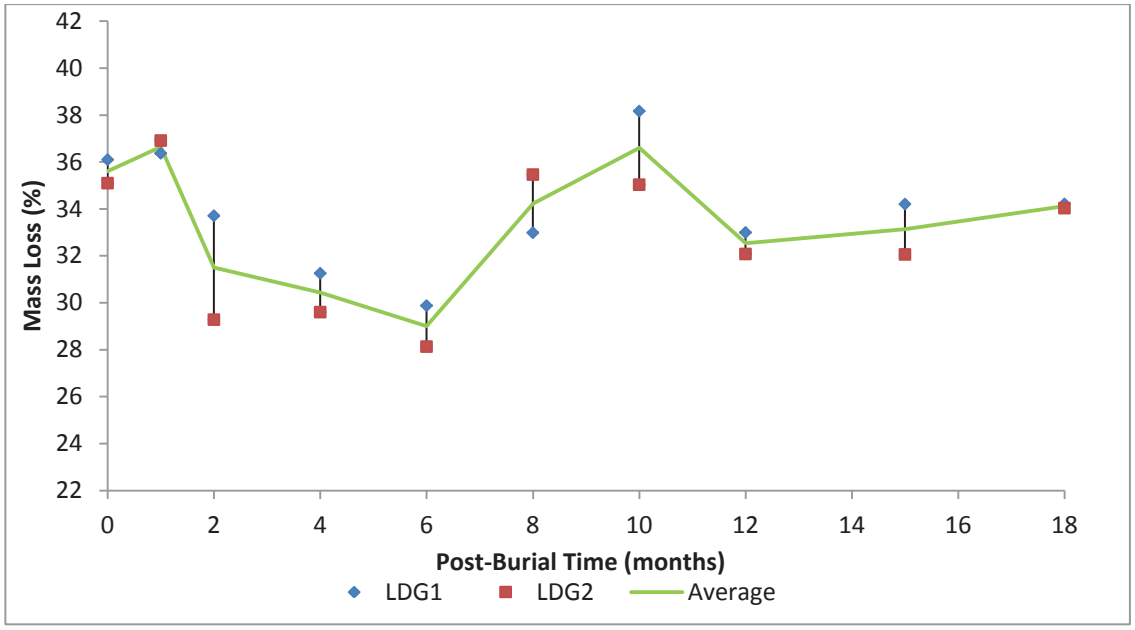

Figure 4-75. Comparison of TG results of Loam Degreased 1 and 2 bone samples

4.4.3 Pyrolysis Gas Chromatography-Mass Spectrometry

The degreased bones were also analysed using Py-GC-MS and linear regression analysis and the results are presented in Figure 4-76.

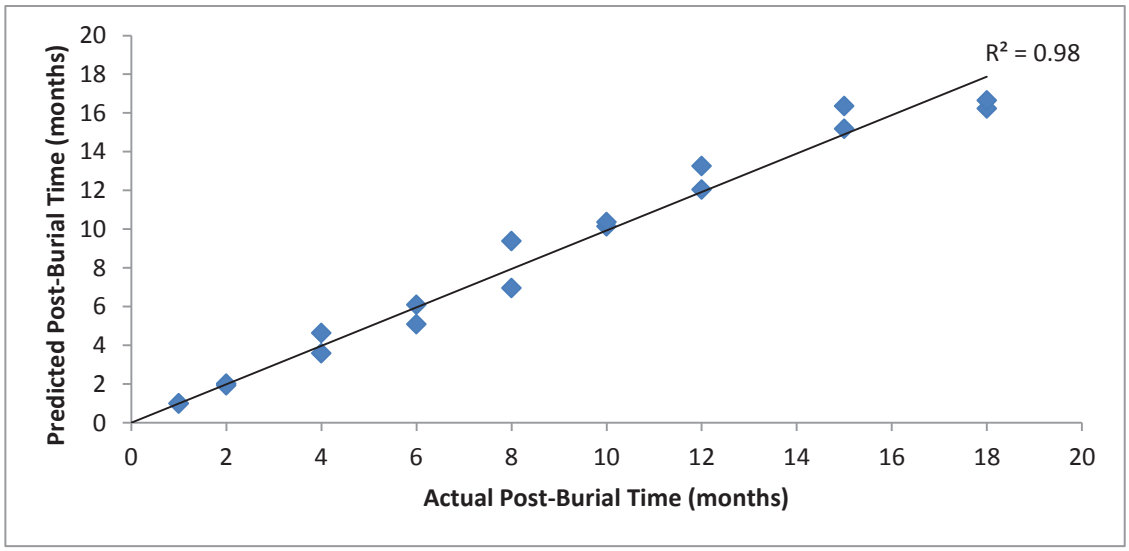

Figure 4-76. Correlation of predicted post-burial time with actual post-burial time for bone samples degreased prior to burial

The results of Py-GC-MS show that there is a strong correlation between the actual and the predicted post-burial times of bone samples that were degreased prior to burial. Only two bone samples out of nine samples showed slight deviation from the linear trend ($R^2 = 0.98$, $p < 0.05$) – bones with post-burial times of 8 and 18 months. Based on these results, it can be stated that the post-burial times of degreased bones can also be estimated using Py-GC-MS up to 18 ± 1.4 months of burial.

The pyrolysis products identified during different stages of burial are presented in Table 4-13. Degreasing a bone involves removing the fats and oils present in the bone. It is interesting to note that many compounds that were present in the bones that were defleshed only are also present in the bones that were defleshed and degreased. It is also important to note that the compound, 1-mono-olein (fat), is only present in the degreased bone samples, during the first month of burial. This indicates that the process of degreasing was not successful in removing all the fats and oils prior to burial.

Table 4-13. Significant retention times and corresponding compounds in bone samples degreased prior to burial at different post-burial times

		Burial Time (months)			
Retention Time (min)	**Compound Name**	**1**	**6**	**12**	**18**
8.3670	Pyrazole	N	N	Y	N
12.683	Benzenepropanenitrile	Y	Y	Y	N
19.142	phenoxy-phenol	N	Y	N	Y
19.250	3-phenoxy-phenol	Y	N	N	N
20.192	isopropyl myristate	N	Y	N	N
20.267	trans-2-methyl-5-(1-methylethyl)-cyclohexanone	N	Y	N	N
20.283	N,N-dipropyl-1-propanamine	Y	N	N	N
20.875	9-octyl-8-heptadecene	N	Y	N	N
21.267	hexadecanenitrile	Y	Y	Y	Y
22.100	octadecanoic acid	Y	Y	Y	N
23.158	hexadecenenitrile	Y	Y	Y	Y
24.017	1-mono-olein	Y	N	N	N
25.900	(Z)-9-octadecenamide	Y	N	N	N

4.5 Boiled Bones

4.5.1 Visual Observations

The boiled bone samples changed in colour from a white-red combination to a white-pale brown/yellow combination after the 2nd month of burial and remained that colour until the end of the study. Small holes started appearing after 4-6 months of burial and were evident in all the samples until the end of the burial study. No odour was present in any of the bone samples. No microbial activity was noticed on any of the bone samples except for the 2-

month Loam Boiled 1 sample. The physical characteristics of the bone samples are reported in Table 4-14.

Table 4-14. Physical characteristics of defleshed and boiled bone samples analysed at each post-burial time (n = 36)

Bone Pre-treatment	Average Length (cm)	Standard Deviation (±)	Average Diameter (cm)	Standard Deviation (±)	Average Mass (g)	Standard Deviation (±)
Defleshed (Control)	8.2	0.49	1.5	0.46	6.5	1.9
Boiled	9.1	0.51	1.6	0.33	8.4	1.4

Images of bones that were defleshed and boiled prior to burial are presented in Figure 4-77. It can be seen that slices obtained from boiled bones appear very similar to slices obtained from defleshed and fleshed bone samples. The bones are all predominantly brown in colour.

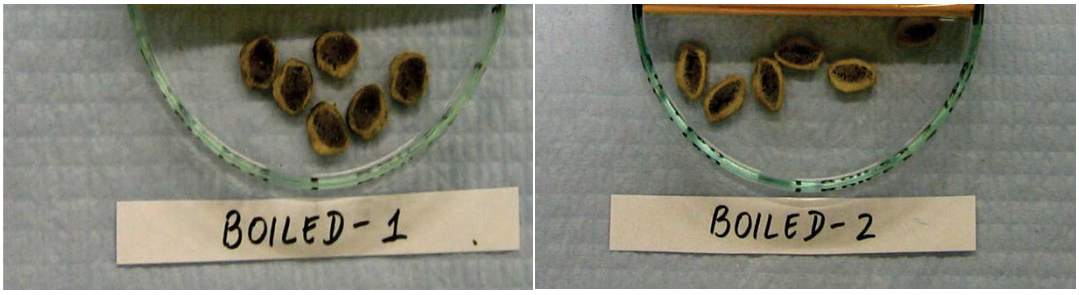

Figure 4-77. Photographs of boiled bone samples

4.5.2 Thermogravimetric Analysis

4.5.2.1 Reproducibility

The results of the bones that were boiled prior to burial are shown in Figure 4-78. The averages of the bones boiled prior to burial ranged from 29.5 ± 1.4 % to 35.7 ± 4.7 %. Generally, the standard deviations of the bones buried for shorter periods of time were lower than the standard deviations of bones with longer burial times. The results of the bones with burial periods up to and including 6 months were more reproducible than older bones; however, there was still some scatter in the results. Again, this could be due to a longer burial time leading to variations occurring in the degradation processes that take place in bones once they are buried in soil. The total mass loss of boiled bones at each of the post-burial times is presented in Table 4-15.

Table 4-15. Total mass loss ± standard deviation (%) at each post-burial time for defleshed and boiled bones (n = 40)

Post-burial time (months)	Loam Defleshed (%)	Loam Boiled (%)
0	35.6 ± 0.7	35.6 ± 0.7
1	35.3 ± 2.8	33.7 ± 1.2
2	35.5 ± 2.7	34.4 ± 0.2
4	29.8 ± 1.3	29.5 ± 1.4
6	30.5 ± 0.0	28.7 ± 0.8
8	32.3 ± 1.6	27.1 ± 6.7
10	32.4 ± 5.6	35.7 ± 4.7
12	30.1 ± 5.1	32.0 ± 1.7
15	38.2 ± 4.4	33.7 ± 3.3
18	33.6 ± 0.6	32.6 ± 1.1

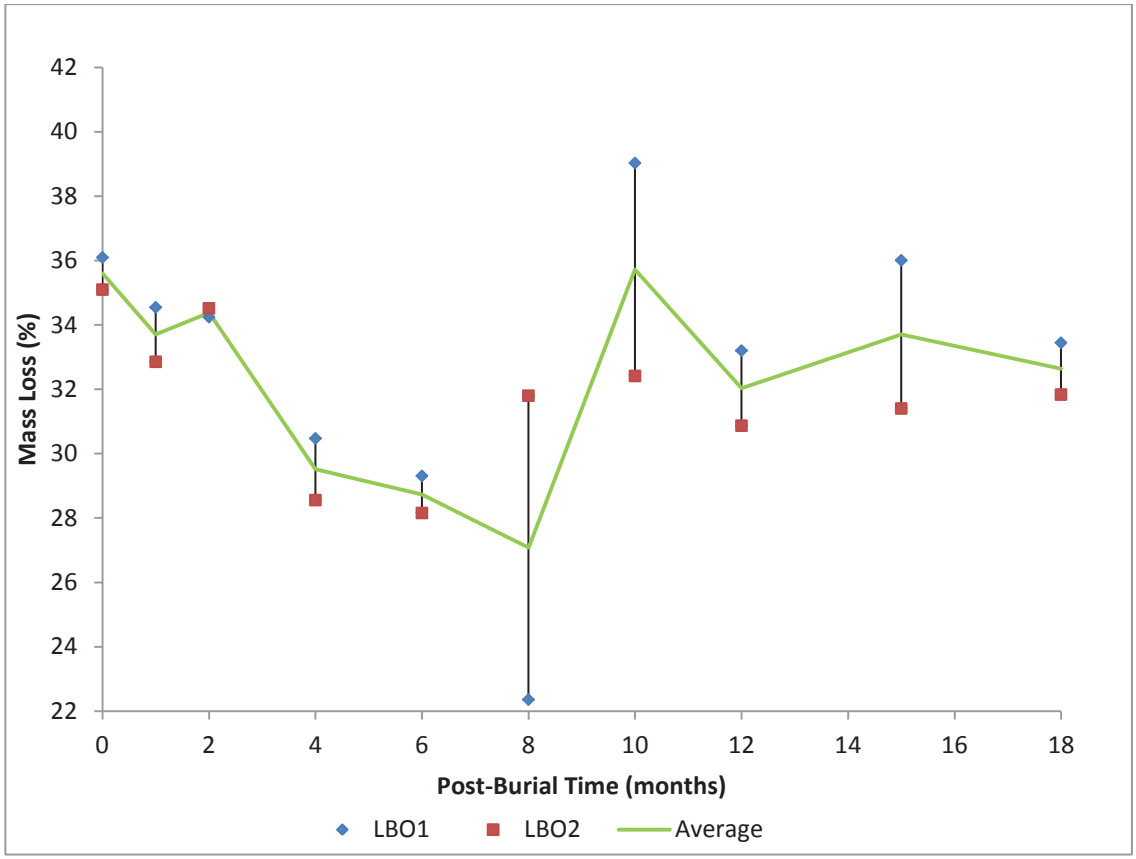

Figure 4-78. Comparison of TG results of Loam Boiled 1 and 2 bone samples

4.5.3 Pyrolysis Gas Chromatography-Mass Spectrometry

Statistical analysis of the Py-GC-MS results of boiled bones showed no peaks or retention times that had a linear correlation with post-burial time when the results were subjected to linear regression analysis using SPSS software. Since no significant peaks or retention times were identified using the statistical software, the software was unable to create a graph representation of the set of data for boiled bones. Therefore, it was noted that no compounds were shown to be changing in a significant manner with post-burial time. The compounds present in bones decompose slowly during the process of burial. The fact that no compounds were shown to be significantly changing in boiled bones could be because the

process of boiling decomposes these compounds prior to burial, and therefore, there are no remaining compounds that could be used for the estimation of the post-burial time of the boiled bones.

4.6 Comparison of Bone Pre-treatments

4.6.1 Environmental Scanning Electron Microscopy

The qualitative assessment of the visual, physical and surface properties of bone samples recovered from the different burial conditions after 1, 6, 12 and 18 months of burial are presented in Table 4-16 and show that differences exist between the samples as a result of the length of burial. SEM micrographs of the loam defleshed (control) bone samples are presented in Figures 4-79 to 4-82.

Table 4-16. Comparison of surface characteristics and porosity in bones with post-burial time

Burial Condition	1 month	6 months	12 months	18 months
Defleshed Bone (Control)	S +	S +	S +++	S/R ++
Fleshed Bone	R +	R +	R ++	S/R ++
Degreased Bone	S +	S +	S ++	S +++
Boiled Bone	S +	S ++	S ++	R ++

S = Smooth R = Rough +/++/+++ = Degree of porosity

4.6.1.1 1 Month Post-Burial

Firstly, there were no micro-pores visible in all of the 1-month bone samples, regardless of burial condition. It is interesting to note that the fleshed bone samples appeared to have a rougher surface when compared to the defleshed bone samples. In terms of bone treatments, Loam Degreased 1 appeared smoother and had fewer pores when compared to Loam Degreased 2. Both the boiled bone samples were smooth in appearance with few pores visible.

4.6.1.2 6 Months Post-Burial

Bones buried with flesh covering them, resulted in a rougher surface when compared to the defleshed bone samples. In terms of bone pre-treatments, Loam Degreased 1 appeared smoother when compared to Loam Degreased 2. Both the boiled bone samples were smooth in appearance, similar to the 1-month bone samples; however, more pores were visible in the 6-month boiled bone samples, when compared to the 1-month samples. After six months of burial, bone pre-treatment methods tend to have an effect on the degradation processes occurring in the bones.

4.6.1.3 12 Months Post-Burial

Analogous to the 1 and 6 month bone samples, Loam Fleshed 1, recovered after 12 months of burial appeared to have a rougher surface when compared to Loam Defleshed 1. However, Loam Fleshed 2 and Loam Defleshed 2 bone samples appeared very similar, with the Loam Defleshed 2 bone sample, showing more pores than the Loam Fleshed 2 bone sample. With regards to bone pre-treatments, Loam Degreased 1 appeared smoother when compared to Loam Degreased 2. Both the boiled bone samples were smooth in appearance with Loam Boiled 2 showing more visible pores than Loam Boiled 1.

4.6.1.4 18 Months Post-Burial

After 18 months of burial, the differences between the fleshed and defleshed bone samples were not as pronounced. Loam Defleshed 1 appeared to be smoother than Loam Fleshed 1; however, in Loam Defleshed 2 and Loam Fleshed 2 bone samples, it was the opposite. This indicates the retardation of the bacterial activity occurring in the bones, since the commencement of burial. Looking at bone pre-treatments, both the degreased bone samples appeared smooth and had many visible pores. Comparatively, the boiled bone samples were rougher in appearance than the degreased bone samples.

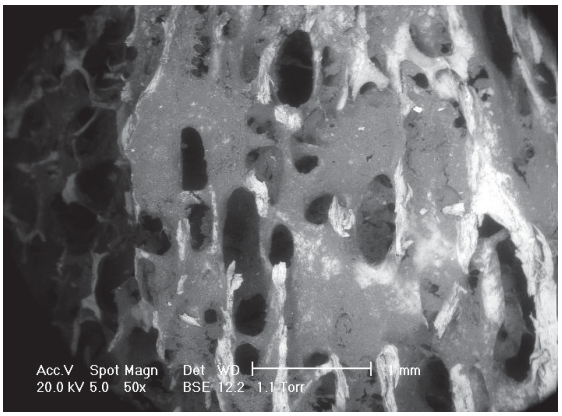

Figure 4-79. 1 month post-burial

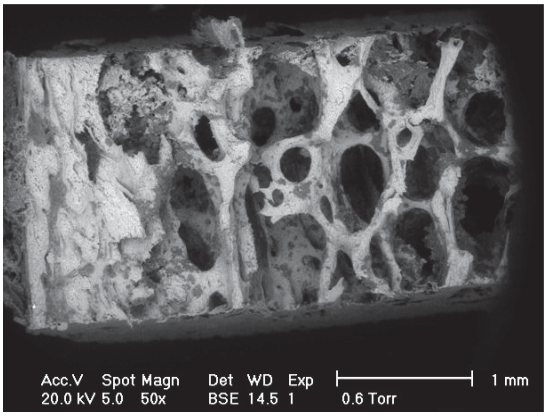

Figure 4-80. 6 months post-burial

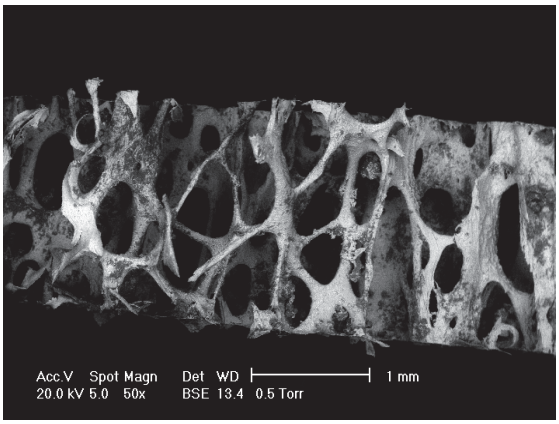

Figure 4-81. 12 months post-burial

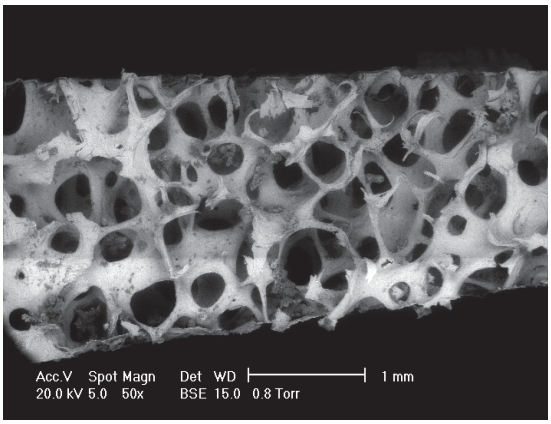

Figure 4-82. 18 months post-burial

4.6.1.5 Summary

Overall, all bone samples increased in bone porosity after a post-burial time of 6 months. Fleshed bone samples appeared to be rougher compared to defleshed bone samples.

In conclusion, several differences were observed in the bone samples at the different post-burial times and in the different burial conditions investigated in this study. The differences observed were in roughness of the surface and the presence of pores and micro-pores in the various samples. Using these differences, the bones recovered can be classified into two different categories – young and old. The 'young' category includes bones with post-burial times of 6 months and less. The 'old' category refers to bones with post-burial times ranging from 8 to 18 months. Therefore, using ESEM analysis, the post-burial time of bones recovered from various burial conditions can be classified into either one of these categories, and hence, estimated with a certain degree of accuracy.

In addition, the increasing degradation of the bones observed using ESEM demonstrated the potential for the successful use of chemical techniques such as Py-GC-MS for the estimation of the post-burial time of discovered skeletal remains based on changes in the chemical compounds present at the different stages of burial.

4.6.2 Thermogravimetric Analysis

The way the bone is treated prior to burial as well as the presence of flesh on the bones have been shown to have an effect on the structure of bones (Connelley et al., 2010; Nicholson, 1996; Mant, 1987). In this study, bones with and without flesh were buried in loam soil. Also, bones subjected to two different pre-treatments – degreasing and boiling – were also buried in loam soil for comparison purposes. The TG curves of the bones subjected to the various

pre-treatment methods after 1 and 18 months of burial are presented in Figures 4-83 and 4-84, respectively.

Overall, a decreasing trend in mass loss is observed for the first six months of burial in all the bones subjected to the different pre-treatment methods. After the first six months of burial; however, two differing trends are observed for the bone samples up to a post-burial time of 18 months. Typically, the defleshed bones and fleshed bones are observed as following similar trends from 8 to 18 months of burial, with the trends in the fleshed bone samples being less pronounced. Similarly, degreased and boiled bone samples follow similar trends between 8 and 18 months of burial.

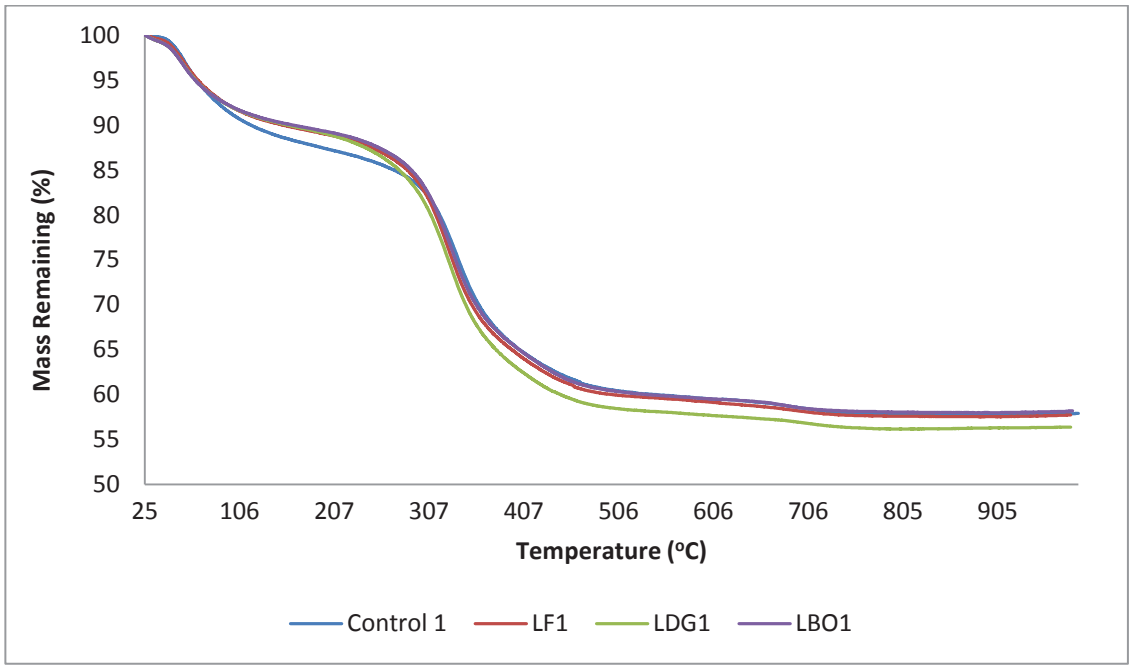

Figure 4-83. TG curves of bone samples subjected to different pre-treatments after 1 month of burial

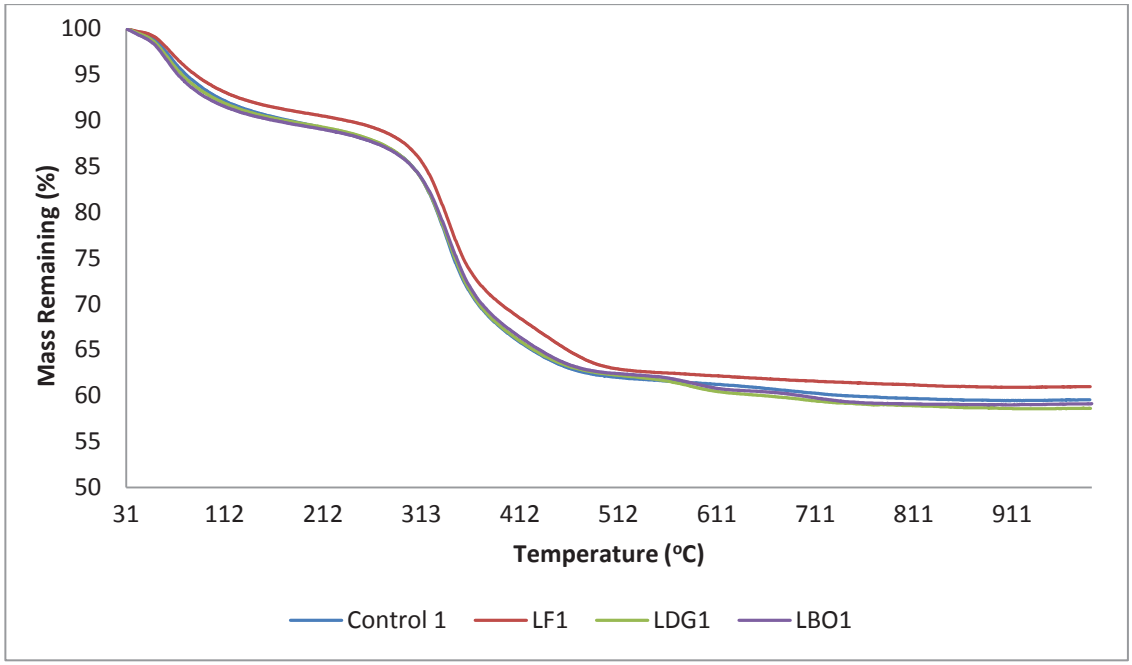

Figure 4-84. TG curves of bone samples subjected to different pre-treatments after 18 months of burial

Additionally, it can be seen that after the first month of burial, all the bones samples resulted in similar mass loss curves; however, the total mass loss in the degreased bones was slightly higher than the other bone samples. This may be a result of the difference in water content in the degreased bone samples compared to the other bones. Another explanation for this observation could be that the process of degreasing releases fats and oils, which might form a protective film on the bone and, therefore, hinder the processes of bone decomposition which normally occur in bone once it is buried in soil. This would mean that there is more organic content present in the degreased bones prior to TG, which would result in these bones having a greater total mass loss. It is interesting to note that after 18 months of burial, the defleshed, degreased and boiled bones follow the same trend in terms of mass loss with temperature. However, the mass loss curve of the fleshed bones appears to be different in shape, which is exemplified by the differences observed in the DTG curves of the fleshed and

defleshed bone samples after 1, 12 and 18 months of burial (Figures 4-85, 4-87 and 4-88). Interestingly, however, the DTG curves of the defleshed and fleshed bone samples after 6 months of burial (Figure 4-86) are very similar.

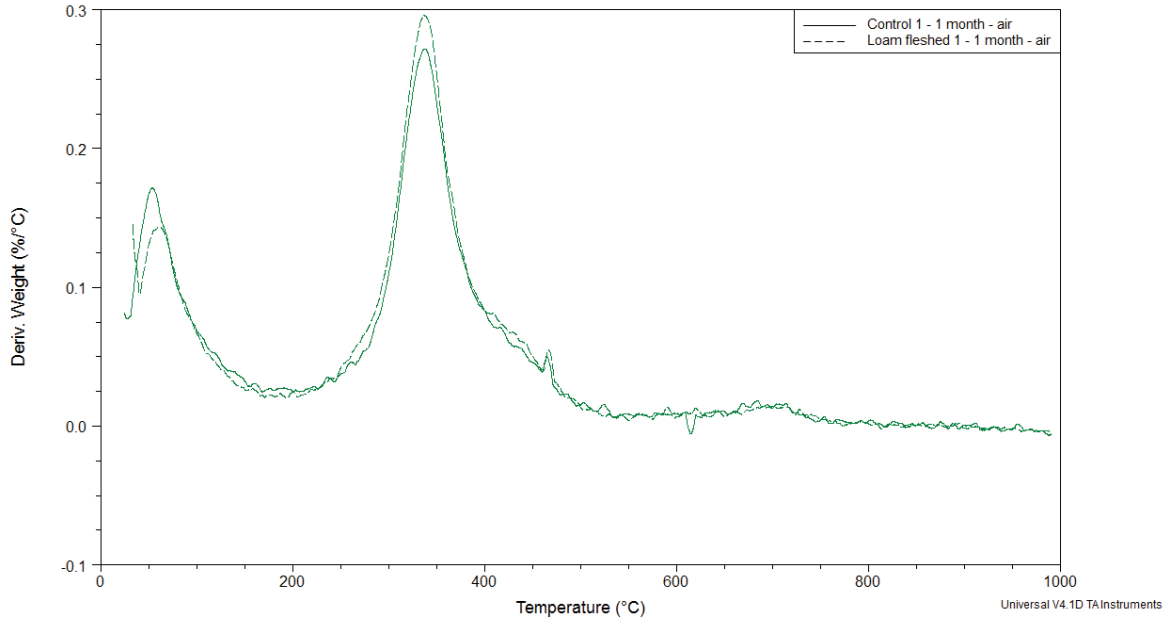

Figure 4-85. DTG curves of defleshed and fleshed samples after 1 month of burial

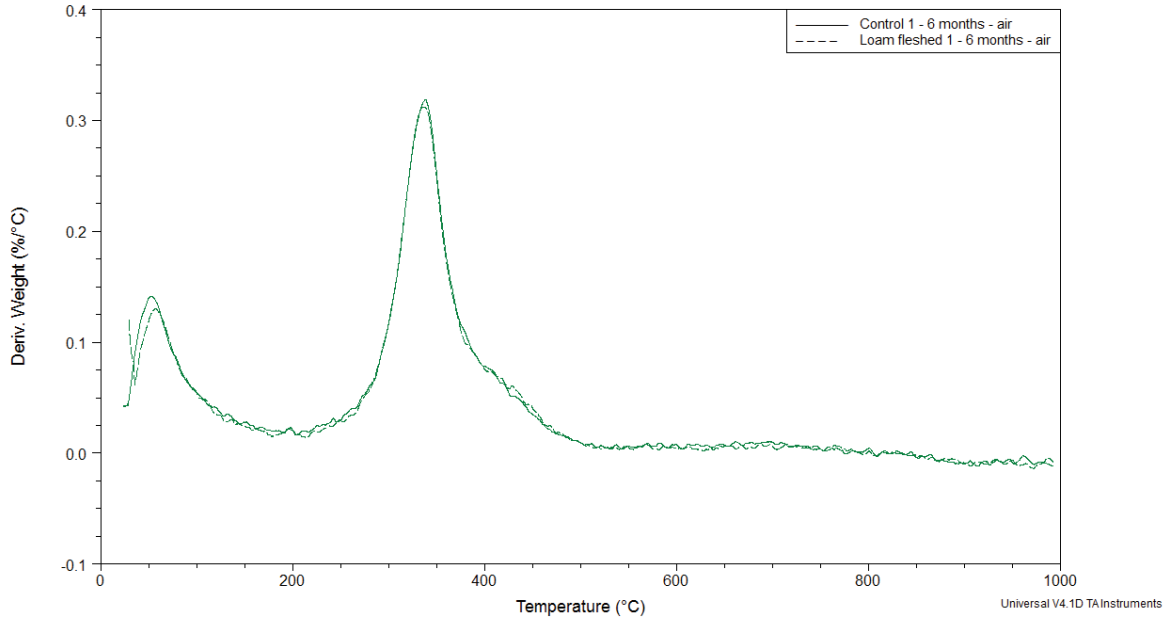

Figure 4-86. DTG curves of defleshed and fleshed samples after 6 months of burial

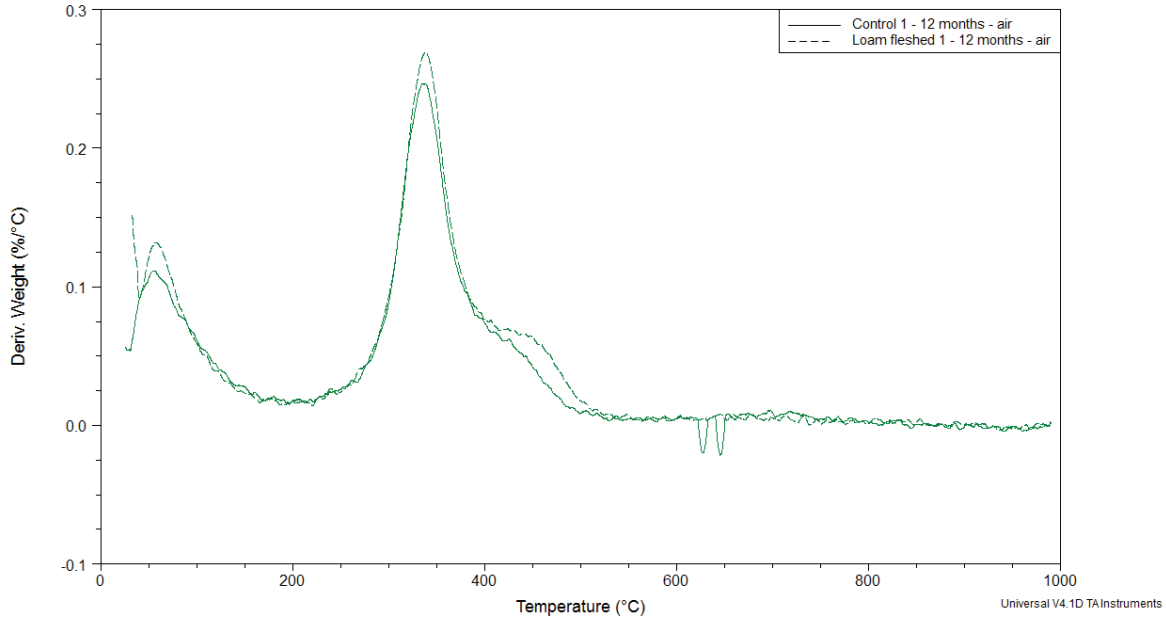

Figure 4-87. DTG curves of defleshed and fleshed samples after 12 months of burial

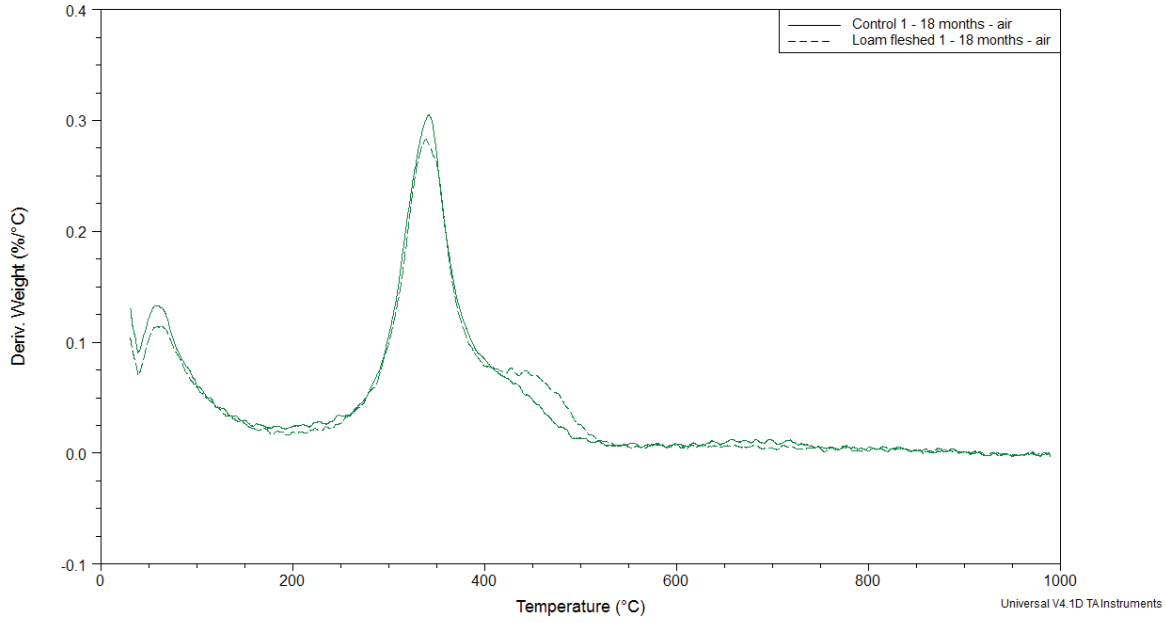

Figure 4-88. DTG curves of defleshed and fleshed samples after 18 months of burial

Figure 4-89 shows an overall decreasing trend in mass loss in all the bone samples up to a post-burial time of 8 months. It also shows that fleshed, defleshed and boiled bones follow similar trends in mass loss for the 18 months of burial investigated, with the exception of a decrease in mass loss between 6 and 8 months of burial in boiled bones when compared to the increase in mass loss observed in the same period for the fleshed and defleshed bones.

It is interesting to note that the changes in mass loss observed in fleshed bones appear to be dampened when compared to the defleshed and boiled bones, which tend to oscillate more. This indicates that the degradation processes in fleshed bones are occurring at a slower rate than in defleshed and boiled bones, suggesting that the flesh acts as a barrier and so hampers bone diagenesis. The degreased bones follow the same mass loss trend as the other bones for a majority of the total burial period of 18 months (between 2 and 15 months post-burial). In the first month of burial, the mass loss of the degreased bones decreased compared to the

other bones. Similarly, during the 15 and 18 month burial period, the mass loss increased for the degreased bone compared to the other bones.

Overall, it was observed that there was a similar difference in mass loss in all the bone samples, with the total mass loss occurring after 18 months of burial being much lower than the total mass loss that occurred after 1 month of burial in all the bone samples, regardless of the presence of flesh or the pre-treatment method.

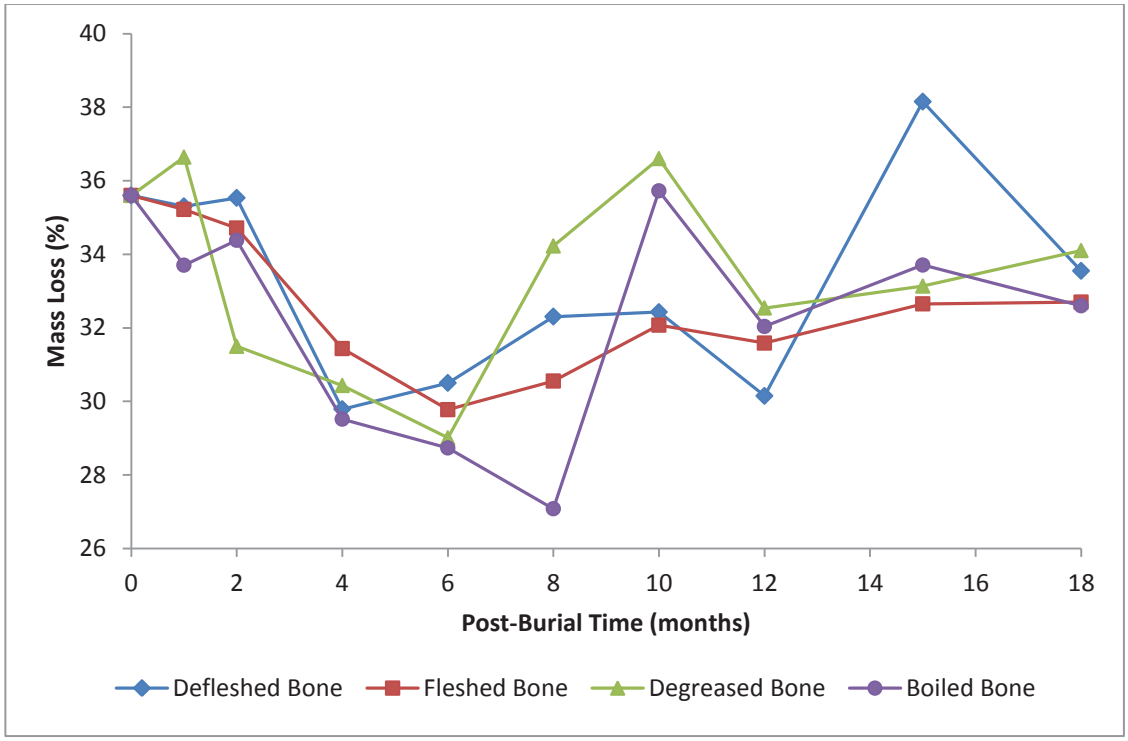

Figure 4-89. Mass loss vs post-burial time for defleshed, fleshed, degreased and boiled bones

4.6.3 Pyrolysis Gas Chromatography-Mass Spectrometry

The Py-GC-MS data shows a direct relationship between the predicted and actual post-burial time of bones for most of the pre-treatments studied except for boiling (Figure 4-90). The greatest variation is observed in the data after 15 months of burial. As a result, the lengths of

burial of most of the bone samples can be estimated up to a post-burial time of 15 ± 3.0 months, even without prior information of the pre-treatments of the recovered samples.

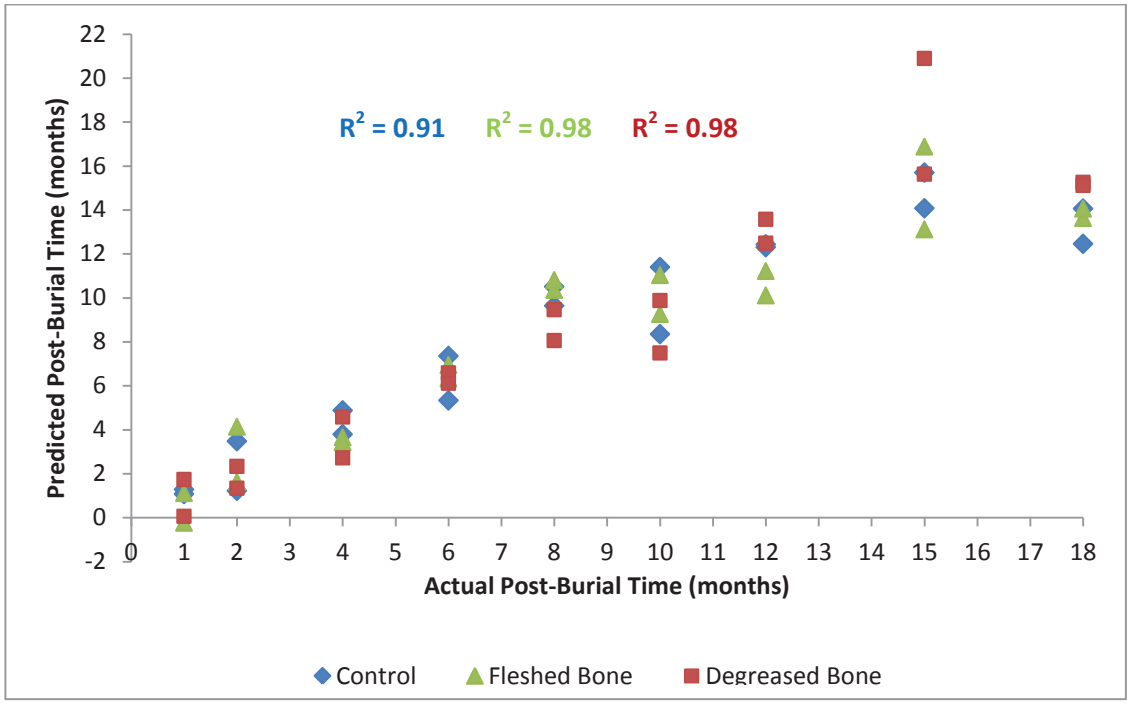

Figure 4-90. Predicted post-burial time vs actual post-burial time for bones subjected to different pre-treatments

4.6.4 The Effect of Preparation Methods on Bone Structure: A Comparative Study Using Infrared Spectroscopy

The effects of bone pre-treatments on bone decomposition were studied using FTIR spectroscopy as part of another project being carried out in the laboratories at UTS (Howes *et al.*, 2012). The FTIR study involved analysis of the same bones analysed for the current study. There were no major differences observed between the samples subjected to the different pre-treatments. It was interesting to note that the FTIR study showed that fleshed bone samples appeared to contain more organic content than the defleshed bone samples. This supports the TG results of the present study and the idea that the presence of flesh on bones

hinders bone decomposition by reducing the exchange of organic bone matter with the surrounding soil. It was also observed that bones that were boiled prior to burial contained the least amount of organic material. This was not seen in the TG results of the present study. Lastly, similar trends were observed for both the fleshed and defleshed bone samples and these confirm the TG results of the present study. FTIR spectroscopy showed decreasing trends in the inorganic content during the first eight months of burial for both the fleshed and defleshed bone samples but no trends were observed in the organic content of the bones.

4.7 Summary

The aim of the present study was to establish a method that can lead to the accurate estimation of the post-burial time of bones once they are recovered. Bone samples subjected to different pre-treatment procedures including defleshing, degreasing and boiling were studied using ESEM, TG, Py-GC-MS and XRD analyses. ESEM analysis of the defleshed (control) samples showed that the bone samples with younger post-burial times were smoother than the bone samples buried for longer periods of time. Overall, all bone samples increased in bone porosity after a post-burial time of 6 months. Fleshed bone samples appeared to rougher compared to defleshed bones samples. Using ESEM analysis, it is possible to classify bones into two different categories – young (post-burial time of 6 months or lower) and old (post-burial time of 8 to 18 months).

TG analysis showed an overall decreasing trend in mass loss in all the bone samples up to a post-burial time of 8 months. It also showed that fleshed, defleshed and boiled bones follow similar trends in mass loss for the 18 months investigated, with the exception of a decrease in mass loss between 6 and 8 months of burial in boiled bones when compared to the increase in mass loss observed in the same period for the fleshed and defleshed bones. The changes in

mass loss observed in fleshed bones appeared to be dampened when compared to the defleshed and boiled bones, which tend to oscillate more. This indicates that the degradation processes are occurring at a slower rate in fleshed bones than in defleshed and boiled bones. The degreased bones follow the same mass loss trend as the other bones between 2 and 15 months of burial.

The technique of Py-GC-MS was observed to be the most useful and accurate in estimating the post-burial time of recovered bone samples. The Py-GC-MS data shows a direct relationship between the predicted and actual post-burial time of bones for all the pre-treatment procedures studied except for boiling. The post-burial times of defleshed, fleshed and degreased bone samples buried in loam soil can be predicted relatively accurately up to 18 ± 4.0, 18 ± 1.4 months and 18 ± 1.4 months, respectively.

XRD analysis is used to monitor changes in the inorganic content of bones. It takes several years for the inorganic content in bones to change. The XRD results of the present study show that no changes in crystallinity can be observed for a post-burial period of 18 months, which supports the notion that changes in the inorganic component of bones occur relatively slowly.

Chapter 5

The Effect of Storage Conditions on Bone Structure

5.1 Introduction

In any forensic investigation, the discovery of bone(s) can be crucial in solving the case. Bone evidence may aid in identifying the decedent(s), determining cause of death and it could also lead to identifying possible offenders. Therefore, it is critical to be aware of the most appropriate method of storage of the bone samples once they are discovered. The samples may need to be stored for a short or long period of time before any analysis is performed depending on the availability of analysts and resources at the time. The ideal method of storage would be such that all information contained in the bone is preserved and any changes to the bone are limited, if not completely halted. In general terms, this means protecting the bones from physical damage and limiting the changes to the inorganic and organic content of bones.

The present study investigated the effects of storage conditions on bone samples. Due to time constraints, the testing of storage conditions on the bone samples was based on establishing a hypothesis and testing its validity. It was hypothesised that storing the bone samples in a freezer at -7 °C to -10 °C (Lamoureux *et al.*, 2011; Park *et al.*, 2003; Linde and Sorensen, 1993) would be an appropriate method of sample storage and would involve limited or no change to the morphological, molecular and histological structures in bones.

Two fresh bone samples (same as bone samples in Section 2.3.1.3) were prepared using the method mentioned in Section 2.3.1.4 and were then analysed using various analytical techniques including TG, Py-GC-MS and XRD analyses before they were stored in a freezer (within a temperature range of -7 °C to -10 °C). The samples were analysed for 18 months at regular time intervals to investigate if any changes had occurred since they were stored in the freezer.

5.2 Thermogravimetric Analysis

The results of TG appear to demonstrate that changes are still occurring to the organic content of the bone samples even after they are stored in the freezer. The results of both the Fresh Samples 1 and 2 analysed at each storage interval are shown in Figures 5-1 and 5-2, respectively. Theoretically, if no changes were occurring in the bone, then the mass loss results would not be significantly different, regardless of when the bone was being analysed. However, the reproducibility study conducted (Section 4.2.4.4) using TG and DTG results demonstrated that the results obtained using TG were not reproducible due to the heterogeneity of the bone samples being analysed. It was also shown that another factor responsible for the variations observed in the results was the poor accuracy in the absolute mass measured by the instrument (0.3 mg in a 2-3 mg sample). This error led to mass loss measurements being inaccurate by 10 % or more as a result of the initial mass of the samples being close to the limit of resolution of the TG instrument.

The mass loss data of both the samples, Fresh Sample 1 and Fresh Sample 2, are presented in Table 5-1 and show differences in mass loss for the samples when analysed at different storage intervals, highlighting the heterogeneous nature of the samples being compared. Based on these results, it is difficult to extrapolate whether storage in a freezer is suitable for bone samples being analysed using TG as the variations in the mass loss results can be attributed to more than one factor.

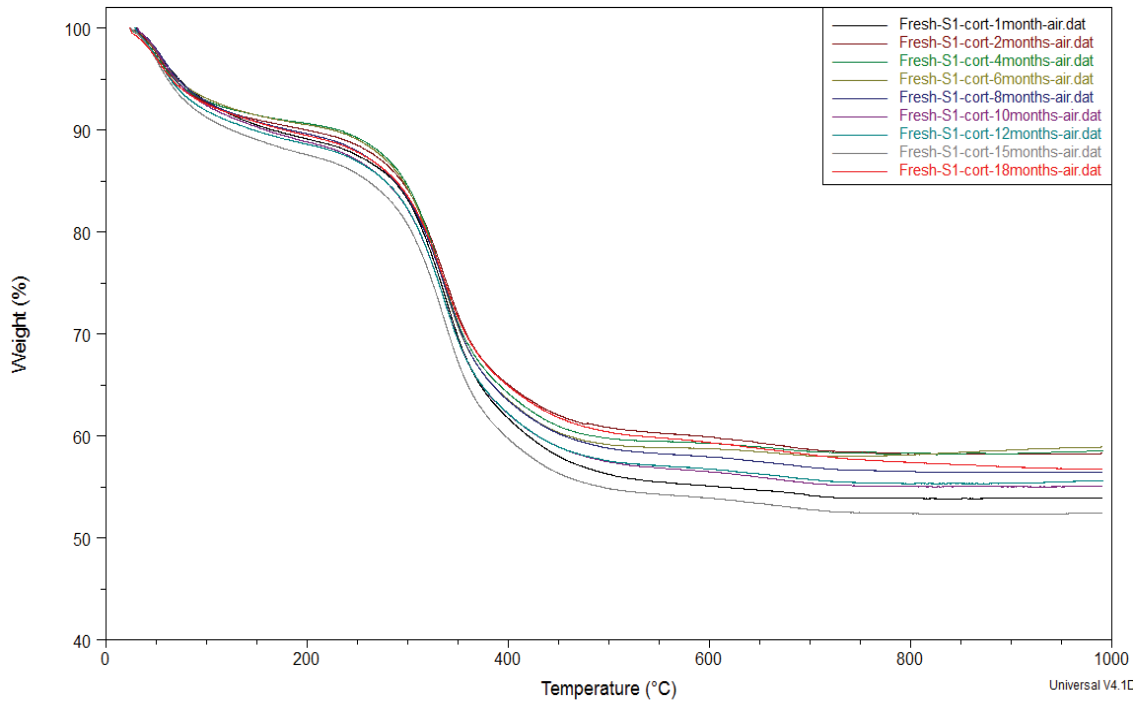

Figure 5-1. TG results of Fresh Sample 1 at each storage interval

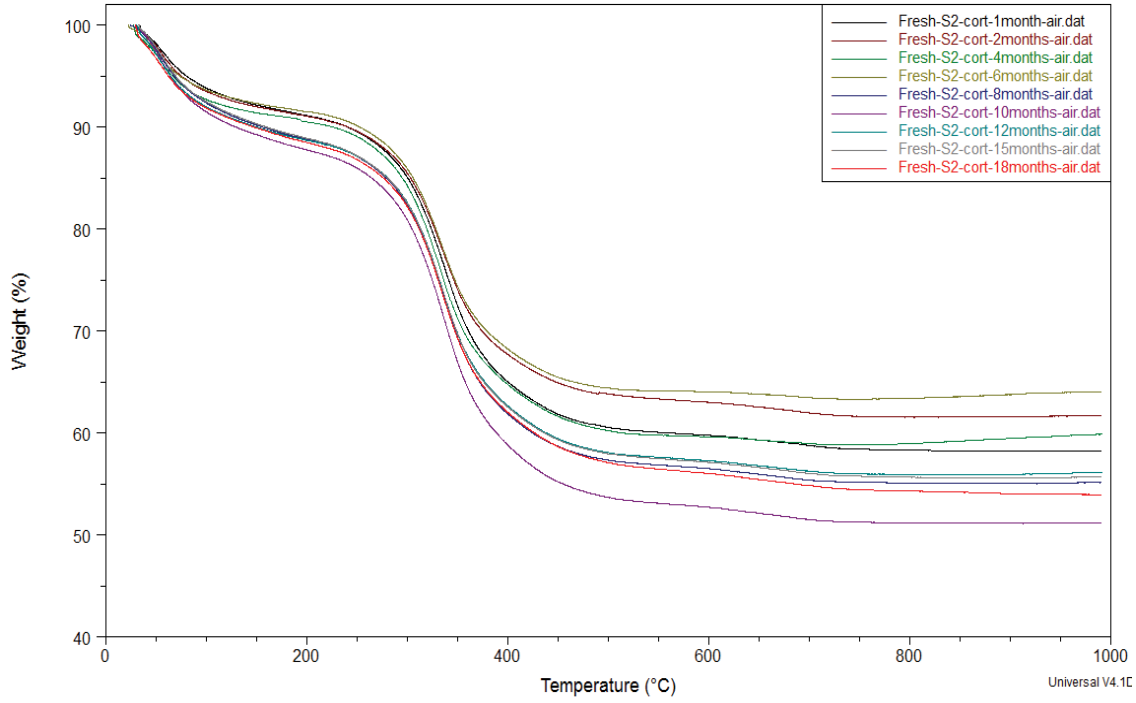

Figure 5-2. TG results of Fresh Sample 2 at each storage interval

Table 5-1. Total mass loss (%) at each storage interval for fresh bone samples stored in the freezer (n = 20)

Storage interval (months)	Fresh Sample 1 (%)	Fresh Sample 2 (%)
0	35.6	35.6
1	39.1	35.8
2	34.9	31.9
4	35.2	33.9
6	34.9	29.8
8	36.6	37.5
10	37.7	41.3
12	36.9	36.4
15	39.7	37.0
18	36.2	38.7

The results of the total mass loss as a function of the storage interval for the fresh samples are presented in Figure 5-3. For analysis, the ideal method of storage of bone samples would involve the complete preservation of the samples. The variation in mass loss (Figure 5-3) demonstrates that this is not the case when storing the bone samples in a freezer. Comparing the results of the fresh bone samples stored in a freezer (Figure 5-3) with the results of the defleshed bone samples buried in a loam soil environment (Figure 5-4) show a similar degree of variation in mass loss at the different time intervals. This indicates that storage in a freezer does not hinder the decomposition processes occurring in the organic component of the bone samples, which is required for a storage method to be effective. The DTG curves of the fresh samples at storage intervals of 1, 6, 12 and 18 months further demonstrate the variations observed in the mass loss results (Figures 5-5 and 5-6). However, due to a lack of reproducibility in the TG results, it is difficult to determine if storage in a freezer is suitable for bone samples being analysed using this technique as it is difficult to establish whether the variations in the mass loss results are due to the heterogeneity of the samples or due to diagenesis still occurring even when the bone samples are stored in the freezer.

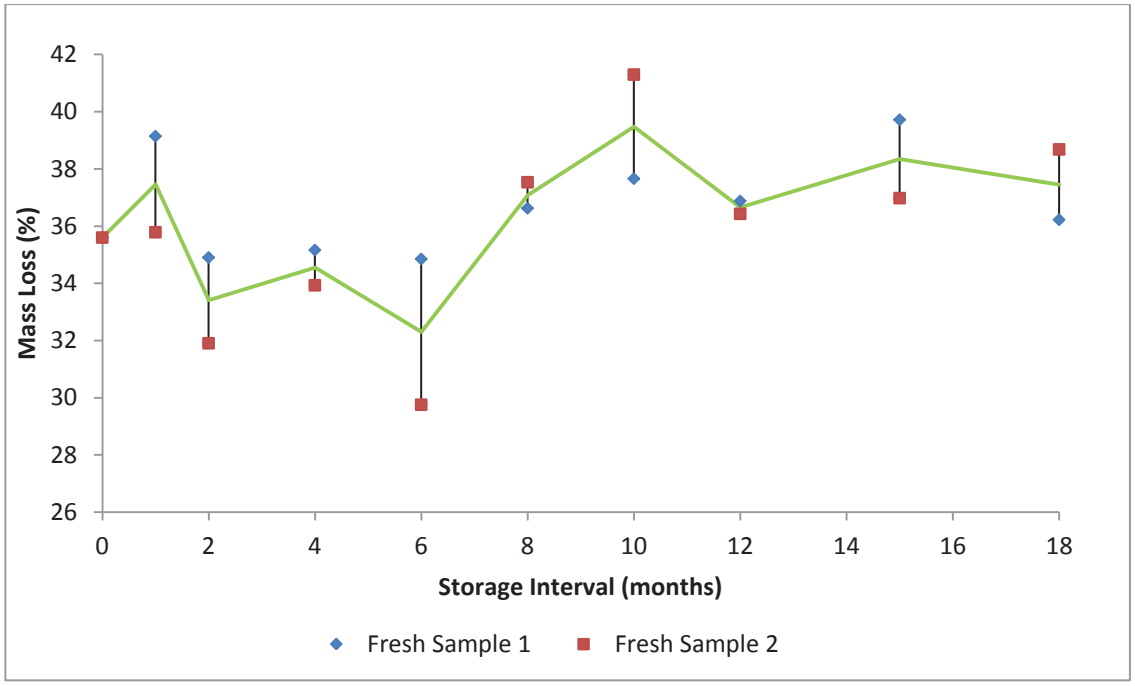

Figure 5-3. Comparison of TG results of Fresh 1 and 2 bone samples

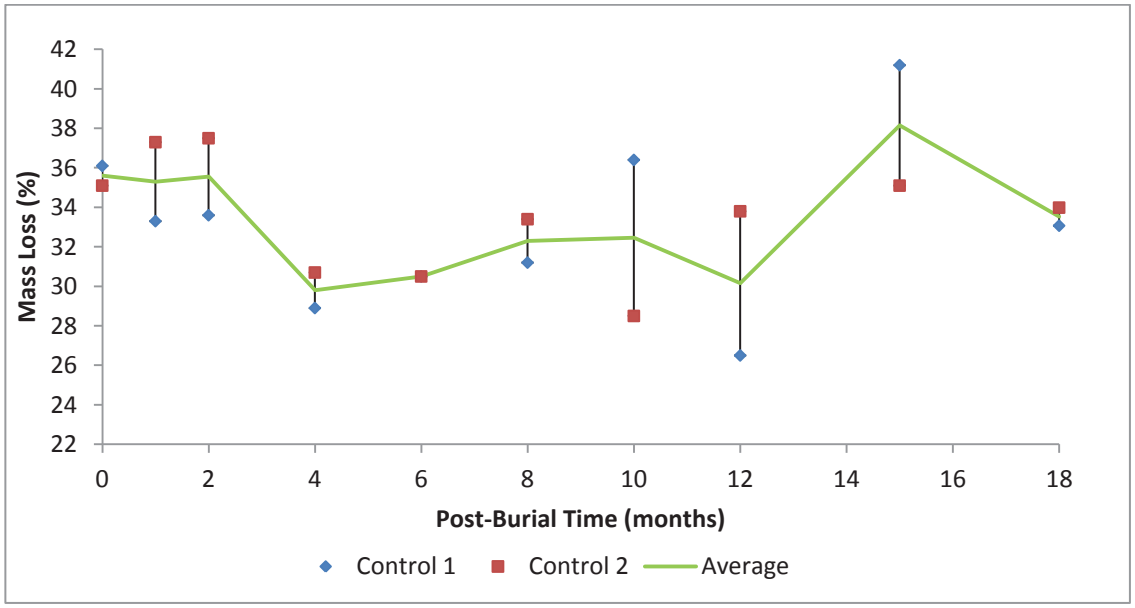

Figure 5-4. Comparison of TG results of Loam Defleshed (Control) 1 and 2 bone samples

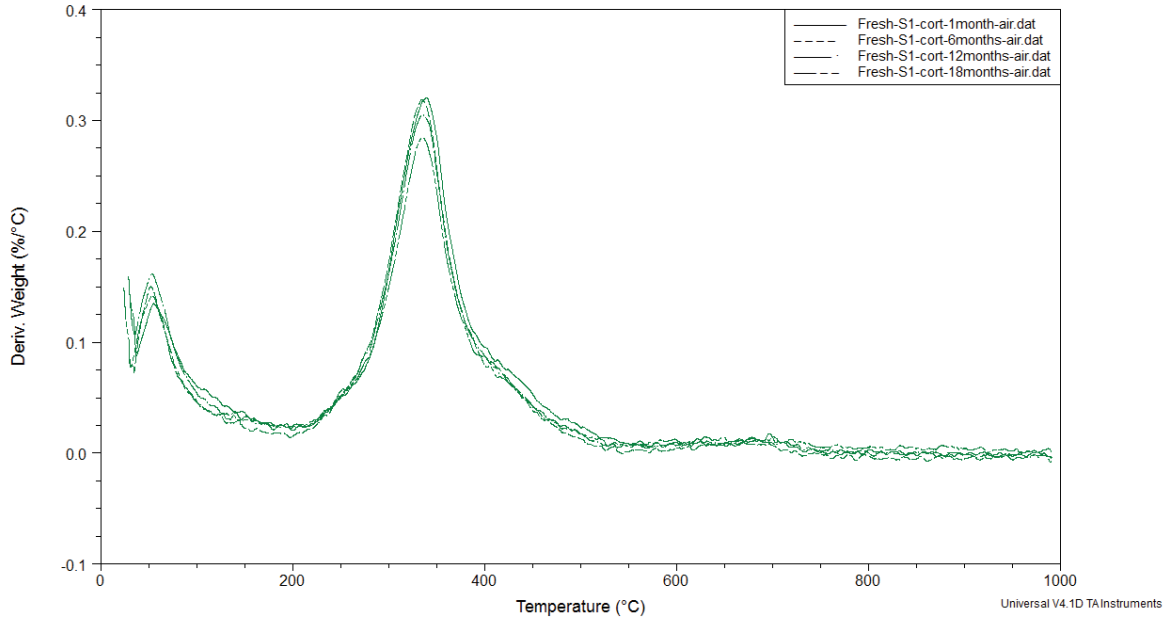

Figure 5-5. DTG curves of Fresh Sample 1 for 1 to 18 months of storage

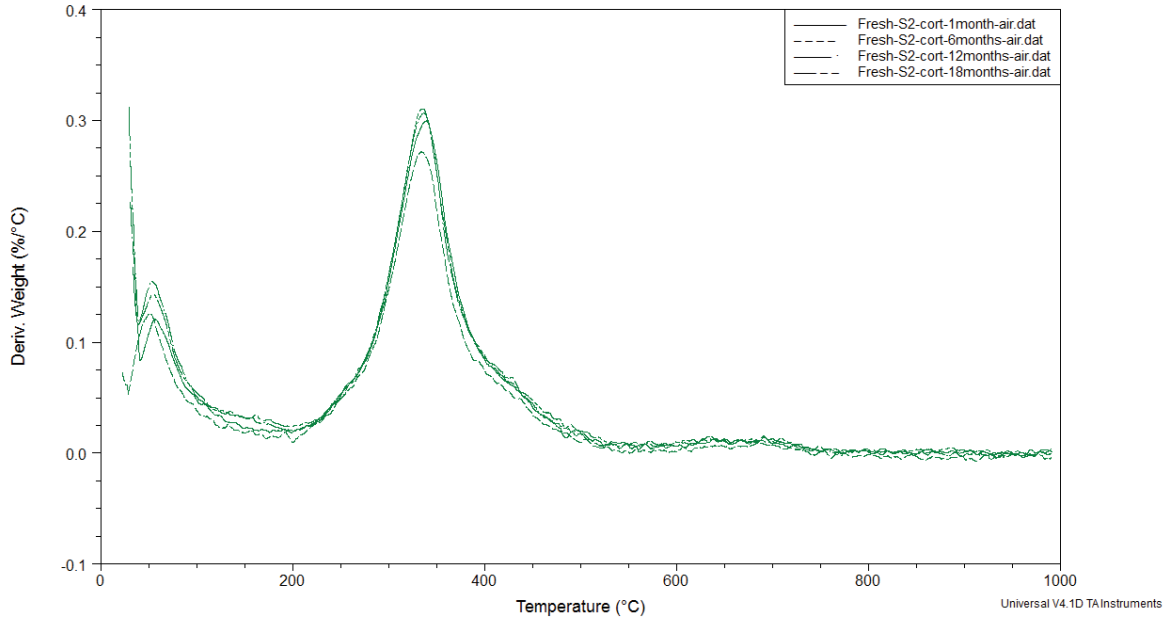

Figure 5-6. DTG curves of Fresh Sample 2 for 1 to 18 months of storage

5.3 Pyrolysis Gas Chromatography-Mass Spectrometry

The Py-GC-MS results of the effect of storage conditions on bone samples confirm that diagenesis appears to be continuing in the organic component of the bones even when the bone samples are stored in the freezer. The pyrograms of the fresh bone samples prior to storage and at different time intervals after storage are shown in Figure 5-7. Figure 5-8 presents the pyrograms of the defleshed bone samples buried in loam soil and examined at the same time intervals as the fresh bone samples. Both sets of pyrograms are similar in that there are many peaks present in the bone samples examined after a period of storage/burial of 1 and 2 months. Also, the number of the peaks decreases significantly in both sets of pyrograms after 2 months of burial. Since the pyrograms of bones buried in a loam soil environment and bones that have been stored in a freezer were similar, it can be concluded that storage in a freezer is not effective in stopping or even minimising the decomposition of the organic component of bones. It appears that bone samples continue to degrade even when they are stored at a low temperature. The results of this study demonstrate that storage in a freezer is not an effective way of preserving the integrity of the organic content of the bone samples once they are recovered and prepared for analysis using Py-GC-MS. However, the technique of Py-GC-MS has proven to be effective in estimating the post-burial time of bones, even without knowledge of the burial environment. This means that the post-burial time of bone samples stored in a freezer could still be estimated by taking into account the time of storage in the freezer and deducting that from the estimated post-burial time determined by Py-GC-MS calculations.

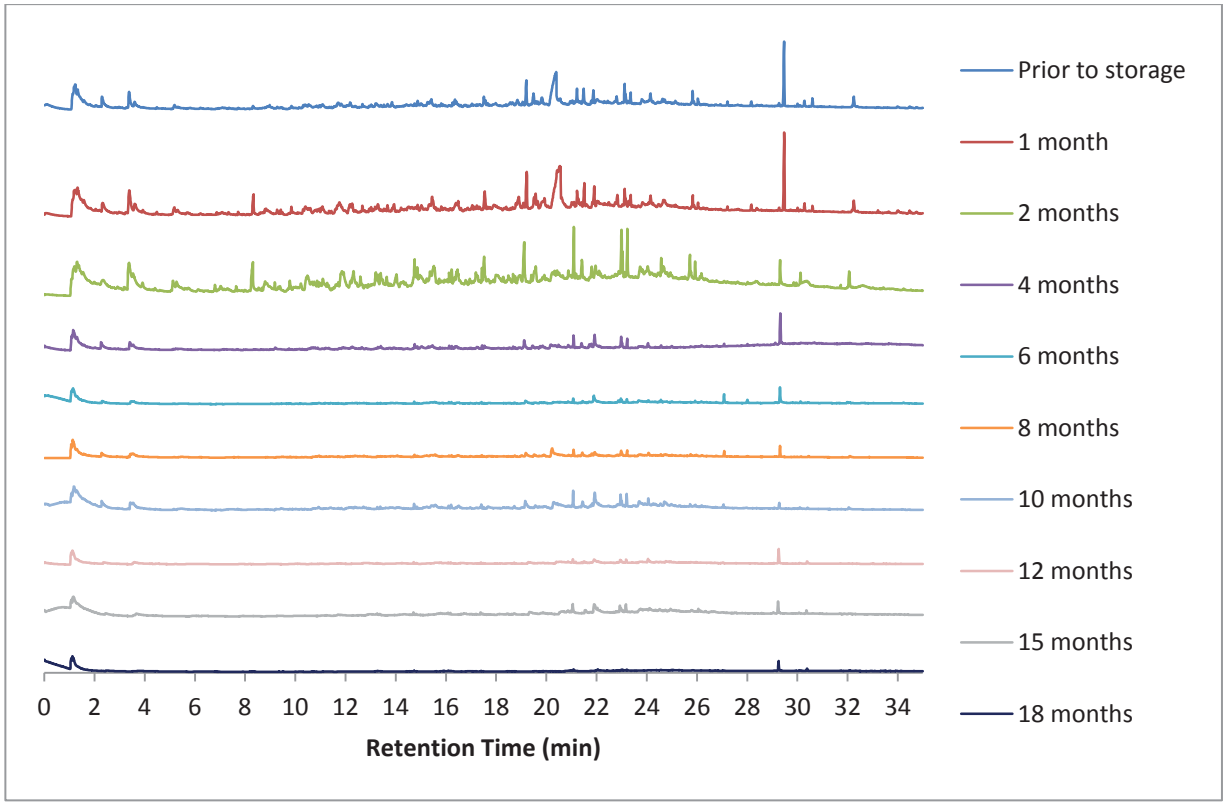

Figure 5-7. Py-GC-MS results of storage conditions testing on fresh bone samples

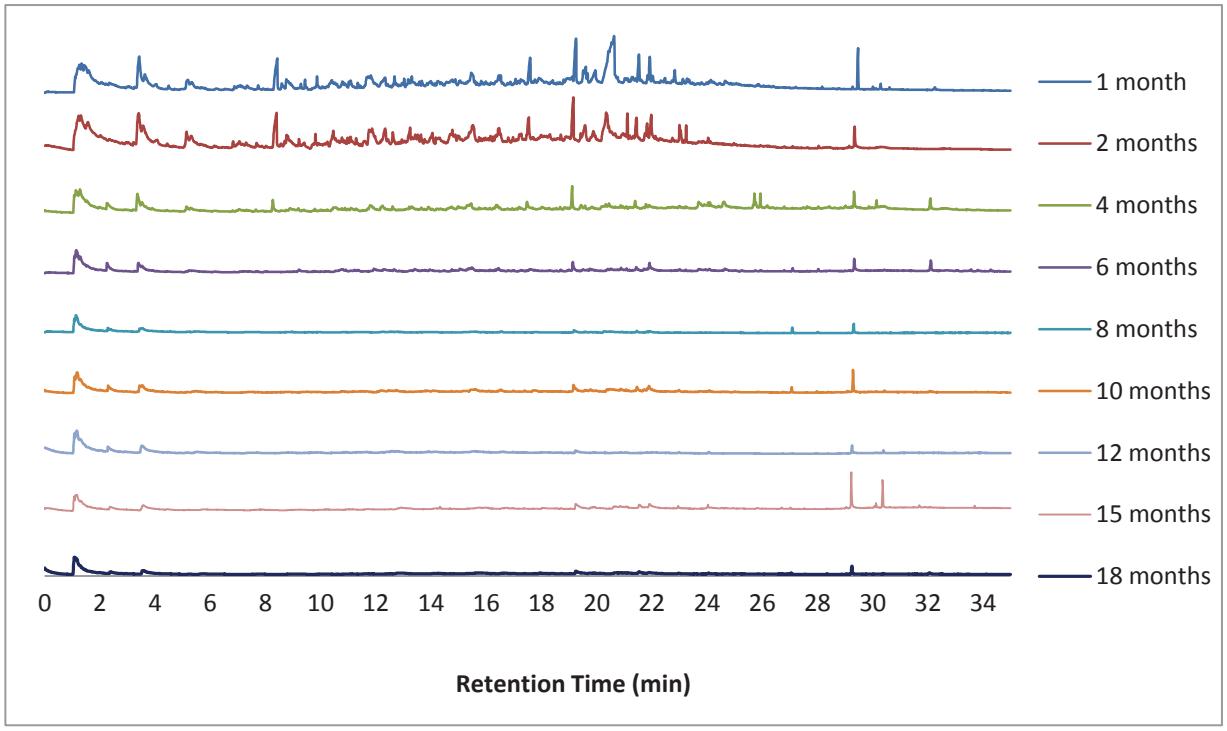

Figure 5-8. Py-GC-MS results of defleshed bone samples buried in a loam soil environment

5.4 X-ray Diffraction

The XRD results of bone samples prior to storage in a freezer and at different time intervals after storage are presented in Figures 5-9 and 5-10. XRD is a technique that provides information on the inorganic content of bone, which takes years to decompose (Bartsiokas and Middleton, 1992; Prieto-Castelló *et al.*, 2007). It was therefore expected that no changes would be observed in the XRD results over a period of 18 months. The results of the calculations of crystallinity indices 1 and 2 for the fresh bone samples stored in the freezer are presented in Table 5-2. For both sample sets, crystallinity index 1 and 2 range from 0.78 to 0.80 and from 0.72 to 0.76, respectively. These results show that the inorganic component of the bone samples has not changed significantly in the 18 months of storage.

Table 5-2. Crystallinity Indices of Fresh 1 and 2 bone samples stored in the freezer

	Crystallinity Index 1		Crystallinity Index 2	
Storage interval (months)	Fresh 1	Fresh 2	Fresh 1	Fresh 2
1	0.79	0.78	0.72	0.76
6	0.78	0.79	0.74	0.76
12	0.80	0.80	0.73	0.73
18	0.78	0.79	0.74	0.72

Even though bone degradation appears to still be occurring when bones are stored in a freezer (evident in the TG and Py-GC-MS results), the results of these degradation processes can only be observed as changes in the organic content of bones rather than the inorganic content. This is because unlike inorganic content, the organic content of bones degrades at a much faster rate (Mkukuma *et al.*, 2004; Onishi *et al.*, 2008). Therefore, since XRD results are not affected by changes in the organic content of bone, storage in a freezer is suitable for bone samples to be analysed using this technique over a period of 18 months.

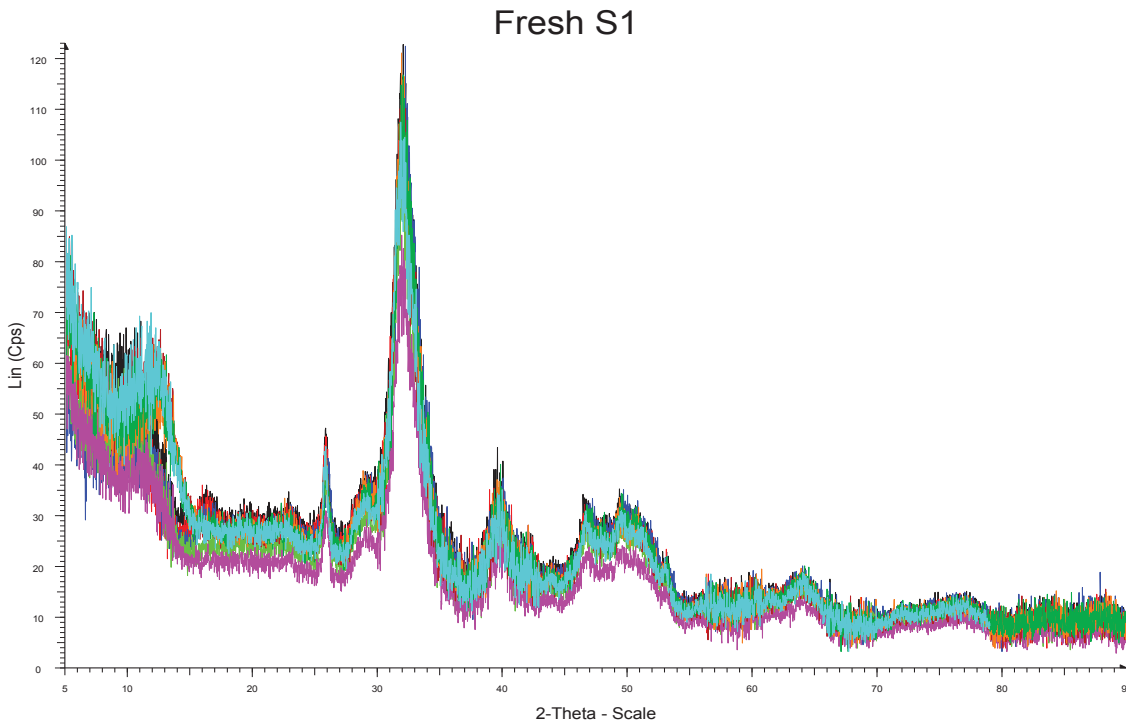

Figure 5-9. XRD results of storage conditions testing on bone samples (Fresh Sample 1)

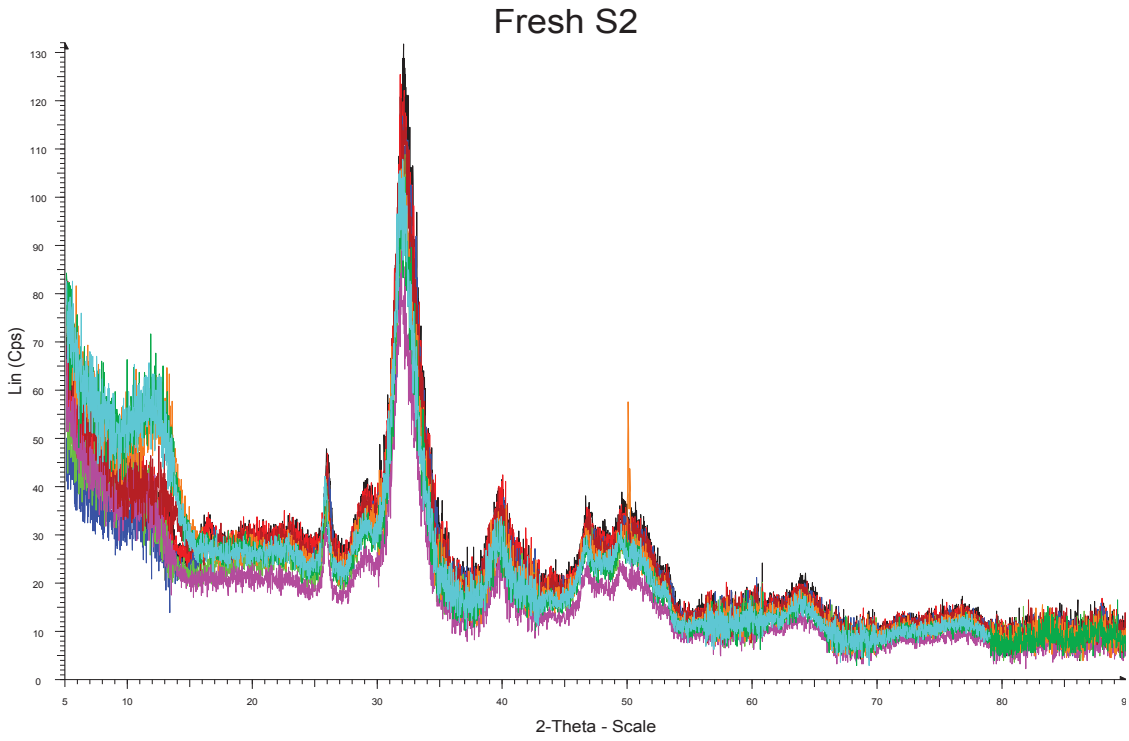

Figure 5-10. XRD results of storage conditions testing on bone samples (Fresh Sample 2)

5.5 Summary

The present study examined the effect of storage conditions on bone structure over a period of 18 months. Using the TG results, it was difficult to determine whether storage in a freezer was suitable for bone samples that are to be analysed using this technique or not. It appeared that the organic component of the bones continued to decompose even when the bones were stored at a low temperature. However, it could not be established whether diagenesis was the sole cause of the mass loss results or whether the heterogeneity of the samples was responsible for the variations observed. The results of Py-GC-MS analysis demonstrated that storage in a freezer was not suitable for bone samples to be analysed using thermal techniques as the organic content of the bones was observed to be degrading even when the samples were stored in a freezer. Both the TG and Py-GC-MS results showed that storage in a freezer does not slow down the decomposition of the organic content of bones. In contrast, for analysis using XRD, bone samples can be stored in a freezer prior to analysis. For a period of 18 months, no differences were observed in the XRD results of bones stored in a freezer. This is because XRD analysis is dependent on changes in the inorganic content of bones, which take years to occur and were therefore not observed in the period examined in this study.

Chapter 6

The Effect of Burial Conditions on Bone Structure

6.1 Introduction

The surrounding burial environment directly affects decomposing remains and can be defined as the "chemical, biological and geological conditions that prevail in that particular location" (Janaway, 1996, p. 58). Firstly, the chemical conditions include burial factors such as soil pH, redox potential, ion exchange capacity and oxygenation. Secondly, the biological conditions involve the microbial activity of bacteria, fungi, algae and protozoa. Lastly, the soil mineral particles that are derived from weathered rock represent the geological conditions.

Depending on the burial environment in which an organism is deposited, the decomposition processes proceed at accelerated or retarded rates. Initially, the organism is subjected to wet and dry stages of decomposition due to the presence of mycota, enteric and local soil bacteria, scavengers, plants and insects. The rate at which this occurs and the time spent in each stage of decomposition is dependent upon environmental factors of type of soil, pH, availability of water and temperature. Once the soft tissues have decomposed and a cadaver is reduced to hard tissues, the pace of deterioration slows somewhat, but is still dependent on the physical factors of soil type, soil pH, moisture content and temperature. (Gill-King, 1997)

As the post-mortem period increases, the deterioration of the remains depends increasingly upon bacterial and plant activity. After the destruction of the organic phase of bone by bacterial activity (predominantly collagenase), the loss of mineral hydroxyapatite occurs as a result of inorganic chemical weathering. (Gill-King, 1997)

Since burial conditions can have a major effect on the decomposition rates in bones, it is important to study these effects in order to find a suitable method of estimating the post-

burial times of bones when they are discovered. In the present study, the effects of various factors including soil type, pH, moisture content and temperature were investigated.

6.2 Soil Type

There are three main soil types that exist in nature, which are distinguished based on particle size and composition. These include silt, sand and clay. Three different burial environments, each containing one of the three soil types, were prepared to represent these principal soil classes. Bones were buried in these three different soil types and compared to the control (loam defleshed) bone samples. Visual observations were recorded for the recovered bones. The bones were also analysed using TG, Py-GC-MS and XRD.

6.2.1 Visual Observations

Bones buried in various soil types were inspected prior to and after slicing. Upon recovery, measurements including length, diameter and mass were recorded for each bone. Physical descriptions including peeling, cracking, colour and odour of each bone were also recorded. The physical characteristics of the bone samples are recorded in Table 6-1.

Table 6-1. Physical characteristics of bone samples buried in different soil types analysed at each post-burial time (n = 72)

Soil Type	Average Length (cm)	Standard Deviation (±)	Average Diameter (cm)	Standard Deviation (±)	Average Mass (g)	Standard Deviation (±)
Loam Defleshed (Control)	8.2	0.49	1.5	0.46	6.5	1.9
Silt	10.6	0.6	1.3	0.24	12.3	3.6
Sand	9.8	0.8	1.3	0.26	9.5	2.6
Clay	8.3	0.8	1.5	0.35	8.1	2.6

The colour of the silty soil bone samples did not change throughout (1st month onwards) and remained a combination of black, white and red for the entire study. The colour of the sandy soil bones changed from a dark brown/black to a dark brown/white colour after about 8-10 months of burial. Clay soil bone samples had an original colour of white, which then changed to a more white-grey colour after 12 months of burial. No holes/cracks, odour or microbial activity were evident in any of the bone samples buried in the silty, sandy and clay soil types throughout the duration of the burial.

Once the bones were recovered (after 1 month), sectioned and dried, photographs were taken of the samples. Differences in physical characteristics were recorded for bone samples buried in the silty, sandy and clay soil environments. Bones buried in silty and sandy soil environments looked very similar when they were recovered, sectioned and dried. They were predominantly brown in colour. However, bones that were buried in a clay soil environment looked quite different to the bones buried in a silty and sandy soil environment. The cancellous section of the bones buried in a clay soil environment was completely dark red/maroon, while the cortical section was white. Images of the slices of bones recovered from these three environments are shown in Figure 6-1.

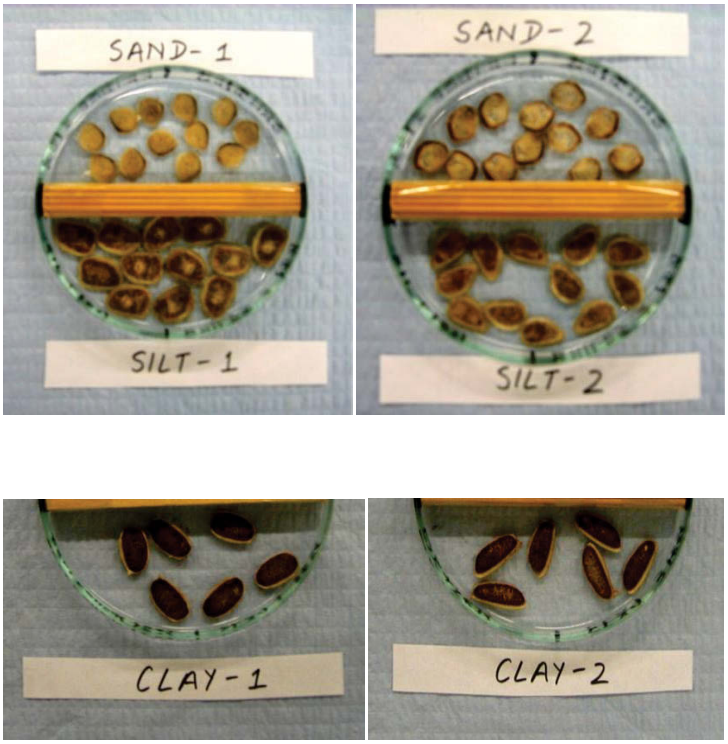

Figure 6-1. Photographs of sandy, silty and clay bone samples

6.2.2 Thermogravimetric Analysis

Bones buried in different soil types were analysed using TG and the results were compared in terms of reproducibility and the effect of soil type on bone structure.

6.2.2.1 Reproducibility

Mass loss data of the samples buried in silt, sand and clay soils are shown Figures 6-2, 6-3 and 6-4, respectively, with the averages and standard deviations for the samples given in Table 6-2. For the silty soil environment, the results of the bone samples buried for shorter periods of time (up to and including 4 months of burial) were similar. Similarly, the results of the bone samples buried in a sandy soil environment for up to 6 months were reproducible. The bone samples buried for longer periods of time, in both the silty soil and sandy soil environment, showed variation in the results. This variation can again be attributed to the variations that

are possible in the degradation processes that occur in bone when bones are buried for a longer period of time.

By contrast, the results of the bone samples buried in a clay soil environment show great variations at all the post-burial times and can be said to not be reproducible. This indicates that the degradation processes occurring in bone can occur at different rates and follow different pathways, even when the bones are buried in the same clay environment.

The results of the bones buried in the silty and sandy soil environments are similar in terms of reproducibility, in comparison to the results of the bones buried in a clay soil environment.

Table 6-2. Total mass loss ± standard deviation (%) at each post-burial time for defleshed bones buried in loam, silty, sandy and clay soils (n = 80)

Post-burial time (months)	Loam Defleshed (%)	Silt (%)	Sand (%)	Clay (%)
0	35.6 ± 0.7	35.6 ± 0.7	35.6 ± 0.7	35.6 ± 0.7
1	35.3 ± 2.8	36.3 ± 0.1	36.2 ± 2.5	40.9 ± 0.8
2	35.5 ± 2.7	35.7 ± 0.5	33.9 ± 0.8	38.9 ± 6.8
4	29.8 ± 1.3	32.4 ± 0.1	31.5 ± 0.6	39.6 ± 3.5
6	30.5 ± 0.0	33.7 ± 2.0	33.7 ± 1.0	36.3 ± 1.5
8	32.3 ± 1.6	39.0 ± 3.4	35.9 ± 9.3	39.1 ± 1.3
10	32.4 ± 5.6	35.6 ± 1.4	50.5 ± 2.6	42.9 ± 1.1
12	30.1 ± 5.1	32.6 ± 1.2	36.3 ± 3.9	36.2 ± 2.3
15	38.2 ± 4.4	36.8 ± 1.9	36.3 ± 0.7	38.3 ± 2.5
18	33.6 ± 0.6	37.2 ± 1.1	37.1 ± 3.0	37.7 ± 0.1

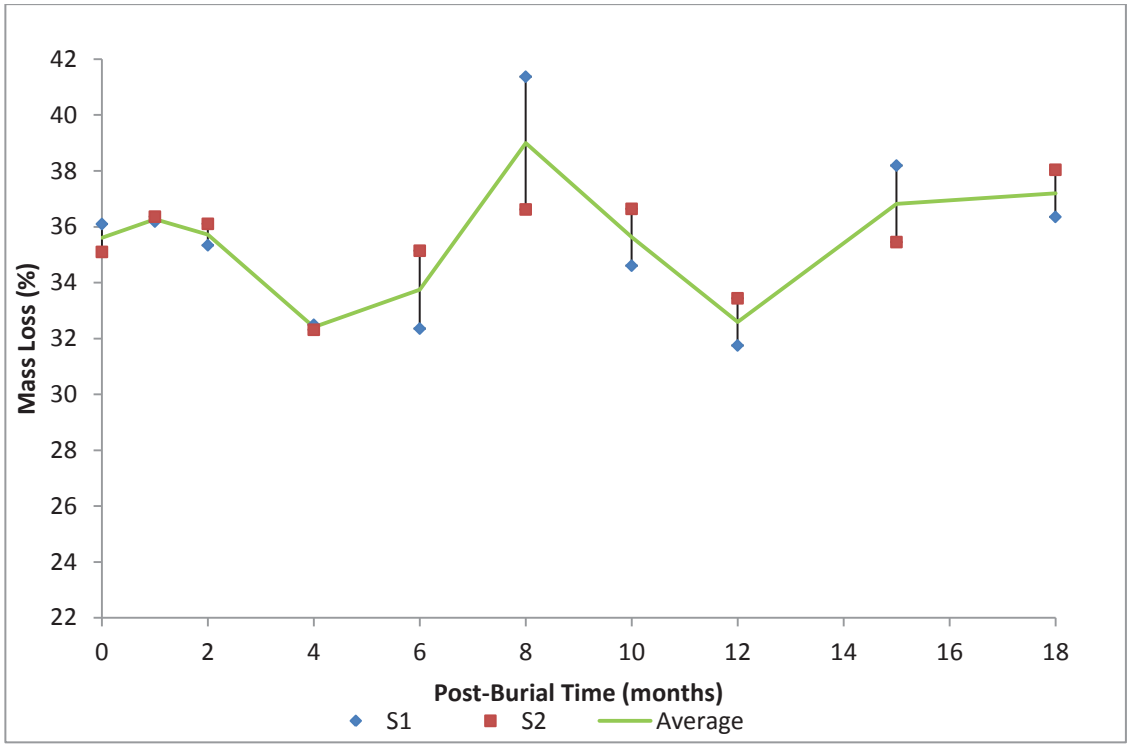

Figure 6-2. Comparison of TG results of Silt 1 and 2 bone samples

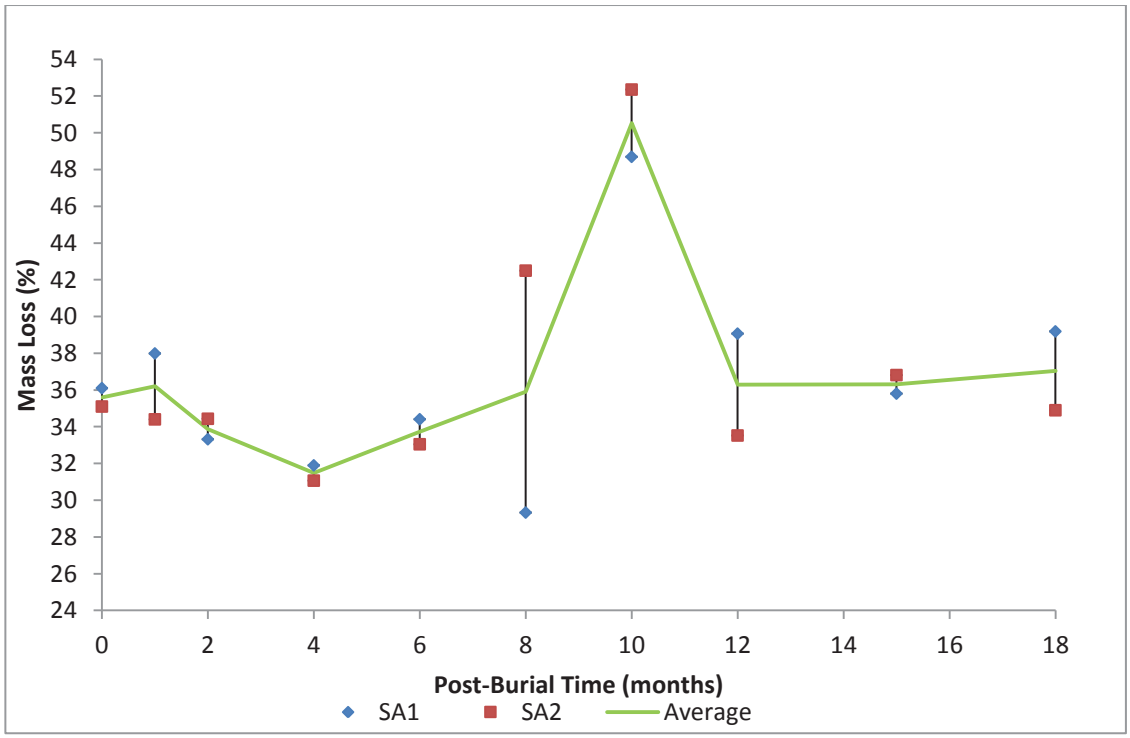

Figure 6-3. Comparison of TG results of Sand 1 and 2 bone samples

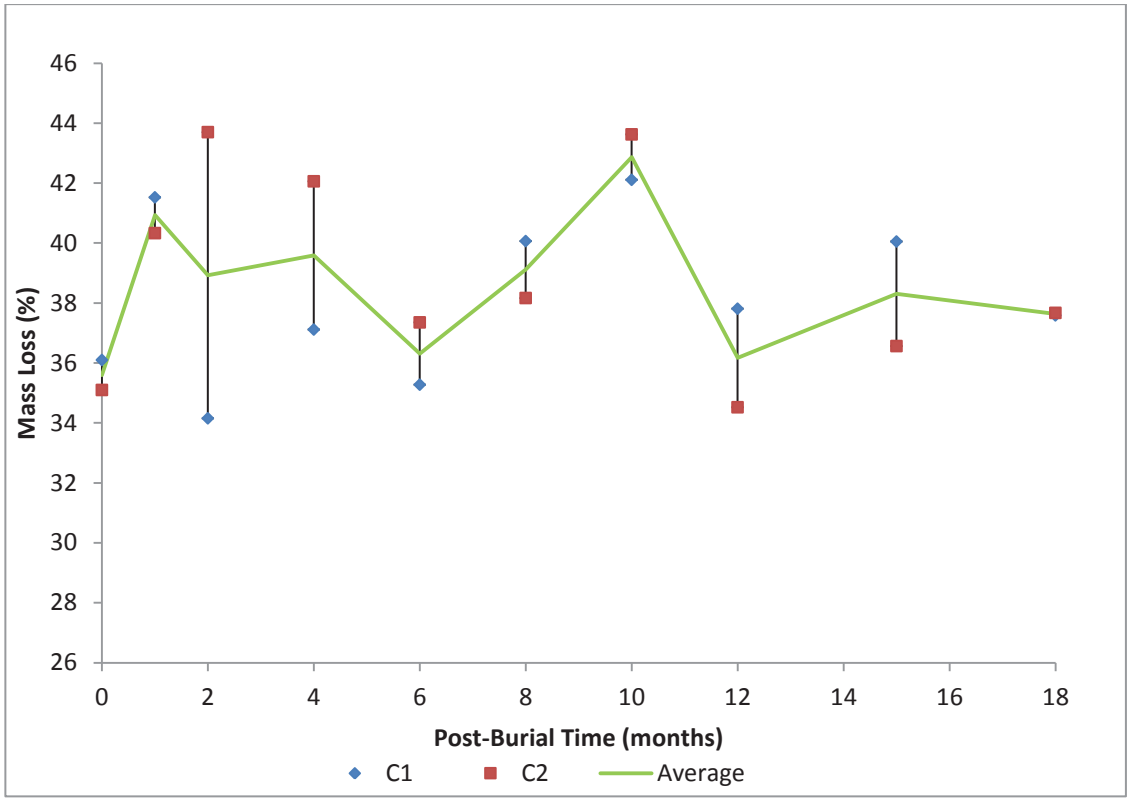

Figure 6-4. Comparison of TG results of Clay 1 and 2 bone samples

6.2.2.2 Effect of Soil Type on Bone Structure and Decomposition

Soil type in itself is a significant aspect to consider in burial decomposition rates. In this study, the loam (control) soil was compared with the silt, sand and clay soils to see the effect of the soil type on bones. The TG curves of the bones buried in these soil types after 1 and 18 months of burial are presented in Figures 6-5 and 6-6, respectively. After the first month of burial, the TG curves of the bones buried in all four soil types followed a similar trend but significant differences existed in the total mass loss that occurred in the different soil types. The total mass loss was the lowest for the bones buried in loam soil, followed by silty soil and then sandy soil, as can be seen in Figure 6-5. The highest mass loss observed was in the bones buried in clay soil. After 18 months of burial, the bones buried in loam, silty and sandy soils

followed the same trend. The TG curve of the bones buried in clay soil followed the same trend as the other soil types up to a temperature of 570 °C. After 570 °C, there was a marked increase in the mass loss occurring, indicating that more organic content may be present and had not decomposed to the same extent as the rest of the soil types during burial. This is confirmed by the DTG curve (Figure 6-7) showing an inflection at a temperature of 570 °C, signifying a more intense mass loss occurring at that temperature, however, the same trend is not observed for the repeat clay samples (Figure 6-8). Interestingly though, inflections suggesting an increase in mass loss can be seen in the repeat samples – inflections for the sandy soil sample at 625 °C and for the clay and silty soil samples at 690 °C and 700 °C, respectively. These results suggest that the silty, sandy and clay soil environments do not degrade certain organic components of bone as aggressively as the loam soil environment.

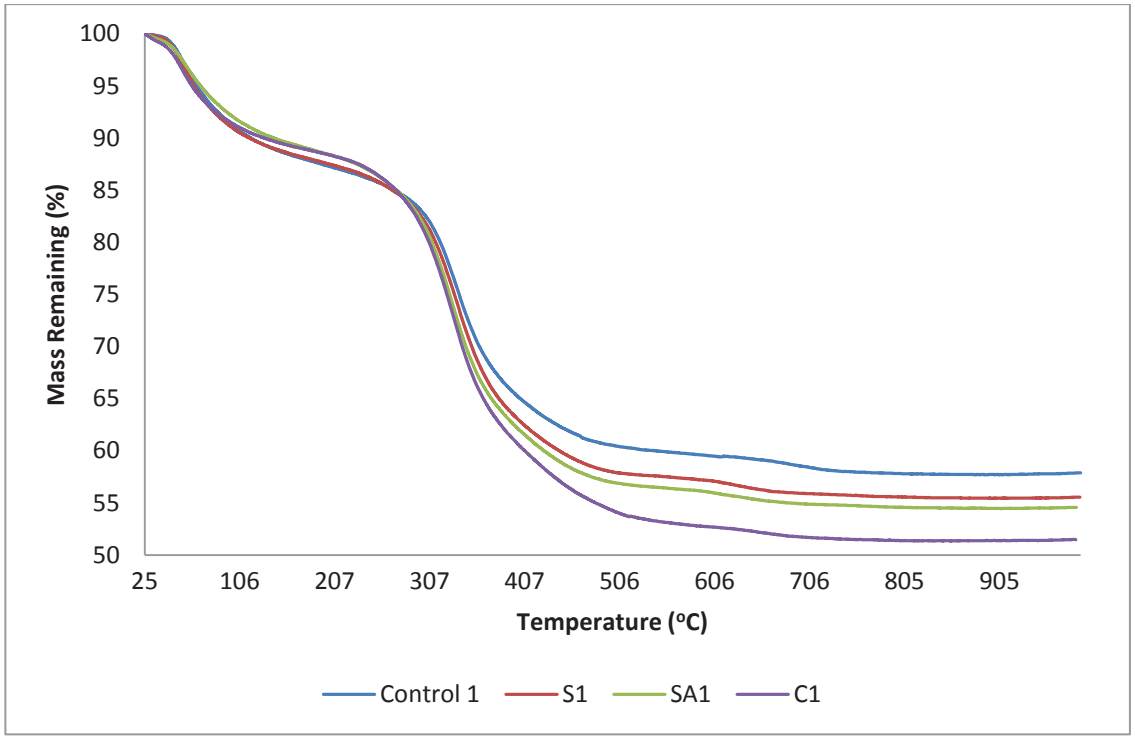

Figure 6-5. TG curves of bones buried in different soil types after 1 month of burial

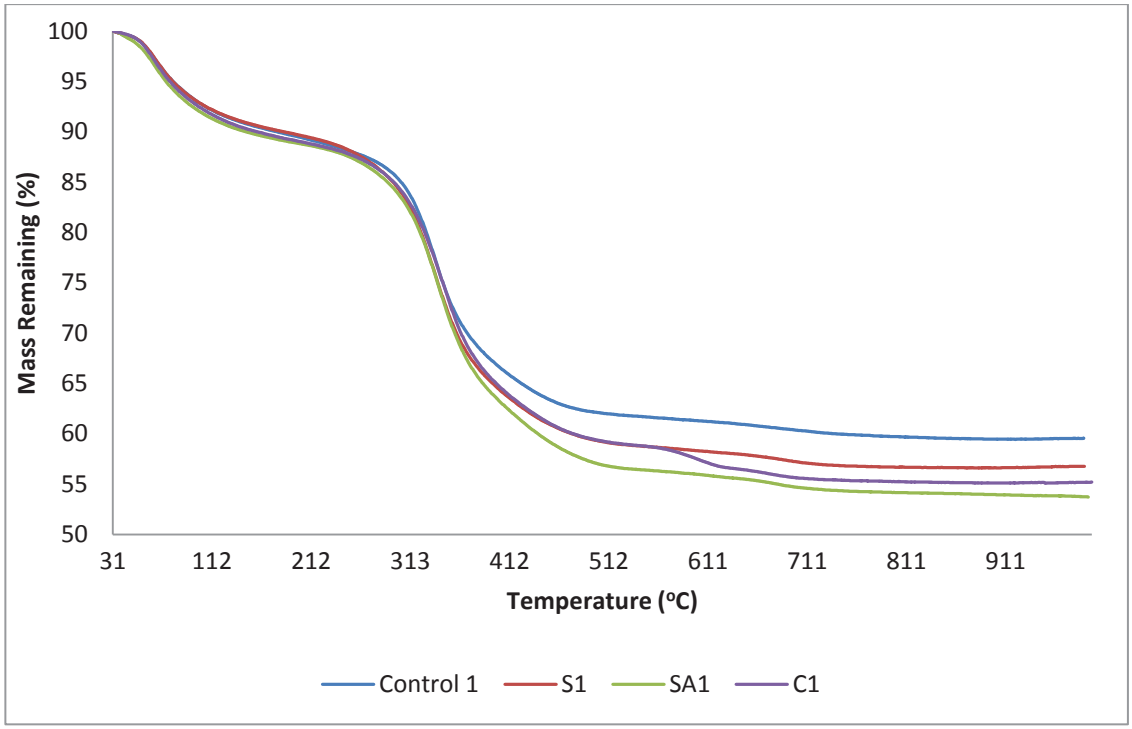

Figure 6-6. TG curves of bones buried in different soil types after 18 months of burial

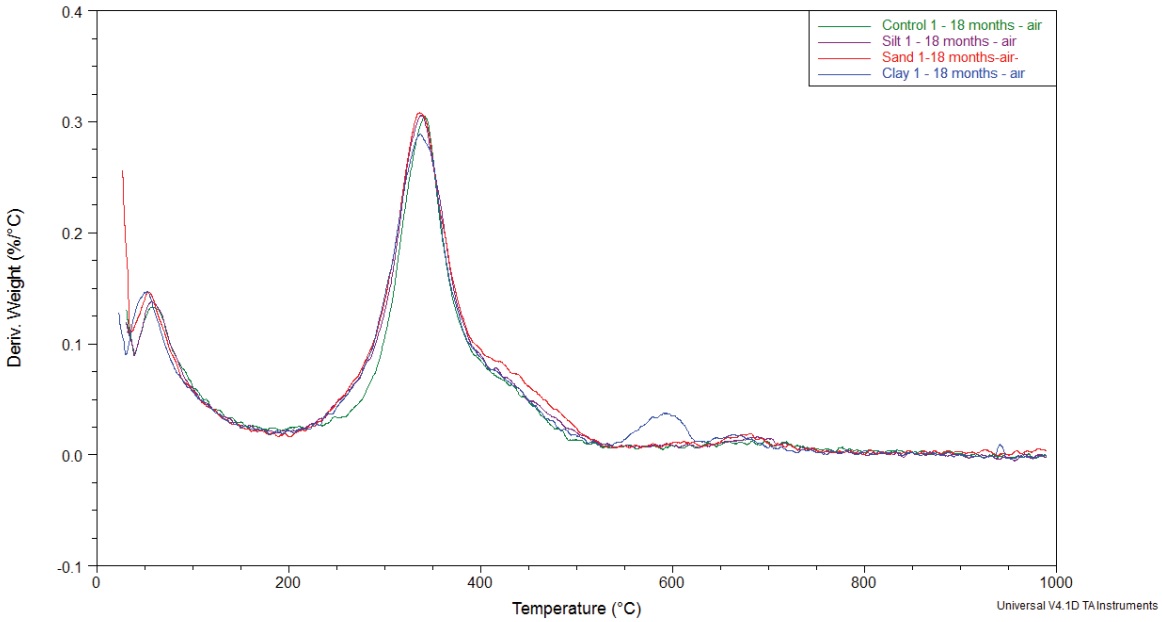

Figure 6-7. DTG curves of bones buried in different soil types after 18 months of burial

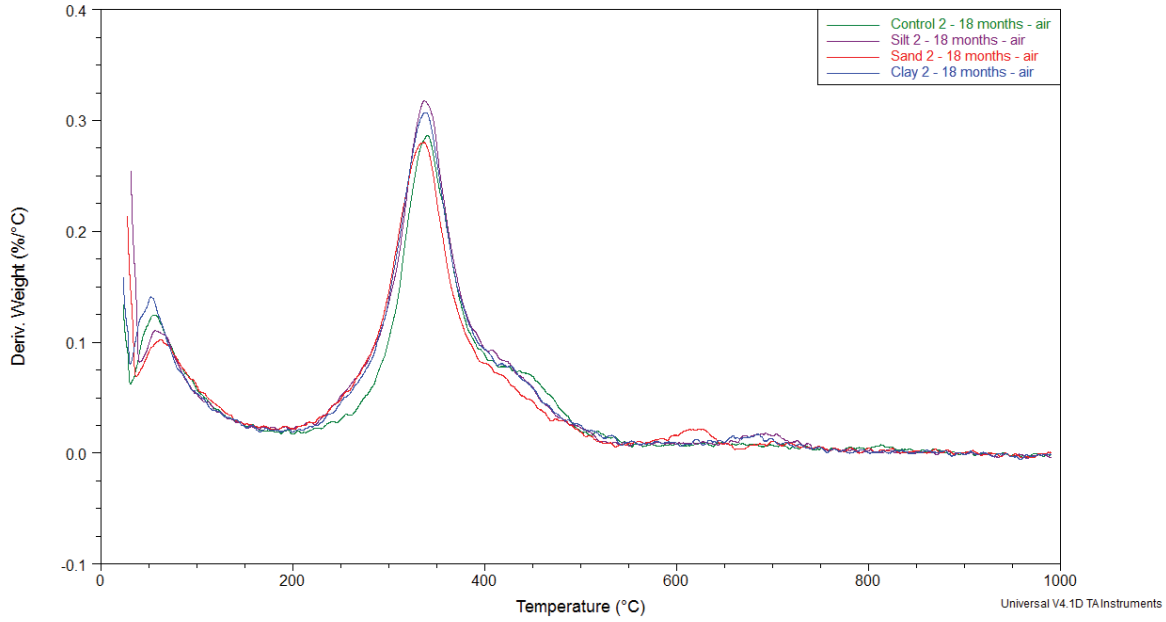

Figure 6-8. DTG curves of bones buried in different soil types after 18 months of burial (repeat samples)

Comparison of the total mass loss occurring in the different soil types is shown in Figure 6-9. Bones buried in the loam, silty and sandy soils show the same mass loss trends up to and including a burial period of 8 months, with the mass loss being the greatest in the loam soil environment. Within a burial period of 8 and 10 months, the loam and silty soil bone samples follow the same trend and the bones buried in sandy and clay soils follow the same trend. After 10 months of burial, bones buried in all four soil types show the same trend in mass loss.

Overall, the total mass loss observed in the bones buried in the loam soil for the 18 months of burial was lower when compared to the other soil types. It may be expected that bone diagenesis would occur more readily in loam soil than silt, sand or clay soils, due to the presence of bacteria in loam soil. When compared to the loam soil, the silt, sand and clay soils preserve the bone samples and therefore, have more mass to lose when heated in TG.

For the first 8 months of burial, clay soils tend to be the most effective in preserving the bone samples, as they show the greatest mass losses, when analysed using TG. For the period between 8 and 12 months of burial, a sandy soil environment appears to extensively preserve the bone, but after 12 months of burial, it is again the clay soil environment which seems to preserve the bone samples. Previous research has demonstrated that coarse-textured soils such as sand generally promote desiccation, which can inhibit decomposition (Fiedler and Graw, 2003; Santarsiero *et al.*, 2000). Similarly, fine-textured soils such as clay have been shown to retard bone decomposition due to low rates of gas diffusivity (Carter, 2005). These findings are supported by the results of the present study in that the sandy and clay soils appear to be more effective in hindering bone decomposition compared to loam and silty soils. It is also interesting to note that after 18 months of burial, the bones buried in silty, sandy and clay soil environments result in the same total mass loss.

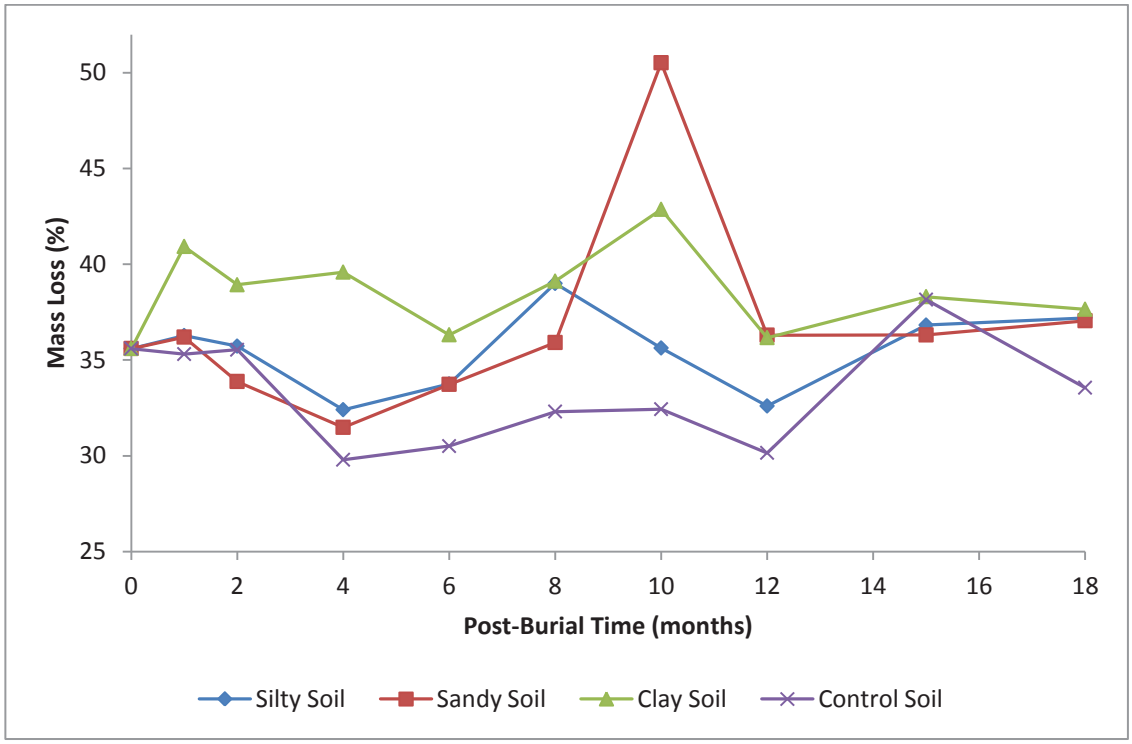

Figure 6-9. Comparison of TG results of loam, silt, sand, clay and loam (control) soils

6.2.3 Pyrolysis Gas Chromatography-Mass Spectrometry

The type of soil in the burial environment was also investigated using Py-GC-MS analysis. The Py-GC-MS analysis results of bone samples buried in a silty soil environment subjected to statistical analysis are presented in Figure 6-10.

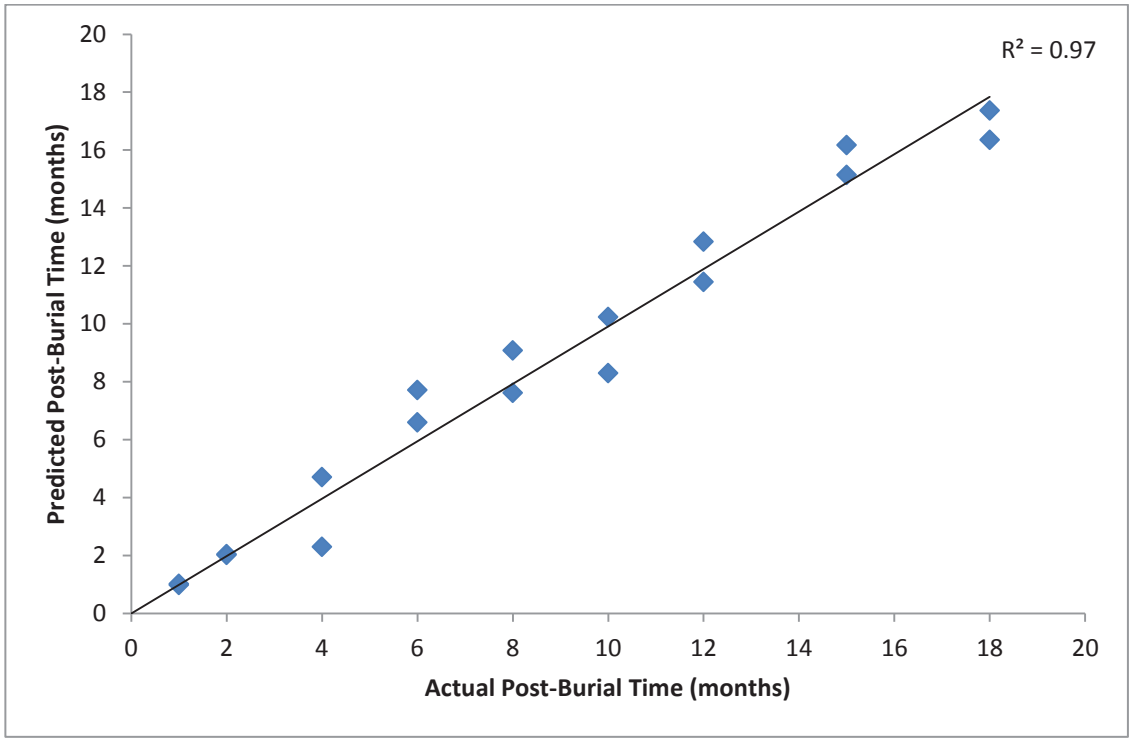

Figure 6-10. Correlation of predicted post-burial time with actual post-burial time for bone samples buried in silt (1 and 2)

Bone samples buried in a silty soil environment produced a high coefficient of determination value (R^2 = 0.97, $p < 0.05$) when the Py-GC-MS results were examined using statistical analysis. Most of the post-burial times of the bone samples can be predicted based on these results. The only bone sample that showed slight variation in the predicted and actual post-burial time values was the 4 month sample. Therefore, it can be concluded that estimation of

the post-burial time of bone samples buried in a silty soil environment can be made using Py-GC-MS results up to 18 ± 2.1 months.

Throughout the burial period of 18 months, it might be expected that many organic compounds present in the bones would have changed or decomposed. The retention times of compounds that significantly changed with post-burial times were highlighted during statistical analysis. It can be said that these compounds contributed to the estimation of the post-burial time of the bone samples buried in a silty soil environment. These compounds are presented in Table 6-3.

Table 6-3. Significant retention times and corresponding compounds in bone samples buried in silt at different post-burial times

		Burial Time (months)			
Retention Time (min)	**Compound Name**	**1**	**6**	**12**	**18**
19.142	phenoxy-phenol	N	Y	Y	N
19.250	3-phenoxy-phenol	Y	N	N	N
20.292	trans-1,1,3,4-tetramethyl-cyclopentane	Y	N	Y	N
22.142	octadecanoic acid	Y	N	N	N
21.267	hexadecanenitrile	N	Y	N	Y
23.383	heptadecanenitrile	Y	N	N	N
24.058	2-hexyl-1-decanol	N	N	Y	N
24.575	1-(1,2-dimethylpropyl)-1-methyl-2-nonyl-cyclopropane	N	Y	N	N

The second type of soil investigated was sand and the bones buried in this soil medium were also analysed using Py-GC-MS. The results were then examined using statistical analysis and are presented in Figure 6-11. The results of the bones buried in a sandy soil environment ($R^2 = 0.88$, $p < 0.05$) show a greater amount of scatter when compared to the results of the silty ($R^2 = 0.97$, $p < 0.05$) and clay ($R^2 = 0.90$, $p < 0.05$) soil environments. This could be because there is minimal decomposition in a sandy soil environment meaning smaller

changes in mass loss, which makes correlation less accurate. Based on these results, only some of bones can be identified with the actual post-burial time (18 ± 4.1 months). Bone samples with younger post-burial times (up to and including 4 months) can be estimated by this method. However, bone samples with older post-burial times (greater than 4 months) show scatter in the results and cannot be accurately estimated. Another possible reason may be that the compounds changing and decomposing in a sandy soil environment are doing so at rates which do not follow a linear trend. Therefore, these result in a lower coefficient of determination as compared to the other burial environments, and so, linear regression analysis cannot be used to accurately predict the post-burial time of bone samples buried in sandy soil.

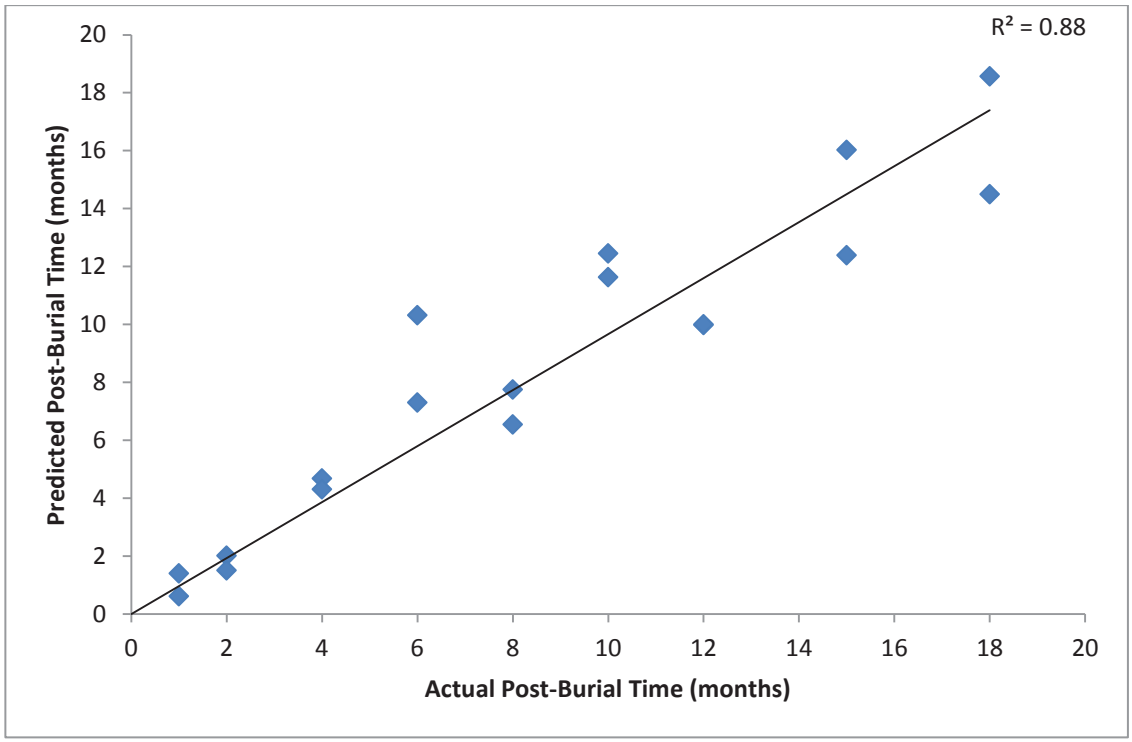

Figure 6-11. Correlation of predicted post-burial time with actual post-burial time for bone samples buried in sand (1 and 2)

The compounds that are changing and decomposing are presented in Table 6-4. The results of the bones buried in a sandy soil environment indicate that the changes in these compounds are occurring at a rate which cannot be accounted for using linear regression analysis. However, it is still important to note which compounds are present during different time periods of the burial.

Table 6-4. Significant retention times and corresponding compounds in bone samples buried in sand at different post-burial times

		Burial Time (months)			
Retention Time (min)	**Compound Name**	**1**	**6**	**12**	**18**
8.3920	2-cyclohexen-1-one	Y	N	N	N
9.2830	2,4-dimethyl-2,4-heptadienal	Y	N	Y	N
9.4250	2,3-dimethyl-1,3-heptadiene	Y	N	N	N
10.792	1,2,3,6-tetramethyl-bicyclo 2.2.2 octane	Y	N	N	N
10.850	N,3-dimethyl-benzenamine	Y	N	N	N
11.617	6-nitro-2-picoline	Y	N	N	N
11.725	(Z)-2-dodecene	N	Y	N	N
12.100	N-ethylidene-1-pyrrolidinamine	Y	N	N	N
12.242	Piperidinone	Y	N	N	N
13.858	1,2-dimethylpiperidine	Y	N	N	N
15.400	trans-2-methyl-5-(1-methylethyl)-cyclohexanone	N	Y	N	N
15.433	cis-tetrahydro-2,5(1H,3H)-pentalenedione	Y	N	N	N
16.375	5-methyl-2-(1-methylethyl)-2-cyclohexen-1-one	Y	N	N	N
16.392	1-(cyanoacetyl)-piperidine	Y	N	Y	Y
17.600	2,6-dihydroxy-benzoic acid	Y	Y	Y	N
18.792	2-ethyl-quinoline	Y	N	N	N
19.142	phenoxy-phenol	Y	Y	N	Y
20.192	isopropyl myristate	N	Y	N	N
20.292	trans-1,1,3,4-tetramethyl-cyclopentane	Y	N	Y	N
23.125	9-cycloheptadecen-1-ol	Y	N	N	N

Bones buried in a clay soil environment were also analysed using Py-GC-MS and then PLS regression analysis using mean-center pre-processing and linear baseline correction was carried out. A coefficient of determination of $R^2 = 0.90$ ($p < 0.05$) was achieved. Results of the statistical analysis of these bone samples are shown in Figure 6-12. Similar to the results of the bone samples buried in a sandy soil environment, bone samples buried in a clay soil environment showed scatter in the results. It was noted, however, that the post-burial times of the younger bone samples (up to and including 4 months) can be predicted using Py-GC-MS. Estimation of the older post-burial time bone samples is more difficult but can still be predicted up to 18 ± 5.6 months.

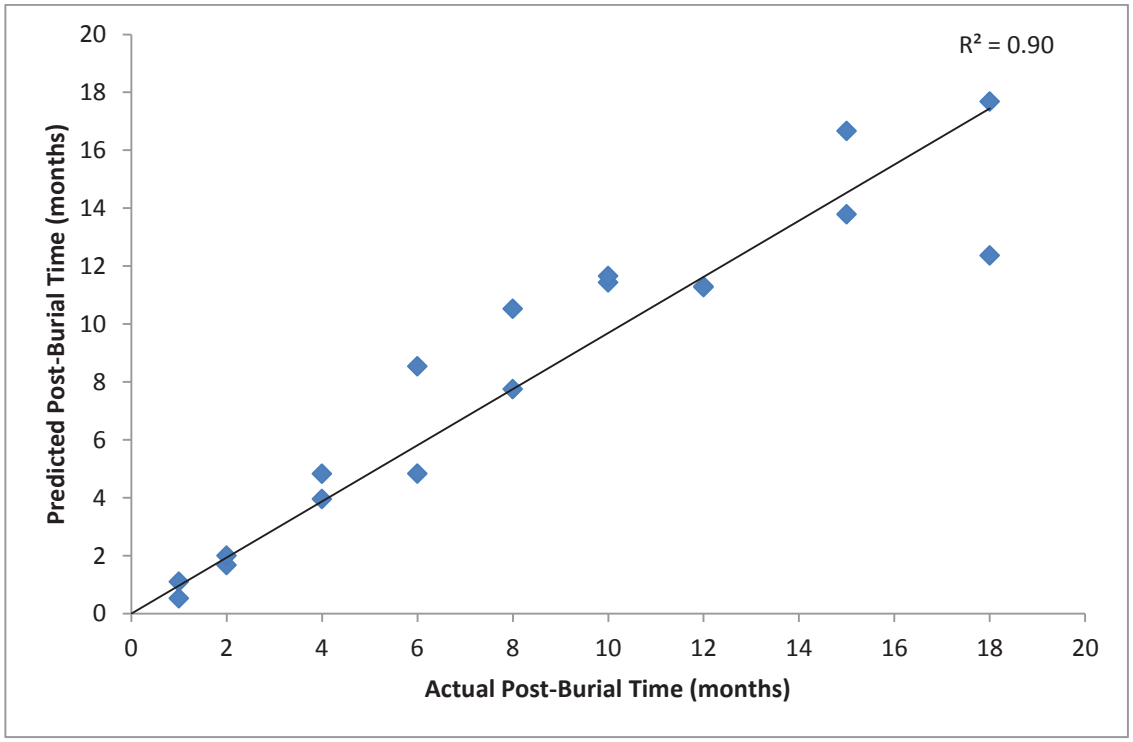

Figure 6-12. Correlation of predicted post-burial time with actual post-burial time for bone samples buried in clay (1 and 2)

The compounds that are changing significantly, even though not following a completely linear trend, are presented in Table 6-5. These are the compounds that enable the estimation of the

post-burial time of younger bone samples. Even though the change in these compounds is not linear, it is still important to take note of the compounds that are present during the different stages throughout the burial.

Table 6-5. Significant retention times and corresponding compounds in bone samples buried in clay at different post-burial times

		Burial Time (months)			
Retention Time (min)	**Compound Name**	**1**	**6**	**12**	**18**
10.867	5-methyl-1-hexene	Y	N	N	N
11.817	Dodecane	N	N	Y	N
13.858	1,2-dimethyl-piperidine	Y	N	N	N
15.000	1-(O-methyloxime)-4-O-3-acetyl-1-(trimethylsilyl)-1H-indolyl-2,3,5,6-tetrakis-O-(trimethylsilyl)-D-glucose	Y	Y	N	N
15.333	2,2,6,6-tetramethyl-4-piperidinamine	N	Y	N	N
15.425	2-amino-4(1H)-pyrimidinone	N	Y	N	N
17.600	2,6-dihydroxy-benzoic acid	Y	Y	N	N
19.142	phenoxy-phenol	Y	Y	N	N
19.650	(Z)-3-methyl-2-undecene	Y	Y	Y	N
20.192	isopropyl myristate	N	Y	N	N
20.292	trans-1,1,3,4-tetramethyl-cyclopentane	Y	Y	Y	Y
20.867	1-heptadecanol	Y	Y	N	N
21.267	hexadecanenitrile	Y	Y	Y	Y
21.442	4-amino-6-methoxy-2-(trifluoromethyl)-pyrimidine	Y	N	N	N
21.900	2-ethyl-1-methyl-3-propyl-cyclobutane	Y	N	N	N
23.158	hexadecenenitrile	Y	N	N	N
23.392	octadecanenitrile	Y	N	N	N
25.900	(Z)-9-octadecenamide	Y	Y	Y	N

6.3 Soil pH

Since bone comprises of both organic and inorganic components, the acidity of the soil is an important factor to consider for the accurate post-burial time estimation of recovered bones. Therefore, this study attempts to monitor the differences between samples buried in acidic (pH = 5-6), basic (pH = 9-10) and neutral (pH = 7) soils using various analytical techniques.

6.3.1 Visual Observations

In this study, bones buried in soils with varying pH were observed and the physical characteristics of the bone samples are recorded in Table 6-6. The colour of the bones buried in acidic soil changed throughout the burial study as described in Table 6-7. From the very first month, tiny holes and cracks started appearing on the bones and became more prominent as the burial time increased, with holes entirely covering the bones by 8 months of burial. Also, after 6 months of burial, the bones started to become brittle and were difficult to cut using the diamond saw as they crumbled. No odour was present in any of the bone samples. No microbial activity was evident in any of the bone samples except for the presence of a small amount of white/pale pink fungus on the 4-month Loam Acidic 2 sample.

For the bone samples buried in basic soil (pH = 9-10), there were no visible colour changes in the bones over the burial period. Tiny holes and cracks began to appear in the bones after 8 months of burial. No odour was present in any of the bone samples. No microbial activity was present in any of the bone samples except for the first 8-month basic sample, which had a minimal amount of white-pink fungus. Interestingly, half of the container of the Loam Basic 1 samples was covered in white fungus-like material at the 8-month burial interval, but disappeared before the 10 month bones were recovered.

Table 6-6. Physical characteristics of bone samples buried in soils varying in pH analysed at each post-burial time (n = 54)

Soil Type	pH	Average Length (cm)	Standard Deviation (±)	Average Diameter (cm)	Standard Deviation (±)	Average Mass (g)	Standard Deviation (±)
Neutral (Control)	7	8.2	0.49	1.5	0.46	6.5	1.9
Acidic	5-6	7.9	0.80	1.6	0.42	6.8	2.8
Basic	9-10	8.9	1.0	1.2	0.19	6.7	2.3

Table 6-7. Observations of bones buried in acidic soil at different post-burial times

Post-burial Time (months)	Colour	Observations
1	Light brown-white	Few tiny holes/cracks present
2	Light brown-white	Few tiny holes/cracks present
4	Light brown-white	Few tiny holes/cracks present
6	Light brown-light pink	Bones started to become brittle
8	Light brown-light pink	Covered in tiny holes/cracks; brittle
10	Light brown-light pink	Covered in tiny holes/cracks; brittle
12	Light brown	Covered in tiny holes/cracks; brittle
15	Light brown-red	Covered in tiny holes/cracks; brittle
18	Light brown-red	Covered in tiny holes/cracks; brittle

Once the bones were recovered, sectioned and dried, photographs were taken of the samples. As Figure 6-13 presents, bones that were buried in acidic (pH = 5-6) and basic (pH = 9-10) soils looked very different once they were recovered, sectioned and dried. Bones buried in acidic soils were predominantly white with small pink areas, while bones buried in a basic soil environment were light brown-pale yellow in colour. Also, bones that were buried in an acidic soil environment crumbled upon sectioning after just one month of burial. After about six months of burial, these bones began to crumble as soon as they were touched. This was not the case for the bones buried in a basic soil environment. These results confirm the

destructive nature of an acidic soil environment on buried bones, when compared to a basic soil environment.

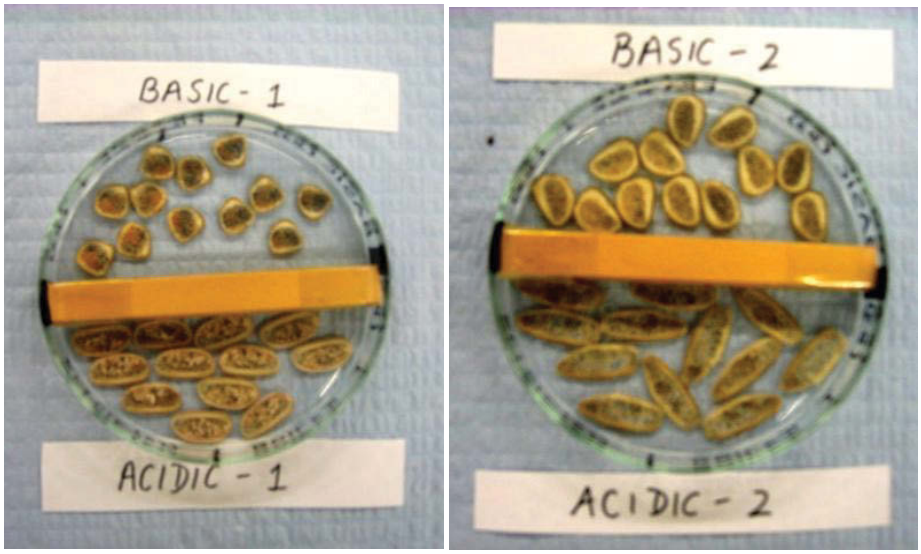

Figure 6-13. Photographs of acidic and basic bone samples

6.3.2 Environmental Scanning Electron Microscopy

The technique of ESEM was used in conjunction with the BSE detector to analyse the longitudinally fractured bone samples recovered from the various burial conditions at the different post-burial times. Using the measurement tool in the ESEM software, the diameters of the visible pores were measured for each sample using the BSE detector. Any outliers in the measurements were excluded using the q-test. The average diameters of both bone samples buried in each burial condition were calculated and then compared to determine if any trends existed within the data.

The results of the calculations for the samples buried in acidic, basic and neutral soils are presented in Figure 6-14. The graph shows a decreasing trend in pore size with increasing post-burial time up to 6 months (Figures 6-15 and 6-16) where upon the pore size appears to

reach a limiting value. These trends are tentative as significant scatter is observed in the data. The bones buried in acidic and basic soils show a more linear trend when compared to the bones buried in neutral soils. However, the scatter in the data is quite large and therefore limits the utility of this technique.

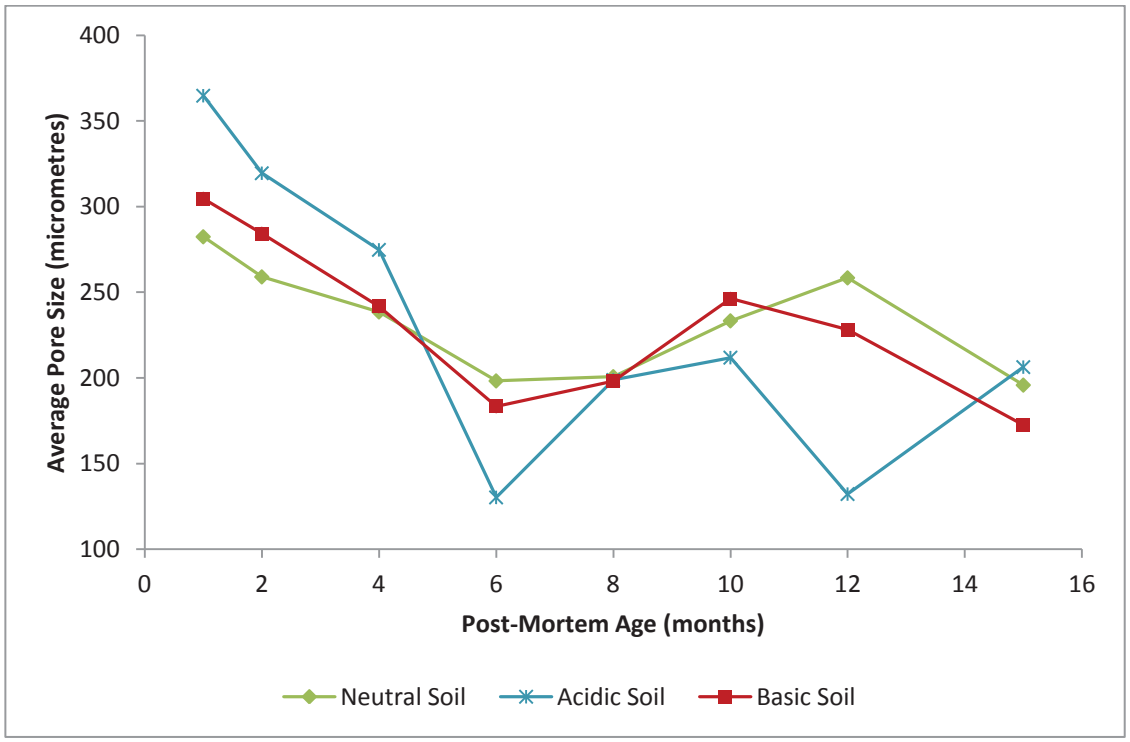

Figure 6-14. Average pore size vs post-burial time of bone buried in acidic, basic and neutral soils

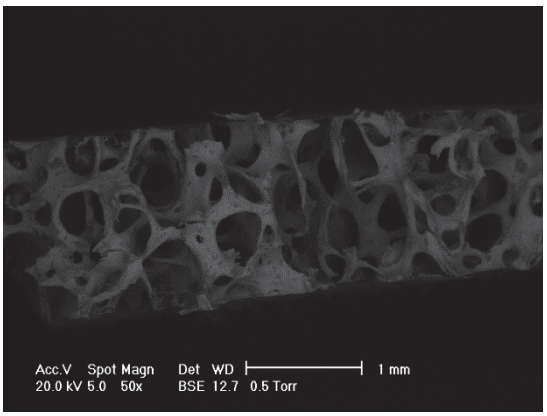

Figure 6-15. Loam Basic 1 – 1 month

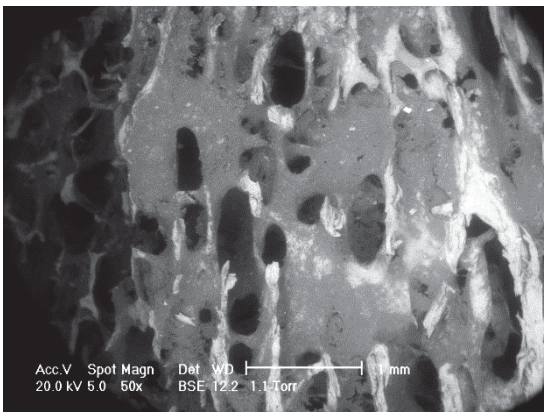

Figure 6-16. Loam Basic 1 – 6 months

6.3.3 Thermogravimetric Analysis

Bones buried in soils of varying pH values were analysed using TG and the effect of acidic and basic soils on the progress of bone diagenesis was explored.

6.3.3.1 Reproducibility

For the acidic soil environment, a pH of 5-6 was maintained for the duration of the study. Figure 6-17 shows the TG results of the defleshed bones buried in acidic loam soil. The results were similar for most of the bone samples, with the exceptions being bones buried for 8, 10, 12 and 18 months. Generally, the standard deviations of the bones buried for shorter lengths of time (less than 8 months) were lower than the standard deviations of the bones buried for longer. As suggested for the neutral soil samples, a possible reason for the variation in the bone samples buried for a longer period in acidic soil could also be that more variation is possible in the degradation processes occurring in bones that have been buried for longer.

Table 6-8. Total mass loss ± standard deviation (%) at each post-burial time for defleshed bones buried in neutral, acidic and basic soils (n = 60)

Post-burial time (months)	Neutral (Control) (%)	LA (%)	LB (%)
0	35.6 ± 0.7	35.6 ± 0.7	35.6 ± 0.7
1	35.3 ± 2.8	35.8 ± 0.5	34.0 ± 0.4
2	35.5 ± 2.7	32.7 ± 0.3	37.0 ± 3.6
4	29.8 ± 1.3	28.8 ± 0.6	29.6 ± 0.6
6	30.5 ± 0.0	29.4 ± 0.1	30.7 ± 0.4
8	32.3 ± 1.6	30.1 ± 4.0	37.6 ± 8.4
10	32.4 ± 5.6	27.0 ± 2.8	31.4 ± 0.6
12	30.1 ± 5.1	22.5 ± 2.1	31.0 ± 1.7
15	38.2 ± 4.4	22.1 ± 0.3	32.3 ± 2.8
18	33.6 ± 0.6	24.0 ± 3.4	33.6 ± 0.1

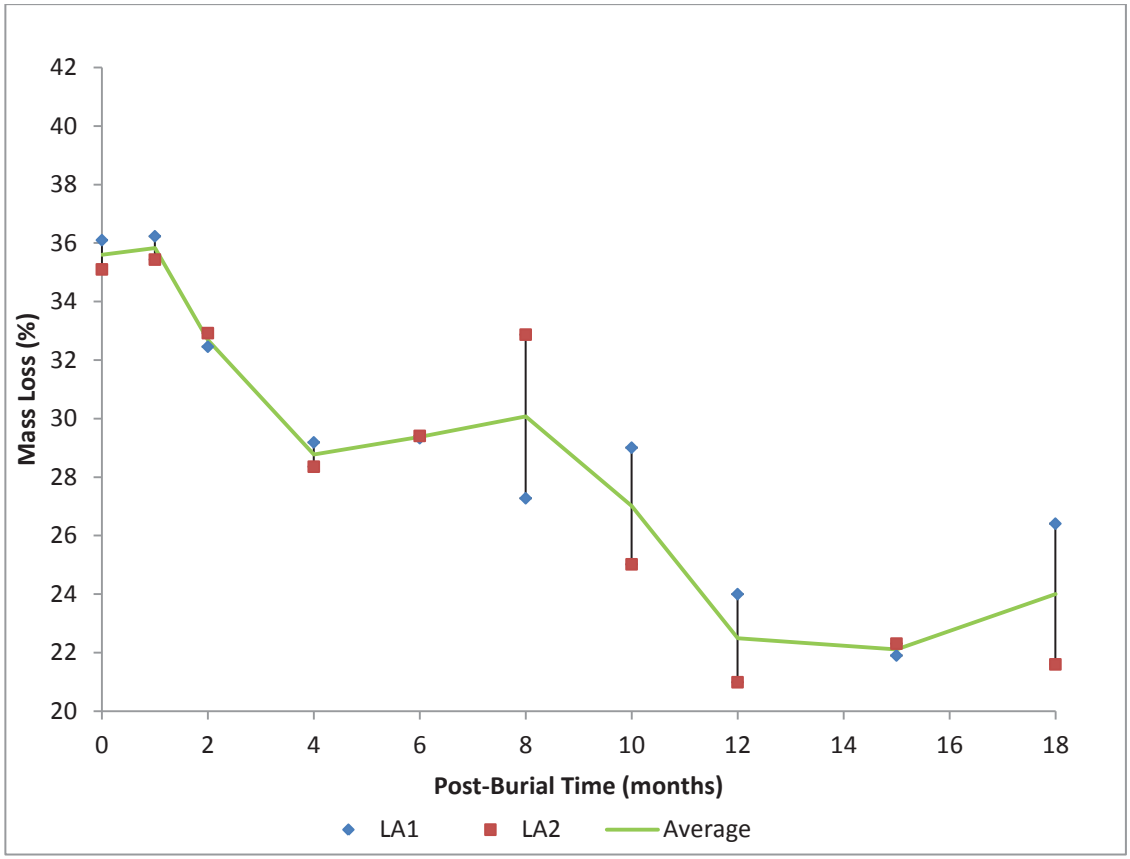

Figure 6-17. Comparison of TG results of Loam Acidic 1 and 2 bone samples

The basic soil environment had a pH of 9-10, which was maintained throughout the 18 months of burial. The results of the defleshed bone samples buried in basic loam soil are shown in Figure 6-18. The averages and standard deviations of the results are recorded in Table 6-8. The results obtained for all the post-burial periods are similar except for the 2, 8, 12 and 15 month samples. The mass loss averages ranged from 29.6 ± 0.6 % to 37.6 ± 8.4 %. Again, greater variation is observed in the bone samples buried for longer periods of time. As proposed for the neutral and acidic soil samples, this variation could be due to more variation being present in the degradation processes occurring in bones that have been buried for longer. The variation in the total mass results of the 2 month samples is difficult to explain since the TG curves of both the bone samples buried in basic soil for 2 months follow a similar shape.

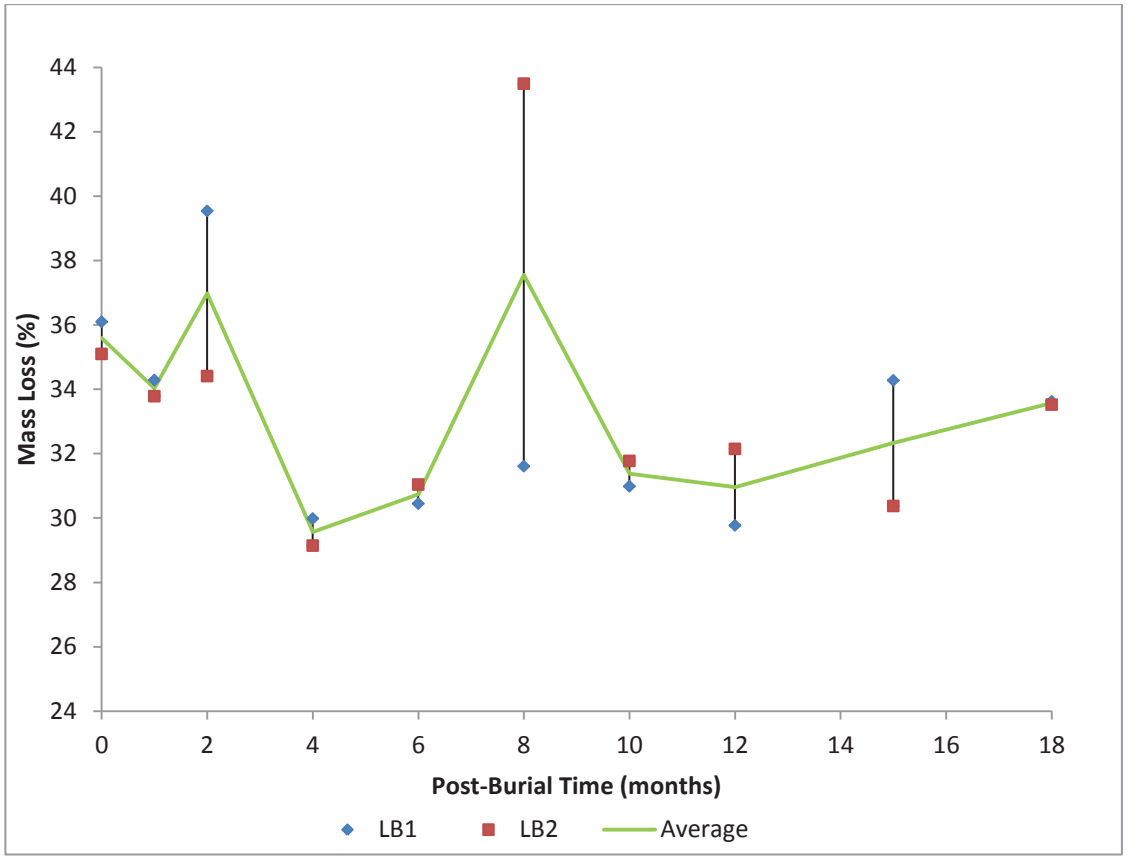

Figure 6-18. Comparison of TG results of Loam Basic 1 and 2 bone samples

6.3.3.2 Effect of Soil pH on Bone Structure and Decomposition

The TG curves of the bone samples buried in soils with varying pH after 1 and 18 months of burial are presented in Figures 6-19 and 6-20, respectively. The mass loss curves of the bones buried in neutral and basic soils follow the same trend and result in a similar total mass loss after 1 and 18 months of burial. The mass loss curves of the bones buried in acidic soil follow the same trend as the bones buried in neutral and basic soils but differences are present in the total mass loss results. After the first month of burial, the bones buried in acidic soils resulted in a higher mass loss during TG, suggesting that there was more organic content in these bone samples compared to the other bones. This might be explained by the fact that an

acidic environment attacks the inorganic content of the bone, rather than degrading the organic content. Research by Howes *et al.* (2012) demonstrated that the carbonate content of bones deteriorates more rapidly in an acidic soil environment during 8 months of burial. Phosphate content does not appear to be affected significantly in an acidic soil environment.

In contrast, after 18 months of burial, the mass loss in the bones buried in an acidic soil environment was much lower compared to the other environments. An explanation for this may be that as the inorganic content of the bone is significantly degraded by the acidic environment, the organic content becomes susceptible to degradation, which can then occur at a much faster rate. This may mean that a large proportion of the organic content is destroyed by the end of the burial duration of 18 months and, therefore, less organic content is present when the bones are analysed by TG.

The mass loss (%) vs post-burial period of defleshed bone samples buried in acidic, basic and neutral soils for a period of 18 months is shown in Figure 6-21. The mass loss results of the bone samples buried in neutral and basic soils follow the same trend up to and including 8 months post-burial. After 8 months of burial, the two burial environments result in different total mass losses in the bone samples.

The results of the bones buried in acidic soil show a decreasing trend with increasing post-burial period as can be seen in Figure 6-21. According to Gordon and Buikstra (1981), soil pH has the largest influence on bone preservation, with preservation generally effective in soils above pH 5.3. In contrast, basic and neutral soils can lead to the preservation of buried skeletal remains for centuries (Nielsen-Marsh et al., 2007). The results of the present study support these results in that acidic environments attack bone in a more rigorous manner compared to neutral and basic soils. In an acidic environment, the carbonate content of bone shows the maximum change, as acidic conditions are more likely to attack this component of

bone. Soils with a highly acidic pH decompose bone more rapidly due to the dissolution of the inorganic matrix of bone (Nafte, 2000). This is indicated by the decrease in mass loss that occurred in Step 3 as burial time increased, shown in Figure 6-22. Mass loss that occurs in Step 3 is a result of the decomposition of the inorganic content in bone. Since the mass loss occurring in Step 3 appears to be minimal, it can be assumed that the mass loss being observed in the TG results for the acidic bone samples is due to the organic phase of bone.

Figure 6-21 shows a decreasing trend of the total mass loss with increasing post-burial time up to 15 months of burial for the acidic bone samples (r^2 = 0.87, C.I. = 95%). As the study continued up to 18 months, a slight increase in the total mass loss was observed between 15 and 18 months of burial but the trend overall for the 18 months of burial was a decreasing one with respect to post-burial time.

This study supports the findings of a previous study that demonstrated the potential of TG in estimating the post-burial period of bones based on a percentage mass loss, when the sample is heated in an air atmosphere, to a temperature of 1000 °C at a rate of 10 °C/min (Onishi *et al.*, 2008). The bone samples used by Onishi *et al.* (2008) were buried in a soil with a pH of approximately 5, which is considered to be acidic. In the present study, the same pH was used for the acidic loam soil samples. The results of the present study strongly agree with the results obtained by Onishi *et al.* (2008) as both show a significant correlation of mass loss with post-burial time. Therefore, in a forensic context, TG is a valuable technique that can be used to estimate the length of burial for up to one and a half years in an acidic soil environment.

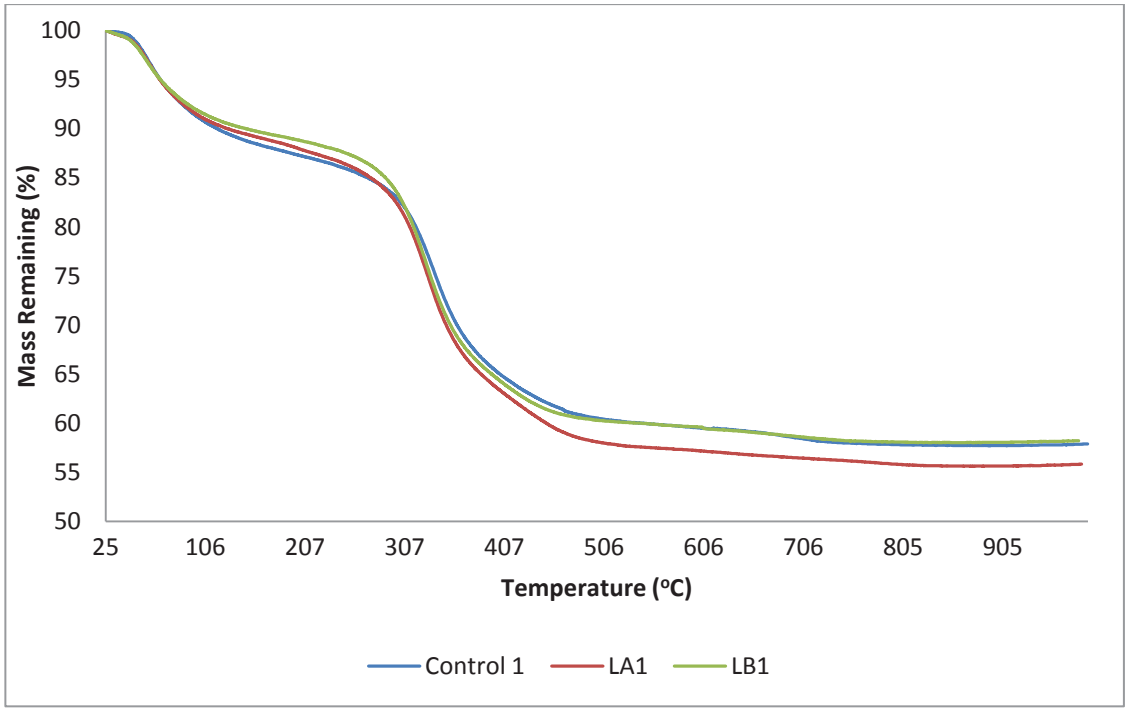

Figure 6-19. TG curves of bones buried in soils with varying pH after 1 month of burial

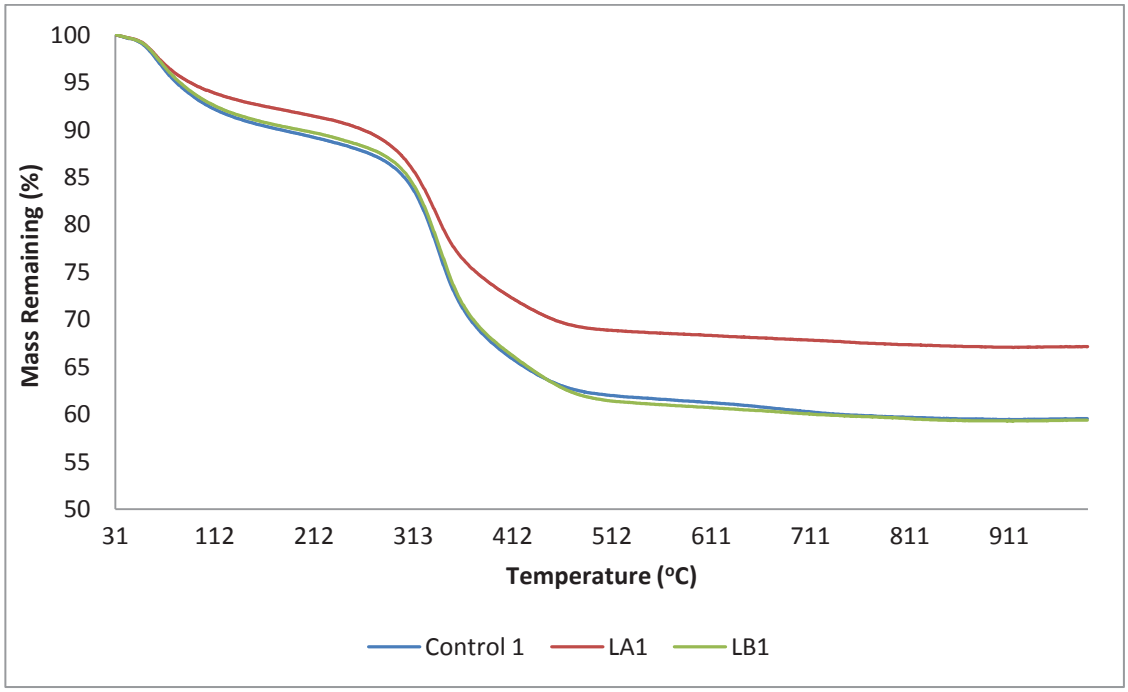

Figure 6-20. TG curves of bones buried in soils with varying pH after 18 months of burial

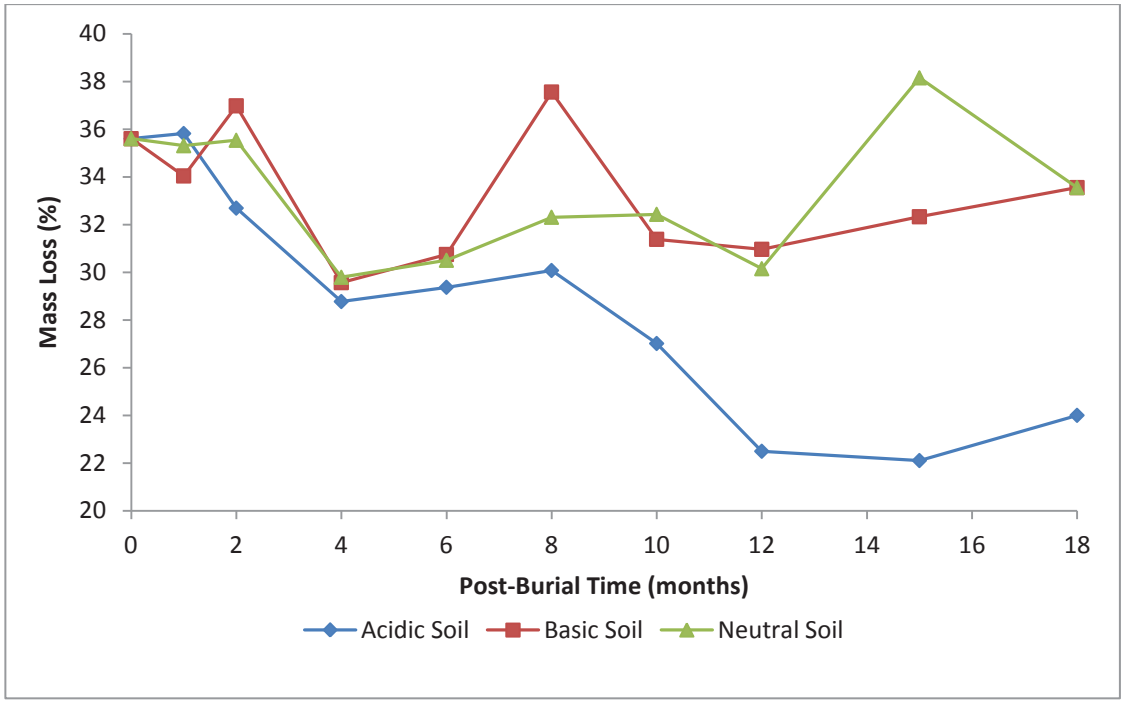

Figure 6-21. Comparison of TG results of bones buried in acidic, basic and neutral soil

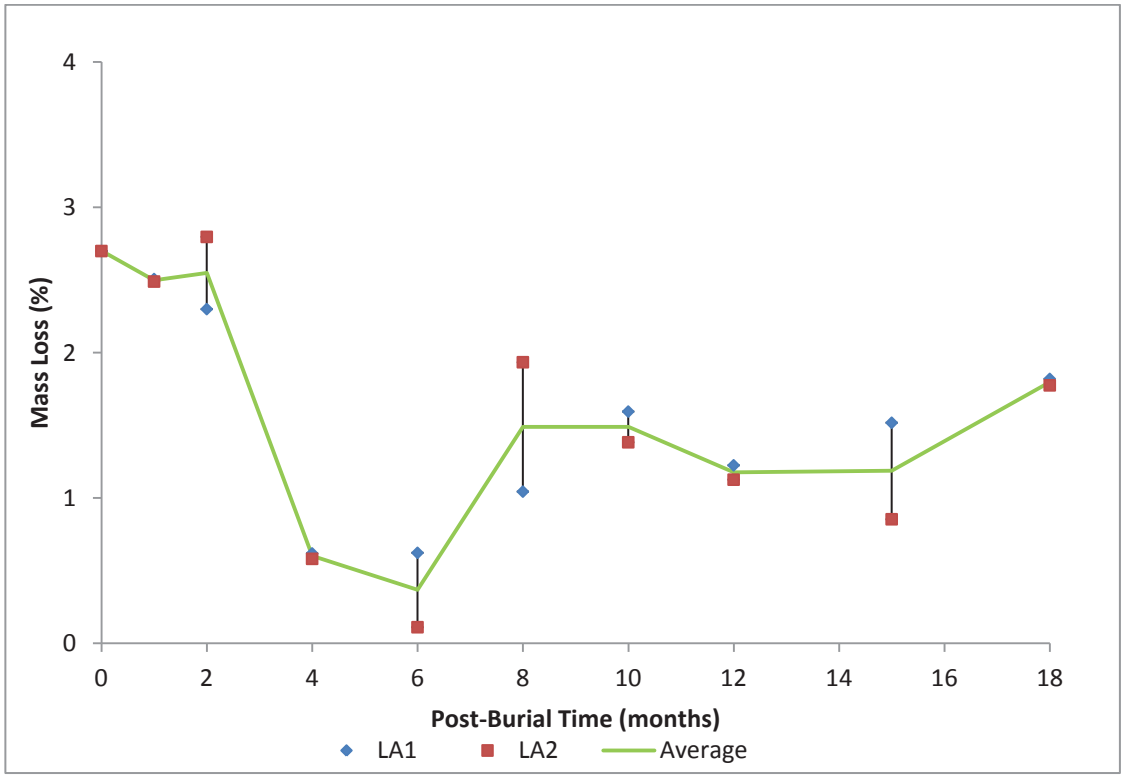

Figure 6-22. Mass loss due to Step 3 for bones buried in acidic soil

6.3.3.3 Effect of Soil pH on Bone Decomposition: A Comparative Study using Infrared Spectroscopy

The study conducted by Howes *et al.* (2012) used FTIR spectroscopy to analyse the effect of the pH of the soil environment on bone decomposition. Results of the study demonstrated that an acidic soil environment accelerated the decomposition processes that occur in bones. A decreasing trend in the carbonate content was observed over the eight months of burial (Howes *et al.*, 2012). Similarly, the results of the current study also illustrate that bone decomposition occurs more readily in an acidic soil environment with a decreasing trend being observed in the organic content of the bones over the 18 months of burial.

6.3.4 Pyrolysis Gas Chromatography-Mass Spectrometry

The effect of the pH of the burial environment on the estimation of the post-burial time of bones was analysed using Py-GC-MS. Bones were buried in an acidic and a basic soil environment with a pH of 5-6 and 9-10, respectively. Both sets of bones were analysed using Py-GC-MS followed by PLS regression analysis using mean-center pre-processing and linear baseline correction. The results of the statistical analysis of the bones buried in an acidic soil environment are shown in Figure 6-23.

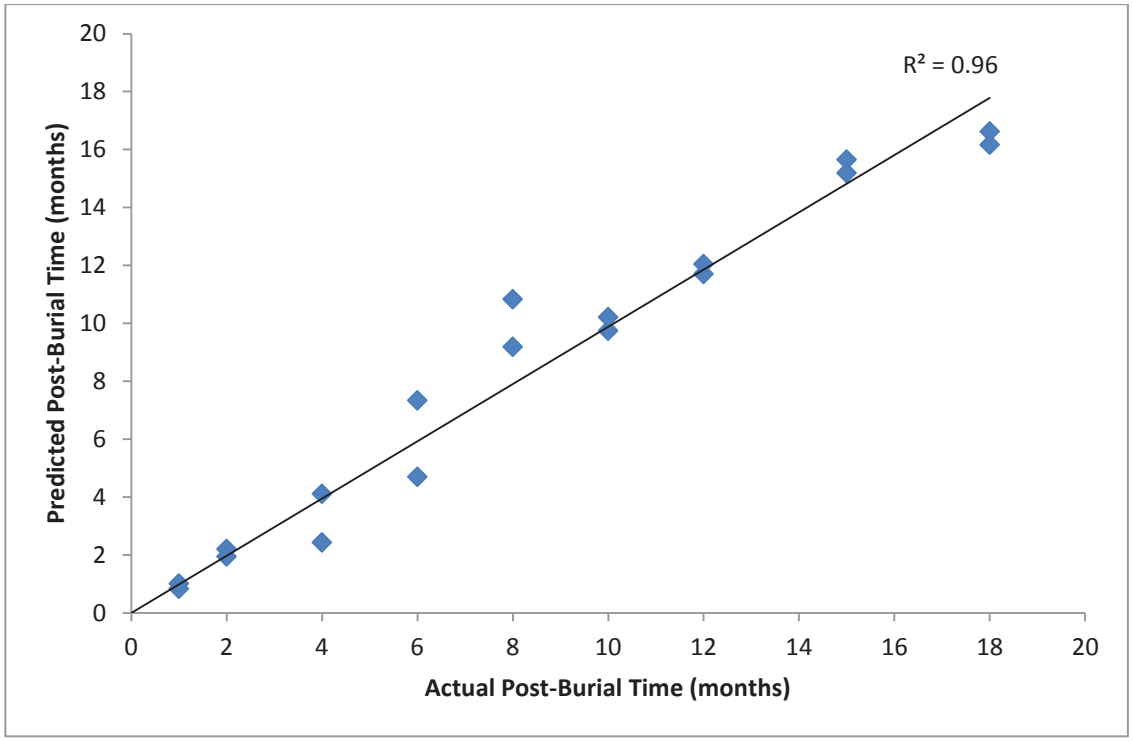

Figure 6-23. Correlation of predicted post-burial time with actual post-burial time for bone samples buried in acidic soil (1 and 2)

Most of the post-burial times of bone samples buried in acidic soil can be predicted using Py-GC-MS and statistical analysis, $R^2 = 0.96$, $p < 0.05$. Some variation can be observed in the actual and predicted post-burial times for samples with post-burial times of 6 and 8 months. Based on these results, one can estimate the post-burial times of bone samples up to 18 ± 2.6 months when they are discovered buried in acidic soil (pH = 5-6). The present study confirms the results of an earlier study as the compounds, phenoxy-phenol, trans-1,1,3,4-tetramethyl-cyclopentane and octadecanoic acid, are shown to be significant in assisting in the discrimination of bone samples with different post-burial times (Raja *et al.*, 2010a).

Since Py-GC-MS only analyses organic compounds in samples, it is not possible to monitor the changes in the inorganic content of the bones as the post-burial time increases. However, the technique of Py-GC-MS provides a promising estimation of the post-burial time of bones

buried in acidic soil, based solely on changes in the organic content of the bones. The organic compounds that are significantly changing as post-burial time increases and their corresponding retention times are presented in Table 6-9. The table shows the compounds that are present at different time periods during the 18 months of burial and any changes in these compounds that have occurred.

Table 6-9. Significant retention times and corresponding compounds in bone samples buried in acidic soil at different post-burial times

		Burial Time (months)			
Retention Time (min)	**Compound Name**	**1**	**6**	**12**	**18**
8.3920	2-cyclohexen-1-one	Y	Y	N	N
12.242	Piperidinone	Y	Y	N	N
17.600	2,6-dihydroxy-benzoic acid	Y	Y	N	N
19.142	phenoxy-phenol	N	Y	Y	Y
19.250	3-phenoxy-phenol	Y	N	N	N
19.650	(Z)-3-methyl-2-undecene	Y	Y	Y	Y
19.675	4-(2-hydroxyphenyl)pyrimidine	Y	Y	N	N
20.292	trans-1,1,3,4-tetramethyl-cyclopentane	Y	N	Y	Y
22.100	octadecanoic acid	Y	Y	N	N
24.583	(Z)-9-octadecenal	N	Y	N	N

The results of the statistical analysis of bones buried in a basic soil environment are shown in Figure 6-24. The post-burial times of bone samples buried in basic soil can be predicted using Py-GC-MS and statistical analysis, $R^2 = 0.96$, $p < 0.05$. Only two of the bone samples – 8 and 15 months – show differences in values of the actual and predicted post-burial times. Therefore, it can be said that bone samples found buried in basic soil can be assessed for estimating their burial times up to 18 ± 2.1 months of burial.

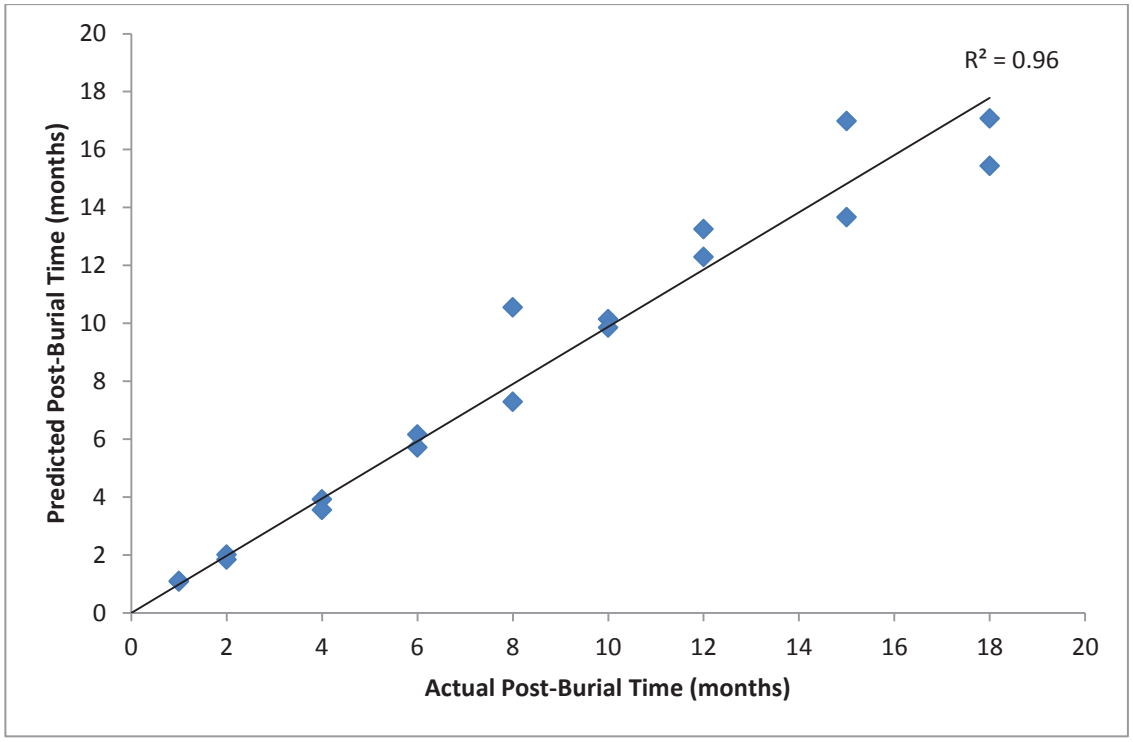

Figure 6-24. Correlation of predicted post-burial time with actual post-burial time for bone samples buried in basic soil (1 and 2)

In comparison to an acidic environment, a basic environment appears to preserve the bone samples rather than attacking the bone. The compounds that are present and changing during the burial time period are presented in Table 6-10. It is interesting to note that the number of compounds changing significantly in the samples belonging to a basic environment is the same as the samples buried in an acidic environment. This indicates that the technique of Py-GC-MS is able to avoid any bias which would be introduced due to the differences in pH of burial environments. Also, both the acidic and basic environments resulted in the same coefficient of determination ($R^2 = 0.96$, $p < 0.05$) of the predicted post-burial time with the actual post-burial time. This confirms that Py-GC-MS is a technique which is able to estimate the post-burial time of bone samples regardless of the pH of the burial environment.

Table 6-10. Significant retention times and corresponding compounds in bone samples buried in basic soil at different post-burial times

		Burial Time (months)			
Retention Time (min)	**Compound Name**	**1**	**6**	**12**	**18**
8.4250	N-methyl-2-propyn-1-amine	Y	N	N	N
15.400	trans-2-methyl-3-(1-methylethyl)-1-(2-propenyl)-aziridine	N	N	Y	N
17.600	2,6-dihydroxy-benzoic acid	Y	Y	Y	N
19.142	phenoxy-phenol	N	Y	N	Y
19.250	3-phenoxy-phenol	Y	N	N	N
20.292	trans-1,1,3,4-tetramethyl-cyclopentane	N	Y	Y	Y
20.617	1-nonadecene	Y	N	N	N
21.850	2,2-dipropyl-N-ethylpiperidine	Y	N	N	N
21.933	3-methylene-undecane	Y	N	N	N
22.100	octadecanoic acid	N	Y	N	N

6.4 Moisture Content

Another factor that was investigated was the moisture content in soil and its potential to affect the accuracy when estimating the post-burial time of a bone. Two additional soil environments were established, wet soil and dry soil, and studied using ESEM, TG, Py-GC-MS and XRD analyses.

6.4.1 Visual Observations

In this study, bones buried in soils with varying moisture contents were also analysed. The measurements including length, diameter and mass of the bone samples as well as physical descriptions of each bone sample were recorded. The recorded measurements are presented in Table 6-11.

Table 6-11. Physical characteristics of bone samples buried in soil varying in moisture content analysed at each post-burial time (n = 54)

Soil Type	Average Length (cm)	Standard Deviation (±)	Average Diameter (cm)	Standard Deviation (±)	Average Mass (g)	Standard Deviation (±)
Loam as supplied (control)	8.2	0.49	1.5	0.46	6.5	1.9
Wet	9.9	1.2	1.4	0.33	12.6	4.4
Dry	8.4	0.9	1.5	0.32	9.2	2.8

The colour of the bones buried in wet soil changed from a dark brown to a light brown colour over the burial study in the Loam Wet 2 samples, but the bones in the Loam Wet 1 soil were light brown until 12 months of burial and then became dark brown again by the end of the study. The soil in the Loam Wet 2 samples was drier than the soil in the Loam Wet 1 samples after 12 months of burial. No visible cracks or holes were present in the Loam Wet 1 samples for the entire duration of the study, but for the Loam Wet 2 samples, tiny cracks and holes began to appear after 10 months of burial. This can be attributed to the soil being drier in the Loam Wet 2 samples than the Loam Wet 1 samples. No odour was present in any of the bone samples, except for the 1-month Loam Wet 1 sample. No microbial activity was evident in any of the bone samples.

Bone samples buried in dry soil did not change in colour and remained brown-white for the entire study. There were no visible cracks or holes in any of the bone samples. Also, no odour was present in any of the bone samples. White fungus was present on all of the bone samples from the first month of burial till the end of the study.

Images of bones buried in wet and dry soil environments are shown in Figure 6-25. Burial in a wet soil environment resulted in bones that were predominantly white in colour, while burial in a dry soil environment led to bones that were much darker in colour. Therefore, it is possible to differentiate between bones that were buried in a wet or a dry soil environment.

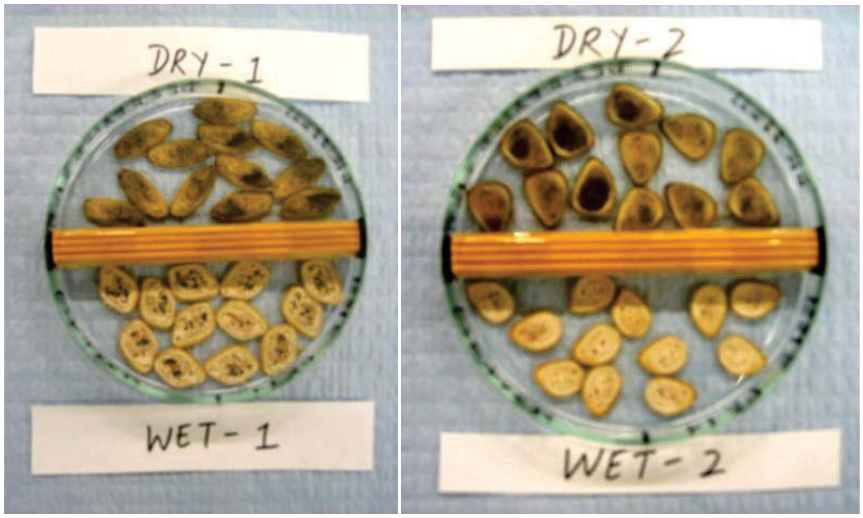

Figure 6-25. Photographs of wet and dry bone samples

6.4.2 Thermogravimetric Analysis

Bones buried in wet and dry soil environments were compared to the loam as supplied (control) soil environment using TG.

6.4.2.1 Reproducibility

The water-logged loam soil used in this study was loam soil that was saturated with water for the entire duration of the study. Figure 6-26 shows the TG results of defleshed bones buried in the wet loam soil. The mass loss averages obtained ranged between 29.7 ± 0.9 % and 37.4 ± 0.4 %, and are presented in Table 6-12. The results for the samples of all the burial periods

were similar with the exceptions of bones buried for 2, 6, 10 and 15 months. Therefore, it can be said that results of bone samples buried in wet loam soil are not reproducible. The presence of excess water could be a reason for the degradation processes occurring at different rates in the bones buried in wet loam soil even when the bones are subjected to similar burial conditions. The soil surrounding the bone was saturated with water, which might affect the rate of uptake of water and other substances present in the soil by the bone.

Table 6-12. Total mass loss ± standard deviation (%) at each post-burial time for defleshed bones buried in as supplied, wet and dry loam soils (n = 60)

Post-burial time (months)	Loam As Supplied (Control) (%)	LW (%)	LD (%)
0	35.6 ± 0.7	35.6 ± 0.7	35.6 ± 0.7
1	35.3 ± 2.8	33.4 ± 0.6	35.6 ± 0.7
2	35.5 ± 2.7	33.3 ± 3.0	38.2 ± 0.4
4	29.8 ± 1.3	29.7 ± 0.9	29.8 ± 4.7
6	30.5 ± 0.0	31.6 ± 1.7	32.2 ± 1.3
8	32.3 ± 1.6	37.4 ± 0.4	30.5 ± 6.0
10	32.4 ± 5.6	32.0 ± 1.9	36.7 ± 5.0
12	30.1 ± 5.1	32.9 ± 0.3	33.5 ± 0.5
15	38.2 ± 4.4	32.2 ± 1.9	36.1 ± 4.1
18	33.6 ± 0.6	33.1 ± 0.4	34.4 ± 2.1

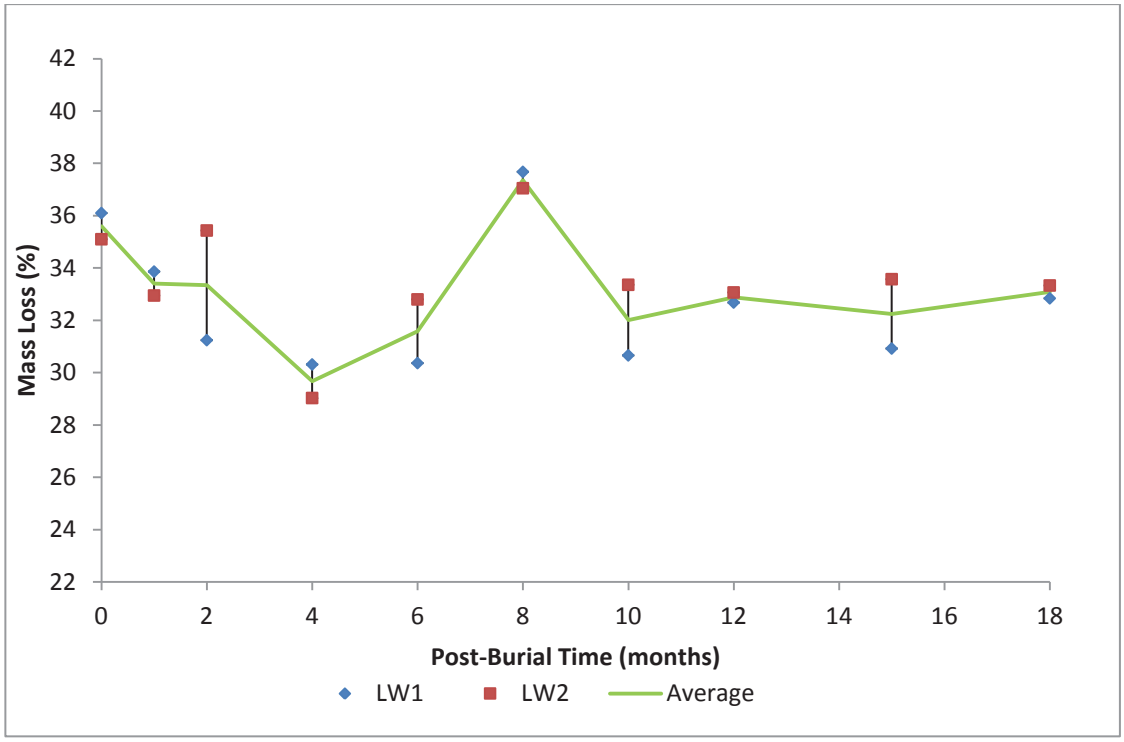

Figure 6-26. Comparison of TG results of Loam Wet 1 and 2 bone samples

In contrast, the results of the defleshed bones buried in dry loam soil show greater variation as can be seen in Figure 6-27, which may be a result of the wet loam soil environment being a less heterogeneous environment. The averages and standard deviations of these results are shown in Table 6-12. The samples with younger post-burial times of 1 and 2 months show similar mass losses. The samples with longer post-burial periods (4, 8, 10, 15 and 18 months) show more variation in the mass loss percentages. The averages of the 4 to 18 month samples ranged from 29.8 ± 4.7 % to 36.7 ± 5.0 %. As explained in the loam as supplied (control) samples, variation in the bone samples buried for longer periods of time can be attributed to a greater possibility of variations in the degradation processes that occur in bones that have been buried for longer.

In the first 2 months of the study, the soil stayed relatively dry but after 4 months, water from the atmosphere caused some areas of the soil to become damp. Visually, the Loam Dry 2 soil was slightly damper than the Loam Dry 1 soil. These changes in the moisture content of

the soil are reflected in the results of the samples. The results of the bones buried in dry loam soil were not reproducible, which could be due to some areas of the dry soil environments becoming damp during the study.

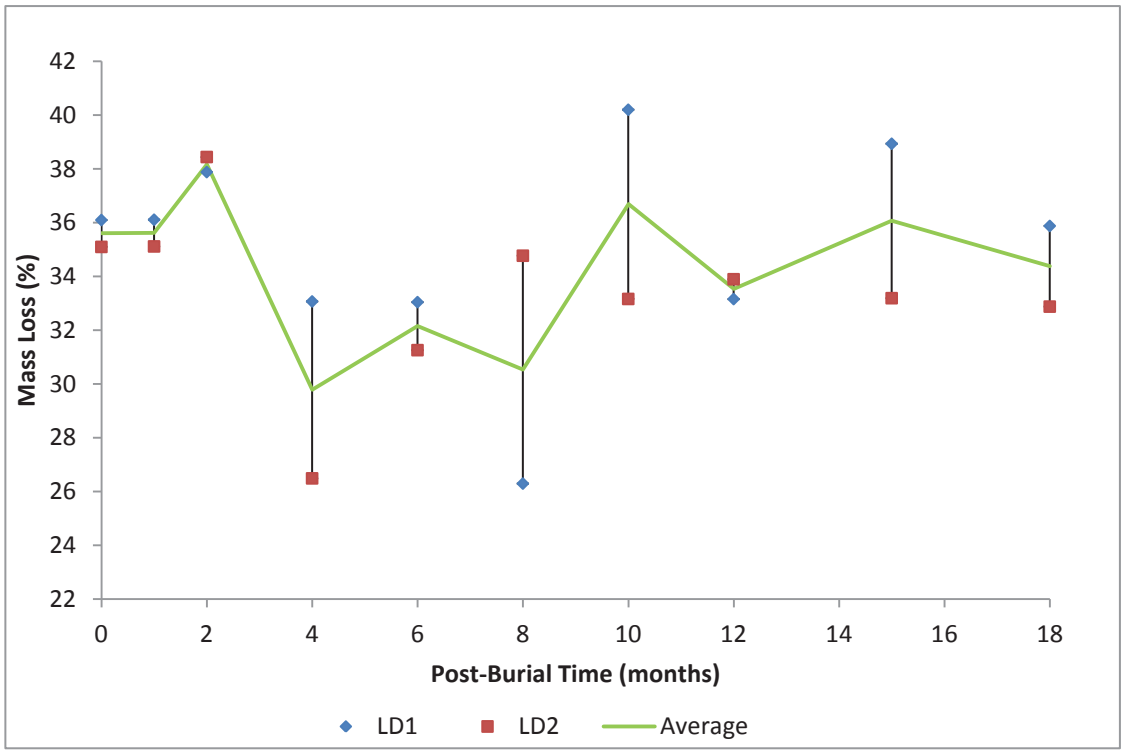

Figure 6-27. Comparison of TG results of Loam Dry 1 and 2 bone samples

6.4.2.2 Effect of Moisture Content on Bone Structure and Decomposition

Another important factor to consider is the moisture content in the soil and so, in this study, bone samples were buried in both wet soil and dry soil environments for comparison purposes. The TG curves of the bone samples buried in wet, dry and loam as supplied (control) soils after 1 and 18 months of burial are presented in Figures 6-28 and 6-29, respectively. Generally, the mass loss curves of the bones buried in all three environments were similar after 1 and 18 months of burial. After 1 month of burial, the total mass loss of

the wet and loam as supplied (control) soils were similar while the total mass of the bones buried in dry soil was greater than the other two burial environments. This indicates that after the first month of burial, there was more organic content present in the bones buried in dry soil compared to the wet soil samples, which supports previous research that showed that a moist environment enhances bone decomposition while a dry environment delays the process (Smith, 1984). Up to a temperature of 475 °C, the total mass loss of the bones buried in wet soil was slightly lower than the total mass loss of the bones buried in the loam as supplied (control) soil. The same trends were also observed after 18 months of burial with the total mass loss of the bones buried in dry soil being greater than the other two burial environments. The only difference observed was that the mass loss in the bones buried in wet soil was slightly lower than the loam as supplied (control) soil bones.

Figure 6-30 shows the trends in mass of the bones buried in wet, dry and loam as supplied (control) soil environments. All the bones followed similar trends between 2 and 6 months of burial. After 8 months of burial, the bones buried in the dry and loam as supplied (control) soil environments followed the same trend in terms of the changes in mass loss. Interestingly, the bones buried in a wet soil environment follow the opposite trend. Generally, the total mass loss in the bones buried in a dry soil environment was higher than in the bones buried in the wet soil environment, except for the 8-month samples. This supports the theory that a dry environment is more effective in preserving bone samples than a moist environment (Smith, 1984). According to Swift *et al.* (1979), soil moisture can have a significant effect on decomposition rates as water is known to leach the organic component of bones. The results of the present study support that the presence of water in the burial environment affects the degradation processes that occur in bone and in particular, in the organic content of bone. Overall, the results of the present study show that moisture content in the soil significantly affects the degradation processes occurring in bone after the initial 6 months of burial.

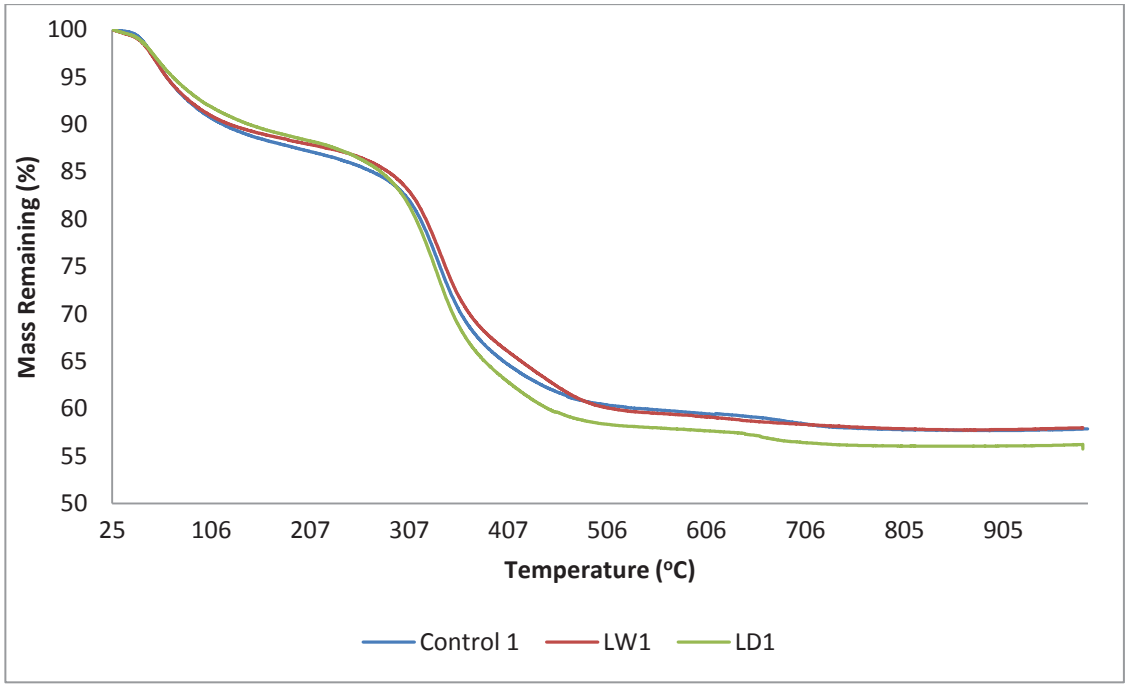

Figure 6-28. TG curves of bones buried in soils with varying moisture contents after 1 month of burial

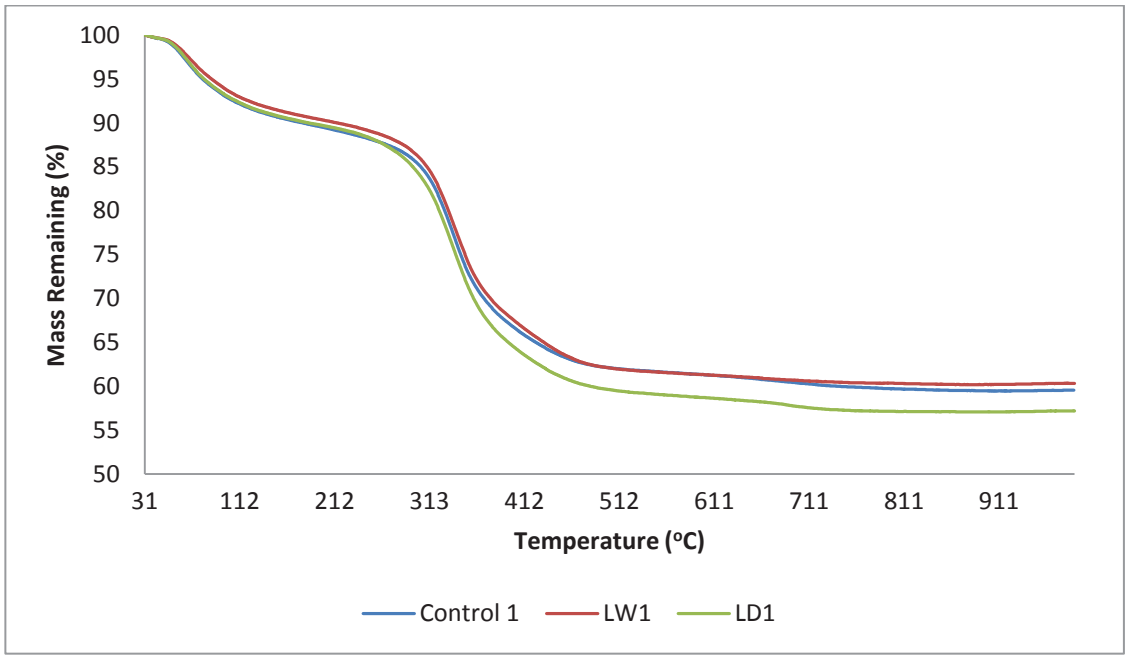

Figure 6-29. TG curves of bones buried in soils with varying moisture contents after 18 months of burial

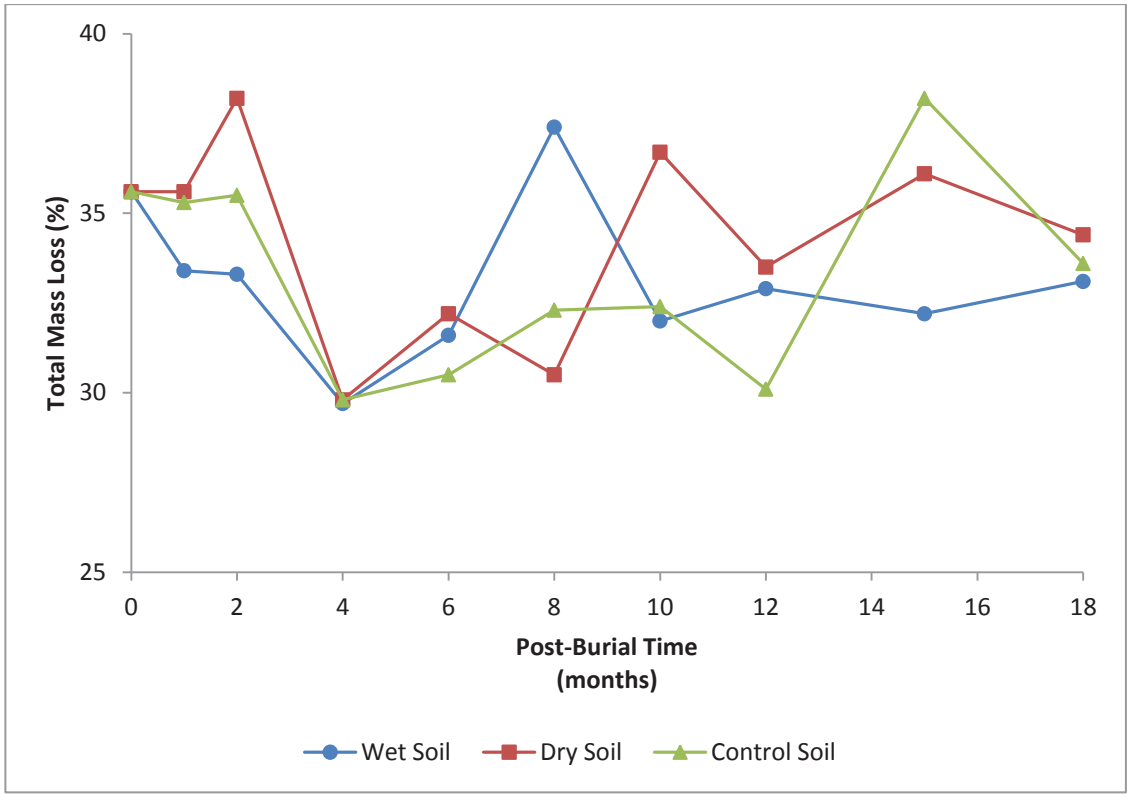

Figure 6-30. Comparison of TG results of bone samples in wet, dry and loam as supplied (control) soils

6.4.3 Pyrolysis Gas Chromatography-Mass Spectrometry

Both the sets of bones buried in wet and dry soil environments were analysed using Py-GC-MS to determine if this technique could be used to estimate post-burial time. The results of PLS regression analysis following mean-center pre-processing and linear baseline correction on the Py-GC-MS data of the bones buried in wet soil are presented in Figure 6-31.

The data (Figure 6-31) shows that the post-burial times of bone samples buried in wet soil can be predicted ($R^2 = 0.93$, $p < 0.05$) for most bone samples using Py-GC-MS. The actual post-burial time and the predicted post-burial times showed high correlation for all bones except

for the 8, 10 and 18 month samples. Hence, it can be concluded that bone samples buried in wet soil can have their burial time estimated using Py-GC-MS up to 18 ± 3.2 months of burial.

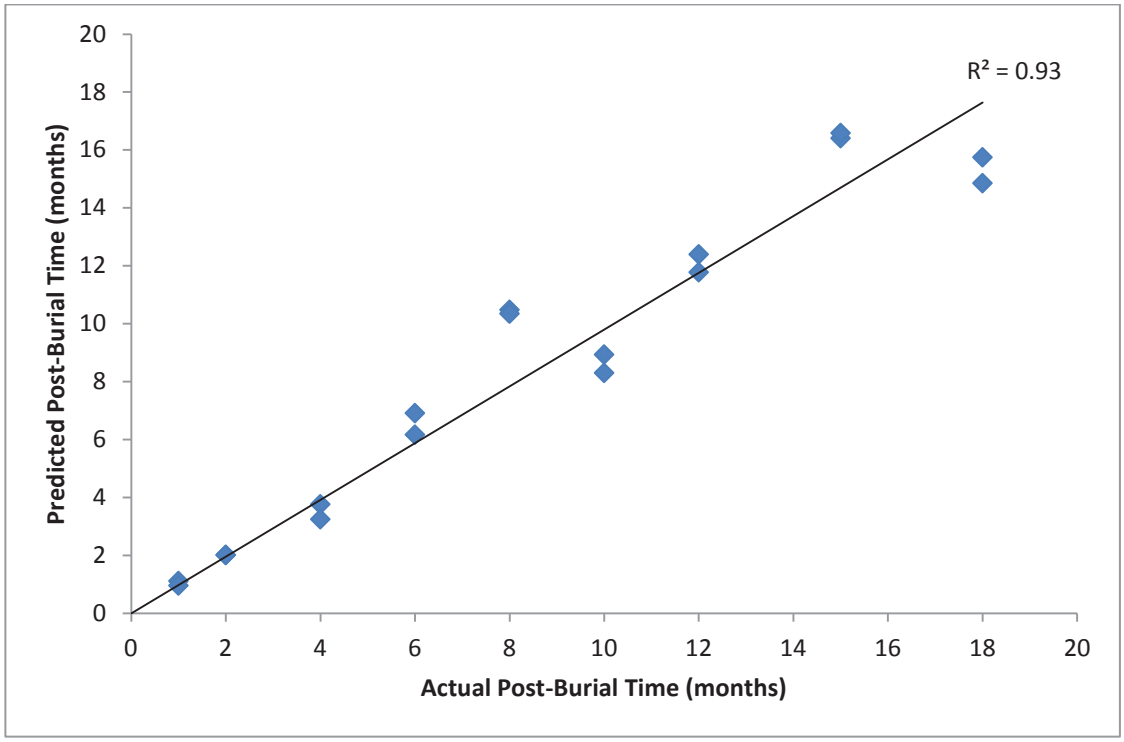

Figure 6-31. Correlation of predicted post-burial time with actual post-burial time for bone samples buried in wet soil (1 and 2)

The compounds that most significantly changed in the 'wet soil' bone samples during the burial time frame and, therefore, had an effect on the estimation of the post-burial time of bone samples are presented in Table 6-13. It is interesting to note that many more compounds are used in the statistical analysis of bone samples buried in wet loam soil for the differentiation of varying burial times, when compared to the loam as supplied (control) soil.

Table 6-13. Significant retention times and corresponding compounds in bone samples buried in wet soil at different post-burial times

		Burial Time (months)			
Retention Time (min)	**Compound Name**	**1**	**6**	**12**	**18**
8.3670	Pyrazole	N	N	Y	N
15.400	trans-2-methyl-3-(1-methylethyl)-1-(2-propenyl)-aziridine	N	N	Y	N
15.492	cis-tetrahydro-2,5(1H,3H)-pentalenedione	N	Y	N	N
17.600	2,6-dihydroxy-benzoic acid	Y	Y	Y	N
17.683	Heneicosane	Y	N	N	N
19.142	phenoxy-phenol	N	Y	N	Y
19.250	3-phenoxy-phenol	Y	N	N	N
19.650	(Z)-3-methyl-2-undecene	Y	Y	Y	N
20.292	trans-1,1,3,4-tetramethyl-cyclopentane	Y	Y	Y	Y
20.667	1,2-diethyl-1-methyl-cyclohexane	Y	N	N	N
21.267	hexadecanenitrile	Y	Y	N	Y
21.925	hexadecanoic acid	N	N	N	Y
21.942	(E)-4-undecene	N	N	Y	N
21.967	trans,cis-1,2,4-trimethyl-cyclopentane	Y	N	N	N
22.100	octadecanoic acid	Y	N	N	N
23.392	Octadecanenitrile	Y	N	N	N
24.058	2-hexyl-1-decanol	N	N	Y	Y
24.592	(E)-9-eicosene	N	Y	N	N

The Py-GC-MS results analysed using PLS regression analysis, mean-center pre-processing and linear baseline correction for bones buried in dry soil are presented in Figure 6-32. Similar to the Py-GC-MS results of the bone samples buried in wet soil, the post-burial times of most bone samples buried in dry soil can also be predicted ($R^2 = 0.90$, $p < 0.05$) using these results. There were only two samples that showed differences in the values of the actual post-burial time and the predicted post-burial time. These were the 4 and 18 month samples. Therefore, the post-burial times of bones buried in dry soil can be predicted up to 18 ± 5.9 months of burial.

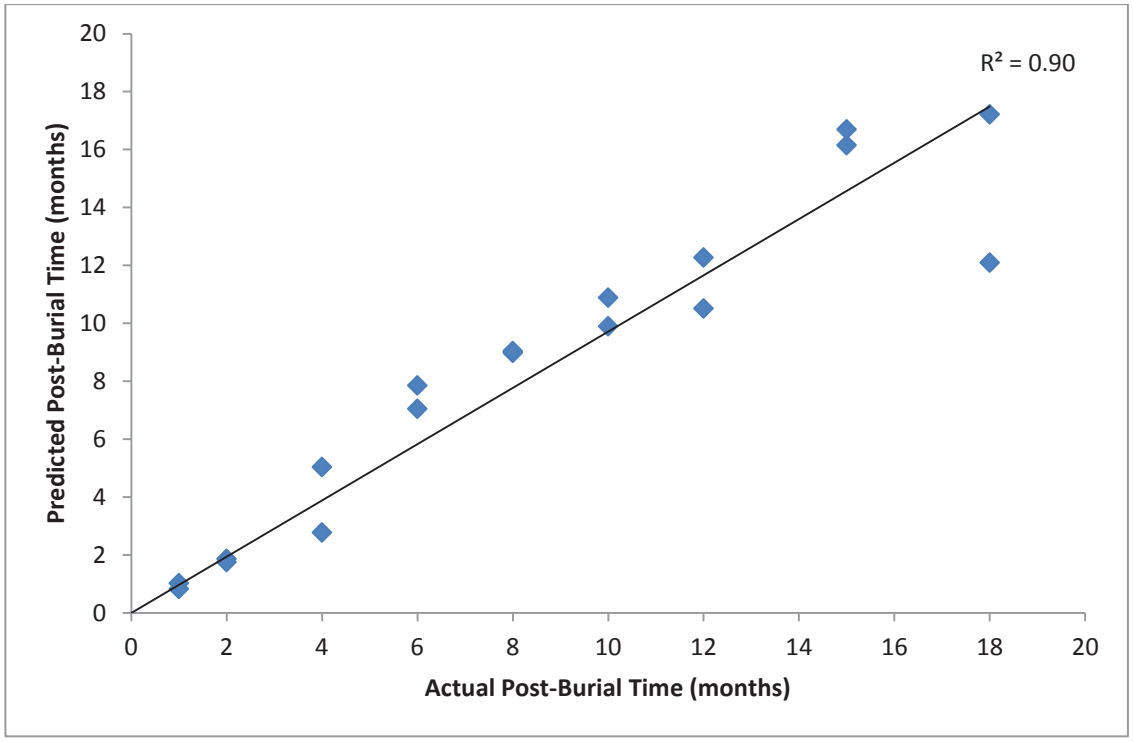

Figure 6-32. Correlation of predicted post-burial time with actual post-burial time for bone samples buried in dry soil (1 and 2)

Interestingly, the coefficient of determination of the bones buried in dry soil ($R^2 = 0.90$, $p < 0.05$) is slightly lower than that of the bones buried in wet soil ($R^2 = 0.93$, $p < 0.05$). This could be due to bones degrading more slowly in a dry soil environment, leading to smaller changes in mass and therefore resulting in a less accurate prediction overall.

The most important compounds that changed during the burial period in a dry soil environment are presented in Table 6-14. The presence and absence of certain organic compounds at different stages during burial is the key in being able to estimate the post-burial time of bone samples.

Table 6-14. Significant retention times and corresponding compounds in bones buried in dry soil at different post-burial times

		Burial Time (months)			
Retention Time (min)	**Compound Name**	**1**	**6**	**12**	**18**
8.4250	N-methyl-2-propyn-1-amine	Y	N	N	N
17.600	2,6-dihydroxy-benzoic acid	Y	Y	Y	N
19.142	phenoxy-phenol	N	Y	Y	N
19.250	3-phenoxy-phenol	Y	N	N	N
20.292	trans-1,1,3,4-tetramethyl-cyclopentane	Y	N	Y	N
20.850	1-pentadecanol	N	N	N	Y
20.858	1-hexadecanol	N	N	Y	N
20.867	1-heptadecanol	N	Y	N	N
21.267	hexadecanenitrile	Y	Y	Y	Y
22.933	3-pentyl-4,5-tetramethylenepyrazole	Y	N	N	N
23.150	Hexadecane	N	Y	N	N
23.158	hexadecenenitrile	Y	N	N	N
23.208	cis-13-octadecenal	Y	N	N	N
23.708	(E)-3-eicosene	N	Y	N	N
24.050	2-butyl-1-octanol	N	N	N	Y

6.5 Temperature

Another factor examined in this study was the temperature of the burial environment and its effect on the decomposition of bones which may in turn affect the accurate estimation of the post-burial time of the bones. Bones were buried in a colder environment and compared to the room-temperature (control) soil environment to observe the effects of varying temperature on bone diagenesis.

6.5.1 Visual observations

The measurements of bones buried in soils with varying temperatures are presented in Table 6-15.

Table 6-15. Physical characteristics of bone samples buried in soils varying in temperature analysed at each post-burial time (n = 36)

Soil Type	Temper-ature (°)	Average Length (cm)	Standard Deviation (±)	Average Diameter (cm)	Standard Deviation (±)	Average Mass (g)	Standard Deviation (±)
Room-temperature (Control)	20-23	8.2	0.49	1.5	0.46	6.5	1.9
Cold	4	8.9	1.5	1.6	0.39	11.3	3.8

For the room-temperature (control) samples, the colour did not change from the initial light brown colour. Tiny cracks and holes started appearing after 2 months of burial and were evident in all the samples up to and including 18 months of burial. No odour was present in any of the bone samples. In terms of microbial activity, white fungus was present on only one of the Control 1 (room-temperature) samples (8-month). In contrast, white fungus was present on all the Control 2 (room-temperature) samples with post-burial times of 6 months and greater. After 4 months of burial, a layer of white, jelly-like mucus was covering the entire surface of soil in the Control 2 (room-temperature) samples and could be the cause of the greater extent of microbial activity in the Control 2 (room-temperature) samples. Also, for both sets of the room-temperature samples, cockroaches were present in the containers from 15 months of burial.

The bone samples buried in a colder environment changed from a dark brown-red colour to a pale brown colour after about 6 months of burial. Small cracks were only evident on the 1 and 8-month refrigerator 2 samples. None of the other samples had any evidence of cracks or holes. No odour was present in any of the bone samples. White fungus started appearing on the bone samples after 4-6 months of burial and was present on all the bone samples for the duration of the study. In both sets of samples, a white jelly-like layer of fungus covered the top layer of the soil during the period of 6-10 months post-burial, which disappeared during the 12-14 months post-burial period, but then re-appeared after 15 months. This change in microbial activity can be attributed to a short refrigerator malfunction (< 1 week at 17-18 °C) during the 10th month of burial and the samples being moved to a cold storage area as a result.

Bone samples recovered from a cold soil environment looked very similar to the fresh bone samples. They were mostly very pale brown and red/orange, while the fresh bone samples were predominantly white and red. Images of the bones buried in a cold soil environment are shown in Figure 6-33. It is interesting to note that these bones are the only ones that look similar to the fresh bones, after being recovered from their burial environment. These results confirm the notion that a cooler environment tends to preserve bones when compared to any of the other burial conditions investigated in this study.

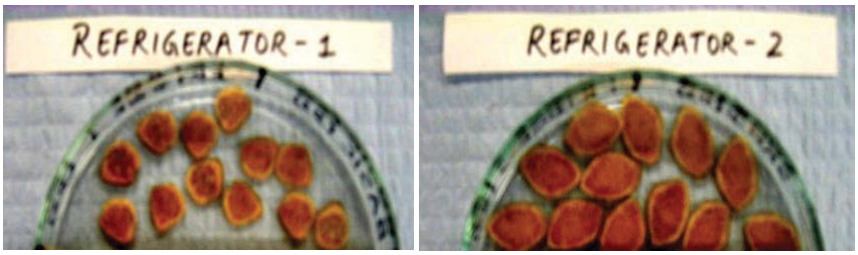

Figure 6-33. Photographs of cold loam (refrigerator) bone samples

6.5.2 Thermogravimetric Analysis

Bones buried in soils of different temperatures were analysed using TG and these were compared to observe the effects of temperature on the decomposition of buried bones.

6.5.2.1 Reproducibility

Both sets of defleshed bones stored in a colder environment, i.e. in a refrigerator maintained at 4 °C, showed similar mass loss results for most of the burial periods investigated in this study. These results are presented in Figure 6-34. Bones buried in a colder environment had mass losses ranging from 31.1 ± 0.1 % to 40.6 ± 10.7 % (Table 6-16). The only bone sample which showed a significant variation in the results was the 8 month sample, with the 2 and 6 month samples only showing a small degree of variation in the results. From the data, it is evident that the mass loss is much greater in the first two months of burial compared to the rest of the burial period of the study. The organic and inorganic changes to bones stored at a much cooler temperature might be expected to occur at a slower rate. This is confirmed by the results of the present study.

Table 6-16. Total mass loss ± standard deviation (%) at each post-burial time for defleshed bones buried in room-temperature and cold soils (n = 40)

Post-burial time (months)	Room-Temperature (Control) (%)	LR (%)
0	35.6 ± 0.7	35.6 ± 0.7
1	35.3 ± 2.8	40.3 ± 0.1
2	35.5 ± 2.7	40.0 ± 3.2
4	29.8 ± 1.3	31.1 ± 0.1
6	30.5 ± 0.0	33.3 ± 2.4
8	32.3 ± 1.6	40.6 ± 10.7
10	32.4 ± 5.6	33.3 ± 1.2
12	30.1 ± 5.1	33.6 ± 0.4
15	38.2 ± 4.4	34.8 ± 0.3
18	33.6 ± 0.6	35.3 ± 0.9

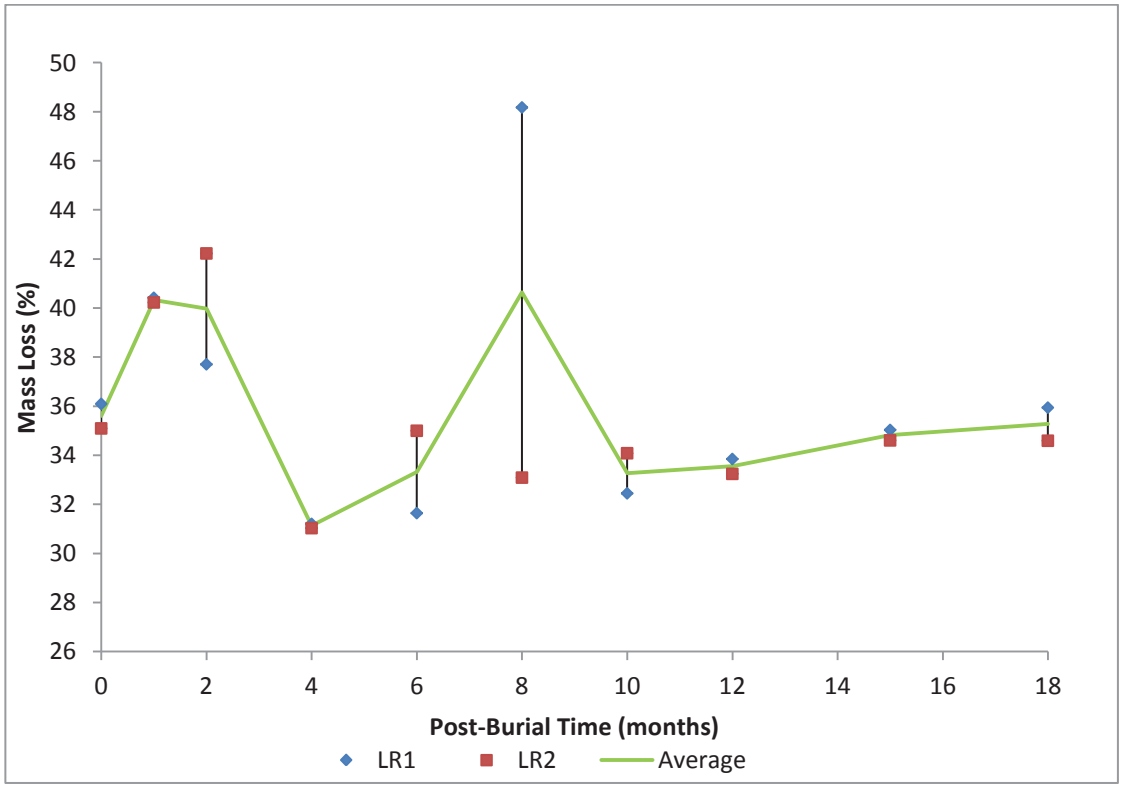

Figure 6-34. Comparison of TG results of Loam Refrigerator 1 and 2 bone samples

6.5.2.2 Effect of Temperature on Bone Structure and Decomposition

The temperature of the burial environment is a factor that needs to be considered when observing bone diagenesis. Colder environments are reported to 'preserve' bone samples and hinder bone decomposition (Smith, 1984; Janaway, 1996; Forbes *et al.*, 2005a). The TG results of bone samples buried in soils at room temperature as well as at a cooler temperature of 4 °C after 1 and 18 months of burial are shown in Figures 6-35 and 6-36. The TG curves of the bones buried in both the soil environments follow the same shape after the first month of burial and after 18 months of burial. The only difference exists in the total mass loss that occurs in the bones after TG analysis is performed. Bones buried in the soil at ambient temperature show a lower mass loss when compared to the bones buried in a cooler environment. This indicates that bones buried in a cooler environment are preserved and the degradation processes occurring in the bones buried in a room temperature environment occur at a faster rate. Therefore, more organic content remains in the bones buried in the cooler soil, which then leads to a greater mass loss observed for these samples during TG analysis.

A comparison of the mass losses observed for the duration of the burial period of 18 months are presented for both sets of bone samples in Figure 6-37. A similar trend in mass loss is observed in both the burial environments up to and including a burial period of 10 months; however, the mass loss observed in the bones buried at room temperature is much lower than the bones buried in a cooler environment. After 10 months of burial, the mass loss increases slightly for the bones buried in cold soil, while the mass loss in the bones buried in room temperature soil tends to oscillate. Overall, the mass loss observed in the bones buried in cold soil is greater for the entire duration of burial with the exception of the bones recovered after 15 months of burial.

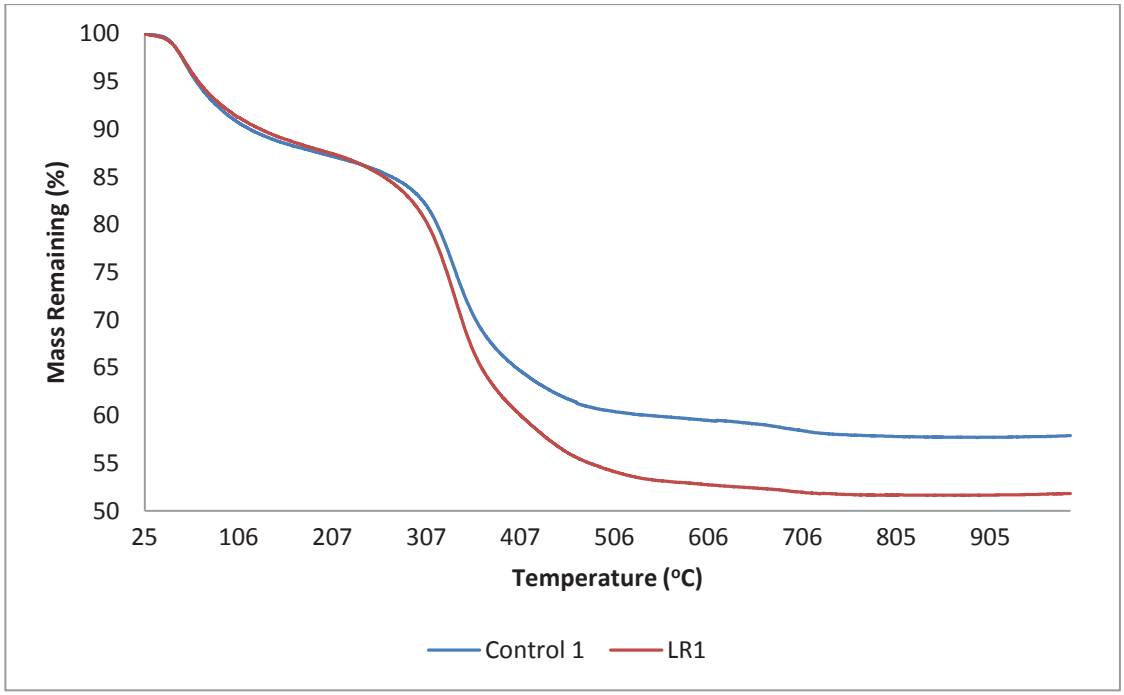

Figure 6-35. TG curves of bones buried in soils of varying temperatures after 1 month of burial

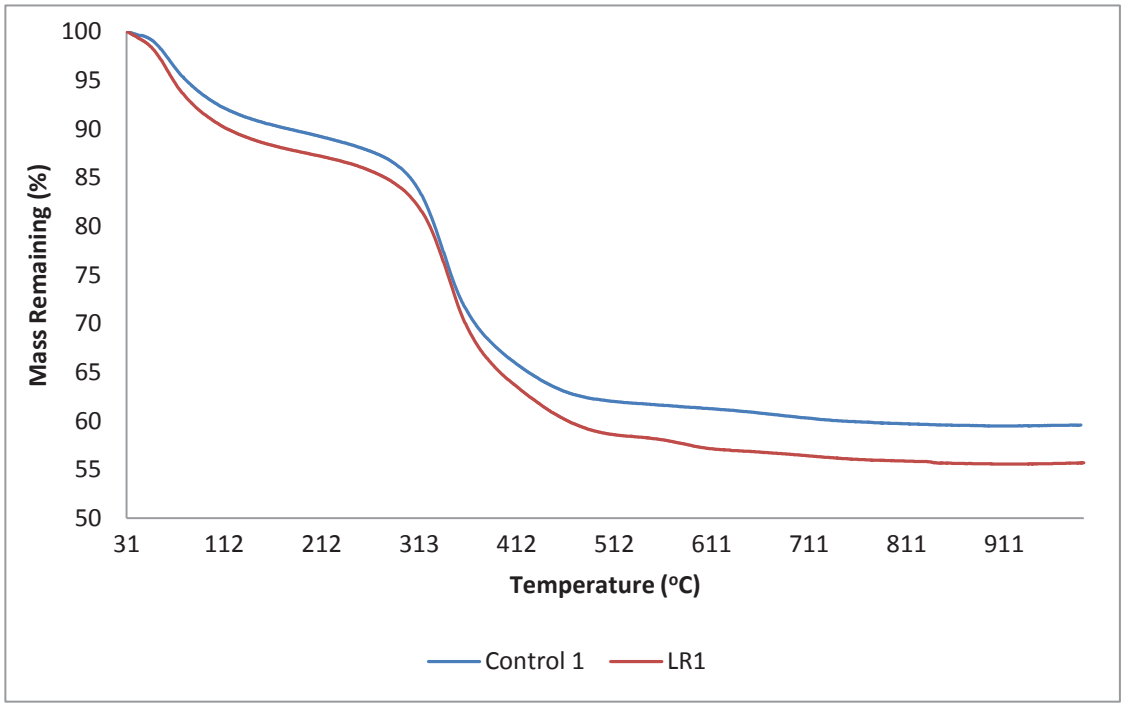

Figure 6-36. TG curves of bones buried in soils of varying temperatures after 18 months of burial

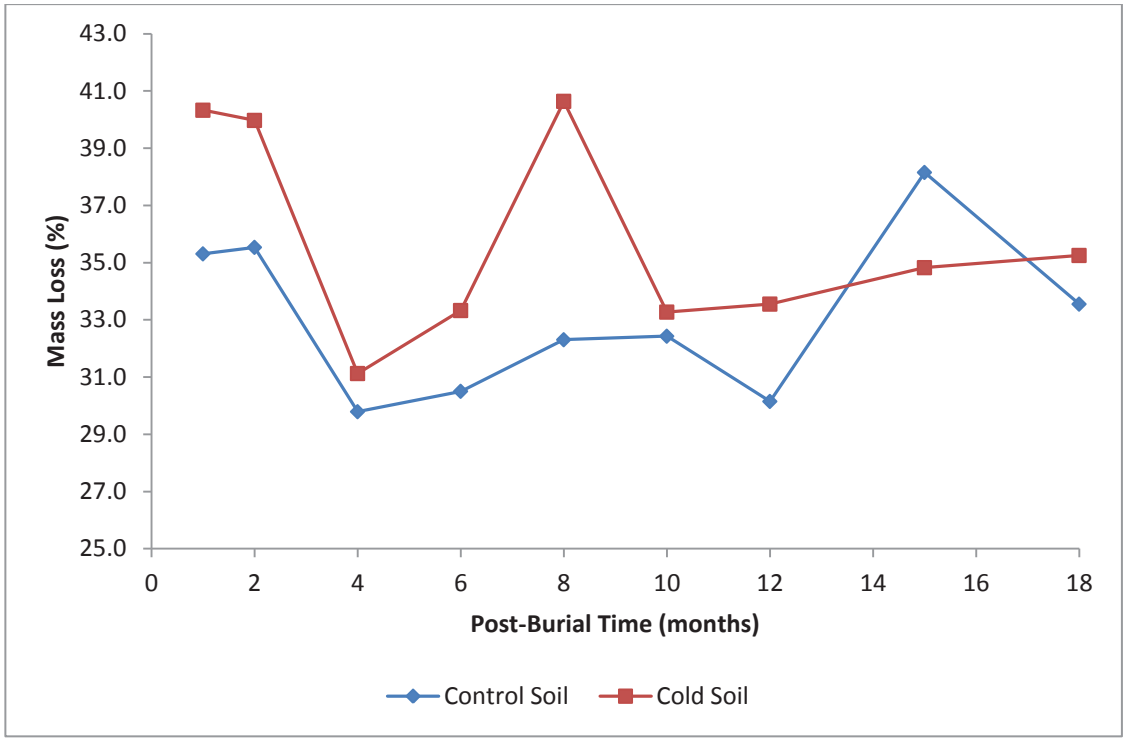

Figure 6-37. Comparison of TG results of bone samples buried in soil at room and colder temperature

Previous research found that a cooler environment preserves bones compared to a room-temperature environment (Smith, 1984; Janaway, 1996; Forbes *et al.*, 2005a). Results of this study confirm these results using the technique of TG. As bones buried at a temperature of 4 °C are better preserved during burial, they tend to have more mass to lose and therefore, show a higher mass loss when analysed using TG compared to the bones buried at room temperature.

6.5.3 Pyrolysis Gas Chromatography-Mass Spectrometry

Bone samples were buried in a colder environment and analysed using Py-GC-MS and PLS regression analysis using mean-center pre-processing and linear baseline correction. Results of the statistical analysis are shown in Figure 6-38.

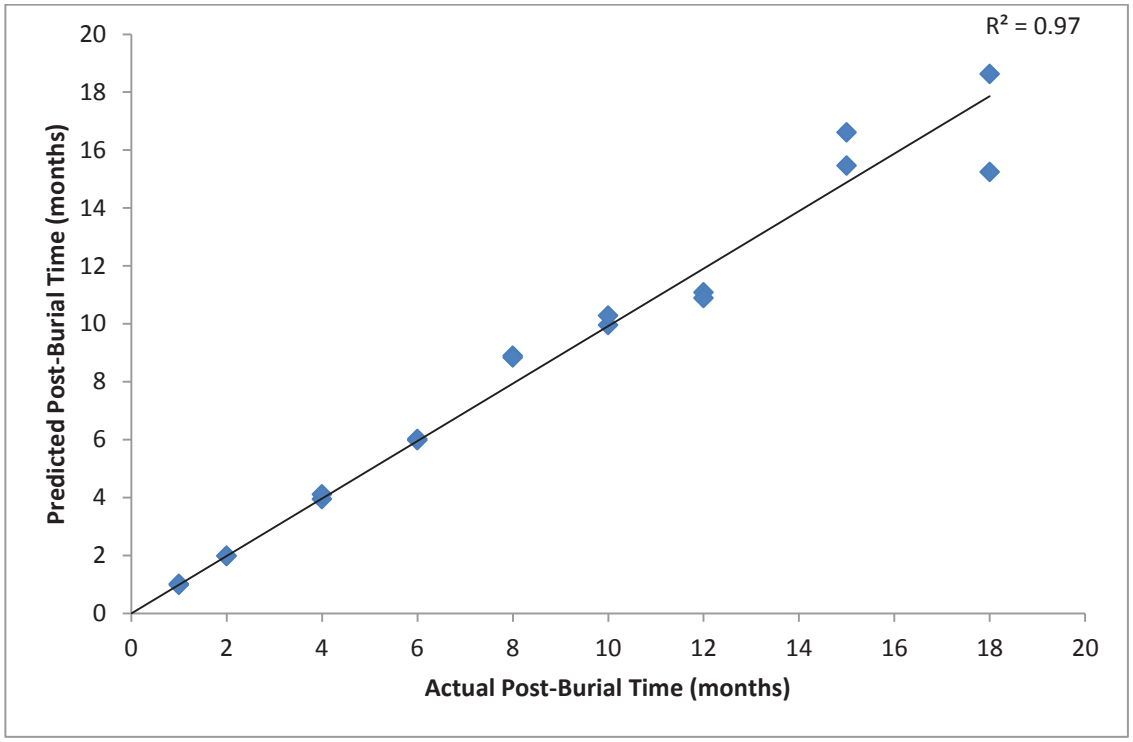

Figure 6-38. Correlation of predicted post-burial time with actual post-burial time for bone samples buried in a cold environment (1 and 2)

Linear regression analysis shows that the post-burial times of bone samples buried in a colder environment can be estimated using Py-GC-MS analysis (R^2 = 0.97, $p < 0.05$). Only the bones with a post-burial time of 18 months showed differences in the values of the actual when compared to the predicted post-burial times. As a result, it can be concluded that it is possible to predict the post-burial times of bones buried in a colder environment up to 18 ± 2.8 months. Interestingly and perhaps, coincidentally, the coefficient of determination

(R^2 = 0.97, $p < 0.05$) of the bones buried in a colder environment is much higher than the coefficient of determination (R^2 = 0.91, $p < 0.05$) of the bones buried in an environment of room-temperature (control) samples.

A list of the compounds that are present during different periods of the duration of the burial are presented in Table 6-17. The table shows that there are many more compounds present in the bone samples buried in a colder environment than the bones buried in a warmer environment. This confirms the notion that bone samples buried in a colder environment would be more preserved than the bones buried in a warmer environment.

Table 6-17. Significant retention times and corresponding compounds in bone samples buried in a cold environment at different post-burial times

		Burial Time (months)			
Retention Time (min)	**Compound Name**	**1**	**6**	**12**	**18**
8.4250	N-methyl-2-propyn-1-amine	Y	N	N	N
14.775	1,3,4,6,9,9a-hexahydro-2H-quinolizine	Y	N	N	N
14.883	(+)-isomenthone	Y	N	N	N
17.600	2,6-dihydroxy-benzoic acid	Y	N	N	Y
19.142	phenoxy-phenol	N	Y	N	Y
19.250	3-phenoxy-phenol	Y	N	N	N
19.550	4-methoxy-3-cyanomethyl-(1H)-indole	Y	N	N	Y
19.650	(Z)-3-methyl-2-undecene	Y	N	Y	Y
20.292	trans-1,1,3,4-tetramethyl-cyclopentane	Y	Y	y	N
20.517	3,5-dihydroxy-4,4-dimethyl-2,5-cyclohexadien-1-one	N	N	Y	N
20.667	1,2-diethyl-1-methyl-cyclohexane	Y	N	N	N
21.267	hexadecanenitrile	Y	Y	N	N
21.950	trans,cis-1,2,4-trimethyl-cyclopentane	Y	N	N	N
23.142	(Z)-13-octadecenal	Y	N	N	N
23.158	Hexadecenenitrile	N	N	N	Y
23.392	Octadecanenitrile	Y	N	N	N
23.975	2-methyl-6-hepten-3-ol	Y	N	N	N
24.267	Cyclopentadecane	Y	N	N	N
25.900	(Z)-9-octadecenamide	Y	N	N	N

6.6 Comparison of Burial Conditions Study

6.6.1 Environmental Scanning Electron Microscopy

Previous research has utilised the technique of ESEM to study changes in bone samples older than 2.5 years (Yoshino et al., 1991; Bell, 1990), however, research in younger bone samples is limited. Therefore, in the present study, the qualitative assessment of the visual, physical and surface properties of bone samples recovered from the different burial conditions after 1, 6, 12 and 18 months of burial was conducted. The results are presented in Table 6-18 and show that differences exist between the samples as a result of the length of burial.

6.6.1.1 1 Month Post-Burial

There were no micro-pores visible in all of the 1-month bone samples, regardless of burial condition. Bones buried in basic soil were rougher than bones buried in acidic soil, and bones buried in dry soil appeared rougher than bones buried in wet soil. The bone samples buried in a silty (Figure 6-39), sandy and clay soil environment, all appeared very similar, with the bones buried in a clay soil environment showing the least number of visible pores. Lastly, bone samples stored in a cool environment (Figure 6-40) appeared to be very smooth, which indicates that a cooler environment appears to act in a preserving manner in the first month of burial.

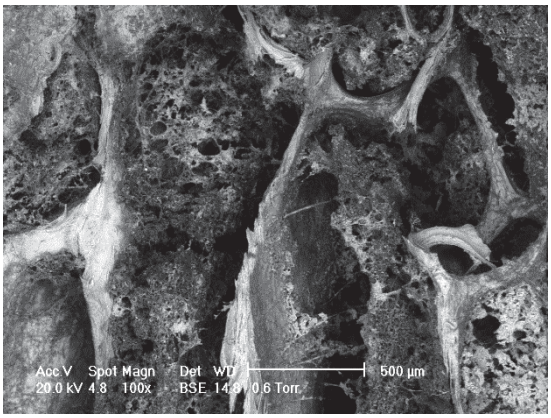

Figure 6-39. Silt 1 – 1 month

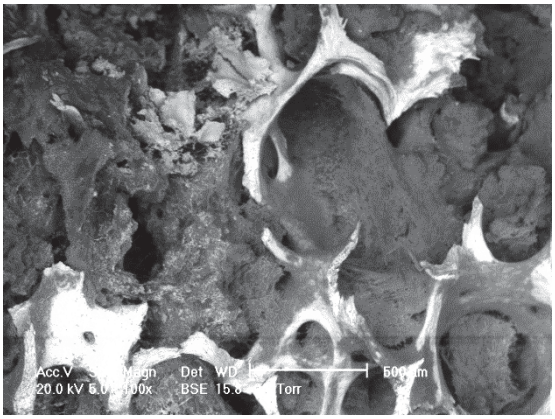

Figure 6-40. Loam Refrigerator 1 – 1 month

6.6.1.2 6 Months Post-Burial

In contrast to the 1-month bone samples, bones buried in an acidic soil environment (Figure 6-42) and recovered after 6 months of burial, appeared to be much rougher than bones buried in a basic soil environment (Figure 6-41). This agrees with observations made during this study, in which bones buried in an acidic soil environment began to crumble when touched. Also, bones buried in basic soil showed more pores than bones buried in acidic soil. Again, in contrast with the 1-month bone samples, bones buried in wet soil looked rougher than bones buried in dry soil. After six months of burial, it appears that the wet soil environment is accelerating the degradation processes occurring in bone, when compared to a dry soil environment. Also, the wet soil bone samples showed fewer pores than the dry soil bone samples.

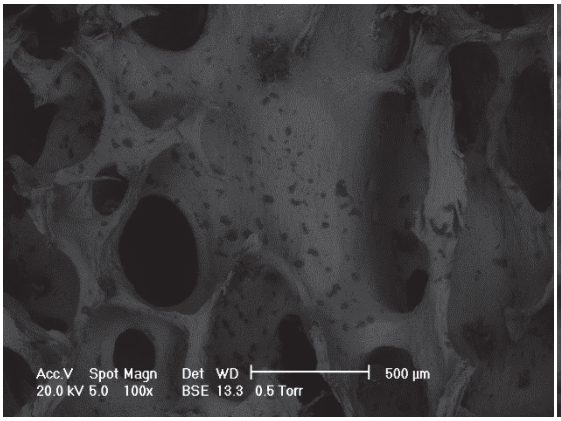

Figure 6-41. Loam Basic 1 – 6 months

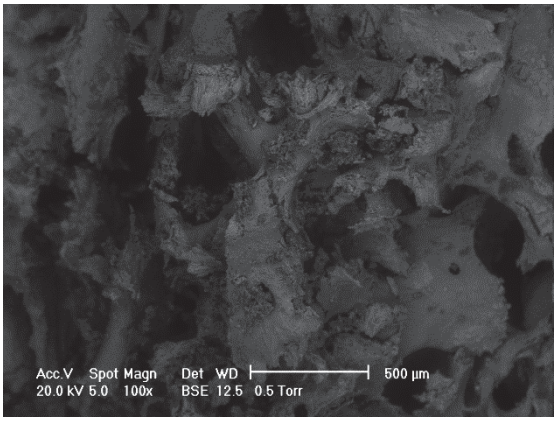

Figure 6-42. Loam Acidic 1 – 6 months

Similar to the 1-month samples, bones buried in a silty, sandy and clay soil environment, all appeared very similar, with the bones buried in a clay soil environment showing the least number of visible pores. Lastly, bone samples stored in a cool environment showed very few visible pores and, therefore, confirm the preserving nature of such an environment.

6.6.1.3 12 Months Post-Burial

Just like the 6-month bone samples, bones buried in acidic soil were much rougher than bones buried in basic soil, and again, the basic soil bone samples showed more pores than the acidic soil bone samples. Bones buried in wet soil (Figure 6-43) appeared rougher and had more visible pores than bones buried in dry soil (Figure 6-44). These results indicate that after a year's burial, basic and dry soil environments tend to preserve the bone sample, rather than destroy it.

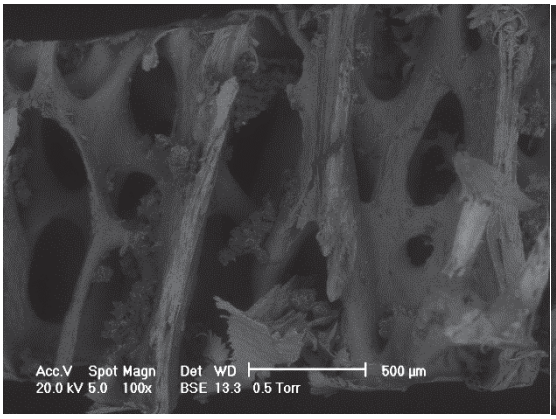

Figure 6-43. Loam Wet 1 – 12 months

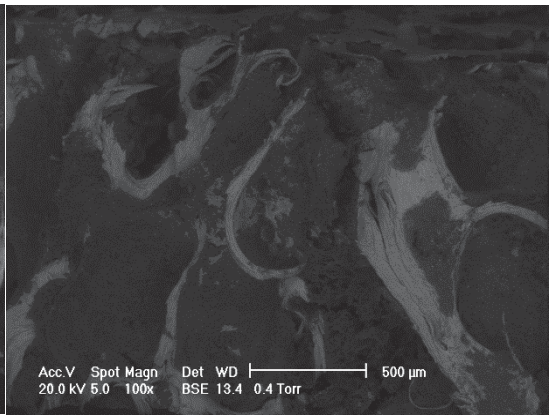

Figure 6-44. Loam Dry 1 – 12 months

After 12 months of burial, bone samples buried in a silty, sandy and clay soil environment, all appeared very similar. The bones buried in a clay soil environment showed the least number of visible pores. Bone samples stored in a cool environment also appeared to have very few pores compared to the bones recovered from the other burial conditions.

6.6.1.4 18 Months Post-burial

Following the trend since 6 months of burial, the bones recovered from an acidic soil environment (Figure 6-46) after 18 months of burial, appear much rougher than bones recovered from basic soil. Again, this confirms that an acidic environment tends to attack bone rather than preserve it. In contrast with the observations of the 6 and 12 month bone samples, but in accordance with the results of the 1-month bone samples, bones buried in dry soil (Figure 6-45) and recovered after 18 months, looked rougher than bones buried in wet soil. This indicates that the effect of the moisture content of the burial environment is not as significant as it was in the earlier months of burial.

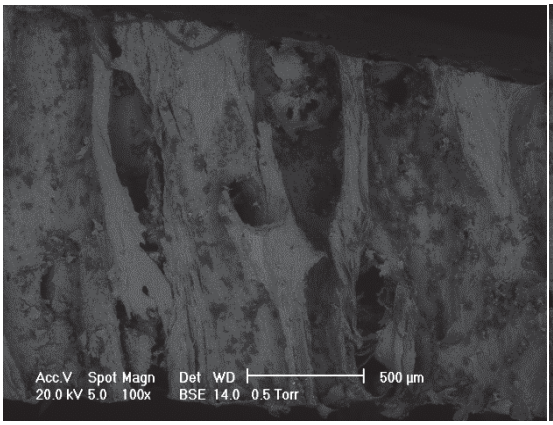

Figure 6-45. Loam Dry 1 – 18 months

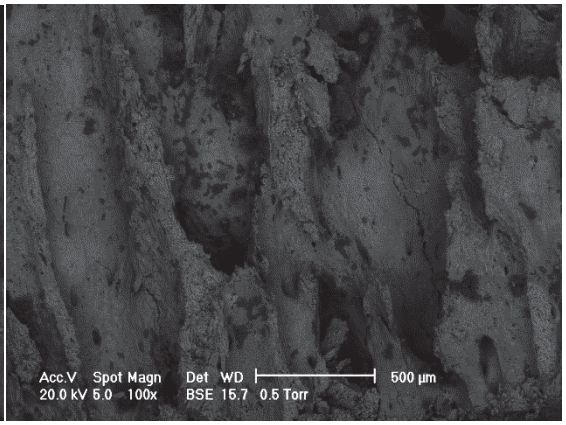

Figure 6-46. Loam Acidic 1 – 18 months

Table 6-18. Comparison of surface characteristics and porosity with post-burial time (n = 72)

Burial Condition	1 month	6 months	12 months	18 months
Neutral, As Supplied, Room-Temperature Loam (Control)	S +	S +	S +++	S/R ++
Silty Soil	S ++	S +	S +++	S +++
Sandy Soil	S ++	S +	S +++	S +++
Clay Soil	S +	S +	S ++	S ++
Acidic Soil	S +	R +	R ++	R ++
Basic Soil	R +	S ++	S +++	S ++
Wet Soil	S +	R +	R +++	S ++
Dry Soil	R +	S ++	S ++	R ++
Cold Soil	S -	S -	S +	S +

S = Smooth R = Rough -/+/++/+++ = Degree of porosity

Similar to the 1, 6 and 12 month bone samples, bones buried in a silty, sandy and clay soil environment, all appeared very similar. Only few pores were visible in all the samples, with the bones buried in a clay soil environment showing the least number of visible pores. The bone sample recovered from the first sandy soil environment, had small, white circular 'platelets' clustered in certain sections of the bone. This was not evident in the other bone sample recovered from a sandy soil environment. Lastly, bone samples stored in a cool environment showed fewer micro-pores than all the other bone samples.

Overall, all bone samples increased in bone porosity after a post-burial time of 6 months. It was observed that a colder soil environment followed by a clay soil environment were the least aggressive environments for bone diagenesis in terms of bone porosity. Bones buried in an acidic soil environment were rougher than bones buried in a basic soil environment, emphasising the aggressive nature of the acidic soil environment. The bones buried in the wet and dry soil environments demonstrate that the presence of water in the soil has an effect on the bone degradation processes but this is not completely understood.

6.6.2 X-ray Diffraction

The crystallinity indices of the bone samples recovered from all the burial conditions including the acidic soil environment, at the different post-burial times, were very similar. These results are in agreement with the results of previous studies, which show that changes to the inorganic phase of a bone take several years to occur (Bartsiokas and Middleton, 1992; Prieto-Castelló *et al.*, 2007). Since the post-burial times studied were for forensic applications (relatively short period of time), no significant differences in the crystallinity index values were observed for any of the bones samples at the different post-burial times. Therefore, in a forensic context, XRD analysis is not an effective tool for post-burial time estimation.

6.6.3 Thermogravimetric Analysis

The aim of the present study was to compare the effects of varying bone pre-treatment methods and burial environments on the degradation processes that occur in buried bones. A comparison of various investigated burial conditions is presented in Figure 6-47. The mass losses shown in the graph for the different burial conditions were normalised to the mass loss that occurred prior to burial.

Overall, the mass losses occurring in the different burial environments after each burial period follow different trends however there are still a few general trends evident in this graph. The bones buried in a cold soil environment and in a clay soil environment show the lowest mass losses at each of the burial periods up to and including 8 months of burial. These two burial environments appear to be the most effective in preserving bones once they are buried. Within a burial period of 8 and 12 months, sandy and clay soil environments tend to act in a preserving manner towards the bones. After 12 months of burial, only the clay soil environment appears to be effective in preserving the bones, with a slightly higher mass loss being observed for bones buried in this environment after TG analysis is performed.

Bones buried in an acidic soil environment show a decreasing trend in mass loss with increasing burial time for the entire duration of burial. This trend becomes more evident after a burial period of 8 months and indicates that an acidic environment is the most destructive compared to the other burial environments.

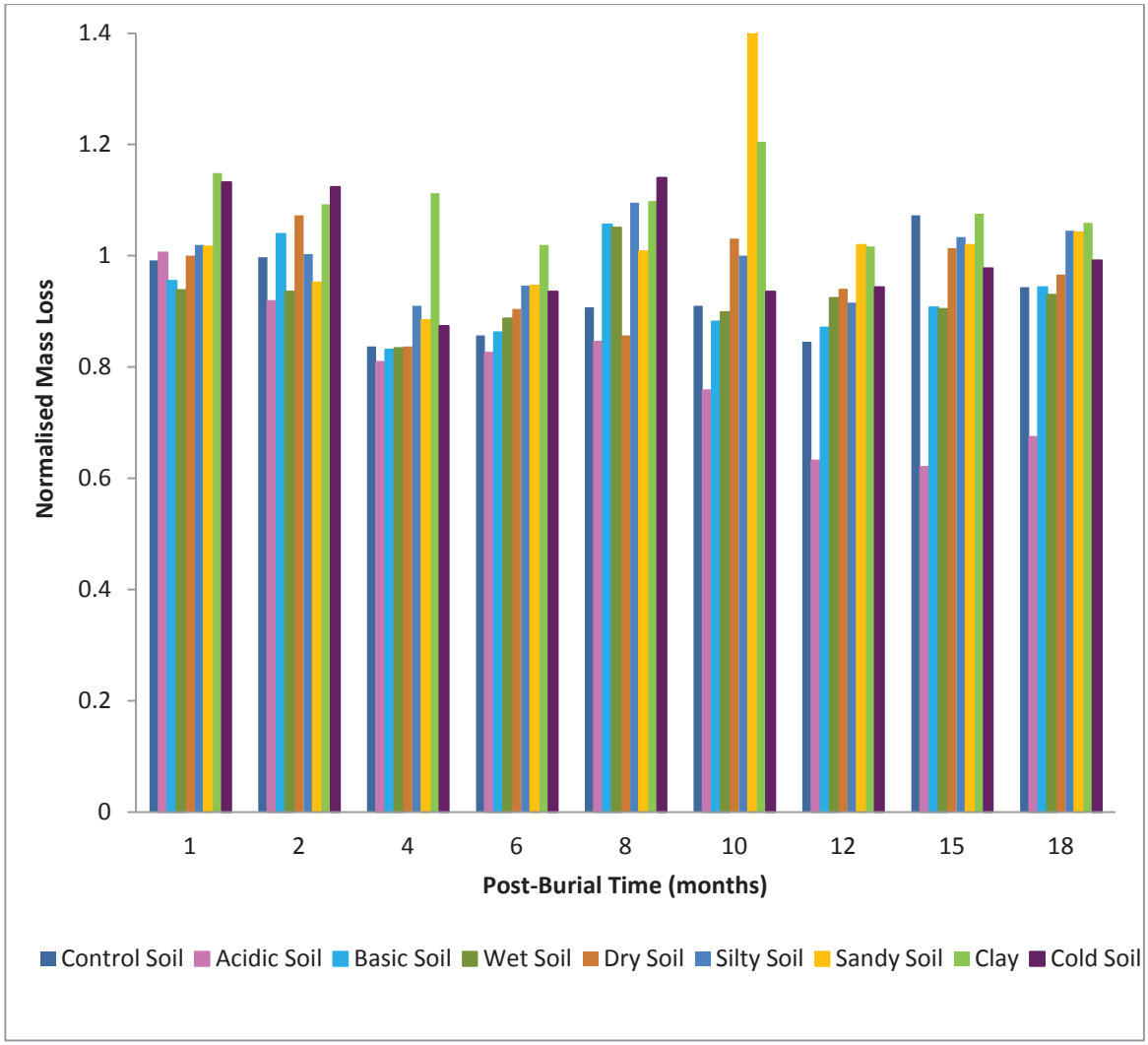

Figure 6-47. Mass loss occurring in the different burial conditions for the duration of 18 months

A comparison of the different burial environments as a function of post-burial time is presented in Figures 6-48 to 6-50. Figure 6-48 shows the mass losses that occurred in bones in the different burial conditions for the first 6 months of burial. The same trend is observed for the first 6 months of burial when bones buried in sandy, clay and cold soil environments and degreased and boiled bones are compared. The mass losses occurring in the bones buried in the varying burial conditions between 6 and 10 months of burial are shown in Figure 6-49. There are no visible trends in this graph, indicating that many different degradation processes are occurring during this burial time in the various burial environments. Figure 6-50

shows the same comparison for the burial period of 10 to 18 months. A similar trend is observed for the 10, 12, 15 and 18 month burial periods when all the burial conditions are compared with the differences between the silty, sandy and clay soil environments being more apparent during the 10 month burial period.

Overall, the differences between the burial conditions are most distinct within the 6 and 10 month burial period. The fewest variations are observed during the 10 and 18 month burial period.

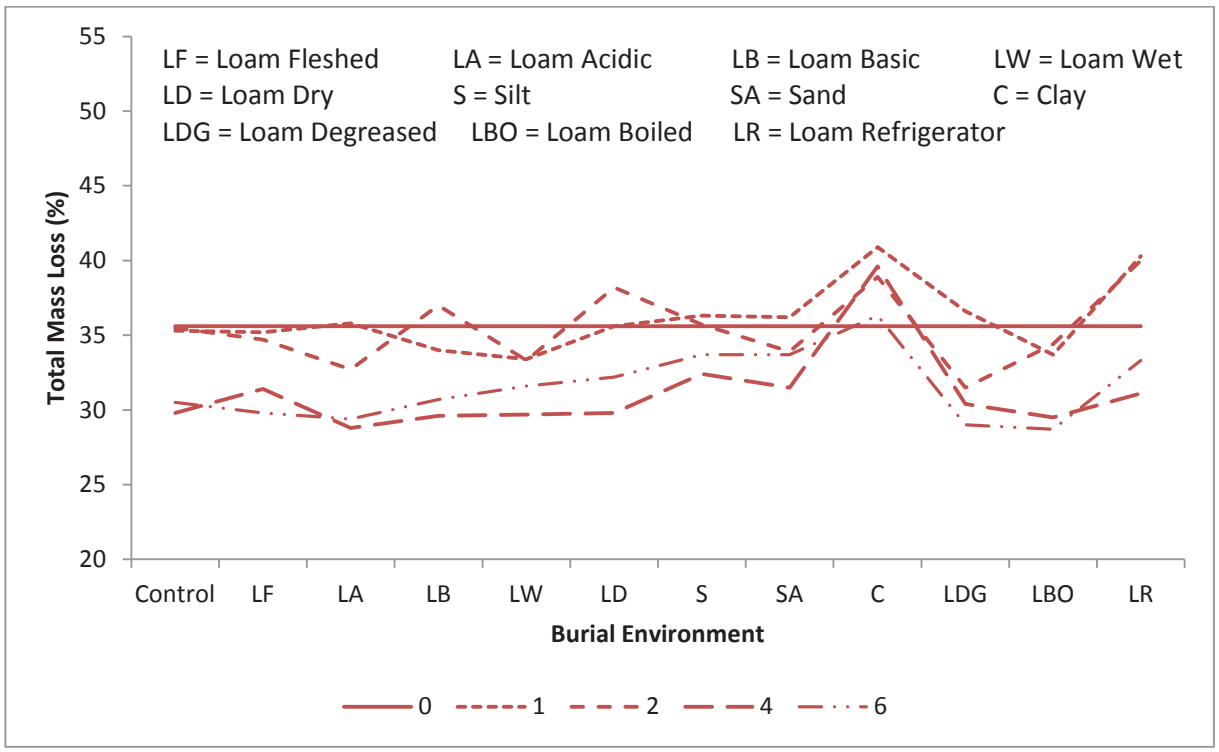

Figure 6-48. Comparison of burial conditions in the first 6 months of burial

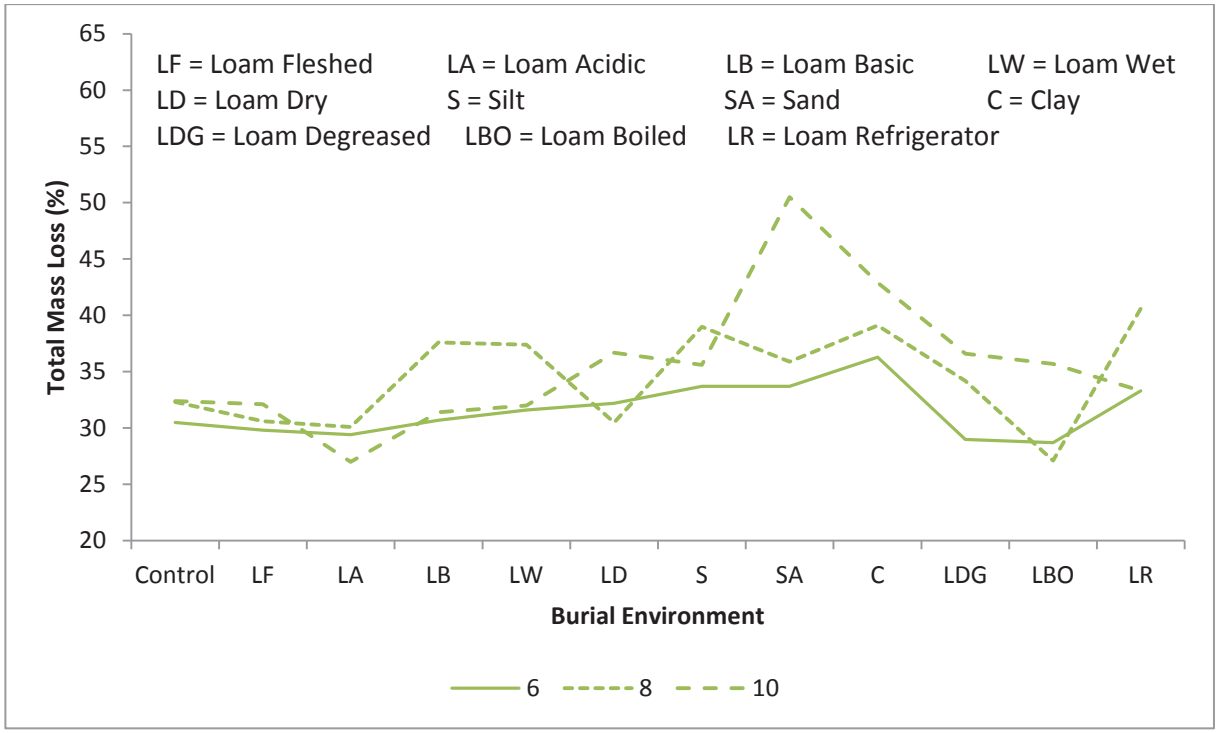

Figure 6-49. Comparison of burial conditions between 6 and 10 months of burial

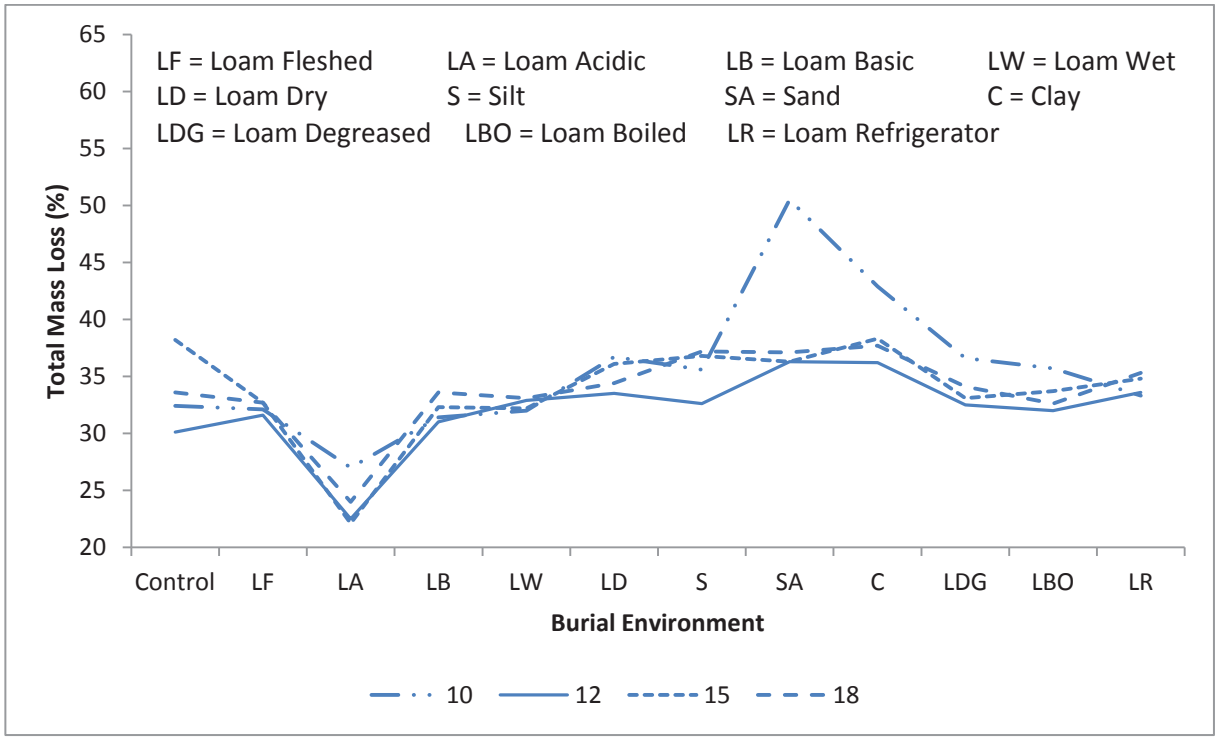

Figure 6-50. Comparison of burial conditions between 10 and 18 months of burial

6.6.4 Pyrolysis Gas Chromatography-Mass Spectrometry

For all the burial conditions investigated in this study, except for the boiled bones, high correlations were observed (R^2 = 0.88 to R^2 = 0.98, $p < 0.05$) between actual and predicted post-burial times, when each burial condition was subjected to linear regression analysis individually. The coefficient of determination and the standard deviation of the results of all the burial conditions are presented in Table 6-19. These results clearly show that, using Py-GC-MS analysis and linear regression analysis, with the data mean-center pre-processed and corrected for baseline using linear fit, the post-burial times of all the bone samples buried in the different conditions, excluding the boiled bones, can be predicted.

Table 6-19. Coefficient of determination and standard deviation of the Py-GC-MS results of bone samples (duplicates) buried in different environments (n = 24)

Samples	Coefficient of Determination	Standard Deviation (months)
Defleshed Bones	0.91	4.0
Fleshed Bones	0.98	1.4
Acidic Soil	0.96	2.6
Basic Soil	0.96	2.1
Wet Soil	0.93	3.2
Dry Soil	0.90	5.9
Silty Soil	0.97	2.1
Sandy Soil	0.88	4.1
Clay Soil	0.90	5.6
Degreased Bones	0.98	1.4
Boiled Bones	--------	-------
Cold Soil	0.97	2.8

6.6.4.1 Comparison of Burial Conditions Based on Statistical Analysis

As the Py-GC-MS results obtained in this study have demonstrated, it is possible to estimate the length of time for which bones have been buried in each of the burial environments described. However, information about the burial environment of recovered bones is not always readily available. Therefore, comparison of the different burial environments using statistical analysis has been performed to investigate the potential of using Py-GC-MS analysis to estimate the post-burial time of buried bones without having any prior knowledge of the burial environment.

The results of linear regression analysis using mean-center pre-processing and linear baseline correction of all the burial environments, after the different lengths of burial are presented in Figure 6-51. This graph shows the relationship between the actual post-burial period (Measured Y) and the length of burial (Pred Cal) predicted as a result of the statistical analysis. As is evident from the graph, most of the bones samples buried for the same amount of time are clustered relatively close together for each post-burial time, regardless of the burial environment. Also, there are only a few distinct outliers in the graph. However, it is important to note that there is still a certain amount of scatter present between the samples recovered from different burial environments. However, it is still possible for a ball park estimation of the post-burial time of the bones within a 6 month range independent of knowledge of the burial environment. Incorporating information about the burial environment improves correlation, resulting in a more accurate estimation of the post-burial time of the bones being examined.

The variation in the results of the bones recovered from different burial environments confirms the idea that the burial environment affects the processes that occur during bone degradation, resulting in different compounds being present in the bones. These compounds

and the intensity of the peaks relating to these compounds are the basis of differentiating between bone samples recovered from varying burial environments.

Data points that lie on or close to the green line in the graph indicate a linear relationship between changes in bones and the length of burial of the bones. Therefore, it is interesting to note that for all of the burial environments except for a few outliers, boiled bones in particular, the length of burial can be roughly estimated, up to a post-burial period of 15 months. For all the burial environments, the burial period of 18 months is under-estimated by the statistical analysis of the Py-GC-MS results. As expected, the boiled bones were the most significant outliers in the graph, as these were the bones that appeared to show the fewest changes with increasing length of burial, when analysed using linear regression analysis.

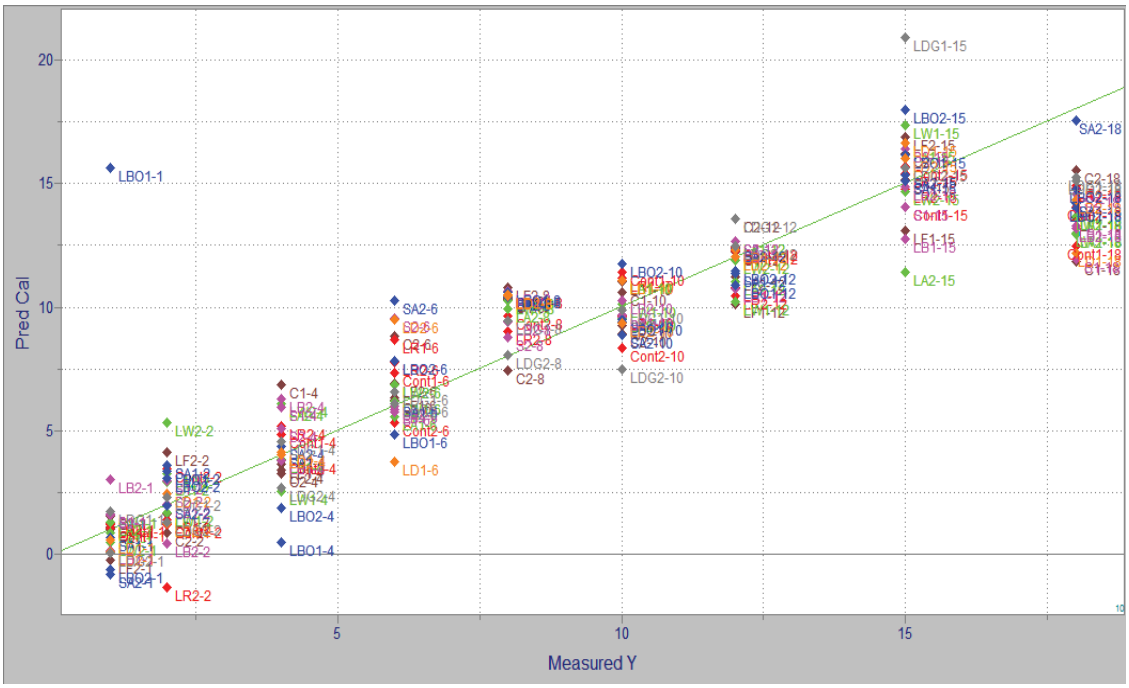

Figure 6-51. PLS analysis of all burial environments and burial periods

The statistical analysis results shown in Figure 6-51 are also more clearly presented in Figures 6-52 to 6-55 to allow for comparison between soil pH, moisture content of soils, soil type and temperature of burial environment, respectively.

The correlation between the predicted and actual post-burial time of bones buried in soils of different pH is presented in Figure 6-52. Most of the data points are clustered together but some variation is evident in the data for 1, 2 and 15 months of burial. Similar to the bones subjected to the different pre-treatments, the lengths of burial of most of the bone samples can be estimated approximately, except for the 18 month bone samples.

The graph in Figure 6-53 shows the relationship between the predicted and actual post-burial time of bones buried in soils with differing moisture contents. Only the data points of 1, 8 and 18 months are clustered together. Variation is present in the results of the remaining burial periods. This large amount of variation could be explained by the presence of water in the soil facilitating the movement of compounds into and out of the bone, in turn, accelerating or decelerating the processes involved in bone diagenesis, depending on if it is a wet or dry environment, respectively. Therefore, it is difficult to estimate the length of burial for these samples, without knowledge of the moisture content of the burial environment.

The statistical analysis of the results of bones buried in different soil types are presented in Figure 6-54. Most of the data points are clustered together, but some variation is evident in the data for 4, 6 and 18 months of burial. A majority of the outliers are due to the bones buried in a sandy soil and clay soil environment. However, despite these variations, it is still possible to approximate the lengths of burial of most of the bone samples.

The relationship between the predicted and actual post-burial time of bones buried in room-temperature and cold soils is shown in Figure 6-55. The data points of 1, 4, 8 and 15 months

are clustered together and variation is present in the results of the remaining burial periods. This variation could be explained due to the retardation of the processes involved in bone degradation as a result of bones being stored in a colder environment. Therefore, it is difficult to estimate the length of burial for these samples, without knowledge of the temperature of the burial environment.

All these results demonstrate that it is possible to estimate the post-burial period of bones without knowledge of the burial environment, however, information about the burial environment makes the estimation of the length of burial more accurate.

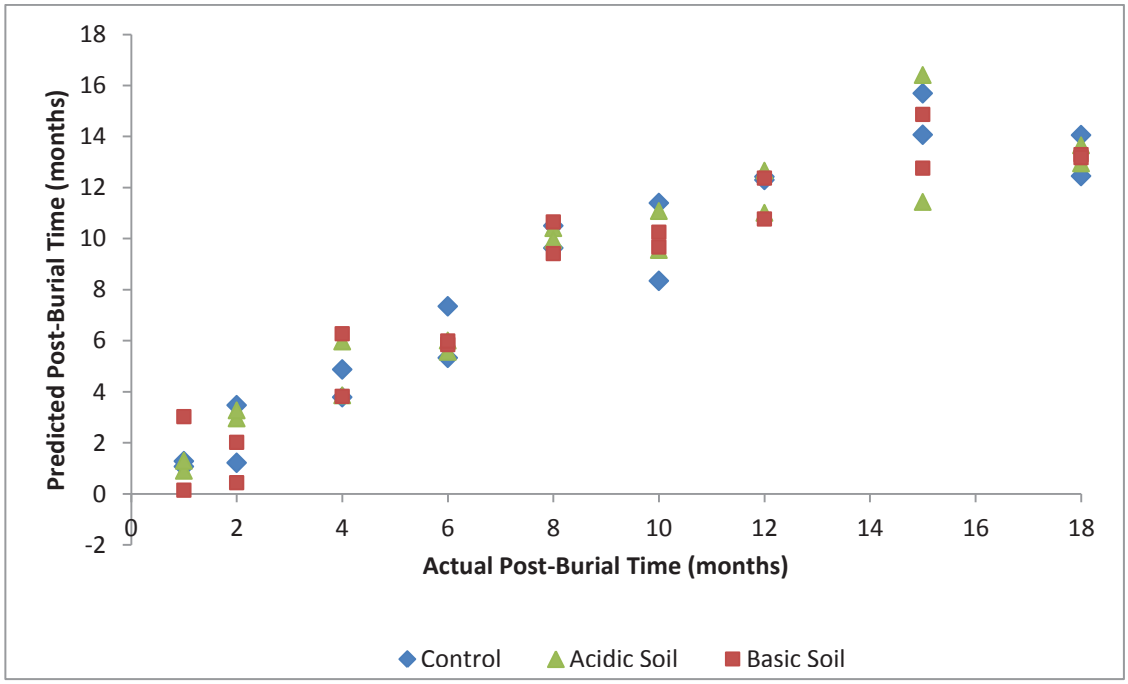

Figure 6-52. Predicted post-burial time vs actual post-burial time for bones buried in soils with varying pH

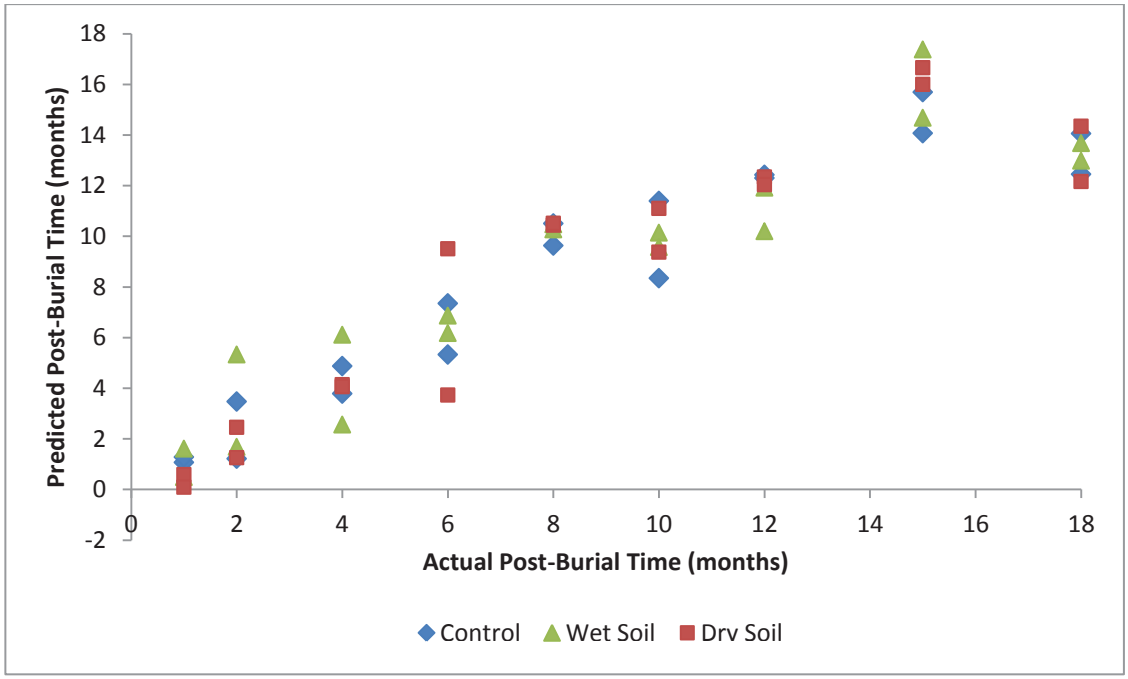

Figure 6-53. Predicted post-burial time vs actual post-burial time for bones buried in soils with varying moisture content

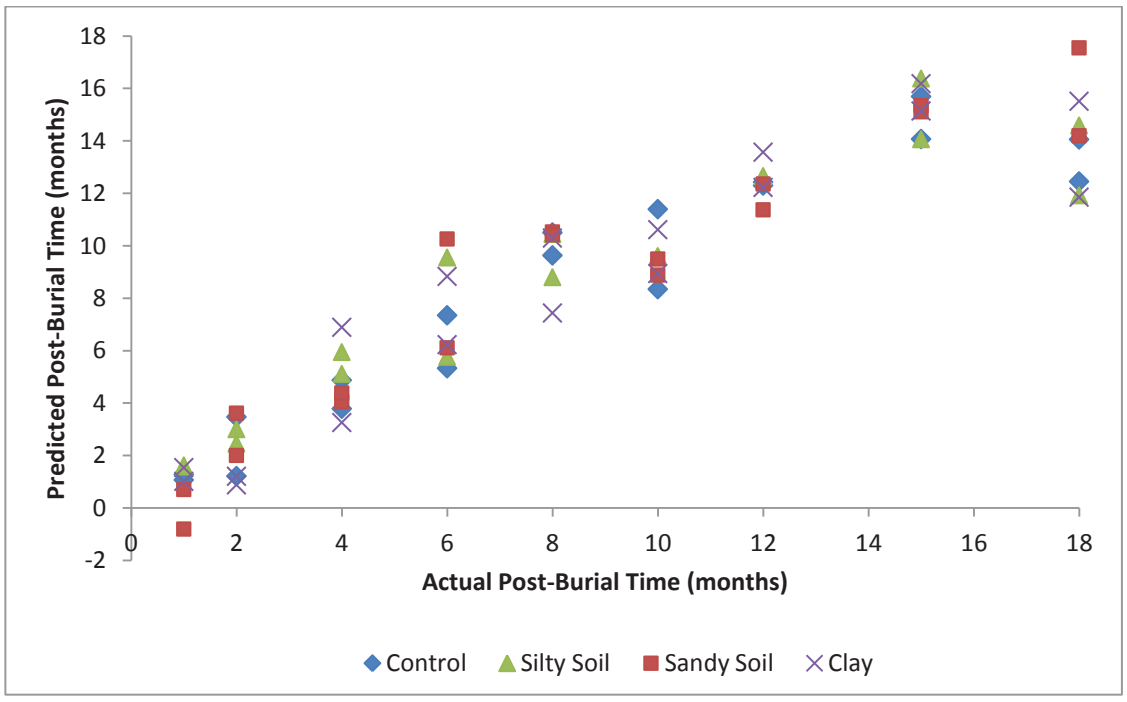

Figure 6-54. Predicted post-burial time vs actual post-burial time for bones buried in different soil types

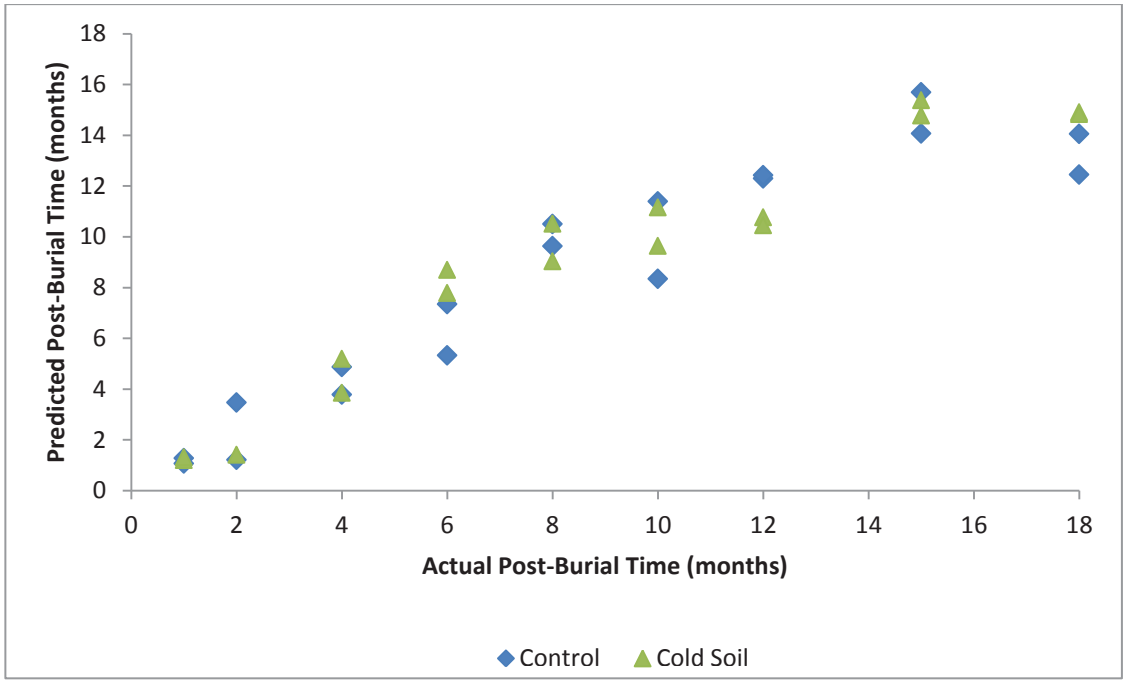

Figure 6-55. Predicted post-burial time vs actual post-burial time of bones buried in soils with varying temperatures

6.6.4.2 Comparison of Burial Conditions Based on Changes in Compounds

Diagenesis includes all the chemical changes and microbial attacks on bone as well as the various processes that are responsible for the destruction of the bone. The major diagenetic change to the organic phase of bone is collagen loss and decay. In the first month of burial, many different compounds are present in the different burial conditions, but as the length of burial increases, the number of compounds found in the bones decreases. This is in accordance with the process of bone diagenesis, in which a bone decomposes as a result of its interaction with the burial environment.

The compounds identified from prominent pyrolysate peaks observed in the pyrograms after 1 month of burial are listed in Table 6-20 for each burial environment. Similarly, the

compounds identified after 6, 12 and 18 months of burial are presented in Tables 6-21, 6-22 and 6-23, respectively. The compounds presented in the table were selected based on a match quality of 70% or greater. The compounds highlighted in the tables represent the pyrolysis products (of bones) that were obtained from more than one type of burial environment after the same burial time. The result is a constant reduction in the number of identifiable pyrolysis products in the bone samples, from the beginning to the end of the burial period. There is also a reduction in the concentration of the identifiable pyrolysis products as the burial time increased, as suggested by the decrease in the intensity of the peaks. After the first month of burial, more than 35 different pyrolysis products were identified in the bones recovered from the various burial conditions. After 6 months of burial, the number of identifiable pyrolysis products present in the bones buried in the different conditions reduced to only 11 compounds. The number of identifiable pyrolysis products found in the bone samples decreased to 7 after 12 months of burial and, then to 5 after 18 months of burial. This demonstrates the process of diagenesis by revealing the constituents of the potential parent compounds most susceptible to degradation based on the identified pyrolysis products. Similarly, the potential parent compounds which persist after longer periods of burial are also identified based on the recovered pyrolysis products.

Previous research has shown that bone tar is mainly composed of aliphatic material with nitrogen functionalities attached as nitriles, pyridines, pyrroles and amides, with a small presence of pyridines and phenols (Purevsuren *et al.*, 2004b). Similarly, the main content of bone tar was found to be organic acids, asphaltanes and preasphaltanes (Purevsuren *et al.*, 2004a). Research by Lodowska *et al.* (2012) showed that the pyrolytic pattern of bone implants consists of derivatives of benzene, pyridine, pyrrole, phenol, nitriles, sulfur compounds, saturated and unsaturated aliphatic hydrocarbons and fatty acids (C12-C20). The results of the present study confirm the occurrence of these compounds in bone pyrolysis

products based on the pyrolyzate peaks in the pyrograms. The presence of nitrogen-containing compounds corresponds to the protein content of bones (Poinar and Stankiewicz, 1999). There is a small presence of benzene-derivative compounds which can be associated with the thermal degradation of the organic component of bone (Lodowska *et al.*, 2012). In terms of fatty acids, only oleic acid derivatives were observed. This is because fatty acid compounds are not volatile and it is therefore difficult to identify their presence using GC-MS (Lodowska *et al.*, 2012).

The pyrolysis products which were produced in bone samples even after 18 months of burial in a few of the burial environments were trans-1,1,3,4-tetramethyl-cyclopentane and hexadecanenitrile, which are produced from the presence of proteins in bones (Poinar and Stankiewicz, 1999). This suggests that the parent compounds of these pyrolysis products are possibly less prone to decomposition during burial and as a result, persist longer than other compounds. After 12 months of burial, the compound still found in the bones buried in most of the burial environments was 2,6-dihydroxy-benzoic acid, with (Z)-3-methyl-2-undecene still present in a couple of the burial environments, which could be due to the presence of fatty acids in bones (Lodowska *et al.*, 2012). Bones analysed after 6 months of burial showed that 2,6-dihydroxy-benzoic acid, phenoxy-phenol and 2-cyclohexen-1-one still existed in a few of the burial environments. Again, these compounds may be attributed to the existence of fatty acids in bones. There were many more compounds present in the samples with the post-burial time of 1 month, which demonstrates that decomposition is still in its early stages in the first month of burial.

The results of Py-GC-MS highlight the process of diagenesis and demonstrate that even though the process is microscopic, it can still be monitored closely using this technique. Py-GC-MS shows that as the burial time of the bones increases, the number of compounds found

in the bones decreases considerably, which supports the use of this technique in monitoring changes in the organic content of bones.

Py-GC-MS results have also proven to be a very effective method for estimating the post-burial time of bone samples buried in all the different burial conditions investigated (excluding the boiled bones which represent accelerated decomposition). High correlation was observed for most of the burial environments, which highlights the ability of Py-GC-MS analysis to estimate the length of time a bone has been buried for, before being recovered. From this study, it can be said that the technique of Py-GC-MS is not affected by the burial environment in its ability to determine the length of burial of bone samples, which is very useful for forensic applications. However, it has also been shown that information about the burial environment of a recovered bone makes the estimation of the length of burial more accurate. Therefore, the burial length of the bone samples buried in any of the burial conditions investigated in this study can be predicted using the technique of Py-GC-MS, which makes this a vital tool for many forensic investigations.

Table 6-20. Compounds identified using Py-GC-MS as being present in the 1st month of burial

Compound Name	Cont	LF	LA	LB	LW	LD	S	SA	C	LDG	LR
N-methyl-2-propyn-1-amine	X			X		X					X
3,4,5,67,8-hexahydro-1,1-dimethyl-2(1H)-naphthalene	X										
2-(2-propenyl)-1,4-benzodioxin	X			X							X
4,4'-oxybis-benzenamine	X										X
Theophylline	X										
4-amino-6-methoxy-2-(trifluoromethyl)-pyrimidine	X										
Cyclododecene		X		X				X			
(Z)-2-undecene		X									
Hexadecanal		X									
Indole		X									
(Z)-3-methyl-2-decene		X									
3,5-dimethyl-2-cyclohexen-1-one			X								
1-nonadecene				X							
2,2-dipropyl-N-ethylpiperidine				X							
3-methylene-undecane				X							
Heneicosane					X						
1,8-dioxide-1,8-diethoxyoctahydro-4,7-phophinidenephosphindole					X						
1,2-diethyl-1-methyl-cyclohexane					X						X
trans,cis-1,2,4-trimethyl-cyclopentane					X						
octadecanoic acid					X		X				
Octadecanenitrile					X				X		X
3-pentyl-4,5-tetramethylenepyrazole						X					
Hexadecenenitrile						X			X		

Compound Name	Cont	LF	LA	LB	LW	LD	S	SA	C	LDG	LR
6,7,8,9-tetrahydro-6-methyl-4-oxo-4H-pyrido 1,2-a pyrimidine-3-acetic acid							X				
Heptadecanenitrile							X				
2-cyclohexen-1-one								X			
2,3-dimethyl-1,3-heptadiene								X			
1,2,3,6-tetramethyl-bicyclo 2.2.2 octane								X			
N,3-dimethyl-benzenamine								X			
6-nitro-2-picoline								X			
N-ethylidene-1-pyrrolidinamine								X			
Piperidinone								X			
1,2-dimethylpiperidine								X	X		
cis-tetrahydro-2,5(1H,3H)-pentalenedione								X			
5-methyl-2-(1-methylethyl)-2-cyclohexen-1-one								X			
2-ethyl-quinoline								X			
9-cycloheptadecen-1-ol								X			
2-butyl-2-methyl-1,1-cyclopropanedicarbonitrile									X		
2-ethyl-1-methyl-3-propyl-cyclobutane									X		
N,N-dipropyl-1-propanamine										X	
3,6-dimethyl-4H-pyrido 1,2-a pyrimidin-4-one										X	X
1-mono-olein										X	
(Z)-9-octadecenamide										X	X
1,3,4,6,9,9a-hexahydro-2H-quinolizine											X
(+)-isomenthone											X
3-phenoxy-phenol			X	X	X	X	X			X	X

Compound Name	Cont	LF	LA	LB	LW	LD	S	SA	C	LDG	LR
2,4-diphenylamino-cyclopent-2-enone											X
2-ethyl-N-(2-ethylbutyl)-1-butanamine											X
trans,cis-1,2,4-trimethyl-cyclopentane											X
(Z)-13-octadecenal											X
2-methyl-6-hepten-3-ol											X
Cyclopentadecane											X

Table 6-21. Compounds identified using Py-GC-MS as being present only in the first 6 months of burial

Compound Name	Cont	LF	LA	LB	LW	LD	S	SA	C	LDG	LR
2,6-dihydroxy-benzoic acid	X								X		
6-methyl-2-(trifluoromethyl)-pyrimidinol	X										
phenoxy-phenol	X								X		
1-nonadecene	X										
2-cyclohexen-1-one		X	X								
2-(2-propenyl)-1,4-benzodioxin		X									
butoxy-benzene		X									
Piperidinone			X								
4-(2-hydroxyphenyl)pyrimidine			X								
octadecanoic acid			X								
3-methylene-tridecane								X			
1-(O-methyloxime)-4-O-3-acetyl-1-(trimethylsilyl)-1H-indolyl-2,3,5,6-tetrakis-O-(trimethylsilyl)-D-glucose									X		
1-heptadecanol									X		
3-phenoxy-benzenemethanol										X	
Hexadecanenitrile											X

Table 6-22. Compounds identified using Py-GC-MS as being present only in the first 12 months of burial

Compound Name	Cont	LF	LA	LB	LW	LD	S	SA	C	LDG	LR
1,3,5-trimethyl-2-octadecyl-cyclohexane	X										
2,6-dihydroxy-benzoic acid		X		X	X	X		X			
4-amino-6-methoxy-2-(trifluoromethyl)-pyrimidine			X	X		X	X				
4,4'-oxybis-benzenamine				X							
(Z)-3-methyl-2-undecene					X				X		
9-dodecyltetradecahydro-phenanthrene									X		
(Z)-9-octadecenamide									X		
Benzenepropanenitrile										X	
octadecanoic acid										X	
trans-1,1,3,4-tetramethyl-cyclopentane											X

Table 6-23. Compounds identified using Py-GC-MS as being present for the entire duration

Compound Name	Cont	LF	LA	LB	LW	LD	S	SA	C	LDG	LR
phenoxy-phenol		X									
9-dodecyltetradecahydro-phenanthrene		X									
(Z)-3-methyl-2-undecene			X								
trans-1,1,3,4-tetramethyl-cyclopentane					X				X		
Hexadecanenitrile						X			X	X	
(Z)-9-octadecenamide											X

6.6.5 Effect of burial conditions on bone decomposition: A comparative study using Infrared Spectroscopy

In a separate FTIR study conducted (Howes *et al.*, 2012) using bone samples from the present study, the burial environment observed to have a significant effect on the decomposition of bones was the acidic soil environment. The examination of the organic and carbonate contents as well as the crystallinity index (CI) of the bones demonstrated that bone decomposition is accelerated in an acidic soil environment. The observed trends indicate that protein content is degraded, and there is ordering of the crystalline phase when the bone is exposed to an acidic environment over a relatively short period of time. The carbonate content of the bones showed the greatest change in an acidic environment. The results of the present study support that acidic soil environments accelerate decomposition processes occurring in bone after burial. The other factors investigated in the FTIR study including a basic soil pH, soil moisture content and temperature were not found to have a significant effect on bone degradation processes during burial. The FTIR study found that a sandy soil environment preserves bone samples while the silty and clay soil environments leached away the phosphate content. The results of the present study demonstrated that particular burial environments – sandy, clay, basic, dry and colder soils – preserve bones. An explanation for the difference in the results observed for the clay soil environments could be possibly due to the techniques employed as each technique utilised is sensitive to the presence of certain compounds, which is reflected in the results.

6.7 Summary

Overall, ESEM analysis showed that all bone samples increased in bone porosity after a post-burial time of 6 months. It was observed that a colder soil environment followed by a clay soil environment were the least aggressive environments for bone diagenesis in terms of bone

porosity. Bones buried in an acidic soil environment were rougher than bones buried in a basic soil environment, emphasising the aggressive nature of the acidic soil environment. The bones buried in the wet and dry soil environments demonstrate that the presence of water in the soil has an effect on the bone porosity but this is not completely understood.

The crystallinity indices (XRD) of the bone samples recovered from all the burial conditions at the different post-burial times were very similar. These results are in agreement with the results of previous studies, which show that changes to the inorganic phase of a bone take several years to occur. Since the post-burial times studied were for forensic applications, no significant differences in the crystallinity index values were observed. Therefore, in a forensic context, XRD analysis is not an effective tool for post-burial time estimation.

Using TG, it was demonstrated that the mass losses occurring in the different burial environments after each burial period follow different trends however there are still a few general trends evident. The bones buried in a cold soil environment and in a clay soil environment show the lowest mass losses at each of the burial periods up to and including 8 months of burial, which makes them the most effective in preserving bones. Within a burial period of 8 and 12 months, sandy and clay soil environments tend to act in a preserving manner. After 12 months of burial, only the clay soil environment appears to be effective in preserving the bones. Bones buried in an acidic soil environment show a decreasing trend in mass loss with increasing burial time for the entire duration of burial. This trend becomes more evident after a burial period of 8 months and indicates that an acidic environment is the most destructive compared to the other burial environments. Generally, it was observed that dry soil environments hinder microbial activity and hence bone decomposition compared to wet soil environments. Similarly, bones buried in a colder soil environment were seen to be preserved more effectively compared to bones buried in room-temperature soil.

Py-GC-MS analysis proved to be the most effective of all the techniques used in the present study for the estimation of the post-burial time of buried bone samples. For all the burial conditions investigated, except for the boiled bones (which represent accelerated decomposition), high correlations were observed (R^2 = 0.88 to R^2 = 0.98, $p < 0.05$) between actual and predicted post-burial times, when each burial condition was subjected to linear regression analysis individually. The results clearly show that, using Py-GC-MS analysis and linear regression analysis, with the data mean-center pre-processed and corrected for baseline using linear fit, the post-burial times of all the bone samples buried in the different conditions, excluding the boiled bones, can be predicted. Comparison of the different burial environments also demonstrates that it is possible to estimate the post-burial period of bones without knowledge of the burial environment, however, information about the burial environment makes the estimation of the length of burial more accurate.

The pyrograms collected for the different post-burial times investigated show a constant reduction in the number of identifiable compounds found in the bone samples, from the beginning to the end of the burial period. There is also a reduction in the concentration of the identifiable compounds present as the burial time increased, as suggested by the decrease in the intensity of the peaks. After the first month of burial, more than 35 different compounds were identified in the bones recovered from the various burial conditions. In contrast, only 5 compounds were able to be identified after 18 months of burial. This demonstrates the process of diagenesis and highlights the identifiable compounds most susceptible to degradation as well as the identifiable compounds which persist after longer periods of burial. The pyrolyzate peaks in the pyrograms confirmed the presence of nitrogen-containing compounds, which corresponds to the protein content of bones. There is also a small quantity of benzene-derivative compounds present, which can be associated with the

thermal degradation of the organic component of bone. In terms of fatty acids, only oleic acid derivatives were observed.

Chapter 7

Conclusions

Forensic investigations are presently limited by the lack of reliable techniques for estimating the post-burial time at crime scenes involving skeletal remains. The differences in burial environments in which crime scenes are discovered make the estimation of the post-burial time of bones an even more difficult task. Therefore, the present study investigated the effects of varying environments and pre-treatment procedures on bones using different analytical techniques over a forensically relevant burial period. The primary aim was to develop a method for accurately estimating the post-burial time of bones by burying bones in diverse burial environments and subsequently examining the buried bones using various analytical techniques.

Pig rib bones were used as an analogue for human bones. Differences between various bone pre-treatment procedures including defleshing, degreasing and boiling were observed. Burial environments varying in soil types, soil pH, moisture content and temperature were also examined as they have been observed to have an effect on bone decomposition. The effect of storage conditions on bone decomposition was studied by comparing fresh bone and bones stored for different periods of time in a freezer.

The technique of ESEM analysis was used to observe changes in porosity as the storage times of bones increased. Bone is a complex material consisting of organic and inorganic components. The changes in these two components were examined over time to understand how decomposition occurs. To study changes in the organic content of bones over time, TG and Py-GC-MS were used. The complementary technique of XRD was employed to study variations in the mineral content of bones.

Generally, the results of each of the analytical techniques were observed to be reproducible. TG results were reproducible for bone samples of younger post-burial times (less than 8 months) in most burial conditions. Py-GC-MS analysis, within the region of interest of 0 – 27

minutes, showed that the results at each post-burial time were reproducible. XRD diffraction patterns of both the loam defleshed (control) samples were very similar at each of the post-burial times.

Firstly, the present study investigated the effect of storage conditions on bone structure over time. It was difficult to determine the effect of storing bone samples in a freezer for samples analysed using the technique of TG since reproducibility of the results was poor, which could be attributed either to heterogeneity of the bone samples or to the initial mass of the bone samples being close to the limit of resolution of the TG instrument. The variation observed in the mass loss results of the samples stored in the freezer could be due to diagenesis continuing to occur in the organic content of the bones or due to the poor reproducibility of the results. Results of Py-GC-MS, however, appear to demonstrate that the organic component of the bones continues to decompose even when the bones are stored at a low temperature. Storage in a freezer does not appear to slow down the decomposition of the organic content of bones using Py-GC-MS analysis. As a result, storage of bone samples in a freezer does not appear to be suitable for bone samples to be analysed using Py-GC-MS or TG analysis as it is difficult to establish whether diagenesis was the sole cause of the mass loss results or whether the heterogeneity of the samples was responsible for the variations observed. In contrast, for analysis using XRD, bone samples can be stored in a freezer prior to analysis. For a period of 18 months, no differences were observed in the XRD results of bones i.e. inorganic content of bones stored in a freezer and therefore, storage in a freezer is suitable for bone samples to be analysed using XRD.

Secondly, the effect of pre-treatment procedures and burial conditions on bone porosity was examined. For some of the burial conditions investigated, macroscopic visual identification can be used to identify the burial environment from which a bone has been recovered. In general, based on differences in colour, it is possible to differentiate fresh/cool-temperature

and buried bone samples. Similarly, colour differences can be used to distinguish defleshed (brown) and degreased (orange/black); acidic (pink/white, brittle) and basic (light brown); wet (mostly white) and dry (brown); and silty or sandy and clay (red/white).

ESEM allowed the classification of bone samples into two categories of young (post-burial time of 6 months or lower) and old (post-burial time of 8 to 18 months) based on the differences between surface roughness and the presence of pores and micro-pores. Older bone samples had random shallow grooves while these were absent from the younger bone samples. Fleshed bone samples appeared rougher compared to defleshed bones samples suggesting that the presence of flesh promotes microbial activity in bones. Bones buried in an acidic soil environment were rougher than bones buried in a basic soil environment, emphasising the aggressive nature of the acidic soil environment.

Overall, all bone samples increased in bone porosity after a post-burial time of 6 months. Bone samples buried in a clay soil environment showed the smallest number of visible pores when compared to the other burial environments. It was observed that a colder soil environment followed by a clay soil environment were the least aggressive environments for bone diagenesis in terms of bone porosity.

For the loam defleshed samples, the relationship between post-burial time and the number of visible pores showed that as post-burial time increased, the number of visible pores also increased. However, there was some scatter present in the data and therefore, using the number of pores as a method for accurately estimating the post-burial time of bone samples may not be a reliable method.

Lastly, the effect of pre-treatment procedures and burial conditions on the inorganic and organic contents of bone was studied. The XRD results showed no changes in crystallinity for

a post-burial period of 18 months, clearly supporting the concept that changes in the inorganic component of bones take much longer, up to several years to occur. Therefore, the technique of XRD cannot be used to estimate the post-burial time of bone samples that are relatively young, as is the case in this study as well as, in most forensic applications.

In terms of the organic content of bone, TG analysis showed an overall decreasing trend in mass loss in all the bone samples up to a post-burial time of 8 months. It also showed that fleshed, defleshed and boiled bones follow similar trends in mass loss for the 18 months investigated, except for 6 and 8 months. The results indicated that the degradation processes are occurring at a slower rate in fleshed bones than in defleshed and boiled bones. The degreased bones followed the same mass loss trend as the other bones between 2 and 15 months of burial but in general the scatter for the 2 mg bone samples was large making sound analysis of the results difficult.

It was observed that the mass losses occurring in the different burial environments after each burial period followed different trends. The bones buried in a cold soil and a clay soil environment showed the highest mass loss up to 8 months of burial, thus making them the most effective in preserving bones. Also, sandy and clay soil environments acted in a preserving manner for the burial period between 8 and 12 months. Interestingly, only the clay soil environment appeared to be effective in preserving the bones after 12 months of burial. Bones buried in an acidic soil environment showed a decreasing trend in mass loss with increasing burial time, indicating that an acidic environment is the most destructive environment. Generally, it was observed that dry soil environments hinder microbial activity and hence bone decomposition compared to wet soil environments. Similarly, bones buried in a colder soil environment were seen to be preserved more effectively compared to bones buried in room-temperature soil.

Upon visual inspection of the pyrograms, it is possible to differentiate between bone samples up to the post-burial times of 6 months. However, after the post-burial time of 6 months, it is not possible to discriminate between samples of different post-burial times using visual inspection, therefore, statistical analysis was employed to interpret the trends.

For all the burial conditions investigated, except for the boiled bones, high correlations were observed between actual and predicted post-burial times, when each burial condition was subjected to linear regression analysis. These results clearly show that, using Py-GC-MS analysis and linear regression analysis corrected for baseline, the post-burial times of all the bone samples buried in the different conditions, except for boiled bones, can be predicted accurately. Comparison of the different burial environments also demonstrated that it is possible to estimate the post-burial period of bones without knowledge of the burial environment, however, information about the burial environment allows for a more accurate estimation of the post-burial time (18 ± 1.4 to 18 ± 5.9).

Py-GC-MS was observed to be the most useful and accurate technique for estimating the post-burial time of recovered bone samples. The data showed a direct relationship between the actual and predicted post-burial time of bones for all the pre-treatment procedures studied except for boiling. The pyrograms collected for the different post-burial times demonstrated the process of diagenesis and highlighted the identifiable compounds most susceptible to degradation, as well as the identifiable compounds which persist after longer periods of burial.

Currently, estimating the post-burial time remains one of the most elusive determinants present at a crime scene, which significantly hinders forensic investigations. In the early post-mortem period, it is possible to determine time since death based on the stages of decomposition of soft tissue. However, these stages of decomposition are not useful for

estimating time since death in the extended post-mortem period i.e. following skeletonisation. The chemistry of archaeological bones has been widely studied, however, the structures of lesser aged bones such as those that are encountered in a forensic context, have not been as extensively studied. The extent of changes in the organic and inorganic content of bones as a result of decomposition can be related to different lengths of burial. However, estimating the post-burial time of bones based on the degree of bone degradation is a difficult task since bone decomposition is affected by environmental factors. To eliminate errors, estimates need to be based on the environment that the body was discovered in and the condition of the remains. Therefore, the present study encompassed these critical factors – skeletal remains, forensic context and burial environment – in the investigation of various analytical techniques in estimating post-burial time at crime scenes. The results of the present study showed that the technique of Py-GC-MS was able to discriminate between bone samples of different post-burial times regardless of the burial environment. Therefore, these techniques, in particular Py-GC-MS, show potential for the development of a method for the determination of time since death in forensic investigations involving skeletal remains without prior knowledge of the burial environment.

Future Recommendations

The current study investigated several factors present in a burial environment that are thought to have an effect on bone diagenesis. The successful findings of the study have led to a greater understanding of bone decomposition and in turn, have provided avenues for the development of an accurate method of PMI determination in crime scenes involving skeletal remains.

This study was successful in achieving its aim of developing a valuable method for estimating the post-burial time of bones in diverse burial environments. Further in-depth research and

statistical analysis into burial factors such as moisture content, soil pH and temperature as covariates would allow for the determination of the quantitative effect of these factors in a forensic context. As soil types vary immensely in different parts of the world, this method can be customized regionally by analysing the various types of soils of that area and therefore, the effectiveness of the developed method can be determined around the world. Intra-variability in porcine rib bone samples may be a factor that needs to be considered in future research. Quantitatively measuring features of the bone, such as the colour and porosity, using histomorphometrics as well as using human bone samples would also be beneficial.

Appendices

Appendix 1

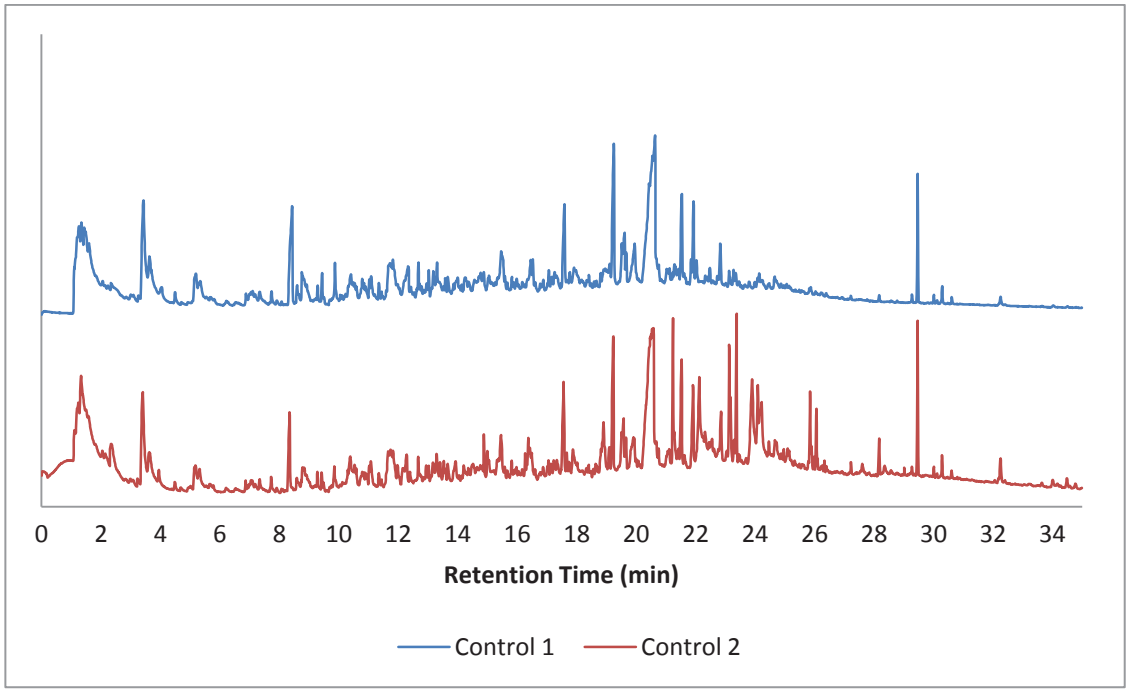

Figure 7-1. Py-GC-MS results of Loam Defleshed (Control) 1 and 2 after 1 month of burial

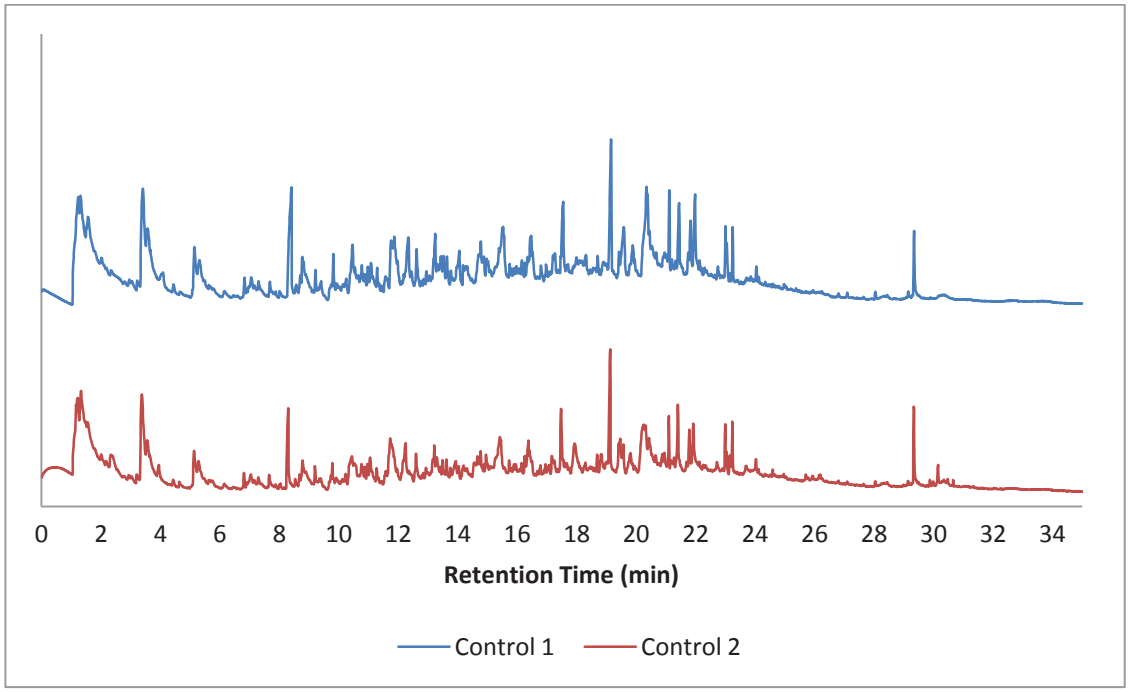

Figure 7-2. Py-GC-MS results of Loam Defleshed (Control) 1 and 2 after 2 months of burial

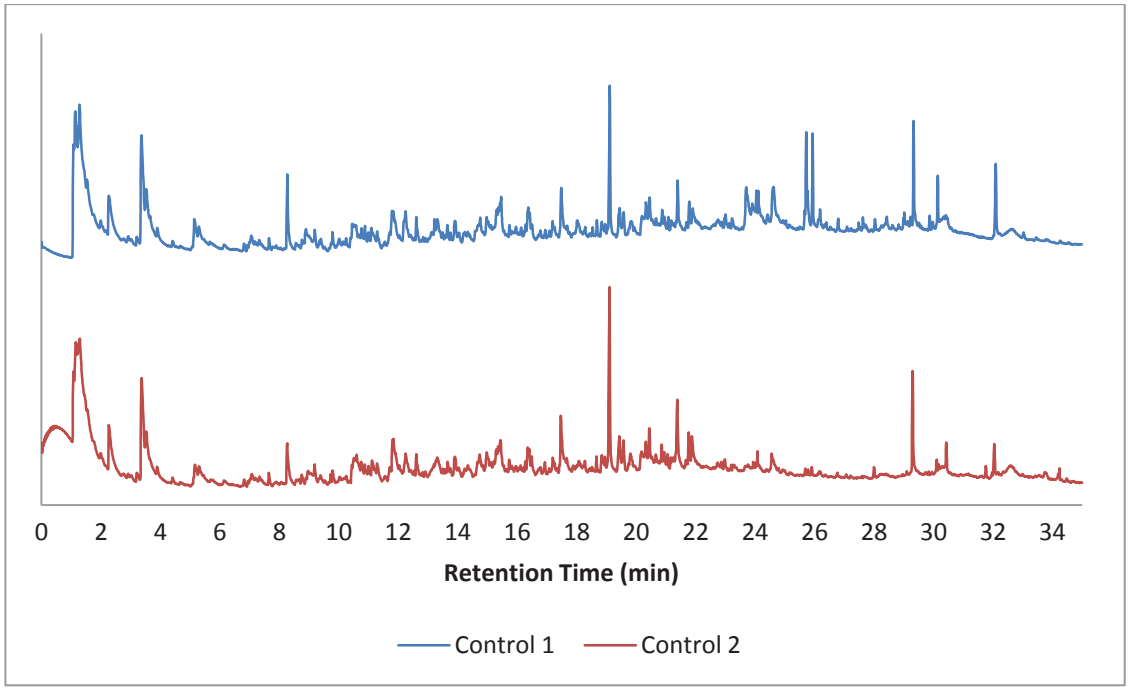

Figure 7-3. Py-GC-MS results of Loam Defleshed (Control) 1 and 2 after 4 months of burial

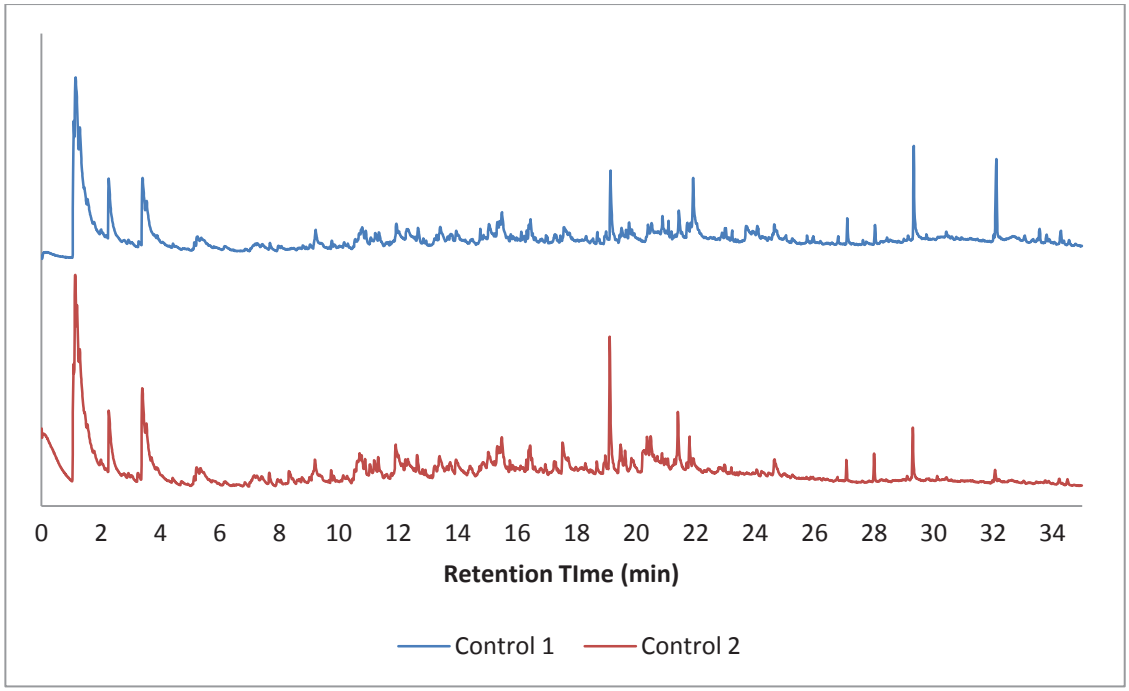

Figure 7-4. Py-GC-MS results of Loam Defleshed (Control) 1 and 2 after 6 months of burial

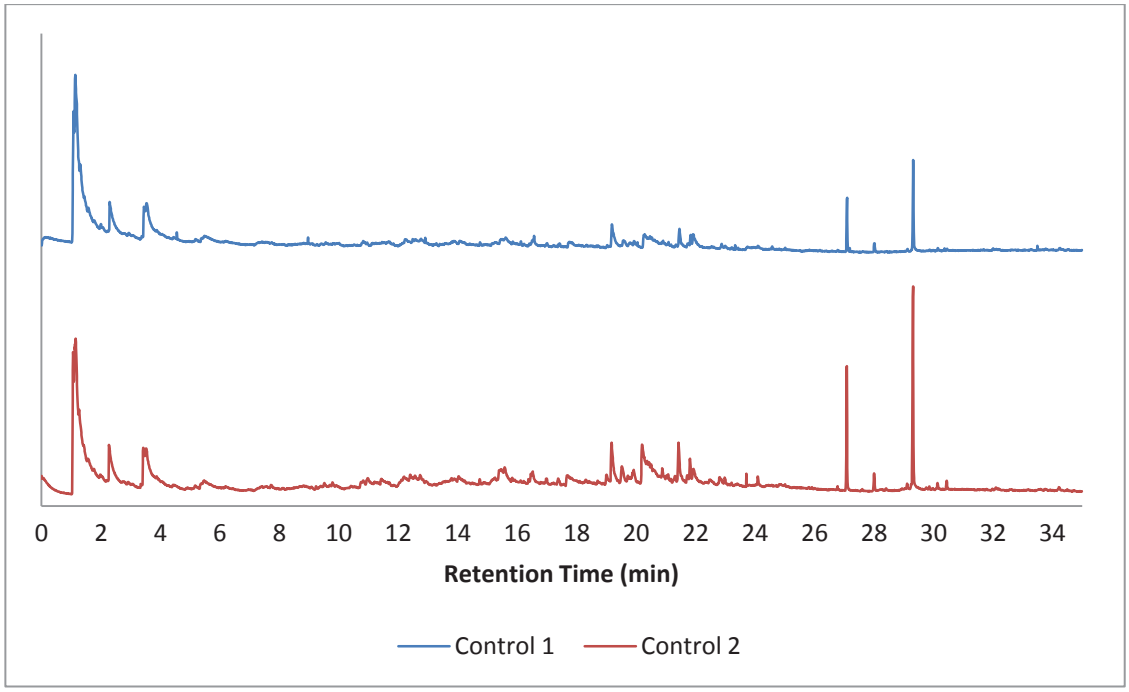

Figure 7-5. Py-GC-MS results of Loam Defleshed (Control) 1 and 2 after 8 months of burial

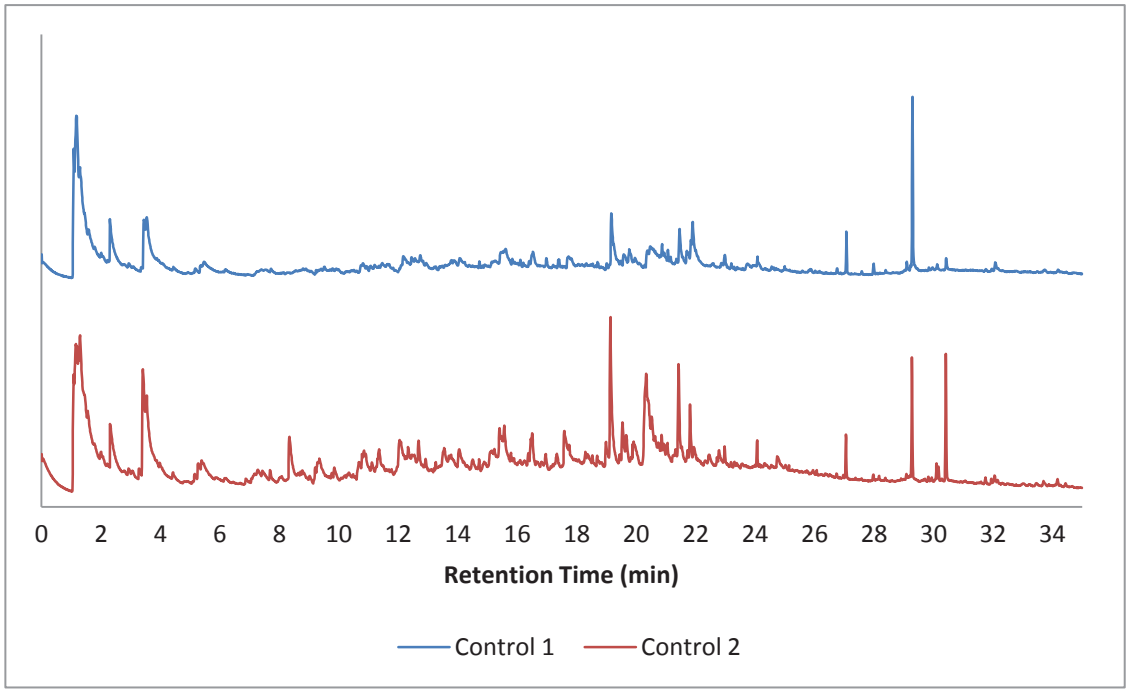

Figure 7-6. Py-GC-MS results of Loam Defleshed (Control) 1 and 2 after 10 months of burial

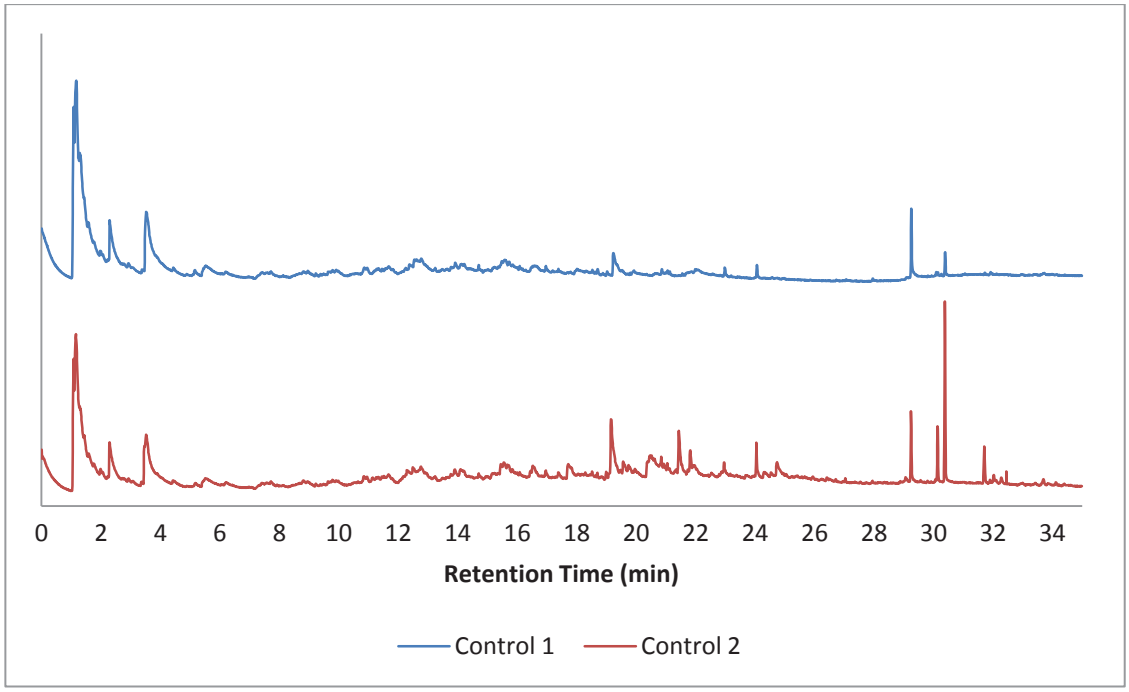

Figure 7-7. Py-GC-MS results of Loam Defleshed (Control) 1 and 2 after 12 months of burial

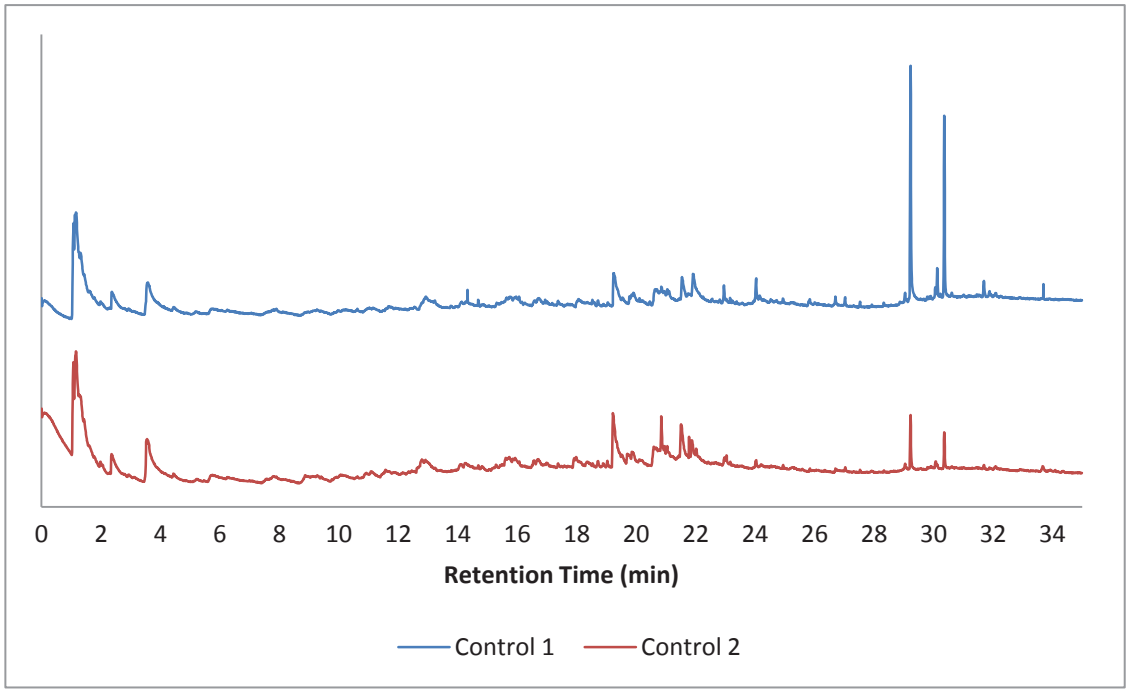

Figure 7-8. Py-GC-MS results of Loam Defleshed (Control) 1 and 2 after 15 months of burial

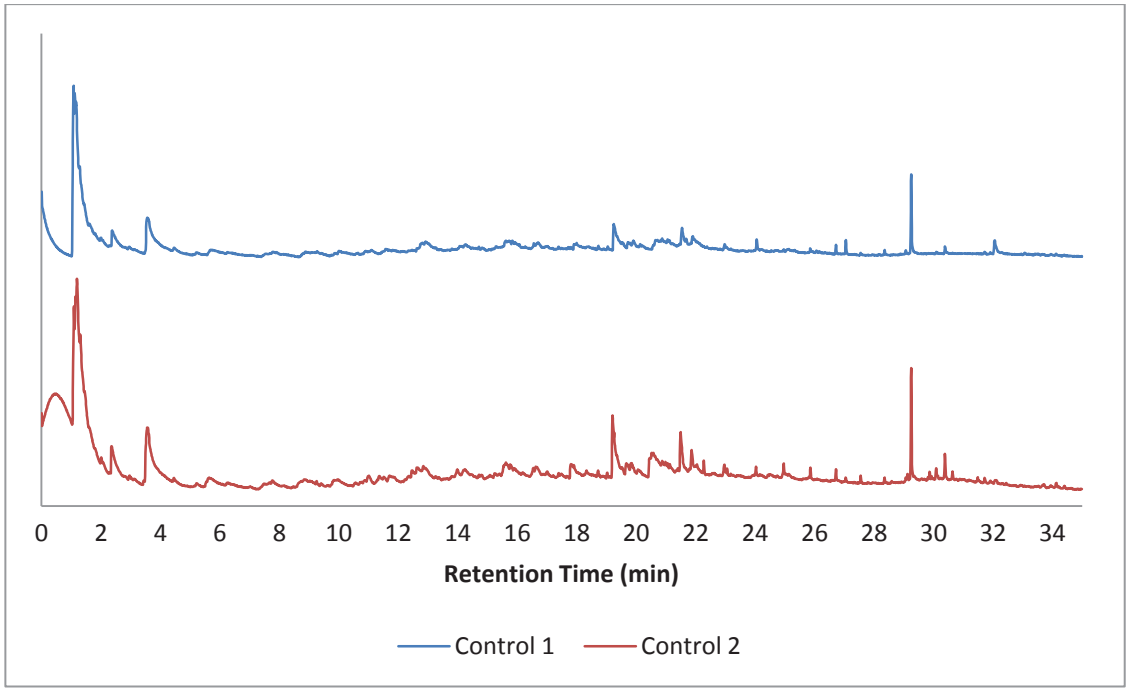

Figure 7-9. Py-GC-MS results of Loam Defleshed (Control) 1 and 2 after 18 months of burial

Appendix 2

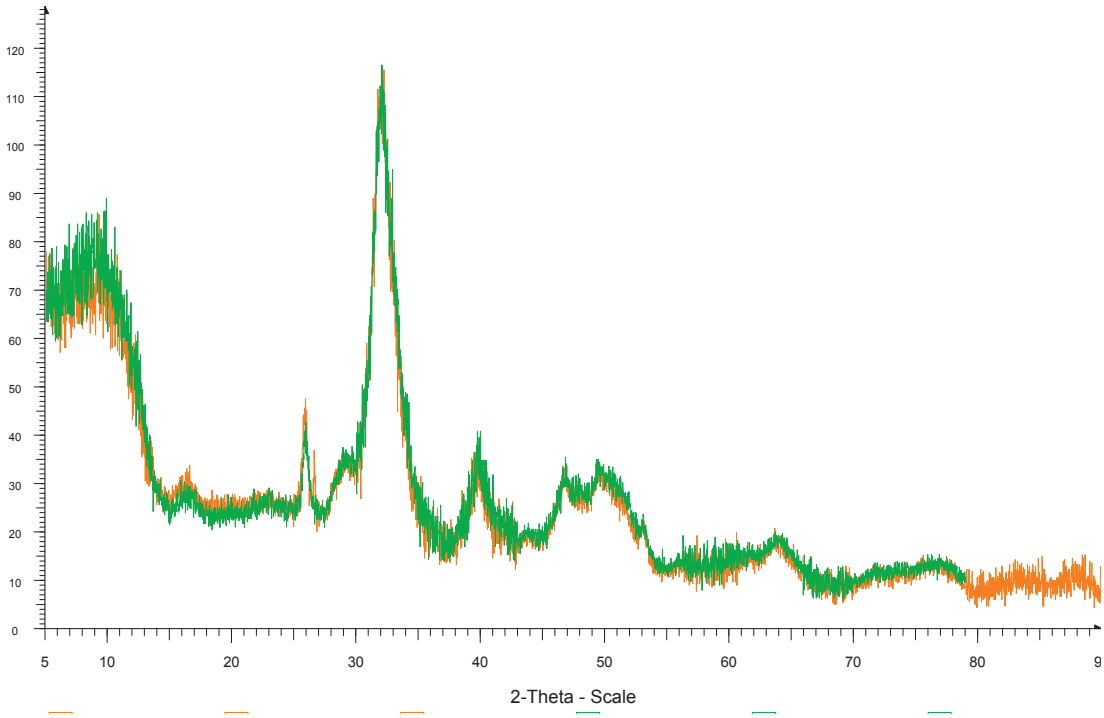

Figure 7-10. XRD results of Loam Defleshed 1 and 2 (orange and green) after 1 month of burial

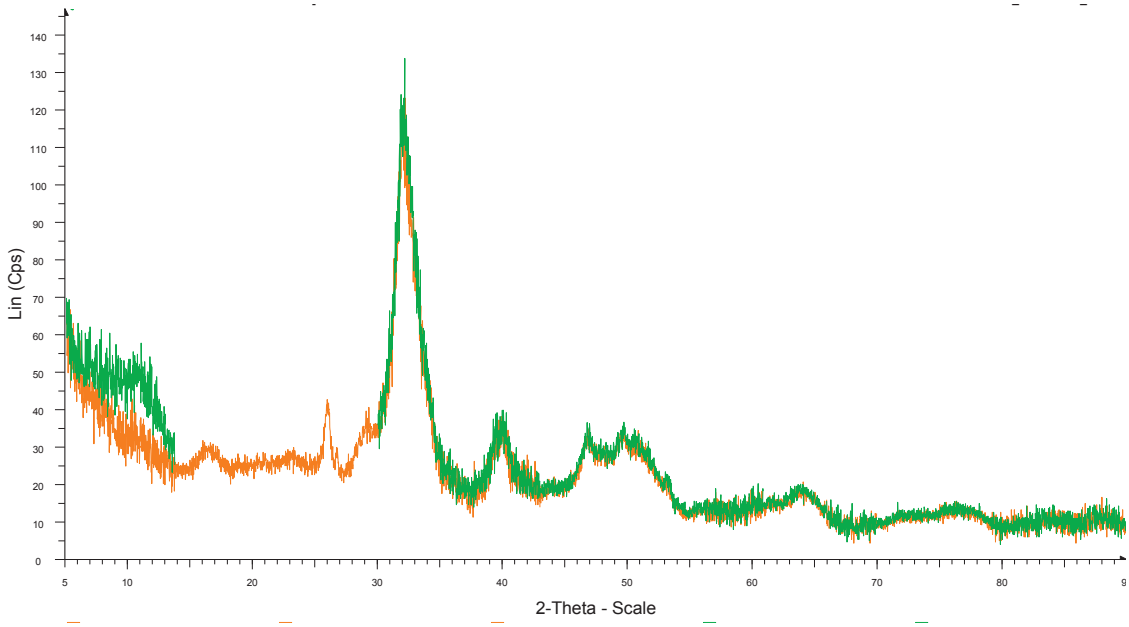

Figure 7-11. XRD results of Loam Defleshed 1 and 2 (orange and green) after 2 months of burial

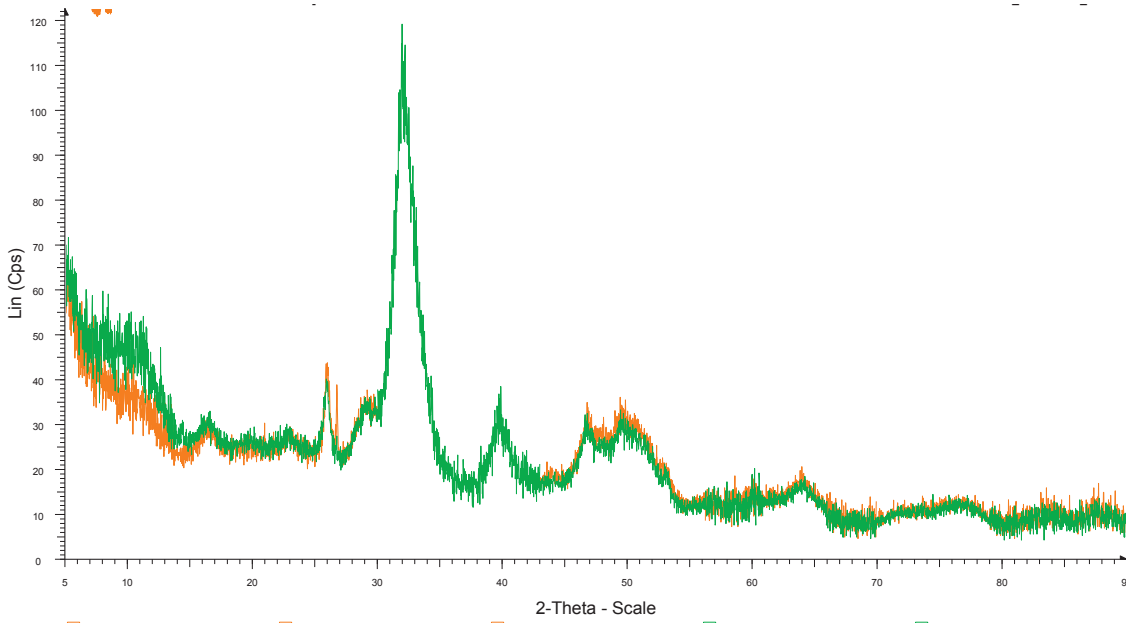

Figure 7-12. XRD results of Loam Defleshed 1 and 2 (orange and green) after 4 months of burial

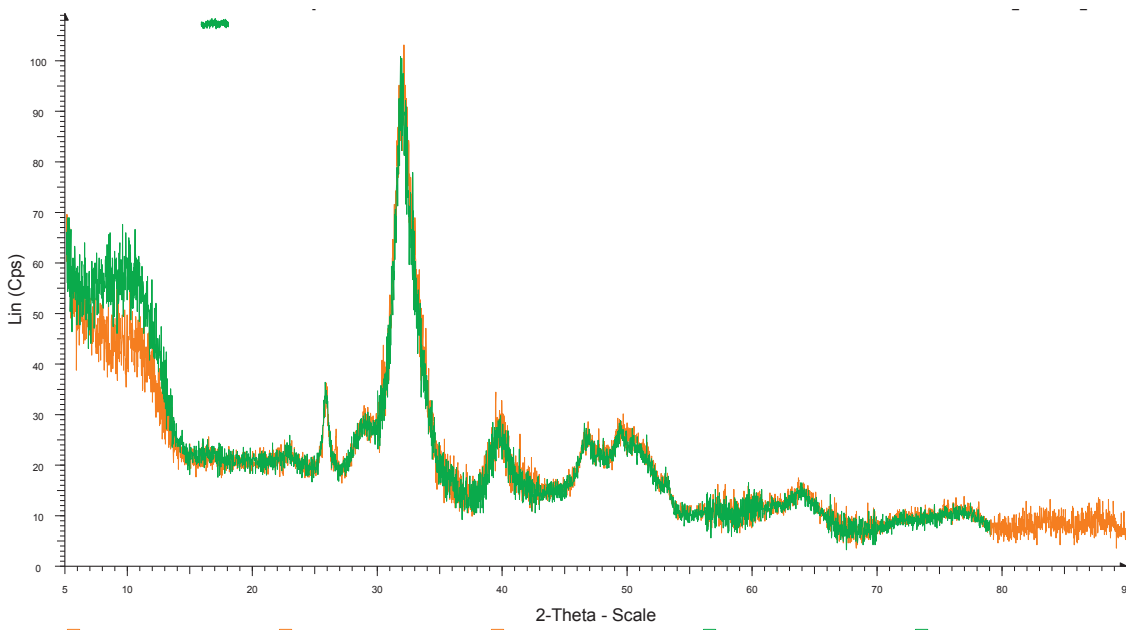

Figure 7-13. XRD results of Loam Defleshed 1 and 2 (orange and green) after 6 months of burial

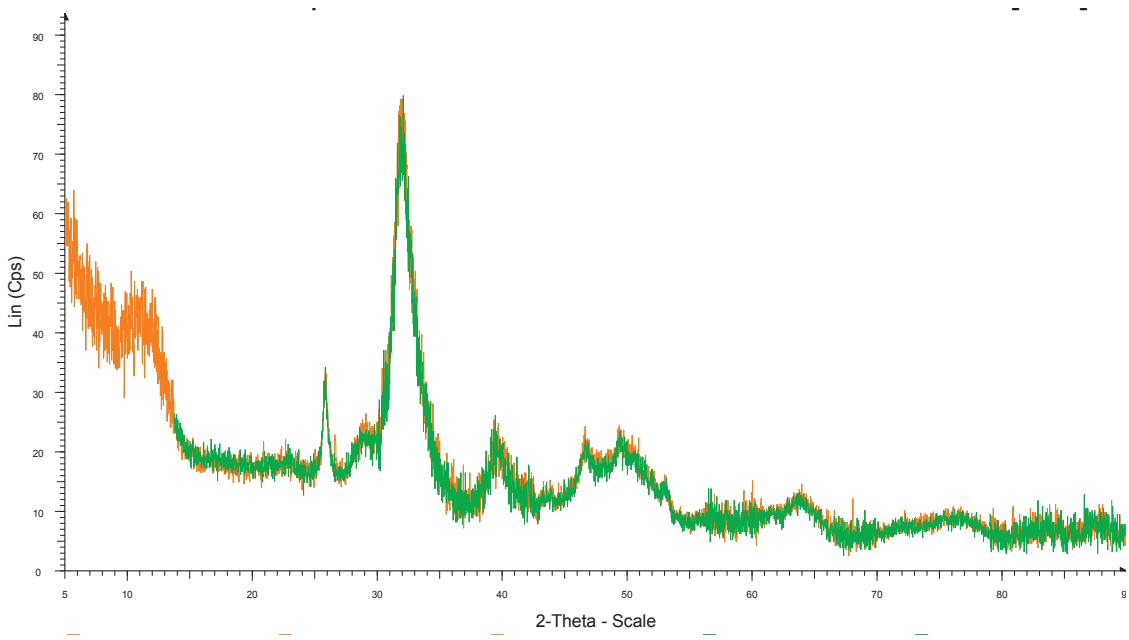

Figure 7-14. XRD results of Loam Defleshed 1 and 2 (orange and green) after 8 months of burial

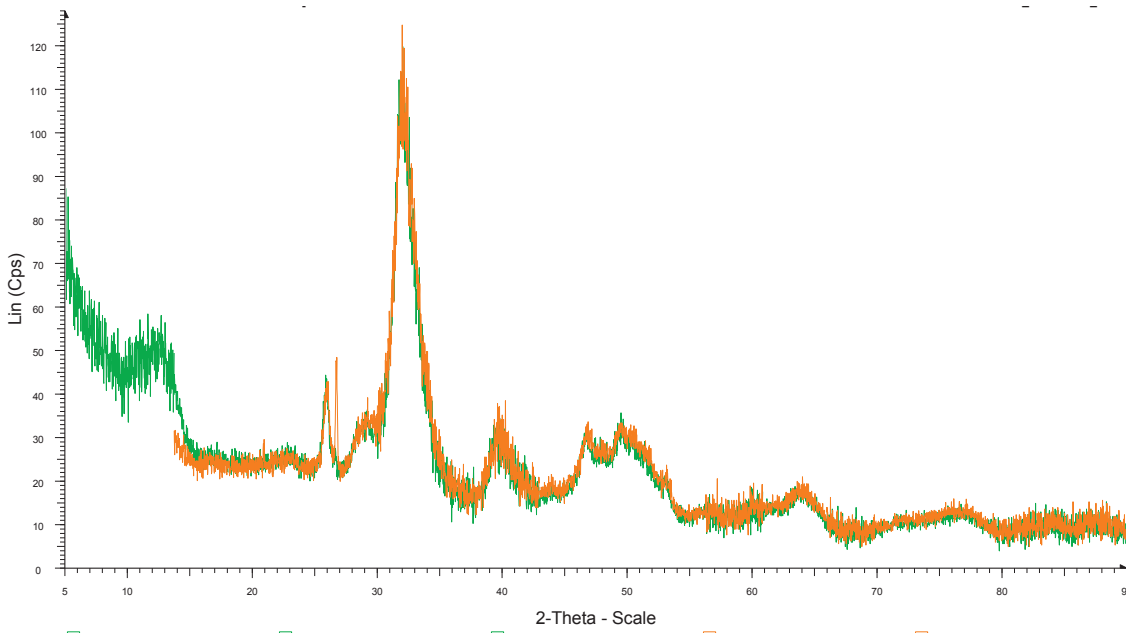

Figure 7-15. XRD results of Loam Defleshed 1 and 2 (orange and green) after 10 months of burial

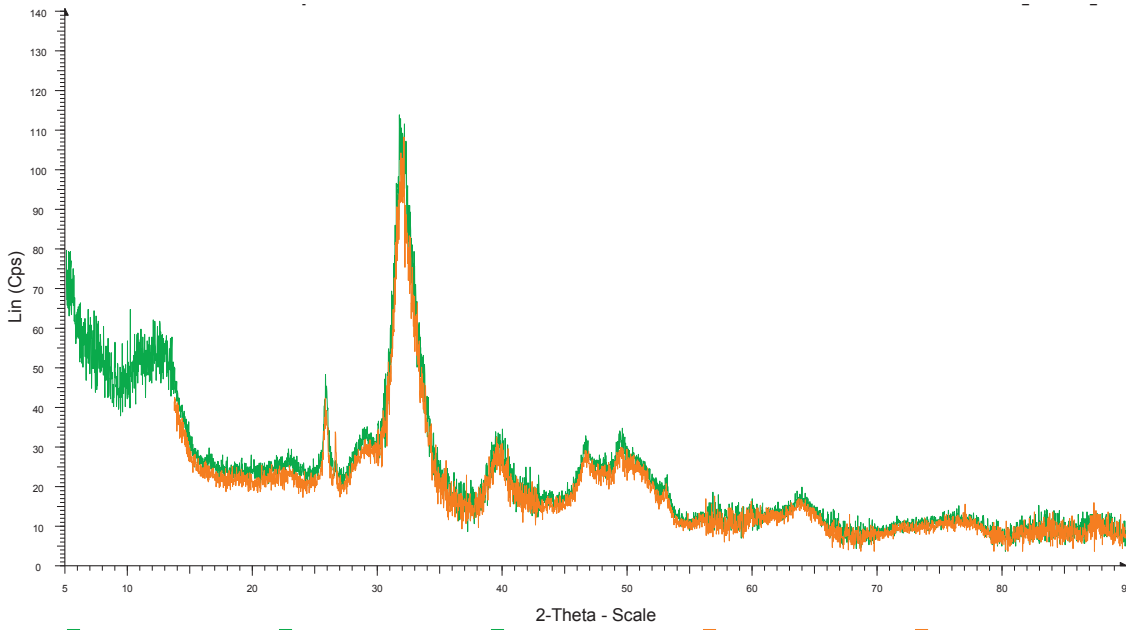

Figure 7-16. XRD results of Loam Defleshed 1 and 2 (orange and green) after 12 months of burial

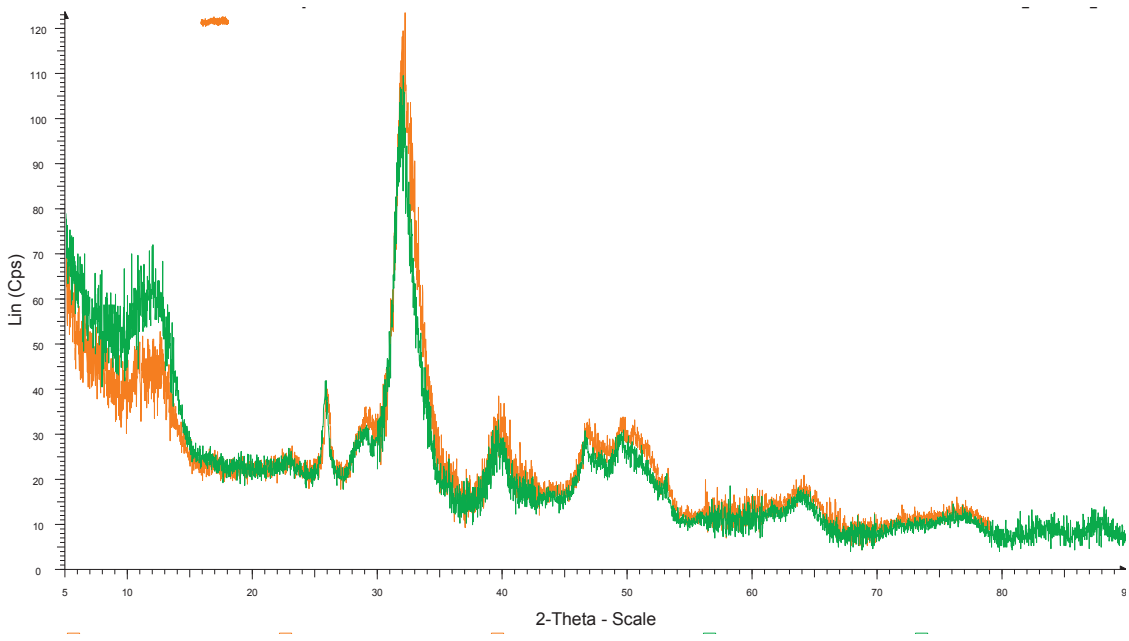

Figure 7-17. XRD results of Loam Defleshed 1 and 2 (orange and green) after 15 months of burial

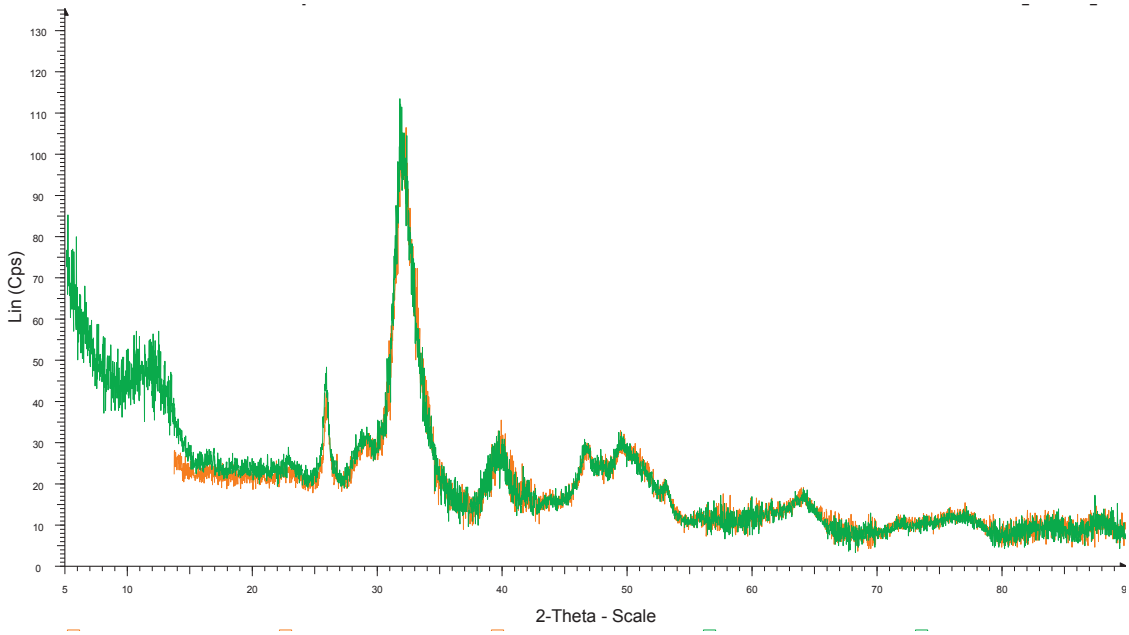

Figure 7-18. XRD results of Loam Defleshed 1 and 2 (orange and green) after 18 months of burial

Appendix 3

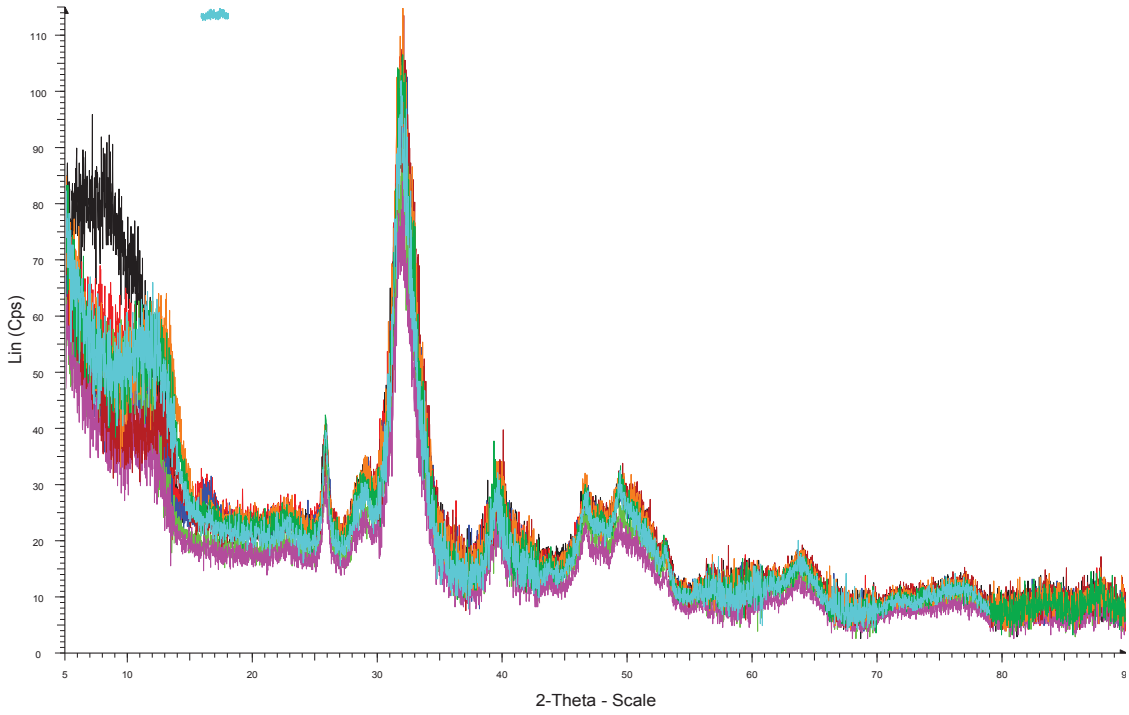

Figure 7-19. XRD results – Loam Fleshed 1 at all post-burial times

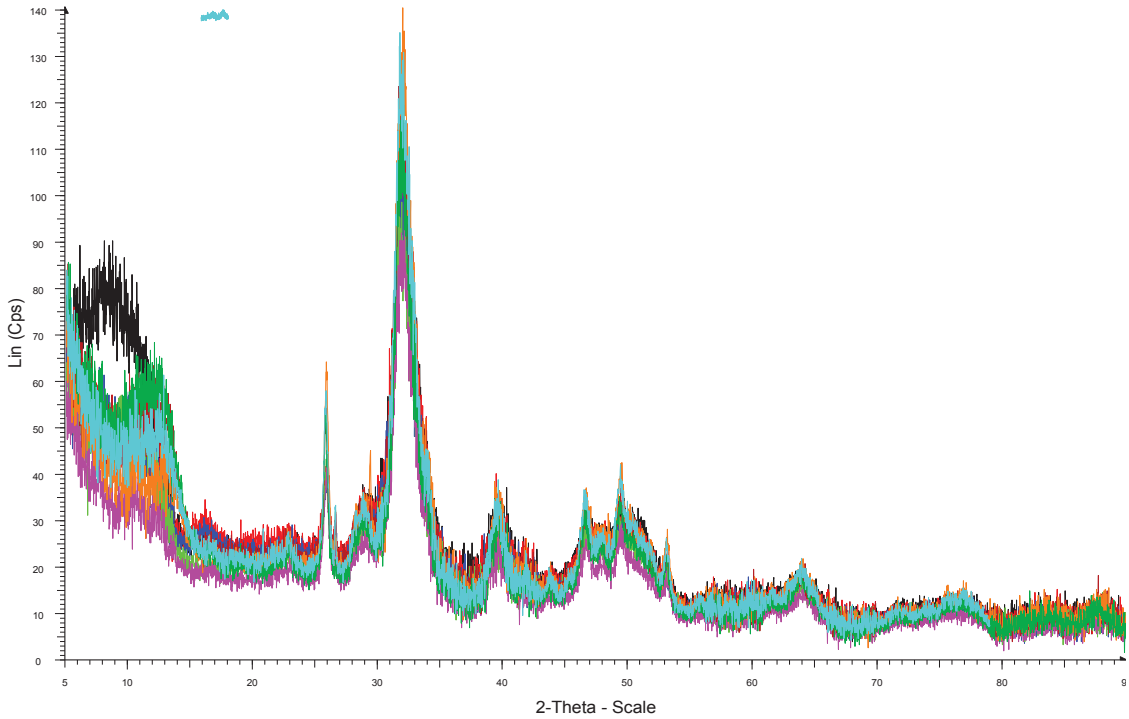

Figure 7-20. XRD results – Loam Acidic 1 at all post-burial times

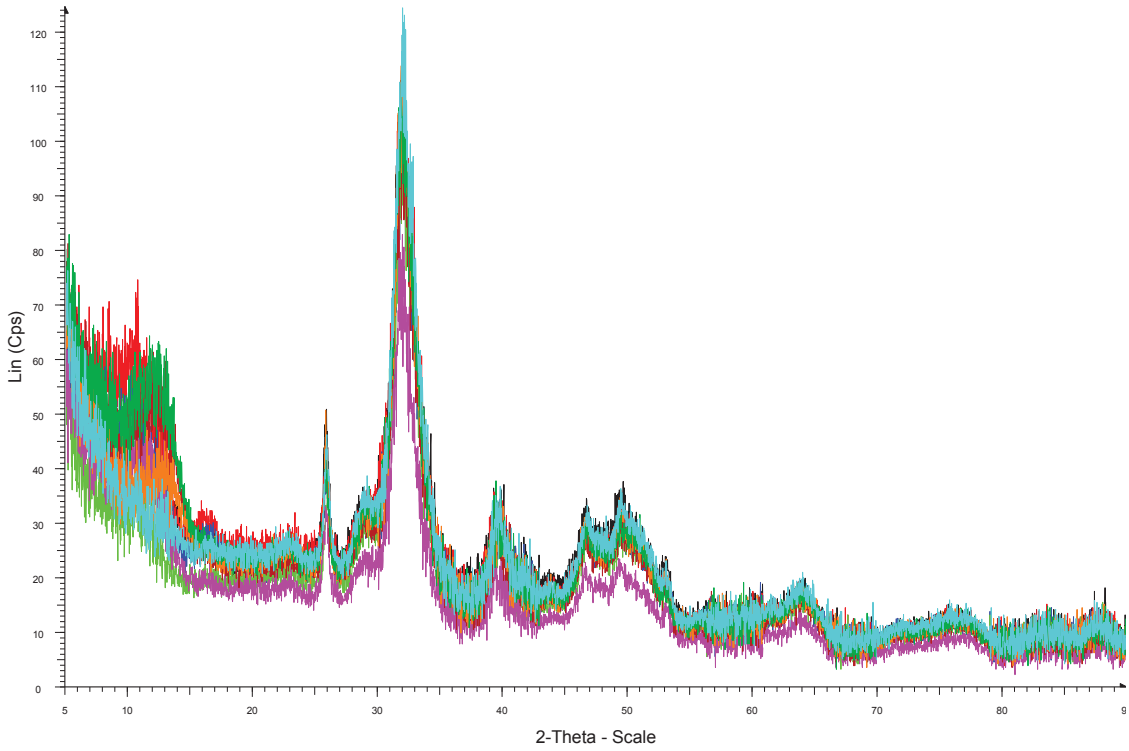

Figure 7-21. XRD results – Loam Basic 1 at all post-burial times

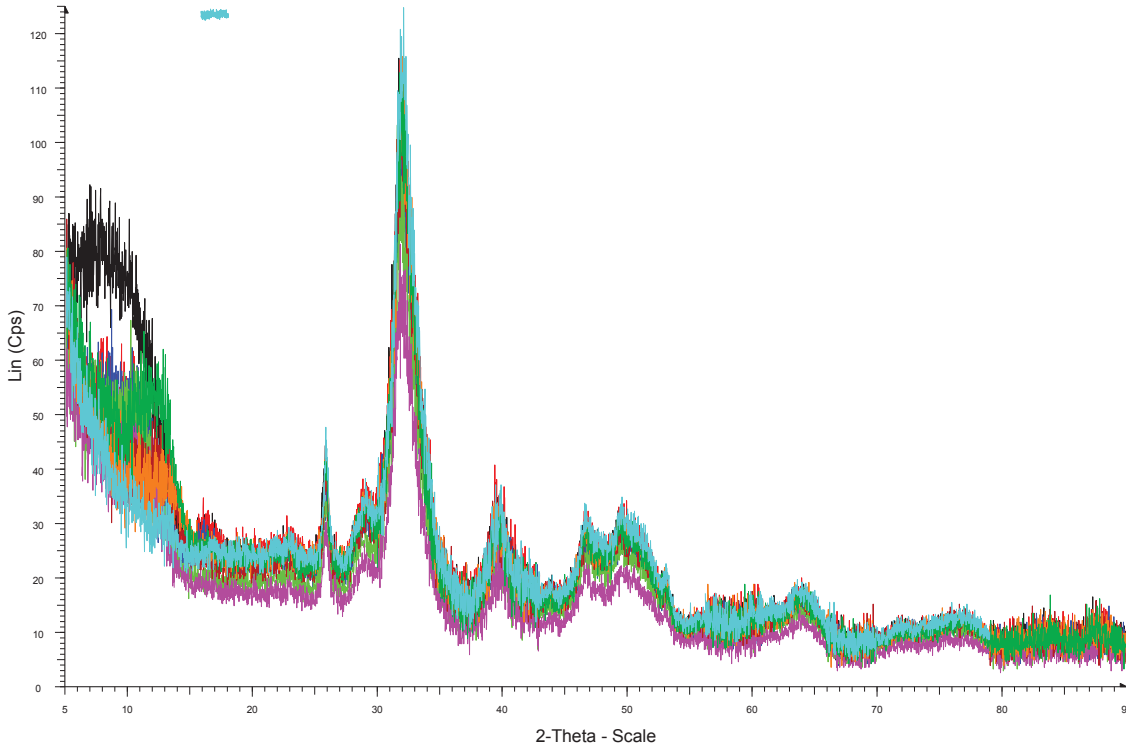

Figure 7-22. XRD results – Loam Wet 1 at all post-burial times

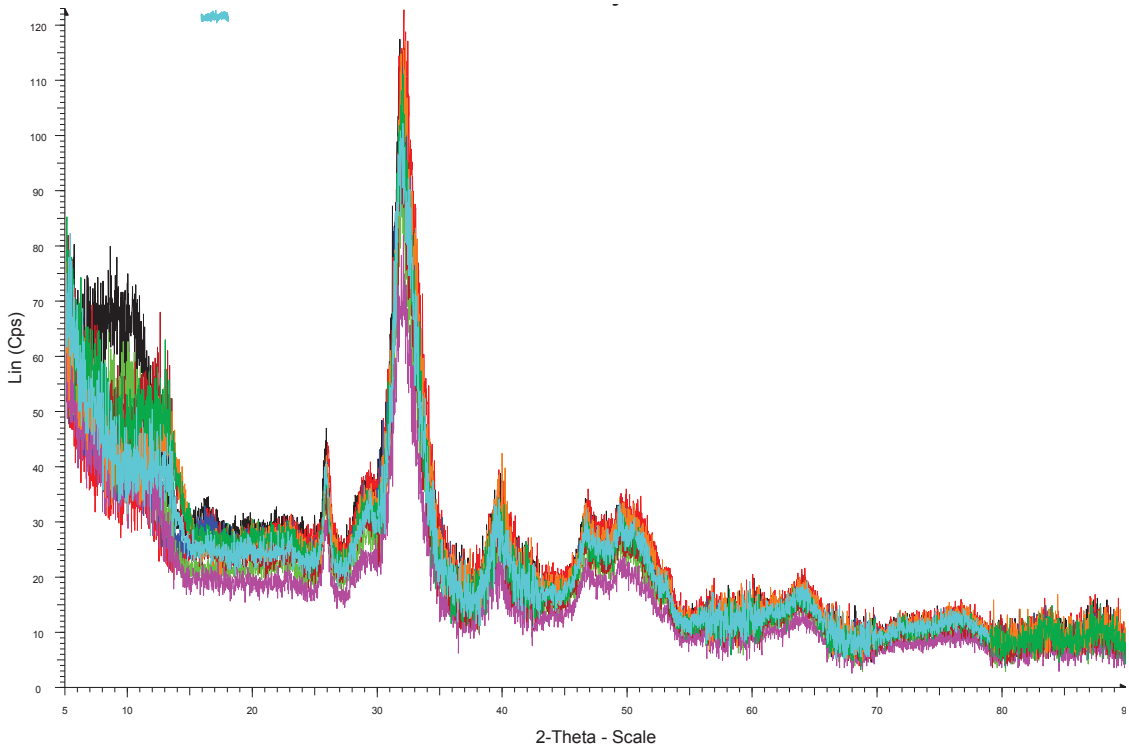

Figure 7-23. XRD results – Loam Dry 1 at all post-burial times

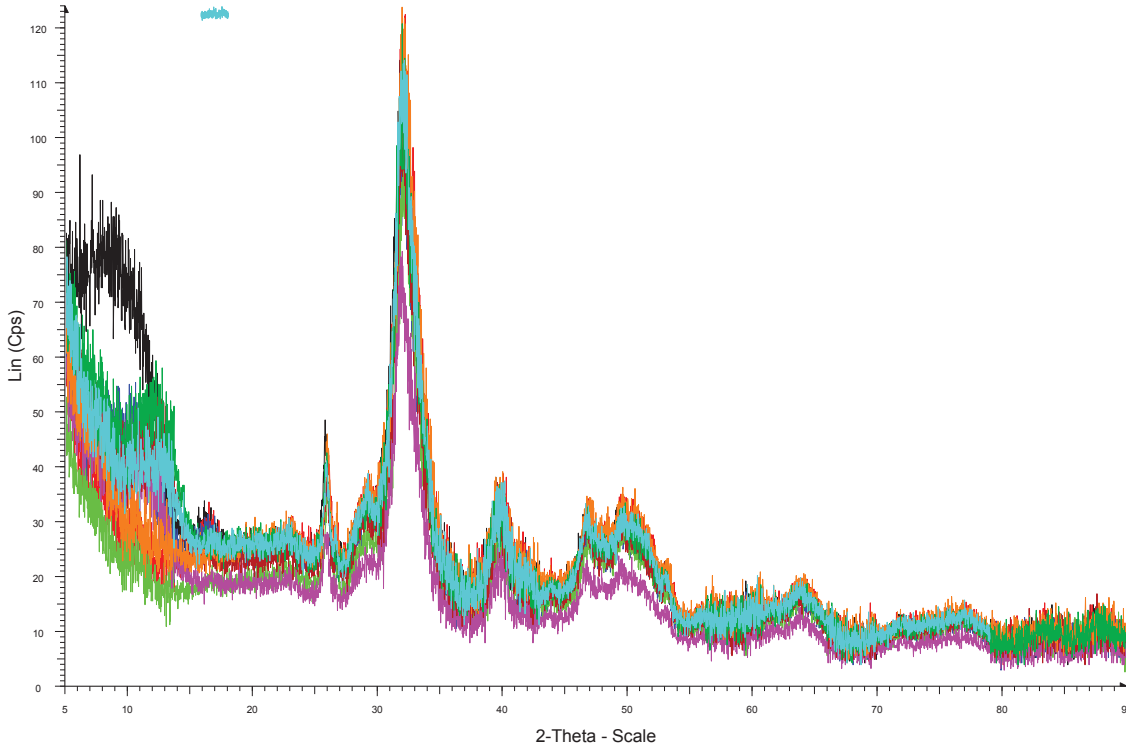

Figure 7-24. XRD results – Silt 1 at all post-burial times

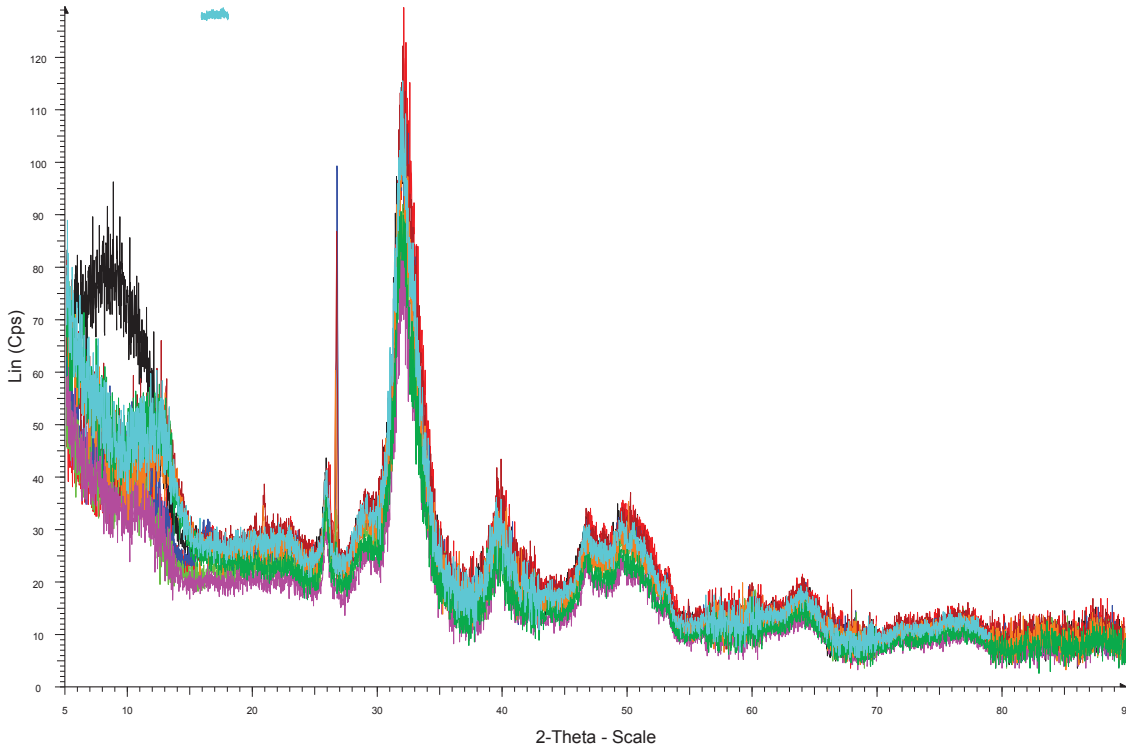

Figure 7-25. XRD results – Sand 1 at all post-burial times

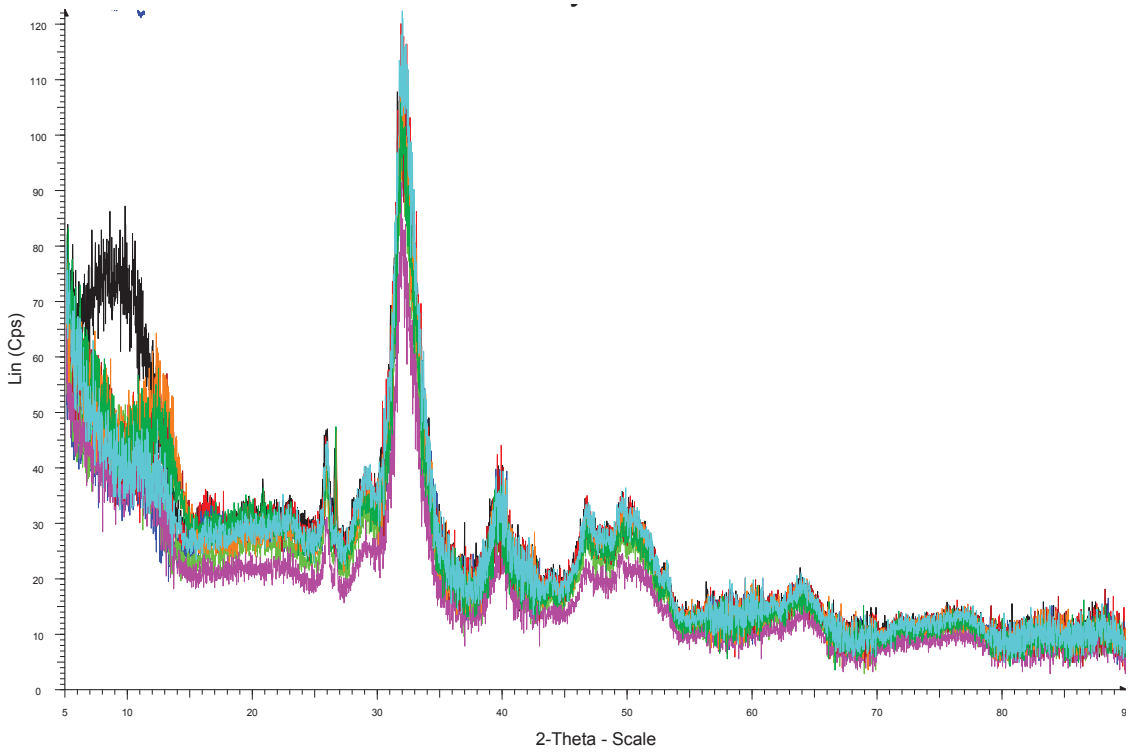

Figure 7-26. XRD results – Clay 1 at all post-burial times

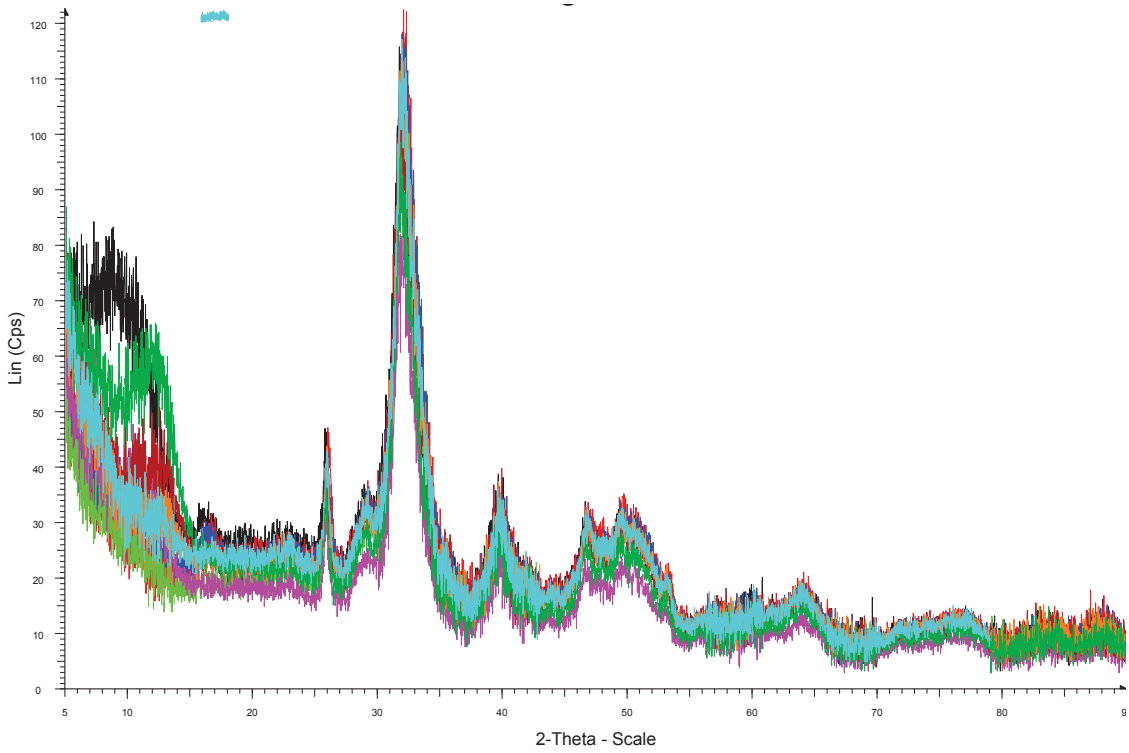

Figure 7-27. XRD results – Loam Degreased 1 at all post-burial times

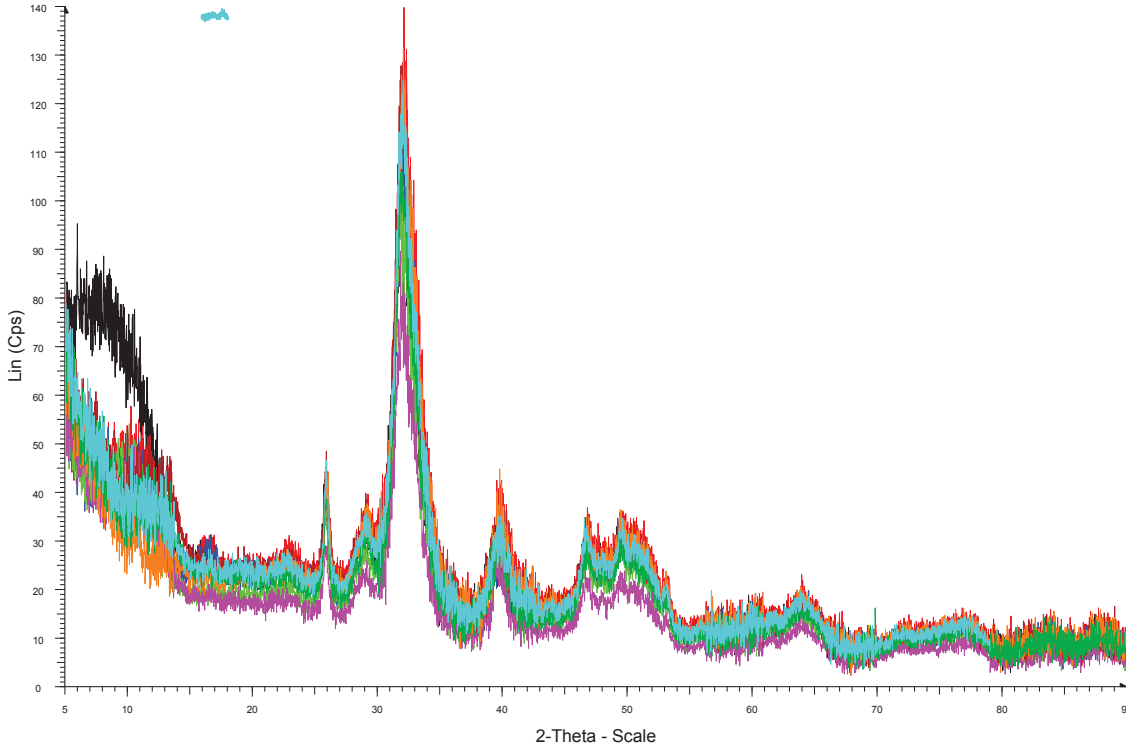

Figure 7-28. XRD results – Loam Boiled 1 at all post-burial times

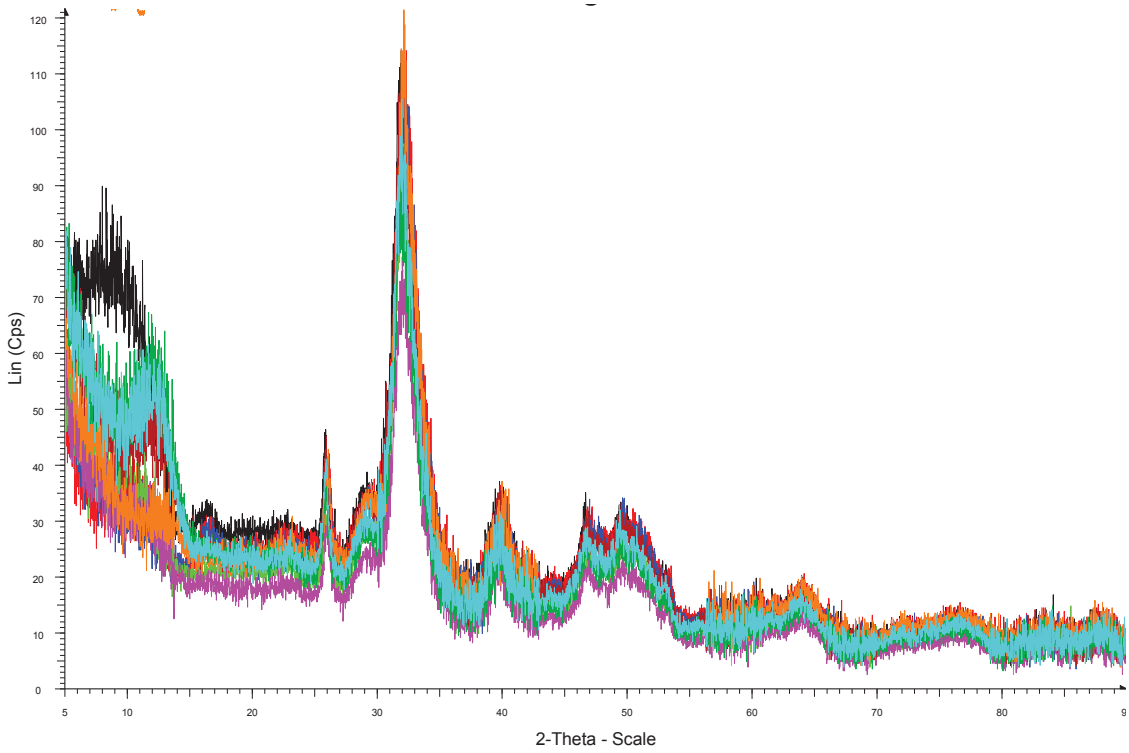

Figure 7-29. XRD results – Loam Refrigerator 1 at all post-burial times

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