

Cranfield University

Department of Applied Science, Security and Resilience

PhD Thesis

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**Development of New Tools for Forensic Analysis of DNA from
Compromised Bone**

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Abstract

Skeletal tissue is an invaluable source of Deoxyribonucleic Acid (DNA) for the generation of an individual specific profile, as it is often the only source of DNA available for identification. Like all forensic samples, skeletal tissue can be exposed to a variety of environmental insults, which can affect the potential of successfully generating a useable DNA profile. Excessive temperatures and unfavourable water conditions are two factors that can influence DNA degradation. There has been very little research into the amplification of DNA from bone compromised by such environmental factors. Studies have often lacked appropriate controls or were based on case reports where accurate details concerning exposure conditions would not be known.

To date, DNA-based identification from badly decomposed remains has often been reliant on the use of mitochondrial DNA (mtDNA), which exists within cells in much higher copy number than nuclear DNA (nDNA). Mitochondrial DNA is inherited through the maternal line; so all offspring from a particular female will share the same mtDNA. This reduces the specificity of identifications made using mtDNA, as matrilineal relatives cannot be distinguished from one another. Nuclear DNA profiling of such samples would greatly improve the reliability of identification.

Using both human and animal bone, this study focused upon characterising the physical and material properties of bone that have endured decaying processes of either water

submersion or heat treatment. Fourier Transform Infra-Red (FTIR) spectroscopy, X-Ray Diffraction (XRD) analysis, microhardness and colourimetry were used to report a comprehensive study into the effect these compromising factors had on collagen, mineral and the physical and morphological properties of bone. A number of properties correlated with the subsequent success or failure of nDNA amplification and produced a diagnostic tool that could predict the probability that nDNA analysis would be a viable option for identification purposes. This study has developed several diagnostic tools that would aid nDNA analysis from compromised bone. They would not only save time and money, but would increase the overall success rate of DNA profiling.

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Abbreviations

AAR	Aspartic acid racemisation
aDNA	Ancient DNA
APR	Amide to Phosphate Ratio
AUROC	Area under the receiver operator characteristic
BLASTn	Basic Local Alignment Search Tool
bHAP	Bio-apatite (Biological Calcium Hydroxyapatite)
CI	Crystallinity Index
CL	Coherence Length
DNA	Deoxyribonucleic acid
DSC	Differential Scanning Calorimetry
EBSD	Electron Backscattering Diffraction
EDTA	Ethylenediaminetetraacetic acid
FWHM	Full Width Half Maximum
FTIR	Fourier Transform Infrared
GC/MS	Gas chromatography-mass spectrometry
HAP	Calcium Hydroxyapatite
HPLC	High-Performance Liquid Chromatography
II	Identity Index
PCR	Polymerase Chain Reaction
PI	Parent/Child Index

MQD	Multivariate Quadratic Discriminant
mtDNA	mitochondrial DNA
ND	Neutron Diffraction
nDNA	nuclear DNA
RGB	Red, Green, Blue
SAXS	Small Angle X-ray Scattering
SEM	Scanning Electron Microscopy
SF	Splitting Factor
SNP	Single Nucleotide Polymorphism
STR	Short Tandem Repeats
TEM	Transmission Electron Microscopy
TGA	Thermogravimetric Analysis
VH	Vickers Hardness
WTC	World Trade Centre
VNTR	Variable nucleotide Tandem Repeats
XRD	X-Ray Diffraction

Chapter 1 Introduction

The identification of unknown individuals can take a number of routes. Relatives can visually identify the deceased through facial recognition, or from specific characteristics or features, such as tattoos. Visual identification may not always be possible or reliable, as the body may have become too disfigured or decomposed to be accurately recognized. If circumstances allow, fingerprints or dental records may be used. However, if no records exist, or the body has undergone extensive decomposition (i.e. teeth or skin are missing), identification attempts may require anthropological or DNA analysis in order to be successful. Forensic anthropologists are often employed to identify individuals from skeletal remains in a wide range of scenarios including individual cases, mass disasters and terror attacks. However, in some cases, where bones are fragmented or incomplete, the anthropologist's methodology of identification may also be unsuccessful. As a result DNA analysis may be the only viable means of identification.

Genetic profiling requires DNA that has not been excessively damaged or degraded. It is relatively straightforward to extract DNA from samples such as hair roots, blood and soft tissue belonging to recently deceased victims or from bodies that have been well preserved. If DNA has not undergone extensive degradation then full DNA profiles would normally be generated. However, in a forensic context, human remains are often compromised by external factors and soft tissue can be adversely affected by

decomposition and contamination. Even after soft tissue has decomposed, DNA within bone can still be obtained. The structural nature of bone provides a physical barrier that protects DNA within it from external factors such as bacterial activity, heat, water and soil pH that may be detrimental to DNA in other tissues. As a result, bone is considered an invaluable source of DNA for the generation of an individual's specific profile. However, DNA degradation in bone still occurs naturally after death, but can be accelerated when specimens are exposed to additional external factors, either in extreme or for prolonged periods. While the role of other environmental factors on DNA degradation should not be ignored, elevated temperature is considered one of the single most important factors that affect the degradation of DNA. An increase in temperature compromises DNA's integrity by promoting fragmentation, which reduces the average length of individual DNA molecules, and hence the maximum amplicon size that can be obtained using PCR. Consequently, the potential for DNA to be used in identification is reduced.

In a forensic context, fire is often used to cause death or to conceal evidence. Genocide, cultural rituals, house and car fires, either accidental or deliberate, and disasters, such as woodland fires, aeroplane crashes and terrorist attacks can all result in the cremation of human remains that require identification.

There are many different types of fires and these vary in temperature and duration. Biological fluids and tissues, which can normally provide DNA for identification purposes

easily, may burn away relatively quickly, rendering them useless for analysis. Nevertheless, until consumed, soft tissues can offer some protection to the underlying bone. However, if a fire continues, skeletal tissue will become exposed to high temperatures for excessive periods of time and DNA degradation will occur.

Another factor that has been problematic to DNA analysts are bodies that have been submerged in water for an extensive period of time. Individuals that have drowned or been deposited in water, deliberately or accidentally, may not be recovered for several months or even years. After such a long period, the bodies may have become skeletonised, leaving bone as the only source of DNA. DNA degradation will occur during this time and can be accelerated by high water temperatures and other unfavourable water conditions, making subsequent genetic profiling challenging.

When DNA has been extracted from burnt and submerged skeletal tissue, the product is often, particularly in the case of burnt samples, not suitable for identification. Both DNA quality and/or quantity can be insufficient for analysis. There is a distinct lack of controlled research, with much of the literature based on case studies, and many examples where the environmental conditions that samples were exposed to, such as temperature or submersion time, were unknown. With most reports of successful DNA analysis usually occurring from bone that had been exposed to lower temperatures or submerged in water for short periods of time, there is a great need to establish the effect of temperature and water submersion on DNA degradation.

In comparison to protocols for extracting DNA from soft tissue, extraction from skeletal tissue is relatively time consuming. Unlike other tissues of the human body, bone is hard and essentially impermeable due to its high mineral component. This means that not only is it difficult for chemical/enzymes to break it down, but that the surface area available for them to act upon is very low relative to the sample volume. Powdering or grinding the bone sample prior to extraction will help address the latter issue, although both can only be fully addressed by initially removing as much of the mineral component as possible; this is a process that can take a significant number of days. The problem of this time/labour requirement can be compounded when dealing with skeletal tissue that has been subjected to adverse environmental factors in that many samples may still fail to yield DNA from which a useable DNA profile can be generated. Bone samples requiring DNA analysis that have been compromised via burning (heat treated) or prolonged submersion in water are relatively common. They can occur in individual cases or in cases where hundreds or even thousands of samples must be identified. Natural disasters such as the Asian tsunami (2004) or the World Trade Centre (WTC) terrorist attacks (2001) have produced thousands of skeletal tissue samples that have needed to be identified. When hundreds of bone samples are processed for DNA analysis, and it is unknown whether the extracted DNA will be viable for profiling, a lot of time and money can be wasted.

This project intended to use a number of existing techniques that measure different properties of bone and develop them into a DNA diagnostic tool that can remove non-

viable samples prior to commencing the process of DNA analysis. Bone is predominantly made from hydroxyapatite (mineral) and collagen (organic), which together with water; act to form a cohesive material that provides multiple functions within the human body. Numerous studies have been published describing the changes that occur to the material and mechanical properties of bone that has been heat-treated. Unlike heat treatment however, there is very little research on the effect of water submersion on the properties of bone.

This project aims to establish whether changes in the material and mechanical properties of bone that occur during heat treatment and submersion in water can be correlated with an increased or decreased success rate of subsequent DNA profiling.

Any strong correlations could then be developed into a diagnostic tool that would be able to reliably predict whether a useable DNA profile could be successfully obtained from compromised (heat treated or submerged in water) skeletal tissue, prior to commencing the DNA extraction process. By producing an effective diagnostic tool that is also cheap, quick and easy to use, both time and money could be saved.

The tool is designed to increase the overall success rate of DNA profiling from compromised bone samples by eliminating non-viable samples. The diagnostic tool will not only benefit the organisation assigned to profiling, but also the law enforcement agency and relatives that may be waiting for a positive identification.

1.1 Aims and Objectives

There are a number of objectives to this project, but ultimately the project is intended to produce a diagnostic tool that can be used in forensic DNA analysis.

The Aim:

To develop a diagnostic tool that can predict, prior to DNA extraction, whether or not a useable DNA profile is likely to be obtained from a compromised (heat treated or submerged in water) bone sample.

The Objectives;

1. To determine whether the pattern of DNA degradation due to heat treatment or submersion in water is a function of duration, temperature or environment.
2. To determine whether heat treatment or submersion in water has any discernable effects on the mechanical properties of bone.
3. To determine whether heat treatment or submersion in water has any discernable effects on the material properties, including the mineral and organic constituents, of bone.
4. To determine whether the changes that occur in bone as a result of heat treatment or water submersion can be correlated with DNA degradation and DNA profiling.

Chapter 2 Literature Review

This chapter seeks to establish an understanding of the aim and the different aspects involved in completing the four objectives outlined in this study (section 1.1). By reviewing the present literature, this chapter will assess the different properties of bone that are available for analysis and evaluate the advantages and disadvantages of different analytical techniques that could be used to fulfil the requirements of this study.

2.1 Biology of Bone

Bone is an essential component of the musculoskeletal system and has multiple functions, both as a tissue and an organ. Bone protects and supports soft tissue, acts as anchors for ligaments, tendons and muscles, and as rigid levers that together with muscles provide the body's movement. Bones also provide facilities for fat storage, red blood cell production as well as supporting reservoirs for essential elements such as calcium (Martin and Burr, 1989).

Bone is not a solid entity but is made of a hierarchy of structures (Figure 2.1), from the macrostructure of cortical and cancellous bone through to the sub-nanostructure of collagen and mineral molecules (Rho et al., 1998).

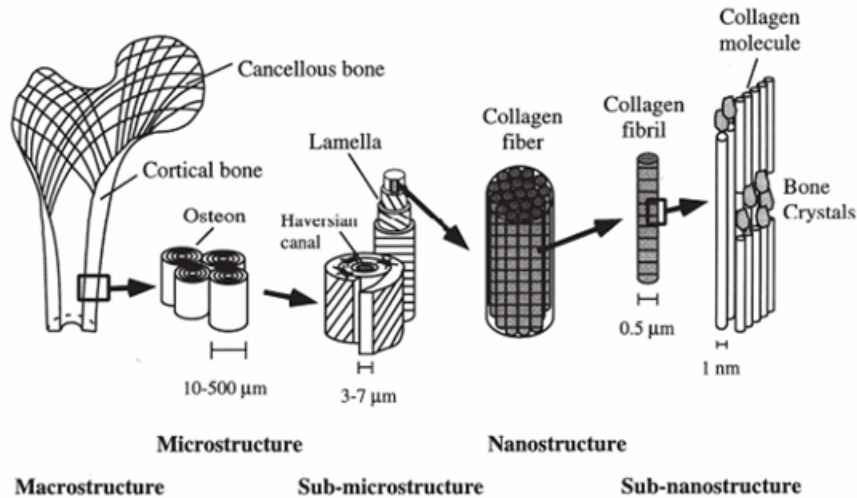


Figure 2.1: Hierarchical structure of bone. Taken from Rho *et al.*, 1998

2.1.1 Macrostructure of Bone

At the gross level, adult bone takes two forms: compact and cancellous. Compact bone is the dense bone found in the walls of bone shafts and on the external bone surfaces. Cancellous, or trabecular bone, is named after the spicules (trabeculae) that form it. In comparison to compact bone, cancellous bone is more spongy and porous with a lightweight honeycomb structure. Although compact and cancellous bones differ in porosity, they are identical in terms of molecular and cellular compositions (Martin and Burr, 1989).

2.1.2 Microstructure of Bone

The microstructures of compact and cancellous bone are very different. The microstructure of compact bone is that of organised osteons or Haversian systems (Martin and Burr, 1989). Haversian systems consist of concentric rings of lamellae that form Haversian canals. The canals allow blood vessels to pass through the bone providing nutrients and removing waste products from cells. Between the layers of lamellae, there are cavities known as lacunae that contain osteocyte cells. Forming a network between the lacunae there are interconnecting pathways known as canaliculi. In contrast, cancellous bone does not contain Haversian systems. Instead, cancellous bone consists of an irregular lattice of thin columns of bone called trabeculae.

2.1.3 Nanostructure of Bone

Bone is a complex, natural composite made from mainly organic materials, minerals and water (Table 2.1), and hence is considered to be a tri-phase system (Yan et al., 2007). The organic phase is predominately type I collagen and makes up the matrix structure of bone. The collagen matrix is permeated and reinforced by the mineral phase, which together with the third component, water, provide the distinctive material characteristics of bone (Burr, 2002).

Table 2.1: Composition of Bone (Yan et al., 2007)

Component of Bone	% of Bone
Inorganic Mineral	50-70
Organic Matrix	20-40
Cellular Elements	5-10
Lipids	3

2.1.4 Summary: The composition of bone

Understanding the composition of each bone phase (organic, mineral and water) is a fundamental aspect of determining the appropriate bone markers to be studied. Although bone is made from a complex hierarchy of structures, its molecular and cellular biology is relatively simple when compared to other tissues. Collagen (organic) and calcium hydroxyapatite (inorganic) are the two main components of bone and as described in this chapter, both appear to show potential as surrogate biomarkers for DNA integrity.

2.2 Collagen

Type I collagen is a member of the collagen protein family responsible for maintaining the structural integrity of vertebrates and other multi-cellular organisms (Viguet-Carrin et al., 2006). It is considered the most abundant family of proteins in humans (Di Lullo et al., 2002). Type I collagen is found in tendons, ligaments, skin and bone (Gineyts et al., 2000, Berthier and Blaineau, 1997, Eghbali and Weber, 1990) as well as organs such as the lungs and liver (Mundy et al., 1981).

Collagen comprises about 90% of bone's organic phase (Knott and Bailey, 1998, Oxlund et al., 1996). The other 10% is made from non-collagenous proteins including osteocalcin and osteonectin. Type I is the predominant form, although there are relatively low levels of type III and V and X collagen present (Bailey et al., 1993, Batge et al., 1992). Type I collagen fibrils are derived from three polypeptide chains (two 1α and one 2α) that form a triple helix structure (Rho et al., 1998). The collagen fibrils are then arranged in parallel to form a lamella matrix. Interfibrillar cross-links form between fibrils which stabilise the matrix (Oxlund et al., 1996). The fibrils are arranged with a quarter-staggered end-overlap that results in the formation of gaps (Figure 2.1). Calcium hydroxyapatite crystals are then deposited into the gaps, resulting in mineralization of the collagen fibres.

There are a number of techniques that can be used to investigate collagen within bone. However, many require complex and/or extensive protocols, making them unsuitable for this study. Table 2.2 summarises the advantages and disadvantages, with regard to this project, of a range of these analytical techniques.

2.2.1 Fourier Transform Infra-red (FTIR) spectroscopy

Infrared (IR) spectroscopy is an analytical technique commonly used to provide structural information on molecular compounds including organic molecules.

Table 2.2: The analytical techniques available to investigate collagen integrity in bone

Method	Description	Advantages	Disadvantages	Reference
Western Blots	Immunochemical assay: Detection of specific proteins, including collagen. Proteins are transferred to a membrane and are detected using antibodies specific to the target protein.	<ul style="list-style-type: none"> • Effective method to detect and characterize proteins. 	<ul style="list-style-type: none"> • Time consuming; demineralisation (20 h) and precipitation in NaCl (20 h) required. • Non-specificity; antibodies can bind to non-target proteins. • Destructive to sample 	(Babraj et al., 2002)
Immuno-histochemistry	Immunochemical assay: Detection of specific proteins, including collagen. Proteins can be tagged with fluorescent antibodies and detected using a microscope. Coupled with flow cytometry, tagged proteins can be quantified.	<ul style="list-style-type: none"> • Relatively inexpensive • Effective method to detect and characterise proteins. • Can determine the localisation of proteins within cells. 	<ul style="list-style-type: none"> • Bone samples undergo a series of fixation protocols; time consuming ~24 hrs. • Non-specificity; antibodies can bind to non-target proteins. • Destructive to sample 	(Sassi et al., 2000) (Shibata et al., 1997) (Nerlich et al., 1993)
HPLC	Chromatographic assay: Detection of hydroxyproline in collagen. Has been used to investigate amino acid racemization in collagen.	<ul style="list-style-type: none"> • Amino acid specific • Quantifies amino acids 	<ul style="list-style-type: none"> • Requires demineralisation of bone taking a number of hours or days. • Measures total amino acid content, irrespective of whether peptides are intact. 	(Collins et al., 2009) (Cloos et al., 2003) (Poinar et al., 1997) (Poinar et al., 1996) (Rydziel and Canalis, 1989)
SEM/TEM	High resolution microscopic imagery using an electron source.	<ul style="list-style-type: none"> • Can visually inspect the integrity of collagen fibrils. • Can be quantified. 	<ul style="list-style-type: none"> • Time consuming; bone fixation, dehydration, infiltration and embedding in resin required • Destructive to sample. 	(Koon et al., 2003) (Richter, 1986)
FTIR Spectroscopy	Infrared analysis; A molecule is radiated with IR. IR absorption is measured across a range of wavelengths (See 2.2.1).	<ul style="list-style-type: none"> • Simple bone preparation: Powdered samples are pressed into a loading KBr Disc. • Scans are very quick ~2-4 min. 	<ul style="list-style-type: none"> • Semi quantitative • Destructive to sample 	(Surovell and Stiner, 2000)
GC/MS	Combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample.	<ul style="list-style-type: none"> • Amino acid specific • Quick analysis of samples 	<ul style="list-style-type: none"> • Bone preparation is complex and time consuming. • Relatively expensive 	(Poinar and Stankiewicz, 1999)

An infrared spectrophotometer contains an infrared radiation beam source, an interferometer, a specimen stage and an IR detector (Figure 2.2). Converting the raw IR data into a spectrum requires a Fourier Transform (FT), hence creating the combined technique known as FTIR spectroscopy.

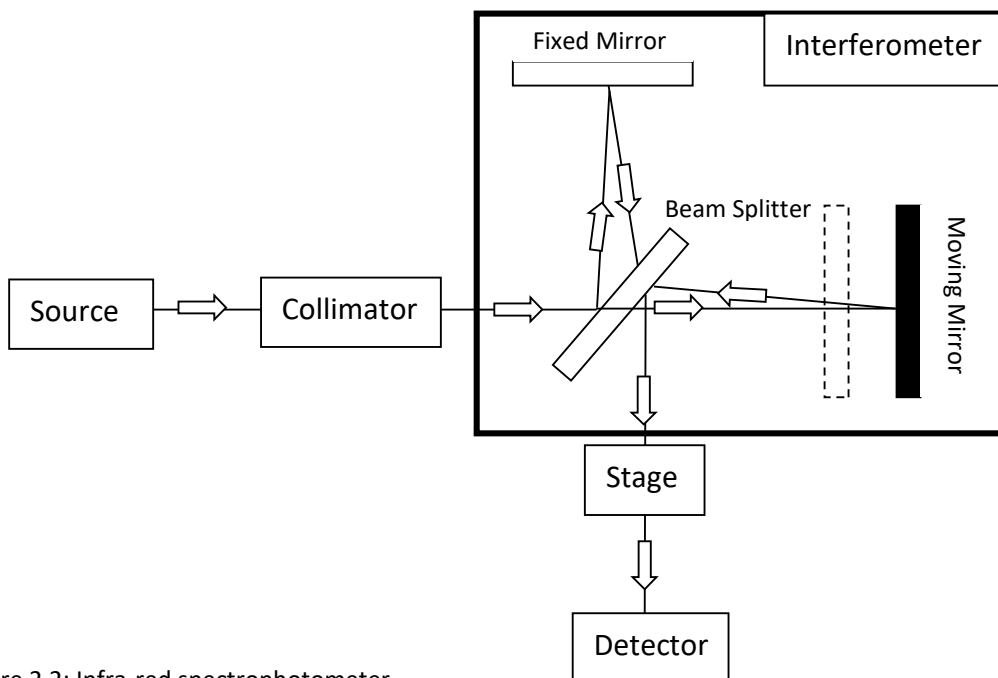
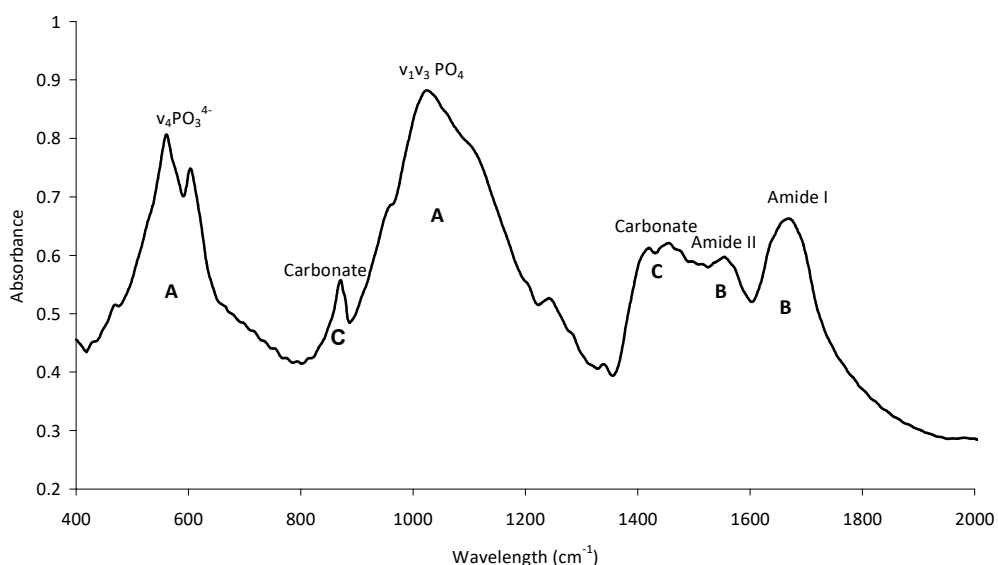


Figure 2.2: Infra-red spectrophotometer

FTIR spectroscopy utilizes the fact that bonds and groups of bonds in a molecule can vibrate at specific frequencies, known as resonant frequencies. When energy, in the form of infrared radiation, passes through a molecule, a portion of that energy, whose frequency matches the resonant energy of bond or groups of bonds within the molecule, is absorbed. IR absorption is measured across a range of wavelengths and the data analysed. The resulting FTIR spectrum is then compared with known signatures of identified materials in a FTIR library.

FTIR spectroscopy has been used to investigate the properties of mineralized and non-mineralized connective tissue, including bone (Boskey and Camacho, 2007). The FTIR spectrum of bone is similar across a range species including human, pig and dog. The amide bands, I and II represent vibrational modes of (C=O) and (C-N) bonds that derive from collagen polypeptides. The amide bands are found at wavelengths between 1500 and 2000 cm^{-1} . The mineral phase can be investigated through the phosphate domains and vibrational modes (ν) $\nu_1\nu_3\text{PO}_4^{3-}$ (at 900-1200 cm^{-1}) and $\nu_4\text{PO}_4^{3-}$ (at 565-605 cm^{-1}). Carbonate can also be detected in two regions between 800-900 cm^{-1} and 1350 -1500 cm^{-1} (Figure 2.3).



Figure

2.3: The FTIR spectrum of fresh bovine bone. Regions of interest, A: phosphate, B: organic phase, C: carbonate.

2.2.2 Using FTIR spectroscopy to investigate collagen integrity

The secondary structure of a polypeptide is defined by the patterns of hydrogen bonds between backbone amide and carboxyl groups. FTIR spectroscopy can be used to investigate the secondary structure of collagen by way of the vibrational transitions of chemical bonds belonging to several amide groups. Consequently, FTIR spectroscopy has been used to analyse several types of collagen in a number of connective tissues including cartilage (Camacho et al., 2001, Bonnier et al., 2008), aortic tissue (Bonnier et al., 2008) and skeletal muscle (Petibois et al., 2006).

FTIR spectroscopy has also been utilized to investigate the integrity of collagen that is found in bone. The amide I band, a vibrational transition due to C=O bond stretch within the peptide carbonyls, has been extensively used to investigate collagen content and maturity. The integration ratio between the amide I ($1600-1700\text{ cm}^{-1}$) and the $\nu_1\nu_3\text{PO}_4^{3-}$ ($900-1200\text{ cm}^{-1}$) band (Figure 2.3), known as the mineral to matrix ratio, has been correlated with fractional ash weight (Boskey and Mendelsohn, 2005) and can be used to evaluate the relative collagen content of a given sample. Maturity of collagen molecules can also be determined through analysis of the amide I peak. Both parameters have been used to defined age-related changes in healthy and osteoporotic bone (Paschalis et al., 1996, Boskey et al., 2005) and characterize the drug-related changes in osteoporosis using animal models such as, dog (Monier-Faugere et al., 1999), rat (Barbier et al., 1997) and monkey (Gadeleta et al., 2000).

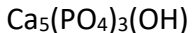
2.2.3 Summary: Collagen, a potential marker for DNA recovery

Although there are a number of analytical techniques that can be used to investigate the collagen content of bone, many are time consuming, and in some cases, carrying out a full DNA analysis would take less time. FTIR spectroscopy has been identified as a possible candidate to measure the collagen content of compromised bone in this study and be developed as a diagnostic tool to aid DNA analysis. The FTIR spectrum of a sample can be generated in minutes and bone preparation prior to analysis is minimal, when compared to the other techniques.

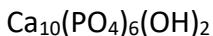
2.3 Calcium hydroxyapatite

Calcium hydroxyapatite (HAP) is a structural prototype for the mineral component of bone and teeth and is also a component of pathological calcifications such as urinary calculi (kidney, bladder and urether stones). HAP consists of calcium ions (Ca^{2+}), phosphate ions (PO_4^{3-}) and hydroxyl ions (OH^-) (Equation 2.1) and can be described as hexagonal in structure. Each crystal of hydroxyapatite is made from a repetitive array of unit cells, consisting of two general formula units of ions that are in specific positions, relative to one another (Elliott, 1973, Harries et al., 1988, Termine, 1972, Mkukuma et al., 2004).

Equation 2.1: General chemical formula of HAP



Equation 2.2: Chemical formula of cell unit



The equations given above describe HAP having assumed stoichiometric composition and a crystal lattice structure devoid of defects and impurities. HAP has a very stable structure as a result of the closely packed arrangement of phosphate ions and is able to tolerate many defects that cause structural disorder and lattice strain within the crystal.

Calcium hydroxyapatite found in biological materials, also known as bio-apatite (bHAP), is described as poorly crystalline with many impurities. The crystal's lattices are deficient in calcium and hydroxyl ions and can contain substitutions including carbonate, sodium, magnesium, potassium, fluoride and chloride ions.

There are a number of techniques that can be used to investigate the composition and structural properties of bHAP in bone. Table 2.3 describes the techniques and their potential advantages and disadvantages when applied to this work. Bone HAP can be examined visually with the use of Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). SEM can be coupled with Electron Backscatter Diffraction (EBSD) that can be used to determine the molecular structure of crystals, while both can be coupled with energy dispersive X-ray spectroscopy (EDS) to investigate the molecular

Table 2.3: The analytical techniques available to investigate the integrity of bone bHAP

Method	Description	Advantage	Disadvantage	Reference
TEM	High-resolution microscopic imagery using an electron source. An image is formed from detecting the electrons transmitted through the specimen.	<ul style="list-style-type: none"> • Can be used to investigate the geometry, size and orientation of apatite crystals. • Can be coupled with EDS. 	<ul style="list-style-type: none"> • Does not provide information regarding the constituents of the apatite. • Bone preparation is complex, requiring fixation, dehydration, infiltration and embedding in resin and is time consuming. • Chemical fixation and staining can affect mineral content and distribution. • Destructive to sample. 	(Carter et al., 2002) (Rubin et al., 2003)
SEM	High-resolution microscopic imagery using an electron source. An image is formed from detecting the interaction of the electrons transmitted through the specimen.	<ul style="list-style-type: none"> • Can be used to investigate the geometry, size and orientation of apatite crystals. • Can be coupled with EDS and EBSD. 	<ul style="list-style-type: none"> • Does not provide information regarding the constituents of the apatite. • Bone preparation is time consuming, requires a sputter coat. • Destructive to sample. 	(Matsuura et al., 2009) (Holden et al., 1995a) (Holden et al., 1995b)
SAXS	Small angle, between 3° and 10°, X ray scattering. Diffracted X-rays from the sample are detected and can be used to determine a number of properties of the molecule.	<ul style="list-style-type: none"> • Can be used to investigate crystal size, shape and orientation. • Non-destructive to sample. • Minimal bone preparation. • Can use whole or powdered bone. 	<ul style="list-style-type: none"> • Time consuming; scans can take up to 9 hours. 	(Hiller et al., 2003) (Wess et al., 2002) (Hiller et al., 2004)
XRD	Wide angle X ray scattering. Diffracted X-rays from the sample are detected and can be used to determine a number of properties of the molecule.	<ul style="list-style-type: none"> • Can be used to investigate crystal size, shape, structure and orientation. • Non-destructive to sample. • Minimal bone preparation. • Can use whole or powdered bone. • Small sample size. 	<ul style="list-style-type: none"> • Scans can take 40-120 mins 	(Piga et al., 2009) (Piga et al., 2008) (Enzo et al., 2007) (Rogers and Daniels, 2002)
ND	Neutron scattering. Diffracted neutrons from the sample are detected and can be used to determine a number of properties of the molecule.	<ul style="list-style-type: none"> • Can be used to investigate crystal size, shape, structure and orientation. • Non-destructive to sample. • Minimal bone preparation. 	<ul style="list-style-type: none"> • Large amount of sample required. • Limited availability of neutron diffractors. 	(Bacon et al., 1984)
FTIR Spectroscopy	Infrared analysis; IR radiation is passed through a sample. Molecule absorption is detected. (See 2.2.1)	<ul style="list-style-type: none"> • Can be used to measure the composition and structure of crystals. • Minimum bone preparation. • Less than 5 mg sample required. 	<ul style="list-style-type: none"> • Analysis can be complex; composition of the $\nu_1\nu_3\text{PO}_4$ domain requires resolution enhancement methods. • Destructive to sample. 	(Lebon et al., 2008)

composition of the crystals. Using microscopy requires an initial preparation that is relatively time consuming. The structure and composition of bHAP can also be investigated through small (SAXS) or wide (XRD) angle X-ray diffraction and neutron diffraction (ND). Such techniques require minimum sample preparation, although the availability of a neutron source and time to scan the sample using SAXS and XRD may be a limiting factor.

XRD and FTIR spectroscopy are two analytical techniques that are commonly reported together when investigating the microstructure of bone bHAP (Figure 2.4). Both can provide compositional and structural information of the mineral. Moreover, both techniques require very little sample preparation and bHAP can be analysed in a relatively short period of time.

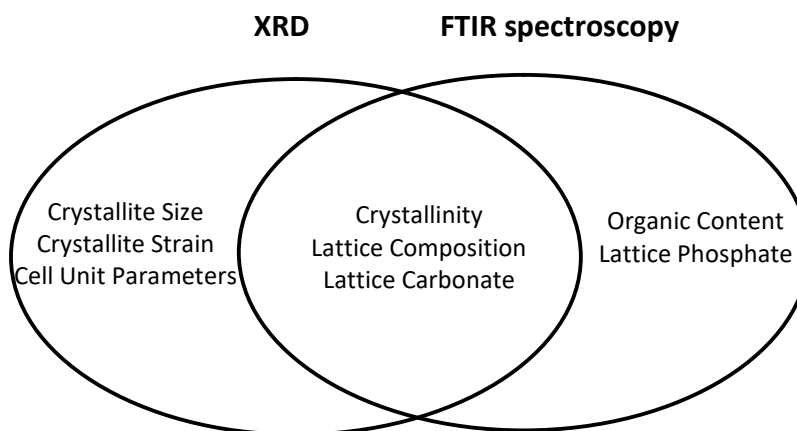


Figure 2.4: Diagrammatic visualization of the individual and overlapping information that can be collected regarding the properties of bone using XRD and FTIR spectroscopy.

2.3.1 Using X-ray Diffraction (XRD) to investigate bone bHAP integrity

X-ray diffraction is an analytical technique commonly used to investigate the compositional and structural integrity of crystalline materials such as bHAP in bone. X-ray diffraction is a result of elastic scattering of X-rays by interaction with the periodic array of electron density (surrounding the nuclei or ions). X-ray diffraction is described by Bragg's law, see equation 2.3. Crystalline materials are made up of atoms that form a series of parallel planes. The planes exist in a number of different orientations. Each parallel plane is separated from one another 'd-spacing'. X-ray wavelengths (λ), with the same direction of propagation, are scattered or 'reflected' from the lattice at an angle theta (θ), the angle of incidence parallel to lattice planes of the crystal (Figure 2.5). Diffraction of the X-rays occurs at angles of θ when the spacing between the planes (d-spacing) satisfies the Bragg's law equation (Cullity, 1956).

Equation 2.3:

$$\lambda = 2d \sin\theta$$

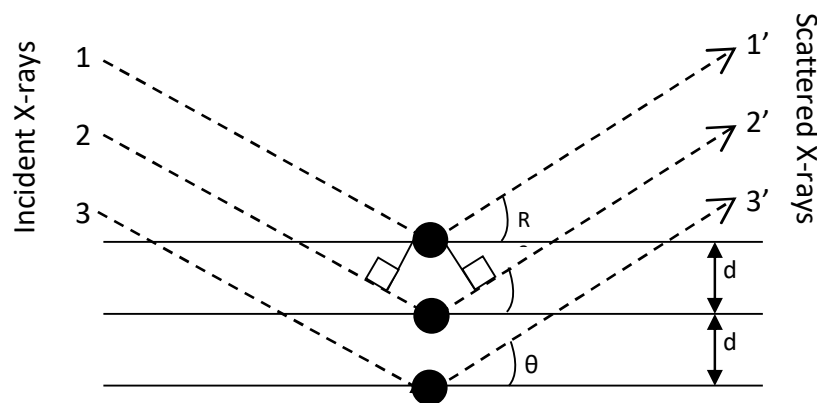


Figure 2.5: X-ray diffraction

Crystalline materials with different compositions and lattice structures differ with regards to the number and orientation of inter-planar spacing or d-spacings. As a result the angles (θ) of X-ray diffraction will vary from material to material and can therefore be used to study a material's composition and structure.

Bone bHAP is a poorly crystalline material. The diffraction peaks that are obtained from fresh bone samples are broad, causing them to overlap with each other. However, the composition and lattice parameters including coherence length, crystallite size/strain and cell unit parameters a/c can be measured and are described below (Table 2.4). Moreover, the bHAP parameters have also been studied in heated bone (Enzo et al., 2007, Etok et al., 2007, Hiller et al., 2003, Piga et al., 2008, Piga et al., 2009, Rogers and Daniels, 2002) and as discussed below (2.8.1.3) have the potential to become a diagnostic tool in DNA analysis from heated bone.

Table 2.4: The bHAP parameters that can be measured by XRD

Parameter	Description
Coherence Length	Measures the crystallinity of the lattice in a given plane. Crystallinity is a function of size and strain.
Crystallite Size	Measures the length of the lattice in a given plane.
Crystallite Strain	Measures the disorder of the lattice.
'a' and 'c' axis	Measures the cell unit size. Cell units are square in shape, only 'a' and 'c' are measured; 'b' is the same length as 'a'.

2.3.2 Using FTIR spectroscopy to investigate crystallinity of bone bHAP

FTIR spectroscopy can be used to investigate the structural integrity and composition of bHAP. A method developed by Weiner and Bar-Yosef (1990) has been extensively used to study the crystallinity of bHAP. The crystallinity is measured using the degree of separation of the two asymmetric bending (a mode of vibration) phosphate bands $\nu_1\nu_3\text{PO}_4^{3-}$ ($900\text{--}1200\text{ cm}^{-1}$) and $\nu_4\text{PO}_4^{3-}$ ($565\text{--}605\text{ cm}^{-1}$) and is known as the splitting factor (SF) (Figure 2.5.). High crystallinity is associated with high SF.

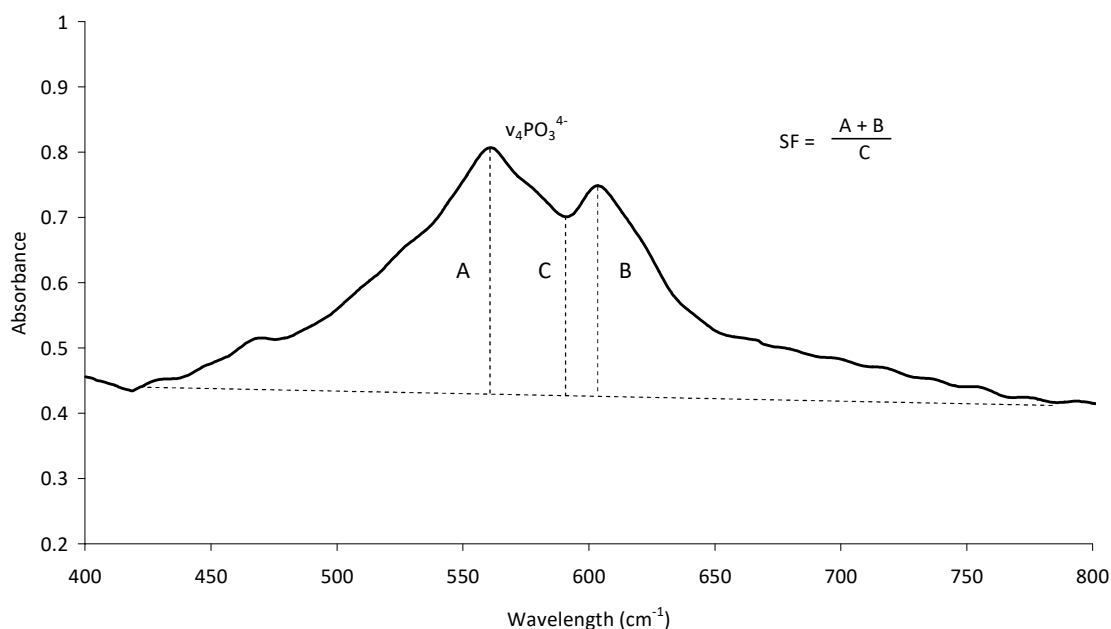


Figure 2.6: A FTIR spectrum of fresh bovine bone between wavelengths 400 and 800 cm^{-1} . The Splitting Factor is calculated by dividing the sum of the height of peaks A and B, by C, the height of the valley in-between.

SF has become an important analytical measurement to investigate the structural integrity of bHAP in skeletal tissue during diagenesis (Reiche et al., 2002), drug

treatment for osteoporosis (Pienkowski et al., 1997) and heat treatment (Surovell and Stiner, 2000, Munro et al., 2007, Mkukuma et al., 2004, Lebon et al., 2008).

The composition of bHAP can also be examined. The $\nu_1\nu_3\text{PO}_4$ domain consists of an intense and broad band which is the result of symmetric (ν_1) and asymmetric (ν_3) P-O bond stretching (Lebon et al., 2008, Paschalis et al., 1996, Paschalis et al., 1997b, Paschalis et al., 1997a). To analyse the band, derivative spectroscopy coupled with curving fitting protocols are required. This determines the separate vibrational modes within the broad peak. However, due to the non-stoichiometric nature of bHAP, the number of vibrational modes that makes up the $\nu_1\nu_3\text{PO}_4$ domain can vary, resulting in overlapping bands that can not be distinguished. Consequently, results can be highly variable, leading to misinterpretation.

2.3.3 Summary: Calcium Hydroxyapatite, a marker for DNA recovery

There are number of techniques that can be used to investigate the structural integrity of bHAP. XRD and FTIR spectroscopy are two techniques that can be used to measure the structural and chemical composition of bone and can provide over-lapping information regarding the characteristics of bHAP. As a technique they are ideal candidates for this study, as both require minimal bone preparation and samples can be analysed in a relatively short period of time. There have also been extensive studies regarding changes in bHAP as a result of heat treatment using FTIR spectroscopy and

XRD. This study will utilise both techniques to report a coherent analysis of the effects of compromising factors on bHAP.

2.4 Biomechanics of Compact Bone

The three main components of bone namely, calcium hydroxyapatite, type I collagen and water, all contribute to its mechanical properties. Disruption to any of these components could lead to mechanical alterations (Burr, 2002, Nyman et al., 2006, Yamashita et al., 2001). The mineral component is correlated with bone strength and stiffness while collagen contributes to the tensile stiffness and toughness of bone (Burr, 2002).

The mechanical properties of bone have been extensively studied using techniques such as 3/4 point bending (Guedes et al., 2006, Yeni et al., 2007), compression and tensile testing (Catanese et al., 1999). Table 2.4 describes the different tests that have been used on fresh bone and their advantages and disadvantages in relation to this study. The majority of the tests required large samples, in some cases whole bones were used. In addition samples usually have to be machined into a particular shape; a requirement that may be problematic when using compromised bone tissues.

Table 2.5: The analytical techniques available to investigate the mechanical properties of bone

Method	Parameter Tested	Advantages	Disadvantages	Reference
Microhardness	Rockwell Vickers Martens scratch hardness	<ul style="list-style-type: none"> • Small samples needed for analysis. • Samples do not need to be machined into shape. 	<ul style="list-style-type: none"> • May need polishing. 	(Lees, 1981)
Nanohardness	Hardness Elastic Modulus	<ul style="list-style-type: none"> • Very small samples needed for analysis. • Samples do not need to be machined into shape. 	<ul style="list-style-type: none"> • Needs polishing using at least 5µm alumina solution. • Very specific, large variation between concentric rings of lamella. 	(Rho and Pharr, 1999) (Zysset et al., 1999) (Rho et al., 2002)
Bending tests	3/4 point bending Stress Strain Young's Modulus	<ul style="list-style-type: none"> • 4 point pure bending at the middle • Whole bones can be used 	<ul style="list-style-type: none"> • 3 point bending can cause transverse shear stresses • 4 point requires bigger sample to accommodate two loads • Bone samples have to be machined 	(Guedes et al., 2006) (Yeni et al., 2007)
Compression	Compression	<ul style="list-style-type: none"> • Small samples needed for analysis. 	<ul style="list-style-type: none"> • Samples required to be machined • Often used to evaluate cancellous bone • Difficult to achieve accurate results 	(Catanese et al., 1999)
Tension	Young's modulus, ultimate strength yield strength	<ul style="list-style-type: none"> • Whole bone 	<ul style="list-style-type: none"> • Machined bone • May not be physiologically relevant; in vivo bone is not primarily loaded in tension 	(Catanese et al., 1999)
Fatigue	Compression-compression Tension-tension	<ul style="list-style-type: none"> • Simple bone preparation 	<ul style="list-style-type: none"> • Samples have to be machined to a certain shape. 	(Kim et al., 2007) (O'Brien et al., 2003)
Fracture	Critical stress intensity factor Critical strain energy release rate	<ul style="list-style-type: none"> • Three different modes I (tension) II (shear) III (tearing) 	<ul style="list-style-type: none"> • Samples have to be machined into a certain shape 	(Yan et al., 2007)
Torsion test	Shear modulus, shear strength, shear toughness	<ul style="list-style-type: none"> • Using whole bones. Structural strength, stiffness energy to fracture can be obtained. 	<ul style="list-style-type: none"> • Samples require machining 	(Kohler et al., 1986)

2.4.1 Microhardness

Microhardness is a measure of a material's resistance to deformation under indentation (Vickers or Rockwell hardness) or scratch (Martens hardness) by an object (Athanasίου et al., 2000). Vickers hardness is calculated using a diamond pyramid indenter; the load placed on the indenter and the resultant area of indented surface is used to calculate the hardness of the surface.

Microhardness has been used to investigate the relationship between the mineral and organic phases of bone (Athanasίου et al., 2000, Lees, 1981), the effect of osteoporosis on bone mechanics (Boivin et al., 2008) and bone modelling (Stea et al., 1995, Boivin et al., 2009). Microhardness of heat treated bone (Lees, 1981, Rogers and Daniels, 2002) has also been studied and is discussed below (2.8.5.1)

2.4.2 Summary: The microhardness of bone, a potential marker for DNA recovery

The mechanical properties of bone have been extensively investigated in order to understand the relationship between bHAP and collagen. Microhardness has been utilised in this study as it requires very small specimens, little preparation and samples can be analysed in a very short period of time.

2.5 Deoxyribonucleic Acid (DNA): A Forensic Tool

The human genome has a large and complex structure. There are roughly six billion base pairs per diploid human cell, these are located in the nucleus and divided across 46 chromosomes (Butler, 2005). The vast majority of the human genome (over 99.7%) is the same between people with only a small fraction (0.3% or ~ 10 million nucleotides) varying between individuals. Nevertheless, it is this variability that makes each human (except identical twins) unique and provides the capacity to use DNA for human identification.

2.5.1 DNA Profiling

DNA profiling utilises variable regions of the genome to generate a unique code that is exclusive to the individual from which the DNA was taken. Today, DNA evidence has taken a central role in identification cases, and carries significant weight in courts of law, both for convicting the guilty and exonerating the innocent (Schneider, 2007).

2.5.2 DNA Profiles

As with all eukaryotic genomes, the human genome contains an extensive amount of repeated sequences known as satellites. Repeated sequences are made from a core repeat unit that can vary in size and contiguous unit number. Satellite DNA makes up

between 20 and 30% of the human genome (Singer, 1982) and is of great importance to forensic applications.

2.5.2.1 Minisatellites

Minisatellites or variable number tandem repeats (VNTR) are interspersed repeats with a core repeat unit that ranges between 10-100 bases in length (Tautz, 1993, Chambers and MacAvoy, 2000). The size of the tandem array is normally from 0.5 to 30 kilobase pairs and can be analysed using the restriction fragment length polymorphism (RFLP) technique and southern blotting (Hviid et al., 1992). Short VNTRs can also be analysed through PCR (Demers et al., 1995)

2.5.2.2 Microsatellites

Microsatellites or short tandem repeats (STRs) are regions of nuclear DNA (nDNA) with repeated units that are typically 2-6 base pairs in length and are amplified as PCR products that range from approximately 100 to 450 base pairs in size (Table 3.14). Figure 2.6 represents a theoretical allele combination at a particular locus. Two allelic forms are present, one that was maternally inherited and one that was paternally inherited. Using PCR, the allelic patterns at multiple (typically 10-15) loci can be typed to produce a profile specific to an individual. Using 13 STR loci, the average probability of another individual matching the exact same profile is approximately one in a trillion (Holden et

al., 1995b, Holden et al., 1995a, Chakraborty et al., 1999). STR analysis has become a popular tool in developing an individual specific profile. Their repeat array range is relatively short, making them very amenable to PCR analysis (Demers et al., 1995). They also have a high power of discrimination due to their high heterozygosity. As a result, extensive databases have been created in the UK (national DNA database (NDNAD)) and other countries, using STR profiles of individuals.

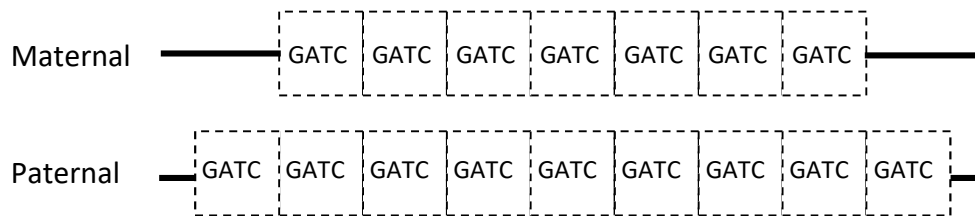


Figure 2.7: Diagrammatic representation of a STR locus. The genotype for this locus would be 7,9.

2.5.2.3 Single nucleotide polymorphisms

Single nucleotide polymorphisms (SNPs) are single base sequence variations between individuals at a particular point in the genome (Huijsmans et al., 2007). Millions of SNPs exist per individual and have been used in linkage studies to track disease genes (Brookes, 1999). SNPs are also of particular interest in forensic DNA analysis. Amplicon size of SNPs are often <100 bp, which is significantly smaller than the size of VNTRs and STRs. Therefore, using SNPs could be beneficial in generating a DNA profile using

degraded DNA samples (Dixon et al., 2006, Westen and Sijen, 2009). However, on average 25-45 SNP loci are needed to yield equivalent random match probabilities to 13 STR loci (Chakraborty et al., 1999).

2.5.2.4 Y-chromosome

The amplification of STRs or SNPs found in Y-chromosomes has been used in various scenarios including sexual assault cases (Cerri et al., 2003, Roewer, 2009), paternity testing (Mansuet-Lupo et al., 2009) and in human migration and evolutionary studies (Su et al., 1999, Cann, 2001). Y-chromosome analysis is limited to males as it is paternally inherited. Consequently family members from the paternal lineage would share the same Y- chromosome profile.

2.5.2.5 Mitochondrial DNA Profiles

Mitochondrial DNA (mtDNA) is found within the cytoplasmic mitochondrial organelles and has been used in DNA typing of individuals. Using PCR, several amplicons ranging in size from 126-170 bp are sequenced and compared to a reference sample. Whereas there are only two copies of nDNA (one paternal and one maternal) per cell, there is on average 500 mtDNA genomes per cell. As a result of this high copy number, there is greater survival potential of mtDNA molecules in comparison to nDNA. Mitochondrial DNA is inherited maternally (Butler, 2005). As result a reference sample can be derived

from the maternal lineage of the individual. However, mtDNA is not autosomal and has a relatively low power of discrimination when compared to nuclear DNA methods.

2.5.3 Short Tandem Repeat (STR) Profiling

STRs are multi-allelic forms of genetic variation, with the number of core repeat units varying considerably at a single locus within a population. As a result, they have a high power of discrimination. The frequency of each allele at a given locus in a particular population has to be determined empirically, before random match probabilities can be calculated. It has been shown that typing about 100-150 individuals per population is sufficient for measuring allele frequencies (Chakraborty, 1992, Evett and Gill, 1991). Once the frequency of each allele at each locus has been determined, the probability of a random individual within the population having the same profile (i.e. same number of alleles at each locus) can be calculated. The more loci used in the calculation, the greater the power of discrimination to define a unique profile.

2.5.4 Reference Samples

In order to identify missing persons, victims or perpetrators of crime and human remains from mass disasters using DNA analysis, a reference sample, ideally from the victim or suspect, is required. A DNA reference sample can be collected from a suspect using mouth swabs and in cases where an individual has died (e.g. from mass disasters),

DNA can be collected from personal effects such as razors, hairbrushes, toothbrushes or other items that were handled by the individual being identified.

DNA from relatives can also be used as a reference sample. As mentioned above, there are two copies of each STR marker, one inherited maternally and the other paternally. As a result, DNA from relatives can be used to indirectly identify the individual. It is possible to estimate the likelihood of STR markers matches using more distant relatives; however immediate relatives namely parents and children are the most valuable 'indirect' reference samples (Butler, 2005). This is because DNA must be shared between them, rather than simply being more likely to be shared between them when compared to random unrelated persons.

2.5.5 Summary: DNA Profiling

This study focused on the amplification of nDNA and STR loci. Table 2.5 describes the advantages and disadvantages of each DNA typing method currently being applied to forensic cases. Although STRs are not the smallest or most abundant type of polymorphism, extensive STR databases already exist in a number of countries, including the UK and they also have a relatively high power of discrimination (Gill et al., 2004). Moreover, by developing a diagnostic tool that could determine the number of STR loci that would be amplified, the appropriate reference sample required for identification could be determined.

Table 2.6: Different methods of DNA analysis

Method	Advantages	Disadvantages	references
VNTR	<ul style="list-style-type: none"> • Can use RFLP or PCR • Using RFLP; Less worry of contamination. Small amounts of contamination will not show in final analysis. 	<ul style="list-style-type: none"> • Using PCR; allelic dropout of larger alleles; due to preferential amplification of smaller alleles. • Using RFLP; can take a number of days. • Only two copies per cell; potential issues with degraded DNA samples. 	(Tully et al., 1993) (Demers et al., 1995)
STR	<ul style="list-style-type: none"> • Autosomal • Multi-allelic; higher power of discrimination per allele • Multiplex; a number of STRs can be amplified in one reaction. 	<ul style="list-style-type: none"> • Only two copies per cell; potential issues with degraded DNA samples. • Potential stutter artefact associated with alleles. • Potential risk of contamination; a relatively small amount of contamination can be amplified. 	(Butler, 2002)
SNP	<ul style="list-style-type: none"> • Autosomal • Potentially higher success rates with degraded DNA samples. • No stutter artefact associated with each allele. • Can use PCR or Microchip-based SNP assay; >1000 SNPs can be examined. 	<ul style="list-style-type: none"> • Majority are bi-allelic; only three possible genotypes. • Mixture interpretation can be challenging. • Less information per SNP; a large number of SNPs are required to obtain a power of discrimination to define a unique profile. • Microchip assays are expensive. • Issue with multiplex PCR amplification. • Potential risk of contamination; a relatively small amount of contamination can be amplified. 	(Gill et al., 2004)
Y-chromosome	<ul style="list-style-type: none"> • Can use STRs or SNPs • Inherited paternally; maternal lineage can be used as a reference sample. 	<ul style="list-style-type: none"> • Inherited paternally, not specific to the individual. 	(Cerri et al., 2003) (Roewer, 2009)
Mitochondrial	<ul style="list-style-type: none"> • ~500 copies per cell • Potentially higher success rates with degraded DNA samples. • Inherited maternally; maternal lineage can be used as a reference sample. 	<ul style="list-style-type: none"> • Inherited maternally, not specific to the individual. 	(Sato and Kuroiwa, 1991)

2.6 Biological Samples

A DNA profile can be generated from a wide variety of biological samples. Using nDNA, DNA profiles have been produced from blood and saliva traces (Adams et al., 1991, Grubwieser et al., 2003), semen samples (Sato et al., 2007, von Wurmb-Schwark et al., 2006) hair roots (Boonen et al., 2008, Gill et al., 1987, Muller et al., 2007) and various cadaveric tissues (Lorente et al., 2001). It was also possible to generate a DNA profile from skeletal tissue (Davoren et al., 2007, Hochmeister et al., 1991, Lee et al., 1991).

2.6.1 DNA Profiles from Skeletal Tissue

Bone is an extremely important source of post-mortem DNA, as it is often the only sample that is available for analysis (Parsons and Walter Weedn, 1996). The soft tissues of corpses can be too decomposed for DNA analysis. DNA has been used to successfully identify victims from a wide range of scenarios including natural disasters (Steinlechner et al., 2006), terror attacks (Holland et al., 2003) and armed conflicts (Alonso et al., 2001).

Identifying individuals using DNA profiles derived from bone samples is becoming increasingly important (Holland et al., 2003). Both nDNA and mtDNA have played a significant role in the identification of skeletal remains of murder victims (Hagelberg et al., 1991) and missing persons (Hochmeister et al., 1995).

DNA from bone samples has also played a significant role in identifying victims of mass disasters; soft tissue and organs of victims can be crushed into small pieces or destroyed and are often not suitable for DNA analysis (Loreille et al., 2007). Using skeletal tissue, victims from the World Trade Centre (WTC) attacks (Holland et al., 2003, Mundorff et al., 2009, Budimlija et al., 2003) and the 2004 South Asian tsunami (Steinlechner et al., 2006, Deng et al., 2005) have been identified using STR markers.

The International Commission on Missing Persons (ICMP) has identified victims that were recovered many years after the wars in Croatia, Bosnia and Herzegovina (Alonso et al., 2001, Davoren et al., 2007, Parsons et al., 2007) and Kosovo (Djuric et al., 2007). Individuals have also been identified from the Spanish civil war (Rios et al., 2010) and members of the Ernesto Che Guevara guerrillas in Bolivia (Lleonart et al., 2000).

Successful identification of individuals from skeletal remains is often dependent on the environment from which they were recovered. This study has focused on two environmental factors, heat and submersion in water, which are two major environmental factors that can affect DNA profiling.

2.6.2 DNA Profiling From Burnt Skeletal Samples

When a human body has been damaged by heat, identification of the individual, either visually or by dental and fingerprint records can be impossible. As such, DNA analysis may be the only option. If a fire has been of sufficient intensity and duration, all soft tissue sources of DNA may have been consumed, leaving the practice of attempting to identify burnt skeletal tissue a common task for DNA analysts (Schwark et al., 2010).

In a forensic context, fire is often used to cause death or to conceal evidence of homicide (Fantoni et al., 2006, Sivolap et al., 2001). It is also possible that heat-compromised bone tissue from mass disasters (Holland et al., 2003, Meyer, 2003), house/car fires (Staiti et al., 2004, Calacal et al., 2005) and even suicides (Lutz et al., 1996) may require identification.

The details of previous attempts to amplify DNA from bone that has been subjected to high temperatures have been mostly communicated through case studies where the exact exposure times/temperatures were unknown (Calacal et al., 2003, Cattaneo et al., 1999, Sivolap et al., 2001, Staiti et al., 2004, Von Wurmb-Schwark et al., 2004b). Results have been variable and there is very little literature where environmental factors have been controlled. An early study from Cattaneo et al, (1999) used cortical bone from fresh cadavers which were then exposed to uncontrolled temperatures ranging from 800 °C to 1200 °C. The study attempted to amplify regions of mtDNA, but reported that

no DNA could be amplified from any of the cadavers and suggested that a further investigation into the rate of loss of detectable DNA in relation to burning could be of great interest.

DNA was successfully amplified from bone fragments remaining after commercial cremations (Von Wurmb-Schwark et al., 2004). DNA was extracted from large fragments of long bones, particularly femora, as they are often the last bones to succumb to cremation (Von Wurmb-Schwark et al., 2004 and Parson and Walter Weedn, 1997). Ten cadavers were exposed to 1000 °C for 70 minutes. From femurs of the ten 10 corpses, nDNA was successfully amplified from seven. Although DNA was successfully amplified, the strategy for identification would have been limited to SNP analysis due to the small amplicon size.

It has been suggested that the use of DNA extracted from burnt bone could not guarantee reliable generation of a useable STR profile (Von Wurmb-Schwark et al., 2004). This statement has been supported by recently published studies that have suggested that nDNA is too degraded once bones have charred and that other avenues, including mtDNA analysis, should be pursued for identification (Schwark et al., 2010, Heinrich et al., 2009a). Mitochondrial DNA analysis was successfully used to identify a charred body found in a burnt out motor vehicle (Lutz et al., 1996)

Some published case studies have contradicted this theory and have identified individuals from cremated skeletal tissue using nDNA. Fire tragedy victims (Calacal et al., 2005) and bone fragments found within a domestic fireplace (Sivolap et al., 2001) have all yielded viable nDNA that was subsequently used to create a STR profile. STR markers have also been amplified successfully and used to identify victims of a house fire in Manila (Staiti et al., 2004), where 18 of the 21 victims were successfully identified. A STR profile was also generated from DNA extracted from bone of a victim who had been killed and burnt (Staiti et al., 2004).

There have been cases where a large number of samples, which have been exposed to high temperatures, were analysed. After the WTC attacks for example, 13,000 bone samples required identification, however, a useable DNA profile could not be generated from 4,550 of them (Holland et al., 2003). As a result a lot of time and money was wasted.

One aspect that is consistent between all the published studies is the lack of documentation regarding the temperatures and conditions that the remains were exposed to. In some cases the identification of human remains using burnt skeletal tissue was performed in conjunction with analysis of other tissues (Calacal et al., 2003, Lutz et al., 1996) or taken from regions of bone that had not been exposed to such high temperatures (Wurmb-Schwark et al., 2004).

It is clear that heating bone promotes DNA degradation processes including hydrolysis and oxidation (Alaeddini et al., 2010). Case studies have suggested that DNA degradation, sufficient to affect the ability to obtain a useful profile, would have occurred once bone had become charred (Heinrich et al., 2009b, Schwark et al., 2010). However, whether degradation mechanisms are dependent on maximum exposure temperature or the duration of exposure remains inconclusive. Moreover, collagen and bHAP have been seen to play a role in DNA degradation in bone (section 2.7).

2.6.3 DNA profiling from skeletal tissue that has been submerged in water

As discussed below there have also been reports of successful DNA typing of bone samples that have been submerged in both fresh water and seawater. However, similar to that of DNA profiling from burnt remains, the literature is based on case studies where accurate details of the submersion period or environmental conditions are not known or have not been reported. Profiles have been successfully generated from bone samples that have been submerged in fresh water for periods of between 18 months (Hochmeister et al., 1991) and 3 years (Crainic et al., 2002). In seawater, profiles have been successfully obtained from bone that has been submerged from a couple of days (Mukaida et al., 2000) to a year (Imaizumi et al., 2005). However, profiling from bone that had been presumed submerged for 11 years was not successful (Imaizumi et al., 2005), and bone samples that had been lying in a damp environment for periods ranging from 1 year to 45 years also failed to generate a profile (Graw et al., 2000).

2.6.4 Summary: DNA Profiling from Skeletal Tissue

Bone acts as a harbour for DNA (Meijer et al., 1992). As well as acting as a physical barrier against environmental insults (Bandelt et al., 2006), the cellular structure and bHAP content of bone stabilises the DNA. The mineralised matrix allows DNA to bind to the cellular matrix, which is thought to protect it from digestive enzymes (Parsons and Walter Weedn, 1996). Skeletal tissue can be an invaluable source of DNA as it can be the only source available for DNA analysis. However, even in the relatively stable environment that bone provides, DNA will continue to break down after death (Lindahl, 1993). DNA degrades through the processes of hydrolysis and oxidation (Lindahl, 1993), which can be accelerated by a variety of environmental factors. Soil pH, air and soil humidity and microbial-mediated decay have all been seen to affect DNA degradation (Ovchinnikov et al., 2001, Smith et al., 2001). A comprehensive review by Alaeddini et al., (2010) describes the forensic implications of DNA degradation

There has been very little controlled research regarding DNA degradation in heated bone or skeletal tissue that has been submerged in aquatic environments. The current literature suggests that DNA can survive in bones that have become charred (Heinrich et al., 2009b, Schwark et al., 2010), which is indicative of temperatures ranging from 150-300 °C (Shipman et al., 1984). In bone that has been submerged in aquatic environments, DNA degradation appears to be dependent on factors including salinity, temperature and duration.

In order to develop a diagnostic tool that can predict, prior to DNA extraction, whether subsequent amplification is likely to be successful in heated bone or bone tissue that has been submerged in an aquatic environment, the property of bone measured should show significant correlation with DNA degradation.

2.7 Predicting the state of DNA preservation in skeletal tissue

Although the processes that cause DNA degradation are well known, their investigation has been restricted to controlled tests and environments and cannot be applied directly to DNA degradation in bones (Lindahl, 1993). It is thought that factors such as the adsorption of DNA to the bone matrix may influence DNA degradation in bone samples (Collins et al., 2000). There has been extensive research that has examined the possibility of using the properties of bone to determine or predict the state of DNA preservation. However, these studies are often restricted to archaeological samples. Nevertheless, the literature presents an insight into the relationship between collagen, bHAP and DNA, and provides valuable information for the development a diagnostic tool that would aid DNA analysis from modern bone.

2.7.1 Using measurements from the mineral phase of bone to predict the likelihood of successful DNA recovery.

DNA is thought to have a high affinity for bone bHAP (Collins et al., 1995, Person, 1992, Gotherstrom et al., 2002, Parsons and Walter Weedn, 1996). As a result of this strong affinity, DNA is thought to be protected from degradation processes as evident by the successful amplification of DNA fragments from archaeological bone (Gotherstrom et al., 2002). DNA affinity to bone bHAP is thought to arise from the negatively charged phosphate groups in the DNA molecule and the adsorption hydroxyl sites on the mineral (Kawasaki et al., 1985). The study by Gotherstrom et al., (2002), reported a significant logistic regression correlation (for more details see section 3.17.1) between bHAP re-crystallisation and DNA survival in both ancient horse bone samples (Figure 1.3) and modern bovine bones that were compromised by different levels of humidity. The likelihood of successful DNA amplification decreased with increased bHAP crystallinity.

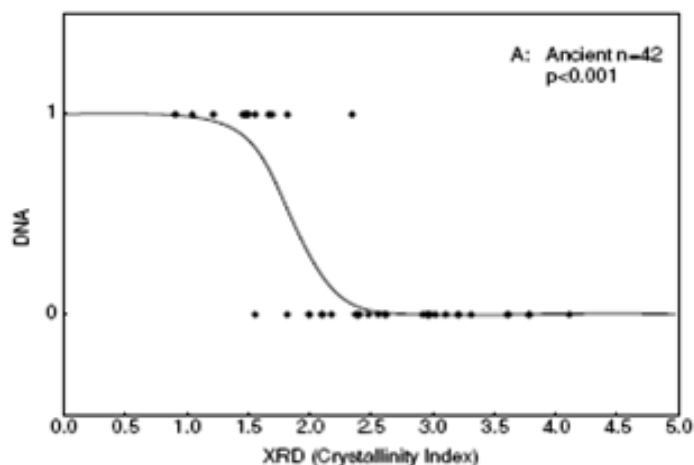


Figure 2.8: The relationship between the presence of DNA (detectable by PCR) and the crystallinity of bHAP in ancient bone (Gotherstrom et al., 2002).

2.7.2 Using measurements from the organic phase of bone to predict the likelihood of successful DNA recovery.

A relationship between collagen and DNA degradation has also been established (Colson et al., 1997, Cooper et al., 1997). Gotherstrom et al, (2002) examined collagen content as a possible marker for DNA recovery. Collagen is a triple helix organic molecule that is thought to have a similar degradation process to DNA (Collins et al., 2002). Although the correlation was significant, there was a distinct overlap between samples from which DNA was successfully amplified and samples from which DNA amplification failed (Figure 2.16). Moreover, the collagen assay used required the prior demineralisation of bone, and the reported amplification was of only a relatively short (83 bp) DNA fragment. In a forensic context, this would only be suitable for SNP analysis.

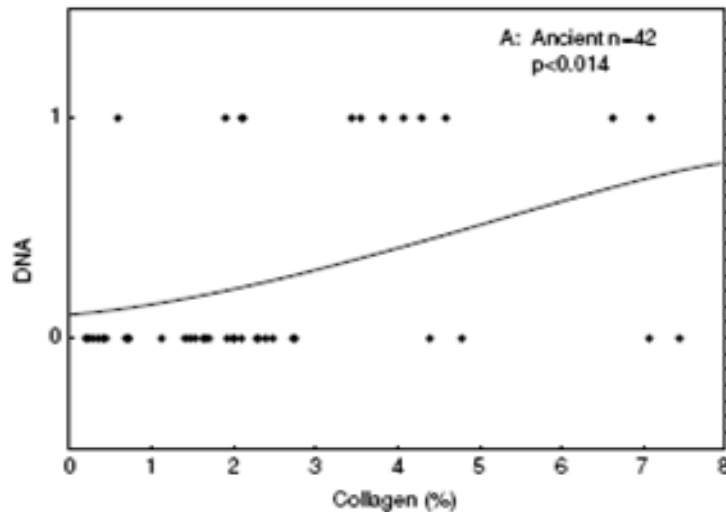


Figure 2.9: The relationship between the presence of DNA (detectable by PCR) and the amount of collagen in ancient bone (Gotherstrom et al., 2002).

Other analytical techniques, which can measure the collagen content of bone, have been more successful as markers for DNA recovery. The rates of aspartic acid racemisation (AAR) in collagen and DNA depurination (hydrolysis) are thought to be similar and the former has been developed as a marker to determine the likelihood of successful DNA amplification (Poinar et al., 1996, Collins et al., 2009). AAR is defined as the ratio of L/D-forms of aspartic acid, an amino acid found in collagen. Proteins, such as collagen are originally synthesized from only the L-forms of amino acids. Once the L-form is incorporated in the polypeptide, it begins to racemize to the D-form. The longer the racemisation process continues the more D-form is present. Threshold AAR ratios that could be used to determine whether or not aDNA would be amplifiable from archaeological skeletal tissue have been reported (Poinar et al., 1996, Collins et al., 2009).

Temperature is one factor that can affect the rate of both racemisation and DNA depurination. As a result, AAR could correlate with DNA degradation in heat-treated bone. However, racemisation requires High Performance Liquid Chromatography (HPLC), which was considered an analytical technique that was too expensive and time-consuming to be used as a diagnostic tool in the context of this study (Table 2.2).

Gas chromatography-mass spectrometry (GC/MS) allows complex organic materials, such as the organic content of bone, to be analysed. GC/MS has been used to detect amino acids and their derivatives, such as proline and hydroxyproline which are

constituents of collagen. Using archaeological bone, estimates of the relative amounts of peptide hydrolysis could be calculated and were found to correlate with DNA amplification (Poinar and Stankiewicz, 1999). However, similar to HPLC, GC/MS is relatively expensive and complex with regard to tissue preparation and analysis, when compared to other analytical techniques (Table 2.2).

Although collagen has been predominantly investigated as a possible marker for successful aDNA amplification, other organic components of bone have also been investigated as possible markers for DNA recovery. Osteocalcin preservation has been correlated with aDNA amplification (Buckley et al., 2008, Smith et al., 2005). However, the analytical techniques used, western blot analysis (Smith et al., 2005) and HPLC (Buckley et al., 2008) were considered unsuitable for this study (Table 2.2).

Other techniques, also deemed unsuitable for this study, have also been correlated with aDNA recovery. Histology coupled with CHN (carbon, hydrogen and nitrogen) microanalysis has been suggested as a possible tool to determine DNA recovery (Jans et al., 2002). However, CHN requires bone samples to be demineralised (Jans et al., 2002). The thermal history (the mean temperature that the bone tissue has been subjected to over time) of the fossilised bone has also been used to assess the likelihood of aDNA amplification (Smith et al., 2003). However, in a forensic context and particularly in cases in which skeletal tissue has been burnt, it may not be always possible to

determine an accurate thermal history. Subjective approaches have also been developed including the gross morphology and histology of bone (Haynes et al., 2002).

The literature regarding the use of bone markers as indicators of DNA recovery has focused on bone tissues that are in the process of diagenesis. Published studies regarding modern bone are limited in number. Gotherstrom et al., (2002) demonstrated that the crystallinity of bHAP might be a marker for DNA recovery in modern bone tissue. Moreover, Gotherstrom et al., (2002) used XRD, an analytical technique that has been considered suitable for this study (Table 2.3).

Other diagenic parameters have also been used to predict the likelihood of successful DNA amplification in modern bone samples, with varying success. The gross morphology and histology have been reported as analytical techniques that could predict whether a DNA profile can be successfully obtained from bone samples that had been buried for approximately 12 years (Al-enizi et al., 2008). However, another factor, nitrogen content, was not successful (Al-enizi et al., 2008).

STR genotyping kits use primer multiplexes that are relatively expensive when compared to standard PCR kits. A simple duplex-PCR that evaluates whether or not 164 bp nDNA and 260 bp mtDNA fragments could be amplified from forensic samples has been developed (von Wurmb-Schwark et al., 2004a). A correlation between successful amplification of these fragments and the successful amplification of useable STR or mitochondrial profiles was reported. Thus, as well as indicating whether or not DNA

analysis would be possible, the duplex would also be able to specify the appropriate direction of analysis (nDNA or mtDNA). Although, the system may save money for the organisation assigned to the case, considerable time would be wasted as non-viable samples would still be processed (von Wurmb-Schwark et al., 2004a).

2.7.3 Summary: Indicators of DNA recovery

Publications have suggested numerous techniques that have been developed to determine whether or not DNA can be successfully amplified from archaeological bone. However, the analytical techniques chosen were often time consuming, required complex analysis procedures or bone preparation protocols and were expensive. Analytical techniques that are time-consuming and labour intensive may be justified in an archaeological context as the time taken from collecting the tissue at the scene to DNA analysis, may not be important. In a forensic context, the time taken to generate DNA profiles is very important. By generating a DNA profile in the shortest possible period of time, perpetrators who would otherwise elude law enforcement agencies may be apprehended and victims could be identified quickly. This would not only benefit criminal investigations, but also the families and communities involved. However, the studies described above, have shown that relationships between collagen, bHAP and DNA exist and it may be possible to use a bone marker to predict the likelihood of successful DNA profile generation.

2.8 Developing a diagnostic tool for DNA analysis from heated bone

To develop a diagnostic tool that can predict whether or not a useable DNA profile could be obtained from heated bone, two factors have to be taken into account.

1. Temperature
2. Time

In a forensic context, it would be difficult to determine an accurate account of exposure conditions (heat). The analytical techniques were not only chosen because of their technical features, including assay completion time and bone preparation procedures, but also due to their application in previous investigations involving heated bone.

Although, the effect that the duration of heat treatment has on DNA degradation in bone remains inconclusive, it has been suggested that DNA degradation can occur when bone is heated to temperatures ranging from 150 and 300 °C. Therefore a diagnostic tool should be able to detect discernable changes in its measured marker at these relatively low temperatures.

2.8.1 Material changes in heated bone

Heating affects both the collagen and apatite phases of bone. At relatively low temperatures (below 100 °C), there are few changes in collagen and apatite characteristics. However, above 100 °C, significant changes occur. At 200 °C, organic

matter, such as collagen, begins to denature (Lees, 1981, Munro et al., 2007, Rogers and Daniels, 2002). The tertiary structure of the collagen becomes disrupted and eventually degrades. The mineral phase has also been shown to change due to heat treatment. Heat treatment facilitates bHAP crystal growth and increased crystallinity (also known as re-crystallisation) (Surovell and Stiner, 2000, Munro et al., 2007, Mkukuma et al., 2004, Lebon et al., 2008).

2.8.1.1 Measuring the effect of temperature on collagen using FTIR spectroscopy

Walker et al, (2007) reported the effect of temperature on collagen degradation using FTIR spectroscopy and suggested it may be possible to correlate collagen integrity with DNA amplification. The mineral to matrix ratio can be used to indirectly measure the collagen content of heat-treated bone (section 2.2.2). This ratio has been strongly correlated with the ash weight of synthetic mixtures of hydroxyapatite and collagen (Boskey and Mendelsohn, 2005) (Figure 2.7).

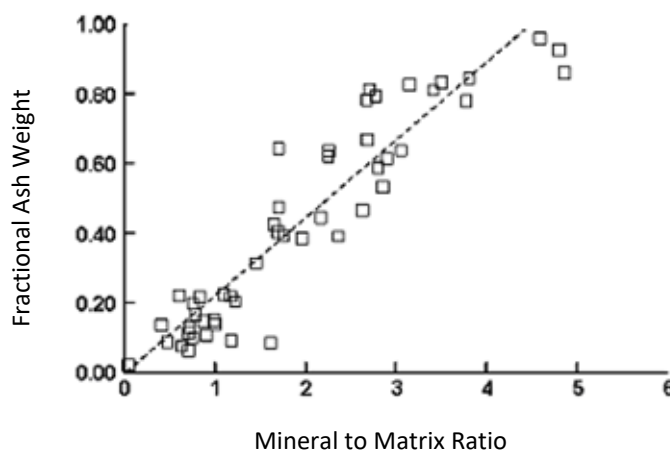


Figure 2.10: Relationship between mineral to matrix ratio and ash weight (Boskey and Mendelsohn, 2005)

The ash weight measures the difference between the weight of bone before and after heat treatment. The change in the weight of bone due to heat treatment has been extensively studied through Thermogravimetric Analysis (TGA). Above 20 °C, the weight of bone begins to decrease and continues as the temperature increases (Figure 2.8). It may be possible to use the mineral to matrix ratio to measure the ash weight and representative weight loss of heated bone. Ash weight may correlate with DNA degradation. Between 20 and 300 °C, the temperature range at which it has been suggested that DNA degrades in bone, there is distinct decrease in the weight of the sample.

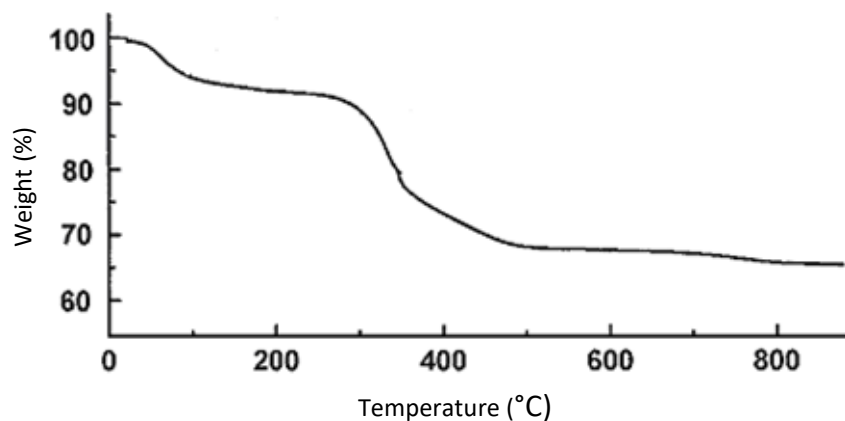


Figure 2.11: % weight loss of fresh human cortical bone when heat-treated, measured with TGA (Lozano et al., 2003)

2.8.1.2 Measuring the effect of temperature on crystallinity using FTIR spectroscopy

FTIR spectroscopy has also been extensively used to investigate the effect of heat treatment on bHAP crystallinity in bone (Surovell and Stiner, 2000, Munro et al., 2007).

The general consensus within the literature is that the SF increases as temperature increases. However, at lower temperatures (50-300 °C) there remains some controversy. Some studies have reported that the SF continues to increase as the temperature increases, while others suggest that SF 'dips' before continuing to increase (Figure 2.9).

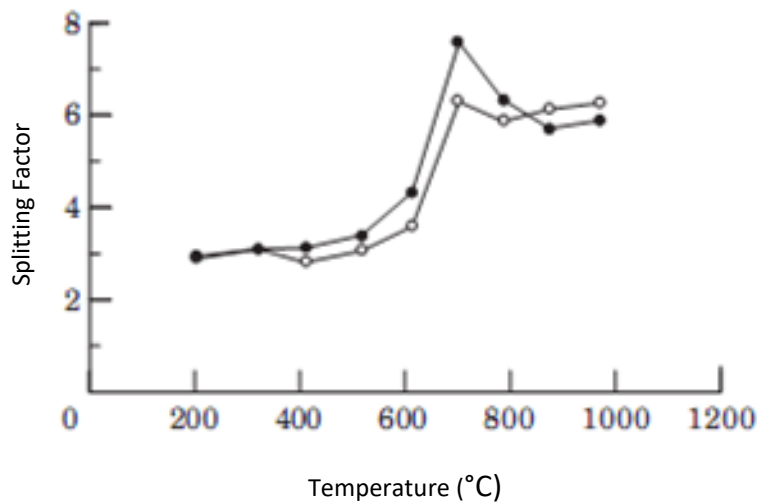


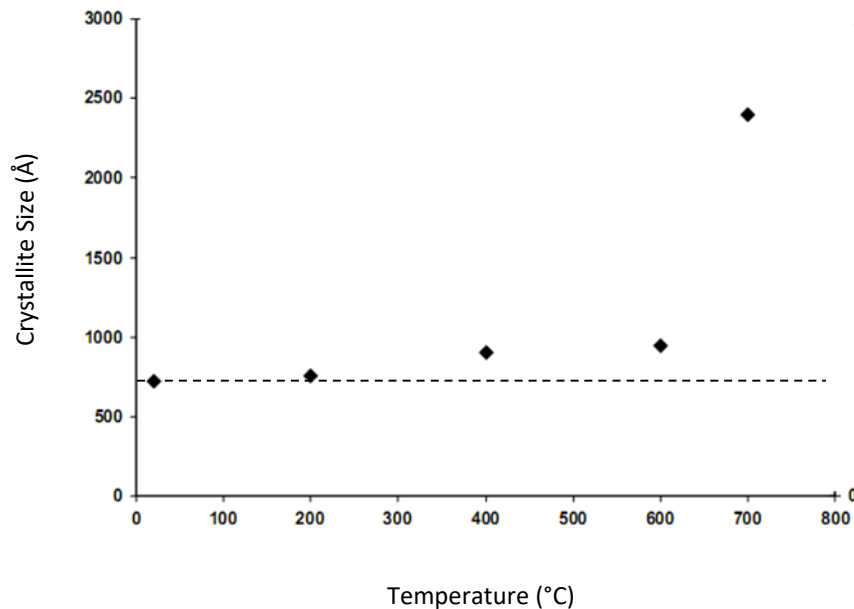
Figure 2.12: The relationship between splitting factor and temperature. (o) Uncontrolled grinding (●) controlled grinding. Modified from Surovell and Stiner (2000).

Surovell and Stiner (2000) have suggested that the dip in SF seen in some studies is the result of issues with bone preparation. The inconsistency inherent in grinding bone using a pestle and mortar may be a contributing factor to the decrease in SF (Figure 2.9). The gradual increase in splitting factor between 50 and 400 °C suggests that measuring crystallinity using FTIR spectroscopy may have potential to be used as a diagnostic tool in this study.

2.8.1.3 Measuring the effect of temperature on bone bHAP integrity using XRD

XRD can be used to measure the crystallinity, crystallite size and strain of the bHAP. The size of each cell unit bHAP can also be measured (section 2.3.1). XRD has been used to investigate the effect of temperature has on bHAP integrity (Rogers and Daniels, 2002, Munro et al., 2007, Etok et al., 2007).

Crystallite size (Figure 2.10) and coherence length (CL) (Figure 2.11) have all shown gradual increases at low temperatures, although there has been some debate as to whether this increase is significant (Rogers and Daniels, 2002).



2.13 Relationship between crystallite size of bHAP in human cortical bone and temperature (modified from Rogers and Daniels 2002))

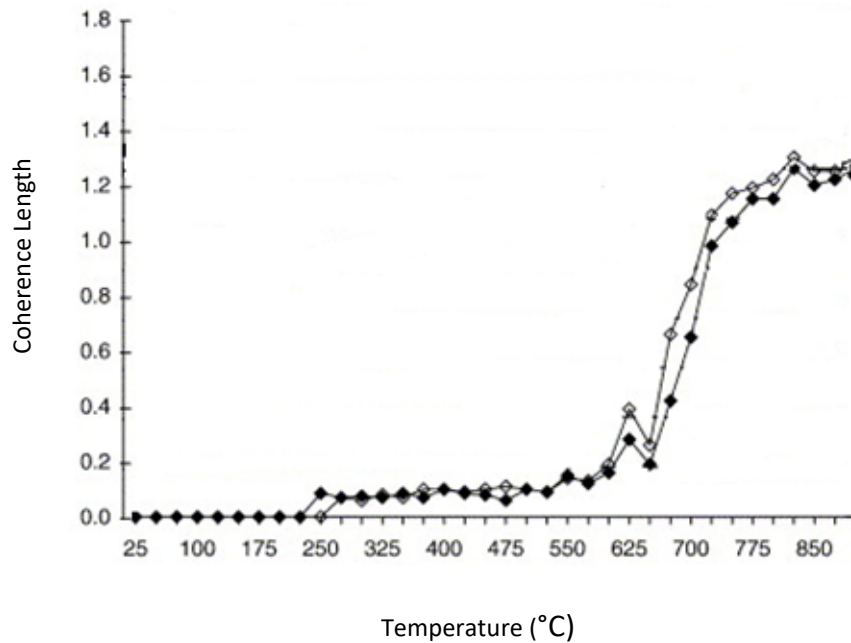


Figure 2.14 Relationship between coherence length of bHAP in deer bone and temperature (modified from (Munro et al., 2007))

There are also other parameters that can be measured using XRD and may have the potential to be developed into diagnostic tools that could aid DNA analysis. The lattice parameters ('a-axis' and 'c-axis') have both shown differences within the temperature range of interest. However, a particular parameter value may result from the sample having been heated to different temperatures. For example, a 'c'-axis value of 6.885 can be the result of being heated to 150 or 700 °C (Figure 2.12). This may be problematic as it is likely DNA would be successfully amplified from bone heated to 150 °C but amplification would certainly fail from bone that had been heated to 700 °C. It may be possible to couple parameters to categorise samples that may not otherwise be distinguished. Using the example above, if the crystallite size or CL was also measured along with 'c', then the two samples could be distinguished as the crystallite size or CL of

bHAP that has been heated to 700 °C (Figure 2.10 and 2.11 respectively) is significantly higher than that of bHAP heated to 150 °C. In bone samples heated to lower temperatures, where significant changes occur in cell unit parameters but few changes are observed in bHAP crystallinity and crystallite size, a similar methodology could be used to distinguish samples.

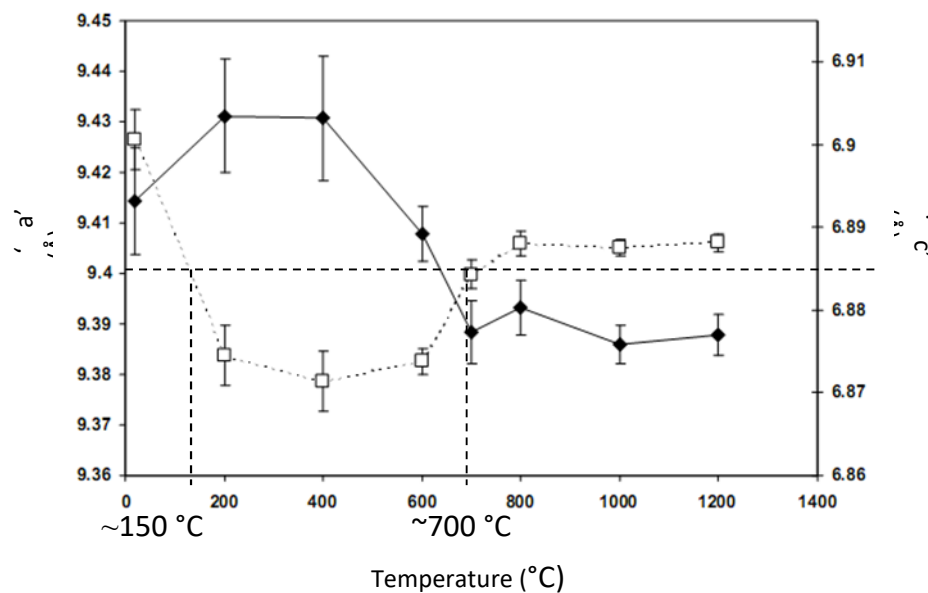


Figure 2.15 Relationship between 'a' and 'c' parameters of bHAP in human cortical bone and temperature (Rogers and Daniels, 2002). (□) 'a' (♦) 'c' Illustrated are the two temperatures (~150 and ~700 °C), which would produce similar 'c' values.

2.8.1.4 Summary: Using the changes in the material properties of heat-treated bone to predict DNA recovery

FTIR spectroscopy and XRD have been used to characterise the mineral and organic changes that occur in heat-treated bone. Currently, there is very little research detailing

such changes and subsequent DNA recovery. Furthermore, both analytical techniques have shown discernable changes within the temperature range of reported DNA degradation. As a result, FTIR spectroscopy and XRD are two analytical techniques that were used in this study.

2.8.2 Mechanical changes in heated bone

There have also been a number of studies that have investigated the changes in mechanical properties, including the fracture toughness (Yan et al., 2007), elastic modulus (Yamashita et al., 2001, Burstein et al., 1975, Rogers and Daniels, 2002) and hardness (Rogers and Daniels, 2002) of bone after heat treatment. The majority of techniques require samples to be machined into a particular shape (Table 2.4) or require extensive polishing. Microhardness is a technique that can be used to analysis relatively sample samples, which do not necessarily have to be embedded in resin or extensively polished. Thus, microhardness analysis was selected for utilisation in this work.

2.8.2.1 Microhardness of heated bone

Amprino (1958) showed that as temperature increased to 200 °C, there was a corresponding increase in the microhardness of bone. This is a result of water loss causing collagen fibres to shrink, thus increasing the amount of free space within the matrix (Amprino, 1958). As a result, crystallites are brought together, which increases

the hardness of bone. Above 200 °C, there was a decrease in microhardness (Lees, 1981, Rogers and Daniels, 2002). Lees (1981) found that the modification and degradation of organic material weakened the ability of the bone to resist the applied forces. There have also been studies that have looked into the microhardness of bone that has been heated to temperatures above 400 °C (Rogers and Daniels, 2002). Rogers and Daniels (2001) found that above 400 °C, there was a further increase in microhardness. This was due to an increase in bHAP size (Rogers and Daniels, 2002), thus reducing the amount of free space and increasing the resistance to an applied force .

2.8.2.2 Summary: Using the microhardness of heated bone to predict DNA recovery

Using microhardness as a diagnostic tool to aid DNA analysis from heated bone could be problematic. A given microhardness value may result from heat treatment at various different temperatures. It would be impossible to distinguish between samples that would or would not be viable for DNA analysis. However, as with the lattice parameters, microhardness could be used in conjunction with other measurements to distinguish samples.

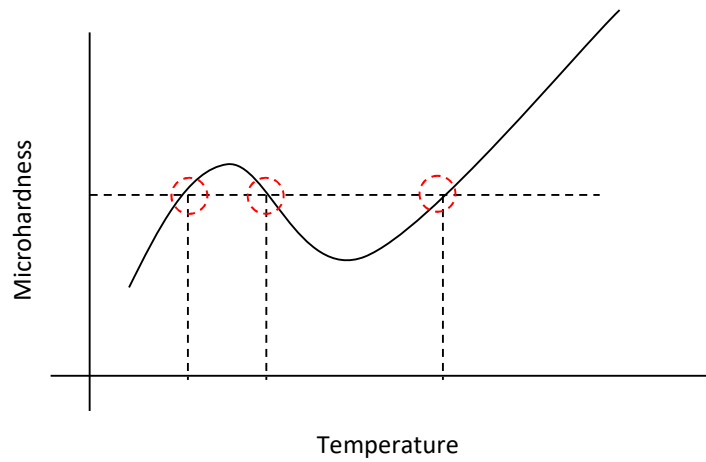


Figure 2.16: A diagrammatic representation of the effect of temperature on microhardness on heat-treated bone. Highlighted are temperatures that correspond to the same microhardness value.

2.8.3 Colour of heated bone

Histological features of bone have been successfully used as an indicator for DNA recovery (Al-enizi et al., 2008, Haynes et al., 2002). However, the Haversian systems, lamellae and lacunae have been shown to retain their integrity when heated to temperatures between 200 and 600 °C (Holden et al., 1995b, Holden et al., 1995a). The colour change of bone as a result of heat treatment has been extensively studied in both laboratory and natural contexts (Devlin et al., 2008, McCutcheon, 1992). A study by Shipman et al., (1984) standardized, with the use of a Munsell® colour chart, the sequence of colour change due to heat treatment, see Figure 2.14.

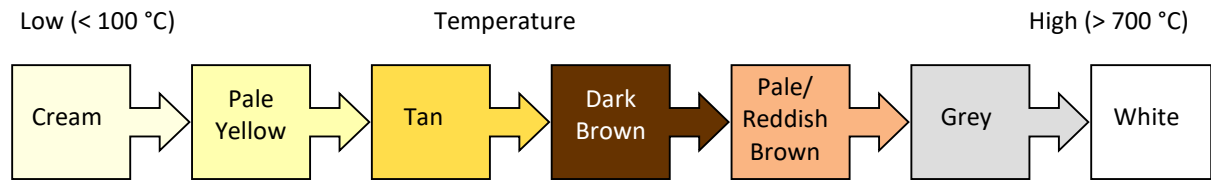


Figure 2.17: General sequence of bone colour change with increased temperature (Shipman et al., 1984).

Although other studies have echoed the correlation between temperature and surface colour, the temperature at which the changes in colour were reported varied (Nicholson, 1993). It has also been observed that other factors, such as blood, soft tissue and bone marrow and increased exposure time can also influence the colour of bone (Devlin et al., 2008).

2.8.3.1 Summary: Using the colour of heated bone to predict DNA recovery

Traditionally anthropologists have used the Munsell® colour chart to examine the colour of burnt bone. Although, Munsell® charts are cheap and very easy to use, the analysis relies on the individual making the decision that a sample is of a particular colour. Other, more quantitative techniques, such as colourimetry (Devlin et al., 2008) and recording the Red Green Blue (RGB) components of digital images (Walker et al., 2008), have been employed to investigate the colour pattern of cremated archaeological and modern bone respectively. Although, sample photographs would have to be taken in very controlled environments (i.e. lighting) in order to be compared.

There have been suggestions of a possible link between colour and DNA recovery, however, the literature on possible correlations is limited (Walker et al., 2008). Assessing the chemical processes that have occurred in heat-treated bone by recording a colour measurement, may provide inexpensive diagnostic tool that could screen samples for the presence of viable DNA quickly.

2.8.4 Summary: Developing a diagnostic tool to aid DNA analysis from heated bone

Based on the current literature, FTIR spectroscopy, XRD, microhardness and colour measurement are analytical techniques that could be developed into diagnostic tools to aid DNA analysis from burnt skeletal tissue. As analytical techniques, they are relatively quick, simple to perform and require very little sample preparation. Moreover, these techniques have already been extensively used to investigate the changes that occur in the organic and mineral phases of heat-treated bone. This study determined whether correlations existed between the parameters measured by each analytical technique and results of attempted DNA amplification.

2.9 Developing a diagnostic tool to aid DNA analysis from bone submerged in different aquatic environments

There are a number of factors that could influence DNA degradation in bone that has been submerged in water. The three main factors to consider are;

1. Water temperature
2. Environment i.e. salinity of water
3. Duration of submersion

In order to develop a diagnostic tool that could predict whether or not a usable DNA profile could be successfully obtained from bone that has been submerged in water, all three factors must be taken into account. In a forensic context, determining accurate details of the water conditions from which the skeletal tissue was recovered could be difficult. The temperature of bodies of water can change with depth and season.

2.9.1 Material changes in bone due to submersion in aquatic environments

The process of bone degradation in water occurs in two phases, early and late diagenesis. Early diagenesis is divided, into three subdivisions. Initially the organic components, including collagen, become degraded through microbial activity. Within a year collagen becomes gelatinised (the formation of a collagen derivative, gelatine) which can cause microcracks to appear in the Haversian systems (Collins et al., 1995). Subsequently pyrite (iron sulphide), particularly in seawater, is deposited in the Haversian canals and microcracks (Pfretzschner, 2004). After approximately a year, apatite exchanges ions with the surrounding water (Hedges and Millard, 1995). Late diagenesis involves the fossilisation of bone. Minerals replace bHAP through pH-

dependent precipitation and later pyrite and silica form within the bone (Pfretzschner, 2004).

2.9.2 Biomechanical changes in bone due to submersion in aquatic environments

There is very little research regarding the effect of water submersion on bone. However, the literature has reported that the integrity of bHAP and collagen can be compromised as the result of being submerged in an aqueous environment (Pfretzschner, 2004). Therefore is it possible that mechanical changes would be seen in bone that has been submerged in aquatic environments.

2.9.3 Summary: Developing a diagnostic tool to aid DNA analysis from bone submerged in water

It is the intention of this study to investigate the changes that occur to the organic and mineral phases of bone when submerged in different aquatic environments. There are a number of changes that occur to collagen and bHAP due to submersion in water that may potentially be developed into a diagnostic tool that could predict, prior to DNA extraction, whether or not amplifiable DNA should be recoverable. Using the array of analytical techniques described in this chapter, a greater understanding of bone decomposition in aquatic environments will be achieved.

2.10 Conclusion

The skeletal tissue of individuals requiring identification can be recovered from a wide range of environments. Although only reported in case studies, factors such as heat and water exposure have been shown to compromise the integrity of DNA in bone. Consequently, subsequent attempts at DNA analysis may be problematic.

Bone comprises a tri-phase system, which together provide its distinct characteristics. As mentioned in the literature, a number of studies have investigated whether different properties of bone can be used to predict the likely success of DNA amplification. However, current studies have primarily focused on aDNA and have not investigated whether such properties can be used to predict the likelihood of successfully producing a usable DNA profile from modern bone.

Processing bone that has been exposed to adverse environmental conditions for subsequent DNA extraction, can be both laborious and time consuming; with effective demineralisation (an important component of many extraction protocols) taking up to a week (dependent upon prior physical processing e.g. whether powdered or not). As a consequence, in large cases, such as mass disasters, specimens that ultimately fail to be identified successfully can consume significant time and resources. An approach that could quickly, reliably and cost effectively screen out such specimens prior to that investment of resources would clearly be of significant value.

Chapter 3 Materials and Methods

3.1 Introduction

This chapter describes in detail the methodology behind each of the selected techniques that were used in this work. Three bone preparation techniques were used to allow a number of different studies on each sample to be carried out. This chapter is divided into sections that relate to how the bone samples were prepared and the subsequent technique used (Table 3.1).

Table 3.1: Overview of the techniques and measurements taken from each type of sample preparation

Chapter	Sample Preparation	Technique	Measurement	Samples Analysed
3.5	Mounted	Micro-hardness	Vickers Hardness	All
		Microscopy	Histology	Submerged Only
3.8	Powdered	XRD	Mineral content	All
		FTIR	Mineral content	All
		Spectroscopy	Organic content	
		Colourimetry	Colour	Heat Treated Only
		DSC	Heat Flux	Fresh Bone only
		TGA	Weight loss	Fresh Bone only
3.14	Cubed	DNA Extraction	DNA	All
		PCR	Quality	All
		DNA typing	Identification	All

3.2 Bone samples

To fulfil the requirements set out in the aims and objectives (Section 1.2), bovine bone was used as a substitute for human bone tissue. *Bos taurus* (cow) was chosen due to the availability of a commercial STR genotyping kit (Applied Biosystems, Warrington, UK) and the ready availability of skeletal tissue. Bovine femur was chosen as it has been reported to produce a significantly higher profiling success rate when compared to other skeletal elements (Milos et al., 2007). This study also utilised human skeletal remains recovered from the English Channel (section 3.3.3).

3.2.1 General sample preparation

Bovine femora were collected from Dury and Sons Abattoir (Wootton Bassett, Swindon, UK). All femora that were used in this work were from the same breed (Jersey) and approximately the same age (18 months). Seven ~5-10 mm thick sections were cut from each femur (Figure 3.1). These sections were taken from the diaphysis, where the diameter was the narrowest (i.e. not the metaphyses), and were cut using a 360 mm bandsaw (Draper Tools, Hampshire, UK). During cutting, the sections were cooled with water, or cut from frozen to reduce the effect of heat (caused by friction) from the bandsaw. Using a scalpel, bone samples were defleshed and washed with water to remove any remaining soft tissue and bone marrow. Human skeletal remains were cleaned and cut from frozen using the same protocol.

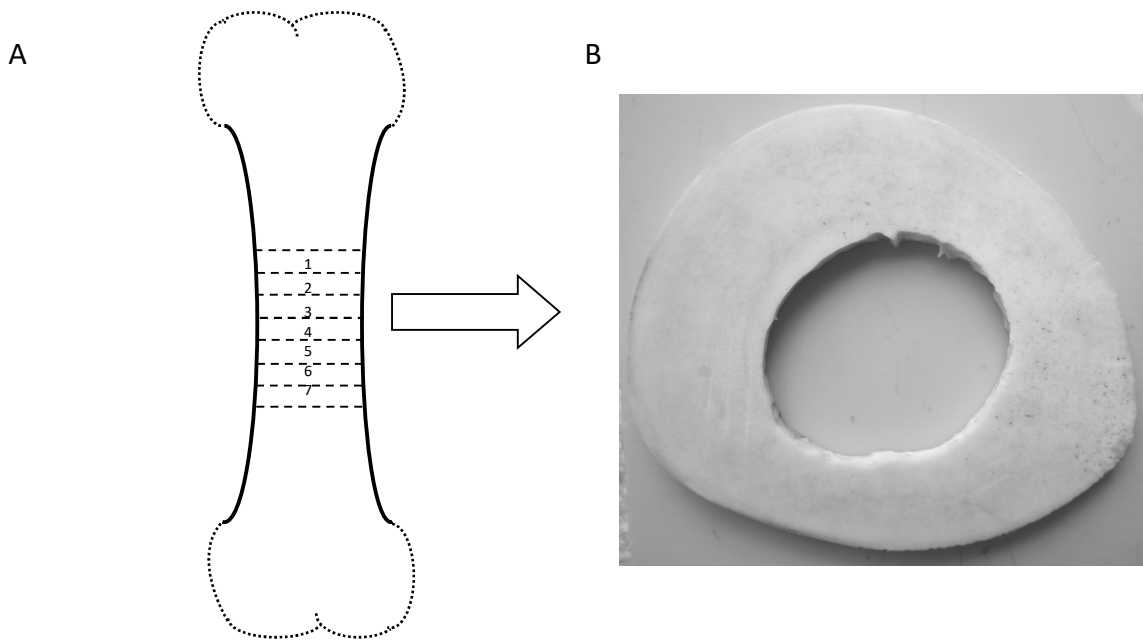


Figure 3.1: Bovine femoral bone. A: Diagrammatic representation of a bovine femoral bone. Highlighted in bold is the diaphysis which was used to produce the bone sections. B: A photograph of a Bovine femoral section.

3.3 Experimental design

In order to accurately determine the effect of heat treatment and water submersion on bone, factors such as water temperature and duration of heat treatment were subject to tight experimental control. The experimental design and the factors that were controlled in each study are described in this section.

3.3.1 Heat treatment study: Investigating the effect of heat treatment on bone

The effects of heat treatment on the properties of bone were investigated over a broad temperature range (50-1000 °C). Two different durations of heat treatment (2 and 4 hours) were used to determine whether the period of heat treatment and/or the maximum temperature reached, had a significant effect on the properties of bone. In each series, the highest temperature at which DNA was detectable (by PCR Section 3.15)) and the lowest temperature at which DNA could not be detected were determined. This represented the temperature range over which DNA degradation occurred. Once the temperature at which DNA began to degrade was determined, the temperature increments used in the experiment were decreased from 50 °C to 10 °C. This enabled the pattern of DNA degradation in both 2 and 4 hour time periods to be determined (Table 3.2).

Table 3.2: Experimental design: heat treatment study

Experiment	Temperature Range (°C)	Temperature Interval (°C)	No. samples per temp.	Time (Hr)
1	50 - 1000	50 (Except between 170 – 220, where 10 °C was used)	3	2
2	50 - 1000	50 (Except between 140 – 200, where 10 °C was used)	3	4

Sections of bovine femur were heat-treated using a Carbolite 2000 furnace (Carbolite Inc, Hope Valley, UK). The furnace was programmed with a ramp rate of 5 °C per minute. Once the required target temperature had been reached, the timer was set. After 2 or 4

hours the samples were removed from the furnace and allowed to cool naturally to room temperature. From each individual bone, one untreated section was used as a control.

3.3.2 Water Submersion study: Investigating the effect of water submersion on bone

Two independent studies, one to simulate a cold-water environment (e.g. The English Channel) and the other to simulate a warm water environment (e.g. The Indian Ocean), were designed to investigate the effect of water submersion on bone (Table 3.3). The purpose was to determine whether water temperature, in addition to the duration of submersion, had a significant effect on the properties of bone.

Table 3.3: Seawater Environments of the English Channel and the Indian Ocean

Factor	English Channel	Indian Ocean
Temperature (°C)	4-15	22-28
Salinity (parts per thousand)	34	30-35
pH	8	7.8-8.3

3.3.2.1 Investigating the effect of warm water submersion on bone

Warm oceans, such as the Indian Ocean, have a temperature between 22-28 °C (Samuel et al., 2006). To simulate such an environment, a series of tanks were set up as shown in Figure 3.2. A water heater (Betta Tech Ltd, Milton Keynes, UK) circulated warm (~27 °C) water throughout a main tank, which in turn heated three independent tanks that were

partially submerged in the warm water (Figure 3.2). The sample tanks containing bone sections were held in place by a Perspex shelf and were covered by a plastic film and a lid in order to reduce evaporation. The main tank was insulated with polystyrene and a lid to maintain the temperature and reduce evaporation.

A

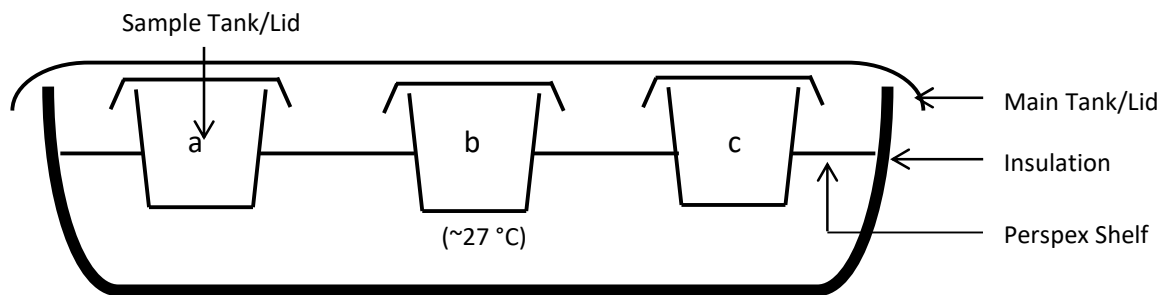
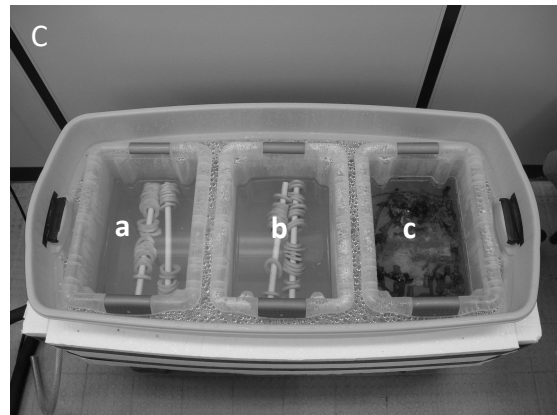


Figure 3.2: Warm water submersion model. A: A diagrammatic representation of the warm water submersion apparatus. Tanks a: bovine bone submerged in distilled water, b: bovine bone submerged in synthetic seawater and c: human bone submerged in synthetic seawater. B: Photograph of the closed tank with surrounding insulation. C: view of the arrangement of sample tanks within the main tank.

3.3.2.2 Investigating the effect of cold water submersion on bone

To maintain a temperature (5-15 °C) similar to that of the English Channel (Uncles and Stephens, 2001), sample tanks were kept in a refrigerator. Due to the differences in temperature that were found within the fridge (temperatures were measured prior to submersion); tanks were manually rotated between shelves every 4 weeks to maintain an average temperature throughout the experiments (Figure 3.3).

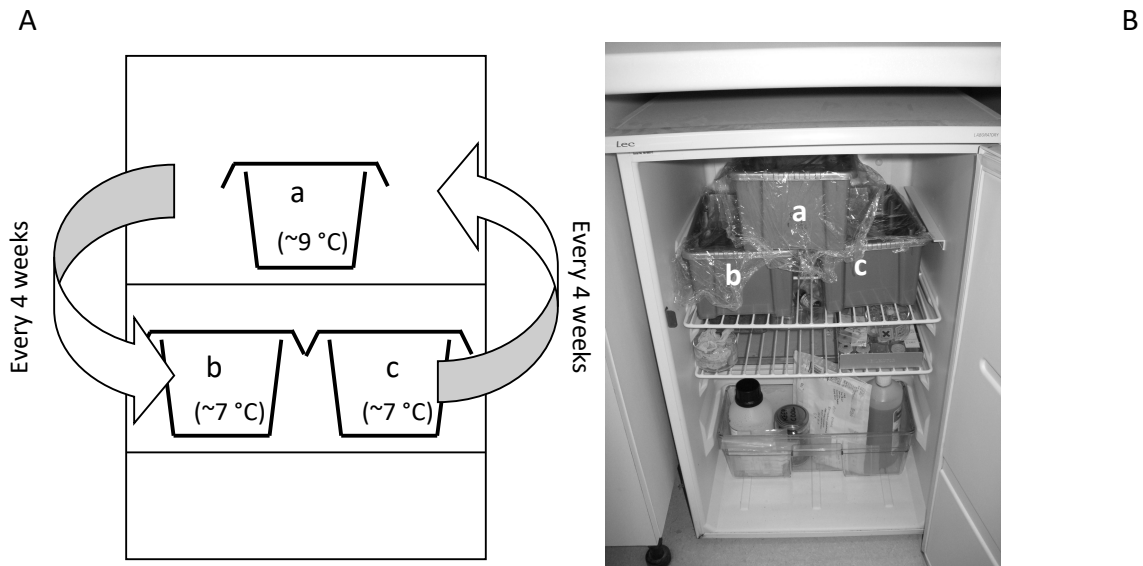


Figure 3.3: Cold water submersion model: A: A diagrammatic representation of the cold water submersion apparatus. Tanks a: bovine bone submerged in distilled water, b: bovine bone submerged in synthetic seawater and c: human bone submerged in synthetic seawater. B: photograph of the sample tanks.

3.3.2.3 Controlling and maintaining water environments

Sample tanks were filled with either distilled water or synthetic seawater. Synthetic seawater was made with Instant Ocean® synthetic sea salt (Instant Ocean, Sarrebourg,

Fr) and distilled water. The salinity of the water was maintained at levels found in ocean seawater, 33-36 parts per thousand (Millero et al., 2008), using a hydrometer (Tetratec, Southampton, UK). The use of Instant Ocean® also meant that the concentrations of other major ions and constituents had a high similarity to natural seawater (Table 3.4). Sample tanks filled with distilled water were used as a control and to determine whether any of the elements found in seawater have an effect on bone degradation. To ensure that a constant environment was maintained, a thermocouple (Fluke, Washington, USA) and pH meter (Jenway, Dunmow, UK) were used to measure temperature and pH respectively every 7 days. The pH of seawater was maintained between 7.5 and 8.5 pH (Prenesti et al., 2007).

Table 3.4: Mean Concentration of the major Ions of natural seawater and Instant Ocean® sea salt

Ion	Natural Seawater (mg/L)	Instant Ocean (mg/L)
Sodium (Na ⁺)	10.781	10.78
Potassium (K ⁺)	0.399	0.42
Magnesium (Mg ²⁺)	1.284	1.32
Calcium (Ca ²⁺)	0.4119	0.4
Strontium (Sr ²⁺)	0.00794	0.0088
Chloride (Cl ⁻)	19.353	19.29
Sulphate (SO ₄ ²⁻)	2.712	2.66
Bicarbonate ((HCO ₃) ⁻)	0.126	0.2
Bromide (Br ⁻)	0.0673	0.056
Boric Acid (B(OH) ₃)	0.0257	-
Fluoride (F ⁻)	0.0013	0.001

3.3.2.4 Bovine femur samples

Thirty-six sections (see, 3.2.1), taken from thirty bovine femora, were placed in four tanks (Table 3.5), suspended in the water by a length of plastic tubing (Figure 3.4).

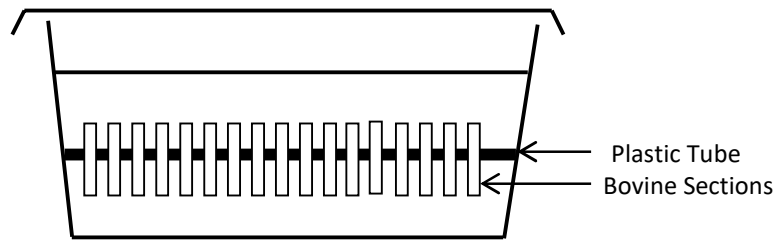


Figure 3.4: Arrangement of bone sections within each tank.

Table 3.5: The temperature and type of water in each tank used in the water submersion study

Tank	Temperature (°C)	Water
1	~8	Distilled
2	~8	Sea
3	~27	Distilled
4	~27	Sea

Three sections were removed from each sample tank after 2, 4 and 8 weeks and then every 12 weeks thereafter. The sections were submerged for a total of 104 weeks. Time increments were initially short in order to determine an appropriate time interval for the project.

3.3.3 Case Study: Human bone samples

As part of this project, human skeletal remains, from a missing person's case, were also studied. The individual, a 26-year-old Caucasian male, had been recovered approximately two years after going missing in the English Channel (missing from August 2005 – August 2007). The individual was found on the seabed only a few hundred metres from the location that he was last seen alive. Therefore, it has been assumed that the body had been submerged for the entire duration. The Cranfield University Health Ethics Committee granted permission for experimental research on this case, and consent was also obtained from the individual's next of kin (Appendix 1). Table 3.6 shows the types of bone that were recovered. Every bone was analysed for DNA STR profiles.

Table 3.6: Bone elements recovered human skeletal remains.

Bone
Clavicle
Calcaneus
Femur
Fibula
Humerus
Intermediate Cuneiforms
Lumbar vertebrae
Lateral Cuneiforms
Medial Cuneiforms
Metatarsals
Navicular
Pelvis
Radius
Rib
Sacrum
Scapula
Talus
Tibia
Thoracic vertebrae
Ulna

3.5 Sample preparation: Bone samples mounted in resin

Bone mounted in hardened resin provides an ideal surface for polishing. This can be used for microscopic imagery and mechanical testing. Samples were cut from the anterior aspect of each section of bone and mounted in hardened resin (Figure 3.5). Samples were mounted using plastic moulds and polyester casting resin (Metprep Ltd, Coventry, UK). The samples were embedded at room temperature and were allowed to set overnight. Mounting the samples using this procedure has been shown to preserve the sample and prevents various cavities from becoming filled with resin (Rho et al., 2002). Mounted samples were then polished using a series of silicon carbide abrasive papers (Metprep Ltd) with decreasing grit size (800, 1200, 2500 and 4000P), on a rotary pre-grinder (Buehler, Illinois, USA). Finally, the samples were metallographically polished using a 0.05 μm alumina suspension (Metprep Ltd). Mounted samples were then washed with distilled water in a sonic bath (Ultratech, Sussex, UK) to remove any debris or alumina residue from exposed bone cavities.

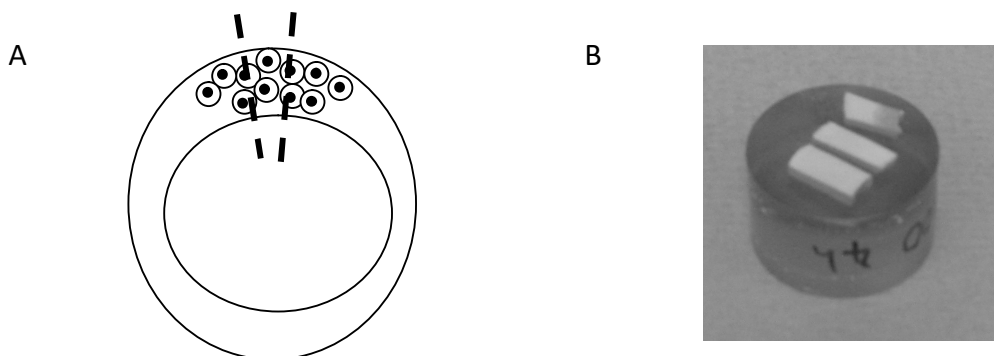


Figure 3.5: Regions of dense Haversian system in bovine sections. A: Diagrammatic representation of regions of dense Haversian systems taken from the anterior aspect of the bovine section B: Photograph of mounted bovine bone.

3.6 Histology

Although numerous studies have investigated the effect of heat treatment on the appearance of Haversian systems, the literature regarding the effect of water submersion on such structures is limited (Section 2.9.2). As a result, only mounted bone samples taken from the water submersion study were assessed with regards to histological features.

3.6.1 Histological: Data collection and analysis

Histological features including Haversian systems, their concentric rings of lamellae and canals were examined under a Polyvor Met light microscope at 50x magnification (Figure 3.6) (Reichert, New York, USA). Images were recorded using a digital camera (Polaroid, Dunbartonshire, UK) and assessed using Paint Shop Pro (JASC, Minnesota, USA).

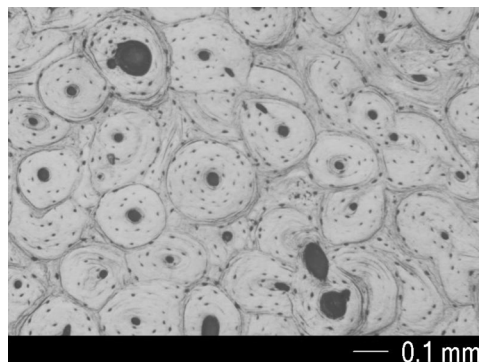


Figure 3.6: Microscopic image of regions of dense Haversian systems in fresh bovine femoral bone. At 50x magnification

3.7 Microhardness

Microhardness has been used to evaluate the mechanical properties of a number of different bones. The method used to measure microhardness has been well documented (Huja et al., 1999) and was used with all samples in this study.

3.7.1 Microhardness: Data collection and analysis

The microhardness of each sample was measured using the Vickers hardness method. The Vickers Hardness (VH) of each bone sample was ascertained using a HWDM-7 microhardness indenter (Indentec, Stourbridge, UK). The indenter was programmed to use a load of 0.1 kg. A load of 0.1 Kg is recommended to record microhardness in wet bone samples as it has been seen to generate the most reproducible results (Johnson and Rapoff, 2007).

Microhardness is measured as the ratio of applied load to indented area. The Vickers hardness (kgf/mm^2) is given as:

Equation 3.1: Vickers Hardness

$$\text{VH} = 2P \sin (\theta/2) / D^2$$

Where, P is the applied load in Kg, D is the mean length of the indented area (mm) and θ is 136° . D is calculated as given by equation 2.

Equation 3.2: Calculating area, D, for Vickers hardness testing

$$D = (w + h) / 2$$

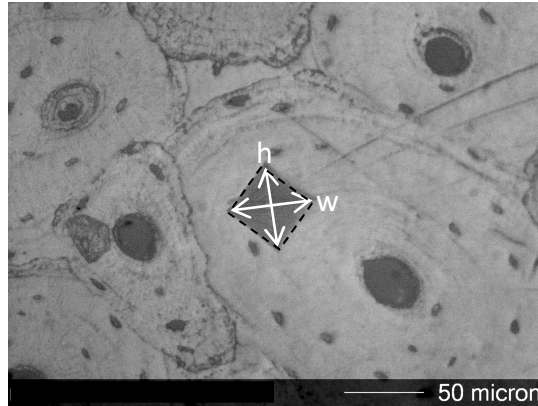


Figure 3.7: Microscopic image of a Microhardness indentation (200x magnification) on a Haversian system in bovine bone.

Bone samples were indented in the regions of dense Haversian systems (Figure 3.7). In order to ensure valid statistical analysis, 12 micro-indents were made per sample.

3.8 Sample preparation: Bone samples milled to a fine powder

Powdered bone samples were analysed using XRD and FTIR spectroscopy. These are two techniques that can be used to provide measures of the structural and chemical composition of bone.

Pieces of bone, measuring $\sim 1 \times 1 \times 5$ mm, were cut from each section and were placed in a 15 ml sterile tube (Fisher Scientific Ltd). The cubes were then cleaned using 10% Decon[®] 90 for 30-60 minutes. After cleaning, the cubes were washed with distilled water and placed in a sonic bath for 15 minutes, a process that was repeated three times. The cubes were then air-dried. Samples that were heated above 200 °C were too brittle to cut into cubes. Instead they were broken manually into pieces of similar size. The cubes were then milled into a powder using a M2000 ball mill and Zirconium mill jars (Retsch Ltd, Leeds, UK). Powdered bone was sieved through a 106 μ m sieve and stored in a specimen tube at room temperature. Mill jars were cleaned prior to use. They were cleaned for 24 hours in 10% Decon[®] 90, washed with 70% ethanol and then air-dried.

3.9 Differential scanning calorimetry (DSC)

Differential Scanning Calorimetry (DSC) is a thermal analytical technique that records differences between endo- and exo-thermic processes occurring in a sample (compared to a reference sample) as the temperature increases.

3.9.1 DSC: Data collection and analysis

Fresh bovine bone powder (20.5 mg) was sealed within an aluminium crucible and using a needle, a hole was pierced in the top to allow gaseous escape. Using a Mettler Toledo M3 DSC, the sample was heated from room temperature at 10°C per minute to the

maximum temperature of 500°C. The M3 DSC measured the heat flux (mW) across the sample as the temperature was increased.

3.10 Thermogravimetric analysis (TGA)

Thermogravimetric Analysis (TGA) is a technique that is used to determine changes in weight in relation to changes in temperature (Section 2.8.1.1).

3.10.1 TGA: Data collection and analysis

Fresh bovine bone powder (50 mg) was sealed within a TGA sample tube. Using a Mettler Toledo TA4000, the sample was heated from room temperature at 10 °C per minute to the maximum temperature of 500°C. The change in weight (%) as the temperature increased was recorded.

3.11 Fourier Transform Infrared (FTIR) spectroscopy

Fourier Transform infrared (FTIR) spectroscopy uses infrared irradiation to study the vibrational modes of bonds in a given molecule and has been extensively used to study the material and structural properties of bone (Section 2.2.1). FTIR spectroscopy was used to analyse both submerged and heat treated samples, plus their controls.

3.11.1 FTIR spectroscopy: Sample preparation

FTIR spectroscopy requires specimens to be mixed with potassium bromide (KBr) and pressed into a disc. KBr is used as a supporting medium that produces sharp absorption spectra which do not contain interfering bands (FernandezBertran and Reguera, 1996). Each disc was made using 0.005 mg bone powder and 0.05 mg of dried powdered (<106 μm sieved) KBr. The bone powder and KBr were mixed thoroughly and pressed in to a pellet using a pressure of 10 tonnes for 30 seconds.

3.11.2 FTIR spectroscopy: Data collection and analysis

The FTIR spectrum of each sample was recorded using a Vector 22 spectrometer (Bruker Optics, Coventry, UK) and analysed using Bruker Optics' OPUS software. Instrument parameters are described in Table 3.7.

Table 3.7: Bruker Optics' OPUS software Instrument parameters

Parameter	FTIR scan
Number of scans	14
Resolution (cm^{-1})	2
Gain	auto
Range (cm^{-1})	400-2000
Mode	absorbance

The FTIR spectra produced (Figure 3.8) were used to measure changes in the mineral and organic properties of the bone. The descriptions of each parameter and how they were calculated can be found in Table 3.8.

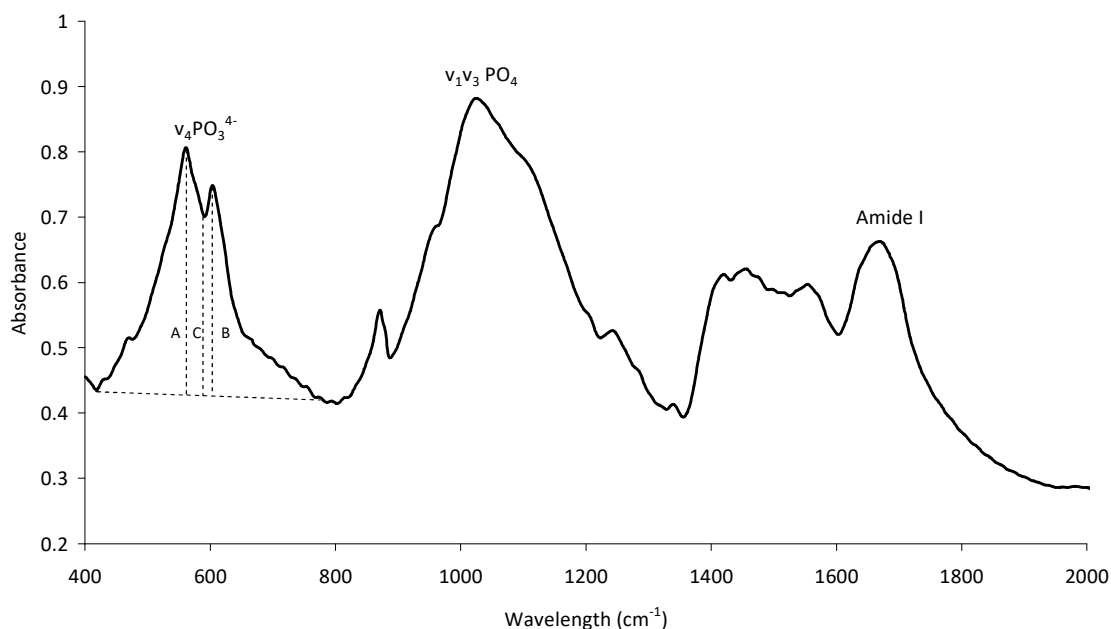


Figure 3.8: The FTIR spectra of fresh bovine bone between 400 and 2000 cm^{-1} . The Splitting Factor was calculated by dividing the sum of the height of peaks A and B, by C, the height of the valley in-between.

Table 3.8: Bone parameters measured by FTIR spectroscopy

Parameter	Definition	Validation
Matrix -to- Mineral	Ratio of area 1585-1720 cm^{-1} (amide I) band/area 900-1200 cm^{-1} phosphate band ($v_1v_3\text{PO}_4$)	Linearly related to ash content of synthetic collagen and apatite mixtures
Splitting Factor	Measured by the sum of the heights of the 563 cm^{-1} (A) and valley between them 603 cm^{-1} (B) phosphate peaks divide by the height of valley between them (C) (Figure 3.8).	Related to crystal size in c-axis dimension determined by X-ray diffraction line broadening.

3.12 X-ray diffraction (XRD)

X-ray Diffraction (XRD) is an analytical technique that can be used to investigate the structural integrity of material, such as calcium hydroxyapatite found in bone (Section 2.3.1). All samples that were heat treated and submerged, including the controls used, were studied using XRD.

3.12.1 XRD: Sample preparation

Bone powder (4.5-5 g) was tightly packed into an XRD specimen plate (Figure 3.9) and the top of the sample was gently flattened, using a microscope cover slide, to produce an even surface.

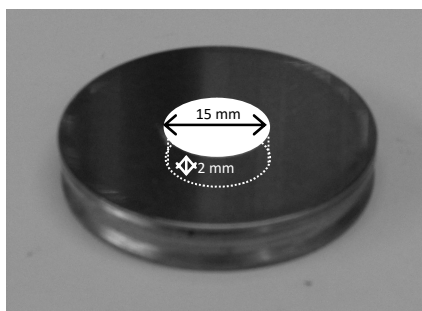


Figure 3.9: XRD specimen plate

3.12.2 XRD: Data collection and analysis

XRD analysis was performed using diffractometers with copper X-ray ($\text{Cu K}\alpha$) radiation (PANalytical, Cambridge, UK). The detectors collected data as stepped scans across an

angular range (Table 3.9). Each sample was scanned across the angular range containing the 002 and 004 peaks (bHAP002 and bHAP004 respectively) and a full diffractogram (bHAP full) of bHAP mineral.

Table 3.9: XRD PANalytical Parameters and profile settings

Parameter	bHAP002	bHAP004	bHAPfull
Angular Range ($^{\circ}/2\theta$)	23-28	50-55	10-600
Count time of each step (sec)	5	5	5
Step size ($^{\circ}/2\theta$)	0.0066	0.0066	0.0066

Topas Software (version 2, 2000) was used to refine each profile. Peaks were fitted using Rietveld Refinement and each peak in the study was fitted using the same protocol. Rietveld Refinement uses 'least square' approach to refine each peak. To do this, the protocol entered into Topas included the emission profile of the Cu K α radiation and instrument details of the diffractometer. Each peak/set of peaks were refined between the angular ranges (Table 3.9). The peaks were refined using a split pseudo-Voigt, which is a method of peak fitting and is an approximation to a Voigt function, which is a combination of Gaussian and Lorentzian peaks (Pecharsky and Zavalij, 2009).

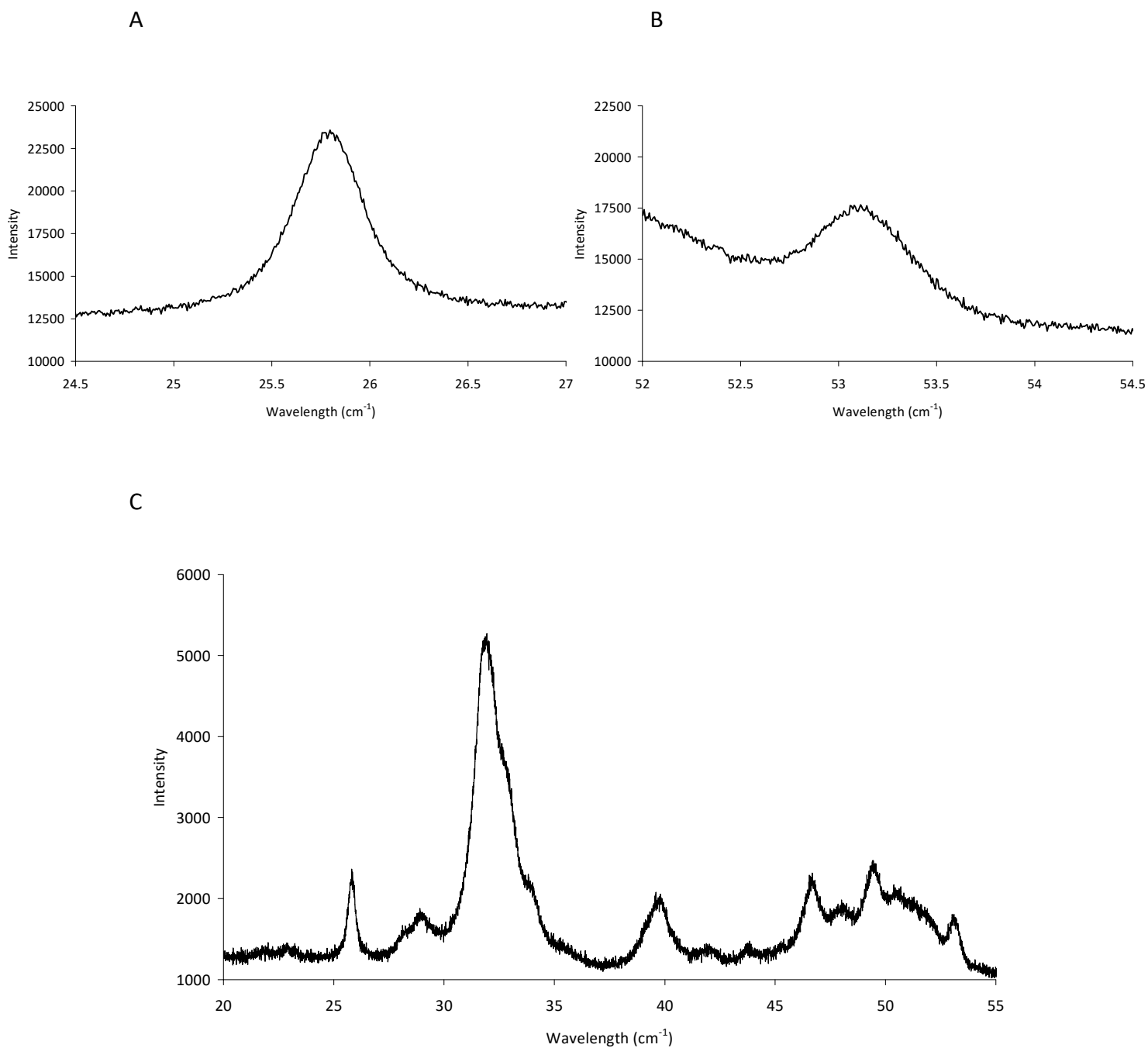


Figure 3.10: Diffractograms of bHAP from fresh bovine bone. A: bHAP 002, B: bHAP 004 and C: bHAP full profile.

The peak broadening of the diffraction profile is a combination of peak broadening from the sample and the diffractometer instrument. The contribution of the bHAP 002 full width half maximum (FWHM) to the measured diffraction profile was calculated using equation 3.3; where F_s , F_0 and F_i are the FWHMs of the sample, diffractometer instrument and measured bHAP 002 diffraction profile (Figure 3.10: A) respectively.

Equation 3.3 Calculation of the bHAP 002 FWHM contribution of a sample to the measured bHAP 002 FWHM:

$$(F_s)^2 = (F_0)^2 - (F_i)^2$$

The bHAP 002 FWHM was used to determine the coherence length of the hydroxyapatite crystals and was calculated using the Scherrer equation (Equation 3.4). F_s is converted to radians, theta (θ) is the 2θ position of the peak (converted into radians), and λ is the wavelength of the Cu K α radiation ($\lambda = 1.54 \text{ \AA}$). A shape factor 0.9 was also used.

Equation 3.4: Scherrer equation

$$CL \text{ (nm)} = 0.01 (0.9\lambda / (F_s) \cos\theta)$$

The crystallite size and strain was measured from Williamson and Hall plots where coordinates (FWHMcos θ , sin θ) from bHAP 002 and 004 are plotted.

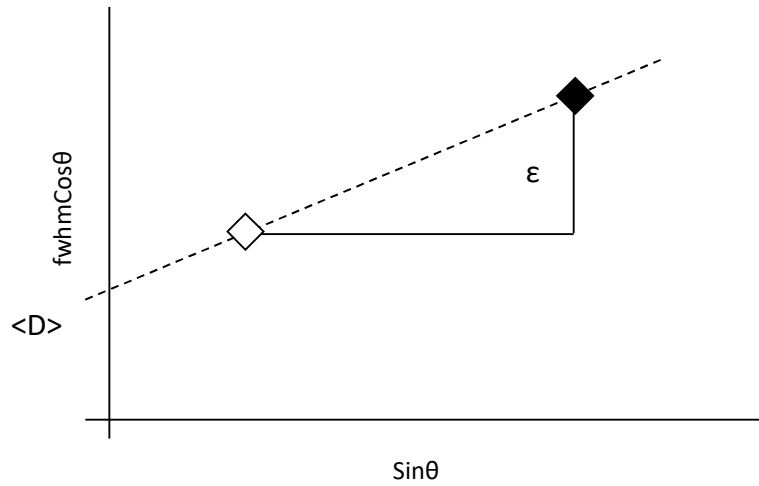


Figure 3.11 Williamson and Hall plot. (◊) 002 (◆) 004

The crystallite size is calculated using equation 3.5, where $\langle D \rangle$ = intercept.

Equation 3.5 Crystallite size

$$\text{Crystallite size (nm)} = 0.1 (\lambda / \langle D \rangle)$$

Equation 3.6 Crystallite strain

$$\text{Crystallite strain (\%)} = \epsilon \times 100$$

3.13 Colour

Due to the distinct colour changes that occur in heated bone (section 2.8.6), the colour of each sample from the heat treatment study was recorded using a colourimeter and the Munsell® colour chart.

3.13.1 Munsell® Colour Chart: Data collection and analysis

The colour of every bone sample in the study was recorded using the Munsell® colour charts (Munsell® Book of colour and Munsell® soil colour charts). Powdered bone was visually matched to a colour pallet and recorded.

3.13.2 Colourimetry: Sample preparation

The smooth area of powdered bone created for the XRD plate was ideal for measuring the colour of the powder (Figure 3.9).

3.13.3 Colourimetry: Data collection and analysis

Using a CR-400 colourimeter (Konica Minolta, Milton Keynes, UK) the colour of each heat-treated sample was recorded. The colourimeter measured Hue (colour), Lightness and saturation which is recorded as $L^*a^*b^*$ (XYZ) coordinates (Figure 3.12: A), where L^*

is lightness and a^* and b^* are chromaticity coordinates (Figure 3.12: B). The colour of each sample was recorded three times to produce an average.

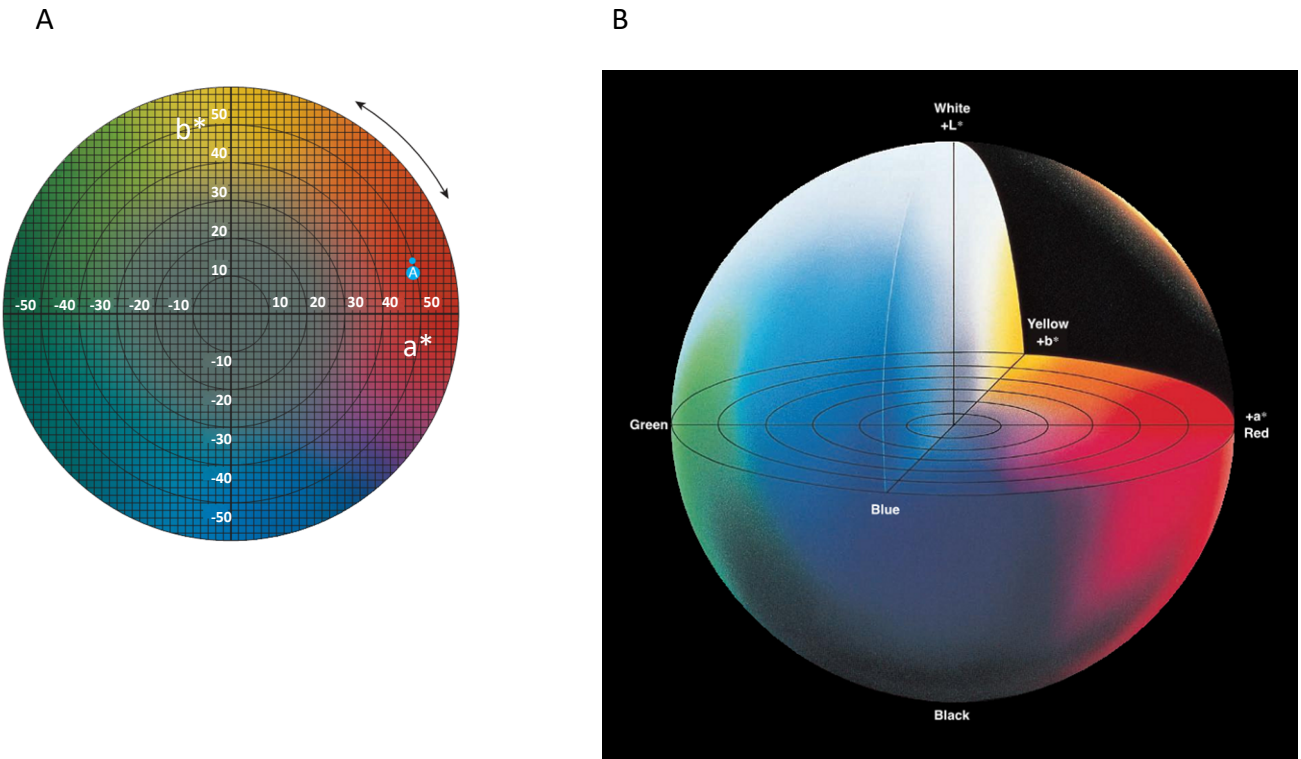


Figure 3.12: Colourimetry: A: $L^*a^*b^*$ colour space chromaticity diagram. B: Representation of colour solid for $L^*a^*b^*$ colour space. Taken with permission from Konica Minolta Inc.

3.14 Sample preparation: Cubed bone samples

Samples for DNA extraction were made by cutting pieces of bone from each section requiring DNA analysis. Using silicon carbide abrasive paper (220P) the surfaces of each cube was cleaned to remove any debris (Metprep Ltd, Coventry, UK). To minimize the

risk of contamination, abrasive paper was replaced after each sample was cleaned. The samples were placed in a 15 ml sterile tube (Fisher Scientific Ltd UK) and then cleaned by submerging the cubes in 10% Decon® 90 (Decon Laboratories Ltd, Sussex, UK) for 30-60 minutes. The bone samples were then washed with distilled water and placed in a sonic bath for 15 minutes. This process was repeated three times. The bone samples were then dried, under UV light, and transferred to a 50 ml sterile tube ready for DNA extraction. Samples that were heated above 200 °C were too brittle to cut into cubes using the bandsaw; instead they were broken manually into pieces of similar size.

3.14.1 DNA extraction

To fulfil the requirements of the proposed hypotheses (Section 1.2), the DNA extraction kit chosen for this work had to yield DNA that was the most desirable (in terms of quality and quantity). Three different DNA extraction kits, which represented different approaches of extracting DNA from forensic samples, were evaluated in order to standardize a protocol that was to be used throughout this work (Table 3.10).

Table 3.10: DNA Extraction Kits

	Invitek: Invisorb® Spin Forensic Kit	Invitek: Invisorb® Forensic Kit	Genial: First DNA; All- tissue DNA-extraction kit
Completion Time	2-24 hrs	15-24 hrs	Up to 1 week

3.14.2 Invitex: Invisorb® sample preparation

- Spin Forensic Kit
- Forensic Kit

The pieces of bone were milled into a powder using a M2000 ball mill (Retsch Ltd, Leeds, UK). Mill cylinders were cleaned prior to use; submerged for 24 hours in 10% Decon®90, washed at least three times with 70% ethanol and then UV treated three times for 20 minutes.

3.14.3 Genial: First DNA; All-tissue DNA-extraction kit Sample preparation

To reduce the risk of contamination, pieces of bone (instead of drill shavings as described in manufacturer's protocol) were submerged in 0.5M, 7.5 pH ethylenediaminetetraacetic acid (EDTA) (Severn Biotech, Worcestershire, UK) for 5 days; the EDTA was changed everyday. After 5 days the cubes were washed with distilled water and 'soft' demineralised bone was removed from each cube using forceps and scalpels (Figure 3.13). To reduce the risk of contamination, disposable sterile scalpels were used. Forceps were washed in 70% ethanol and heated under a flame prior to use and non-sterile plastic weighing boats were treated for at least 30 minutes under UV light.

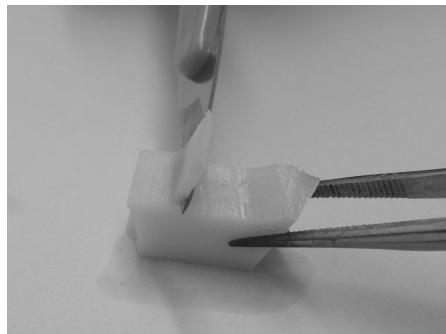


Figure 3.13: Photograph depicting the removal of demineralised bone that had been incubated in EDTA.

3.14.4 Modification to DNA extraction protocols

Unless stated (Table 3.11) each protocol was followed as directed by the manufacturer's manual.

Table 3.11: Modifications made to each protocol.

Modification	Genial	Invisorb	Spin
Amount of Sample (mg)	500 (after demineralisation)	500	500
Proteinase K (20 mg/ μ l)	75 (Roche, Sussex, UK)	As in protocol	As in protocol
Incubation period (hr)	24 hr	24 hr	24 hr
Elution Volume (μ l)	60	60	60

3.15 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is an established molecular biological technique that is used to amplify specific regions of an intact DNA strand (Kleppe et al., 1971, Mullis et al., 1986). DNA primers were designed to amplify nuclear DNA sequences that were conserved across three species, human, cow and pig (Table 3.12). The primers were designed to the c-myc gene. The c-myc gene is a single copy gene found in *H.sapiens* (Accession No. NM_002467.3, chromosome 8), *B.Taurus* (Accession No. NM_001046074.1, chromosome 14) and *S.Scrofa* (Accession No. X297040.1, chromosome 4). Using Bioedit sequence alignment Editor (Ibis Therapeutics, California, UK), the c-myc gene sequence was aligned across the target species and primers amplifying ~100, ~300 and ~500 bp in length were designed. Primers were ordered from Invitrogen Corp, Paisley, UK. Prior to ordering the primers, the Basic Local Alignment Search Tool (BLASTn) on the NCBI website was used to compare potential primer sequences to all available DNA sequences. This was to avoid the amplification of non-target DNA sequences.

Table 3.12: Primer Sequences and amplified fragment sizes

Primer	Primer Set	Sequence (5' - 3')	Amplicon size (bp)
NucFor1	1,2,3	GAA-CTA-TGA-CCT-CGA-CTA-CG	N/A
NucRev1	1	CGA-ATT-TCT-TCC-AGA-TAT-CCT-C	131
NucRev3	2	GCT-CGG-TCA-CCA-TCT-CCA	290
NucRev5	3	GCA-GGT-ACA-AGC-TGG-AGG-T	506

3.15.1 PCR conditions

DNA fragments (Table 3.12) were amplified using the Polymerase Chain Reaction (PCR). Reactions were performed in 0.2 mL PCR tubes (Fisher Scientific, Leicestershire, UK) and each contained the following; 7.5 μ L 2 $\times$ ReddyMix™ PCR Master Mix (ABgene, Surrey, UK), 4.5 μ L PCR grade water (Severn Biotech Ltd, Worcestershire, UK), 1.0 μ L forward primer (10 μ M), 1.0 μ L reverse primer (10 μ M) and 1.0 μ L template DNA. The same thermal cycling parameters were used for all primer sets and were as follows; an initial denaturation at 94 °C for 5 minutes, then 34 cycles of 94 °C for 30 seconds / 55 °C for 30 seconds / 72 °C for 30 seconds, with a final extension period at 72 °C for 10 minutes (Figure 3.14). After completion, tubes were stored at 4 °C until the PCR products were analysed by agarose gel (2%) electrophoresis. DNA extracted from calf thymus (Invitrogen Corp, Paisley, UK) was used as a positive control. A PCR master mix without sample DNA was also used as a negative control. The results were then recorded using a Photodoc-it gel recording system (UVP, Cambridge, UK).

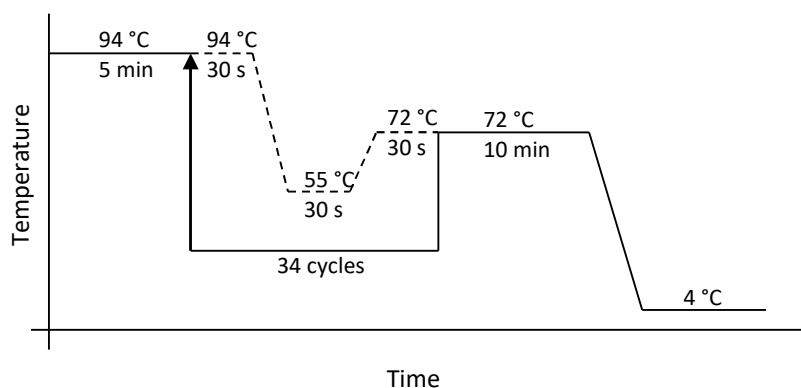


Figure 3.14: Thermal cycle parameters for PCR primers amplifying ~100, ~300 and ~500 bp.

3.15.2 PCR optimisation

Serial dilutions using control bovine DNA (10 to 0.001 ng/ μ l) were used to measure the sensitivity of the DNA primers sets (Table 3.12) and aid in their optimisation (Figure 3.15). The PCR conditions were optimised so that all three primer sets were sensitive enough to detect 0.01 ng template DNA concentrations per reaction.

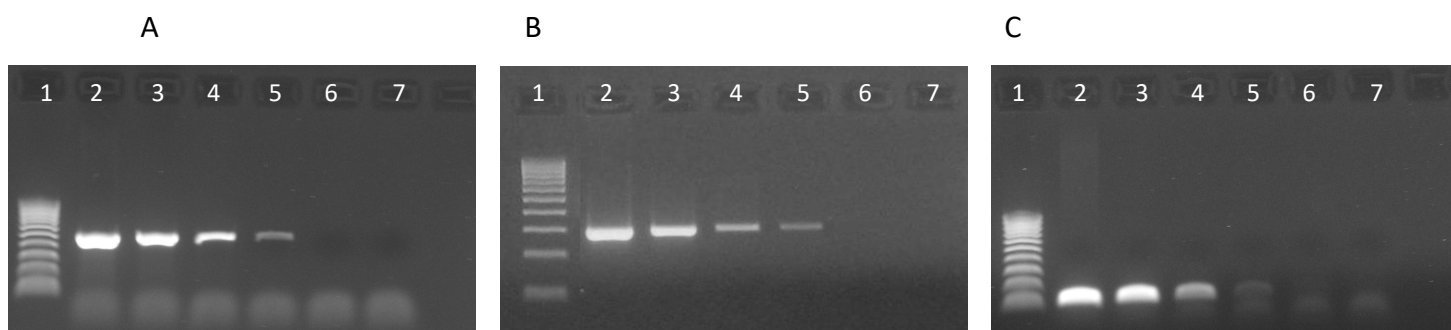


Figure 3.15: Optimisation of PCR primers. DNA fragments; A: 500 bp B: 300 bp and C: 100 bp. Well numbers; 1: 100 bp ladder, 2: control bovine DNA (10ng/ μ l), 3: control bovine DNA (1ng/ μ l), 4: control bovine DNA (0.1ng/ μ l), 5: control bovine DNA (0.01ng/ μ l), 6: control bovine DNA (0.001ng/ μ l) and 7: negative control. Note 1 μ l of template DNA was added per reaction.

3.15.3 PCR: Data collection and analysis

PCR was used to determine whether each amplicon could be successfully amplified from each sample. Therefore, PCR results were recorded as either positive (+) or negative (-).

All DNA primer sets were designed to the same target sequence and all used a common forward primer. It was, therefore, assumed that if the 500 bp DNA fragment could be amplified from a sample, the 300 bp and 100 bp DNA fragments would have also been

amplifiable. Therefore, 300 and 100 bp fragments were not necessarily amplified using PCR. Similarly, when the 300 bp DNA fragment was amplified; it was assumed that the 100 bp fragment would have been.

3.16 DNA profiling

Commercially available DNA typing kits, PlowerPlex® 16 HS (Promega, Southampton, UK) and StockMarks® for Cattle Bovine (ABI Biosystems, California, USA) were used to genotype human and cow samples respectively.

3.16.1 Bovine genotyping: Data collection

The StockMarks® for Cattle Bovine Genotyping Kit, utilises PCR to amplify a total of 11 unlinked microsatellite loci (Table 3.17). Bovine DNA samples were genotyped using the thermal cycling parameters (Figure 3.16) and PCR protocol described in the manufacturer's manual. The number of loci that amplified successfully from each sample was recorded, as were any 'allele drop outs' (loci which appeared homozygous, but were known to be heterozygous).

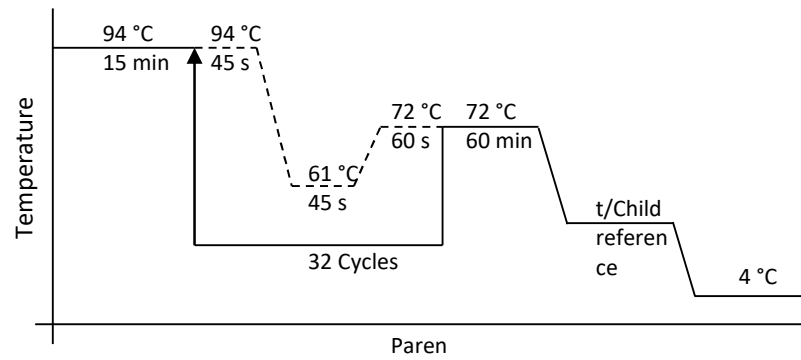


Figure 3.16: StockMarks® for Cattle Bovine Genotyping Kit Thermal Cycling parameters.

Table 3.13: Bovine-Specific Loci

Locus	Name	Size Range (bp)
1	TGLA227	64-115
2	BM2113	116-146
3	TBLA53	147-197
4	ETH10	198-234
5	SPS115	104-131
6	TGLA126	235-265
7	TGLA122	134-193
8	INRA23	193-235
9	ETH3	90-135
10	ETH225	135-165
11	BM1824	170-218

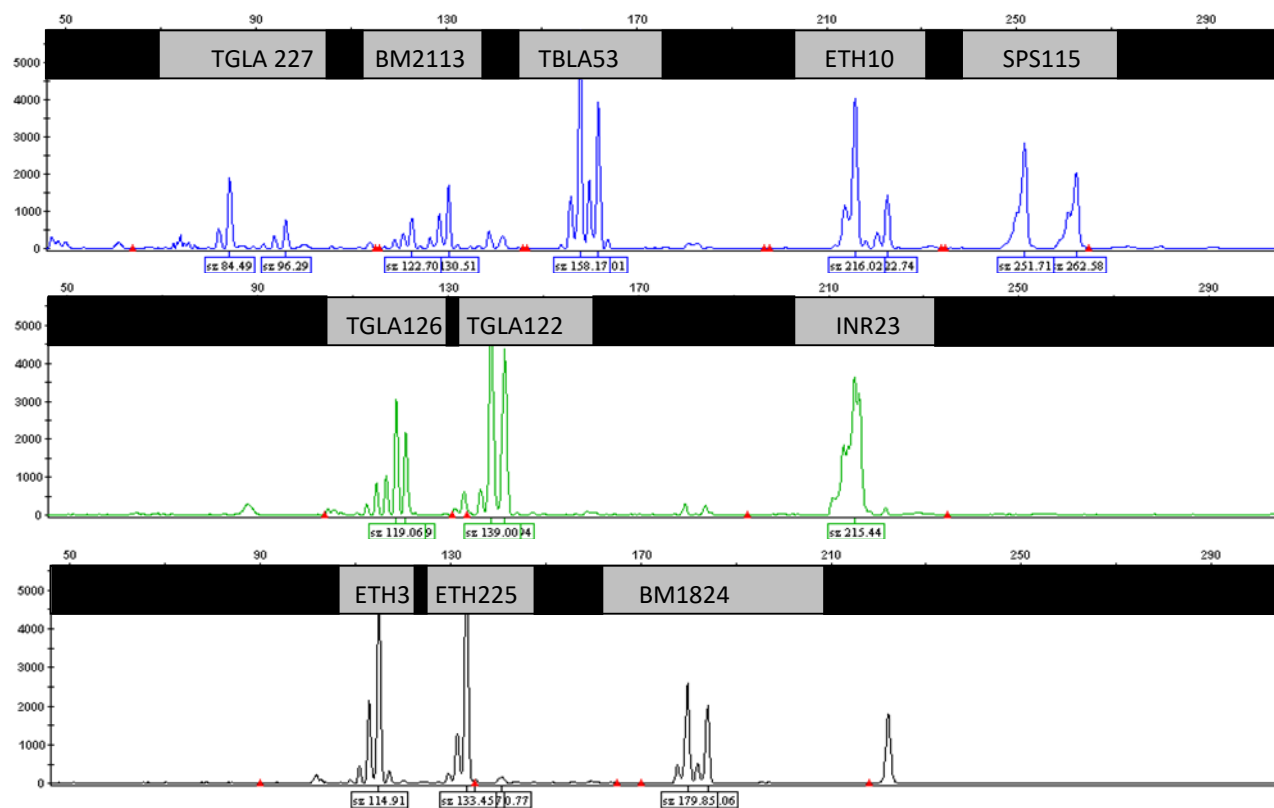


Figure 3.17: Genotype software analysis of PCR amplification products amplified from bovine control DNA (ABI Biosystems, California, USA) separated on an ABI Prism 310 Genetic Analyzer. A: FAM (Blue) alleles; 1-5, B: JOE (Green) alleles; 6-8, C: TAMRA (yellow) alleles; 9-11.

3.16.2 Human Genotyping: data collection

The PlowerPlex® 16 HS Human Genotyping Kit utilises PCR to amplify 15 unlinked microsatellite loci and amelogenin (a gender specific marker) (Figure 3.18). Human DNA samples were genotyped using the thermal cycling parameters (Figure 3.17) and PCR protocol as described in the manufacturer's manual. The number of loci successfully amplified and any 'allele drop outs' were recorded (Figure 3.18).

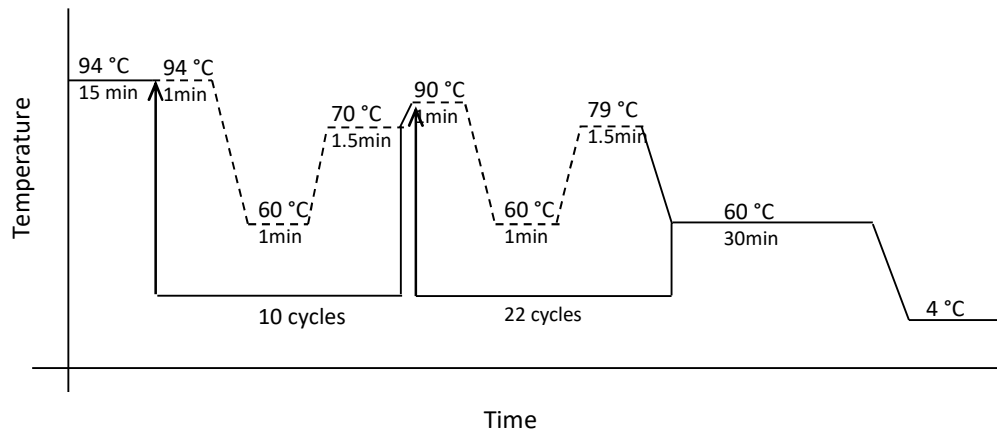


Figure 3.18: PlowerPlex® 16 HS Human Genotyping Kit Thermal Cycling parameters.

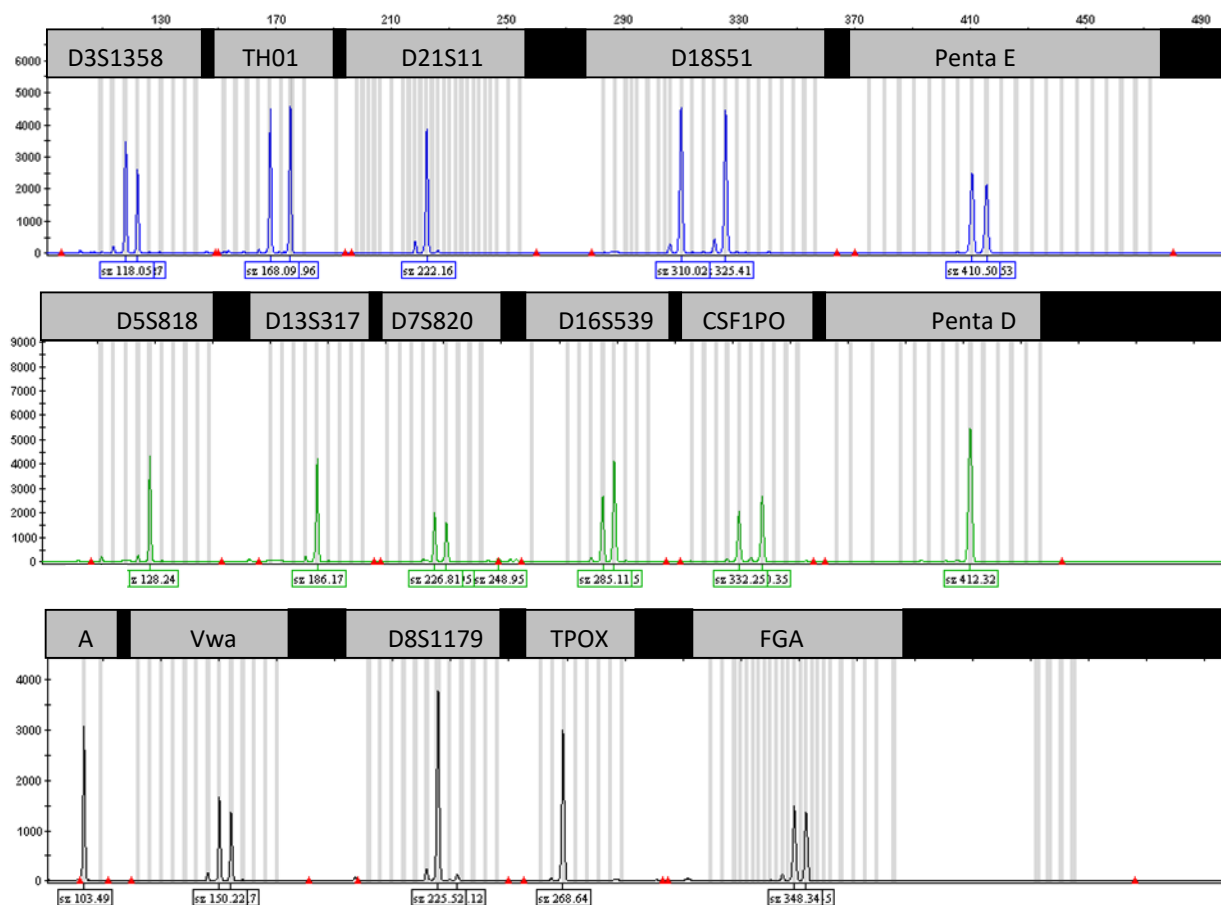


Table 3.14: Human-Specific Loci

Locus	Name	Size Range (bp)
1	D3S1358	115-147
2	TH01	156-195
3	D21S11	203-259
4	D18S51	290-366
5	Penta E	379-474
6	D5S818	119-155
7	D13S317	176-208
8	D7S820	215-247
9	D16S539	264-304
10	CSF1PO	321-357
11	Penta D	376-449
12	Amelogenin (A)	106,112
13	Vwa	123-171
14	D8S1179	203-247
15	TPOX	262-290
16	FGA	322-444

Figure 3.19: Genotype software analysis of PCR amplification products using human control DNA (ABI Applied Biosystems) separated on an ABI Prism 310 Genetic Analyzer. Alleles; 1-16 (Table: 3.14) are labelled. A: FAM (Blue) alleles; 1-5, B: JOE (Green) alleles; 6-8, C: TAMRA (yellow) alleles; 9-11.

3.16.3 Using bovine STR markers as a model for human STR markers

The Bovine Stockmark® kit was used to investigate the effect of heat treatment on STR loci amplification. However, as the intention was for this to serve as a model in a human forensic context, human allelic frequency data was used to calculate the minimum number of loci that would be required to establish an individual's identity with >99% confidence. Two common scenarios were considered, one where the DNA profile was matched to a reference sample originating from the same individual (e.g. a toothbrush) and another where it was matched to the profile obtained from a parent/child. As allele frequency and locus heterozygosity vary considerably between STR markers, a 'worst case average' allele frequency was calculated by taking the mean of the highest Caucasian allele frequency at each locus with Powerplex16® marker set (Promega.com). This produced a 'worst case average' allele frequency value of 0.285. Using this value, the corresponding identity (II) and parent/child (PI) indices for a single locus were calculated using the formulae shown in Table 3.14. These PI and II values could then be used to determine the minimum number of similar loci that would be required for positive identification (with >99% confidence) in each scenario (Table 3.15). It was calculated that to match a profile, when using a reference sample originating either from the same individual or a parent/child, a minimum of three and seven STR loci were required (with >99% confidence) respectively.

Table 3.15: Identity and parent/child index formulae for different genotype combinations

Direct individual match			
unknown		known	
p	p	$1/p^2$	12.31
p	q	$1/2pq$	6.16
Average:			9.24

Parent/child match			
Alleged Father	Child	PI	
q	q	$1/q$	3.51
q	pq	$1/2q$	1.75
qr	q	$1/2q$	1.75
		$(p+q) /$	
pq	pq	$4pq$	1.75
qr	pq	$1/4q$	0.877
Average:			1.93

Table 3.16: Profile match probability using reference samples. A: From a parent/child, B: from the individual

A				B			
No Loci	II	CII	Probability at 50% prior	No Loci	PI	CPI	Probability at 50% prior
1	9.24	9.24	90.23	1	1.93	1.93	65.87
2	9.24	85.3776	98.84	2	1.93	3.72	78.84
3	9.24	788.889	99.87	3	1.93	7.19	87.79
4	9.24	7289.335	99.99	4	1.93	13.87	93.28
5	9.24	67353.45	>99.99	5	1.93	26.78	96.4
6	9.24	622345.9	>99.99	6	1.93	51.68	98.1
7	9.24	5750476	>99.99	7	1.93	99.75	99.01
8	9.24	53134399	>99.99	8	1.93	192.51	99.48
9	9.24	4.91E+08	>99.99	9	1.93	371.55	99.73
10	9.24	4.54E+09	>99.99	10	1.93	717.09	99.86
11	9.24	4.19E+10	>99.99	11	1.93	1383.98	99.93
12	9.24	3.87E+11	>99.99	12	1.93	2671.08	99.96
13	9.24	3.58E+12	>99.99	13	1.93	5155.19	99.98
14	9.24	3.31E+13	>99.99	14	1.93	9949.52	>99.99
15	9.24	3.06E+14	>99.99	15	1.93	19202.58	>99.99

*Highlighted are the minimum number of loci need to match a profile with 99% confidence for each scenario.

3.16.3.1 Bovine Stockmark® genotyping: data analysis

Depending on the scenario (see below), each sample that was genotyped using the bovine Stockmark® genotyping kit was recorded as either a positive or a negative.

Scenario 1: Reference sample originating from the same individual

Samples from which ≥ 3 STR loci were successfully amplified were recorded as positives (+). Samples from which < 3 STR loci were amplified were recorded as negatives (-).

Scenario 2: Reference sample originating from parent/child

Samples from which ≥ 7 STR loci were successfully amplified were recorded as positives (+). Samples from which < 7 STR loci were amplified were recorded as negatives (-).

3.17 Statistical Analysis

The data from each analytical technique used (e.g. FTIR, XRD etc.) was correlated with the amplification of specific PCR products (see 3.15) and the number of successfully amplified STR loci (see 3.16.3). To do so, a binary code was applied to the DNA positive and negatives results. Positives (+) were recorded as one (1) and negatives as zero (0).

Two statistical methods were chosen to carry out the analysis, logistic regression and multivariate quadratic discriminant (MQD) analysis.

3.17.1 Logistic Regression

Logistic regressions are used to calculate the probability of a particular event occurring. In this study, the results of DNA amplification (both amplicon and STR loci amplification) have been translated into a binary code (see above). By fitting data to a logit function the probability of successfully amplifying the amplicon/minimum number STR loci can be calculated (Cooper, 1983). Further details are described in Cooper, 1983.

The logistic regression functions usually take the following form:

$$y = 1 / (1 + \exp[-(a-bx)])$$

Where, y is probability of the event occurring, x is the parameter measured and a and b are constants.

3.17.2 Multivariate quadratic discriminant (MQD) analysis

Multivariate quadratic discriminant (MQD) analysis is a method that can be used to predict a certain outcome using a number of variables (Seber, 2008). The colourimetry results required MQD analysis. Further details are described in Seber, 2008.

Equation 3.7: Multivariate quadratic discriminant function

$$\frac{f_1(0)}{f_2(1)} = \frac{|\Sigma_2|^{\frac{1}{2}}}{|\Sigma_1|^{\frac{1}{2}}} \exp \left[\left\{ \nu'(\Sigma_1^{-1} - \Sigma_2^{-1})\nu - 2\nu'(\Sigma_1^{-1}\mu_1 - \Sigma_2^{-1}\mu_2) + \mu_1'\Sigma_1^{-1}\mu_1 - \mu_2'\Sigma_2^{-1}\mu_2 \right\} \right]$$

μ_1 = mean parameter value of samples from which DNA was successfully amplified (1)

μ_2 = mean parameter value of samples from which DNA was unsuccessfully amplified (0)

ν_1 = covariance parameter values of samples from which DNA was successfully amplified (1)

ν_2 = covariance parameter value of samples from which DNA was unsuccessfully amplified (0)

3.17.3 Sample classification

For each model, a probability, of whether the specified amplicon or minimum number of STR loci would be successfully amplified, was calculated for every sample. The samples were then classified as follows;

Probability 0-0.5 = negative sample (i.e. would not successfully amplify DNA fragments)

Probability 0.5-1.0 = positive sample (i.e. would successfully amplify DNA fragments)

The number of true positives and true negatives were then calculated to determine the performance of the model. From this the percentages of false positives and negatives can be devised.

3.17.4 Area under the receiver operating characteristic curve

The area under the receiver operating characteristic (AUROC) curve is a statistical measure that determines the performance of a model. The ROC curve plots sensitivity and specificity, where sensitivity is the percentage of correctly classified positive samples and specificity is the percentage of correctly classified negative samples. AUROC curve is scored between 0 and 1. An optimal model would achieve 100% sensitivity and specificity; with an AUROC curve of one.

Chapter 4 Results

Chapter 3 describes the analytical techniques that were used to investigate various properties of bone that had been heated or submerged in water and their subsequent correlation with DNA amplification. In addition to these techniques, a number of secondary techniques were used to support any correlations that were found. This chapter presents the results of this work in four sections (Table 4.1).

Table 4.1: Sections of chapter 4

Section	Study
4.1	Preliminary
4.2	Heat Treatment
4.3	Submerged
4.4	Case

4.1 Comparison of three commercial DNA extraction protocols

To compare the quantity of DNA extracted using each protocol, a 10 fold serial dilution was used and the minimum dilution factor at which no DNA (500 bp amplicon) could be amplified was recorded (section 3.14.1). The Genial®: First DNA; all tissue kit underwent 5-6 fold serial dilutions (1:100,000 – 1:1,000,000) before the amplification of the 500 bp amplicon failed. In comparison, both Invitex: Invisorb® DNA extraction kits underwent only 2-3 fold serial dilutions (1:100-1:1000), before amplification failure was observed (Table 4.2). The Genial®: First DNA; all tissue kit extracted was therefore chosen as the standard DNA extraction protocol for use in this study.

Table 4.2: Serial Dilution results

Protocol	Bone	No. dilution
Genial: First DNA; All tissue kit	1	6
	2	5
	3*	6
Invitek: Invisorb® Forensic kit	1	2
	2	2
	3*	3
Invitek: Invisorb® Spin Forensic kit	1	3
	2*	3
	3	3

*Illustrated in Appendix 2.

4.2 Heat treatment study

The results from the heat treatment study are sequentially presented in this section as shown in Table 4.3. The heat-treated sample set used in this investigation incorporated two series; samples that were heat-treated for two and for four hours (section 3.3.1).

Table 4.3: Presented heat treatment study

Section	Technique	Factor Measured
4.2.1	DNA amplification	Amplicon amplification STR Profiling
4.2.2	Colourimetry	Colour
4.2.3	FTIR spectroscopy	Crystallinity (Splitting Factor) Amide to Phosphate Ratio
4.2.4	XRD	Crystallinity (Coherence Length) Crystallite Size Crystallite Strain Lattice Parameters
4.2.5	Microhardness	Vickers Hardness
4.2.6	DSC	Heat Flow
4.2.7	TGA	Weight Loss

4.2.1 Amplification of PCR products: Heat treatment study

The polymerase chain reaction (PCR) can be used to detect (within the optimised sensitivity limits of the assay) whether a given sequence of intact DNA is present in a sample (section 3.15.2). Samples that had been heated over a range of temperatures were tested for specific DNA fragment amplification (section 3.3.1).

4.2.1.1: PCR amplification of DNA from bone heated to different temperatures for two and four hours

A similar pattern of PCR fragment amplification was found between samples which were heat-treated for two (Table 4.4, A) and four (Table 4.4, B) hours. In both sample series the maximum length of amplifiable PCR product decreased as temperature increased. In samples that were heated for four hours, this effect was observed at a lower temperature, when compared to samples heated for two hours. The 500 bp DNA fragment was successfully amplified from all control samples. However, when samples were heated above 180 or 160 °C for two and four hours respectively, the 500 bp fragment could not be amplified. Correspondingly, the 300 bp DNA fragment could not be amplified from samples that were heated above 200 °C (2 hrs) or 190 °C (4 hrs), whilst above 210 °C (2 hrs) and 190°C (4 hrs) the 100 bp DNA fragment was no longer amplifiable. DNA degradation was therefore a function of both temperature and duration.

Table 4.4: PCR Products from heated bone. Heat-treated for; A: two hours, B: four hours

A

Temp. (°C)	DNA Amplicon (bp)		
	500	300	100
Control (x9)	+	+	+
50	+	+	+
50	+	+	+
50	+	+	+
100	+	+	+
100	+	+	+
100	+	+	+
140	#	#	#
140	#	#	#
140	#	#	#
150	+	+	+
150	+	+	+
150	+	+	+
160	#	#	#
160	#	#	#
160	#	#	#
170	+	+	+
170	+	+	+
170	+	+	+
180	+	+	+
180	+	+	+
180	-	+	+
190	-	-	+
190	-	+	+
190	-	+	+
200	-	-	+
200	-	-	+
200	-	-	+
210	-	-	-
210	-	-	-
210	-	-	-
220	-	-	-
220	-	-	-
220	-	-	-

B

Temp. (°C)	DNA Amplicon (bp)		
	500	300	100
Control (x9)	+	+	+
50	+	+	+
50	+	+	+
50	+	+	+
100	+	+	+
100	+	+	+
100	+	+	+
140	+	+	+
140	+	+	+
140	+	+	+
150	+	+	+
150	+	+	+
150	+	+	+
160	+	+	+
160	+	+	+
160	-	+	+
170	-	-	+
170	-	-	+
170	-	-	+
180	-	-	+
180	-	-	-
180	-	-	+
190	-	-	-
190	-	-	-
190	-	-	-
200	-	-	-
200	-	-	-
200	-	-	-
210	#	#	#
210	#	#	#
210	#	#	#
220	#	#	#
220	#	#	#
220	#	#	#

Key: + positive result, - negative result,

Denotes samples that were not analysed but were assumed would have amplified successfully (grey), or have failed to amplify (black).

4.2.1.2 DNA profiling from heated bone

DNA profiling, using STRs, has been developed to aid individual identification (section 2.5.3). A full profile was successfully amplified from each individual animal that was used as a control in the heat treatment study. The control profiles were then used as a reference to calculate the number of loci successfully amplified from each heat-treated bone sample.

The correlation between the number of amplified loci and temperature was similar between the two sample series. Once a particular temperature was reached, there was a decrease in the number of STR loci that were successfully amplified. This effect occurred at a lower temperature when samples were heated for four hours when compared to those heated for two (Figure 4.1).

From samples that were heat treated for two hours, the number of STR loci that were successfully amplified decreased from 11 (full profile) to zero within a 30 °C range; the number of STR loci amplified began to decrease above 170 °C and no profile could be detected above 200 °C. A similar trend was observed in samples that were heat treated for four hours. The number of loci that were successfully amplified dropped from 11 to zero in a 30 °C range; the decrease began after 160 °C and no profile could be detected above 190 °C (Figure 4.1).

In both sample series, it was also observed that the initial decrease in the number of STR loci amplified was due to the unsuccessful amplification of larger loci, including SPS115, ETH10 and INRA23. As the temperature increased, allele drop out was also observed from the smaller loci e.g. TLGA53 and TGLA126 (Tables 4.5 and 4.6). The STR loci successfully amplified decreased in number and size, as the temperature and duration increased.

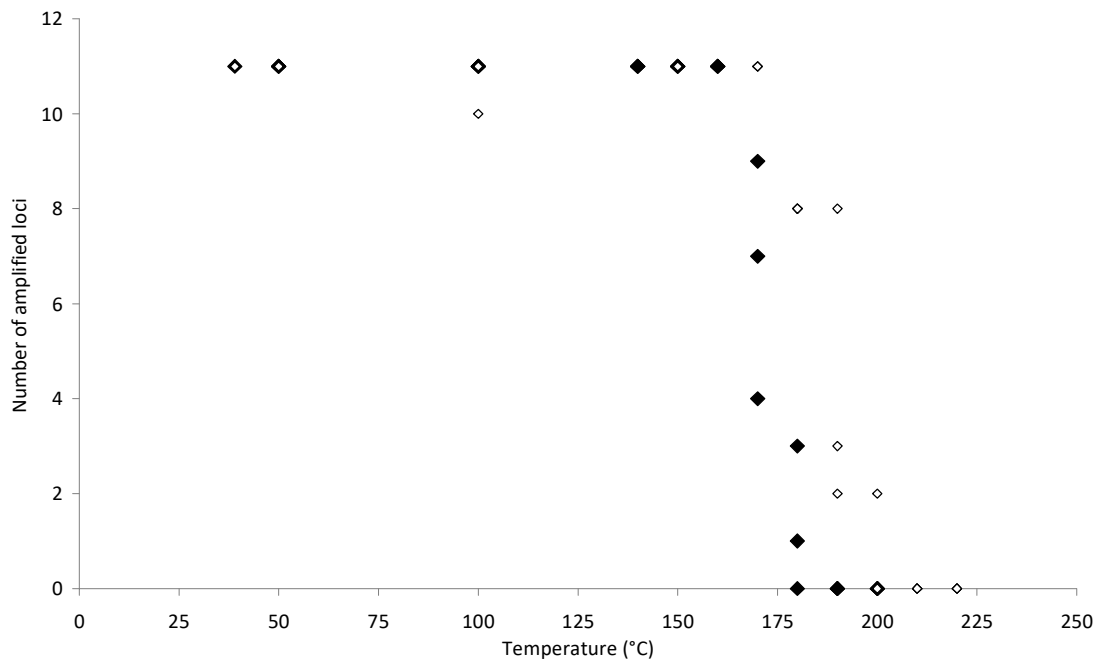


Figure 4.1: The relationship between the number of successfully amplified STR loci from bone, heated at different temperatures for (◇) 2 hours and (◆) 4 hours.

Table 4.5: DNA profiles from bovine femora heat-treated between 39 and 220 °C for two hours

		Stockmark® Genotype for Cattle STR Loci (size range bp)												
Bone No.	Temp	TGLA227 (64-115)	ETH3 (90-135)	TGLA126 (104-131)	BM2113 (116-146)	TGLA122 (134-193)	ETH225 (135-165)	TGLA53 (147-197)	BM1824 (170-218)	INRA23 (193-235)	ETH10 (198-234)	SPS115 (235-265)	Loci	
1-9	39	+	+	+	+	+	+	+	+	+	+	+	11	
4	50	+	+	+	+	+	+	+	+	+	+	+	11	
5	50	+	+	+	+	+	+	+	+	+	+	+	11	
6	50	+	+	+	+	+	+	+	+	+	+	+	11	
1	100	+	+	+	+	+	+	+	+	+	+	-	10	
2	100	+	+	+	+	+	+	+	+	+	+	+	11	
3*	100	+	+	+	+	+	+	+	+	+	+	+	11	
4	150	+	+	+	+	+	+	+	+	+	+	+	11	
5	150	+	+	+	+	+	+	+	+	+	+	+	11	
6	150	+	+	+	+	+	+	+	+	+	+	+	11	
7*	170	+	+	+	+	+	+	+	+	+	+	+	11	
8	170	+	+	+	+	+	+	+	+	+	+	+	11	
9	170	+	+	+	+	+	+	+	+	+	+	+	11	
7	180	+	+	+	+	+	+	+	+	+	+	+	8	
8*	180	+	+	+	+	+	+	+	+	-	-	-	8	
9	180	+	+	+	+	+	+	+	+	-	-	-	8	
7	190	+	+	-	+	-	-	+	-	-	-	-	2	
8*	190	+	+	+	-	+	+	-	+	-	-	-	3	
9	190	+	+	+	+	-	+	+	+	+	+	-	8	
1*	200	+	+	+	+	-	-	-	-	-	-	-	2	
2	200	-	-	-	-	-	-	-	-	-	-	-	0	
3	200	-	-	-	-	-	-	-	-	-	-	-	0	
4*	210	-	-	-	-	-	-	-	-	-	-	-	0	
5	210	-	-	-	-	-	-	-	-	-	-	-	0	
6	210	-	-	-	-	-	-	-	-	-	-	-	0	
4	220	-	-	-	-	-	-	-	-	-	-	-	0	
5	220	-	-	-	-	-	-	-	-	-	-	-	0	
6	220	-	-	-	-	-	-	-	-	-	-	-	0	

+ = Allele Present, - = Allele not present,

N.B. Examples of raw data (samples denoted with *) can be found in Appendix A4.1

Table 4.6: DNA profiles from bovine femora heat-treated between 39 and 200 °C for four hours

		Stockmark® Genotype for Cattle STR Loci (size range bp)												
Bone No.	Temp	TGLA227 (64-115)	ETH3 (90-135)	TGLA126 (104-131)	BM2113 (116-146)	TGLA122 (134-193)	ETH225 (135-165)	TGLA53 (147-197)	BM1824 (170-218)	INRA23 (193-235)	ETH10 (198-234)	SPS115 (235-265)	Loci	
1-9	39	+	+	+	+	+	+	+	+	+	+	+	11	
4	50	+	+	+	+	+	+	+	+	+	+	+	11	
5	50	+	+	+	+	+	+	+	+	+	+	+	11	
6	50	+	+	+	+	+	+	+	+	+	+	+	11	
1	100	+	+	+	+	+	+	+	+	+	+	+	11	
2	100	+	+	+	+	+	+	+	+	+	+	+	11	
3	100	+	+	+	+	+	+	+	+	+	+	+	11	
7	140	+	+	+	+	+	+	+	+	+	+	+	11	
8	140	+	+	+	+	+	+	+	+	+	+	+	11	
9	140	+	+	+	+	+	+	+	+	+	+	+	11	
4	150	+	+	+	+	+	+	+	+	+	+	+	11	
5	150	+	+	+	+	+	+	+	+	+	+	+	11	
6	150	+	+	+	+	+	+	+	+	+	+	+	11	
4	160	+	+	+	+	+	+	+	+	+	+	+	11	
5	160	+	+	+	+	+	+	+	+	+	+	+	11	
6*	160	+	+	+	+	+	+	+	+	+	+	+	11	
7*	170	+	+	+	+	+	+	+	+	+	+	-	9	
8	170	+	+	+	+	+	+	+	+	+	+	-	7	
9	170	+	-	+	+	+	+	+	+	+	+	-	4	
7*	180	+	+	+	-	+	+	+	-	+	-	-	3	
8	180	+	+	+	+	-	+	-	-	-	-	-	1	
9	180	+	-	-	-	-	-	-	-	-	-	-	0	
1	190	-	-	-	-	-	-	-	-	-	-	-	0	
2*	190	-	-	-	-	-	-	-	-	-	-	-	0	
3	190	-	-	-	-	-	-	-	-	-	-	-	0	
4	200	-	-	-	-	-	-	-	-	-	-	-	0	
5	200	-	-	-	-	-	-	-	-	-	-	-	0	
6	200	-	-	-	-	-	-	-	-	-	-	-	0	

+ = Allele Present, - = Allele not present,

N.B. Examples of raw data (samples denoted with *) can be found in Appendix A4.2

4.2.2 Colour: Heat treatment study

The colour of every sample in the heat-treated study was measured and recorded as a three axis coordinate (L , a^* , b^*), where ' L ' represents the brightness and ' a^* ' and ' b^* ' are chromaticity coordinates (section 3.13). Using the Munsell® colour chart, the colour of each sample was also visually recorded.

4.2.2.1 Colour of bone heated to different temperatures for two hour and four hours

The sequence of colour change was very similar between both sample series (Figure 4.2). The sequence began $\sim 10^\circ\text{C}$ earlier, in the samples heated for four hours.

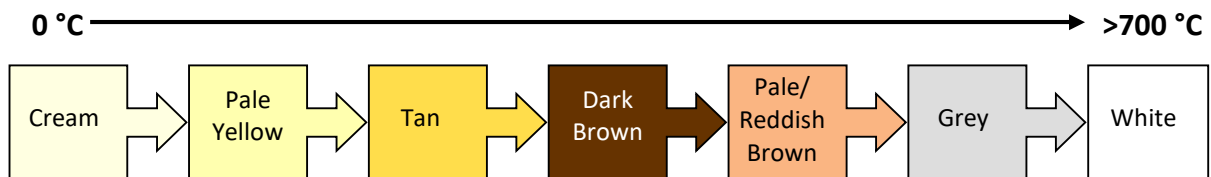


Figure 4.2: General sequence of colour change in heated bone.

Figure 4.3 describes the changes in colour using the colourimetry coordinates (L , a^* , b^*) and the equivalent Munsell® Colour chart code. To simplify the figures, samples that shared a similar (L , a^* , b^*) colour and could not be distinguished visually, were grouped together.

A change from the cream colour of fresh bone was observed visually, in samples that were heat-treated for two hours when the temperature reached 180 °C (Figure 4.3: A). The same change was seen at 160 °C in samples heat-treated for four hours (Figure 4.3: B). As the temperature increased the cream colour became increasingly dark. From a yellow/cream colour, samples from both series changed to a tan colour, which occurred at 250 and 200°C in samples that were heat treated for two and four hours respectively. From a tan colour, the bone changed to a dark brown. This change occurred at an earlier temperature in samples that were heat treated for four hours (250°C) than the samples heated for two (300°C). In both series, between 400 and 600 °C, changes from a pale/reddish brown to grey were observed. Samples had turned brilliant white after being heated to 650 °C for four hours. In contrast, samples heated for two hours were still grey in colour. Above 700 °C, the samples in both samples series turned brilliant white in colour.

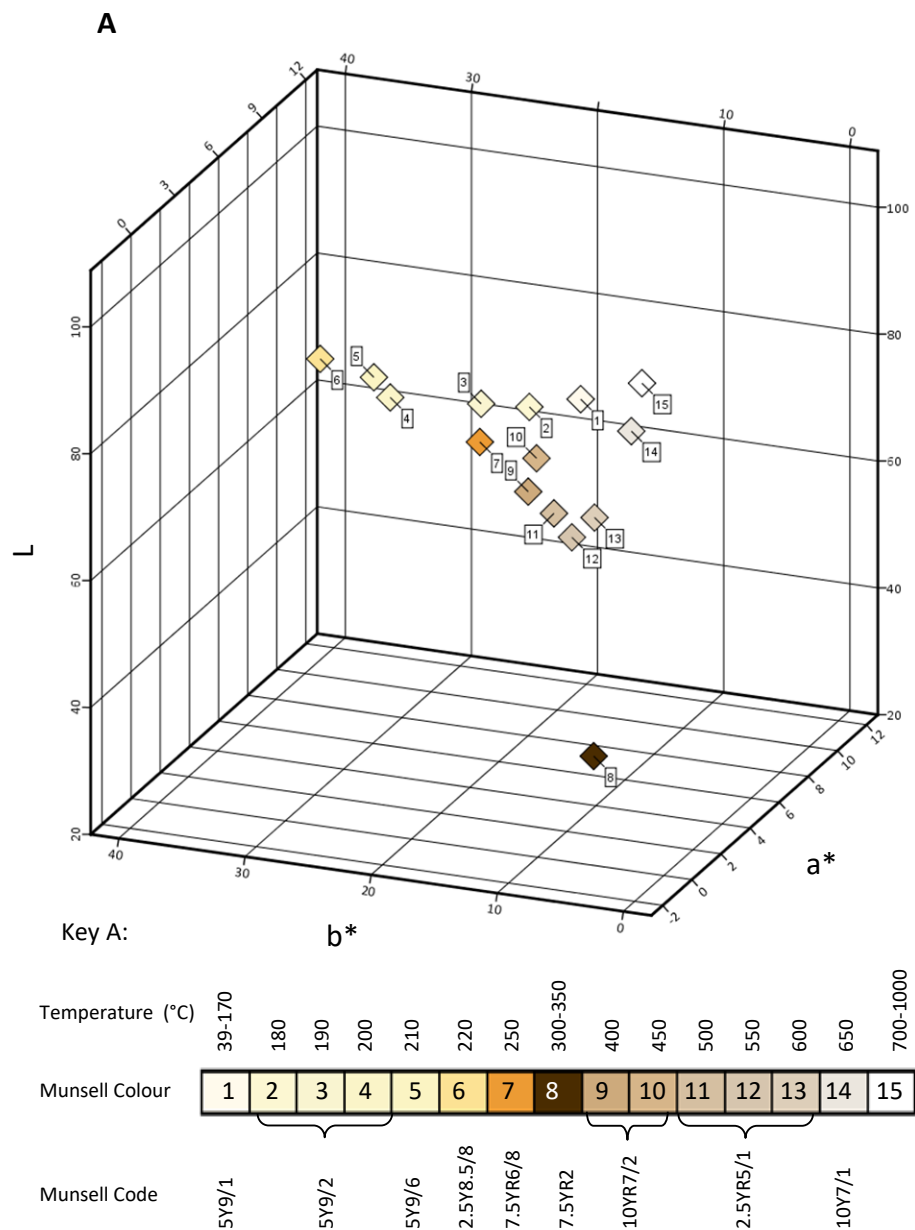
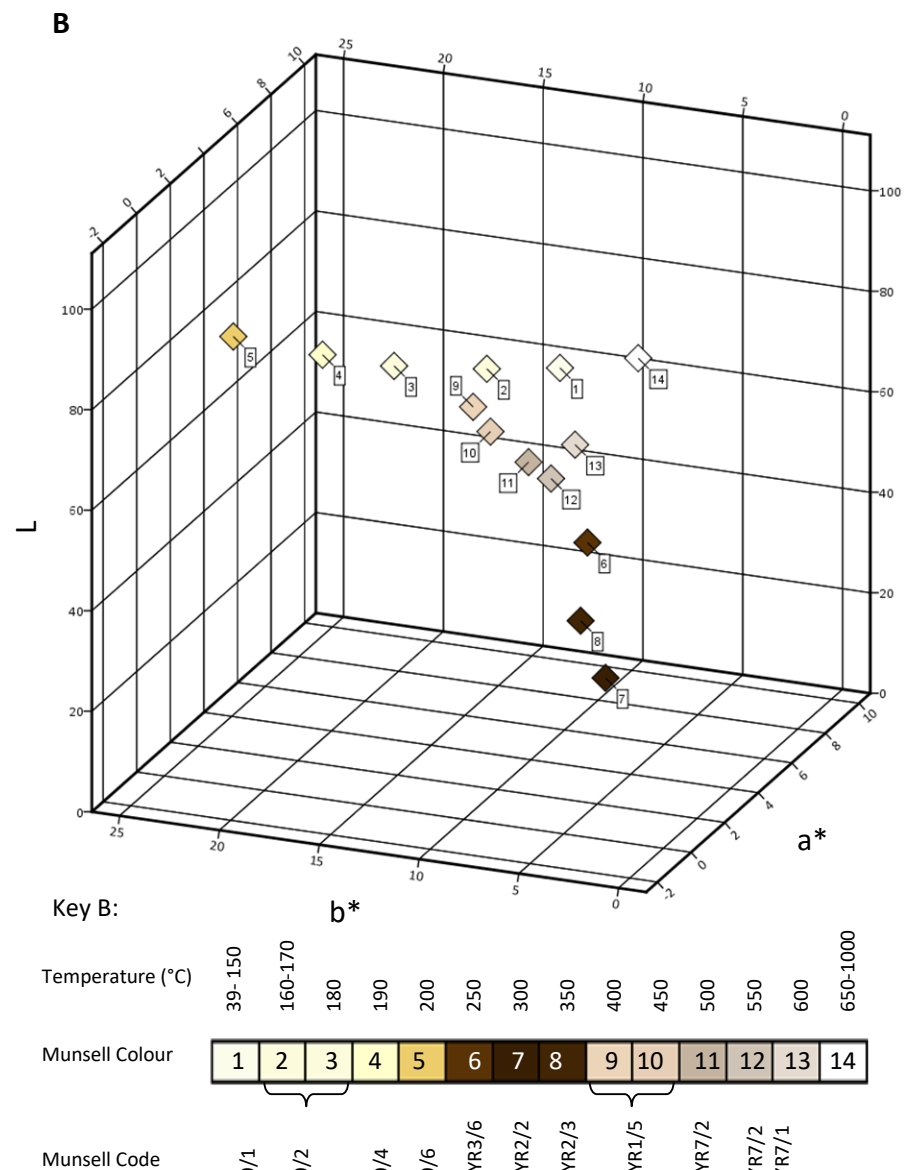


Figure 4.3: Colour change of bovine femur heated at various temperatures for A: Two hours, B: four hours. The (L, a*, b*) coordinate of each sample was presented as their Munsell® chart equivalent colour. To simplify the figures, samples that were recorded as having the same Munsell® colour and similar (L,a,b) coordinates were grouped (see keys).

Using a colourimeter, colour changes that were not visually resolvable were detected. Figures 4.4, 4.5 and 4.6, describe the changes in brightness (L) and chromaticity coordinates (a^* and b^*), respectively, in samples heated at various temperatures for two and four hours. A colour change was first observed after samples had been heated at 150 and 140 °C for two and four hours respectively. As the temperature increased, the samples decreased in brightness and changed colour; the a^* and b^* coordinates of the samples moved towards the red/yellow region of the colour spectrum. As the temperature continued to increase the samples in both series became brighter and whiter in colour. The colour of bone was a function of both temperature and duration.

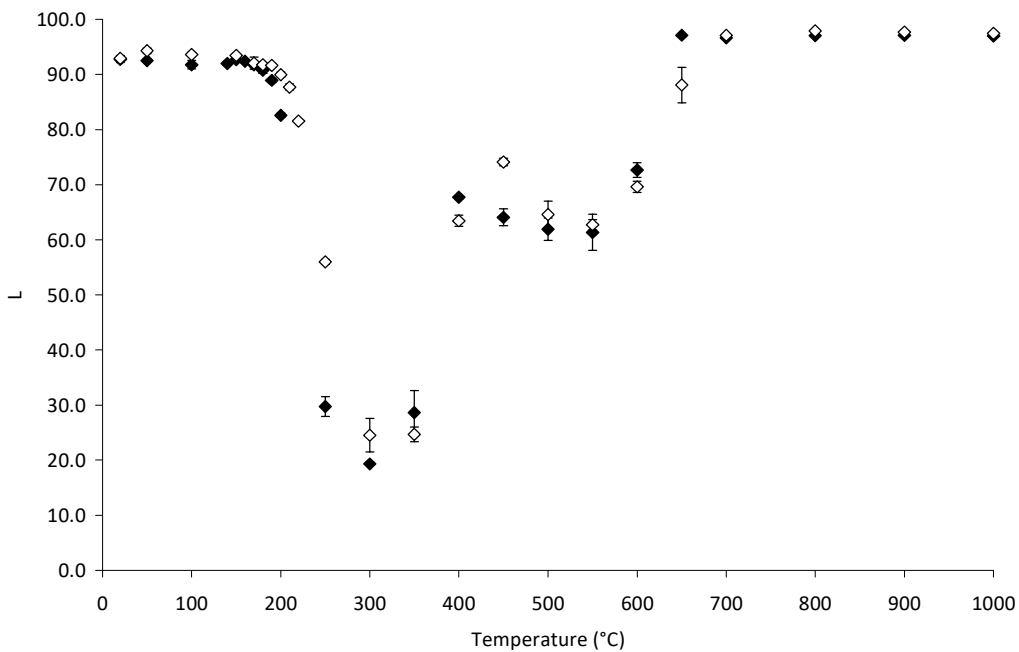


Figure 4.4: The average brightness of bone heated at various temperatures (50-1000°C) for (◇) 2 hours and (◆) 4 hours. Error bars (± 1 standard error).

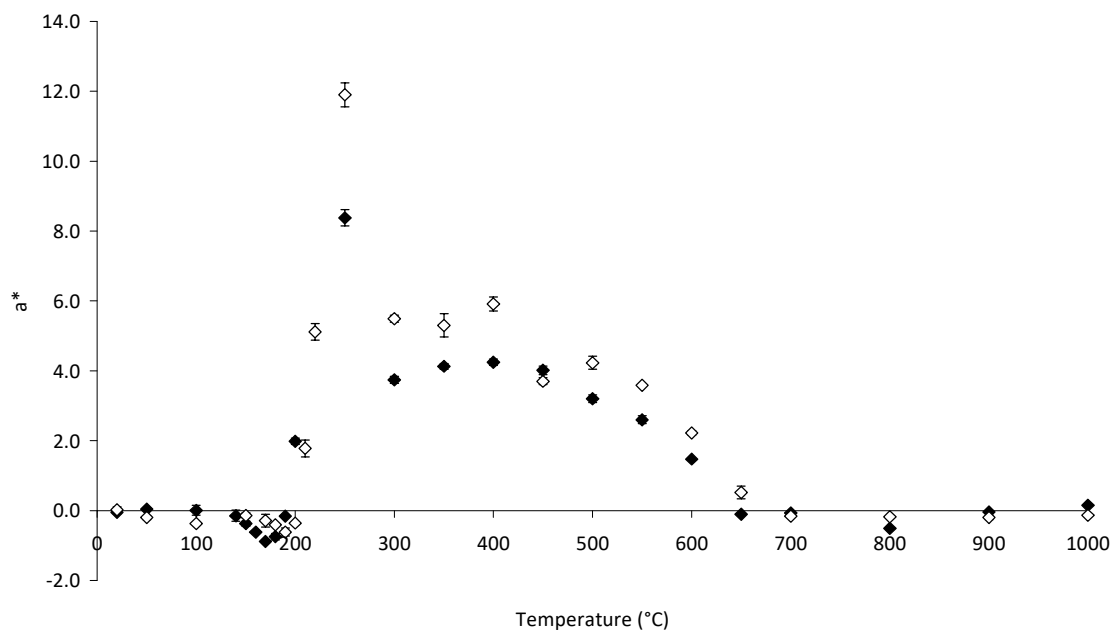


Figure 4.5: The average a^* chromaticity coordinate of bone heated at various temperatures (50-1000°C) for (◇) 2 hours and (◆) 4 hours. Error bars (± 1 standard error).

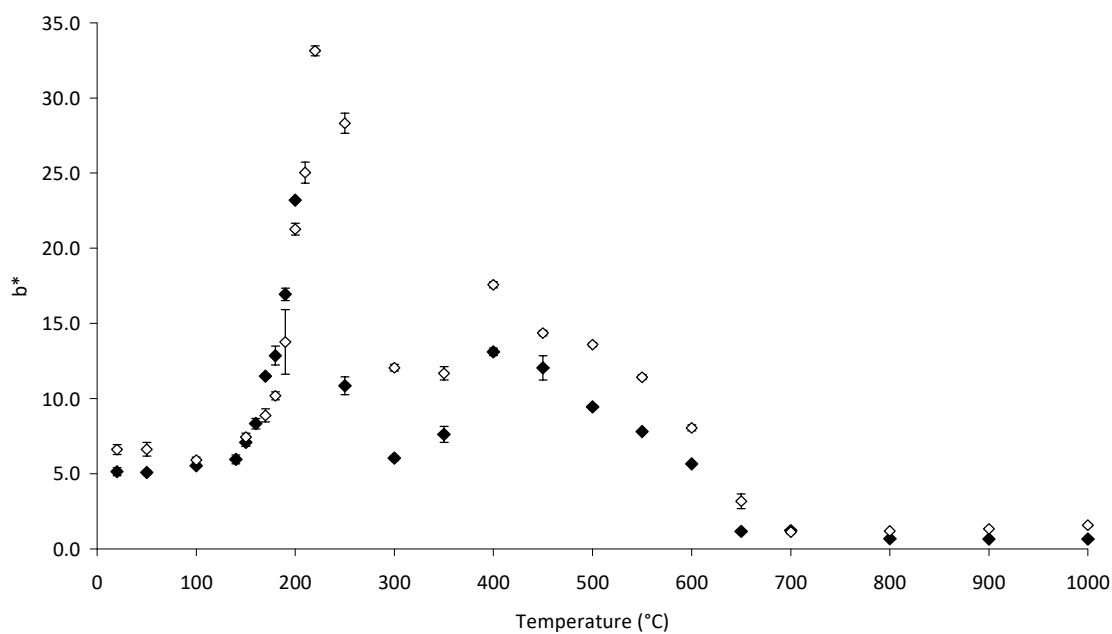


Figure 4.6: The average b^* chromaticity coordinate of bone heated at various temperatures (50-1000°C) for (◇) 2 hours and (◆) 4 hours. Error bars (± 1 standard error).

4.2.2.2 Assessing the likelihood of successful PCR product amplification using the colour of heated bone

Significant ($P < 0.05$) quadratic multivariate discriminant (QMD) functions (Equations 4.1-4.3) were applied to the colourimetry data (section 3.12.2) in order to calculate the probability of successfully amplifying 500, 300 or 100 bp DNA fragments (Figures 4.7, 4.8 and 4.9 respectively). The grey area in each figure denotes the model's boundary. Samples within the area were classified as positive results; samples outside the area were classified as negative results (section 3.17.3). Table 4.7 shows the percentages of true positives, true negatives and the total percentage of samples that were correctly classified. All three QMD functions classified the same percentage of samples correctly (~93%). The percentages of correctly classified true positives and true negatives were also very similar between the three functions although all three models, a number of samples heated to 650-700 °C were incorrectly classified. The probability of successfully amplifying each amplicon from each sample was calculated and can be viewed in Appendix A8.1-A8.3. View Appendix CD1 for animated 3D graphs (Figure 4.7-4.9).

Table 4.7: Sample classification and AUROC curves for DNA amplification as determined by colour

Model Classification	DNA amplicon (bp)		
	500	300	100
True negatives (%)	92.3	92	87.9
True positives (%)	95.7	96	96.1
Total (%)	93.3	93.3	93.3
AUROC curve	0.96	0.96	0.97

Equation 4.1: Multivariate discriminant function 500bp DNA by colour

$$\frac{f_1(0)}{f_2(1)} = |\Sigma_2|^{\frac{1}{2}} |\Sigma_1|^{-\frac{1}{2}} \exp \left[\left\{ \nu' (\Sigma_1^{-1} - \Sigma_2^{-1}) \nu - 2\nu' (\Sigma_1^{-1} \mu_1 - \Sigma_2^{-1} \mu_2) + \mu_1' \Sigma_1^{-1} \mu_1 - \mu_2' \Sigma_2^{-1} \mu_2 \right\} \right]$$

$\Sigma_1 =$	1.06679	-0.04959	-0.31485	$\Sigma_2 =$	586.217	-53.1573	-41.1007
	-0.04959	0.04896	-0.15365		-53.1573	8.60065	12.5404
	-0.31485	-0.15365	2.15328		-41.1007	12.5404	70.1138
$\mu_1 =$	92.763	-0.14717	6.505	$\mu_2 =$	74.2666	2.28385	10.5607

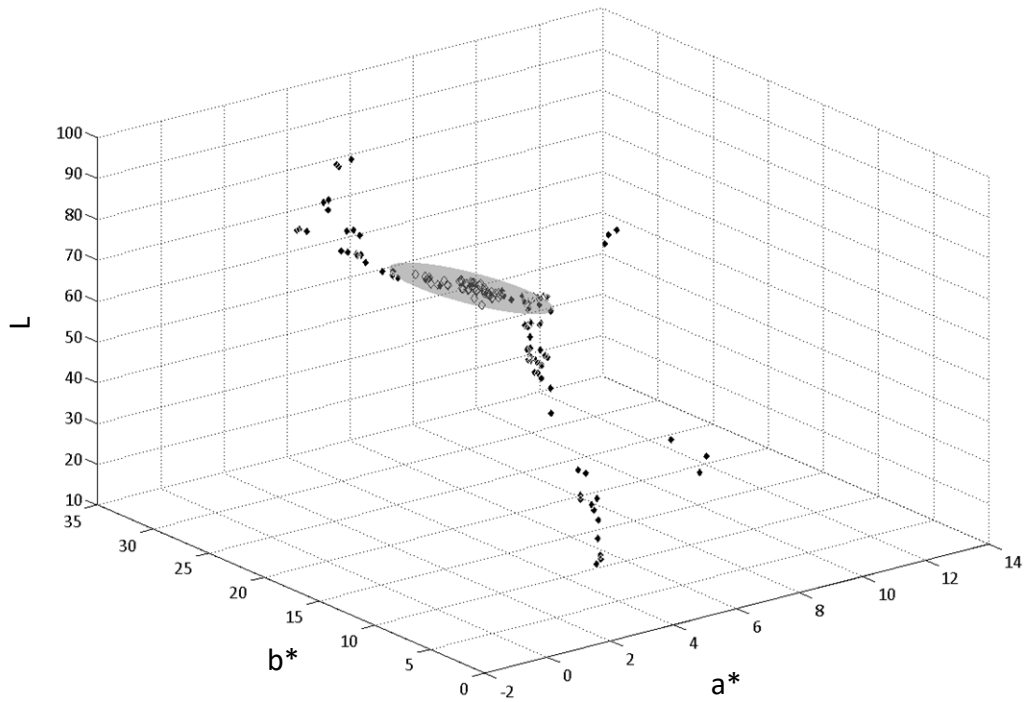


Figure 4.7: Relationship between the amplification of 500 bp DNA and colour. Calculated using the L, a*, b* coordinates recorded from bone heated (50-1000 °C) for 2 hours and 4 hours. (◊) Positive results (♦) negative results. The grey area is the function of equation 4.1.

Equation 4.2: Multivariate discriminant function 300bp DNA by colour

$$\frac{f_1(0)}{f_2(1)} = |\Sigma_2|^{\frac{1}{2}} |\Sigma_1|^{-\frac{1}{2}} \exp \left[\left\{ \nu'(\Sigma_1^{-1} - \Sigma_2^{-1})\nu - 2\nu'(\Sigma_1^{-1}\mu_1 - \Sigma_2^{-1}\mu_2) + \mu_1'\Sigma_1^{-1}\mu_1 - \mu_2'\Sigma_2^{-1}\mu_2 \right\} \right]$$

$\Sigma_1 =$	1.046	-0.0295	-0.5453	$\Sigma_2 =$	596.5921	-53.1823	-411007
	-0.0295	0.0598	-0.2835		-53.1823	8.6089	12.5404
	-0.5453	-0.15365	3.8183		-42.7659	13.0598	70.1138
$\mu_1 =$	92.7064	-0.2835	6.832	$\mu_2 =$	73.5551	2.28385	10.5607

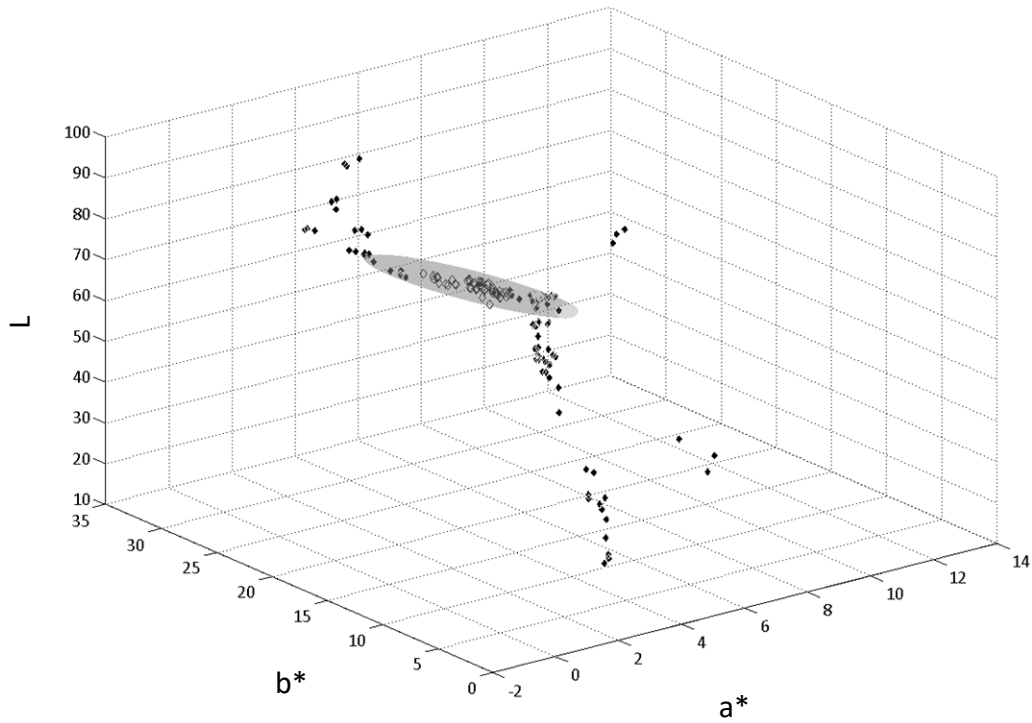


Figure 4.8: Relationship between the amplification of 300 bp DNA and colour. Calculated using the L, a*, b* coordinates recorded from bone heated (50-1000 °C) for 2 hours and 4 hours. (◊) Positive results (●) negative results. The grey area is the function of equation 4.2.

Equation 4.3: Multivariate discriminant function 100bp DNA by colour

$$\frac{f_1(0)}{f_2(1)} = |\Sigma_2|^{\frac{1}{2}} |\Sigma_1|^{-\frac{1}{2}} \exp \left[\left\{ \nu' (\Sigma_1^{-1} - \Sigma_2^{-1}) \nu - 2\nu' (\Sigma_1^{-1} \mu_1 - \Sigma_2^{-1} \mu_2) + \mu_1' \Sigma_1^{-1} \mu_1 - \mu_2' \Sigma_2^{-1} \mu_2 \right\} \right]$$

$\Sigma_1 =$	1.5175	0.0595	-3.0862	$\Sigma_2 =$	623.4527	-52.7165	-56.4738
	0.0595	0.0873	-0.6423		-52.7165	8.4483	15.9873
	-3.0862	-0.6423	16.3525		-56.4738	15.9873	75.2143
$\mu_1 =$	92.4163	-0.2503	8.181	$\mu_2 =$	71.8491	2.6980	10.0534

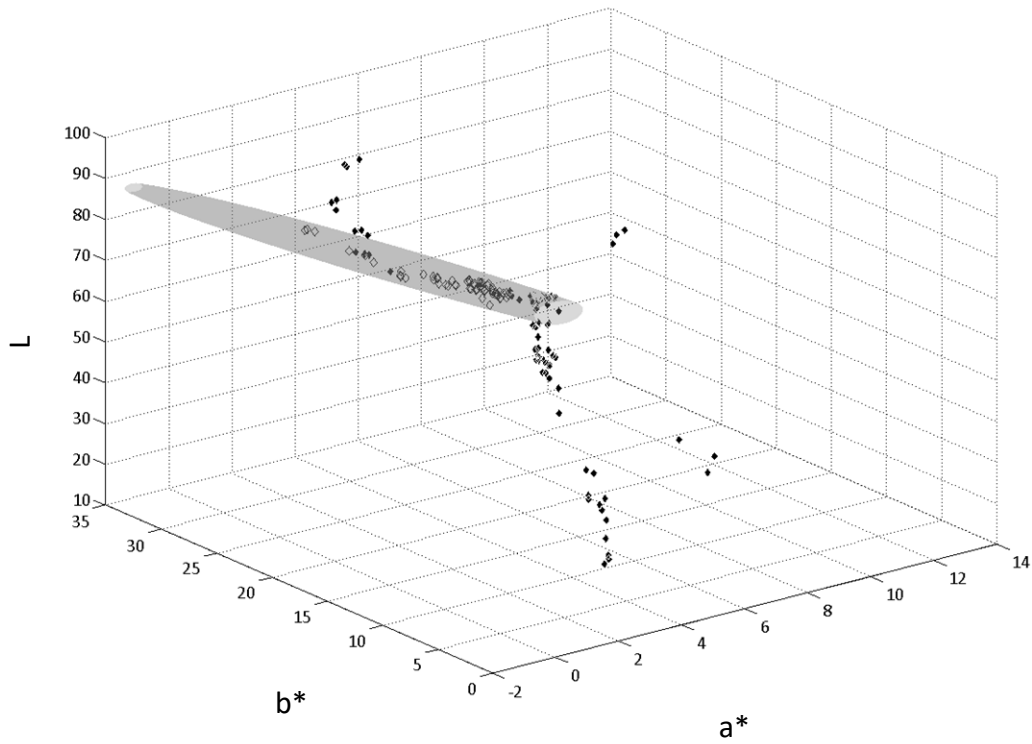


Figure 4.9: Relationship between the amplification of 100 bp DNA and colour. Calculated using the L, a*, b* coordinates recorded from bone heated (50-1000 °C) for 2 hours and 4 hours. (◊) Positive results (♦) negative results. The grey area is the function of equation 4.3.

4.2.2.3 Assessing the likelihood of successful STR loci amplification using the colour of heated bone

Section 3.16.3.1: describes the minimum number of loci that would be needed ‘on average’ to identify an individual; with a $\leq 1\%$ chance that another random (i.e. unrelated) individual would show the same match by chance alone. Two common scenarios were considered, one where the comparative reference sample is believed to originate from the individual in question (requiring ≥ 3 loci), and another where it is believed to originate from a parent or child of the individual in question (requiring ≥ 7 loci).

Significant ($P < 0.05$) QMD functions (Equations 4.4-4.5) were also applied to the colourimetry data to calculate the probability of successfully amplifying a minimum of 3 or 7 STR loci from the heat-treated samples (Figures 4.10 and 4.11 respectively). Both functions classified (Table 4.8) $>95\%$ of true positive samples correctly, although in both models, samples heated to 650-700 °C were incorrectly classified. The probability of successfully amplifying ≥ 3 or ≥ 7 loci from each sample was calculated and can be viewed in Appendix A8.4 and A8.5.

Table 4.8: Sample classification and AUROC curves for STR loci amplification by colour

Model Classification	No. STR Loci	
	≥ 3	≥ 7
True negatives (%)	95.8	94.00
True positives (%)	96.4	98.00
Total (%)	96.00	95.30
AUROC curve	0.96	0.96

Equation 4.4: Multivariate discriminant function amplification of ≥ 3 STR loci by colour

$$\frac{f_1(0)}{f_2(1)} = \left| \Sigma_2 \right|^{\frac{1}{2}} \left| \Sigma_1 \right|^{-\frac{1}{2}} \exp \left[\left\{ \nu' \left(\Sigma_1^{-1} - \Sigma_2^{-1} \right) \nu - 2\nu' \left(\Sigma_1^{-1} \mu_1 - \Sigma_2^{-1} \mu_2 \right) + \mu_1' \Sigma_1^{-1} \mu_1 - \mu_2' \Sigma_2^{-1} \mu_2 \right\} \right]$$

$\Sigma_1 =$	1.1027	0.0336	-1.1771	$\Sigma_2 =$	610.2096	-52.7583	-47.5195
	0.0336	0.0910	-0.5836		-52.7583	8.4824	14.1976
	-1.1771	-0.5836	7.1355		-47.5195	14.1976	75.9381
$\mu_1 =$	92.5996	-0.2389	7.3982	$\mu_2 =$	72.3089	2.5673	10.4278

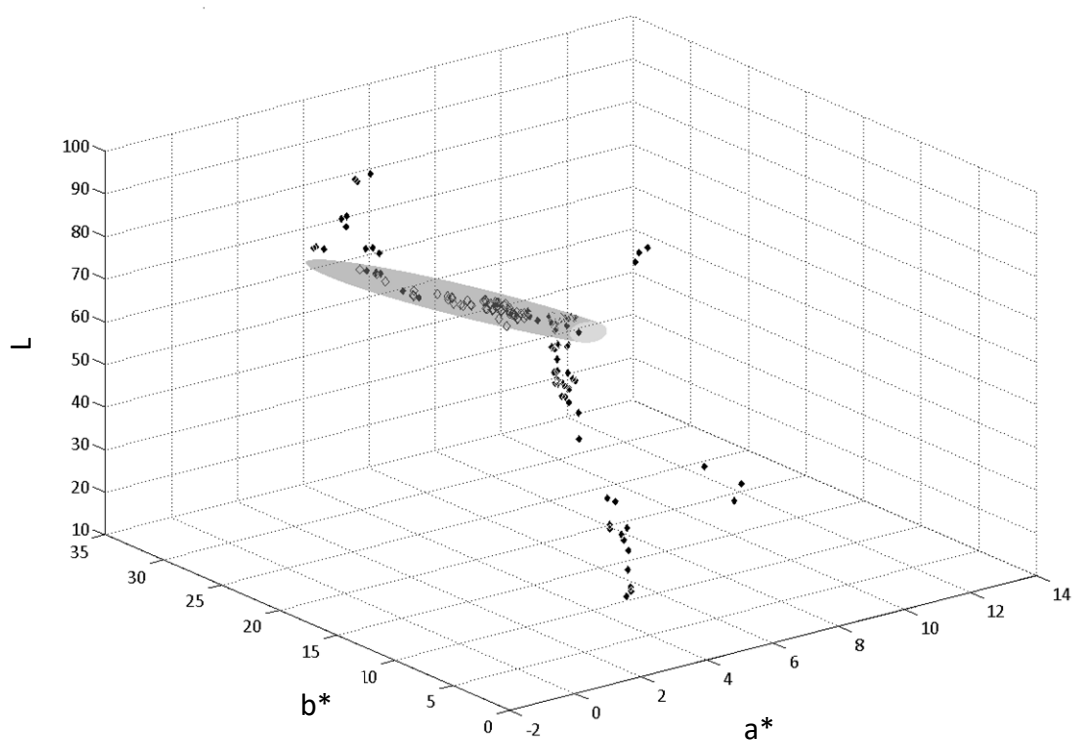


Figure 4.10: Relationship between the amplification of ≥ 3 STR loci and colour. Calculated using the L, a^* , b^* coordinate recorded from bovine femurs heat treated (50-1000 °C) for 2 hours and 4 hours. (◇) Positive results (◆) negative results. The grey area is the function of equation 4.4.

Equation 4.5: Multivariate discriminant function amplification of ≥ 7 STR loci by colour

$$\frac{f_1(0)}{f_2(1)} = \left| \Sigma_2 \right|^{\frac{1}{2}} \left| \Sigma_1 \right|^{-\frac{1}{2}} \exp \left[\left\{ u'(\Sigma_1^{-1} - \Sigma_2^{-1})u - 2u'(\Sigma_1^{-1}\mu_1 - \Sigma_2^{-1}\mu_2) + \mu_1'\Sigma_1^{-1}\mu_1 - \mu_2'\Sigma_2^{-1}\mu_2 \right\} \right]$$

$\Sigma_1 =$	1.1131	-0.0129	-0.6311	$\Sigma_2 =$	597.0537	-53.2180	-42.1385
	-0.0129	0.0606	-0.2470		-53.2180	8.6069	12.9552
	-0.6311	-0.2470	2.9386		-42.1385	12.9552	72.8418
$\mu_1 =$	92.6808	-0.1802	6.7664	$\mu_2 =$	73.5679	2.3976	10.5922

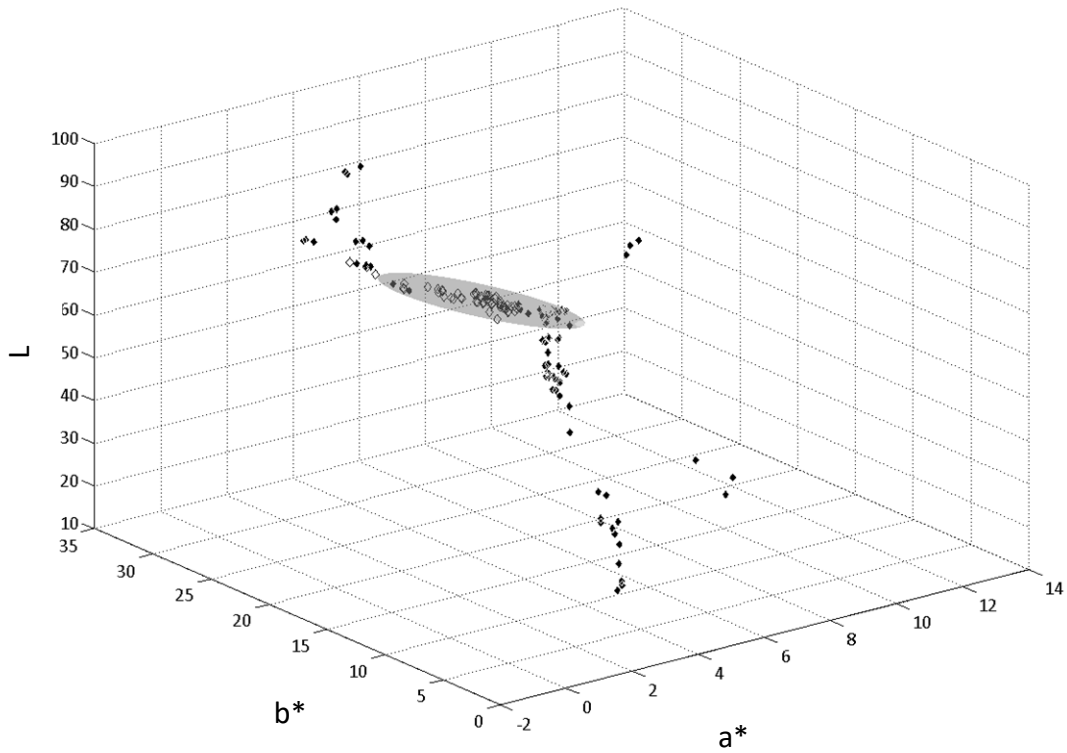


Figure 4.11: Relationship between the amplification of ≥ 7 STR loci and colour. Calculated using the L, a^* , b^* coordinate recorded from bovine femurs heat treated (50-1000 °C) for 2 hours and 4 hours. (◇) Positive results (◆) negative results. The grey area is the function of equation 4.5.

4.2.3 FTIR spectroscopy: Amide I to Phosphate Ratio (APR): Heat treatment study

The phosphate to amide I ratio has been used in previous studies to semi quantify the amount of collagen present in bone samples (section 2.2.2). However, this study has presented the reciprocal, namely, the amide I to phosphate ratio (APR). By inverting the ratio, every sample, including those that had no detectable amide I band, could be assigned a value. If the ratio had not been inverted, samples with no amide I peak the ratio would have generated an infinite value.

4.2.3.1 APR of bone heated to different temperatures for two and four hours

In both sample series, the APR did not change significantly between 39 °C (control) and 150 °C, remaining at approximately 0.4. As the temperature increased above 150 °C, a decrease in the APR was observed in both sample series and continued to decrease until ~600 °C. The initial decrease in the APR occurred at a lower temperature in samples heated for four hours (from 160 °C), when compared to samples which were heated for two hours (from 170 °C). Above 600 °C, the amide I peak was not detectable in samples that had been heated for four hours. However, in samples heated for two hours, the amide I band remained detectable up to 650 °C (Figure 4.12). The phosphate band intensity remains stable, while the intensity of the amide I band decreased as a function of temperature and duration of heat treatment. Therefore, the APR decreased as temperature and time increased.

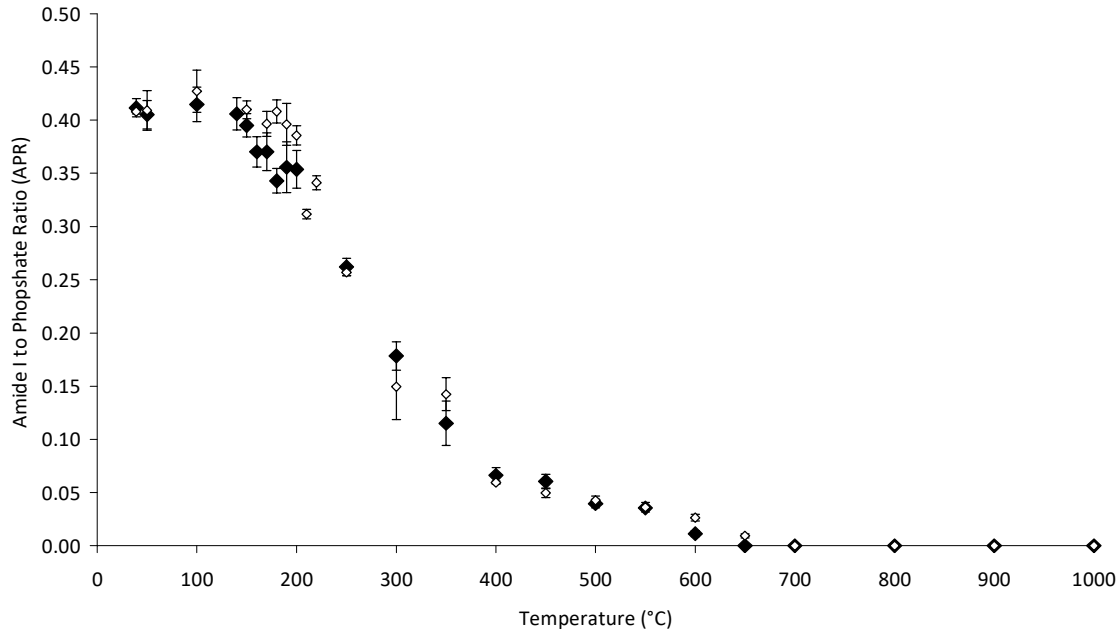


Figure 4.12: The average APR of bovine femurs after heat treatment (50-1000 °C) for (◊) 2 hours and (◆) 4 hours. Error bars (± 1 standard error).

4.2.3.2 Assessing the likelihood of successful PCR product amplification using the APR of heated bone

Significant ($P < 0.05$) logistic regression functions (Equations 4.6-4.8) were applied to the APR data (section 3.17.1) in order to calculate the probability of amplifying 500, 300 or 100 bp DNA fragments from heated bone (Figures 4.13, 4.14 and 4.15 respectively).

$$\text{Probability} = 1 / (1 + \exp[-(a - b\text{APR})])$$

Equation	Amplicon (bp)	<i>a</i>	<i>b</i>
4.6	500	28.80	75.96
4.7	300	28.08	75.46
4.8	100	26.36	75.16

The probability of successfully amplifying each amplicon from each sample was calculated can be viewed in Appendix A8.1-A8.3 and were then classified as either a positive or negative (section 3.17.3). Table 4.9 shows the percentages of true positives, true negatives and the total percentage of samples that were correctly classified. Over 90% of the true positives and negatives were classified correctly by each logistic regression. The areas under the receiver-operating characteristic (AUROC) curve for each model are all close to one (section 3.17.3.1).

Table 4.9: Sample classification and AUROC curve of the logistic regressions of each amplicon by APR

Model Classification	DNA amplicon (bp)		
	500	300	100
True negatives (%)	92.3	92.0	95.6
True positives (%)	91.3	96.0	96.6
Total (%)	92.0	93.3	96.0
AUROC curve	0.98	0.98	0.99

Table 4.10 shows the APR value required to produce a probability of successful amplification equal to 0.5 for each fragment size. The logistic regression curves, although similar in shape, shifted towards zero and became increasingly separated as the length of PCR product decreased (Figure 4.16).

Table 4.10: APR values producing a probability of successful amplification equal to 0.5

Amplicon (bp)	0.5 Probability (APR)
500	0.38
300	0.37
100	0.35

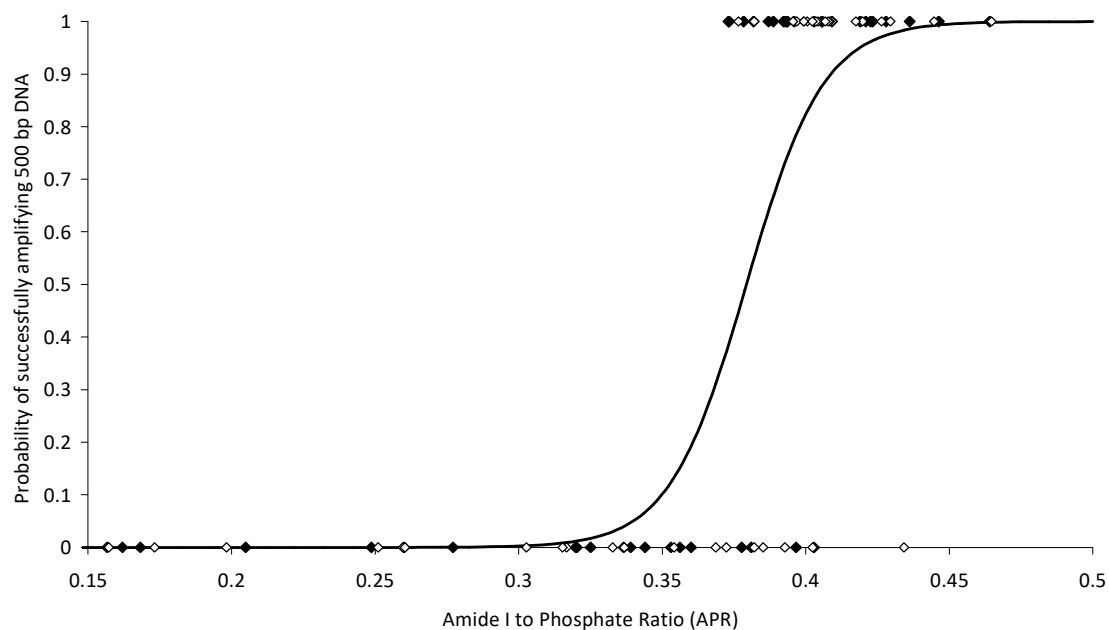


Figure 4.13: Relationship between the amplification of 500 bp DNA and APR. Calculated using the APR of bone heated (50-1000 °C) for (◇) 2 hours and (◆) 4 hours. The curve is the function of Equation 4.6.

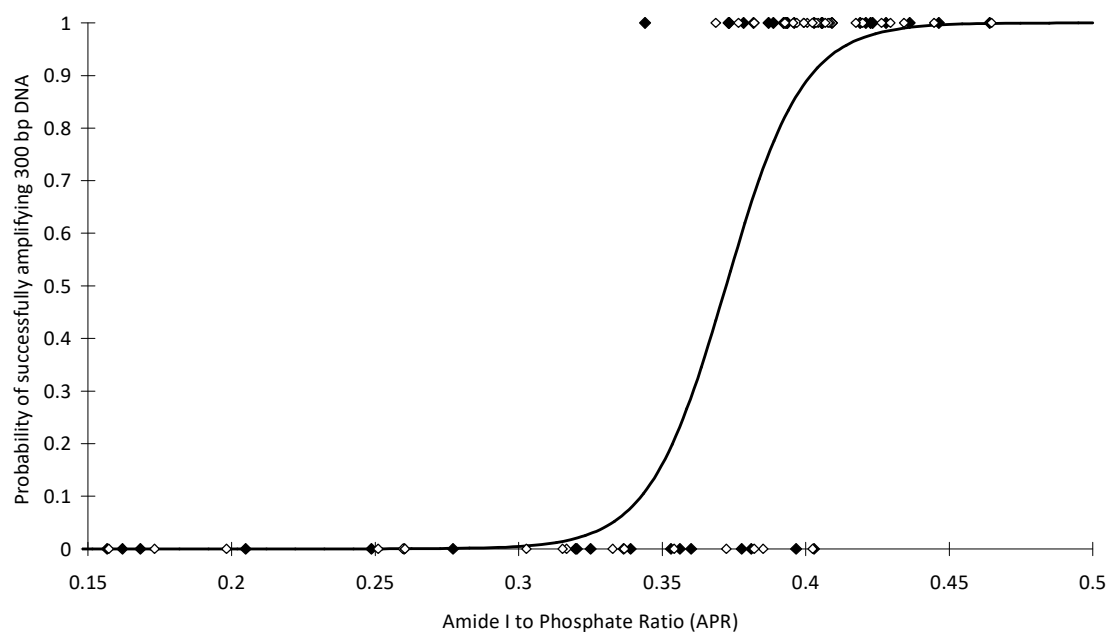


Figure 4.14: Relationship between the amplification of 300 bp DNA and APR. Calculated using the APR of bone heated (50-1000 °C) for (◇) 2 hours and (◆) 4 hours. The curve is the function of Equation 4.7.

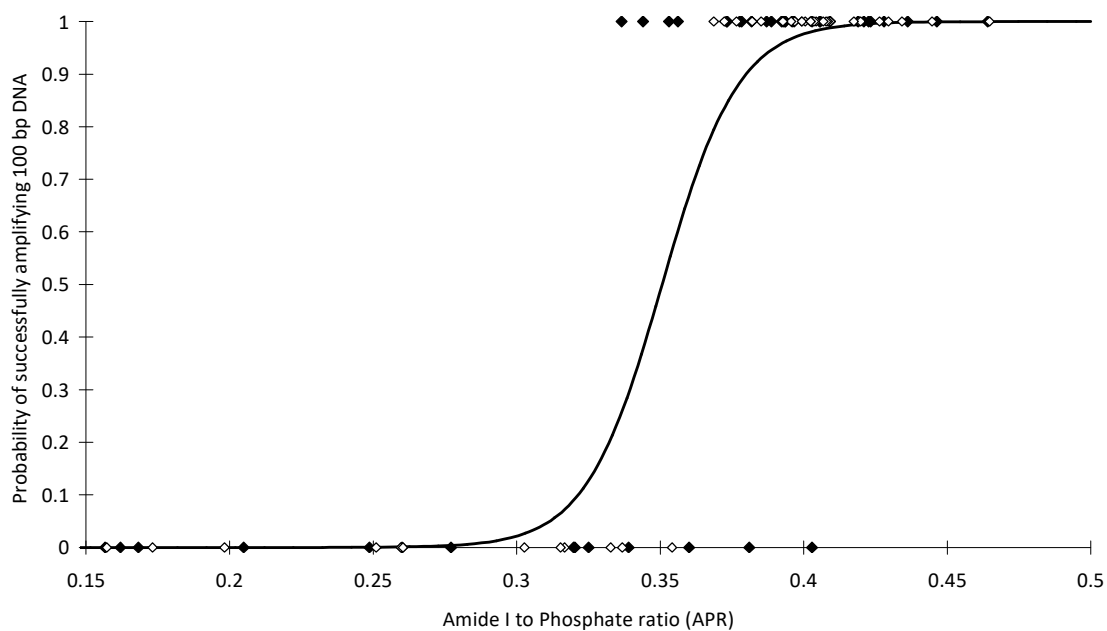


Figure 4.15: Relationship between the amplification of 100 bp DNA and APR. Calculated using the APR of bone heated (50-1000 °C) for (◇) 2 hours and (◆) 4 hours. The curve is the function of Equation 4.8.

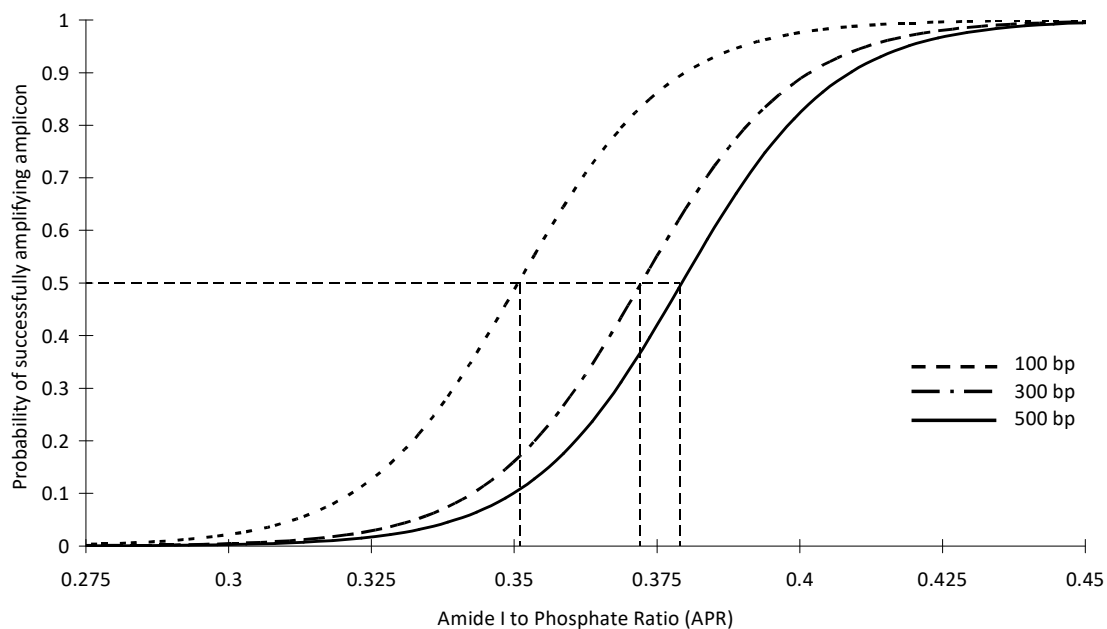


Figure 4.16: Relationship between the amplification of 500, 300 and 100 bp DNA and the APR of heat-treated bone. Illustrated are the APR values described in Table 4.10.

4.2.3.3 Assessing the likelihood of successful STR loci amplification using the APR of heated bone

Significant logistic regression functions (Equations 4.9 and 4.10) were applied to the APR data to calculate the probability of successfully amplifying a minimum of 3 or 7 loci (Figures 4.17 and 4.18, respectively) from the heat-treated samples (Appendix A8.4-A8.5). Both logistic regressions classified 94% of the samples correctly and the AUROC curves were all close to one (Table 4.11).

$$\text{Probability} = 1 / (1 + \exp[-(a-b\text{APR})])$$

Equation	No. STR loci	<i>a</i>	<i>b</i>
4.9	≥ 3	22.21	61.48
4.10	≥ 7	25.88	70.06

Table 4.11: Sample classification and AUROC curve of STR loci amplification by APR

Model Classification	No. STR loci	
	≥ 3 loci	≥ 7 loci
True negatives (%)	93.8	92.9
True positives (%)	94.4	96.1
Total (%)	94.0	94.0
AUROC curve	0.97	0.99

Table 4.12 the APR value required to produce a probability of successful amplification equal to 0.5 for ≥ 3 and ≥ 7 loci respectively. The logistic regression curves, although similar in shape, shifted towards zero as the minimum required number of loci increased (Figure 4.19).

Table 4.12: APR values required to produce a probability of successful amplification equal to 0.5 for ≥ 3 and ≥ 7 STR loci

Min. loci	0.5 Probability (APR)
≥ 3	0.36
≥ 7	0.37

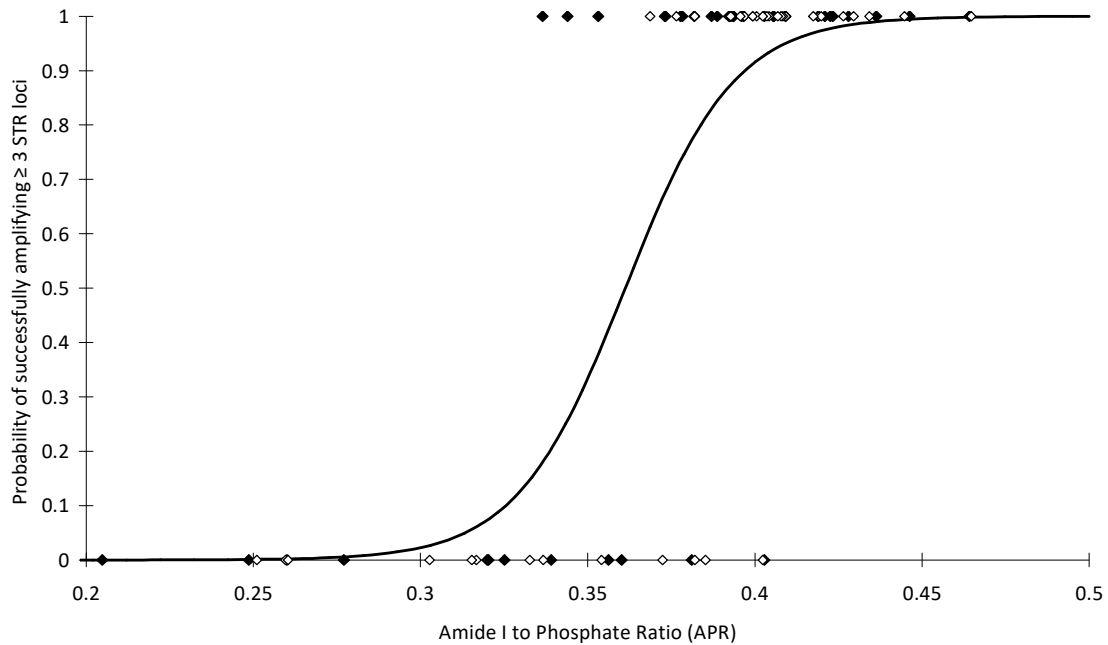


Figure 4.17: Relationship between the amplification of ≥ 3 STR loci and APR. Calculated using the APR of bone heated (50-1000 °C) for ($\diamond$) 2 hours and ($\blacklozenge$) 4 hours. The curve is the function of Equation 4.9.

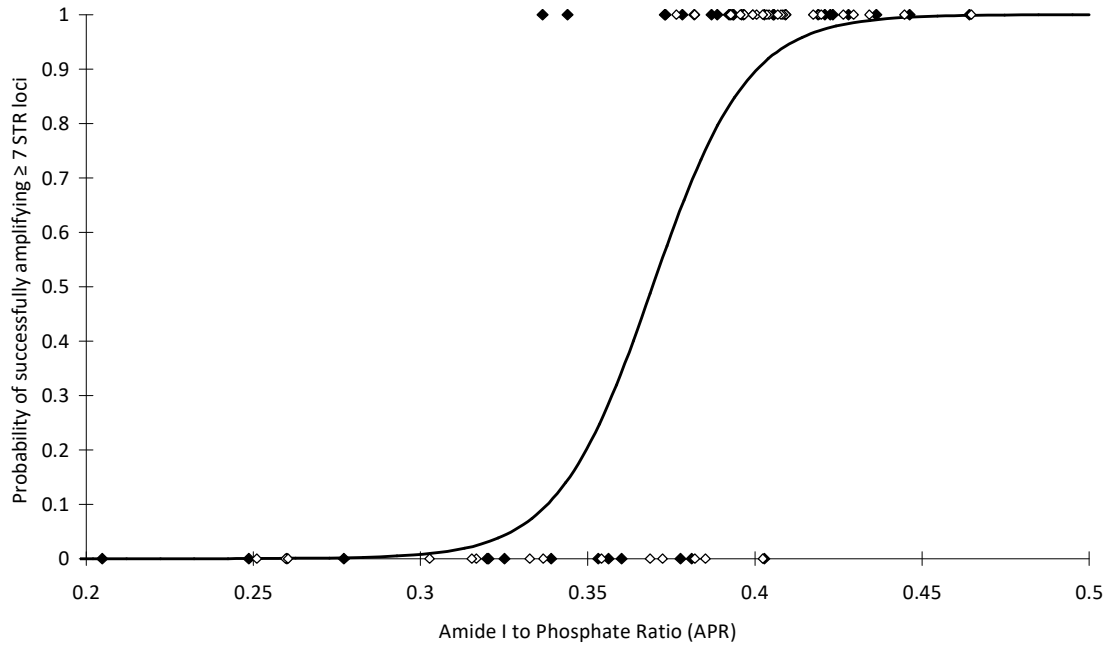


Figure 4.18: Relationship between the amplification of ≥ 7 STR loci and APR. Calculated using the APR of bone heated (50-1000 °C) for ($\diamond$) 2 hours and ($\blacklozenge$) 4 hours. The curve is the function of Equation 4.10.

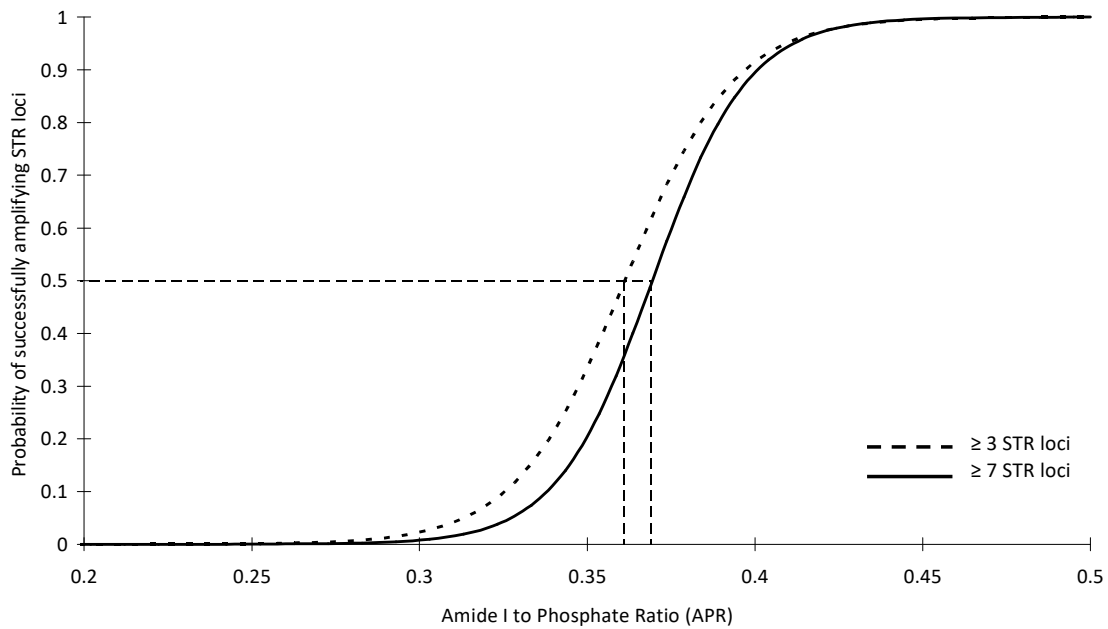


Figure 4.19: Relationship between the successful amplification of ≥ 3 and ≥ 7 STR loci and the APR of heated bone. Illustrated are the APR values described in Table 4.12.

4.2.4 FTIR spectroscopy: Splitting Factor (SF): Heat treatment

The Splitting Factor (SF) of bone bHAP was measured (section 2.3.2) for each sample in both the two and four hour sample series.

4.2.4.1 SF of bHAP in bone heated at various temperatures for two and four hours

There were very few differences in the SF of bHAP measured from samples in both series, when heated between 39 °C (control) and 450 °C. The SF in both sample series remained at approximately 2.6 below 150 °C. In both series, an increase in SF was observed between 160 and 250 °C. Although there were fluctuations, demonstrated by the relatively large error bars, between each sample heated to temperatures within this range. As the temperature increased above 250 °C, there were again very few changes in SF. Above 400 °C there was a gradual increase, with the SF values reaching a plateau at ~700 °C. Above 450 °C, the average SF recorded in samples heat treated for 4 hours was consistently higher (except at 600 °C) than the average SF recorded from samples heat treated for two hours at the corresponding temperature (Figure 4.20).

The SF of bone bHAP increased as temperature increased. The increase in SF was only a function of duration when bone was heated to temperatures above 450 °C.

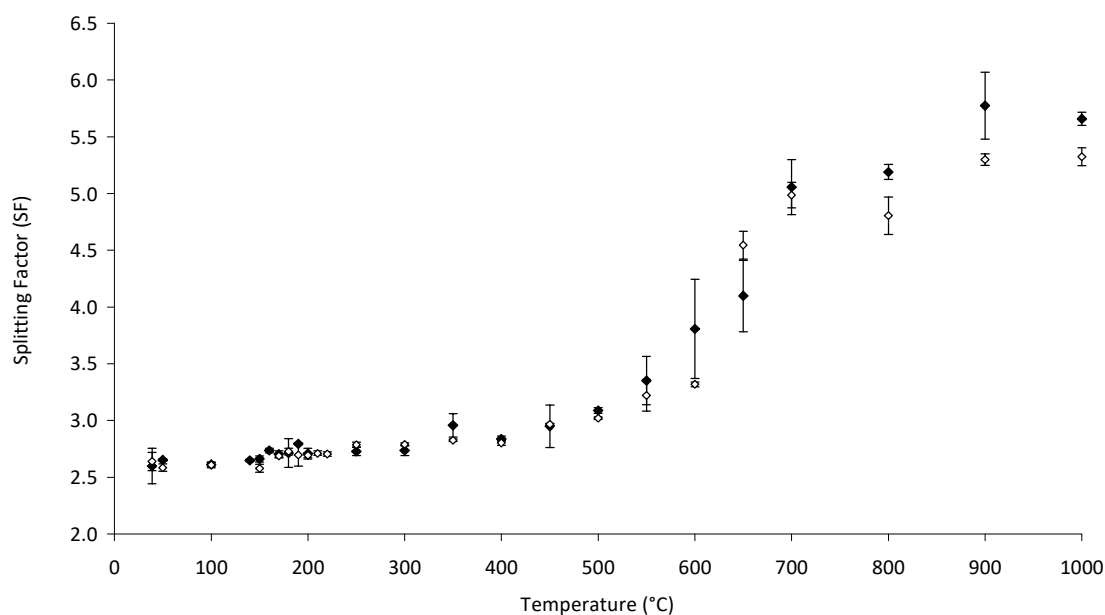


Figure 4.20: The average SF of bHAP in bone after heat treatment (50-1000 °C) for (◇) 2 hours and (◆) 4 hours. Error bars (± 1 standard error).

4.2.4.2 Assessing the likelihood of successful PCR product amplification using the SF of bHAP in heated bone

Significant ($P < 0.05$) logistic regression functions (Equations 4.11-4.13) were applied to the SF data in order to calculate the probability of successfully amplifying 500, 300 or 100 bp DNA fragments (Figures 4.21, 4.22 and 4.23 respectively).

$$\text{Probability} = 1 / (1 + \exp[-(a-b\text{SF})])$$

Equation	Amplicon (bp)	<i>a</i>	<i>b</i>
4.11	500	108.66	40.46
4.12	300	77.85	28.83
4.13	100	97.17	35.67

The probability of amplifying each amplicon successfully was calculated for each sample (Appendix A8.1-A8.3). Table 4.13 describes the percentages of true positives, true negatives and the total percentage of samples that were correctly classified. For each amplicon over 80% of the true positives were correctly classified and the AUROC curves for each model were close to one.

Table 4.13: Sample classification and AUROC curve of logistic regressions of each amplicon by SF

Model Classification	DNA amplicon		
	500	300	100
True negatives (%)	95.2	88.0	87.9
True positives (%)	80.4	82.0	86.4
Total (%)	90.7	86.0	87.3
AUROC curve	0.97	0.95	0.97

Table 4.14 shows the SF value required to produce a probability of successful amplification equal to 0.5 for each fragment size. Figure 4.24 shows that the SF value required to produce a probability of successful amplification equal to 0.5, increases as the amplicon size decreases.

Table 4.14: SF values required to produce a probability of successful amplification equal to 0.5

DNA (bp)	0.5 Probability (SF)
500	2.68
300	2.70
100	2.72

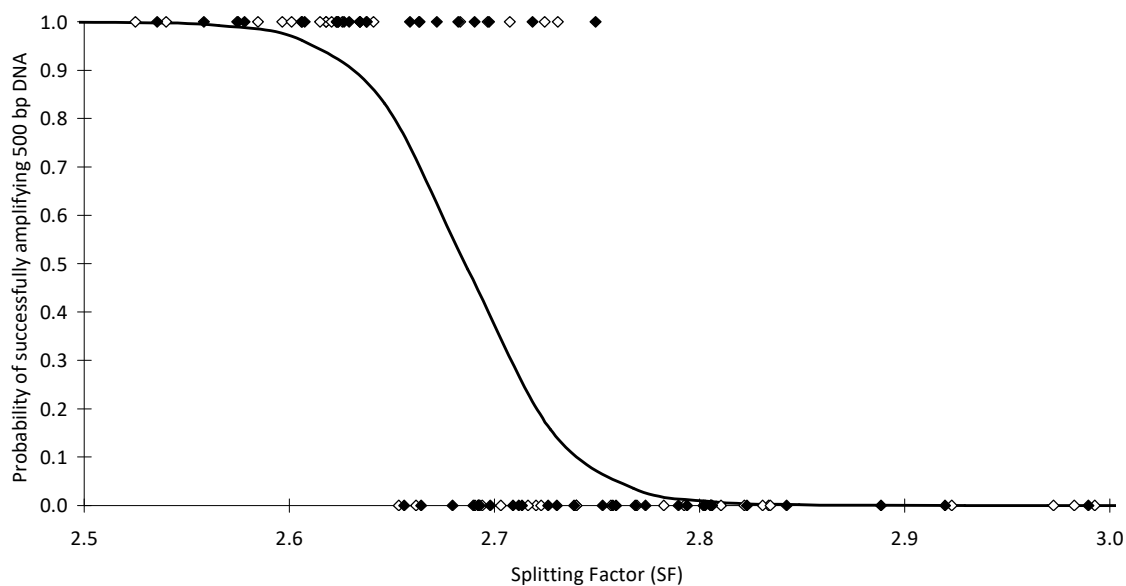


Figure 4.21: Relationship between the amplification of 500 bp DNA and SF. Calculated using the SF in bovine femurs heated (50-1000 °C) for (◇) 2 hours and (◆) 4 hours. The curve is the function of equation 4.11.

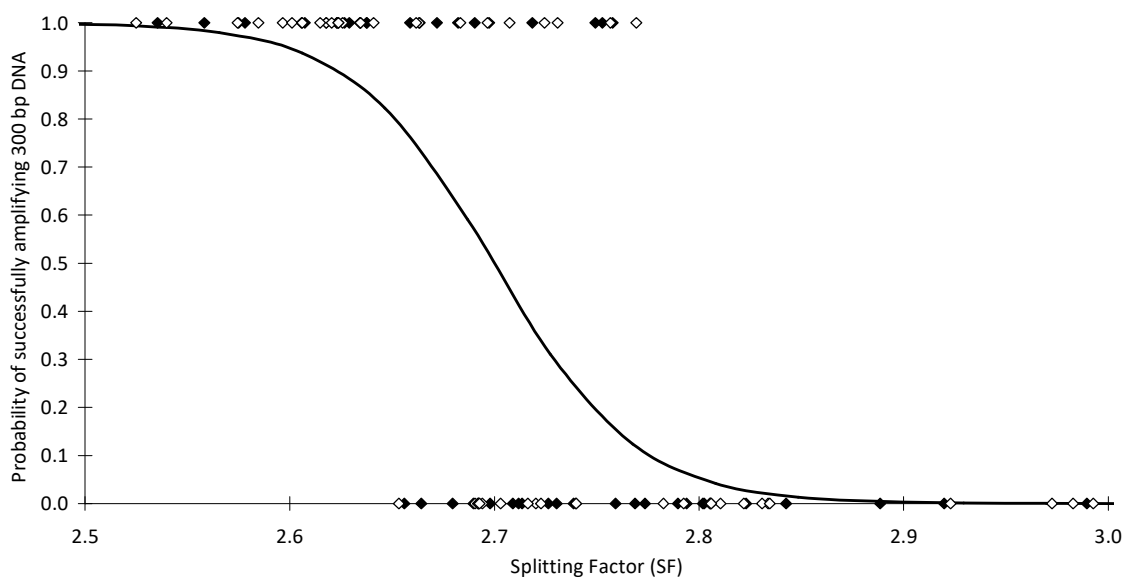


Figure 4.22: Relationship between the amplification of 300 bp DNA and SF. Calculated using the SF in bone heated (50-1000 °C) for (◇) 2 hours and (◆) 4 hours. The curve is the function of equation 4.12.

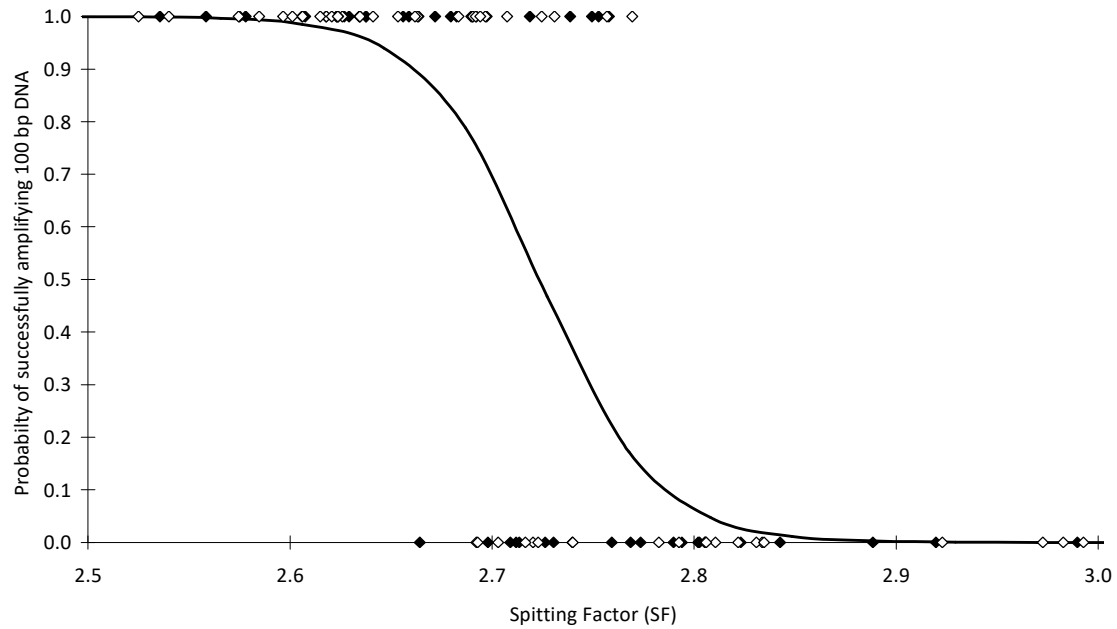


Figure 4.23: Relationship between the amplification of 100 bp DNA and SF. Calculated using the SF in bone heated (50-1000 °C) for (◇) 2 hours and (◆) 4 hours. The curve is the function of equation 4.13.

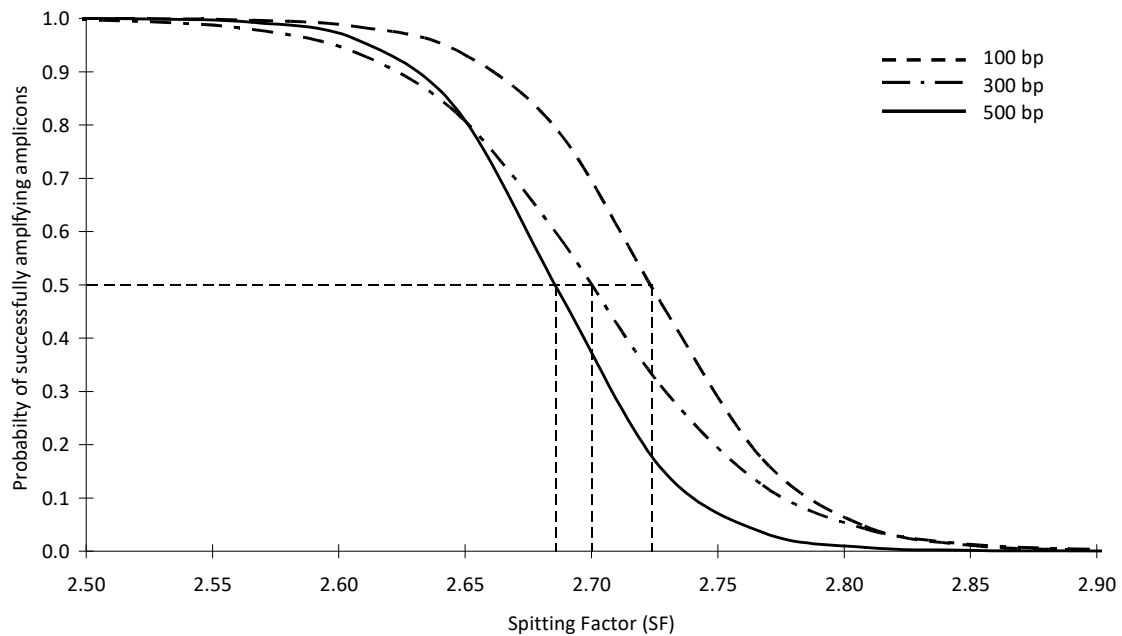


Figure 4.24: Relationship between the amplification of 500, 300 and 100 bp DNA and the SF of bHAP of heat-treated bone. Illustrated are the SF values described in table 4.14.

4.2.4.3 Assessing the likelihood of successful STR loci amplification using the SF of bHAP in heated bone

Significant logistic regression functions (Equations 4.14 and 4.15) were applied to the SF data to calculate the probability of successfully amplifying a minimum of 3 or 7 loci (figures 4.25 and 4.26, respectively) from heat-treated samples. Both logistic regressions classified ~86% of the samples correctly (Table 4.15) and recorded an AUROC curve above 0.95. The probability of successfully amplifying ≥ 3 or ≥ 7 STR loci from each sample was calculated (Appendix A8.4-A8.5).

$$\text{Probability} = 1 / (1 + \exp[-(a-bSF)])$$

Equation	No. STR loci	<i>a</i>	<i>b</i>
4.14	≥ 3	22.95	63.84
4.15	≥ 7	76.28	28.25

Table 4.15: Sample classification and AUROC curve of the logistic regressions of STR loci by SF

Model Classification	No. STR loci	
	≥ 3	≥ 7
True negatives (%)	87.9	88.5
True positives (%)	80.4	83.3
Total (%)	85.3	86.7
AUROC curve	0.96	0.95

Table 4.16 shows the SF value required to produce a probability of successful amplification equal to 0.5 for ≥ 3 and ≥ 7 STR loci respectively. Figure 4.27, shows that

the SF value required to produce a probability of successful amplification equal to 0.5, increased as the number of STR loci decreased.

Table 4.16: SF values required to produce a probability of successful amplification equal to 0.5 for ≥ 3 and ≥ 7 STR loci

Min loci	0.5 Probability (SF)
≥ 3	2.71
≥ 7	2.70

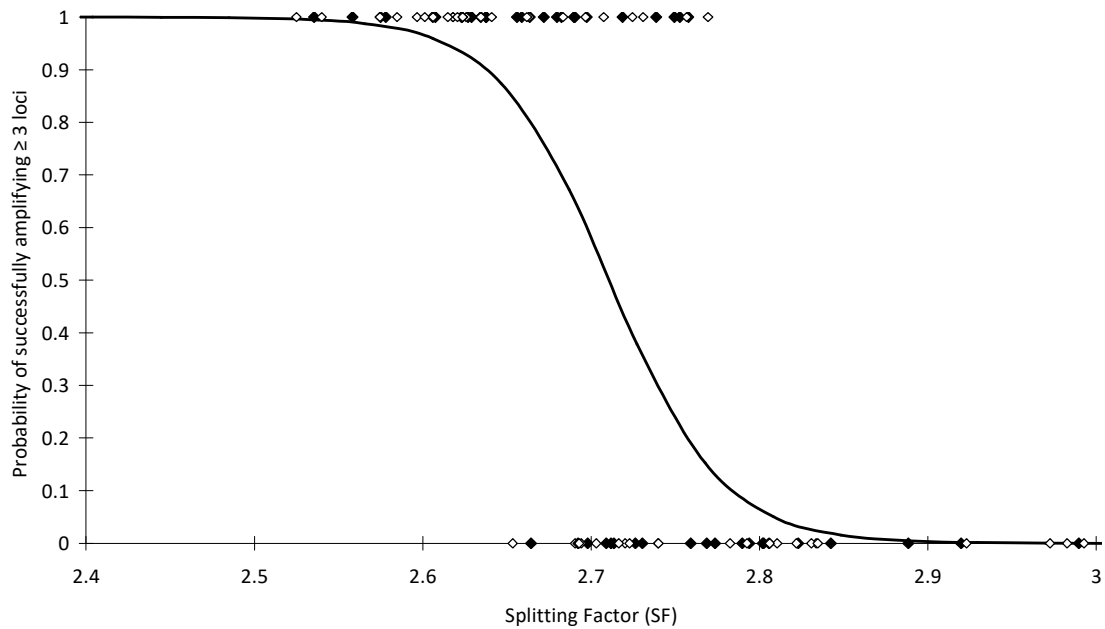


Figure 4.25: Relationship between the amplification of ≥ 3 loci and SF. Calculated using the SF in bone heated (50-1000 °C) for (◇) 2 hours and (◆) 4 hours. The curve is the function of Equation 4.14.

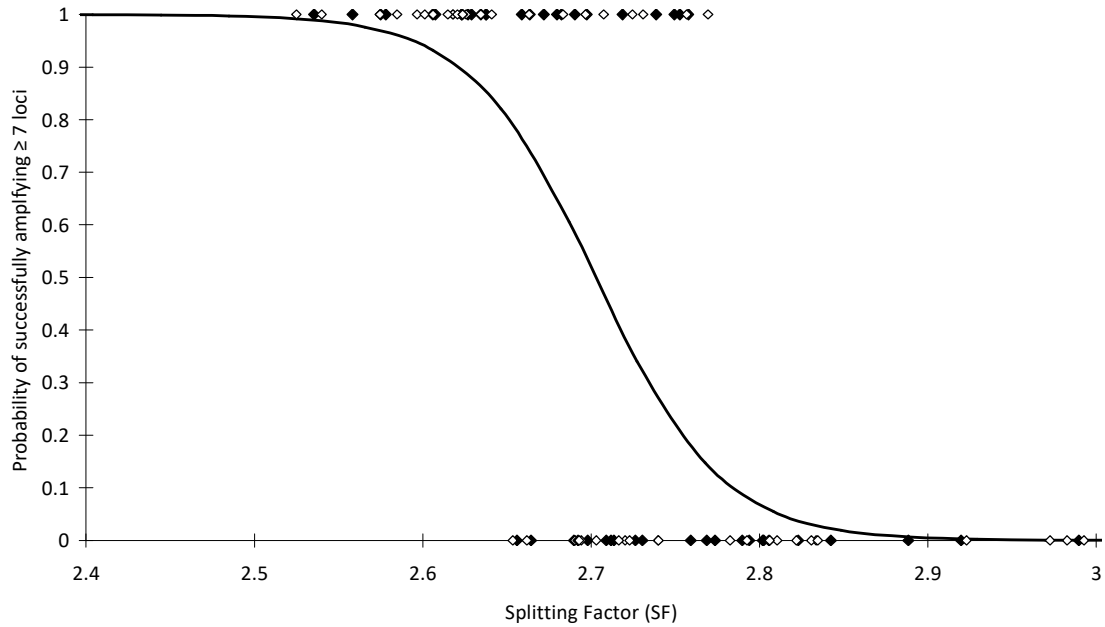


Figure 4.26: Relationship between the amplification of ≥ 7 loci and SF. Calculated using the SF in bone heated (50-1000 °C) for (◇) 2 hours and (◆) 4 hours. The curve is the function of Equation 4.15

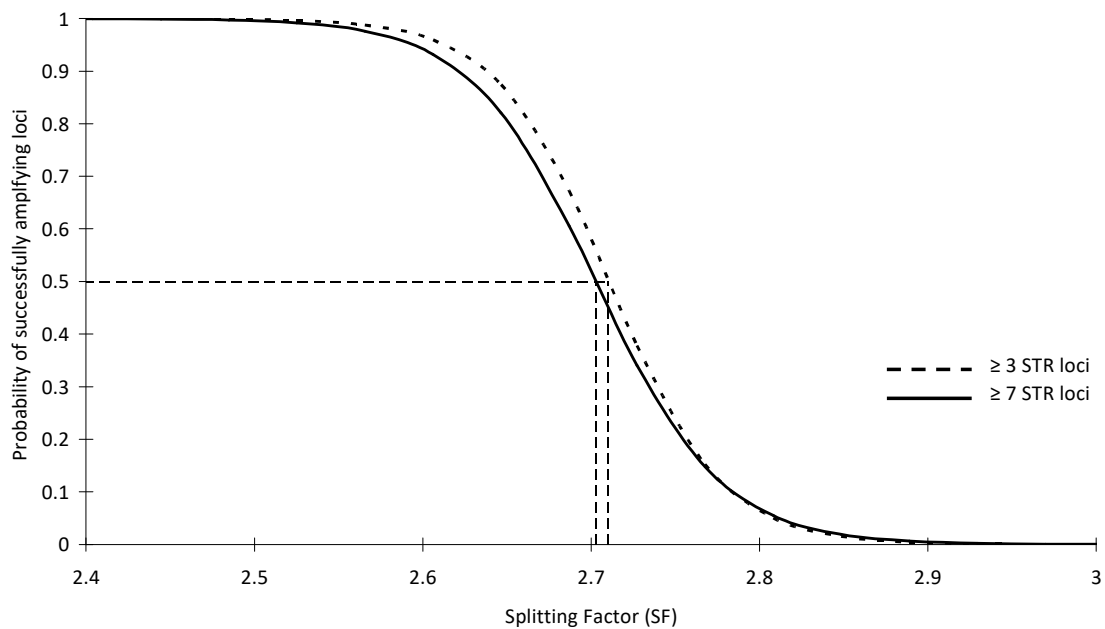


Figure 4.27: Relationship between the successful amplification of ≥ 3 and ≥ 7 loci and the SF of heat-treated bone. Illustrated are the SF values described in Table 4.16.

4.2.5 X-ray diffraction (XRD): Heat treatment study

XRD was used to investigate the effect of heat treatment on the structure of calcium hydroxyapatite (bHAP) in bovine femur. The coherence length, crystallite size/strain and the 'a'/'c' parameters of the mineral were measured for each sample. The sample set used in the heat treatment study was made from two series: samples heat-treated for two hours and samples heat-treated for four hours.

4.2.5.1 Effect of temperature on the Coherence Length (CL) and crystallite size of bone bHAP

The coherence length (CL) of bHAP did not change significantly in samples heated to temperatures between 39 and 600 °C for either two or four hour periods (Figure 4.28). In both sample series there was a significant increase in the CL of bHAP when samples were heated to 650 °C, this increase continued up to 1000 °C.

A similar trend was observed with regards to temperature and crystallite size (Figure 4.29). A significant increase in crystallite size occurred at 650 °C in both sample series, but did not continue to increase up to 1000°C.

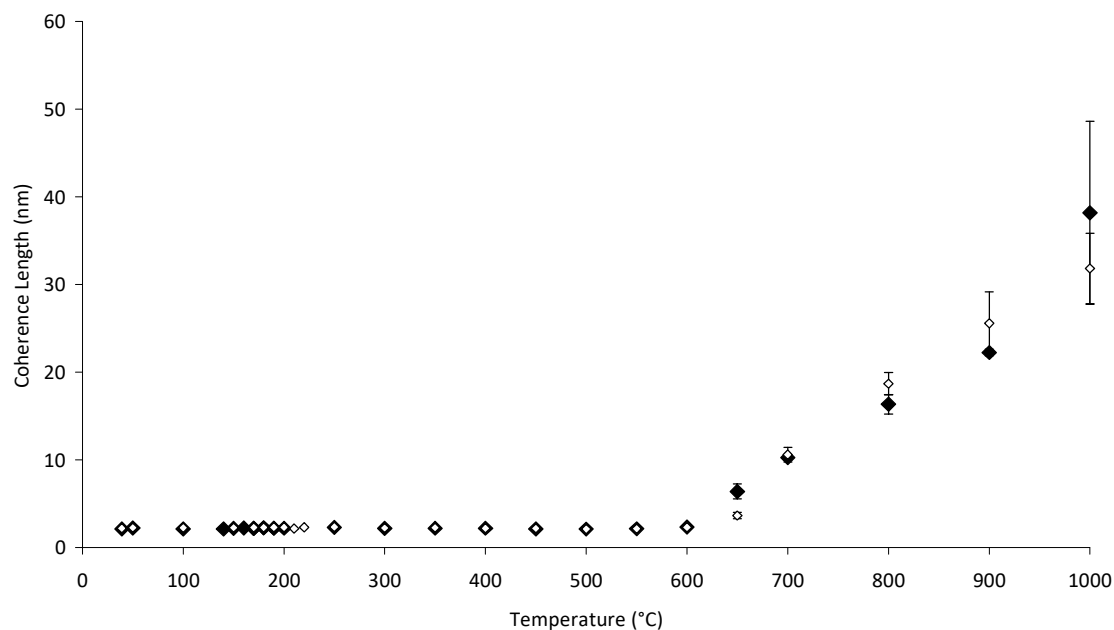


Figure 4.28: CL of bHAP in bone heated at various temperatures for (◇) 2 hours and (◆) 4 hours. Error bars (± 1 standard error).

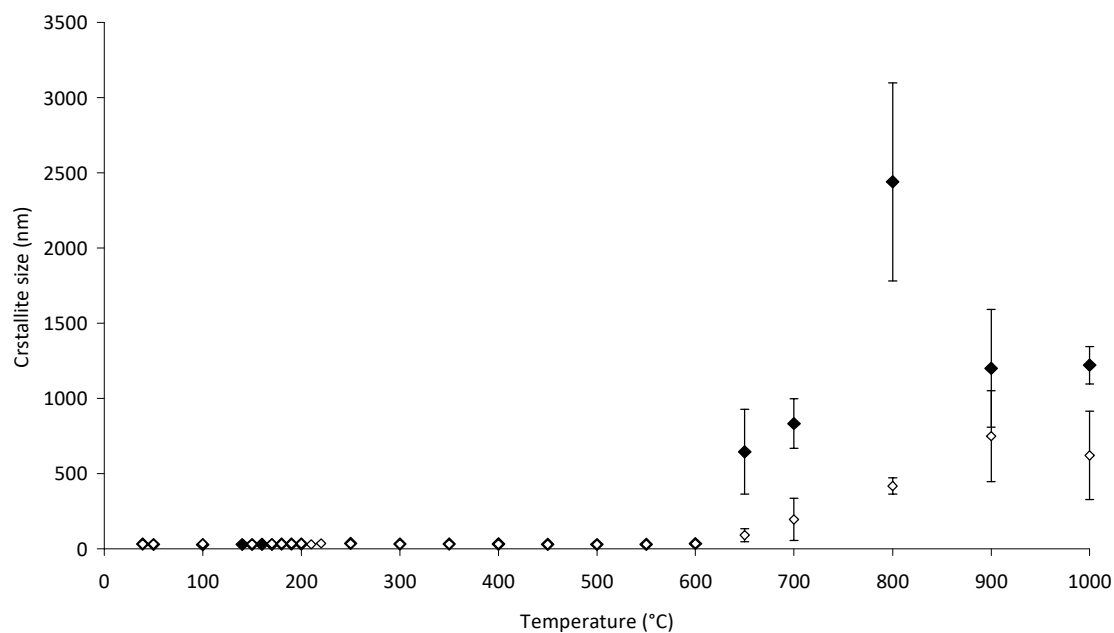


Figure 4.29: Crystallite size of bHAP in bone heated at various temperatures for (◇) 2 hours and (◆) 4 hours. Error bars (± 1 standard error).

4.2.5.2 Effect of temperature on the crystallite strain of bHAP in bone

The changes in crystallite strain of bHAP in samples heated for two and four hours were very similar over the temperature range (Figure 4.30). There is an overall increase in strain between control samples and samples heated to 250 °C. Above 250 °C there is an overall decrease, which continues, with the exception of samples heated to 650 °C, to decrease up to 1000 °C.

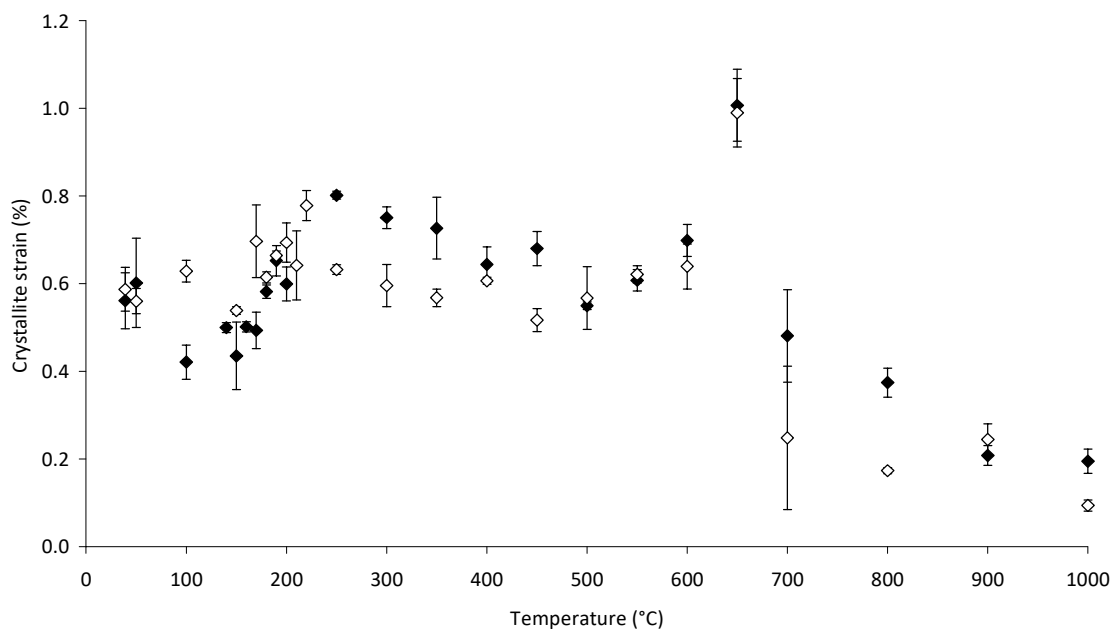


Figure 4.30: Crystallite strain of bHAP in bone heated at various temperatures for (◇) 2 hours and (◆) 4 hours. Error bars (± 1 standard error).

4.2.5.3 Effect of temperature on the lattice parameter 'a-axis' of bHAP in bone

A decrease of 'a-axis' was observed when bone was heated to 600 °C for two and four hours. Between 600 and 700 °C there was a sharp increase in 'a' and from 700 °C there were no significant changes, in either sample series, in the lattice parameter (Figure 4.31).

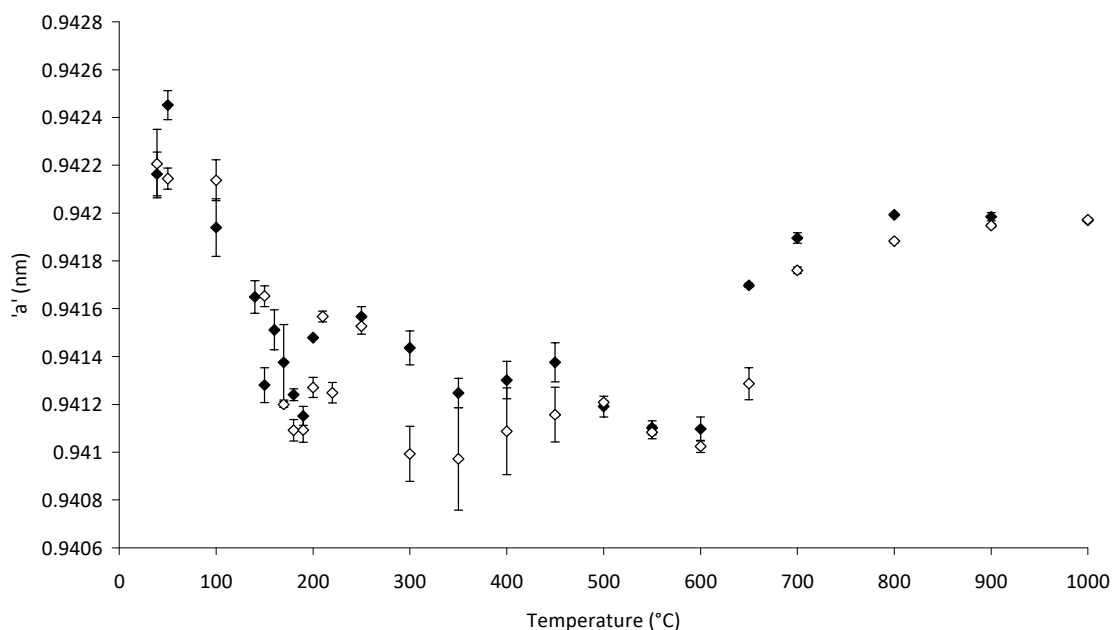


Figure 4.31: Lattice parameter 'a-axis' of bHAP in bone heated at various temperatures for (◇) 2 hours and (◆) 4 hours. Error bars (± 1 standard error).

4.2.5.4 Effect of temperature on the lattice parameter 'c-axis' of bHAP in bone

An increase of 'c-axis' was observed when bone was heated to 350 °C for two and four hours. Between 400 and 700 °C there was a gradual decrease in 'c-axis' and from 700 °C there were no significant changes, in either sample series, in the lattice parameter (Figure 4.32).

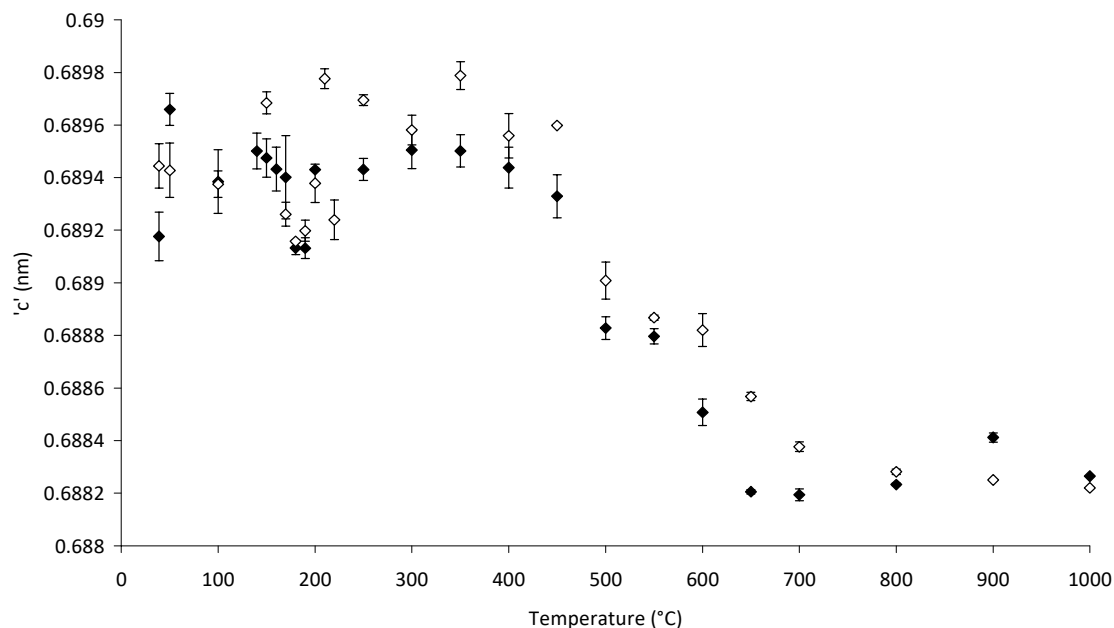


Figure 4.32: Lattice parameter 'c-axis' of bHAP in bone heated at various temperatures for (◇) 2 hours and (◆) 4 hours. Error bars (± 1 standard error).

4.2.5.5 Assessing the likelihood of PCR product amplification using the different XRD parameters from heated bone

Significant ($P < 0.05$) logistic regression models, using four analytical techniques; CL, crystallite size and lattice parameters 'a' and 'c', could be applied and used to calculate the probability of successfully amplifying 500, 300 and 100 bp DNA fragments from a heat treated sample.

The logistic regression models calculated using each parameter to predict successful amplification of the different sized fragments classified over 80 % of true negatives correctly (Equations 4.16-4.19). The models were relatively poor at classifying true positives and this is reflected by the low AUROC curve values (Table 4.16-4.4.18). Due to the low AUROC curve of each model and the fact that across the three amplicons and $\geq 3/\geq 7$ STR loci, the logistic regression curves were similar in shape for each parameter, only the logistic regression models that were calculated to predict the probability of successfully amplifying 500 bp are presented in this section. The logistic regressions that were calculated using XRD parameters to predict the probability of successfully amplifying 300 and 100 bp fragments and $\geq 3/\geq 7$ loci are presented in Appendix 7.

$$\text{Probability} = 1 / (1 + \exp[-(a-b\text{XRD parameter})])$$

Equation	XRD parameter	<i>a</i>	<i>b</i>
4.16	CL	8.54	4.09
4.17	Size	2.93	0.11
4.18	'a'	-2155.76	-2288.66
4.19	'c'	-1227.91	-1780.44

Table 4.17: Sample classification and AUROC curve of the logistic regressions of 500 bp fragment by measured XRD parameters

Classification	CL	Size	Parameter		
			Strain	'a'	'c'
True negatives (%)	97.21	99.04	-	86.54	89.42
True positives (%)	10.87	6.52	-	52.17	19.57
Total (%)	70.67	70.67	-	76.00	68.00
AUROC curve	0.709	0.707	-	0.797	0.694

Table 4.18: Sample classification and AUROC curve of the logistic regressions of 300 bp fragment by measured XRD parameters

Classification	CL	Size	Parameter		
			Strain	'a'	'c'
True negatives (%)	98	98	-	86	82.00
True positives (%)	8	6	-	48.00	18.00
Total (%)	68	67.33	-	73.33	60.67
AUROC curve	0.682	0.691	-	0.745	0.68

Table 4.19: Sample classification and AUROC curve of the logistic regressions of 100 bp DNA fragment by measured XRD parameters

Classification	CL	Size	Parameter		
			Strain	'a'	'c'
True negatives (%)	72.53	72.53	-	78.02	70.33
True positives (%)	42.37	50.85	-	44.07	37.29
Total (%)	60.67	64	-	64.67	57.33
AUROC curve	0.661	0.69	-	0.684	0.684

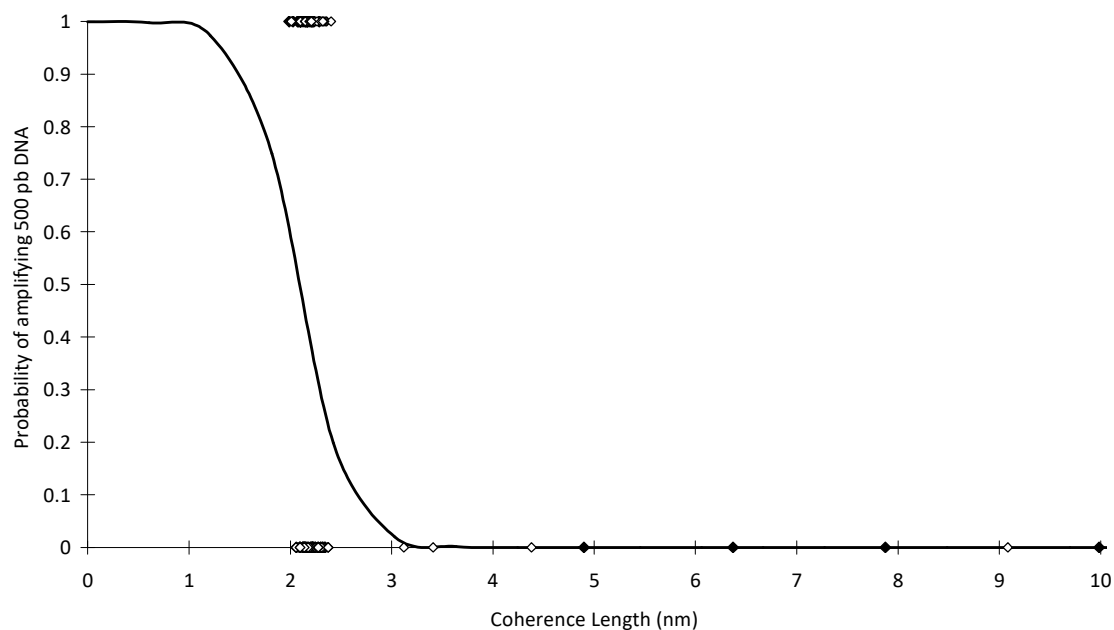


Figure 4.33: Relationship between the amplification of 500 bp DNA and CL of bHAP. Calculated using the coherence length of bHAP from bone heated (50-1000 °C) for (◇) 2 hours and (◆) 4 hours. The curve is the function of equation 4.16.

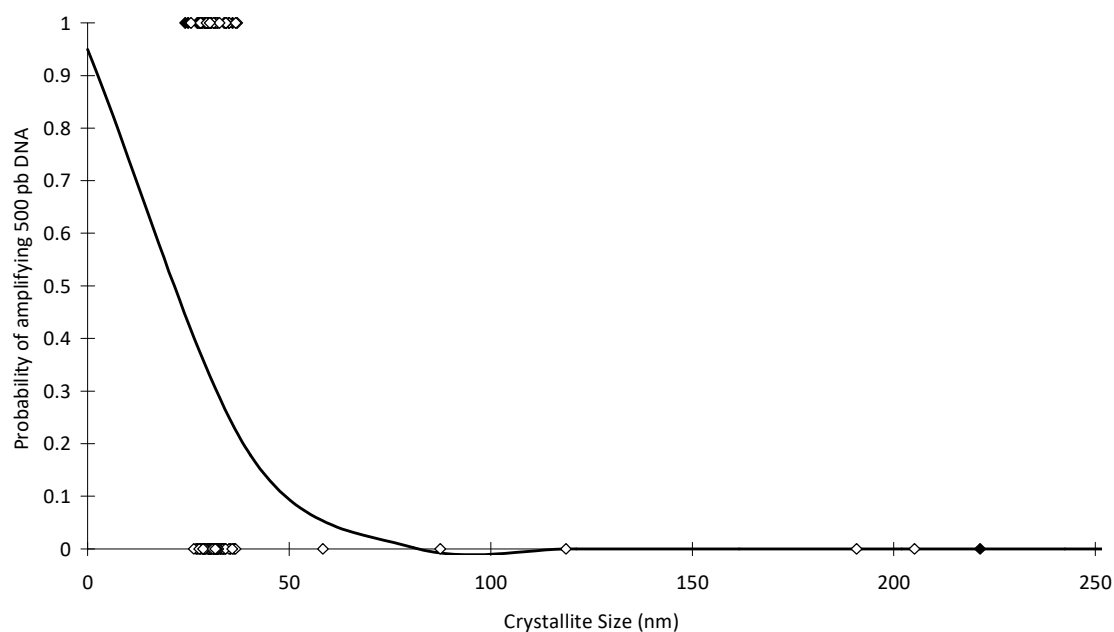


Figure 4.34: Relationship between the amplification of 500 bp DNA and crystallite size of bHAP. Calculated using the crystallite size of bHAP from bone heated (50-1000 °C) for (◇) 2 hours and (◆) 4 hours. The curve is the function of equation 4.17.

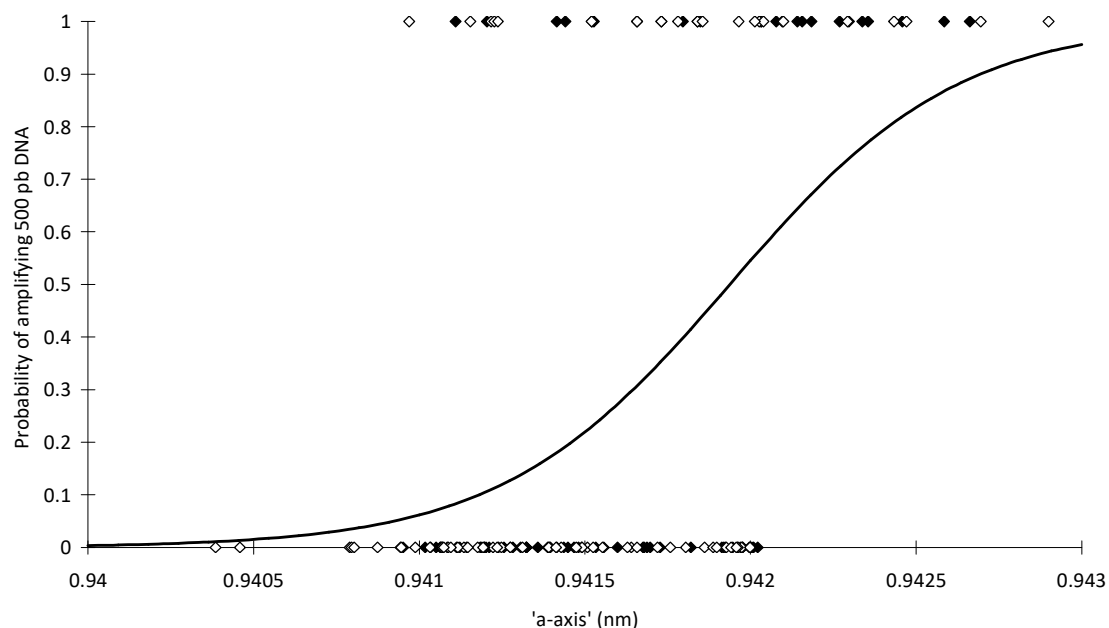


Figure 4.35: Relationship between the amplification of 500 bp DNA and the 'a-axis' parameter of bHAP. Calculated using the 'a-axis' parameter of bHAP from bone heated (50-1000 °C) for (◇) 2 hours and (◆) 4 hours. The curve is the function of equation 4.18.

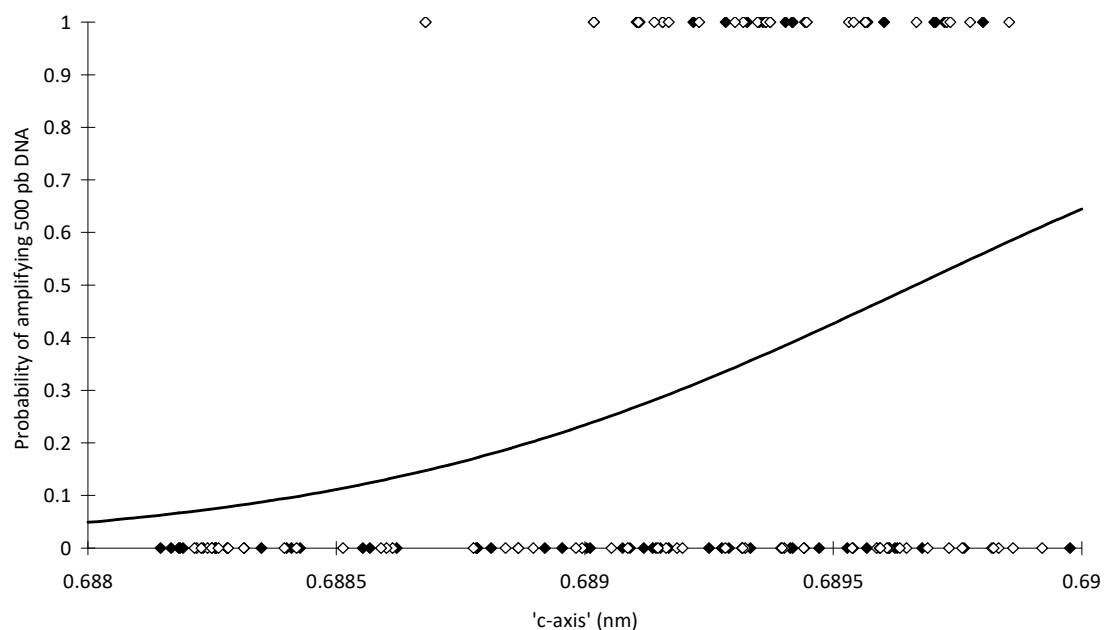


Figure 4.36: Relationship between the amplification of 500 bp DNA and the 'c-axis' parameter of bHAP. Calculated using the 'c-axis' parameter of bHAP from bone heated (50-1000 °C) for (◇) 2 hours and (◆) 4 hours. The curve is the function of equation 4.19.

4.2.5.6 *Assessing the likelihood of successful STR loci amplification using the different XRD parameters from heated bone*

Significant ($P < 0.05$) logistic regression models, using four XRD parameters CL, crystallite size and lattice parameters 'a' and 'c', could be applied and used to calculate the probability of successfully amplifying ≥ 3 or ≥ 7 STR loci from a heat-treated sample. The equations of each logistic regression can be seen in Appendix A7.3 and A7.4.

The models for each parameter and amplicon classified over 75 % of true negatives correctly, but were relatively poor at classifying true positives in comparison to colourimetry and FTIR spectroscopy logistic regressions (Tables 4.19 - 4.20). This is reflected by the low AUROC curve values. The logistic regression curves of each model can be found in Appendix A7.3 and A7.4.

Table 4.20: Sample classification and AUROC curve of the logistic regression, ≥ 3 loci by measured XRD parameters

Classification	Parameter				
	CL	Size	Strain	'a'	'c'
True negatives (%)	85.42	84.38	-	82.29	75.00
True positives (%)	24.07	35.19	-	46.30	35.19
Total (%)	63.33	66.67	-	69.33	66.67
AUROC curve	0.685	0.711	-	0.791	0.677

Table 4.21: Sample classification and AUROC curve of the logistic regression, ≥ 7 loci by measured XRD parameters

Classification	CL	Size	Parameter		
			Strain	'a'	'c'
True negatives (%)	91.9	92.9	-	82.85	78.79
True positives (%)	23.5	25.5	-	49.02	25.49
Total (%)	68.7	70.00	-	71.33	60.67
AUROC curve	0.69	0.70	-	0.76	0.70

4.2.6 Microhardness: Heat treatment study

The microhardness of heat-treated bone has been studied in numerous publications (section 2.8.5.1). All samples, including controls, from the two and four-hour heat treatment series were analysed (section 3.7).

4.2.6.1 Effect of temperature on the microhardness of bovine femur

The same trend between microhardness and temperature was observed in both sample series. A slight initial increase then decrease was observed between 39 and 450 °C; this initial decrease was greater in samples that had been heat treated after four hours when compared with samples heated for two. In samples heated to temperatures above 450 °C there was a gradual increase in microhardness until 700 °C; above which there was a significant increase (Figure 4.37).

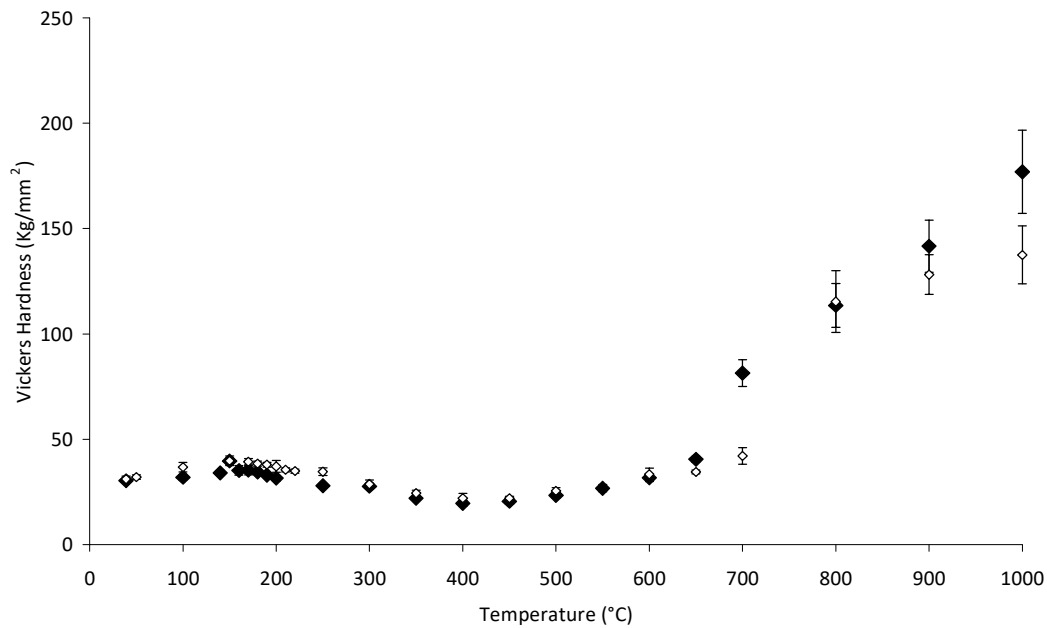


Figure 4.37: The microhardness of Haversian systems in bone femoral bone heated at various temperatures for (◇) 2 and (◆) 4 hours.

4.1.6.2 Assessing the likelihood of PCR product amplification using microhardness

Significant logistic regression functions could not be applied to the microhardness data in order to calculate the probability of successfully amplifying any of the DNA targets from heated bone (Table 4.21).

Table 4.22: P-values of logistic regression functions predicting PCR products using microhardness

logistic regression	p-value
500 bp	0.6
300 bp	0.6
100 bp	0.6
≥ 3 STR Loci	0.5
≥ 7 STR Loci	0.5

4.2.7 Assessing the likelihood of successful PCR product amplification using multiple different physical parameters of heated bone

By combining parameters, it may be possible to improve the accuracy of sample classification. When using colourimetry and FTIR spectroscopy data together, 100 % of true positives were correctly classified, irrespective of the amplicon size (Tables 4.22 - 4.24). The equation of each model can be found in the Appendix A8. The probability of successfully amplifying each amplicon, from each sample was calculated (Appendix 8). Using the APR/SF and colourimetry it was possible to correctly classify samples that were incorrectly classified by one another e.g. samples heated between 650-700 °C that were incorrectly classified as false positives, were correctly classified when colourimetry was used in conjunction with the APR/SF.

Table 4.23: Sample classification and AUROC curve of the logistic regression, 500 bp DNA by FTIR spectroscopy and colourimetry

Classification (%)	Parameter						
	SF	AP	SF + AP	colour	colour+AP	colour+SP	colour+AP+SF
True negatives	95.24	92.31	96.15	92.30	95.24	93.33	94.29
True positives	82.22	91.3	86.95	95.65	100.00	100.00	100.00
Total	91.33	92	93.33	93.33	96.67	95.33	96.00
AUROC curve	0.97	0.98	0.99	0.96	0.99	0.99	0.99

Table 4.24: Sample classification and AUROC curve of the logistic regression, 300 bp DNA by FTIR spectroscopy and colourimetry

Classification (%)	Parameter						
	SF	AP	SF + AP	colour	Colour+AP	Colour+SP	Colour+AP+SF
True negatives	88	92	93	92.00	94.00	94.00	94.00
True positives	82	96	94	96.00	98.00	98.00	98.00
Total	86	93.3	93.33	93.33	95.33	95.33	95.33
AUROC curve	0.95	0.98	0.99	0.96	0.99	0.99	0.99

Table 4.25: Sample classification and AUROC curve of the logistic regression, 100 bp DNA by FTIR spectroscopy and colourimetry

Classification (%)	Parameter						
	SF	AP	SF + AP	colour	Colour+AP	Colour+SP	Colour+AP+SF
True negatives	87.91	95.6	95.6	87.91	95.60	95.60	95.60
True positives	86.44	96.61	96.61	96.10	100.00	100.00	100.00
Total	87.33	96	96	93.33	97.33	97.33	97.33
AUROC curve	0.97	0.99	0.99	0.97	0.99	0.99	0.99

4.2.8 Assessing the likelihood of successful STR loci amplification using multiple different physical parameters of heated bone

When using colourimetry and FTIR spectroscopy data together, 100 % of true positives were correctly classified, irrespective of the minimum number alleles being amplified (Table 4.25 and 4.26). The equations of each model can be found in Appendix 8.

Table 4.26: Sample classification and AUROC curve of the logistic regression, ≥ 3 STR loci amplification by FTIR spectroscopy and colourimetry

Classification (%)	Parameter						
	SF	AP	SF + AP	colour	Colour+AP	Colour+SP	Colour+AP+SF
True negatives	87.88	93.93	93.68	95.8	96.9	96.9	97.69
True positives	80.39	96.08	96.36	96.4	100.00	100.00	100.00
Total	85.33	94.00	94.67	96.00	98.00	98.00	98.00
AUROC curve	0.96	0.97	0.98	0.96	0.99	0.99	0.99

Table 4.27: Sample classification and AUROC curve of the logistic regression, ≥ 7 STR loci amplification by FTIR spectroscopy and colourimetry

Classification (%)	Parameter						
	SF	AP	SF + AP	colour	Colour+AP	Colour+SP	Colour+AP+SF
True negatives	88.54	92.93	91.92	93.9	96.0	96.00	96.00
True positives	83.33	96.08	94.12	98.0	100	100.00	100.00
Total	86.67	94.00	92.67	95.3	97.3	97.3	97.3
AUROC curve	0.95	0.99	0.98	0.96	0.99	0.99	0.99

4.2.9 Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) was used to investigate the endo and exothermic processes that occurred within bone during heat treatment. The heat flow (mW) through the sample dropped significantly between 25 and 110 °C. Above 110 °C there was an increase in heat flow which reached -5.5 mW by 250 °C. Between 250 and 350 there was a gradual decrease in heat flow. Above 350 °C there was a steady increase in heat flow.

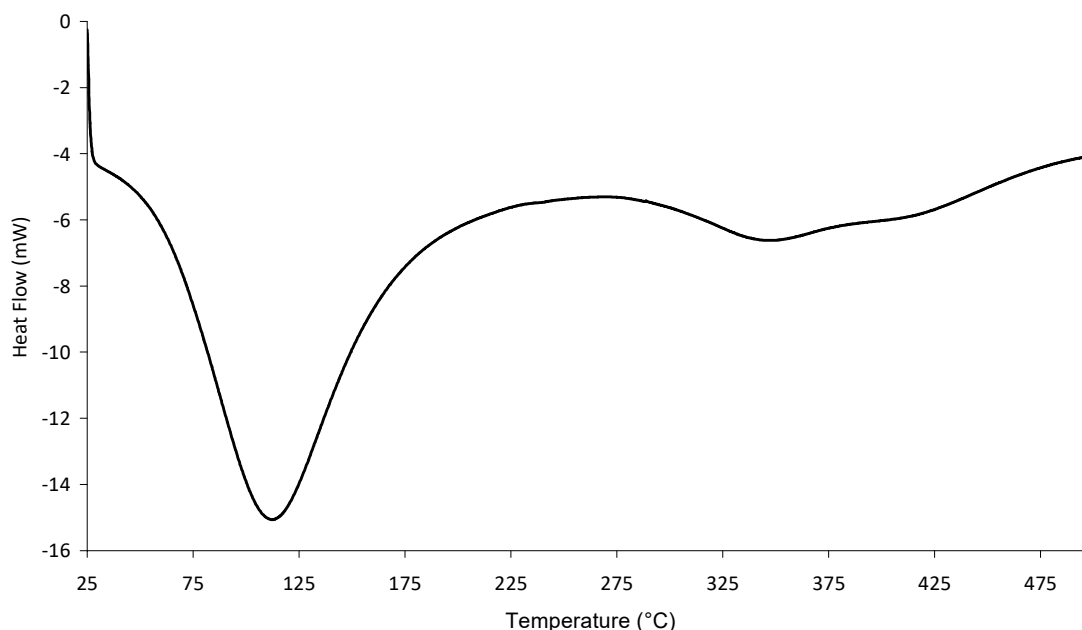


Figure 4.38: DSC analysis of fresh bovine femoral bone powder.

4.2.10 Thermogravimetric Analysis (TGA)

Thermogravimetric Analysis (TGA) was also used to investigate the effect temperature had on the weight of bone. As the temperature increased, the weight of the sample decreased. This decrease began at ~40 °C and by 190 °C, 10% of the samples original weight was lost. Above 190 °C, the weight of bone continued to decrease as the temperature increased. At 280 °C, there was a sharp decrease in weight loss. At 500 °C, the bone sample had lost 28% of its initial weight.

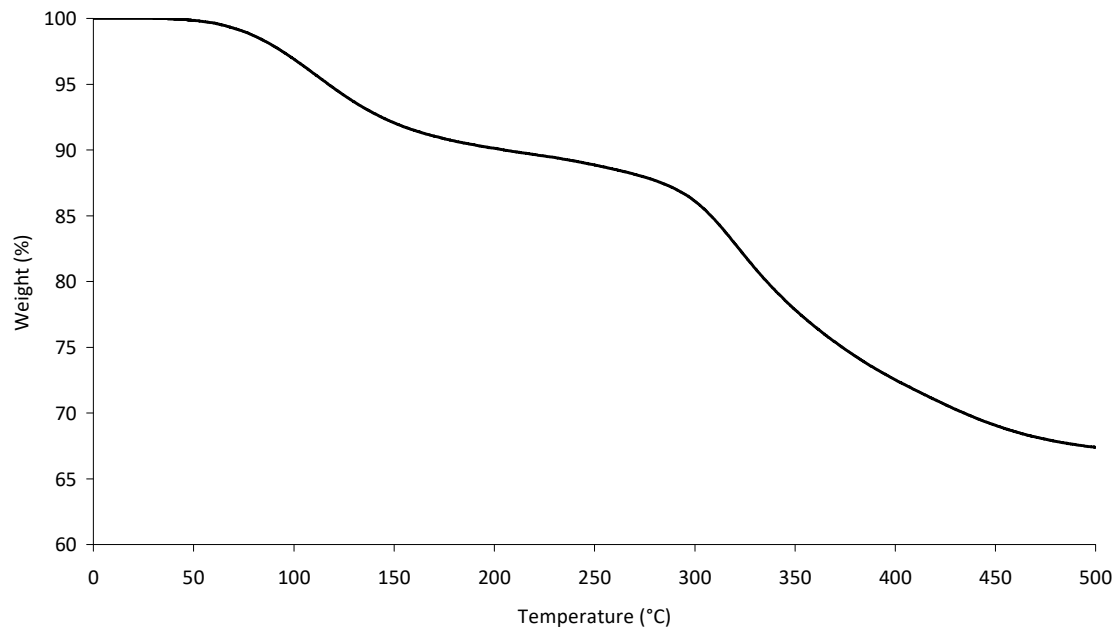


Figure 4.39: TGA of fresh bovine femoral bone powder

4.3 Water submersion study

Chapter 2 outlined the techniques that were chosen for the water submersion study; the methods of which can be found in chapter 3. The results from the water submersion study are sequentially presented in this section as shown in Table 4.26.

None of the measured parameters could be correlated with DNA fragment amplification. The number of samples in each environment where DNA amplification (300-500 bp fragments) failed was insufficient for statistical analysis.

Table 4.28: Presented water submersion study results

Technique	Description
DNA amplification	PCR
FTIR	Crystallinity (Splitting Factor)
Spectroscopy	Amide to Phosphate Ratio
XRD	Crystallinity (Coherence Length)
	Crystallite Size
	Crystallite Strain
	Lattice Parameters
Microhardness	Vickers Hardness
Microscopy	Histology

4.3.1 Amplification of PCR products: Water submersion Study

Standard PCR was used to determine the pattern of DNA degradation in bone samples submerged in either distilled water or synthetic seawater, and maintained at two different temperatures (section 3.3.2).

4.3.1.1 Effect of water submersion on PCR product amplification from bone

The 500 bp fragment was successfully amplified from control bone samples and from samples submerged in cold distilled water or synthetic seawater for up to 104 weeks. Amplification of the 500 bp fragment consistently failed in samples that were submerged in warm water (distilled and seawater) for 80 weeks or longer, although there were a number of samples that failed to amplify the amplicon when submerged for a shorter period of time. The amplification 300 and 100 bp fragments also failed in samples submerged in warm synthetic seawater for 92 weeks or longer (Table 4.27).

Table 4.29: PCR product amplification from submerged bone. A: Cold distilled water, B: warm distilled water, C: cold synthetic seawater, D: warm synthetic seawater.

A				B				C				D			
DNA (bp)				DNA (bp)				DNA (bp)				DNA (bp)			
Week	500	300	100	Week	500	300	100	Week	500	300	100	Week	500	300	100
Control x8	+	+	+	Control x8	+	+	+	Control x8	+	+	+	Control x8	+	+	+
2	+	+	+	2	+	+	+	2	+	+	+	2	+	+	+
2	+	+	+	2	+	+	+	2	+	+	+	2	+	+	+
2	+	+	+	2	+	+	+	2	+	+	+	2	+	+	+
4	+	+	+	4	+	+	+	4	+	+	+	4	+	+	+
4	+	+	+	4	+	+	+	4	+	+	+	4	+	+	+
4	+	+	+	4	+	+	+	4	+	+	+	4	+	+	+
20	+	+	+	20	-	+	+	20	+	+	+	20	+	+	+
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32	+	+	+	32	+	+	+	32	+	+	+	32	+	+	+
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92	+	+	+	92	+	+	+	92	+	+	+	92	-	-	+
104	+	+	+	104	-	+	+	104	+	+	+	104	-	-	-
104	+	+	+	104	-	+	+	104	+	+	+	104	-	+	+
104	-	+	+	104	-	-	+	104	+	+	+	104	-	-	+

4.3.2 FTIR spectroscopy analysis: Water submersion Study

The Amide I to Phosphate ratio and Splitting factor are two parameters that can be used to measure of the integrity of collagen (section 2.1.1) and bone bHAP crystallinity respectively.

No significant correlations were found between the APR (Figure 4.40) or SF (Figure 4.41) and the number of weeks the bovine femoral bone samples were submerged for, regardless of the temperature or type of water.

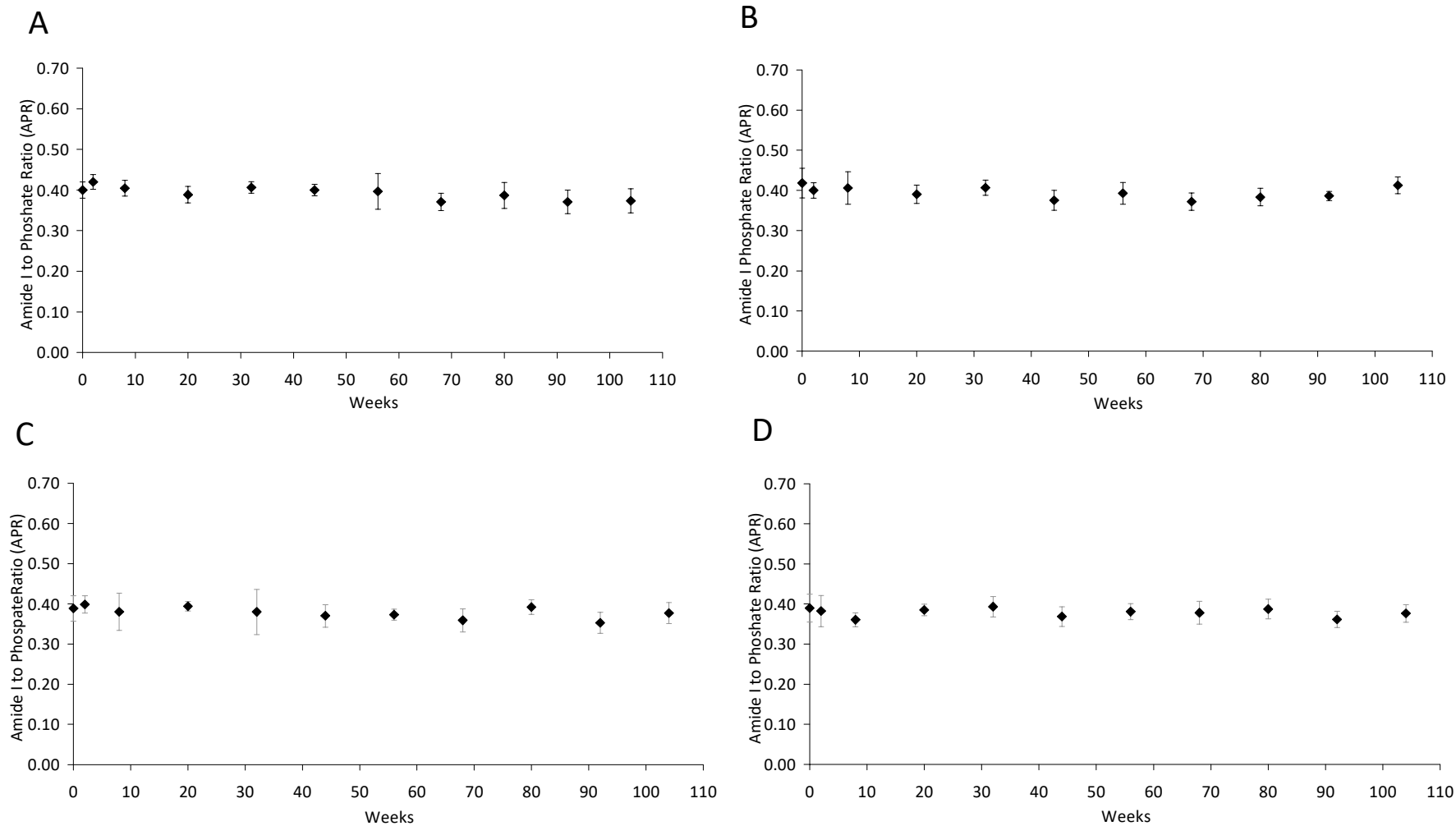


Figure 4.40: APR of bovine femur that had been submerged in four aquatic environments for up to 104 weeks. A: Cold (~7 °C) distilled water, B: Warm (~27 °C) distilled water, C: Cold (~7 °C) synthetic seawater, D: Warm (~27 °C) synthetic seawater. Error bars (± 1 standard error).

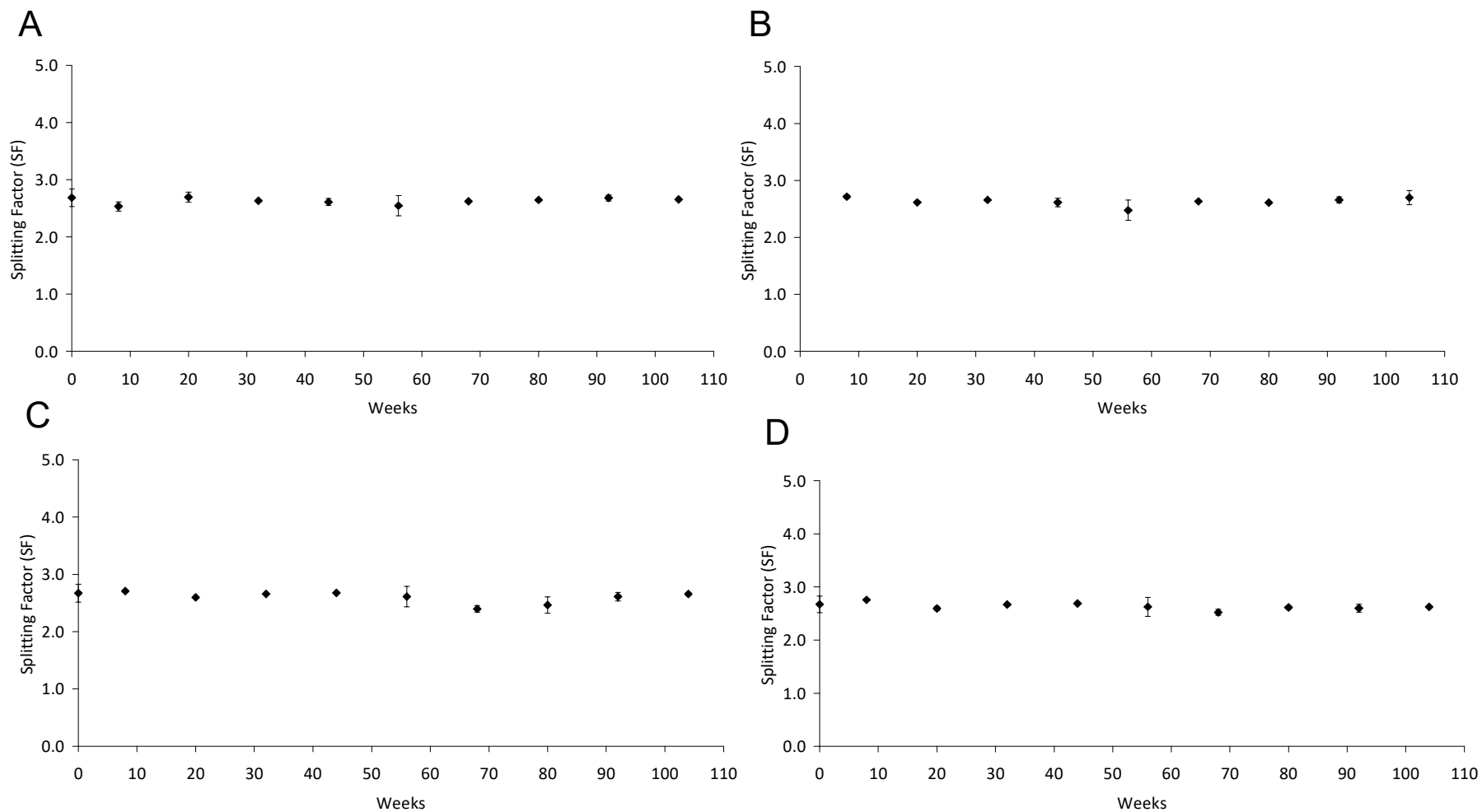


Figure 4.41: SF of bHAP in bovine femur that had been submerged in four aquatic environments for up to 104 weeks. A: Cold ($\sim 7^\circ\text{C}$) distilled water, B: Warm ($\sim 27^\circ\text{C}$) distilled water, C: Cold ($\sim 7^\circ\text{C}$) synthetic seawater, D: Warm ($\sim 27^\circ\text{C}$) synthetic seawater. Error bars (± 1 standard error).

4.3.3 X-ray Diffraction: Water submersion Study

XRD was used to investigate the effect of water submersion on the structure of bone bHAP in bovine femoral bone.

There were no significant correlations between the coherence length (Figure 4.33), the crystallite size (Figure 4.34) and the cell unit parameters 'a-axis' and 'c-axis' (Figure 4.35) of bone bHAP and the duration of submersion, regardless of the temperature and type of water. A similar trend between all four aquatic environments was observed between the crystallite strain and the period of submersion. In all four aquatic environments, there was an initial increase in microstrain after two weeks. The microstrain then gradually declined as the duration of submersion increased (Figure 4.34).

4.3.4 Microhardness: Water submersion study

The Vickers hardness (VH) of each sample was also investigated. In each aquatic environment there was a decline in VH as the duration of submersion increased. This trend is particularly evident in bone samples that were submerged in warm temperatures or in seawater.

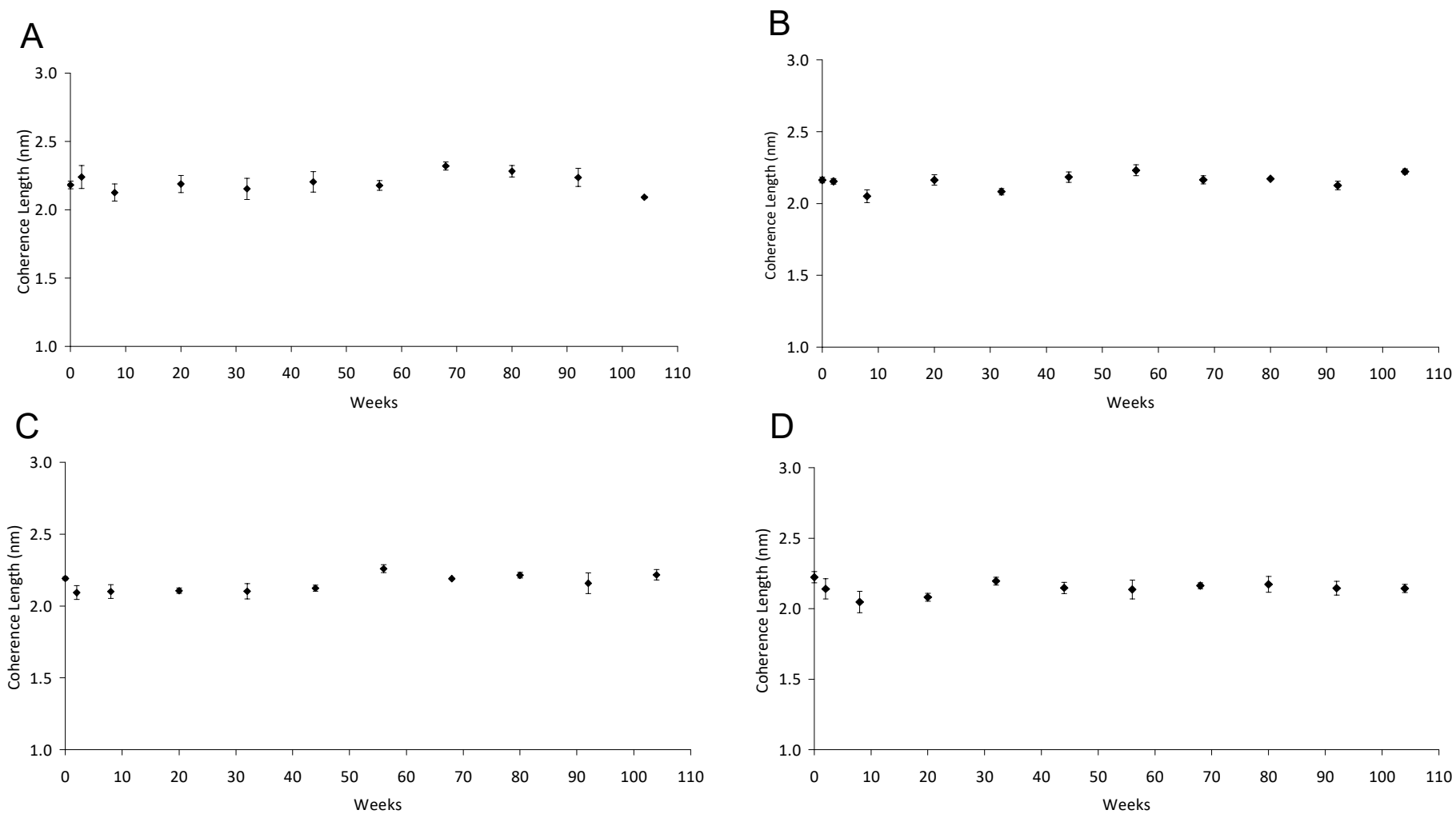


Figure 4.42: CL of bHAP in bovine femur that had been submerged in four aquatic environments for up to 104 weeks. A: Cold ($\sim 7^\circ\text{C}$) distilled water, B: Warm ($\sim 27^\circ\text{C}$) distilled water, C: Cold ($\sim 7^\circ\text{C}$) synthetic seawater, D: Warm ($\sim 27^\circ\text{C}$) synthetic seawater. Error bars (± 1 standard error).

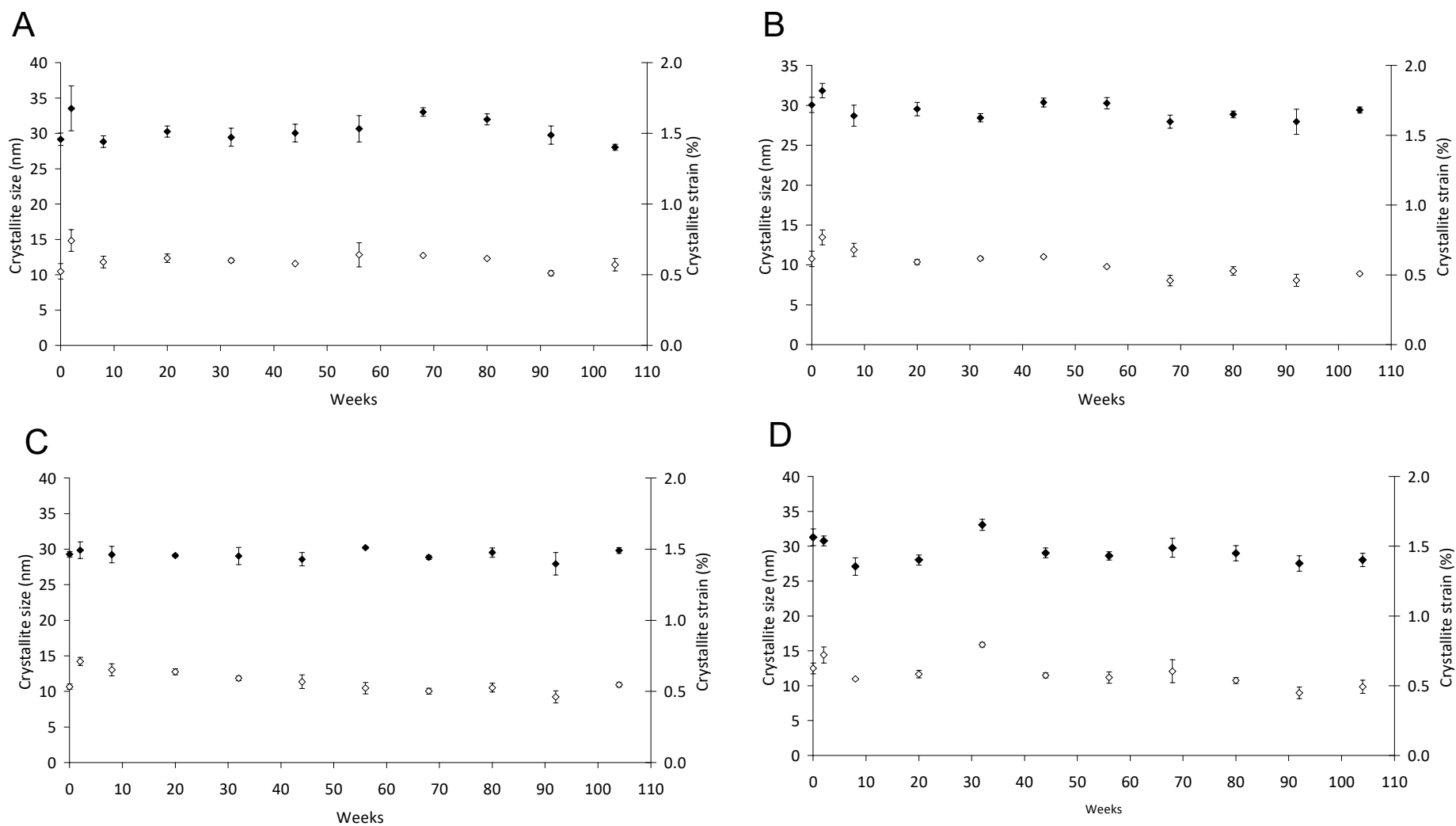


Figure 4.43: Crystallite size (◆) and microstrain (◇) of bHAP in bovine femur that had been submerged in four aquatic environments for up to 104 weeks. A: Cold (~7 °C) distilled water, B: Warm (~27 °C) distilled water, C: Cold (~7 °C) synthetic seawater, D: Warm (~27 °C) synthetic seawater. Error bars (± 1 standard error).

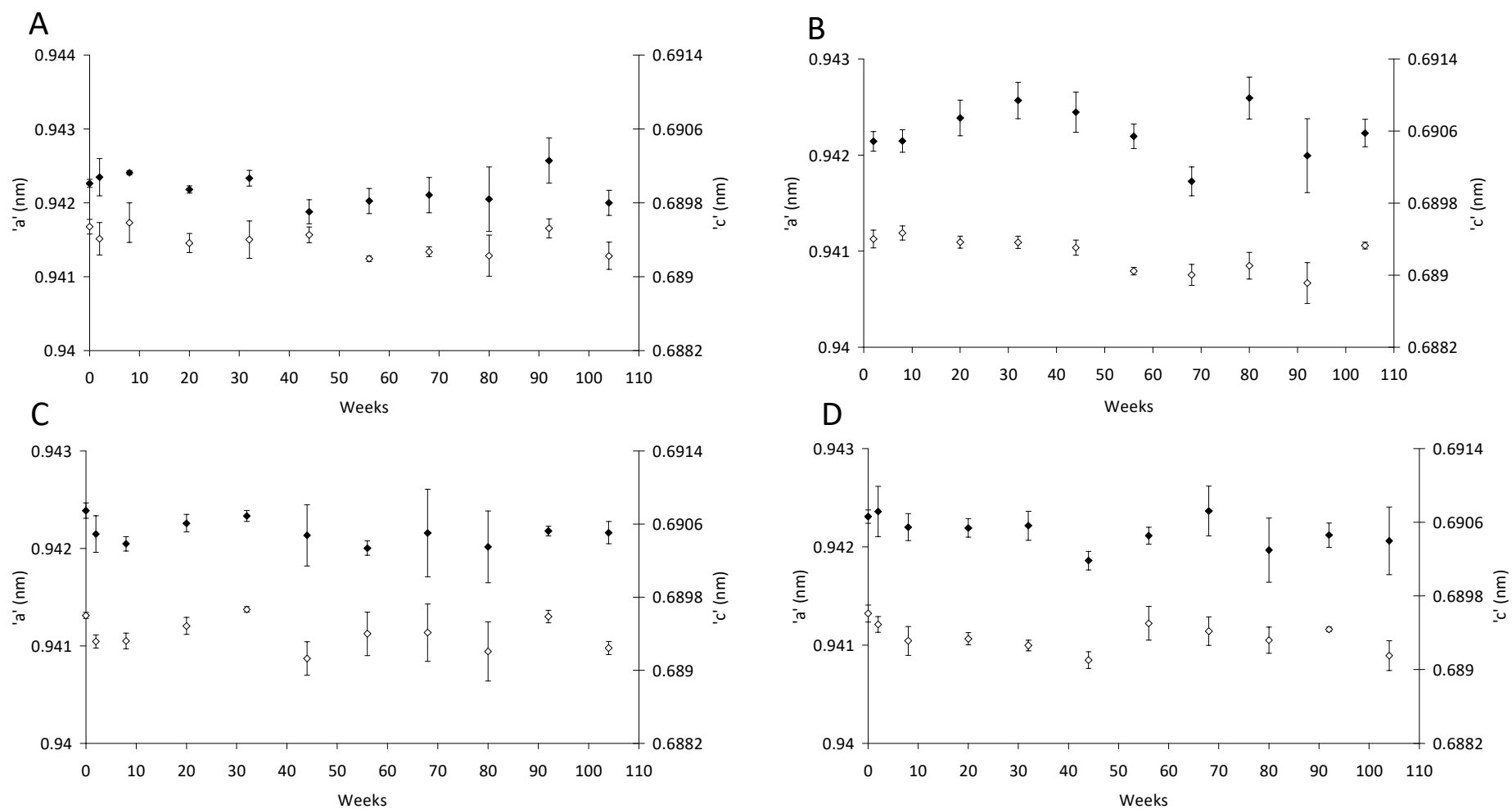


Figure 4.44: lattice parameter 'a-axis' (◆) and 'c-axis' (◇) of bHAP in bovine femur that had been submerged in four aquatic environments for up to 104 weeks. A: Cold ($\sim 7^\circ\text{C}$) distilled water, B: Warm ($\sim 27^\circ\text{C}$) distilled water, C: Cold ($\sim 7^\circ\text{C}$) synthetic seawater, D: Warm ($\sim 27^\circ\text{C}$) synthetic seawater. Error bars (± 1 standard error).

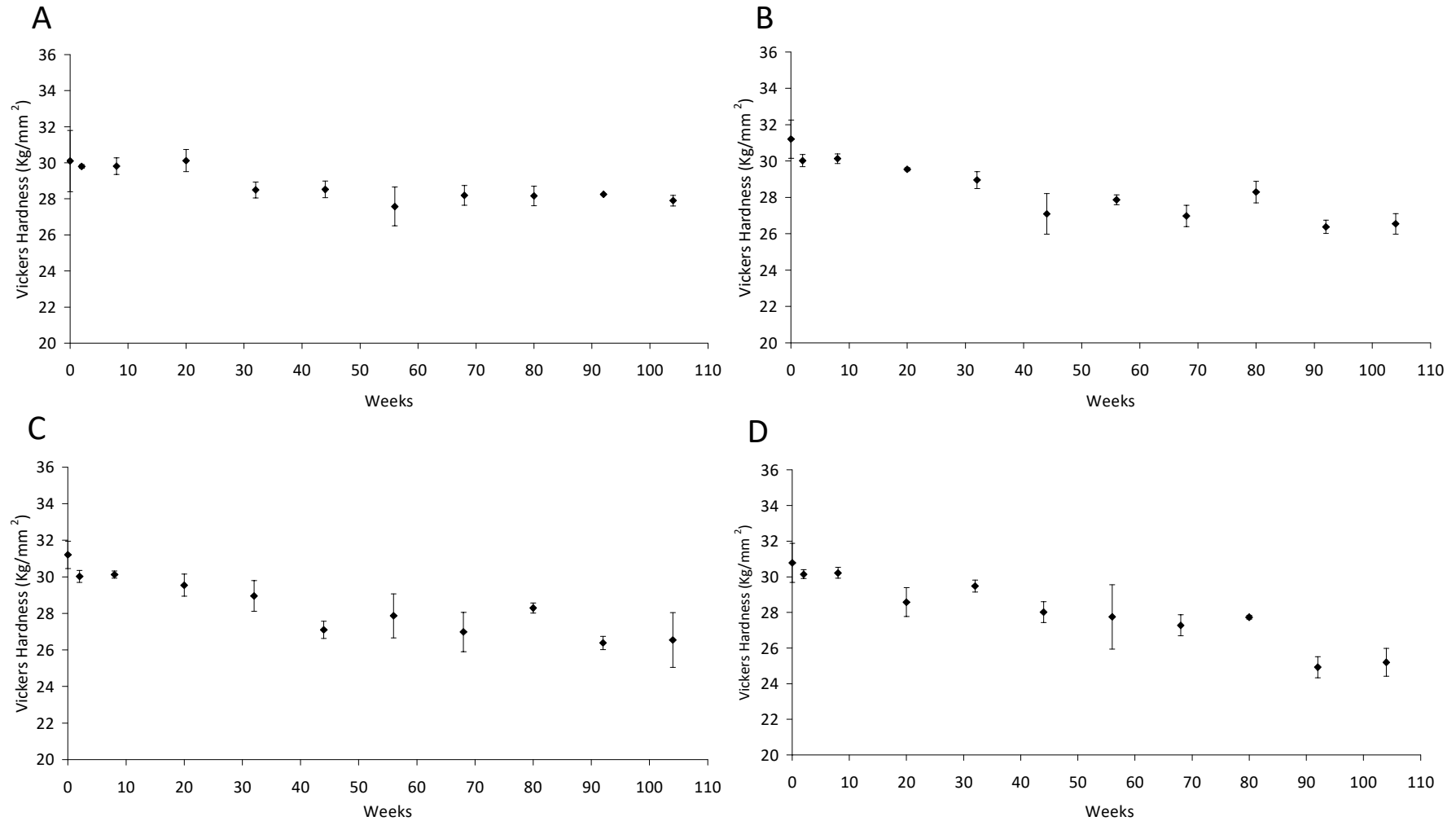


Figure 4.45: VH of Haversian systems in bovine femur that had been submerged in four aquatic environments for up to 104 weeks. A: Cold (~7 °C) distilled water, B: Warm (~27 °C) distilled water, C: Cold (~7 °C) synthetic seawater, D: Warm (~27 °C) synthetic seawater. Error bars (± 1 standard error).

4.3.4 Histology: Water submersion Study

Bone samples, taken from the water submersion study, were viewed for histological features.

There were no visual changes in the histological features of the Haversian systems in bovine femoral bone, when compared to fresh samples (Figure 4.36), regardless of the duration and type of water that they were submerged in (Figure 4.37). The Haversian systems remained their visual integrity and retained their irregular shape.

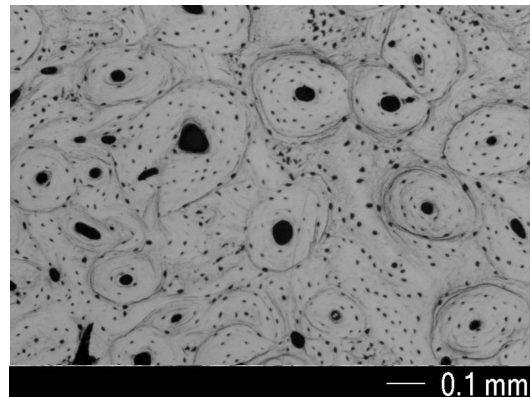


Figure 4.46: Microscopic image (50x magnification) of regions of dense Haversian systems in fresh bovine femur.

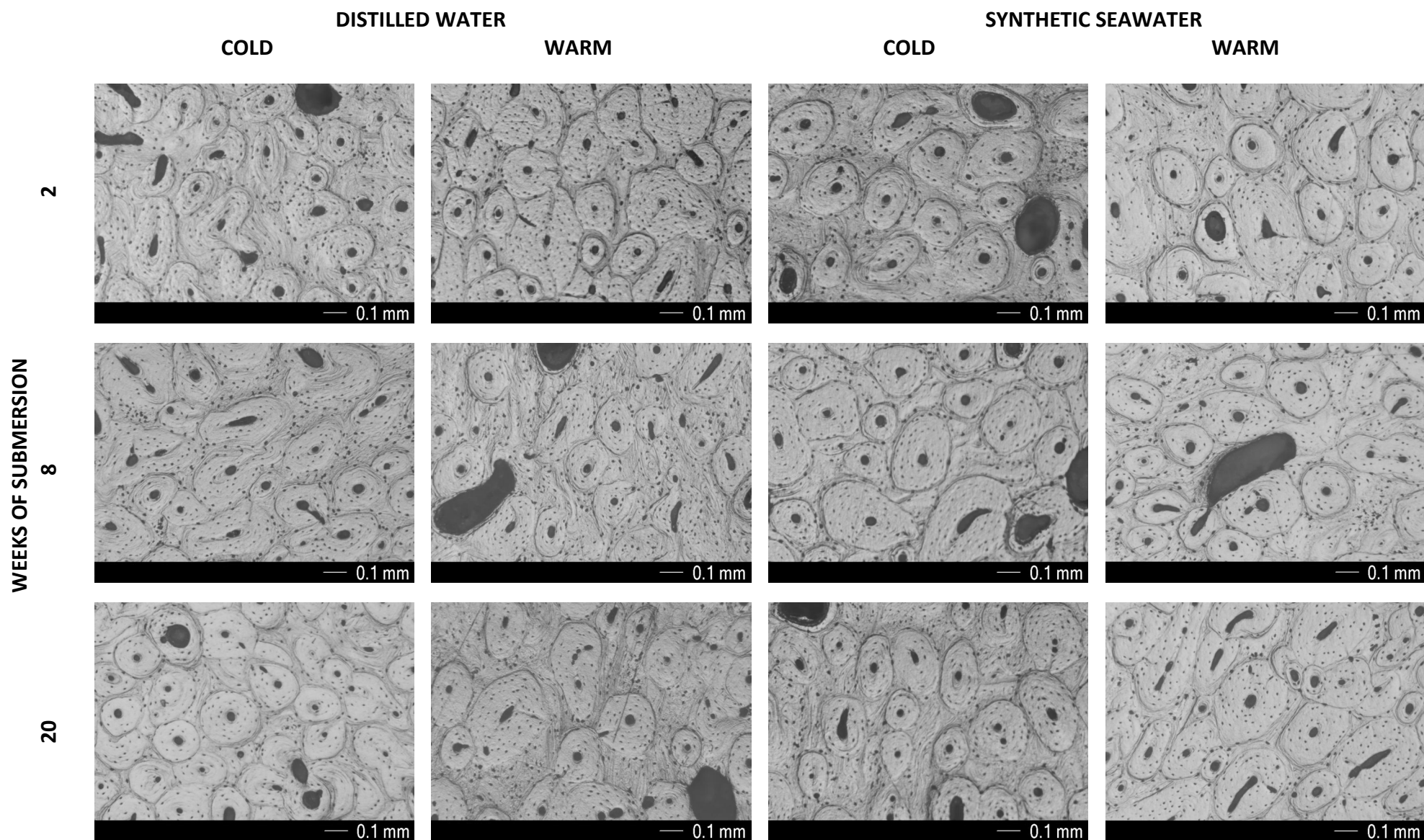


Figure 4.47: Microscopic images (50x magnification) of regions of dense Haversian systems in bovine femur that had been submerged in four aquatic environments for up to 104 weeks.

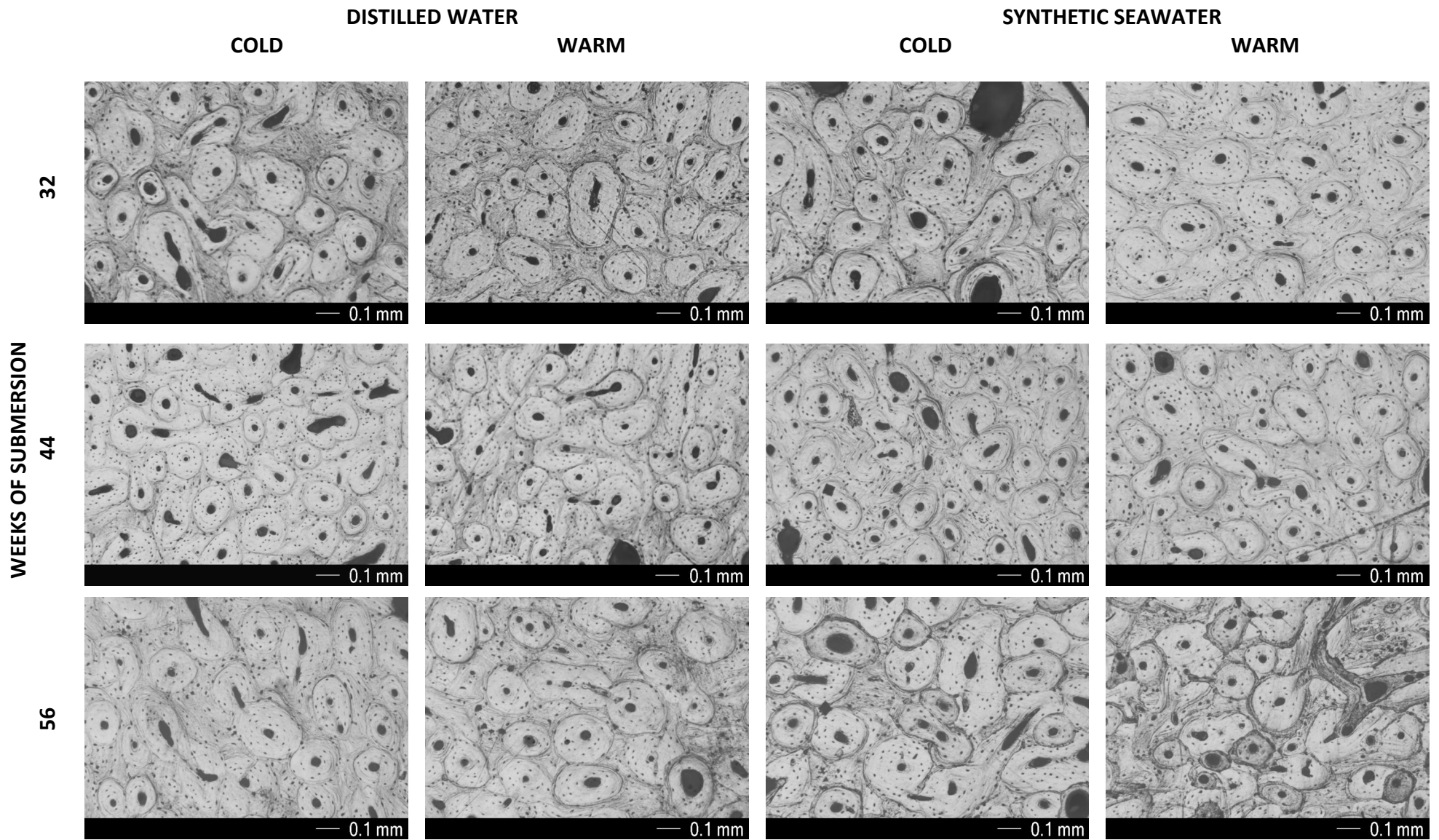


Figure 4.47 continued: Microscopic images (50x magnification) of regions of dense Haversian systems in bovine femur that had been submerged in four aquatic environments for up to 104 weeks.

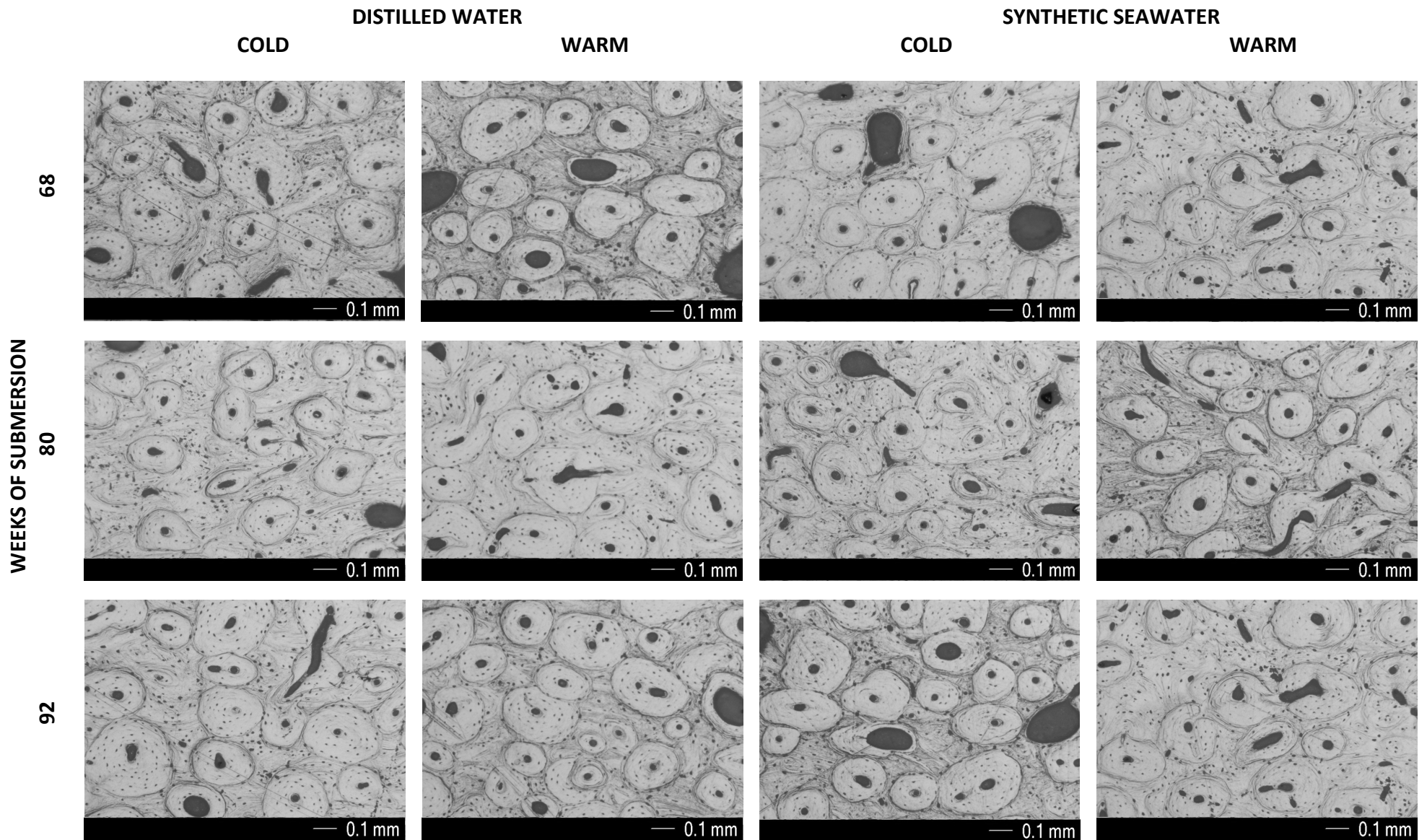


Figure 4.47 continued: Microscopic images (50x magnification) of regions of dense Haversian systems in bovine femur that had been submerged in four aquatic environments for up to 104 weeks.

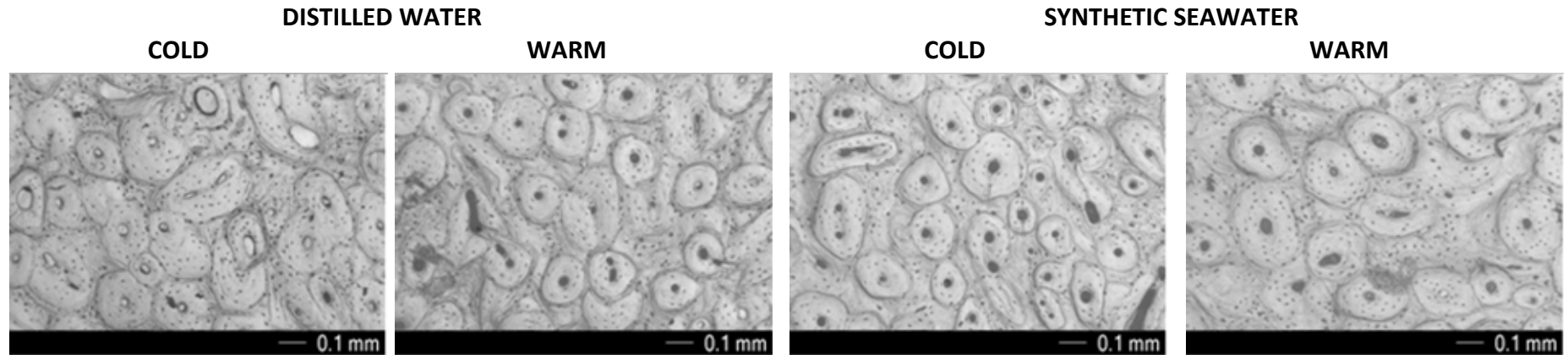


Figure 4.47 continued: Microscopic images (50x magnification) of regions of dense Haversian systems in bovine femoral bone that have been submerged in four aquatic environments for up to 104 weeks.

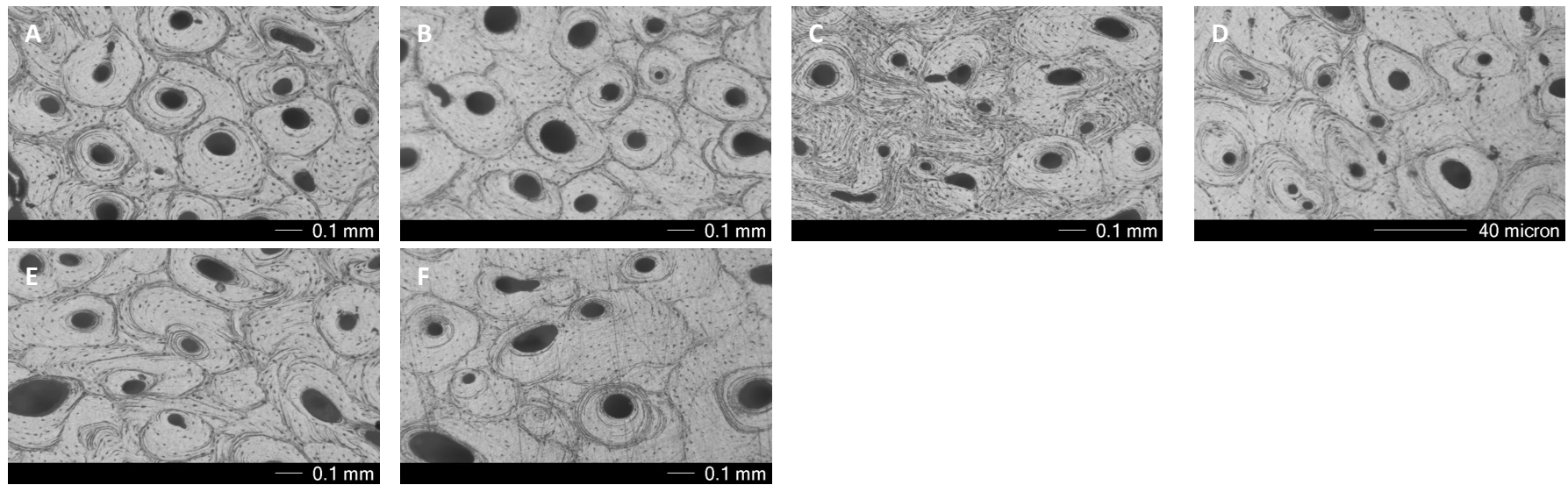


Figure 4.48 Microscopic images (50x magnification) of regions of dense Haversian systems in human long bones. A: Femur, B: Fibula, C: Tibia, D: Humerus, E: Ulna, F: Radius.

4.4 Case Study

Every skeletal element of the individual recovered from the English Channel (section 3.3.3) was genotyped and examined histologically.

4.4.1 DNA genotyping

The human remains recovered from the English Channel (section 3.3.3) were genotyped using the PowerPlex® 16 STR multiplex profiling kit (Table 4.28). For the majority of the bone elements, a full profile of STR markers and amelogenin were amplified. STR locus and allele drop occurred when genotyping DNA from the clavicle, humerus, pelvis and rib. No STR markers were amplified from the radius.

4.4.2 Histology

There were no visual changes in the histological features of the Haversian systems in long bones of the individual (Figure 4.48) when compared to previously taken images (section 4.36). The Haversian systems retained their visual integrity and irregular shape.

Table 4.28: DNA profiles from different bone elements of the case study

	PowerPlex 16 (size range bp)																												
	D3S1358	D5S818	Vwa	THO1	D13S317	D21S11	D8S1179	D7S820	TPOX	D16S539	D18S51	CSF1PO	FGA	Penta D	Penta E	Amelogenin													
Bone	(115-147)	(119-155)	(123-171)	(156-195)	(176-208)	(203-259)	(203-247)	(215-247)	(262-290)	(264-304)	(290-366)	(321-357)	(322-444)	(376-449)	(379-474)	X	Y	Loci											
Clavicle	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	11											
Calcaneus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	15											
Femur	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	15											
Fibula	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	14											
Humerus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	11											
Intermediate Cuneiforms	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	15											
Lumbar body	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	15											
Lumbar processes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	15											
Lateral Cuneiforms	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	15											
Medial Cuneiforms	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	15											
Metatarsals	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	15											
Navicular	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	15											
Pelvis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	13											
Radius	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0											
Rib	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	11											
Sacrum	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	14											
Scapula	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	15											
Talus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	15											
Thoracic body	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	15											
Thoracic processes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	15											
Tibia	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	14											
Ulna	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0											

Key: + = Allele Present, - = Allele not present,

Chapter 5 Discussion

This study utilised a large number of analytical techniques to characterise changes in the material and physical properties of bones that had endured decaying processes, namely heat treatment or water submersion. In heat-treated bone, the changes that occurred in the mineral and organic content could be correlated with the subsequent successful amplification of different sized DNA fragments and Short Tandem Repeat (STR) loci. However, similar statistically significant correlations could not be made from bone submerged in different aquatic environments, although a number of interesting observations are discussed. This chapter will discuss the findings of the results presented in Chapter 4.

5.1 Choosing an appropriate DNA extraction kit

These projects (heat treatment and water submersion) considered the extraction of DNA from compromised bone samples. Given that DNA degradation was likely to have occurred, determining the optimal DNA extraction protocol for routine usage was fundamental.

Three different protocols were evaluated (section 3.14.1) and the relative amount of DNA extracted was determined by limiting serial dilutions. The dilution factor at which PCR amplification failed was dependent on the concentration of DNA in the original

sample. Therefore, the protocol permitting the highest dilution factor before DNA amplification failed, extracted the most DNA.

The Genial® First-DNA all-tissue DNA-extraction kit was chosen as the standard DNA extraction protocol for this study. The dilution factor at which DNA amplification ceased was 10-100 times higher than the dilution factor at which amplification failed using the Invitek: Invisorb® forensic/forensic spin kits (section 4.1).

One aspect common to each protocol tested was the use of Ethylenediaminetetraacetic acid (EDTA). EDTA is a chelating agent of divalent metal cations and is used to remove the calcium content of bone (demineralisation). This allows subsequent digestion of collagen and DNA extraction. However, the extraction kits differed with regard to their recommended EDTA incubation periods. The Invitek: Invisorb® kits recommended overnight incubation, while the Genial® All tissue DNA extraction kit suggested a 5-7 day soak, where the EDTA was changed every day.

Incubating bone in EDTA for a week allowed the extensive demineralisation of the tissue, which could then be removed easily using a scalpel (section 3.14.3). The remaining protein could then be digested and DNA extracted. It is possible that within this incubation period DNA could have been lost and discarded in the EDTA solution (Loreille et al., 2007). However, the results presented show that, even with the potential loss of DNA, the Genial® all-tissue kit still extracted more DNA than the other kits tested.

Moreover, to reduce the risk of losing DNA with each wash, bone samples were not powdered. This not only reduced the surface area from which DNA could be lost but also reduced the risk of contamination.

5.1.1 Summary: Two is always better than one

It may not always be possible to visually determine whether a bone sample has been compromised. The results presented (section 4.1) suggest that it would be possible to extract DNA, which is suitable for DNA analysis, from fresh bone when using any of the three protocols. The diagnostic tool may permit the identification of non-compromised bone allowing a less time-consuming DNA extraction protocol, such as the Invitex: Invisorb kits, to be chosen. This would further reduce the time to generate DNA profiling (Figure 5.1).

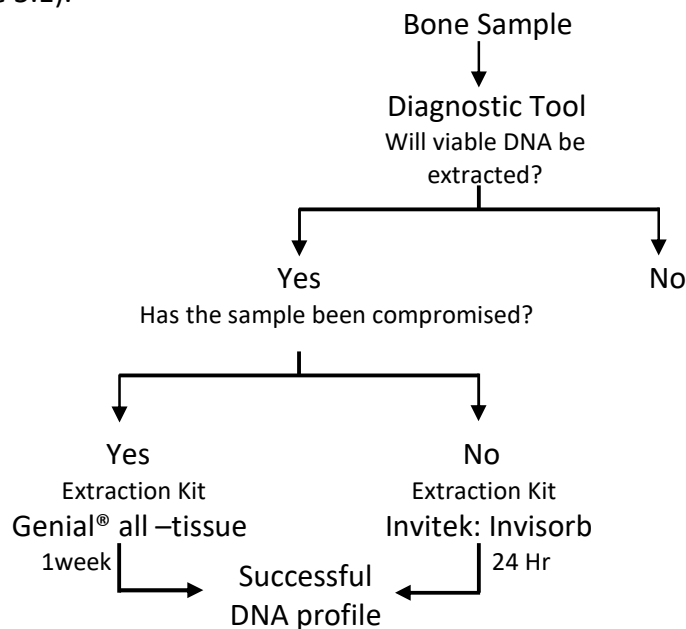


Figure 5.1: Potential diagnostic tool. Determining whether the bone sample has been compromised could indicate the appropriate DNA extraction protocol, thus saving time.

5.2 Developing a diagnostic tool to aid DNA analysis from heated bone

The heat treatment study was designed to determine whether a biomarker in bone, be it mineral or organic, could be used to predict the probability of successfully amplifying different sized amplicons/STR loci from heated bone. A number of analytical techniques were utilised and have shown varying degrees of potential. This section will discuss each technique and their potential utility as a diagnostic tool in DNA analysis.

5.2.1 DNA degradation in heated bone

The present literature regarding DNA amplification from burnt skeletal remains is limited (section 2.7). This study provides an insight into the effect temperature has on the integrity of DNA in bone in controlled conditions, by evaluating the effect that heat treatment (50-1000 °C) had on the success of amplification.

5.2.1.1 Amplification of DNA fragments from heated bone

Different DNA-based identification strategies require varying minimal levels of DNA integrity (at least with regards to mean fragment length). Three different DNA fragments lengths; 500, 300 and 100 bp were designed to represent different approaches including full STR, mini-STR and single nucleotide polymorphism (SNP) respectively (Section 2.5). DNA degradation is a function of temperature and to a lesser

extent duration of heat treatment (Table 4.3). Irrespective of duration and amplicon size, the results suggest that DNA is relatively stable in bone at temperatures below 150°C and supports previous studies that have reported a loss of DNA integrity in charred bone (Schwark et al., 2010, Heinrich et al., 2009b), which is indicative of temperatures between 200-300 °C (Shipman et al., 1984).

Although the detailed mechanisms of DNA degradation were not investigated in this study, the results do suggest that processes such hydrolysis and oxidation are affected by temperature. Strand fragmentation caused by breakages in the phosphate backbone of DNA (oxidation) or depurination, the loss of organic bases (hydrolysis), will both inhibit PCR amplification (Alaeddini et al., 2010). Visualising PCR fragments using gel electrophoresis cannot quantify the absolute amount of DNA present in a sample. However, as temperature and duration increased, the intensity of each amplicon band decreased prior to complete failure, suggesting that DNA was becoming increasingly degraded and hence unsuitable for amplification.

The initial decline in amplicon intensity occurred later as target size decreased. This was most likely due to the simple fact that for any given rate of fragmentation it would take longer for the mean fragment length to fall below the target length, and hence have any detrimental effect upon amplification efficiency.

5.2.1.2 Amplification of STR loci from heated bone

Stockmark® for Cattle Bovine genotyping kit was used to determine the number of loci that could be successfully amplified from each heat-treated bovine sample, hopefully mimicking the pattern of STR profiling that would have occurred in burnt human tissue. The number of loci amplified from each sample heated for 2 and 4 hours is depicted in Tables 4.4 and 4.5 respectively. The longer STR markers (e.g. SPS115 and ETH10) were the first to show degraded amplification, while short loci (TGLA227 and ETH3) were the last.

The peak intensities of all STR loci within a profile declined relative to controls. In addition, the intensities of peaks within a profile declined relative to each other as their size increased (Appendix 4). This suggests amplification of STR loci from heated bone is a function of temperature, duration of heat treatment and STR size and is consistent with the findings of fragment amplification using PCR coupled with gel electrophoresis imaging as described above.

5.2.1.3 Summary: DNA degradation in heated bone

Currently, there is very little literature regarding DNA degradation in heated bone using carefully controlled experiments. Previous publications have often used case studies where exposure temperatures were unknown (Calacal et al., 2003, Cattaneo et al., 1999,

Sivolap et al., 2001, Staiti et al., 2004, Von Wurmb-Schwark et al., 2004b) or investigated the amplification of very short DNA (<100 bp) fragments, unsuitable for STR analysis (Von Wurmb-Schwark et al., 2004). Our results provide a comprehensive insight into DNA degradation in heat-treated bone and have shown that above 150 °C it is a function of temperature and duration.

5.2.2 Colour of heated bone

For anthropologists, the colour of bone has been an important factor when characterising burnt skeletal remains. Previous studies have reported that the colour of bone undergoes a sequence of changes as the temperature increased. Although differences were reported regarding the temperature at which precise colour changes occurred, the colour sequence is very similar throughout the literature. An extensive study by Shipman et al (1984) standardised the sequence of colour changes in heated bone (Figure 5.2) and has been supported by recent publications (Walker et al., 2008).

The results presented in this study (Figure 4.3) are consistent with the results of previous studies (Devlin et al., 2008, Nicholson, 1993, Shipman et al., 1984). The colour of bone reflects the ongoing chemical processes and can be divided into four main categories. Bone underwent an initial stage of dehydration where water evaporated. As the temperature increased the colour changed from cream to a pale yellow, representing the beginning of organic carbonisation. As collagen became increasingly

carbonised the bone darkened to brown/black. With a further increase in temperature the colour became pale/reddish brown, which was indicative of carbonates being oxidised and released as carbon dioxide (inversion). At temperatures above 700 °C the process of calcination occurred, whereby mineral becomes fused and the colour of bone changes from a blue/grey colour to white (Devlin et al., 2008, Nicholson, 1993, Shipman et al., 1984).

It is clear from the results that the changes in colour were dependent on both temperature and the duration of heat treatment. These results are supported by those of McCutcheon (1992), who suggested that although the colour of bone can be helpful in a forensic context, it couldn't be used as a sole indicator of maximum exposure temperature. This theory was supported by the results of this study and can be demonstrated using the results that showed that bone heated to temperatures between 300 and 350 °C for two hours was the same colour as samples heated at 250 °C for four hours (Figure 4.3). The colour of bone did not change when heated to temperatures below 150 °C. This suggested that collagen was stable when heat to temperatures below 150 °C for up to four hours. This was supported by the amide I to phosphate ratio that showed collagen began to degrade above approximately 150 °C (Figure 4.12).

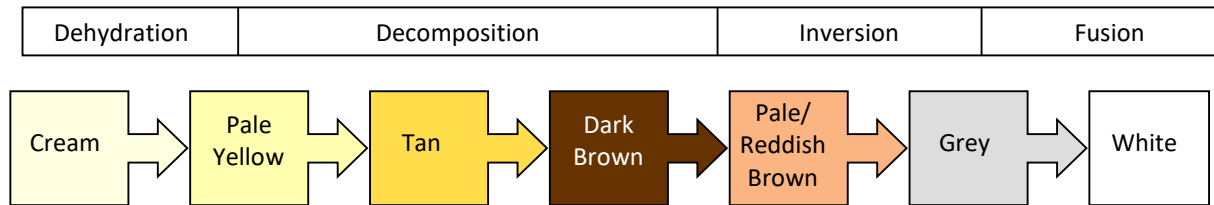


Figure 5.2: General sequence of colour change in heat-treated bone

Recording colour using the Munsell[®] colour chart is a subjective approach as it relies on the examiner matching the colour of bone to the correct colour pallet (Shipman et al., 1984). There have also been several ‘digital’ approaches, such as recording the RGB components of photographs (Walker et al., 2008). The use of a colourimeter removes examiner error and variation in ambient light conditions. Moreover, the results of this study have shown colourimetry to be accurate and reliable tool that could distinguish very subtle differences in colour.

5.2.2.1 Munsell[®] Colour Chart: A possible diagnostic tool?

Using the Munsell[®] colour chart it was impossible to distinguish between samples from which DNA was amplified successfully and those from which DNA amplification failed. For example, the results of this study showed that the 500 bp fragment was successfully amplified from bone heated to a maximum temperature of 180 °C for two hours. The colour code given to the samples heated at 180 °C was the same as the code given to samples heated to 200 °C from which the 500 bp fragment amplification failed (Figure

4.3). Therefore, using the Munsell® colour chart as a diagnostic tool, would result in unsuitable samples (samples from which DNA amplification would fail) being processed.

5.2.2.2 Colourimetry: A possible diagnostic tool?

Colourimetry could distinguish very subtle differences in colour within the temperature region of DNA degradation. Using Figure 4.3a and samples that were heated between 180 and 200 °C (numbers 2-4) as an example, although there was a clear difference in the L, a* and b* coordinates (particularly in b*), no visual differences were recorded (all were recorded as 5Y9/2).

The subtle changes in bone colour within the region of DNA degradation appear to be a function of temperature and time and can be detected using a colourimeter. As a result, the colour changes, recorded by colourimetry significantly correlated with successful DNA amplification (Figures 4.7 – 4.11). Within the temperature region of DNA degradation, bone becomes darker in colour and was indicative of organic material oxidation. It has been suggested that DNA and collagen share a similar degradation pattern as they are both organic (Gotherstrom et al., 2002). Colour indirectly correlates with DNA degradation by reflecting the chemical processes that occur.

5.2.2.3 Colourimetry and amplification of different DNA fragments

Using colourimetry, significant multivariate quadratic discriminant (MQD) functions were produced to predict the probability of successfully amplifying 500, 300 and 100 bp DNA fragments (Figures 4.7, 4.8 and 4.9 respectively). The performance of each model, to classify (whether or not DNA would be amplified successfully) each sample correctly, was very similar (Table 4.7). While only two samples were incorrectly classified as false negatives by each model, they both incorrectly classified samples that were heated between 650 and 700 °C (Appendix 8: Tables A8.1-A8.5). DNA amplification failed in samples that were heated between 650 and 700 °C. However, as they shared comparable L, a* and b* values with that of fresh bone and samples heated in the region of DNA degradation (50-200 °C), the colourimetry model classified them as false positives.

5.2.2.4 Colourimetry and STR Loci amplification

As with colour change and DNA fragment amplification, the number of loci amplified successfully decreased as a function of time and temperature in heated bone. As a result, significant models were produced to predict the probability of amplifying a minimum of 3 and 7 complete STR loci. In similarity to the models produced using different sized DNA fragments, both models incorrectly classified the small number of samples that were heated between 650 and 700 °C as false positives (Appendix 8).

5.2.2.5 Summary: Colour

This study has shown that measuring the colour of heated bone using a colourimeter can be used to predict the probability of successfully amplifying three different sized amplicons and a minimum of ≥ 3 or ≥ 7 STR loci. Colourimetry is a technique that is relative inexpensive, can be performed in seconds and requires minimal bone preparation. Moreover, colourimeters are relatively inexpensive and portable, making large scale pre-screening in an 'in-field' setting a possibility. This study, alone, presents a new and novel diagnostic tool that can reliably predict the probability of successfully amplifying DNA from heat compromised skeletal tissue.

5.2.3 FTIR spectroscopy: Collagen

Measurement of the amide I to phosphate ratio (APR) provides a method that can be used to semi-quantify the collagen content of bone. It detects the vibrational transition of the C=O stretch of the peptide carbonyls that contribute to the helical structure of collagen. As collagen begins to denature, the intensity of the band decreases (Sionkowska et al., 2006).

The results presented in this study suggest that the pattern of collagen degradation is sigmoidal and consists of five phases (Figure 5.3). Collagen degradation did not begin until samples were heated to temperatures above 150 °C (Figure 4.12) and is

represented by the lag phase. This is in-line with a previous study which suggested that mineralised collagen begins to denature at approximately 130 -170 °C (Kronick and Cooke, 1996). Changes in collagen morphology are seen as the temperature approaches 100 °C and become beaded or dumbbell shaped (Koon et al., 2003). However, even with these morphological changes, the majority of the collagen remains intact suggesting that such changes would not necessarily be detected by FTIR spectroscopy or TGA.

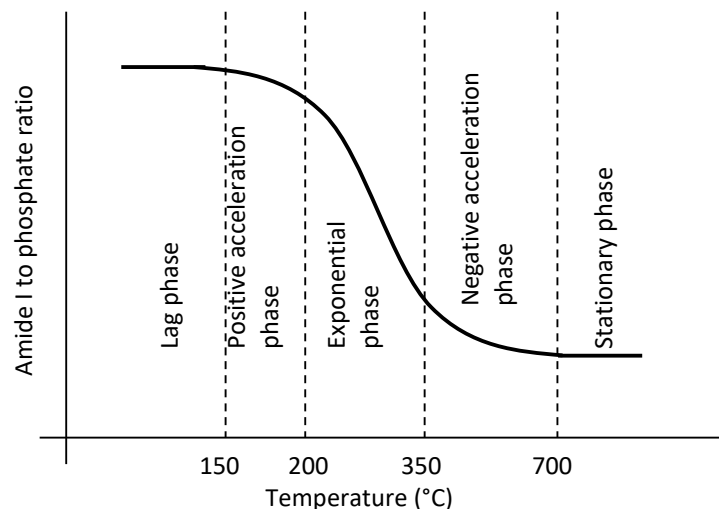


Figure 5.3: Diagrammatic representation of the changes in APR of bone with temperature. Illustrated are the approximate temperatures at which each phase occurs.

The APR began to decrease in both sample series (samples that were heated for two and four hours) when heated to temperatures above 150 °C. The gradual decrease in APR represented the positive acceleration of collagen degradation and occurred at a lower temperature in samples that were heated for four hours. Collagen is relatively stable

when heated to temperatures below 150 °C and has a degradation pattern that is temperature and time dependent.

The decrease in APR did not correlate with the initial decrease in percentage weight that was measured using TGA (Figure 4.34). The TGA recorded an initial weight loss at approximately 40 °C, roughly 100 °C prior to when the APR began to decrease. This has previously been assigned to the evaporation of moisture (Etok et al., 2007, Lees, 1981) and is supported by the broad endothermic peak that was seen using DSC analysis (Figure 4.35).

Between 150 and 350 °C there was a significant decrease in the APR, which correlates with the results obtained from TGA and DSC analysis. This temperature range has been associated with maximum weight loss (Deydier et al., 2005, Etok et al., 2007, Lozano et al., 2003) and represents the exponential phase of collagen degradation (Figure 4.12). However, as shown in the TGA results, between 150 and 350 °C, the rate of weight loss is not linear. This has previously been accounted for by the production of oxidative products that affect the net weight of the sample (Kaminska and Sionkowska, 1996). The correlation between mineral to matrix ratio and ash weight determined by Boskey and Mendelson (2005) was calculated using different fractions of collagen and synthetic hydroxyapatite. The APR ratio would not detect the oxidative products. During this temperature range collagen molecules are undergoing oxidation and carbonisation. At low temperatures the inter- and intra-molecular hydrogen bonds, which are responsible

for the triple helix, structure break and the collagen fibrils begin to lose their structure. As the temperature increases, covalent bonds between C-C, C-H, C-HN, C=O and C-COOH break, increasing the disorder of the single polypeptides and becoming fragmented (Kaminska and Sionkowska, 1996).

The negative acceleration phase occurred between 350 and 650 °C. It could be possible that at this stage, the collagen fibrils could have completely lost their integrity and only fragments of peptides, with intact C=O domains, were detected. In samples heated to temperatures between 650 and 1000 °C (stationary phase), no amide I band was detected. This was consistent with previous studies that have shown that between 600 and 900 °C, the organic material of bone has completely degraded (Etok et al., 2007) and supported by the TGA. Although the TGA only recorded percentage weight loss up to 500 °C, due to the limitation of the machine, it is clear that the weight loss was reaching a plateau. It could be assumed that by 600 °C, the weight loss would have levelled at approximately 30 % of the original weight of bone. Bone bHAP makes up 70 % of the weight of bone (Xiao and Webster, 2005).

5.2.3.1 APR: A possible diagnostic tool?

The results presented in this work suggest that the degradation of collagen and DNA are very similar. Both have similar degradation patterns as their molecular structure remains stable when heated to temperatures below 150 °C. Above 150 °C their

degradation was a function of temperature and time. Although the mechanisms of degradation may be different, the results suggest that the breakage of hydrogen bonds and covalent bonds in collagen and DNA occurred at a similar temperature.

5.2.3.2 APR and amplification of different DNA fragments

Three significant logistic regression models were produced to predict the probability of successfully amplifying 500, 300 and 100 bp (Figures 4.13, 4.14 and 4.15 respectively). DNA and collagen degradation was a function of increased temperature and time, as was represented in the APR logistic regression curves (Figure 4.16), which shifted towards lower APR values as the length of the amplicon decreased (Table 4.9).

All three models were able to classify over 90% of the true positives and true negatives correctly (Table 4.8). Furthermore, statistically, the models were highly accurate and reliable, as the AUROC curves of each model were very close to one (section 3.17.3.1).

5.2.3.3 APR and STR loci amplification

The APR also correlated with the successful amplification of 3+ and 7+ loci from heated bone (Figures 4.17 and 4.18 respectively). In heated bone, it was observed that STR loci were degraded (similar to amplicon size) in order of their size (long to short) as temperature/duration increased. Therefore, the level of DNA integrity required (in

terms of mean DNA length), increased as the minimum number of STR loci necessary for a statistical significant match increased. As a result, the logistic regression curves shifted towards zero as the minimum number of loci required decreased. Both models correctly classified over 90% of the sample and AUROC curve score for each model are very close to one, suggesting that models are highly accurate and reliable.

5.2.3.4 Summary: FTIR spectroscopy Collagen

The initial cost of setting up a FTIR spectrometer is considerably more than that of a colourimeter, although data collection and analysis is relatively inexpensive. Moreover, bone preparation is minimal, and a FTIR spectrum of bone can be generated in minutes. FTIR spectroscopy is destructive to the sample, however, only a few milligrams are required. As a result, APR analysis, through FTIR spectroscopy, has demonstrated that it can be used as an accurate and reliable diagnostic tool in DNA analysis from heated bone.

5.2.4 FTIR spectroscopy: Bone mineral

The crystallinity index (CI) measures the order of atoms within a crystalline structure; the higher the order of atoms, the more crystalline the structure. It is a function of both crystallite size and strain and can be measured, as the Splitting Factor (SF) using FTIR spectroscopy and a method developed by Weiner and Bar-Yosef (1990).

Bio-apatite (bHAP) in bone is believed to become more crystalline (known as re-crystallisation) over time due to diagenesis effects which alter the physicochemical properties of bone (Hedges, 2002). Re-crystallisation involves the growth of bHAP (crystallite size) and the removal of defects and impurities (such as carbonate ions) from the lattice (Rogers and Daniels, 2002). The order of atoms within the lattice increases (crystallinity) and microstrain decreases (Rogers and Daniels, 2002). Heat-treatment facilitates re-crystallisation and the results of this study are consistent with previously published work that has investigated bHAP re-crystallisation using SF (Surovell and Stiner, 2000, Munro et al., 2007, Mkukuma et al., 2004, Lebon et al., 2008, Weiner and Baryosef, 1990). There was an increase in SF (crystallinity) as the temperature increased (Figure 4.20).

The effect of temperature on the SF of bHAP within temperature range of DNA degradation in bone (150-200 °C) remains an area of debate. Munro et al., (2007) described that within this temperature range there was a 'dip' in SF, while other studies have shown a gradual increase (Stiner et al., 1995, Surovell and Stiner, 2000). The difference found in the SF has been attributed to sample preparation. Heated bone becomes more brittle when compared to fresh bone (Ramrakhiani et al., 1980). Thus, fresh bone is considerably more difficult to grind compared to heated bone. It is possible to manually powder heated bone using a pestle and mortar, where as fresh bone may require mechanical assistance. Mechanical grinding it thought to decrease mineral

crystallinity (Nakano et al., 2001) Munro et al., (2007) used a variety of techniques (ball mill and pestle and mortar) to powder bone samples which may explain the 'dip' in $\sim$ SF. In this study, the grinding protocol was standardised, similar to that of the grinding protocol established by Surovell and Stiner (2000). The results presented in this study, regarding the effect of low temperatures (150-200 °C) on SF, are consistent with similar publications by Stiner et al., (1995) and Surovell and Stiner, (2000). The SF of bHAP increased as the temperature increased and there was no significant 'dip'.

The SF of bHAP, with regard to temperature, follows a positive sigmoidal pattern (Figure 5.4). The initial lag phase in SF occurred below 200 °C, after which there was a gradual increase until 500 °C (positive acceleration phase). Between 500 and 700 °C an exponential increase in SF as present and above 700 °C the increase in SF begins to level (negative acceleration/stationary phase).

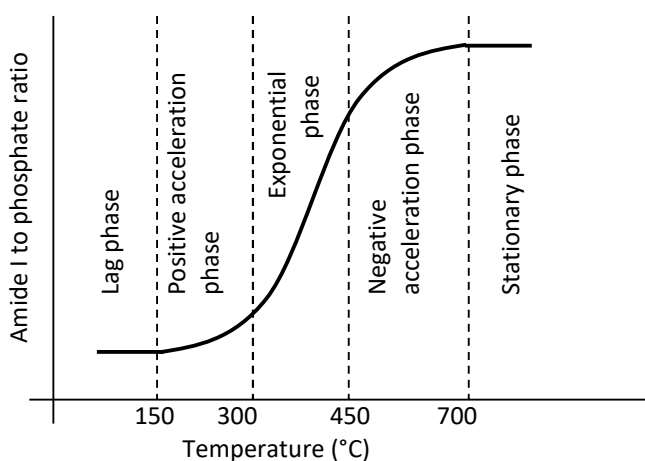


Figure 5.4 Diagrammatic representation of the relationship between bone bHAP crystallinity and temperature.

In fresh bone the peaks of the phosphate domain ($\nu_4\text{PO}_3^{4-}$) used for calculating the SF were broad and the valleys between them shallow. This is the result of poor crystallinity and the presence of ion substitutions (Etok et al., 2007). As the temperature increased the resolution of the peaks increased, resulting in higher peaks and deeper valleys; an attribute of improved crystallinity (Mkukuma et al., 2004). Representative FTIR spectra of samples heated to each temperature can be found in Appendix 5; Figures A5.1 and A5.2. The increase in crystallinity is the result of ion exchanges (Rogers and Daniels, 2002). Above 700 °C, a hydroxyl (OH) peak appeared (635 cm^{-1}) and increased in intensity as the temperature increased (Etok et al., 2007, Haberko et al., 2006, Haberko et al., 2009). Within this temperature range (700 - 1000 °C), there was also a decrease in lattice carbonate (CO_3^{2-}) (850 cm^{-1}). The increase in hydroxyl and decrease in carbonate peaks has been previously reported by Etok et al., (2007) and Haberko et al., (2006) and are attributed to substitution of CO_3^{2-} ions by OH^- ions.

5.2.4.1 SF: A possible diagnostic tool?

Over the temperature region of DNA degradation (150-200 °C), the increase in SF is not linear. Discernable changes in SF occurred during the later stages of DNA degradation, approximately 200 °C, and appear not to be a function of duration of heat treatment. These results suggest that SF may be correlated with the amplification of small fragments of DNA or indirectly correlated with DNA degradation.

5.2.4.2 SF and amplification of different DNA fragments

Significant logistic regression models were calculated for all three amplicons (Figures 4.21, 4.22 and 4.23 respectively). Although there was a general trend that suggested that the logistic regression curves shifted towards higher SF values as the length of the amplicon decreased, there was overlap between the curves (Figure 2.24).

In ancient skeletal tissue it is believed that DNA preservation is closely linked with the degree of bHAP crystallinity (Gotherstrom et al., 2002). DNA has a strong affinity to hydroxyapatite; this is due to the negatively charged phosphate groups in the DNA and hydroxyl sites on the bHAP. It may be possible that DNA and bHAP in bone interact during heat treatment. As the temperature increases cells will burst, releasing their contents, including DNA, into the surrounding environment. DNA molecules may become available to adhere to bone bHAP at temperatures as low as 50 °C, as it has been found that the cell membranes can fragment when heated to temperatures above 48 °C (Ponder, 1949). This may explain the fluctuations in crystallinity between 150 and 200 °C. It may be the case that at lower temperatures in this range, small DNA fragments adhere to bHAP, decreasing the SF of bHAP. At higher temperatures (~200 °C), the conditions within the bone results in the precipitation of the adhered DNA fragments that consequently become degraded and increase the SF of bHAP.

DNA's affinity for bHAP may preserve its integrity in compromised bone; however, there are inconsistencies that should be taken into account. It has been assumed that the affinity of DNA to bHAP can occur over a very short period of time. Publications have focused on archaeological bone, where such interactions can occur over a number of years. Also the fluctuations did not appear to be dependent on the duration of heat treatment. If DNA degradation was correlated with SF, fluctuations should have appeared earlier in samples heated for four hours. It could be possible that other mechanisms may be responsible for the resultant correlation. The interaction between collagen degradation and crystallinity may indirectly correlate with DNA degradation.

Our results also support the theory that bHAP re-crystallisation could be dependent on the organic content of bone. There have been numerous studies that have hypothesised such associations; however their precise relationship remains a topic for debate. The presence of mineral has a profound effect on collagen degradation and vice versa. It has been found that collagen in bone begins to denature at 90 °C higher than collagen in skin (Kronick and Cooke 1996), while collagen degradation promotes mineral re-crystallisation (Etok et al., 2007, Gothenstrom et al., 2002, Rogers and Daniels, 2002). As the APR decreased there was an increase in crystallinity and crystal growth suggesting a relationship between collagen degradation and bHAP re-crystallisation (Appendix 9).

Although the mechanism of this was not investigated, the results suggest that complete removal of the organic content of bone is required for bHAP re-crystallisation; only

when no amide I peak could be detected was there a significant increase in re-crystallisation. All organic matter is thought to be destroyed by 650 °C (Etok et al., 2007). This suggests that although collagen may be fragmented, the presence of amino acids may inhibit crystal growth. It may be the case that the hydroxyl group in collagen plays a fundamental role in bHAP re-crystallisation.

The fluctuations in SF within the temperature region of DNA degradation resulted in fewer discernable changes between positive and negative samples when compared to curves using the APR. This was demonstrated by the low classification percentages and AUROC curve values for each amplicon (Table 4.8).

5.2.4.3 SF and STR loci amplification

The SF of bHAP also correlated with the amplification of ≥ 3 and ≥ 7 STR loci (Figure 4.25 and 4.26 respectively). A lower percentage of samples were classified correctly when compared to the classification percentages using APR models. As mentioned above, the SF of bHAP fluctuates within the temperature region of DNA degradation. This explains the relatively low classification rate and the fact that there small difference between the two logistic regression curves (Figure 4.27).

5.2.4.4 Summary: FTIR spectroscopy: Bone mineral

Although using the SF of bHAP produced respectable logistic regression curves that could predict the probability of successfully amplifying different DNA targets, the models are not as accurate or reliable when comparing the results to APR or colourimetry. This suggests that as a standalone measurement, for the identification of viable samples, it would not be as useful; it would preferable to use FTIR spectroscopy to measure the APR.

5.2.5 XRD: Bone mineral

Bone is considered a poorly crystalline material as it has many impurities incorporated into its mineral structure. X-ray diffraction can measure a number of parameters regarding the integrity of bHAP (Table 2.4). The CI of a material can be determined using XRD analysis by measuring the coherence length (CL) of bHAP. The work presented in this study was consistent with previous publications that have investigated the lattice parameters of bHAP in heated bone (Etok et al., 2007, Piga et al., 2008, Piga et al., 2009, Rogers and Daniels, 2002, Shipman et al., 1984).

Fresh bone consisted of rod-like bHAP crystals that were small in size (Figure 4.29) and were in a relatively strained state (Figure 4.30), which is indicative of poor crystallinity (Figure 4.28). The values that were recorded for fresh bovine bone in this study were

similar to that of human cortical bone (Rogers and Daniels, 2002). It has been reported that there are a number of possible reasons for the microstrain including the presence of lattice substitutions. Bone heated to temperatures up to 600 °C, showed no discernable changes in the CL or crystallite size, remaining approximately 2.0 nm and 30.0 nm respectively. The 002 and 004 peaks of the samples that were heat treated up to 600 °C remained broad, which is indicative of a poorly crystalline material (Appendix 6; Figures A6.1 and A6.2).

The substantial increase in crystallite size and crystallinity and decrease in crystallite strain of bHAP in bone heated to temperatures above 600 °C (demonstrated by the increase in peak resolution [Appendix 6.0]) is indicative of re-crystallisation. This is consistent with previous reports that have suggested that re-crystallisation occurs above 600 °C (Etok et al., 2007, Piga et al., 2008, Piga et al., 2009, Rogers and Daniels, 2002).

The results of this study, regarding the cell unit parameters 'a' and 'c' of bHAP in heated bone (Figures 4.31 and 4.32 respectively), were similar to that of heat-treated human cortical human bone (Rogers and Daniels, 2002). The cell unit decreased in the 'a-axis', as bone was heated to 200 °C. This initial decrease has been assigned to the changes in ionic lattice substitutions during uniaxial crystal growth normal to the 'c-axis' (Rogers and Daniels, 2002). Below 200 °C there are other processes occurring, such as collagen degradation and water loss, as a result, a single mechanism cannot account for the initial decrease. Between 400 and 800 °C there was an increase in 'a' and a decrease in

'c', although between 600 and 800 °C the most significant structural changes occurred. The changes have been attributed to the exclusion of foreign ions, including carbonate (CO_3^{2-}) ions. However, no one single ion loss is thought to be responsible (Rogers and Daniels, 2002).

5.2.5.1 XRD analysis: A possible diagnostic tool?

CL, crystallite size and cell unit parameters 'a'/'c' all produced significant logistic regressions when predicting the probability of successful amplification of the different DNA targets (Figures 4.33-4.36 and Appendix 7). However, the models calculated were not useable in the context of this study. From the results it is clear that, although a large number of true negatives were classified by each parameter, the percentage of true positives correctly classified was relatively low (Tables 4.16 – 4.20) when compared to other techniques, including FTIR spectroscopy and colourimetry analysis. This was supported by the relatively poor AUROC curve values. If XRD analysis were to be used as a diagnostic tool, there would be the possibility of a large percentage of samples being incorrectly classified. There are two reasons why the XRD parameters measured could not produce logistic regression models that were useable in the context of this work.

1. Discernable changes within the temperature region of DNA degradation

To develop a usable diagnostic tool to aid in the analysis of DNA from heated bone, there have to be discernable changes within the temperature region of

DNA degradation. DNA degradation occurred between 150 and 200 °C (Table 4.3). The CL, crystallite size and 'c-axis' showed no discernable changes within this range. Discernable changes in CL and crystallite size only occurred when bone was heated to temperatures above 600 °C, while changes occurred above 500 °C when measuring the 'c-axis' of HAP. There was substantial overlap between positive and negative results (Appendix 7).

2. Different temperatures producing the same parameter value

A suitable marker not only has to show discernable changes within the temperature region of DNA degradation but these changes must also be discrete across the relevant range. Discernable changes occurred in the 'a-axis' parameter within the temperature region of DNA degradation. However, there was increase in 'a' in bone heated to temperatures above 600 °C which resulted in an overlap of 'a' values with bone heated to temperatures below 200 °C and above 600 °C. Samples heated to temperatures below 200 °C and above 600 °C could have been classified as false negatives and false positives respectively. The logistic regression curves (Appendix 7; Figures A7.3, A7.7, A7.11 and A7.15) show that there was substantial overlap of positive and negative results.

5.2.5.2 Summary: XRD: Bone mineral

As described above and demonstrated by the results, XRD as a 'standalone' technique could not be used as a reliable or accurate diagnostic tool to predict the probability of successfully amplifying different DNA targets. Moreover, in comparison with the other analytical techniques that have shown significant correlations with DNA amplification, XRD machines are expensive and analysis of XRD data is complex and time-consuming. The parameters measured using XRD would, therefore, not be suitable 'markers' for this study.

5.2.6 Microhardness of heated bone

Microhardness measures a material's resistance to deformation under indentation. The results of this study are consistent with those of previous studies that have investigated the effect of temperature on the microhardness of bone (Amprino, 1958 and Lees, 1981). An initial increase in microhardness was seen in samples heated to up to 150 °C. This has been ascribed to water evaporation (Amprino 1958) and is supported by the FTIR spectroscopy, XRD, TGA and DSC analysis. FTIR spectroscopy and XRD analysis showed that there were no discernable changes in collagen or bHAP, therefore the initial weight loss and endothermic processes, (measured by TGA and DSC respectively), could also be ascribed to water evaporation. It is thought that the removal of water

from the sample brings together bHAP crystals, which increases bone's resistance to the applied force (Lees, 1981).

Above 150 °C there was a decrease in microhardness, which is believed to be a result of collagen degradation, as it was consistent with the decrease in the APR at similar temperatures. Moreover, the decrease in microhardness appears to be a function of temperature and duration. The disruption of collagen molecules results in the inability of the bone sample to resist the applied force; therefore the microhardness decreases.

The microhardness of bone continued to decrease until samples were heated to temperatures of between 450-500 °C. Above 650 °C there was significant increase in microhardness which corresponded to an increase in crystallite size. It is thought that the increase in crystallite size compensates for the loss of collagen. The spaces left by collagen degradation are filled by the growth of bHAP. The bHAP crystals come together, increasing the resistance to the applied force (Lees, 1981).

5.2.6.1 Microhardness: A possible diagnostic tool?

Using the microhardness of heated bone as a marker for the preservation of DNA may be problematic (section 2.8.5.2) as a single microhardness value may result from heat treatment at one of many different temperatures (Figure 4.37). Therefore, it was not surprising that no significant logistic regression could be applied to DNA amplification (PCR fragment or STR loci).

5.2.6.2 Summary: microhardness

Studying the microhardness of heated bone proved of little value in terms of the objectives of this study. However, the results are consistent with previous studies and supplement the findings of the results presented.

5.2.7 Developing a diagnostic tool using multiple analytical techniques

To test each model, the calculated function was used to re-classify the sample set into expected positive and negative results (section 3.17.3). A 'perfect' diagnostic tool that could predict the probability of successfully amplifying DNA targets from heated bone, would classify every sample with 100% accuracy.

As individual techniques, FTIR spectroscopy and colourimetry could be used as diagnostic tools that can predict the probability of amplifying different DNA targets. Both analytical techniques produced significant statistical models that were accurate and reliable, thus would classify the majority of samples correctly. However, an optimal model was not achieved.

When using FTIR spectroscopy, both the APR and particularly the SF, incorrectly classified samples within the temperature region of DNA degradation. By comparison,

colourimetry was superior at correctly classifying samples within the region of DNA degradation but incorrectly classified samples at high temperature (within 600 – 650 °C).

A model that used colourimetry coupled with the APR/SF increased the percentage of correctly classified samples (Tables 4.23-4.26). By using such models, samples that were incorrectly classified (particularly those misclassified in the 600 – 650 °C region) by the colourimeter were classified correctly. Correspondingly, samples that were incorrectly classified when using the APR or SF on their own, were now classified correctly (Appendix 8; Tables A8.1-A8.5).

5.2.8 Conclusion: Heat treatment study

This study demonstrated that changes occurring to the colour, collagen content and bHAP crystallinity as a result of heat treatment, could be correlated with the subsequent success of DNA amplification. The results of this study show that colourimetry and FTIR spectroscopy (APR and to a less extent SF) can be used individually or together as a possible tool for ‘screening’ compromised samples prior to attempting DNA extraction and analysis. Not only can colourimetry and/or FTIR spectroscopy analysis be useful for the screening of viable samples, but they also have the potential to indicate the appropriate DNA identification method that should be used after extraction (Figure 5.5). Moreover, they have the potential to determine the proportion of STR markers that are likely to be amplified successfully. This would not only suggest possible avenues for STR

analysis, i.e. full or mini STR, that would be successful, but could also determine the necessary reference samples likely to be required (Figure 6.1).

Both analytical techniques can be performed in minutes and require minimal bone preparation. Moreover, colourimeters and FTIR spectrometers are relatively inexpensive and portable, making large scale pre-screening in an 'in-field' setting a possibility. It may even be possible to specifically design a single instrument, coupling colourimetry and FTIR spectroscopy, although the development of such a piece of equipment will not be discussed. This study presents a new and novel diagnostic tool that can reliably predict the probability of successfully amplifying DNA from heat compromised skeletal tissue.

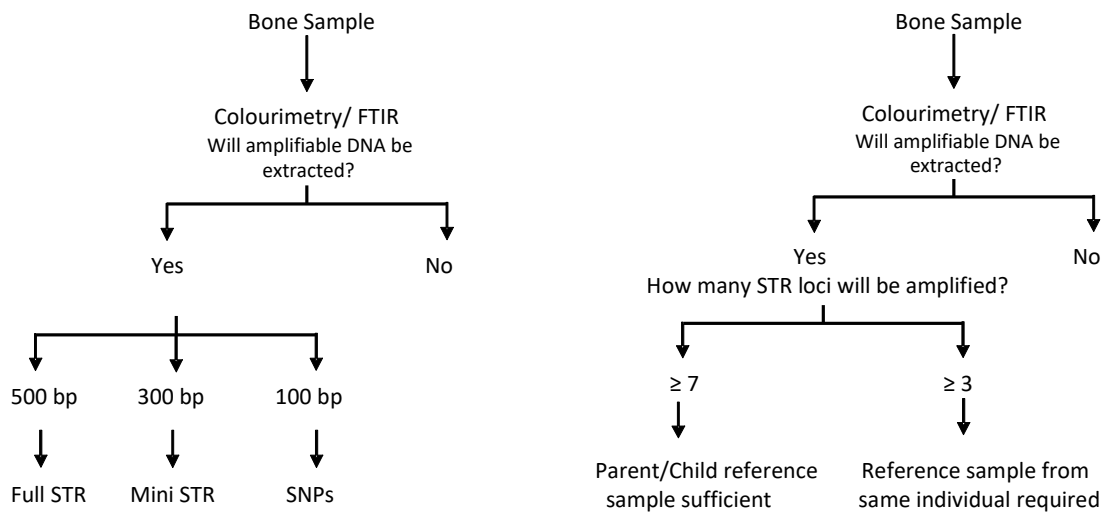


Figure 6.1: Potential diagnostic tools developed. The diagnostic tools developed have the potential determine the most the appropriate strategy for DNA analysis and could also determine the appropriate reference sample that is required.

5.2.8.1 Limitations and future directions

A number of novel techniques that have the potential to be used as an aid in DNA analysis from heated bone have been developed. However, there are a number of limitations of this study. This study has focused on the effect of heat treatment on bovine bone and not human tissue. The changes and trends in collagen degradation and bHAP integrity are consistent and are in most cases comparable to similar studies using human bone. With such comparable results, it could be said with some confidence that similar correlations would likely be found when using human tissue. However, there is the potential that the correlations would not be identical and the models calculated in this study may not be quite as useful in a human forensic context, as the proportion of organic material in bone can differ from species to species (Mkukuma et al., 2004). Nevertheless, developing a similar diagnostic tool for human bone should not take an excessive period of time.

This study was controlled using bone of the same element and age. There have been studies that have reported differences in mineral size in different bone elements and with increasing age (Boskey, 2003). Therefore, it is important that these factors are recognised as a potential limitation to the study. To develop a universal diagnostic tool to aid a DNA analysis from compromised samples such factors would have to be taken into account. In a forensic context, it would not always be possible to analyse the same bone element from individuals of the same age.

Considering the limitations of this study, there are a number of directions the project could follow in the future. Repeating the study using human tissue (from all bone elements and ages) and the analytical techniques that have shown useful potential (colourimetry and FTIR spectroscopy), a comprehensive universal diagnostic tool to aid human DNA analysis from heated bone could be developed.

The diagnostic tools presented in this study could also be developed regarding the type of DNA targets for amplification. This study has focused on DNA fragments and STRs amplification, but could be developed to predict the probability of successfully obtaining mtDNA, Y-chromosome and SNPs profiles. By developing the diagnostic tool to include a variety of different DNA identification strategies, further time and money could be saved.

Advancements in DNA repair technologies have meant that it is now possible to repair certain types of DNA damage. DNA repair kits such as PreCr™ from New England Biolabs® Inc. are commercially available and have been shown to offer some degree of opportunity to repair DNA damaged by heat. Although, these kits can do nothing for DNA strand breakage, they are capable of restoring (the appropriate) organic base to DNA strands that have been subjected to limited depurination (hydrolysis). Analysing the effect that using such kits had on the amplification of heat-degraded DNA, would not only determine whether they would be a useful tool in DNA analysis from heat-

treated bone but could also provide some insight into the primary mechanisms of DNA degradation. This is because amplification failure due primarily to template fragmentation would not be recoverable whereas failure due to depurination might be.

The potential for the diagnostic tools developed in this study is enormous. We have shown that it is possible to use colourimetry/FTIR spectroscopy as a potential tool for 'screening' heat compromised bone samples prior to attempting DNA extraction and analysis.

5.3 Water submersion Study

The literature regarding the effect of heat treatment on the material and physical properties of bone is very extensive. Therefore, it was possible to focus on a set of analytical techniques that have already shown some potential for becoming diagnostic aids in DNA analysis (section 2.8). However, from this set, the techniques ultimately used were primarily chosen as they are relatively cheap, quick to perform and required minimal bone preparation, all of which are fundamental requirements for a technique to become an inexpensive and fast diagnostic tool.

The literature regarding the material and physical changes in bone as a result of water submersion is limited. However, there are a number of changes that occur, such as collagen degradation and mineral re-crystallisation, which it was hoped could also be

investigated using the chosen analytical techniques. It was anticipated that one or more of the chosen techniques could be developed into a diagnostic tool to aid DNA analysis from submerged bone. Moreover, if the correlation between DNA degradation and a marker was comparable to that found in the heat treatment study it might have been possible to develop a 'universal' diagnostic tool to aid DNA analysis for submerged and heated bone samples.

Unfortunately no parameter could be correlated with DNA fragment amplification. Statistical analysis of these data was not a viable option as DNA degradation was only seen in a small number of samples. Moreover, except for bHAP microstrain and microhardness there were very few changes in the measured parameters over the course of the study.

5.3.1 DNA degradation in bone submerged in different aquatic environments

The literature regarding DNA profiling from skeletal tissue that has been submerged in an aquatic environment is very limited, however in both fresh and seawater, DNA profiling was successful in samples that were submerged for over two years (Crainic et al., 2002). The details surrounding the temperatures at which the samples were maintained during submersion are unknown. However, they would be consistent with the results of this study if they had been submerged in cool waters. Regardless of

whether the environment was distilled or seawater, 500 bp amplicons were successfully amplified from bovine bone that had been submerged for up to 104 weeks (Table 4.27).

The results of this study suggest that DNA is relatively stable in bovine bone for at least two years in cool (~7 C) water environments. It was been suggested that DNA stability is maintained only by the low temperature and oxygen levels, which reduce rates of oxidation and hydrolysis (Alaeddini et al., 2010), but also low light levels; conditions mimicked by the cool submersion assay. Low light and oxygen levels would reduce the rate of DNA degradation through microbial activity (Niemcunowicz-Janica et al., 2007).

In contrast to the cool water submersion assay, DNA degradation was evident in the warm water submersion study. The 500 bp fragment could not be amplified from the majority of samples that were submerged in warm distilled and seawater beyond 68 weeks. It should also be noted, that 500 bp amplification failure was sporadic between 44 and 68 weeks in samples that were submerged in warm synthetic seawater. The result suggests that temperature, duration and to a lesser extent salinity of water are factors that could affect DNA amplification, either by direct or indirect mechanisms. DNA degradation in bone that has been submerged in water was not influenced by the integrity of collagen and bHAP. The results suggest that temperature dependent processes were a fundamental contributor to the rate of DNA degradation in bone that has been submerged in water.

5.3.2 Changes in bone properties due to water submersion

Diagenesis of bone submerged in water can take an extensive period of time and is represented by two main stages, early and late diagenesis. There are a number of changes to the properties of bone that should have occurred within the 'time frame' of the experiment (section 2.9.2).

Early diagenesis is divided into three main stages, collagen degradation, collagen gelatinisation and finally the appearance of microcracks and the formation of pryorite, (particularly in seawater). It has been determined that within one year of submersion 5-15 % of collagen would have degraded in large bones such as femora, that have been submerged in +20 °C water (Pfretzschner, 2004). The results of this study show that there was no significant decrease in the APR in all four aquatic environments (Figure 4.40). This suggests that collagen degradation had not occurred. However, there was a decrease in microhardness.

The microhardness of bone is a function of both collagen and bone mineral properties. Both can have an effect on the microhardness of bone, which can be demonstrated by the microhardness of heated bone (section 4.2.6), where collagen degradation and bHAP re-crystallisation was shown to effect microhardness. As there were few changes in bHAP in the submerged bone samples (Figures 4.41-4.44), it could be assumed that the decrease in microhardness is a result of collagen integrity.

Collagen gelatinisation may be a possible explanation for the decrease in microhardness while minimal change occurred in the APR. The partial hydrolysis of collagen fibrils results in the production of gelatine. Therefore, it may be plausible that, although collagen may not have become degraded, the structural integrity of the fibrils may be altered. This would not necessarily be detected by measuring the APR.

Gelatinisation is temperature dependent (Nielsen-Marsh et al., 2000) which may explain the differences in the decline of microhardness across 104 weeks between cool and warm aquatic environments. Gelatine is also soluble in warm ~30 °C and forms a gel (Singh et al., 2009). Although, it was not tested, the surface of bone samples that were removed from the warm water tanks were slippery when held, suggesting the possible presence of gelatine.

In the presence of water, gelatinised collagen fibres swell, which causes microcracks to appear in the Haversian systems. During this study, no microcracks appeared in the Haversian systems (Figure 4.47) suggesting that the rate of diagenesis had decreased or delayed and the samples were still in the early stages of gelatinisation. The rate of diagenesis could of been decreased through two attributing factors.

1. The initial loss of collagen during the early diagenesis has been attributed to bacterial activity. Each tank of water was made from distilled water or synthetic seawater (made from distilled water) and the bone sections were cleaned, to

remove soft tissue. Therefore, although not tested, the potential for substantial bacterial activity was minimal.

2. The water in the tanks was also changed every few months. Gelatinisation is pH dependent and is accelerated by high pH. The water in each tank was maintained at pH 7.5-8.5. As collagen degrades, ammonia is released, causing pH levels to rise. By changing the water, the pH level of water was maintained at a relatively neutral level. Thus, the rate of gelatinisation would not have increased.

After the appearance of microcracks, apatite exchanges ions with the surrounding water and bHAP re-crystallisation occurs (Hedges and Millard, 1995). As the samples (from all four aquatic environments) did not show any signs of microcracks, it was not surprising that the bHAP showed no significant changes in crystallinity, in terms of SF and CL, crystallite size and cell unit parameters 'a' and 'c'. There was a slight decrease in microstrain, particularly in bone that was submerged at warm conditions, suggesting that some ion exchange had occurred. Phosphate ions have been shown to exchange with carbonate, fluoride and chloride ions (Pfretzschner, 2004).

5.3.3 Case Study

The DNA results of the case study were comparable with the results from the controlled experiment using bovine bone in cool synthetic seawater. After two years of submersion

of the remains in the English Channel, it was possible to obtain 13 full profiles from the 22 bones recovered. Loci and allele drop out occurred in the clavicle, humerus, tibia, fibula and rib, while all markers failed to amplify from the radius and ulna. Interestingly, STR markers failed to be amplified from low load-bearing bones. The high load-bearing bones, such as femora, tibiae, fibulae and foot bones had relatively high amplicon band (Appendix 10; Figure A10.1) and STR peak height intensities (Appendix 10; Figures A10.2-A10.4) when compared to low load-bearing bones such as the clavicle and humerus. This is consistent with previous studies that have reported that high load-bearing bones should be the preferable source of DNA, particularly in mass disasters such as the Asia Tsunami (Milos et al., 2007, Prinz et al., 2007). Moreover, the intensities of amplicons bands and STR height peaks from foot bones, such as the talus and navicular, were relatively high when compared to high load-bearing long bones.

A number of additional experiments, such as absolute quantitation by real-time PCR, would have to be performed to determine whether bones from the foot would become the preferred choice for DNA analysis in this particular case study. However, the results from the case study are consistent with recent related publications. The percentage of individuals that were successfully identified from the World Trade Centre (WTC) terror attacks was highest when using DNA extracted from bones, including metatarsals and phalanges (Mundorff et al., 2009). Although also load bearing, these skeletal elements have a higher ratio of cancellous to compact bone, than long bones such as the femur. It is thought that cancellous bone may have a higher cell content than compact bone due

to increased vascularisation (Milos et al., 2007). The results of this case study support the sampling guidelines set out by Mundorff et al., (2009). Given the success rate of STR profiling and relative ease of removing bone elements from the foot, it may be preferable to select these elements for DNA analysis. This would ultimately save, time, money and effort in identifying individuals from skeletal remains.

After a submersion period of two years there were no visual histological changes in the long bones of the individual. The process of gelatinisation is pH-dependent. The water surrounding the human bone would have been constantly changing and it would be unlikely the pH level would have increased. This may explain the lack of microcracks in the human skeletal tissue. To establish the stage of diagenesis the recovered bones are in, further analysis regarding collagen and bHAP integrity would be required.

5.3.4 Conclusion: Water submersion study

Unfortunately no statistical analysis could be carried out between DNA amplification and the different properties of bone that were investigated. However, the results of this study have provided insight into the degradation patterns of DNA and collagen and the effect of water submersion on the integrity of bHAP. It was clear from these results, that temperature plays an important role in DNA degradation in water-submerged bone. However, temperature had very little effect on collagen degradation and HAP integrity.

DNA degradation may not be correlated with collagen degradation or HAP re-crystallisation in water-submerged bone.

5.3.4.1 Limitations and future directions

The current literature regarding bone diagenesis in water suggests that within a year, changes in the organic and mineral phases will begin to appear. Unfortunately, within the two-year period of this experiment very few changes had occurred. The presumed lack of micro organism activity and the continued refreshment of water may have been contributing factors that delayed the effect of water submersion on these particular bone specimens. Future studies should address these issues, as it would be possible to repeat a similar experiment using water from natural water bodies including ponds, lakes and oceans.

With the exception of microhardness, few changes in bHAP and collagen integrity occurred in the bovine bone, when submerged for up to 104 weeks in different aquatic environments. Whether microhardness is directly or indirectly related to DNA degradation remains unknown. The APR remains constant throughout the study, suggesting that the hydroxyl groups of collagen are still present. Further analysis, by SEM for example, may show morphological changes that could have occurred to the collagen fibrils. Although a statistical analysis could not be made, there were very few changes in the parameters measured by each analytical technique, even when DNA

degradation occurred. The analytical techniques chosen for this study may not have been appropriate. Further analysis using additional techniques may result in strong correlations with DNA amplification that may lead to the development of a useful diagnostic tool.

Chapter 6 Conclusions

The intention of this study was to utilise a variety of established analytical techniques to develop a diagnostic tool that could predict, prior to DNA extraction, whether DNA for forensic analysis, could be amplified from compromised bone tissue. The integrity of bone can be compromised by a variety of different factors. DNA analysis from either heated bone or bone submerged in water has been shown to be problematic and these two factors were consequentially chosen for this project. Moreover, the current literature regarding DNA analysis from heated or submerged bone is limited and as discussed in this thesis are often restricted to case studies, where accurate accounts of the environmental conditions are often not described.

The ultimate goal of this study was to develop a universal diagnostic tool that, regardless of the compromising factor, could predict whether DNA analysis was a viable option for identification. This study suggests that it would be difficult to develop such a universal tool using a single analytical technique. Further studies would be required in order to establish a suitable marker to predict the probability of successful DNA amplification from submerged bone, or bone compromised by other factors including pH. Once suitable markers have been developed it might be possible to combine each technique used into a single instrument that could be used to give real-time results in the field.

The experiments designed in this study utilised controlled environments that closely mimicked the conditions regularly found in reality. This thesis provides comprehensive insight into the integrity of bone material when heated or submerged in water. We report that the temperature and duration of each compromising factor are fundamental contributors to DNA degradation in bone. However, the relationship between DNA degradation and the changes in the organic and mineral phases caused by each factor appear to be different. It was clear that collagen and bHAP in bone remain relatively stable, regardless of the period of submersion or water temperature. This suggests that DNA degradation is not due to a loss of bone integrity that may promote or coincide with the degradation of DNA.

Femoral bone is the preferred choice of bone when identifying individuals using DNA analysis from skeletal remains. The DNA analysis of the individual recovered from the English Channel has questioned, but not necessarily answered, whether femur should be the favoured choice. In concordance with current literature, the results from the case study have suggested that DNA analysis from skeletal tissue should use bones from the feet.

Heat treatment, which is a compromising factor that can have an immediate effect on bone integrity, showed significant correlations with DNA degradation. This study demonstrated that it would be possible to predict the probability of amplifying a variety of DNA targets from heated bone. This study also showed that in addition to

determining the most appropriate strategy for DNA analysis, it was also possible to obtain information regarding appropriate reference samples, likely to be required to achieve a significant profile match.

Burnt skeletal remains that require identification can result from a number of different events be they criminal, natural or accidental. The results of this study show that colourimetry and FTIR spectroscopy (APR and to a lesser extent SF) can be used individually or together as a tool to aid DNA analysis from heated bone. Both analytical techniques are relative inexpensive on a cost per sample basis, can be performed in minutes and require minimal bone preparation. Moreover, colourimeters and FTIR spectrometers themselves are both relatively inexpensive and portable. Taken together, these facts makes large scale pre-screening 'in-field' a realistic possibility.

There have been cases where hundreds of samples that have been exposed to excessive heat conditions require identification. This study presents a new and novel diagnostic tool that can reliably predict the probability of successfully amplifying DNA from heat compromised skeletal tissue. The diagnostic tool will not only benefit the organisation assigned to DNA profiling, but also the law enforcement agency and relatives that may be waiting for a positive identification. Consequently, the diagnostic tool could ultimately support criminal investigations and the community.

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Appendix 1

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15 July 2008

Mr Jamie D Fredericks
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Dear Jamie

Project Reference No 02/08: PhD: The development of new tools to aid in the forensic analysis of DNA from compromised bone

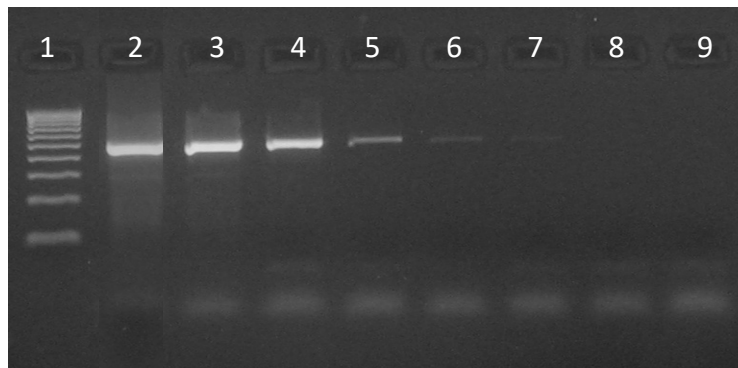
Thank you for submitting the requested amendments to the above project, I can confirm that I am happy to take Chairman's Actions approving the stuffy can go ahead.

Yours sincerely

Dr Sean Tyrrel
Acting Chairman, Cranfield Health Ethics Committee

Appendix 2

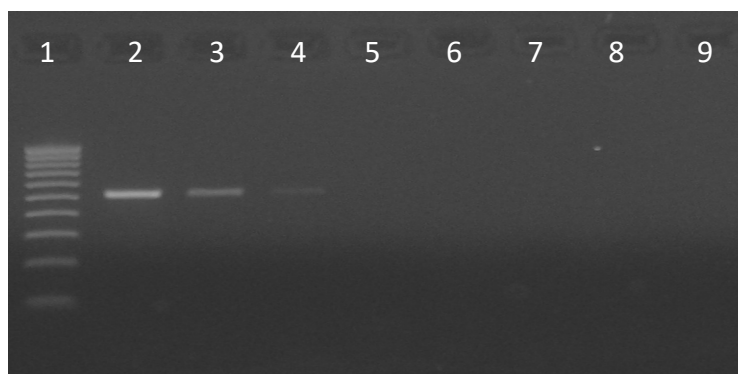
A



Key A1.1:

- 1: 100 bp ladder
- 2: 1:1
- 3: 1:10
- 4: 1:100
- 5: 1:1,000
- 6: 1:10,000
- 7: 1:100,000
- 8: 1:1,000,000
- 9: neg control

B



C

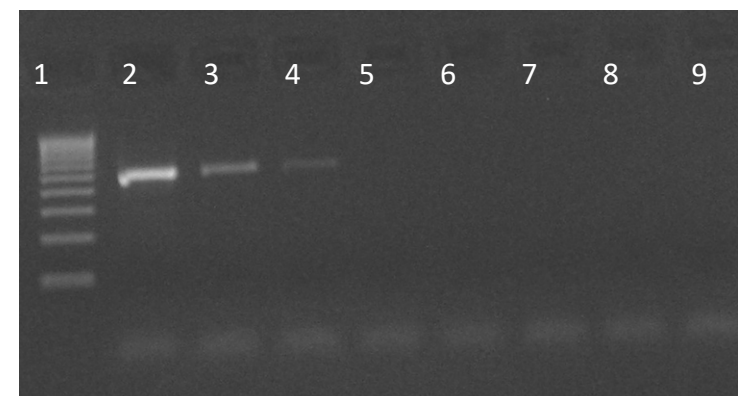


Figure A2.1: Serial dilutions of DNA extracted from fresh bovine bone. A: Genial: First DNA; All-tissue DNA-extraction kit, B: Invitex: Invisorb® Spin Forensic Kit, C: Invitex: Invisorb® Forensic Kit

Appendix 3

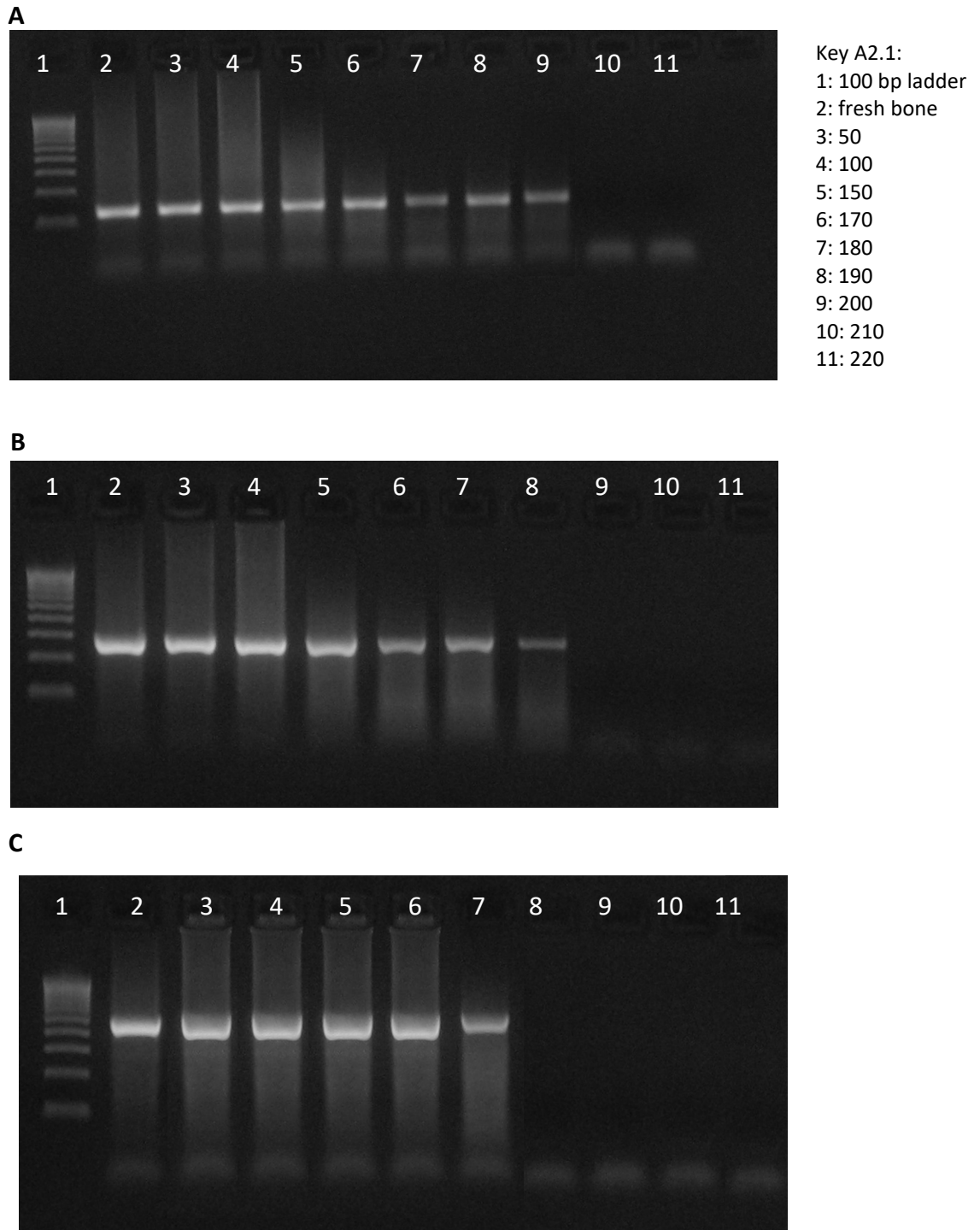
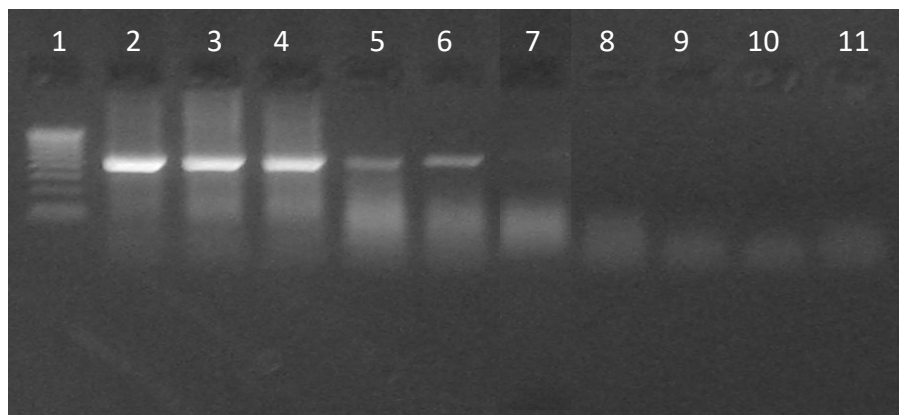


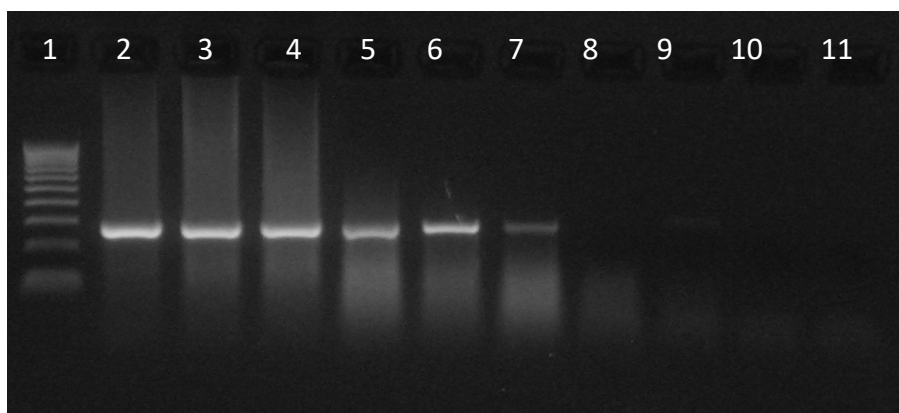
Figure A3.1: Gel electrophoresis images of PCR products taken from samples heated a various temperatures (see key) for two hours. A: 500 bp, B: 300 bp, C: 100 bp.

A



Key A2.2:
1: 100 bp ladder
2: fresh bone
3: 50
4: 100
5: 140
6: 150
7: 160
8: 170
9: 180
10: 190
11: 200

B



C

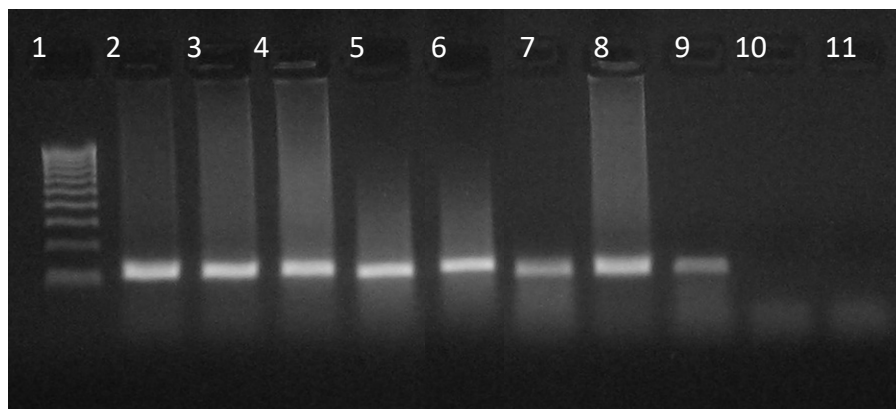
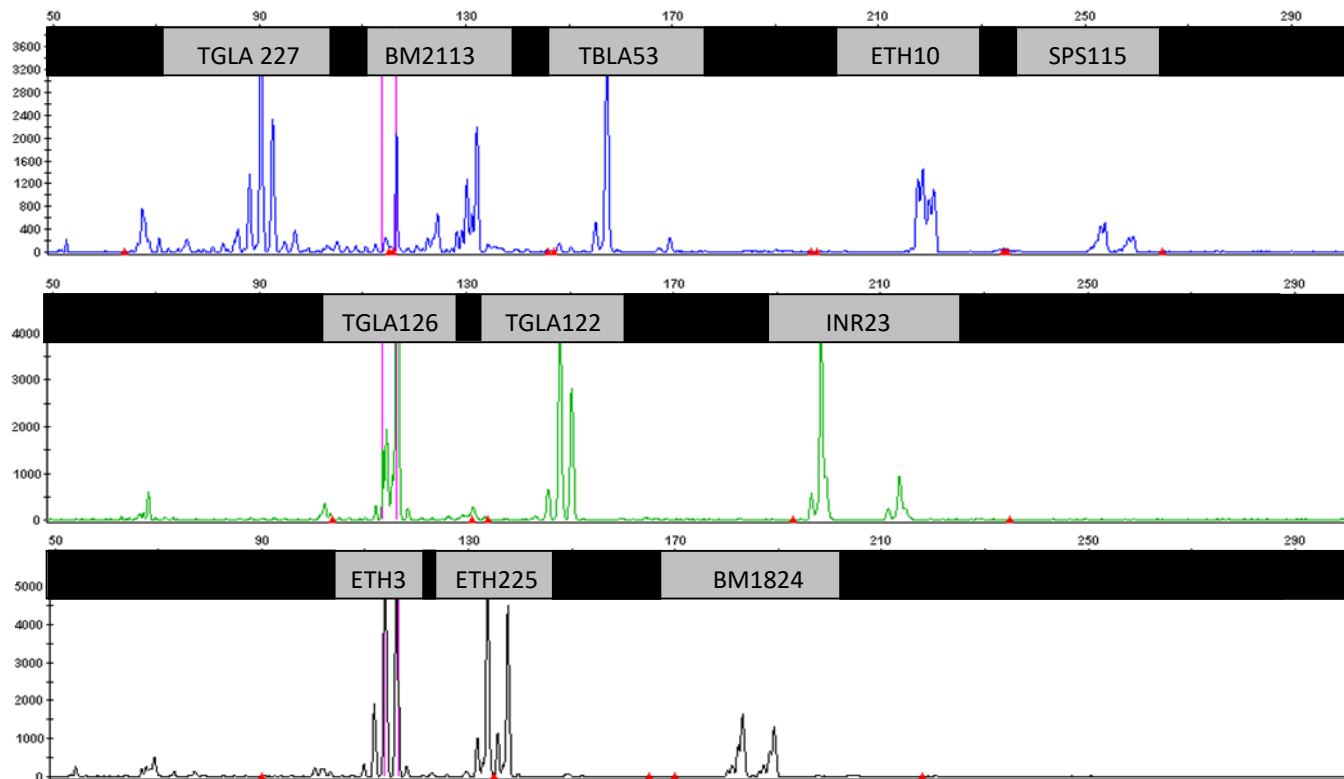


Figure A3.2: Gel electrophoresis images of PCR products taken from samples heated a various temperatures (see key) for four hours. A: 500 bp, B: 300 bp, C: 100 bp.

Appendix 4

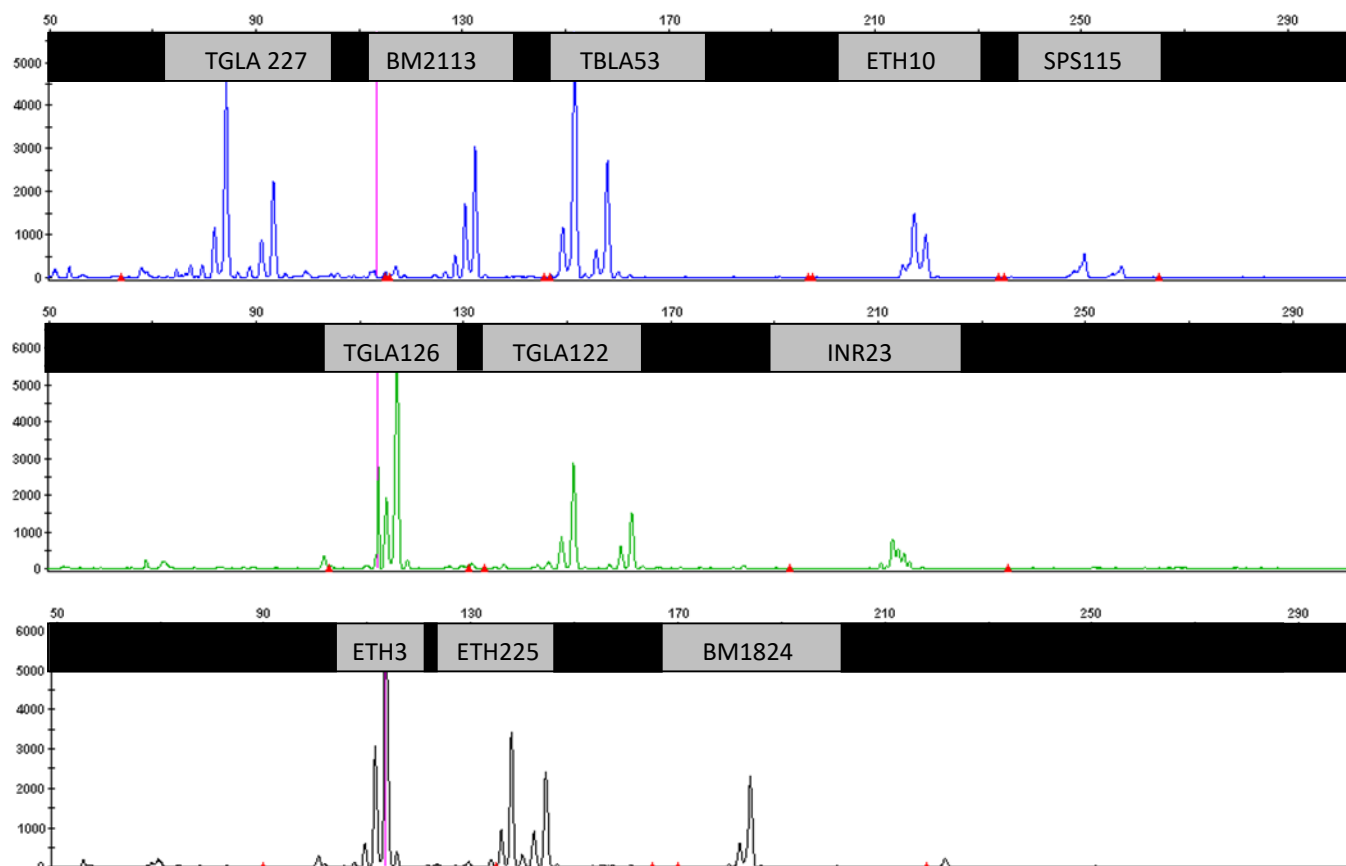
Table A4.1: Bone 3 STR loci size



Locus	Name	loci Size (bp)	
		1	2
1	TGLA227	90.28	92.6
2	BM2113	116.54	132.13
3	TBLA53	157.38	H
4	ETH10	218.58	220.73
5	SPS115	253.87	259.32
6	TGLA126	116.75	H
7	TGLA122	148.11	150.35
8	INRA23	198.54	213.69
9	ETH3	113.94	133.7
10	ETH225	137.62	H
11	BM1824	183.06	189.24

Figure A4.1: DNA profile of bone 3, heated to 100 °C for two hours. Genotype software analysis of STR loci size (Table A3.1).

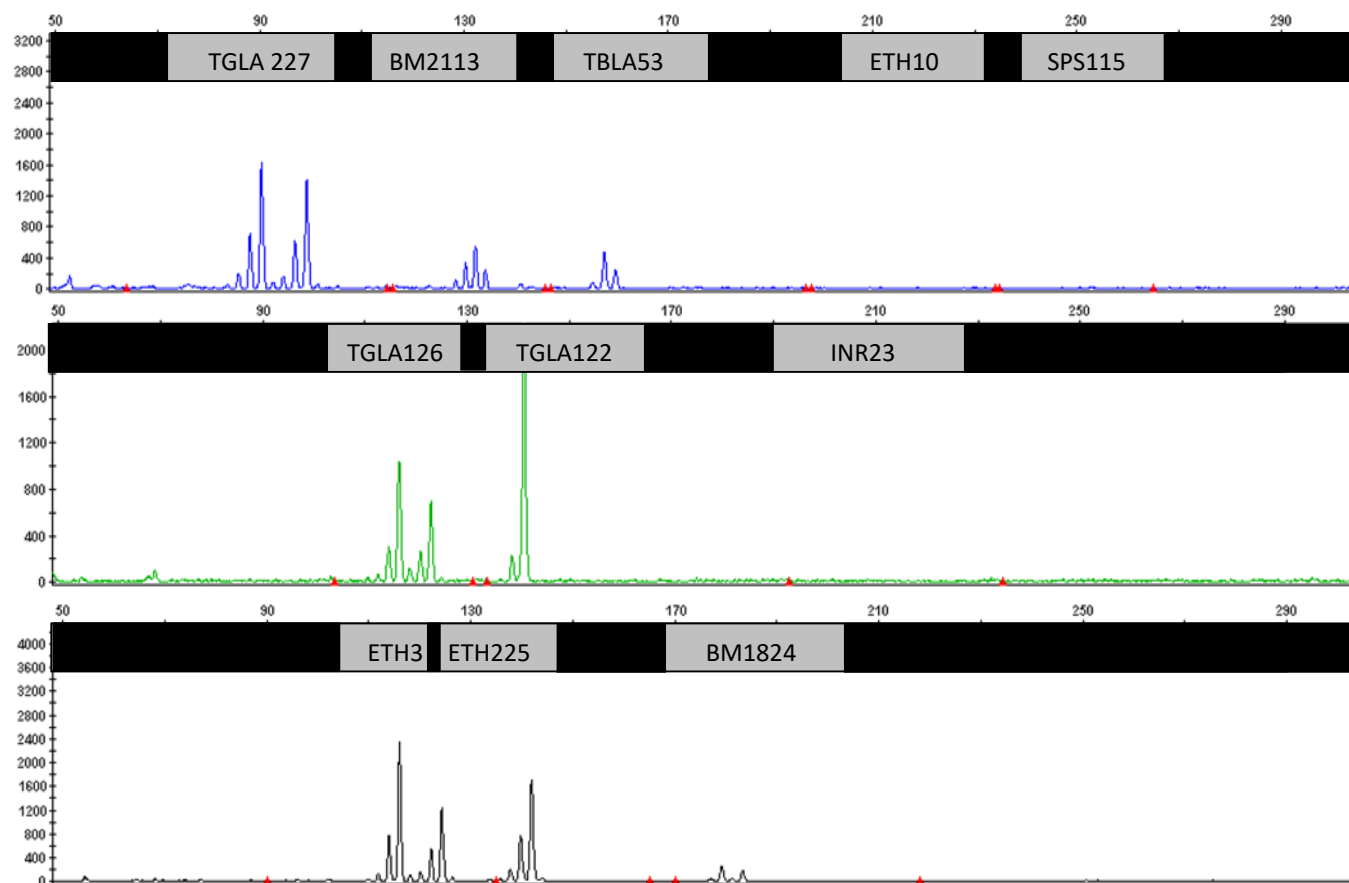
Table A4.2: Bone 7 STR loci size



Locus	Name	Allele Size (bp)	
		1	2
1	TGLA227	84.23	93.4
2	BM2113	132.52	H
3	TBLA53	151.79	158.17
4	ETH10	217.65	219.79
5	SPS115	250.98	257.8
6	TGLA126	117.19	H
7	TGLA122	151.24	162.45
8	INRA23	212.75	215.02
9	ETH3	113.79	H
10	ETH225	137.86	144.44
11	BM1824	184.03	H

Figure A4.2: DNA profile of bone 7, heated to 170 °C for two hours. Genotype software analysis of STR loci size (Table A3.2).

Table A4.3: Bone 8 STR loci size



Locus	Name	Loci Size (bp)	
		1	2
1	TGLA227	90.35	99.26
2	BM2113	132.29	134.21
3	TBLA53	157.44	159.67
4	ETH10	-	-
5	SPS115	-	H
6	TGLA126	156.67	-
7	TGLA122	141.11	H
8	INRA23	-	-
9	ETH3	116.0	124.31
10	ETH225	141.84	H
11	BM1824	179.8	183.3

Figure A4.3: DNA profile of bone 8, heated to 180 °C for two hours. Genotype software analysis of STR loci size (Table A3.3).

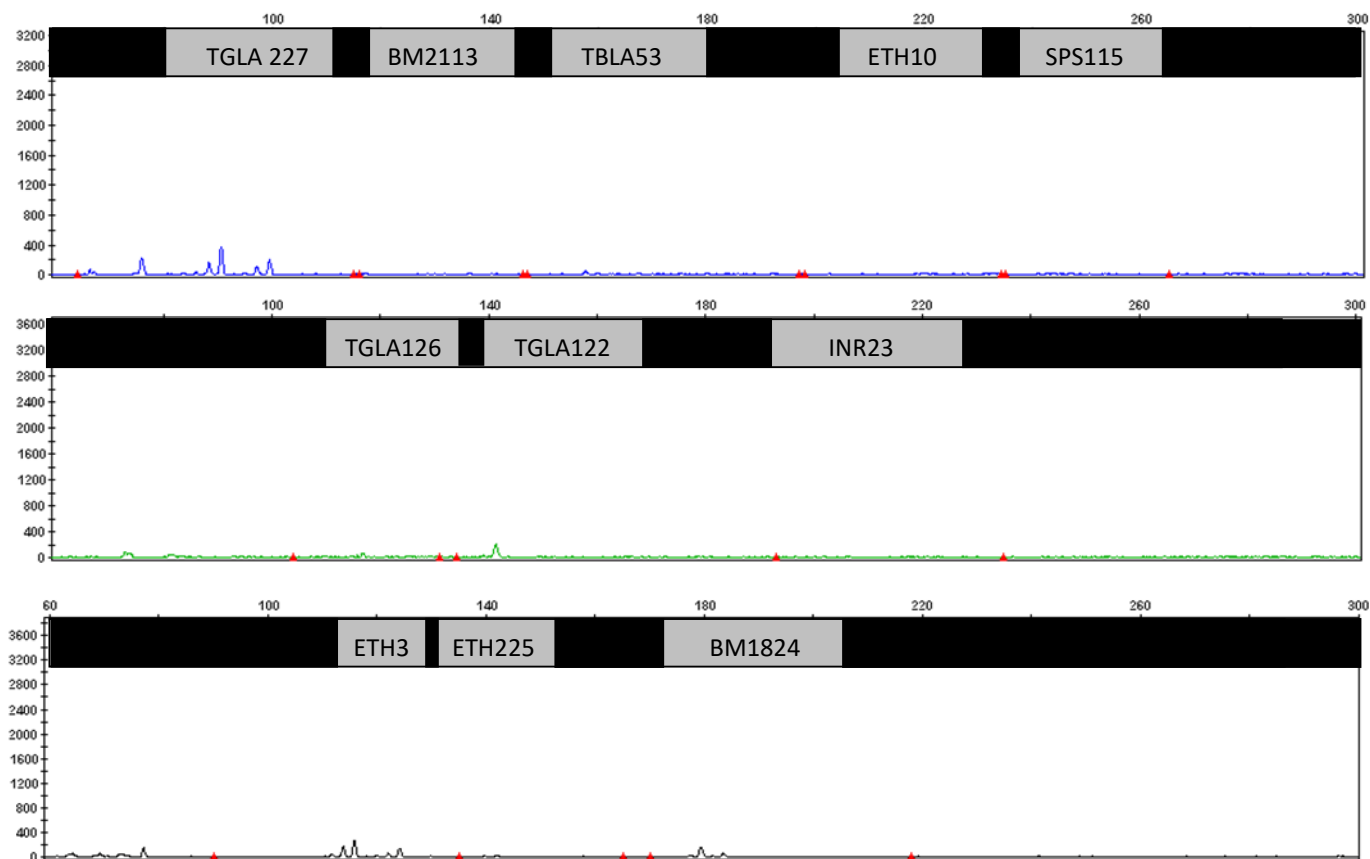


Table A4.4: Bone 8 STR loci size

Locus	Name	Allele Size (bp)	
		1	2
1	TGLA227	90.35	99.26
2	BM2113	-	-
3	TBLA53	-	-
4	ETH10	-	-
5	SPS115	-	H
6	TGLA126	-	-
7	TGLA122	141.11	H
8	INRA23	-	-
9	ETH3	115.9	124.14
10	ETH225	-	H
11	BM1824	179.28	-

Figure A4.4: DNA profile of bone 8, heated to 190 °C for two hours. Genotype software analysis of STR loci size (Table A3.4).

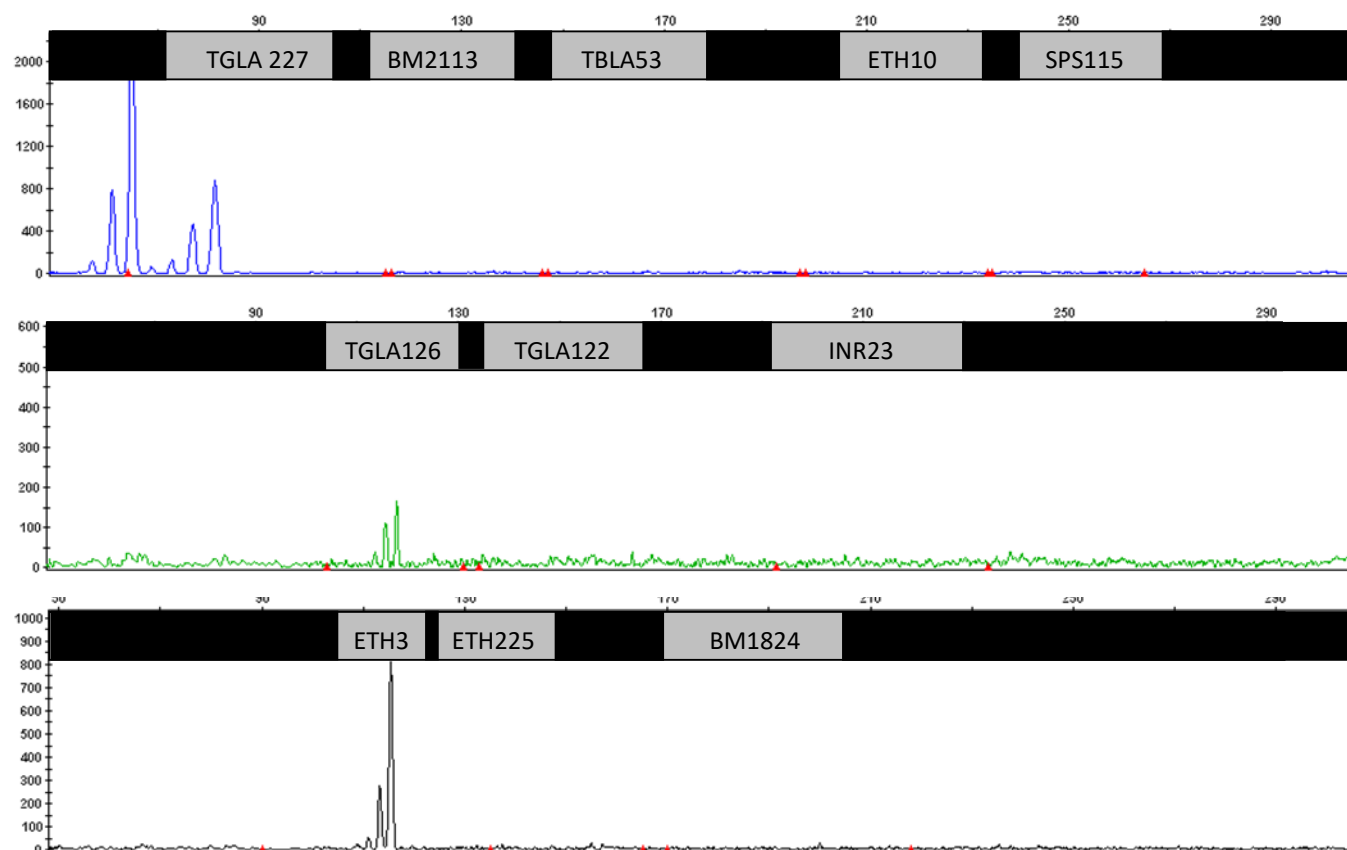


Table A4.5: Bone 1 STR loci size

		Allele Size (bp)	
Locus	Name	1	2
1	TGLA227	64.84	91.11
2	BM2113	-	-
3	TBLA53	-	H
4	ETH10	-	-
5	SPS115	-	-
6	TGLA126	117.78	H
7	TGLA122	-	H
8	INRA23	-	H
9	ETH3	115.49	-
10	ETH225	-	H
11	BM1824	-	-

Figure A4.5: DNA profile of bone 1, heated to 200 °C for two hours. Genotype software analysis of STR loci size (Table A3.5).



Table A4.6: Bone 4 STR loci size

Locus	Name	Allele Size (bp)	
		1	2
1	TGLA227	-	-
2	BM2113	-	-
3	TBLA53	-	-
4	ETH10	-	-
5	SPS115	-	-
6	TGLA126	-	H
7	TGLA122	-	-
8	INRA23	-	H
9	ETH3	-	-
10	ETH225	-	H
11	BM1824	-	-

Figure A4.6: DNA profile of bone 4, heated to 210 °C for two hours. Genotype software analysis of STR loci size (Table A3.6).

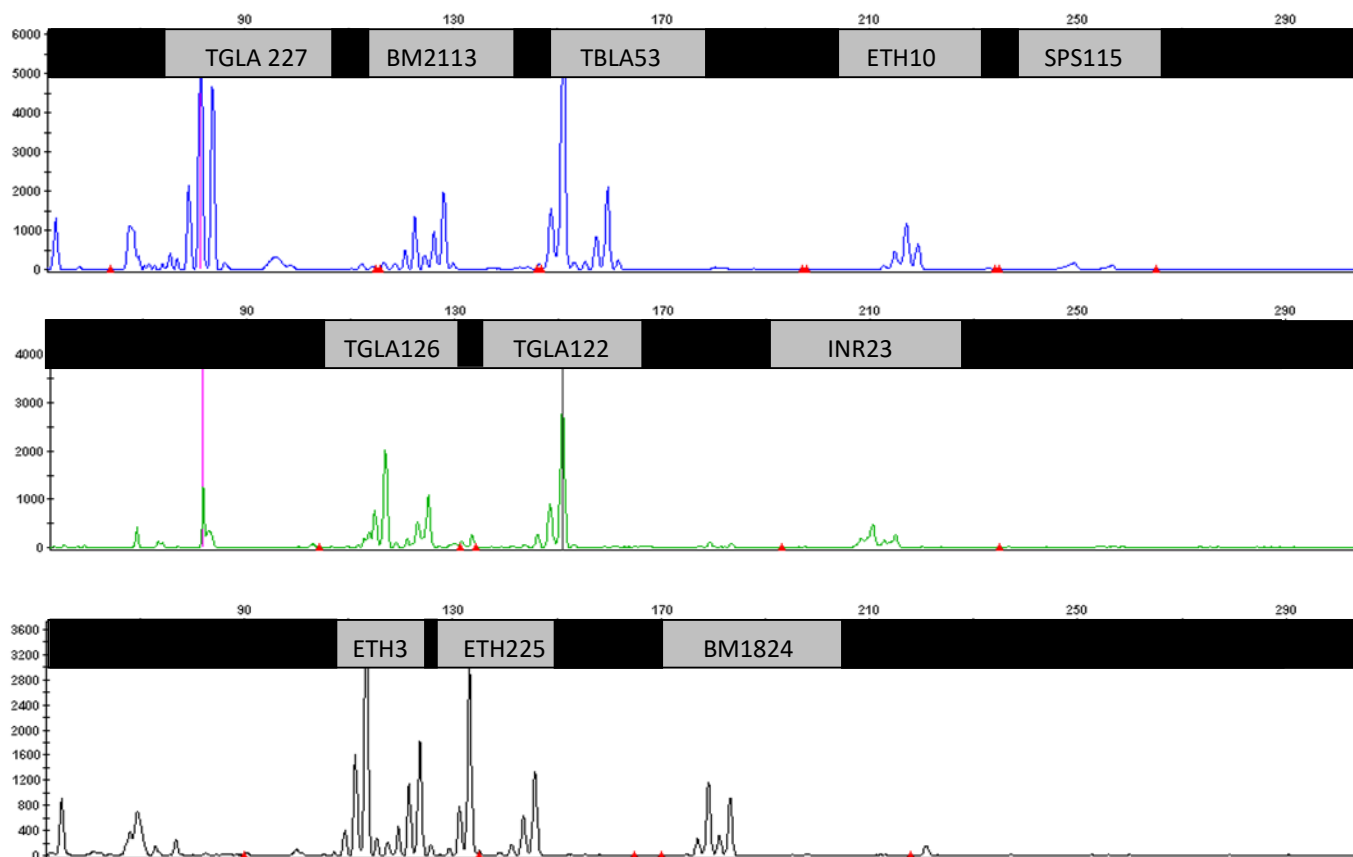


Table A4.7: Bone 6 STR loci size

Locus	Name	Allele Size (bp)	
		1	2
1	TGLA227	81.42	83.7
2	BM2113	122.59	128.1
3	TBLA53	151.07	159.65
4	ETH10	217.01	219.32
5	SPS115	249.42	256.67
6	TGLA126	116.59	124.84
7	TGLA122	150.71	H
8	INRA23	210.45	214.89
9	ETH3	113.35	133.26
10	ETH225	145.76	H
11	BM1824	179.07	183.34

Figure A4.7: DNA profile of bone 6, heated to 160 °C for four hours. Genotype software analysis of STR loci size (Table A3.7).

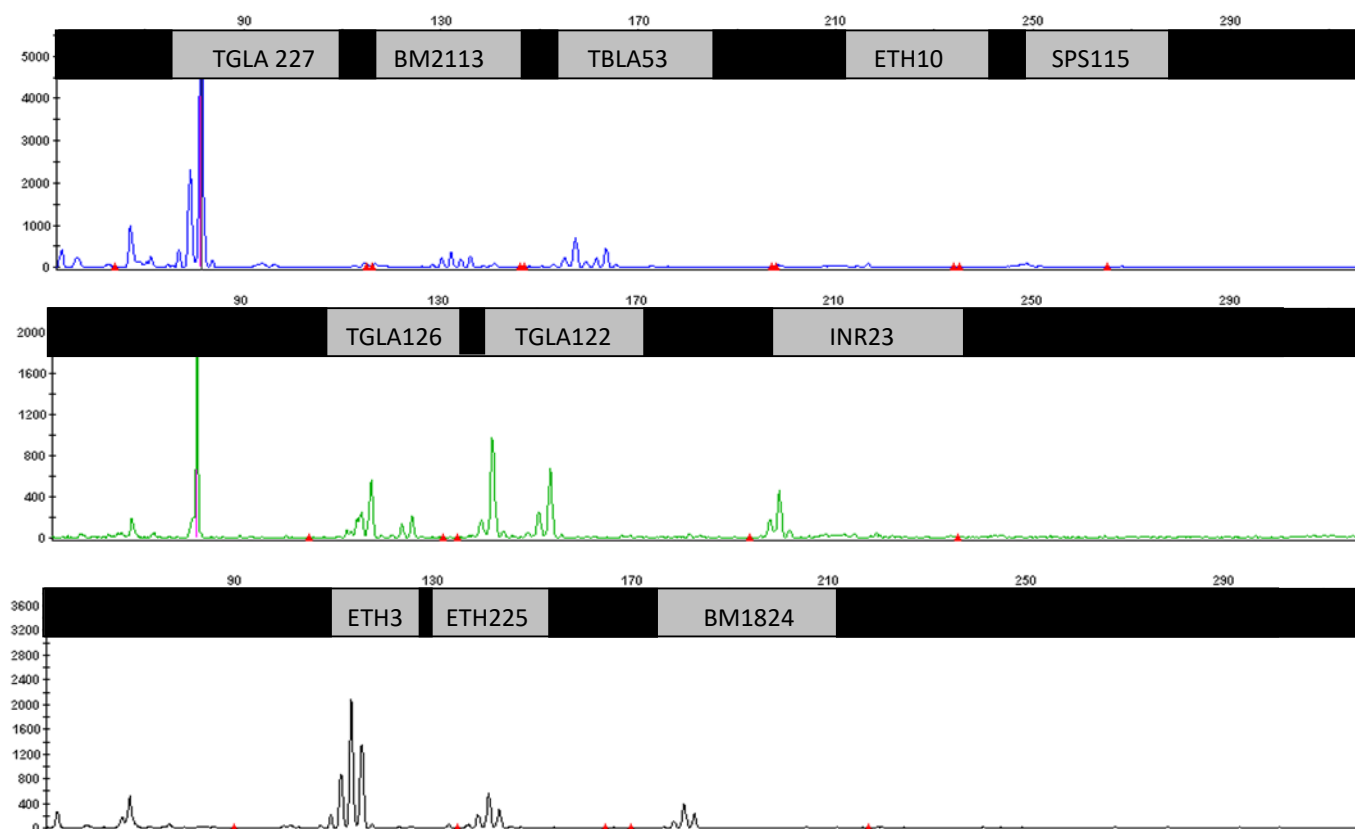
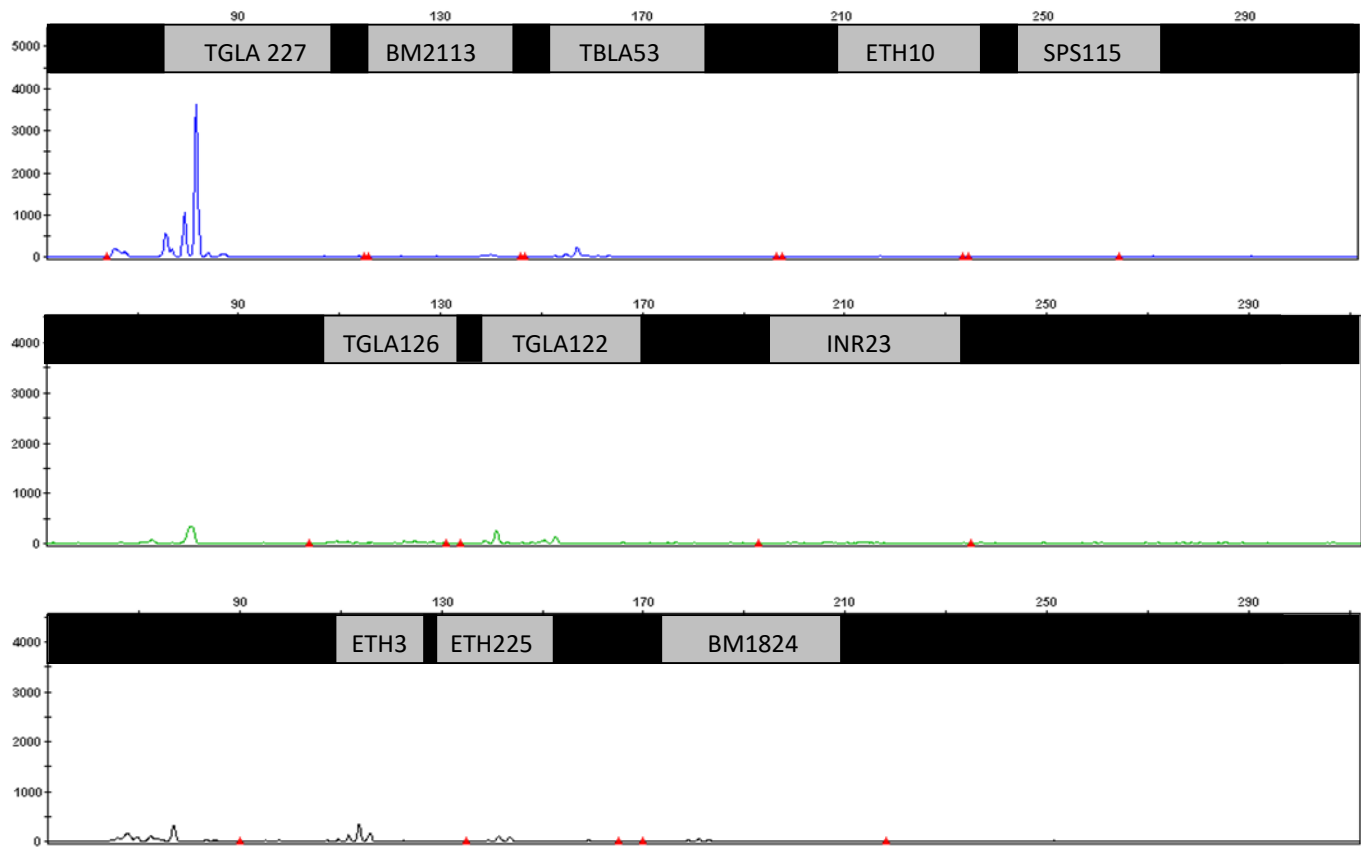


Table A4.8: Bone 7 STR loci size

Locus	Name	Allele Size (bp)	
		1	2
1	TGLA227	81.42	H
2	BM2113	131.97	135.94
3	TBLA53	157.16	163.45
4	ETH10	-	-
5	SPS115	-	H
6	TGLA126	116.56	124.78
7	TGLA122	140.96	152.7
8	INRA23	199.01	H
9	ETH3	113.57	115.81
10	ETH225	141.32	143.47
11	BM1824	180.85	182.99

Figure A4.8: DNA profile of bone 7, heated to 170 °C for four hours. Genotype software analysis of STR loci size (Table A3.8).

Table A4.9: Bone 7 STR loci size



Locus	Name	Allele Size (bp)	
		1	2
1	TGLA227	81.66	H
2	BM2113	-	-
3	TBLA53	157.39	-
4	ETH10	-	-
5	SPS115	-	H
6	TGLA126	-	-
7	TGLA122	141.1	152.65
8	INRA23	-	H
9	ETH3	113.70	115.91
10	ETH225	-	-
11	BM1824	-	-

Figure A4.9: DNA profile of bone 7, heated to 100 °C for four hours. Genotype software analysis of STR loci size (Table A3.9).

Table A4.10: Bone 2 STR loci size



Locus	Name	Allele Size (bp)	
		1	2
1	TGLA227	-	H
2	BM2113	-	-
3	TBLA53	-	-
4	ETH10	-	-
5	SPS115	-	H
6	TGLA126	-	-
7	TGLA122	-	-
8	INRA23	-	H
9	ETH3	-	-
10	ETH225	-	-
11	BM1824	-	-

Figure A4.10: DNA profile of bone 2, heated to 190 °C for four hours. Genotype software analysis of STR loci size (Table A3.10).

Appendix 5

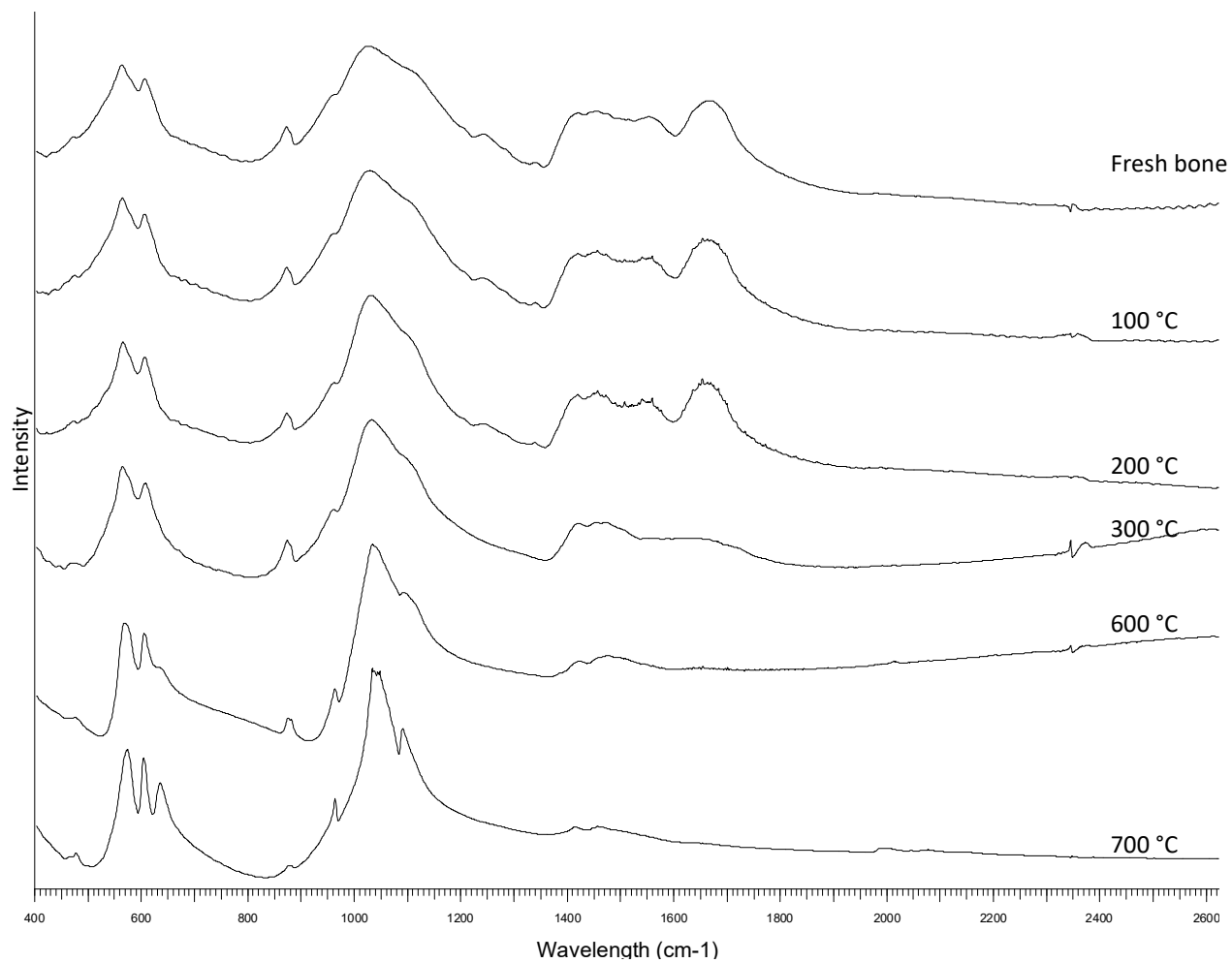


Figure A5.1: FTIR spectra of bovine bone heated to 39 – 700 °C for two hours.

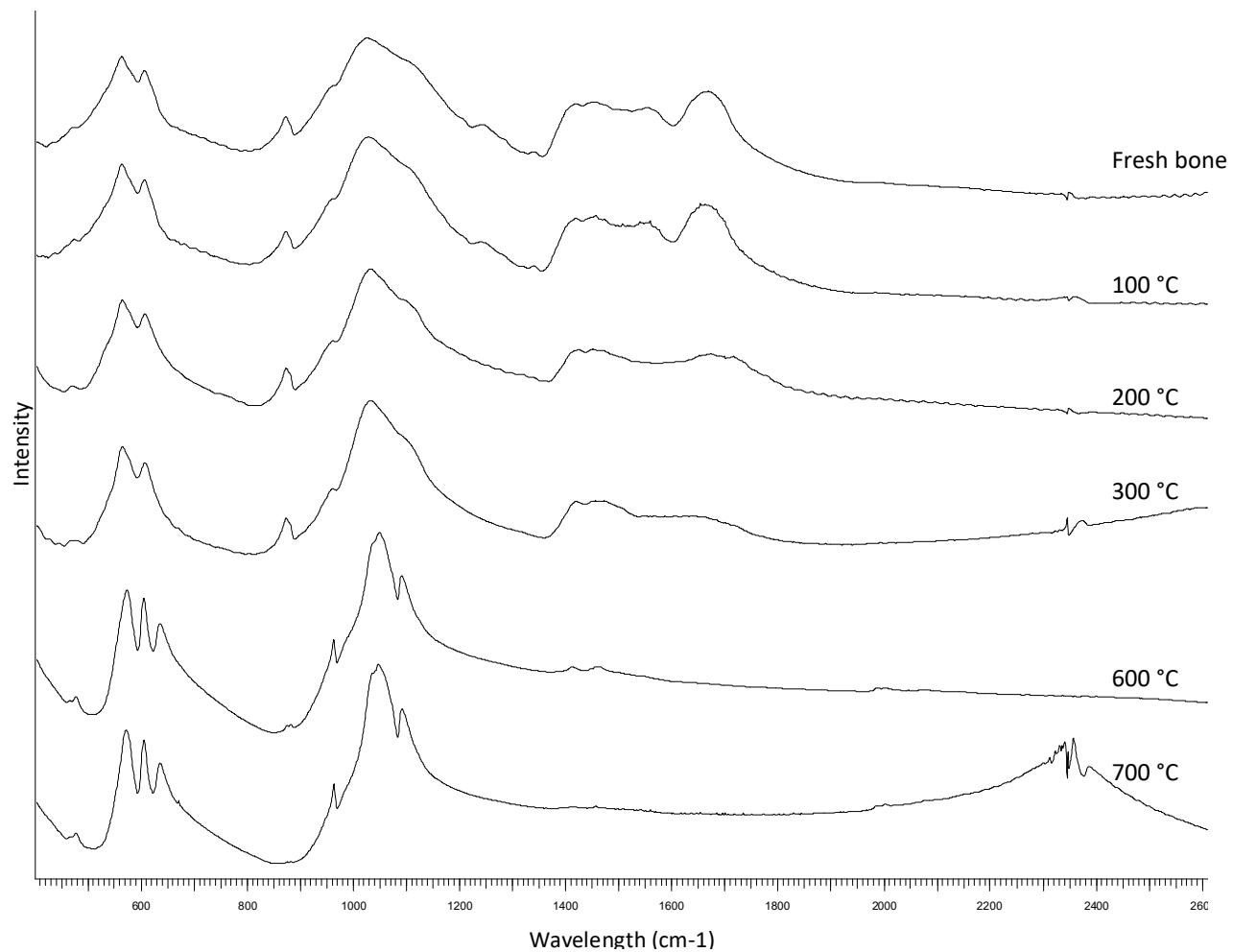


Figure A5.2: FTIR spectra of bovine bone heated to 39 – 700 °C for four hours.

Appendix 6

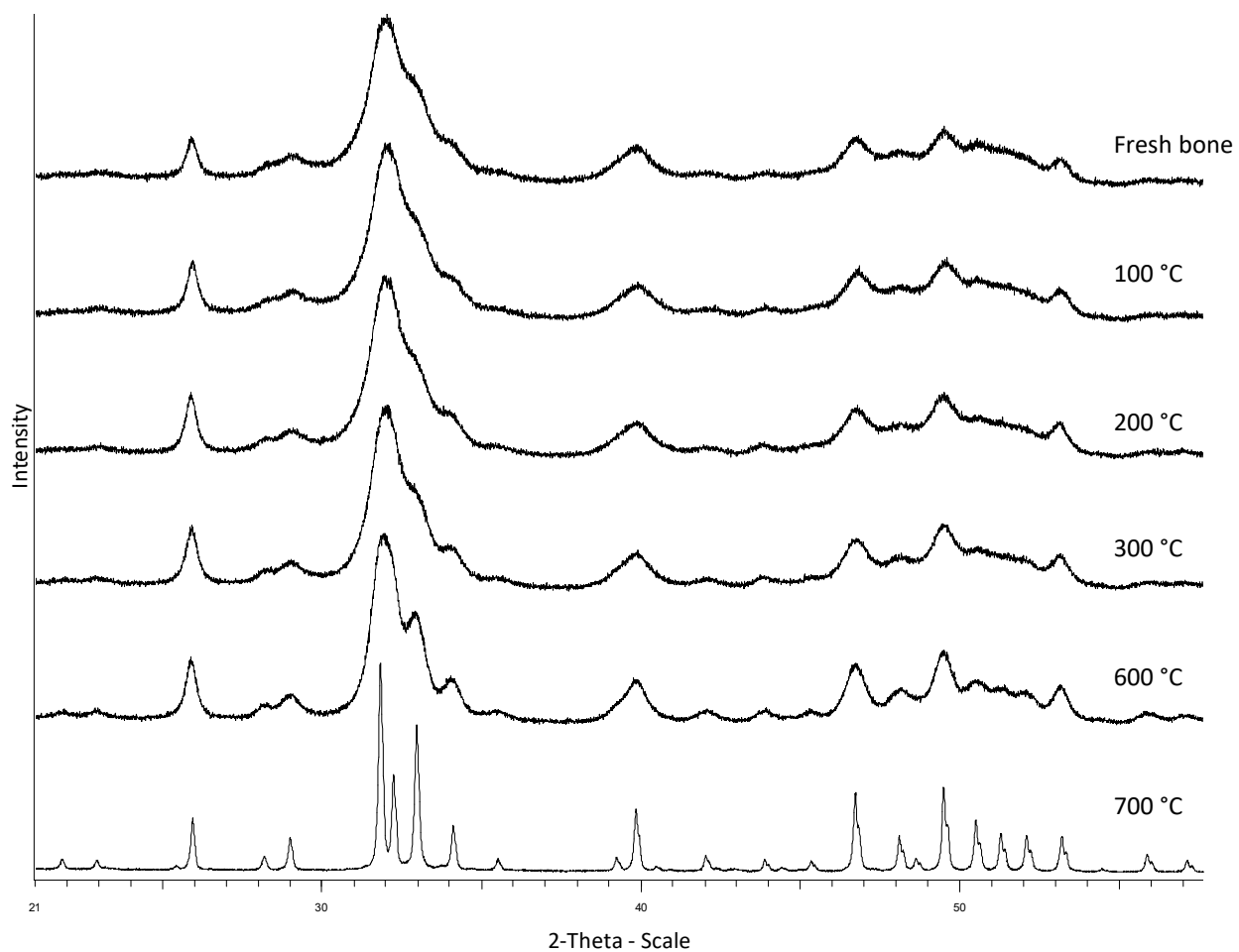


Figure A6.1: Full profile Diffractograms of bovine bone heated to 39 – 700 °C for two hours.

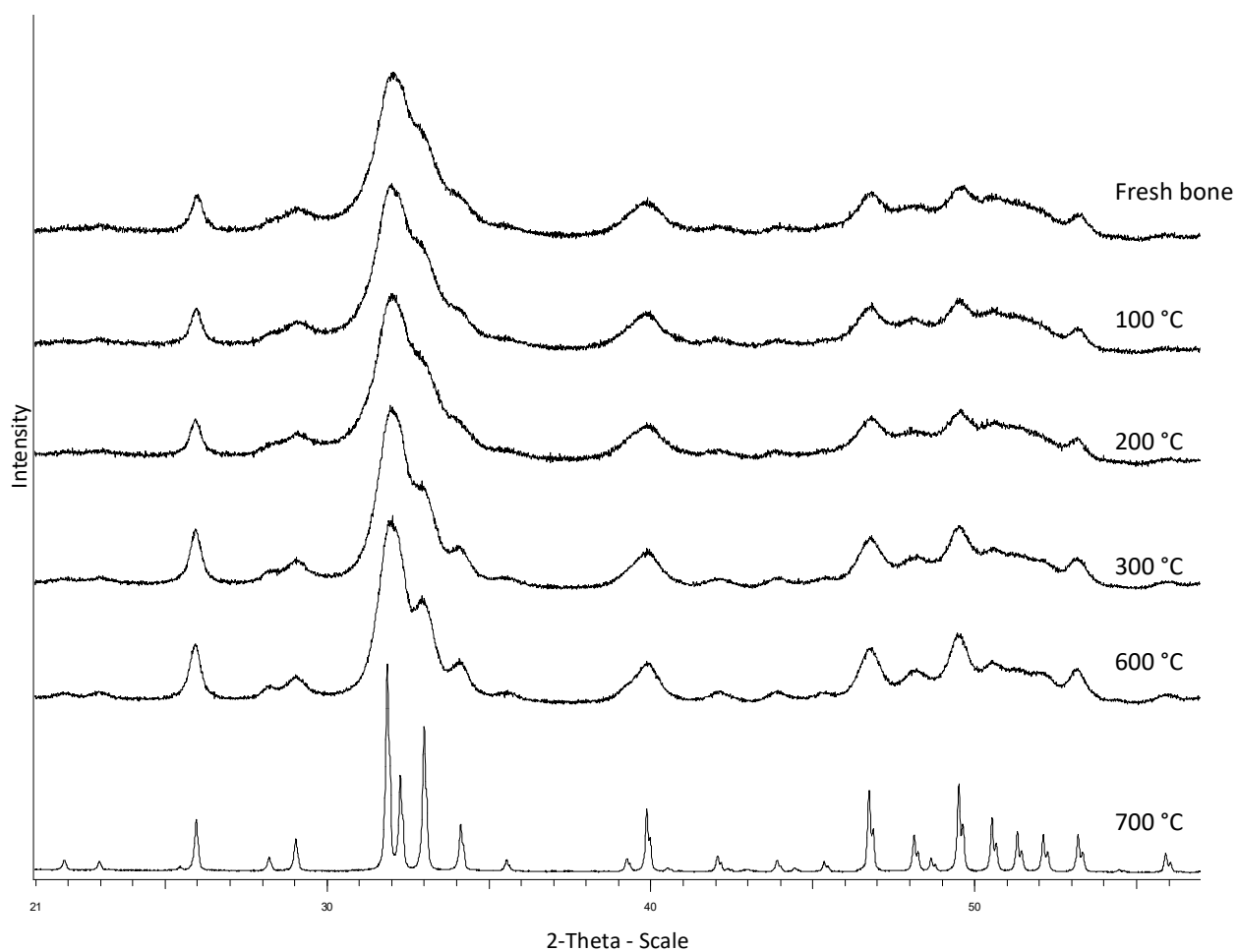


Figure A6.2: Full profile Diffractograms of bovine bone heated to 39 – 700 °C for four hours.

Appendix 7

A7.1 Logistic regression models predicting the successful amplification of 300 bp using different XRD parameters.

$$\text{Probability} = 1 / (1 + \exp[-(a-b\text{XRD parameter})])$$

Equation	XRD parameter	<i>a</i>	<i>b</i>
A7.1	CL	8.54	4.09
A7.2	Size	2.93	0.11
A7.3	'a'	-2732.99	-2901.45
A7.4	'c'	-1227.91	-1780.44

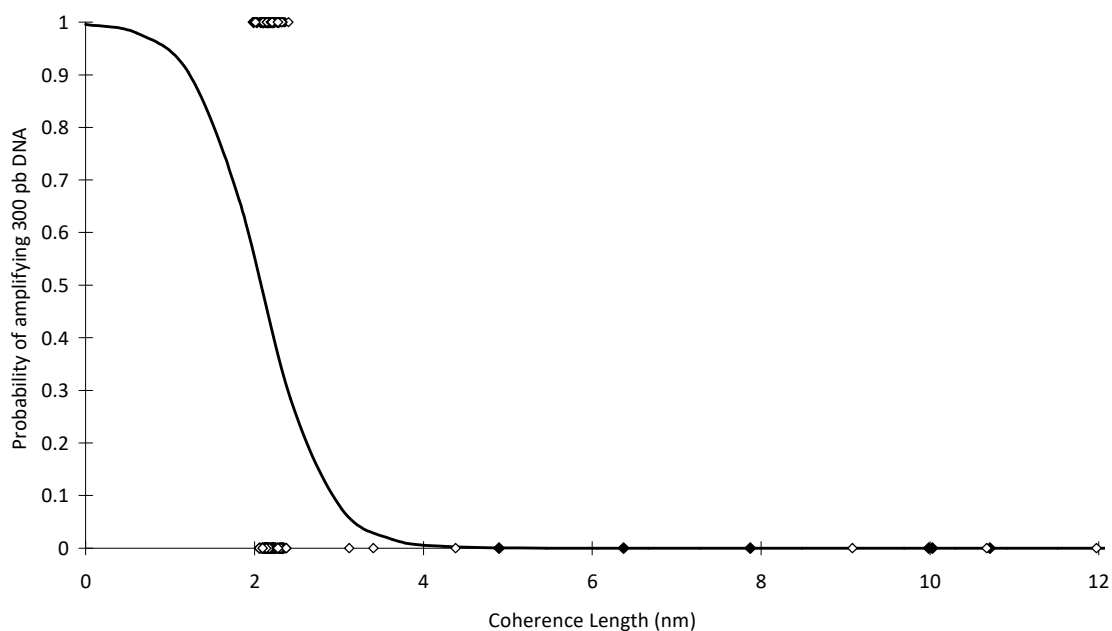


Figure A7.1: Relationship between the amplification of 300 bp DNA and CL of bHAP from bone heated bone (50-1000 °C). (◇) 2 hours and (◆) 4 hours. Logistic regression curve is a function of equation A7.1.

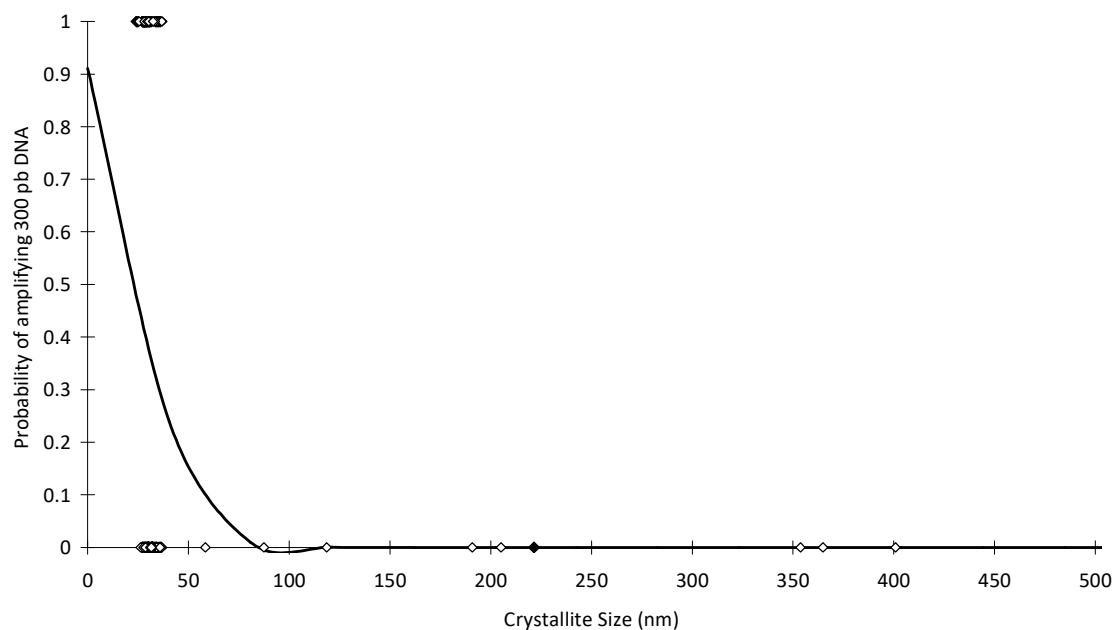


Figure A6.2: Relationship between the amplification of 300 bp DNA and crystallite size of bHAP from heated bone (50-1000 °C). (◊) 2 hours and (◆) 4 hours. Logistic regression curve is a function of equation A7.2.

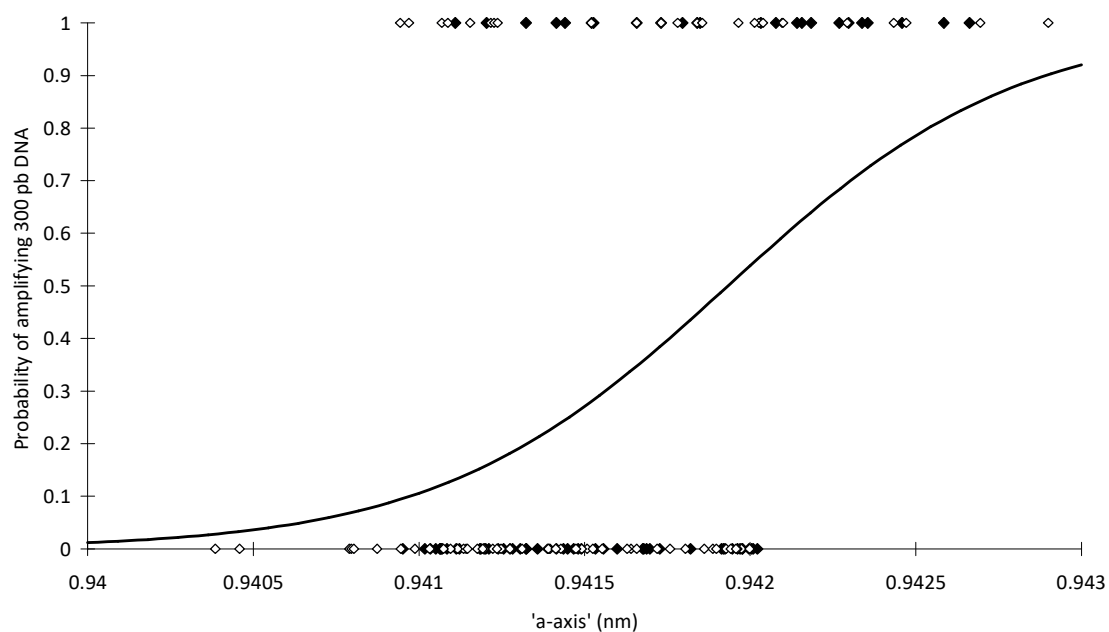


Figure A7.3: Relationship between the amplification of 300 bp DNA and the cell unit 'a-axis' parameter of bHAP from heated bone (50-1000 °C). (◊) 2 hours and (◆) 4 hours. Logistic regression curve is a function of equation A7.3.

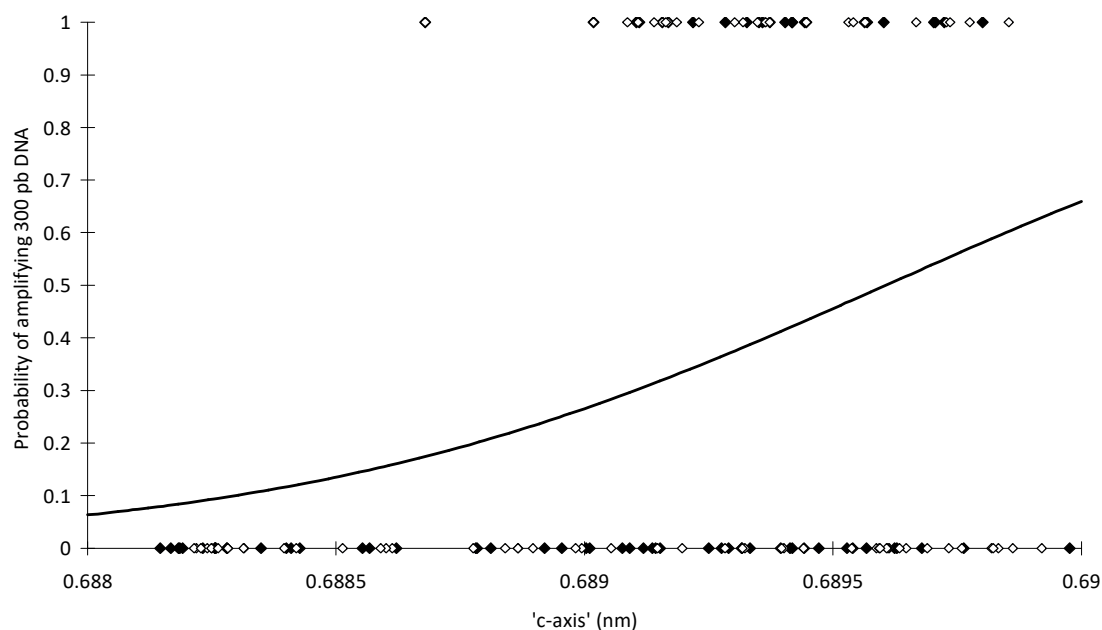


Figure A7.4: Relationship between the amplification of 300 pb DNA and the cell unit 'c-axis' parameter of bHAP from heated bone (50-1000 °C). (◊) 2 hours and (◆) 4 hours. Logistic regression curve is a function of equation A7.4.

A7.2 Logistic regression models predicting the successful amplification of 100 bp using different XRD parameters.

$$\text{Probability} = 1 / (1 + \exp[-(a - b\text{XRD parameter})])$$

Equation	XRD parameter	<i>a</i>	<i>b</i>
A7.5	CL	5.43	2.62
A7.6	Size	2.31	8.59
A7.7	'a'	-2155.76	-2288.66
A7.8	'c'	-1156.74	-1677.39

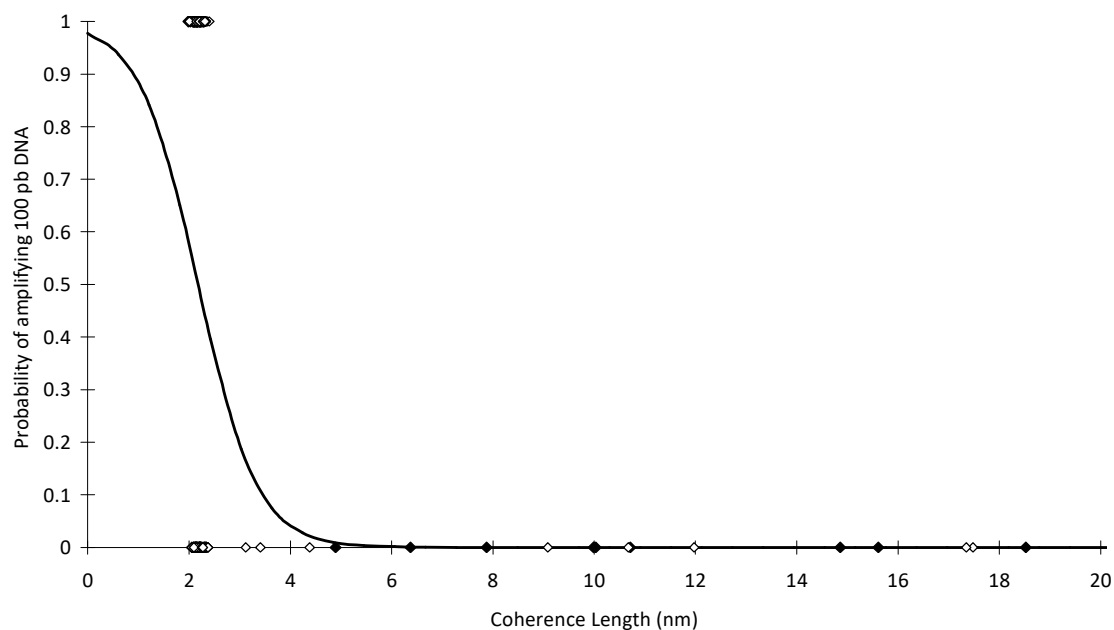


Figure A7.5: Relationship between the amplification of 100 bp DNA and CL of bHAP from heated bone (50-1000 °C). (◇) 2 hours and (◆) 4 hours. Logistic regression curve is a function of equation A7.5.

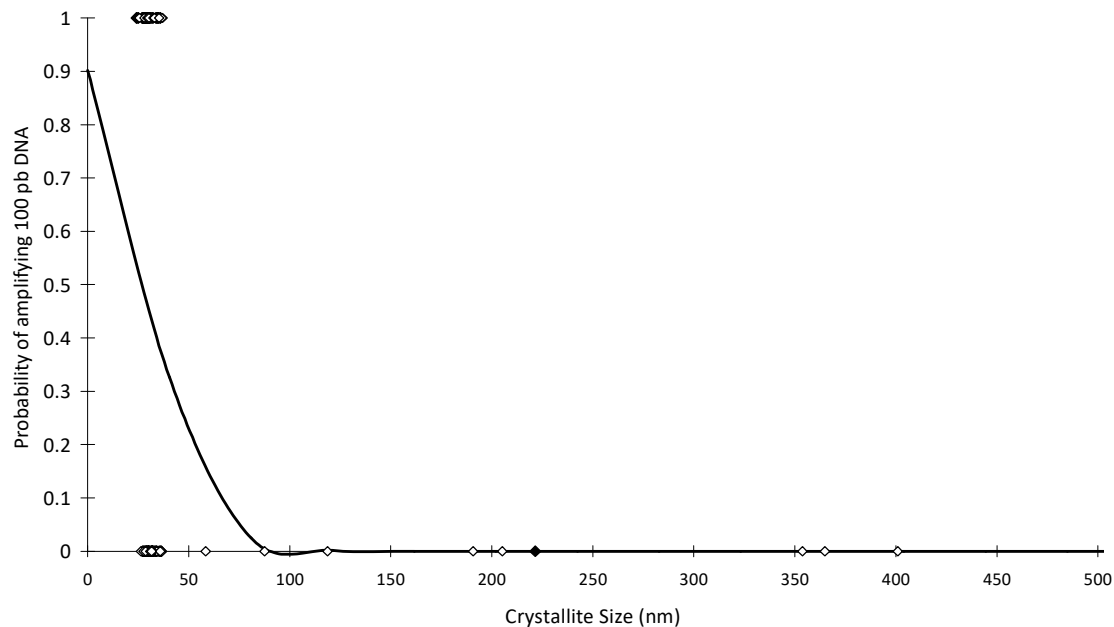


Figure A7.6: Relationship between the amplification of 100 bp DNA and crystallite size of bHAP from heated bone (50-1000 °C). (◇) 2 hours and (◆) 4 hours. Logistic regression curve is a function of equation A7.6.

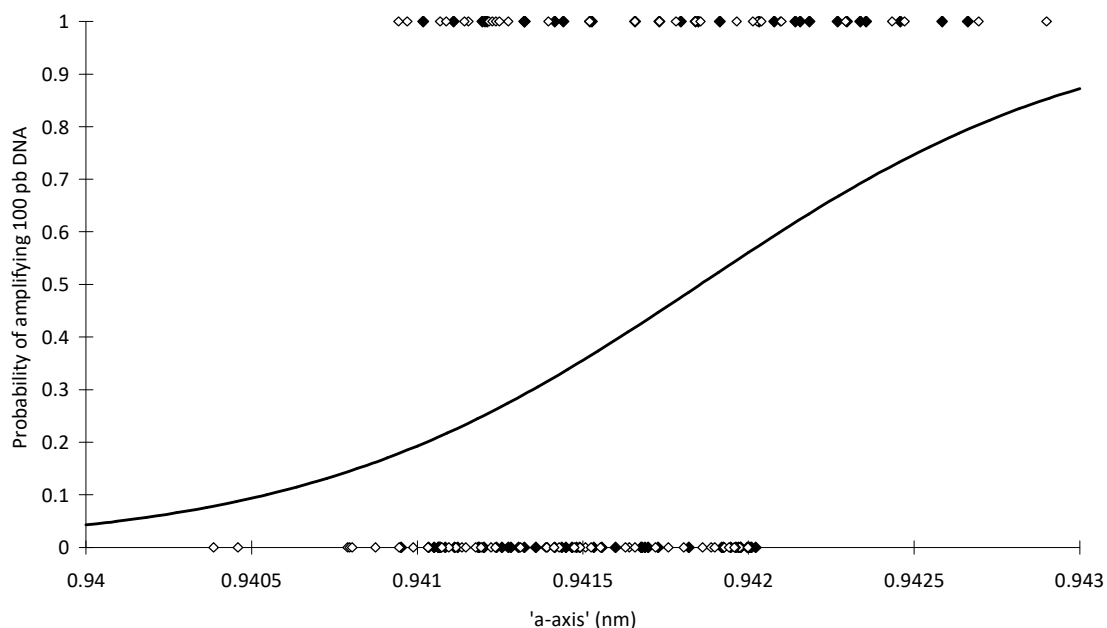


Figure A7.7: Relationship between the amplification of 100 bp DNA and the cell unit 'a-axis' parameter of bHAP from heated bone (50-1000 °C). (◊) 2 hours and (◆) 4 hours. Logistic regression curve is a function of equation A7.7.

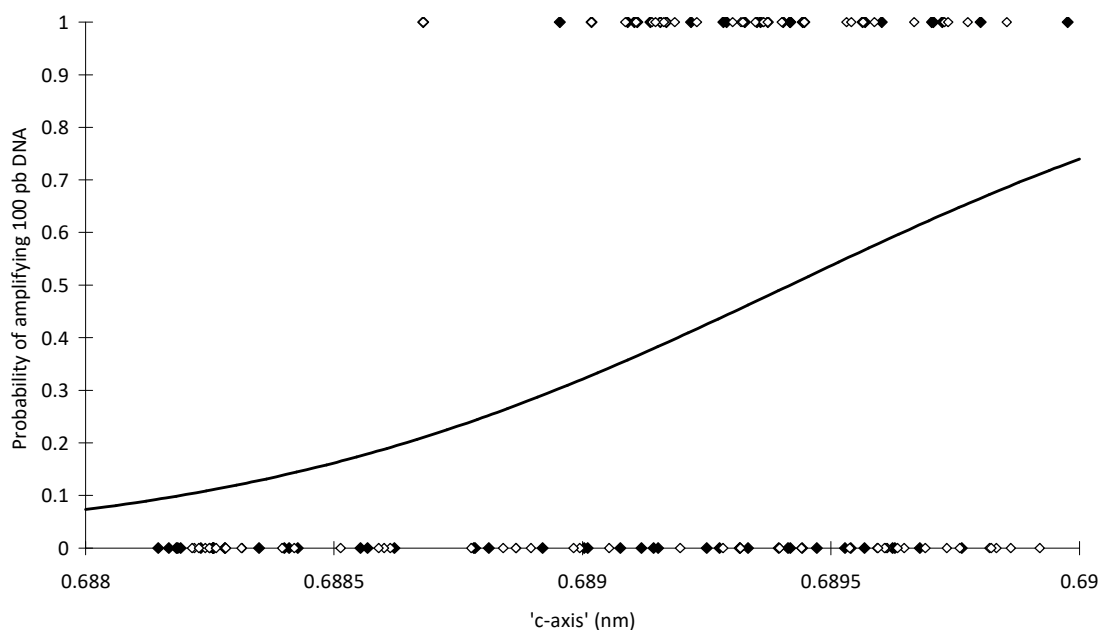


Figure A7.8: Relationship between the amplification of 100 bp DNA and the cell unit 'c-axis' parameter of bHAP from heated bone (50-1000 °C). (◊) 2 hours and (◆) 4 hours. Logistic regression curve is a function of equation A7.8.

A7.3 Logistic regression models predicting the successful amplification of ≥ 3 STR loci using different XRD parameters.

$$\text{Probability} = 1 / (1 + \exp[-(a - b \text{XRD parameter})])$$

Equation	XRD parameter	<i>a</i>	<i>b</i>
A7.9	CL	6.09	2.85
A7.10	Size	3.46	0.11
A7.11	'a'	-1892.58	-2009.32
A7.12	'c'	-1171.02	-1698.31

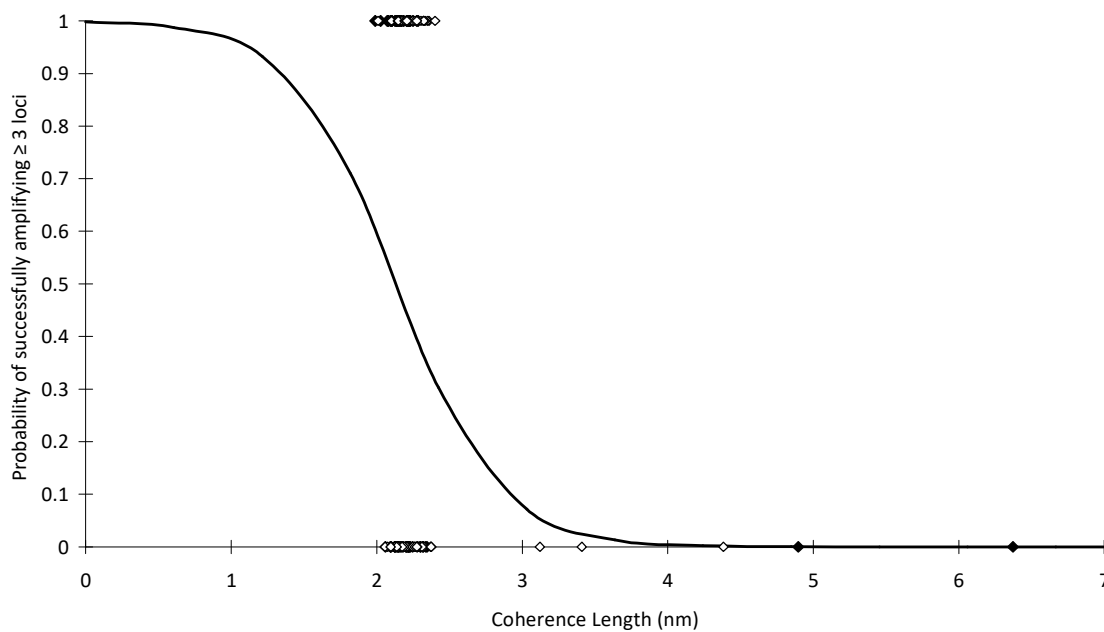


Figure A7.9: Relationship between the amplification of ≥ 3 STR loci and CL of bHAP from heated bone (50-1000 °C). (◇) 2 hours and (◆) 4 hours. Logistic regression curve is a function of equation A7.9.

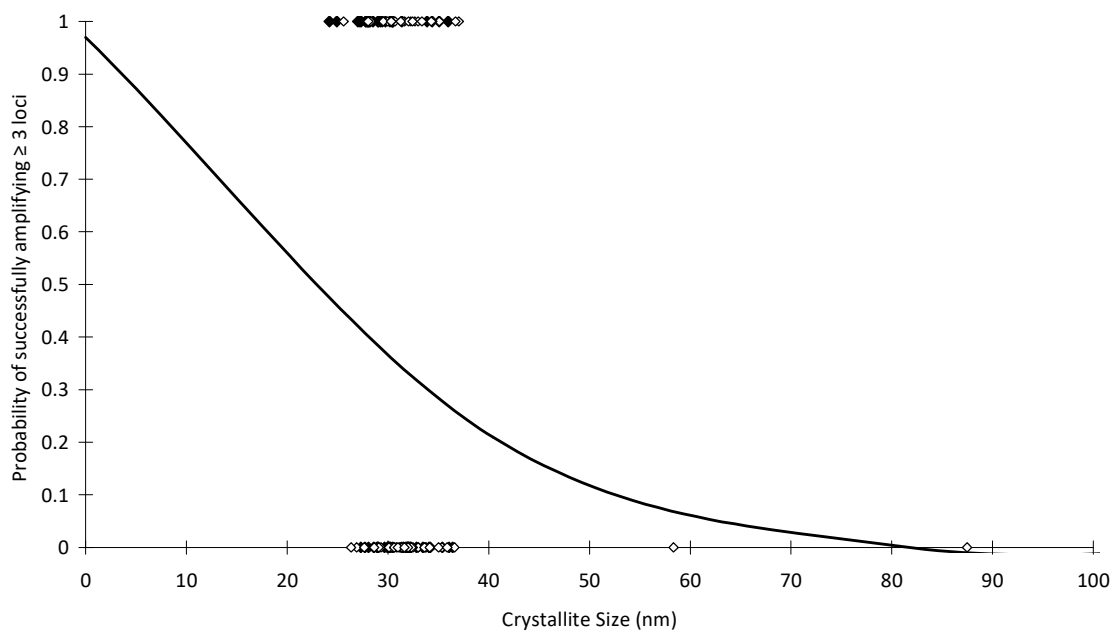


Figure A7.10: Relationship between the amplification of ≥ 3 STR loci and crystallite size of bHAP from heated bone (50-1000 °C). ($\diamond$) 2 hours and ($\blacklozenge$) 4 hours. Logistic regression curve is a function of equation A7.10.

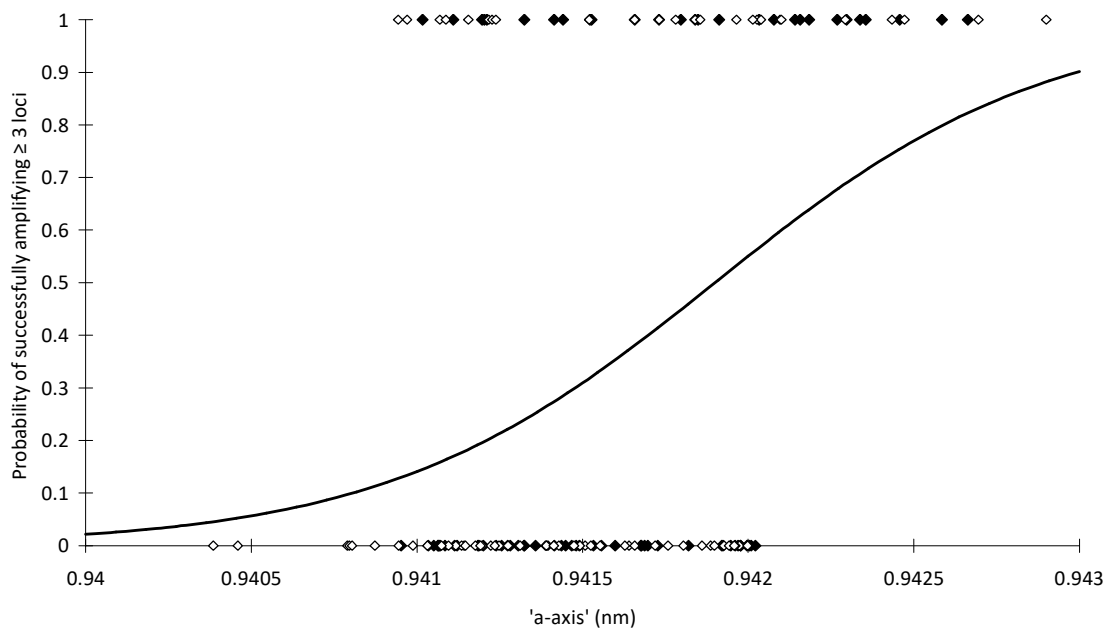


Figure A7.11: Relationship between the amplification of ≥ 3 STR loci and the cell unit 'a-axis' parameter of bHAP from heated bone (50-1000 °C). ($\diamond$) 2 hours and ($\blacklozenge$) 4 hours. Logistic regression curve is a function of equation A7.11.

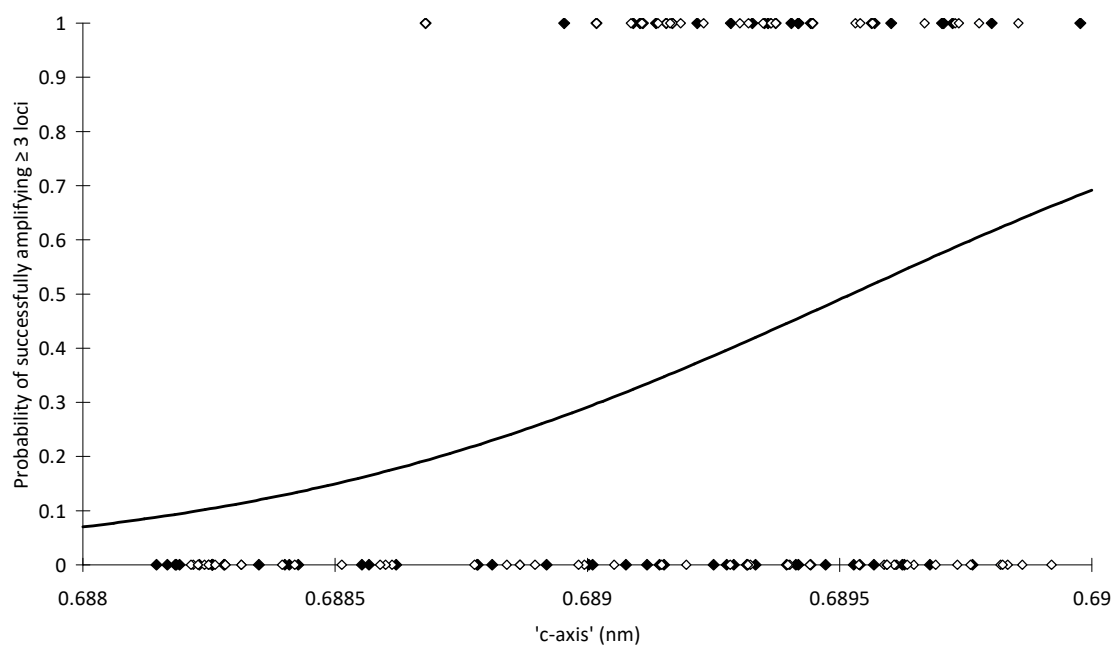


Figure A7.12: Relationship between the amplification of ≥ 3 STR loci and the cell unit 'c-axis' parameter of bHAP from heated bone (50-1000 °C). (◇) 2 hours and (◆) 4 hours. Logistic regression curve is a function of equation A7.12.

A7.4 Logistic regression models predicting the successful amplification of ≥ 7 STR loci using different XRD parameters.

$$\text{Probability} = 1 / (1 + \exp[-(a - b \text{XRD parameter})])$$

Equation	XRD parameter	<i>a</i>	<i>b</i>
A7.13	CL	6.51	3.09
A7.14	Size	3.07	0.10
A7.15	'a'	-2192.18	-2327.37
A7.16	'c'	-1243.78	-1803.72

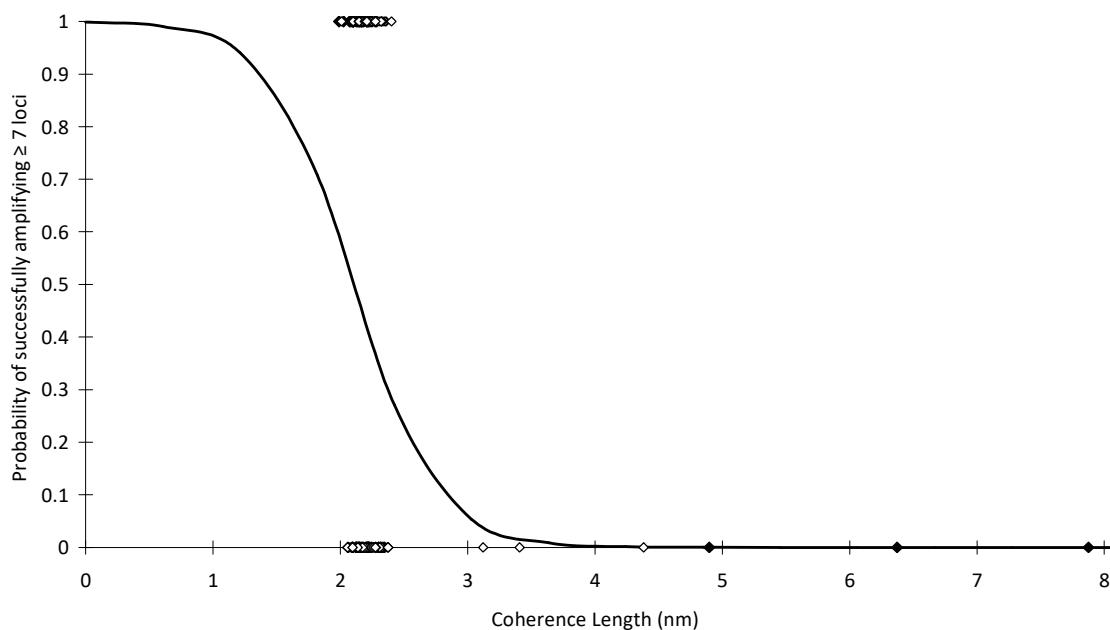


Figure A7.13: Relationship between the amplification of ≥ 7 STR loci and CL of bHAP from heated bone (50-1000 °C). (◇) 2 hours and (◆) 4 hours. Logistic regression curve is a function of equation A7.13.

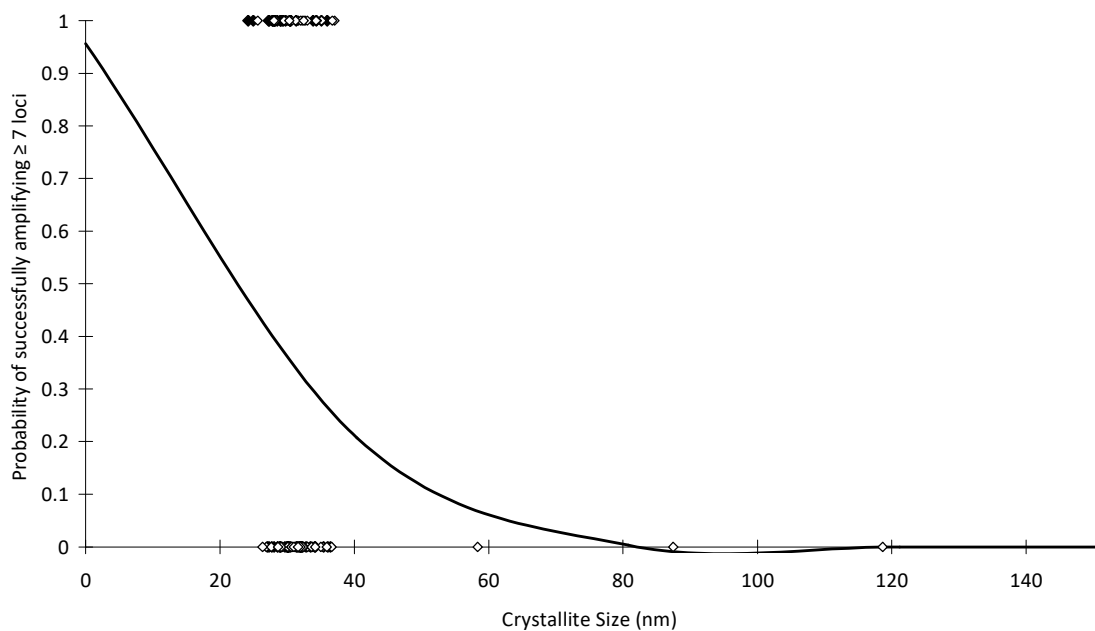


Figure A7.14: Relationship between the amplification of ≥ 7 STR loci and crystallite size of bHAP from heated bone (50-1000 °C). (◇) 2 hours and (◆) 4 hours. Logistic regression curve is a function of equation A7.14.

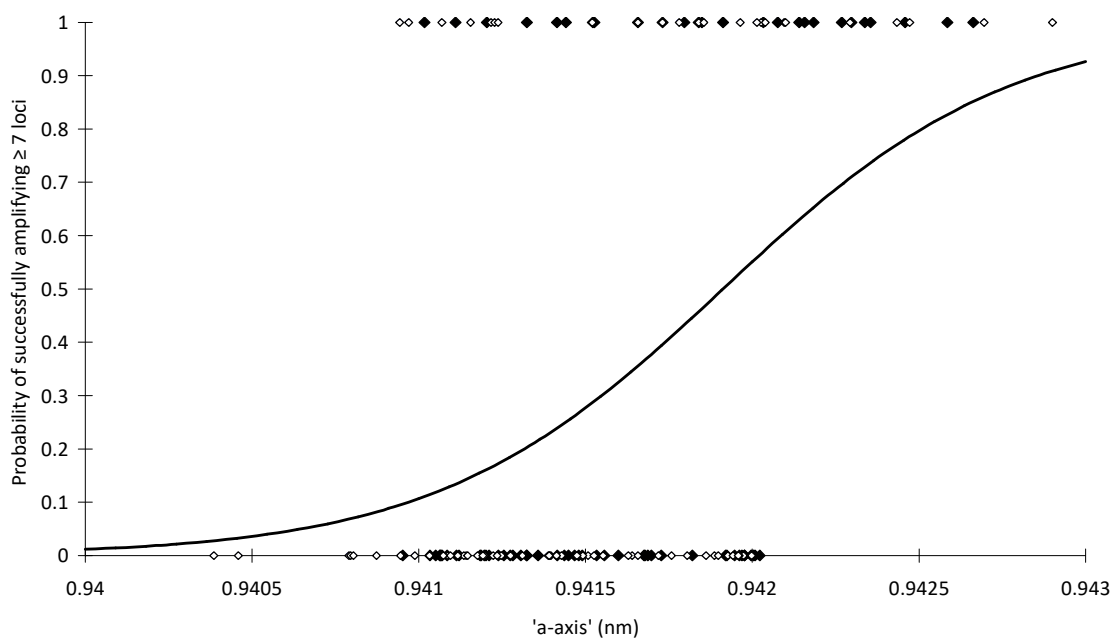


Figure A7.15: Relationship between the amplification of ≥ 7 STR loci and the cell unit 'a-axis' parameter of bHAP from heated bone (50-1000 °C). (◇) 2 hours and (◆) 4 hours. Logistic regression curve is a function of equation A7.15.

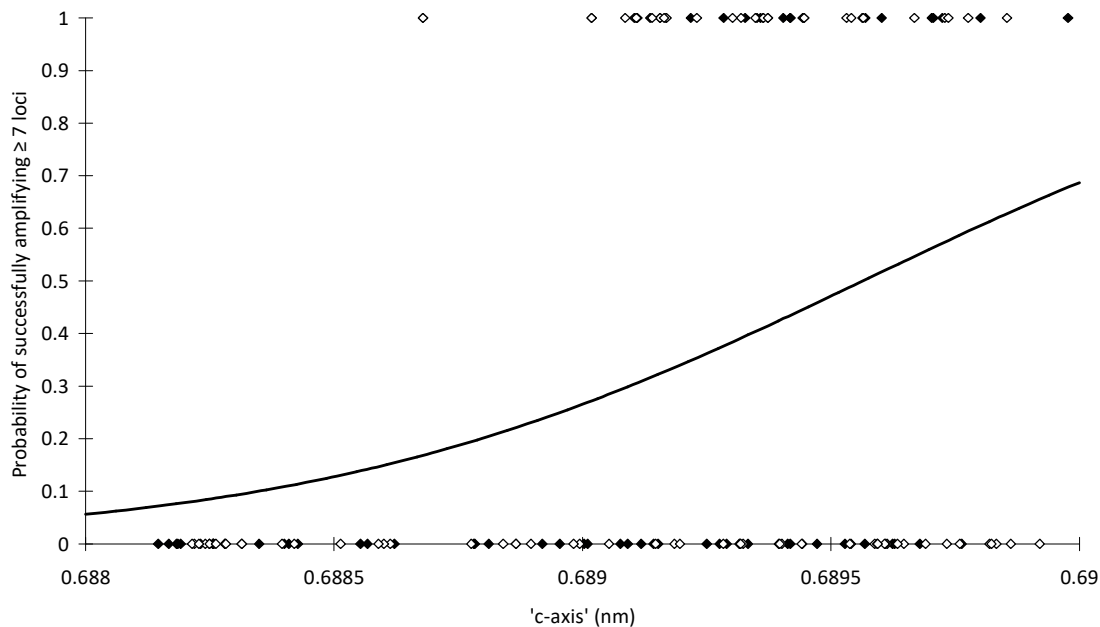


Figure A7.16: Relationship between the amplification of ≥ 7 STR loci and the cell unit 'c-axis' parameter of bHAP from heated bone (50-1000 °C). (◇) 2 hours and (◆) 4 hours. Logistic regression curve is a function of equation A7.16.

Appendix 8

Equations A8.1 –A8.5: Logistic Regression functions of the different DNA targets by SF and APR

$$\text{Probability} = 1 / (1 + \exp[-(a-b\text{SF}+c\text{APR})])$$

Equation	DNA Target	<i>a</i>	<i>b</i>	<i>c</i>
A7.1	500 bp	47.3	65.12	26.81
A7.2	300 bp	11.33	65.32	13.25
A7.3	100 bp	54.63	63.6	28.37
A7.4	≥ 3	22.94	50.43	15.24
A7.5	≥ 7	2.09	61.62	9.23

$$\frac{f_1(0)}{f_2(1)} = |\Sigma_2|^{\frac{1}{2}} |\Sigma_1|^{-\frac{1}{2}} \exp \left[\left\{ \nu'(\Sigma_1^{-1} - \Sigma_2^{-1})\nu - 2\nu'(\Sigma_1^{-1}\mu_1 - \Sigma_2^{-1}\mu_2) + \mu_1'\Sigma_1^{-1}\mu_1 - \mu_2'\Sigma_2^{-1}\mu_2 \right\} \right]$$

Equation A8.6: Multivariate discriminant function 500bp DNA by colour and APR

Σ₁ =	1.0668	-0.0496	-0.3148	0.0025	Σ₂ =	586.217	-53.1573	-41.1007	-0.1581
	-0.0496	0.049	-0.1537	0.0013		-53.1573	8.6007	12.5404	0.026
	-0.3148	-0.1537	2.1533	-0.0047		-41.1007	12.5404	70.1138	0.8619
	0.0025	0.0013	-0.0047	0.0005		-0.1581	0.026	0.8619	0.0217
μ₁ =	92.763	-0.1472	6.505	0.408	μ₂ =	74.2666	2.2838	10.5607	0.1339

Equation A8.7: Multivariate discriminant function 300bp DNA by colour and APR

Σ₁ =	1.046	-0.0295	-0.5453	0.0027	Σ₂ =	596.5921	-53.1823	-42.7659	-0.3498
	-0.0295	0.0598	-0.2835	0.0023		-53.1823	8.6089	13.0598	0.0565
	-0.5453	-0.2835	3.8183	-0.0119		-42.7659	13.0598	72.6566	0.8963
	0.0027	0.0023	-0.0119	0.0006		-0.3498	0.0565	0.8963	0.0199
μ₁ =	92.7064	-0.1796	6.832	0.406	μ₂ =	73.5551	2.3973	10.5594	0.124

Equation A8.8: Multivariate discriminant function 100bp DNA by colour and APR

Σ₁ =	1.5175	0.0595	-3.0862	0.0087	Σ₂ =	623.4527	-52.7165	-56.4738	-0.8586
	0.0595	0.0873	-0.6423	0.0041		-52.7165	8.4483	15.9873	0.1457
	-3.0862	-0.6423	16.3525	-0.0419		-56.4738	15.9873	75.2143	0.8419
	0.0087	0.0041	-0.0419	0.0007		-0.8586	0.1457	0.8419	0.015
μ₁ =	92.4163	-0.2503	8.181	0.4012	μ₂ =	71.8491	2.698	10.0534	0.0992

Equation A8.9: Multivariate discriminant function ≥ 3 STR loci by colour and APR

$\Sigma_1 =$	1.0693	0.02	-0.8984	0.0044	$\Sigma_2 =$	607.279	-52.8223	-45.8191	-0.5577
	0.02	0.089	-0.5156	0.004		-52.8223	8.5029	13.8353	0.0938
	-0.8984	-0.5156	5.6003	-0.0261		-45.8191	13.8353	75.5512	0.9175
	0.0044	0.004	-0.0261	0.0007		-0.5577	0.0938	0.9175	0.0182
$\mu_1 =$	92.6307	-0.2307	7.2256	0.4031	$\mu_2 =$	72.7997	2.5334	10.4933	0.1139

Equation A8.10: Multivariate discriminant function ≥ 7 STR loci by colour and APR

$\Sigma_1 =$	1.0921	-0.0091	-0.6428	0.0027	$\Sigma_2 =$	599.4829	-53.1281	-42.7507	-0.3975
	-0.0091	0.0684	-0.3056	0.0028		-53.1281	8.5854	13.1189	0.0654
	-0.6428	-0.3056	3.3247	-0.0126		-42.7507	13.1189	73.576	0.9112
	0.0027	0.0028	-0.0126	0.0006		-0.3975	0.0654	0.9112	0.0195
$\mu_1 =$	92.6757	-0.1935	6.8598	0.4053	$\mu_2 =$	73.3775	2.4305	10.5827	0.1215

Equation A8.11: Multivariate discriminant function 500 bp by colour and SF

$\Sigma_1 =$	1.0668	-0.0496	-0.3148	-0.0205	$\Sigma_2 =$	586.217	-53.1573	-41.1007	13.8257
	-0.0496	0.049	-0.1537	-0.0058		-53.1573	8.6007	12.5404	-1.5145
	-0.3148	-0.1537	2.1533	0.04		-41.1007	12.5404	70.1138	-6.5789
	-0.0205	-0.0058	0.04	0.0042		13.8257	-1.5145	-6.5789	1.1006
$\mu_1 =$	92.763	-0.1472	6.505	2.6313	$\mu_2 =$	74.2666	2.2838	10.5607	3.5329

Equation A8.12: Multivariate discriminant function 300 bp by colour and SF

$\Sigma_1 =$	1.046	-0.0295	-0.5453	-0.0244	$\Sigma_2 =$	596.5921	-53.1823	-42.7659	14.9796
	-0.0295	0.0598	-0.2835	-0.0081		-53.1823	8.6089	13.0598	-1.6709
	-0.5453	-0.2835	3.8183	0.0602		-42.7659	13.0598	72.6566	-6.8392
	-0.0244	-0.0081	0.0602	0.0049		14.9796	-1.6709	-6.8392	1.1183
$\mu_1 =$	92.7064	-0.1796	6.832	2.6397	$\mu_2 =$	73.5551	2.3973	10.5594	3.5648

Equation A8.13: Multivariate discriminant function 100 bp by colour and SF

$\Sigma_1 =$	1.5175	0.0595	-3.0862	-0.0318	$\Sigma_2 =$	623.4527	-52.7165	-56.4738	18.1403
	0.0595	0.0873	-0.6423	-0.0097		-52.7165	8.4483	15.9873	-2.1311
	-3.0862	-0.6423	16.3525	0.1042		-56.4738	15.9873	75.2143	-7.0285
	-0.0318	-0.0097	0.1042	0.0045		18.1403	-2.1311	-7.0285	1.1455
$\mu_1 =$	92.4163	-0.2503	8.181	2.647	$\mu_2 =$	71.8491	2.698	10.0534	3.6516

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Equation A8.14: Multivariate discriminant function ≥ 3 STR loci by colour and SF

$\Sigma_1 =$	1.0693	0.02	-0.8984	-0.0253	$\Sigma_2 =$	607.279	-52.8223	-45.8191	16.3046
	0.02	0.089	-0.5156	-0.01		-52.8223	8.5029	13.8353	-1.8664
	-0.8984	-0.5156	5.6003	0.0729		-45.8191	13.8353	75.5512	-7.0655
	-0.0253	-0.01	0.0729	0.0047		16.3046	-1.8664	-7.0655	1.1319
$\mu_1 =$	92.6307	-0.2307	7.2256	2.6435	$\mu_2 =$	72.7997	2.5334	10.4933	3.6012

Equation A8.15: Multivariate discriminant function ≥ 7 STR loci by colour and SF

$\Sigma_1 =$	1.0921	-0.0091	-0.6428	-0.0257	$\Sigma_2 =$	599.4829	-53.1281	-42.7507	15.278
	-0.0091	0.0684	-0.3056	-0.0095		-53.1281	8.5854	13.1189	-1.7153
	-0.6428	-0.3056	3.3247	0.0685		-42.7507	13.1189	73.576	-6.9315
	-0.0257	-0.0095	0.0685	0.005		15.278	-1.7153	-6.9315	1.123
$\mu_1 =$	92.6757	-0.1935	6.8598	2.642	$\mu_2 =$	73.3775	2.4305	10.5827	3.573

Equation A8.16: Multivariate discriminant function 500 bp by colour, SF and APR

$\Sigma_1 =$	1.0668	-0.0496	-0.3148	-0.0205	-0.0007	$\Sigma_2 =$	586.217	-53.1573	-41.1007	13.8257	-0.10268
	-0.0496	0.049	-0.1537	-0.0058	0.0025		-53.1573	8.6007	12.5404	-1.5145	-0.1581
	-0.3148	-0.1537	2.1533	0.04	0.0013		-41.1007	12.5404	70.1138	-6.5789	0.026
	-0.0205	-0.0058	0.04	0.0042	-0.0047		13.8257	-1.5145	-6.5789	1.1006	0.8619
	-0.0007	0.0025	0.0013	-0.0047	0.0005		-0.10268	-0.1581	0.026	0.8619	0.0217
$\mu_1 =$	92.763	-0.1472	6.505	2.6313	0.408	$\mu_2 =$	74.2666	2.2838	10.5607	3.5329	0.1339

Equation A8.16: Multivariate discriminant function 300 bp by colour, SF and APR

$\Sigma_1 =$	1.046	-0.0295	-0.5453	-0.0244	-0.0008	$\Sigma_2 =$	596.5921	-53.1823	-42.7659	14.9796	-0.09849
	-0.0295	0.0598	-0.2835	-0.0081	0.0027		-53.1823	8.6089	13.0598	-1.6709	0.0027
	-0.5453	-0.2835	3.8183	0.0602	0.0023		-42.7659	13.0598	72.6566	-6.8392	0.0023
	-0.0244	-0.0081	0.0602	0.0049	-0.0119		14.9796	-1.6709	-6.8392	1.1183	-0.0119
	-0.0008	0.0027	0.0023	-0.0119	0.0006		-0.09849	-0.3498	0.0565	0.8963	0.0006
$\mu_1 =$	92.7064	-0.1796	6.832	2.6397	0.406	$\mu_2 =$	73.5551	2.3973	10.5594	3.5648	0.406

Equation A8.16: Multivariate discriminant function 100 bp by colour, SF and APR

$\Sigma_1 =$	1.5175	0.0595	-3.0862	-0.0318	-0.0009	$\Sigma_2 =$	623.4527	-52.7165	-56.4738	18.1403	-0.08441
	0.0595	0.0873	-0.6423	-0.0097	0.0087		-52.7165	8.4483	15.9873	-2.1311	-0.8586
	-3.0862	-0.6423	16.3525	0.1042	0.0041		-56.4738	15.9873	75.2143	-7.0285	0.1457
	-0.0318	-0.0097	0.1042	0.0045	-0.0419		18.1403	-2.1311	-7.0285	1.1455	0.8419
	-0.0009	0.0087	0.0041	-0.0419	0.0007		-0.08441	-0.8586	0.1457	0.8419	0.015
$\mu_1 =$	92.4163	-0.2503	8.181	2.647	0.4012	$\mu_2 =$	71.8491	2.698	10.0534	3.6516	0.0992

Equation A8.15: Multivariate discriminant function ≥ 3 STR loci by colour, SF and APR

$\Sigma_1 =$	1.0693	0.02	-0.8984	-0.0253	-0.0009	$\Sigma_2 =$	607.279	-52.8223	-45.8191	16.3046	-0.09204
	0.02	0.089	-0.5156	-0.01	0.0044		-52.8223	8.5029	13.8353	-1.8664	-0.5577
	-0.8984	-0.5156	5.6003	0.0729	0.004		-45.8191	13.8353	75.5512	-7.0655	0.0938
	-0.0253	-0.01	0.0729	0.0047	-0.0261		16.3046	-1.8664	-7.0655	1.1319	0.9175
	-0.0009	0.0044	0.004	-0.0261	0.0007		-0.09204	-0.5577	0.0938	0.9175	0.0182
$\mu_1 =$	92.6307	-0.2307	7.2256	2.6435	0.4031	$\mu_2 =$	72.7997	2.5334	10.4933	3.6012	0.1139

Equation A8.15: Multivariate discriminant function ≥ 7 STR loci by colour, SF and APR

$\Sigma_1 =$	1.0921	-0.0091	-0.6428	-0.0257	-0.0009	$\Sigma_2 =$	599.4829	-53.1281	-42.7507	15.278	-0.09749
	-0.0091	0.0684	-0.3056	-0.0095	0.0027		-53.1281	8.5854	13.1189	-1.7153	-0.3975
	-0.6428	-0.3056	3.3247	0.0685	0.0028		-42.7507	13.1189	73.576	-6.9315	0.0654
	-0.0257	-0.0095	0.0685	0.005	-0.0126		15.278	-1.7153	-6.9315	1.123	0.9112
	-0.0009	0.0027	0.0028	-0.0126	0.0006		-0.09749	-0.3975	0.0654	0.9112	0.0195
$\mu_1 =$	92.6757	-0.1935	6.8598	2.642	0.4053	$\mu_2 =$	73.3775	2.4305	10.5827	3.573	0.1215

Development of New Tools for Forensic Analysis of DNA from Compromised Bone

Table A8.1: Calculated probability of successfully 500 bp amplification from heated bone samples using different analytical techniques. A: Two hours, B: Four Hours.

A

Temp.	PCR	APR	SF	Colour	Colour+APR	Colour+SF	Colour+APR+SF
39	1.00	0.91	0.97	1.00	1.00	1.00	1.00
39	1.00	0.91	0.93	1.00	1.00	1.00	1.00
39	1.00	0.91	0.93	1.00	1.00	1.00	1.00
39	1.00	0.87	0.92	1.00	1.00	1.00	1.00
39	1.00	0.90	0.91	1.00	1.00	1.00	1.00
39	1.00	0.82	0.88	0.98	1.00	1.00	1.00
39	1.00	0.95	0.88	1.00	1.00	1.00	1.00
39	1.00	0.90	0.71	1.00	1.00	1.00	1.00
39	1.00	0.89	0.55	1.00	1.00	1.00	1.00
50	1.00	0.84	1.00	1.00	1.00	1.00	1.00
50	1.00	0.99	0.98	1.00	1.00	1.00	1.00
50	1.00	0.55	0.85	0.99	1.00	1.00	1.00
100	1.00	1.00	0.96	1.00	1.00	1.00	1.00
100	1.00	0.79	0.95	1.00	1.00	1.00	1.00
100	1.00	0.96	0.94	1.00	1.00	1.00	1.00
150	1.00	0.86	1.00	1.00	1.00	1.00	1.00
150	1.00	0.82	0.98	1.00	1.00	1.00	1.00
150	1.00	0.97	0.91	1.00	1.00	1.00	1.00
170	1.00	0.78	0.91	0.45	0.99	0.99	1.00
170	1.00	0.45	0.33	1.00	1.00	1.00	1.00
170	1.00	0.95	0.18	0.99	1.00	1.00	1.00
180	1.00	0.85	0.43	0.92	1.00	1.00	1.00
180	1.00	0.98	0.21	0.99	1.00	1.00	1.00
180	0.00	0.74	0.08	0.99	1.00	1.00	1.00
190	0.00	0.61	0.78	0.00	0.00	0.00	0.00
190	0.00	0.31	0.72	0.00	0.00	0.00	0.00
190	0.00	0.98	0.05	1.00	1.00	1.00	1.00
200	0.00	0.85	0.48	0.00	0.00	0.00	0.00
200	0.00	0.37	0.47	0.00	0.00	0.00	0.00
200	0.00	0.55	0.45	0.00	0.00	0.00	0.00
210	0.00	0.00	0.46	0.00	0.00	0.00	0.00
210	0.00	0.01	0.24	0.00	0.00	0.00	0.00
210	0.00	0.01	0.22	0.00	0.00	0.00	0.00
220	0.00	0.03	0.46	0.00	0.00	0.00	0.00
220	0.00	0.13	0.37	0.00	0.00	0.00	0.00
220	0.00	0.04	0.26	0.00	0.00	0.00	0.00
250	0.00	0.00	0.13	0.00	0.00	0.00	0.00
250	0.00	0.00	0.03	0.00	0.00	0.00	0.00
250	0.00	0.00	0.01	0.00	0.00	0.00	0.00
300	0.00	0.00	0.13	0.00	0.00	0.00	0.00
300	0.00	0.00	0.01	0.00	0.00	0.00	0.00
300	0.00	0.00	0.01	0.00	0.00	0.00	0.00
350	0.00	0.00	0.01	0.00	0.00	0.00	0.00
350	0.00	0.00	0.00	0.00	0.00	0.00	0.00
350	0.00	0.00	0.00	0.00	0.00	0.00	0.00
400	0.00	0.00	0.02	0.00	0.00	0.00	0.00
400	0.00	0.00	0.01	0.00	0.00	0.00	0.00
400	0.00	0.00	0.01	0.00	0.00	0.00	0.00
450	0.00	0.00	0.00	0.00	0.00	0.00	0.00
450	0.00	0.00	0.00	0.00	0.00	0.00	0.00
450	0.00	0.00	0.00	0.00	0.00	0.00	0.00
500	0.00	0.00	0.00	0.00	0.00	0.00	0.00
500	0.00	0.00	0.00	0.00	0.00	0.00	0.00
500	0.00	0.00	0.00	0.00	0.00	0.00	0.00
550	0.00	0.00	0.00	0.00	0.00	0.00	0.00
550	0.00	0.00	0.00	0.00	0.00	0.00	0.00
550	0.00	0.00	0.00	0.00	0.00	0.00	0.00
600	0.00	0.00	0.00	0.00	0.00	0.00	0.00
600	0.00	0.00	0.00	0.00	0.00	0.00	0.00
600	0.00	0.00	0.00	0.00	0.00	0.00	0.00
650	0.00	0.00	0.00	0.00	0.00	0.00	0.00
650	0.00	0.00	0.00	0.76	0.00	0.00	0.00
650	0.00	0.00	0.00	0.95	0.00	0.00	0.00
700	0.00	0.00	0.00	0.00	0.00	0.00	0.00
700	0.00	0.00	0.00	0.01	0.00	0.00	0.00
700	0.00	0.00	0.00	0.00	0.00	0.00	0.00
800	0.00	0.00	0.00	0.00	0.00	0.00	0.00
800	0.00	0.00	0.00	0.00	0.00	0.00	0.00
800	0.00	0.00	0.00	0.00	0.00	0.00	0.00
900	0.00	0.00	0.00	0.00	0.00	0.00	0.00
900	0.00	0.00	0.00	0.00	0.00	0.00	0.00
900	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1000	0.00	0.00	0.00	0.02	0.00	0.00	0.00
1000	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1000	0.00	0.00	0.00	0.00	0.00	0.00	0.00

B

Temp.	PCR	APR	SF	Colour	Colour+APR	Colour+SF	Colour+APR+SF
39	1.00	0.96	1.00	1.00	1.00	1.00	1.00
39	1.00	1.00	1.00	1.00	1.00	1.00	1.00
39	1.00	0.97	0.95	1.00	1.00	1.00	1.00
39	1.00	0.96	0.99	1.00	1.00	1.00	1.00
39	1.00	0.95	0.98	1.00	1.00	1.00	1.00
39	1.00	0.75	0.71	1.00	1.00	1.00	1.00
39	1.00	0.78	0.10	1.00	1.00	1.00	1.00
39	1.00	0.48	0.48	0.29	1.00	1.00	1.00
39	1.00	0.65	0.95	1.00	1.00	1.00	1.00
50	1.00	0.98	0.65	1.00	1.00	1.00	1.00
50	1.00	0.88	0.91	1.00	1.00	1.00	1.00
50	1.00	0.55	0.75	1.00	1.00	1.00	1.00
100	1.00	0.88	0.90	1.00	1.00	1.00	1.00
100	1.00	0.73	0.88	0.99	1.00	1.00	1.00
100	1.00	0.99	0.98	0.99	1.00	1.00	1.00
140	1.00	0.67	0.56	0.99	1.00	1.00	1.00
140	1.00	0.74	0.86	1.00	1.00	1.00	1.00
140	1.00	0.99	0.92	1.00	1.00	1.00	1.00
150	1.00	0.39	0.25	1.00	1.00	1.00	1.00
150	1.00	0.91	0.90	1.00	1.00	1.00	1.00
150	1.00	0.86	0.86	1.00	1.00	1.00	1.00
160	1.00	0.75	0.09	0.99	1.00	1.00	1.00
160	1.00	0.39	0.42	0.99	1.00	1.00	1.00
160	0.00	0.06	0.07	0.99	1.00	1.00	1.00
170	0.00	0.79	0.58	0.26	0.61	0.52	0.96
170	0.00	0.04	0.14	0.62	0.10	0.98	0.76
170	0.00	0.47	0.49	0.28	0.01	0.01	0.01
180	0.00	0.12	0.77	0.01	0.01	0.00	0.00
180	0.00	0.01	0.02	0.12	0.39	0.38	0.02
180	0.00	0.15	0.46	0.12	0.00	0.39	0.21
190	0.00	0.02	0.04	0.00	0.00	0.00	0.00
190	0.00	0.86	0.02	0.00	0.00	0.00	0.00
190	0.00	0.05	0.01	0.00	0.00	0.00	0.00
200	0.00	0.19	0.29	0.00	0.00	0.00	0.00
200	0.00	0.01	0.30	0.00	0.00	0.00	0.00
200	0.00	0.54	0.41	0.00	0.00	0.00	0.00
250	0.00	0.00	0.71	0.00	0.00	0.00	0.00
250	0.00	0.00	0.20	0.00	0.00	0.00	0.00
250	0.00	0.00	0.02	0.00	0.00	0.00	0.00
300	0.00	0.00	0.18	0.00	0.00	0.00	0.00
300	0.00	0.00	0.05	0.00	0.00	0.00	0.00
300	0.00	0.00	0.32	0.00	0.00	0.00	0.00
350	0.00	0.00	0.00	0.00	0.00	0.00	0.00
350	0.00	0.00	0.00	0.00	0.00	0.00	0.00
350	0.00	0.00	0.00	0.00	0.00	0.00	0.00
400	0.00	0.00	0.01	0.00	0.00	0.00	0.00
400	0.00	0.00	0.00	0.00	0.00	0.00	0.00
400	0.00	0.00	0.02	0.00	0.00	0.00	0.00
450	0.00	0.00	0.00	0.00	0.00	0.00	0.00
450	0.00	0.00	0.07	0.00	0.00	0.00	0.00
450	0.00	0.00	0.00	0.00	0.00	0.00	0.00
500	0.00	0.00	0.00	0.00	0.00	0.00	0.00
500	0.00	0.00	0.00	0.00	0.00	0.00	0.00
500	0.00	0.00	0.00	0.00	0.00	0.00	0.00
550	0.00	0.00	0.00	0.00	0.00	0.00	0.00
550	0.00	0.00	0.00	0.00	0.00	0.00	0.00
550	0.00	0.00	0.00	0.00	0.00	0.00	0.00
600	0.00	0.00	0.00	0.00	0.00	0.00	0.00
600	0.00	0.00	0.00	0.00	0.00	0.00	0.00
600	0.00	0.00	0.00	0.00	0.00	0.00	0.00
650	0.00	0.00	0.00	0.97	0.00	0.00	0.00
650	0.00	0.00	0.00	1.00	0.00	0.00	0.00
650	0.00	0.00	0.00	1.00	0.00	0.00	0.00
700	0.00	0.00	0.00	0.00	0.00	0.00	0.00
700	0.00	0.00	0.00	0.04	0.00	0.00	0.00
700	0.00	0.00	0.00	0.04	0.00	0.00	0.00
800	0.00	0.00	0.00	0.03	0.00	0.00	0.00
800	0.00	0.00	0.00	0.00	0.00	0.00	0.00
800	0.00	0.00	0.00	0.00	0.00	0.00	0.00
900	0.00	0.00	0.00	0.00	0.00	0.00	0.00
900	0.00	0.00	0.00	0.00	0.00	0.00	0.00
900	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1000	0.00	0.00	0.00	0.01	0.00	0.00	0.00
1000	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1000	0.00	0.00	0.00	0.00	0.00	0.00	0.00

N.B. PCR represents the ‘true’ result. Grey and black boxes denotes positive or negative classified samples.

Development of New Tools for Forensic Analysis of DNA from Compromised Bone

Table A8.2: Calculated probability of successfully 300 bp amplification from heated bone samples using different analytical techniques. A: Two hours, B: Four Hours.

A

Temp°C	PCR	APR	SF	Colour	Colour+APR	Colour+SF	Colour+APR+SF
39	1	0.94	0.95	1.00	1.00	1.00	1.00
39	1	0.94	0.91	1.00	1.00	1.00	1.00
39	1	0.94	0.91	1.00	1.00	1.00	1.00
39	1	0.92	0.91	1.00	1.00	1.00	1.00
39	1	0.94	0.89	1.00	1.00	1.00	1.00
39	1	0.89	0.87	0.97	1.00	1.00	1.00
39	1	0.97	0.87	1.00	1.00	1.00	1.00
39	1	0.94	0.74	1.00	1.00	1.00	1.00
39	1	0.93	0.62	1.00	1.00	1.00	1.00
50	1	0.89	0.99	1.00	1.00	1.00	1.00
50	1	1.00	0.96	1.00	1.00	1.00	1.00
50	1	0.68	0.84	0.99	1.00	1.00	1.00
100	1	1.00	0.94	1.00	1.00	1.00	1.00
100	1	0.87	0.94	1.00	1.00	1.00	1.00
100	1	0.97	0.92	1.00	1.00	1.00	1.00
150	1	0.91	0.99	1.00	1.00	1.00	1.00
150	1	0.89	0.97	1.00	1.00	1.00	1.00
150	1	0.98	0.90	1.00	1.00	1.00	1.00
170	1	0.85	0.90	0.75	1.00	1.00	1.00
170	1	0.58	0.44	1.00	1.00	1.00	1.00
170	1	0.97	0.29	1.00	1.00	1.00	1.00
180	1	0.91	0.52	0.99	1.00	1.00	1.00
180	1	0.99	0.33	1.00	1.00	1.00	1.00
180	1	0.83	0.16	1.00	1.00	1.00	1.00
190	0	0.73	0.79	0.01	0.07	0.01	0.05
190	1	0.43	0.75	0.00	0.01	0.00	0.00
190	1	0.99	0.12	1.00	1.00	1.00	1.00
200	0	0.91	0.57	0.00	0.00	0.00	0.00
200	0	0.50	0.56	0.00	0.00	0.00	0.00
200	0	0.68	0.54	0.00	0.00	0.00	0.00
210	0	0.01	0.55	0.00	0.00	0.00	0.00
210	0	0.01	0.36	0.00	0.00	0.00	0.00
210	0	0.01	0.34	0.00	0.00	0.00	0.00
220	0	0.05	0.55	0.00	0.00	0.00	0.00
220	0	0.20	0.48	0.00	0.00	0.00	0.00
220	0	0.06	0.38	0.00	0.00	0.00	0.00
250	0	0.00	0.24	0.00	0.00	0.00	0.00
250	0	0.00	0.08	0.00	0.00	0.00	0.00
250	0	0.00	0.02	0.00	0.00	0.00	0.00
300	0	0.00	0.24	0.00	0.00	0.00	0.00
300	0	0.00	0.05	0.00	0.00	0.00	0.00
300	0	0.00	0.03	0.00	0.00	0.00	0.00
350	0	0.00	0.04	0.00	0.00	0.00	0.00
350	0	0.00	0.02	0.00	0.00	0.00	0.00
350	0	0.00	0.02	0.00	0.00	0.00	0.00
400	0	0.00	0.06	0.00	0.00	0.00	0.00
400	0	0.00	0.05	0.00	0.00	0.00	0.00
400	0	0.00	0.04	0.00	0.00	0.00	0.00
450	0	0.00	0.00	0.00	0.00	0.00	0.00
450	0	0.00	0.00	0.00	0.00	0.00	0.00
450	0	0.00	0.00	0.00	0.00	0.00	0.00
500	0	0.00	0.00	0.00	0.00	0.00	0.00
500	0	0.00	0.00	0.00	0.00	0.00	0.00
500	0	0.00	0.00	0.00	0.00	0.00	0.00
550	0	0.00	0.00	0.00	0.00	0.00	0.00
550	0	0.00	0.00	0.00	0.00	0.00	0.00
550	0	0.00	0.00	0.00	0.00	0.00	0.00
600	0	0.00	0.00	0.00	0.00	0.00	0.00
600	0	0.00	0.00	0.00	0.00	0.00	0.00
600	0	0.00	0.00	0.00	0.00	0.00	0.00
650	0	0.00	0.00	0.00	0.00	0.00	0.00
650	0	0.00	0.00	0.85	0.00	0.00	0.00
650	0	0.00	0.00	0.98	0.00	0.00	0.00
700	0	0.00	0.00	0.01	0.00	0.00	0.00
700	0	0.00	0.00	0.08	0.00	0.00	0.00
700	0	0.00	0.00	0.02	0.00	0.00	0.00
800	0	0.00	0.00	0.00	0.00	0.00	0.00
800	0	0.00	0.00	0.00	0.00	0.00	0.00
800	0	0.00	0.00	0.00	0.00	0.00	0.00
900	0	0.00	0.00	0.01	0.00	0.00	0.00
900	0	0.00	0.00	0.00	0.00	0.00	0.00
900	0	0.00	0.00	0.01	0.00	0.00	0.00
1000	0	0.00	0.00	0.05	0.00	0.00	0.00
1000	0	0.00	0.00	0.02	0.00	0.00	0.00
1000	0	0.00	0.00	0.01	0.00	0.00	0.00

B

Temp°C	PCR	APR	SF	Colour	Colour+APR	Colour+SF	Colour+APR+SF
39	1	0.98	0.99	1.00	1.00	1.00	1.00
39	1	1.00	1.00	1.00	1.00	1.00	1.00
39	1	0.98	0.94	1.00	1.00	1.00	1.00
39	1	0.98	0.98	1.00	1.00	1.00	1.00
39	1	0.97	0.97	1.00	1.00	1.00	1.00
39	1	0.83	0.74	1.00	1.00	1.00	1.00
39	1	0.86	0.19	1.00	1.00	1.00	1.00
39	1	0.61	0.57	0.26	1.00	1.00	1.00
39	1	0.75	0.93	1.00	1.00	1.00	1.00
50	1	0.99	0.69	1.00	1.00	1.00	1.00
50	1	0.93	0.90	1.00	1.00	1.00	1.00
50	1	0.68	0.76	1.00	1.00	1.00	1.00
100	1	0.93	0.89	1.00	1.00	1.00	1.00
100	1	0.82	0.87	0.99	1.00	1.00	1.00
100	1	1.00	0.97	0.99	1.00	1.00	1.00
140	1	0.78	0.62	0.99	1.00	1.00	1.00
140	1	0.83	0.86	1.00	1.00	1.00	1.00
140	1	0.99	0.90	1.00	1.00	1.00	1.00
150	1	0.52	0.37	1.00	1.00	1.00	1.00
150	1	0.94	0.88	1.00	1.00	1.00	1.00
150	1	0.91	0.86	1.00	1.00	1.00	1.00
160	1	0.83	0.18	1.00	1.00	1.00	1.00
160	1	0.52	0.52	1.00	1.00	1.00	1.00
160	1	0.11	0.16	0.99	1.00	1.00	1.00
170	0	0.86	0.64	0.90	1.00	0.98	1.00
170	0	0.06	0.24	0.97	0.99	1.00	1.00
170	0	0.60	0.57	0.87	1.00	0.99	1.00
180	0	0.19	0.78	0.76	0.94	0.87	0.87
180	0	0.02	0.07	0.90	0.71	1.00	0.97
180	0	0.23	0.55	0.77	0.98	0.97	0.99
190	0	0.03	0.11	0.00	0.00	0.00	0.00
190	0	0.91	0.05	0.00	0.00	0.00	0.00
190	0	0.08	0.04	0.00	0.00	0.00	0.00
200	0	0.29	0.40	0.00	0.00	0.00	0.00
200	0	0.02	0.41	0.00	0.00	0.00	0.00
200	0	0.66	0.51	0.00	0.00	0.00	0.00
250	0	0.00	0.74	0.00	0.00	0.00	0.00
250	0	0.00	0.32	0.00	0.00	0.00	0.00
250	0	0.00	0.06	0.00	0.00	0.00	0.00
300	0	0.00	0.29	0.00	0.00	0.00	0.00
300	0	0.00	0.12	0.00	0.00	0.00	0.00
300	0	0.00	0.43	0.00	0.00	0.00	0.00
350	0	0.00	0.02	0.00	0.00	0.00	0.00
350	0	0.00	0.00	0.00	0.00	0.00	0.00
350	0	0.00	0.00	0.00	0.00	0.00	0.00
400	0	0.00	0.03	0.00	0.00	0.00	0.00
400	0	0.00	0.00	0.00	0.00	0.00	0.00
400	0	0.00	0.05	0.00	0.00	0.00	0.00
450	0	0.00	0.00	0.00	0.00	0.00	0.00
450	0	0.00	0.15	0.00	0.00	0.00	0.00
450	0	0.00	0.00	0.00	0.00	0.00	0.00
500	0	0.00	0.00	0.00	0.00	0.00	0.00
500	0	0.00	0.00	0.00	0.00	0.00	0.00
500	0	0.00	0.00	0.00	0.00	0.00	0.00
550	0	0.00	0.00	0.00	0.00	0.00	0.00
550	0	0.00	0.00	0.00	0.00	0.00	0.00
550	0	0.00	0.00	0.00	0.00	0.00	0.00
600	0	0.00	0.00	0.00	0.00	0.00	0.00
600	0	0.00	0.00	0.00	0.00	0.00	0.00
600	0	0.00	0.00	0.00	0.00	0.00	0.00
650	0	0.00	0.00	0.14	0.00	0.00	0.00
650	0	0.00	0.00	0.02	0.00	0.00	0.00
650	0	0.00	0.00	0.02	0.00	0.00	0.00
700	0	0.00	0.00	0.21	0.00	0.00	0.00
700	0	0.00	0.00	0.19	0.00	0.00	0.00
700	0	0.00	0.00	0.15	0.00	0.00	0.00
800	0	0.00	0.00	0.00	0.00	0.00	0.00
800	0	0.00	0.00	0.00	0.00	0.00	0.00
800	0	0.00	0.00	0.00	0.00	0.00	0.00
900	0	0.00	0.00	0.00	0.00	0.00	0.00
900	0	0.00	0.00	0.00	0.00	0.00	0.00
900	0	0.00	0.00	0.00	0.00	0.00	0.00
1000	0	0.00	0.00	0.00	0.00	0.00	0.00
1000	0	0.00	0.00	0.00	0.00	0.00	0.00
1000	0	0.00	0.00	0.00	0.00	0.00	0.00

N.B. PCR represents the 'true' result. Grey and black boxes denotes positive or negative classified samples.

Development of New Tools for Forensic Analysis of DNA from Compromised Bone

Table A8.3: Calculated probability of successfully 100 bp amplification from heated bone samples using different analytical techniques. A: Two hours, B: Four Hours.

A								B							
Temp°C	PCR	APR	SF	Colour	Colour+APR	Colour+SF	Colour+APR+SF	Temp°C	PCR	APR	SF	Colour	Colour+APR	Colour+SF	Colour+APR+SF
39	1	0.99	0.99	1.00	1.00	1.00	1.00	39	1	0.99	1.00	1.00	1.00	1.00	1.00
39	1	0.99	0.98	0.99	1.00	1.00	1.00	39	1	1.00	1.00	1.00	1.00	1.00	1.00
39	1	0.99	0.98	1.00	1.00	1.00	1.00	39	1	1.00	0.99	1.00	1.00	1.00	1.00
39	1	0.98	0.98	1.00	1.00	1.00	1.00	39	1	1.00	1.00	1.00	1.00	1.00	1.00
39	1	0.99	0.97	0.99	1.00	1.00	1.00	39	1	0.99	0.99	1.00	1.00	1.00	1.00
39	1	0.97	0.96	0.98	1.00	1.00	1.00	39	1	0.96	0.90	1.00	1.00	1.00	1.00
39	1	0.99	0.96	0.99	1.00	1.00	1.00	39	1	0.97	0.29	1.00	1.00	1.00	1.00
39	1	0.99	0.90	1.00	1.00	1.00	1.00	39	1	0.89	0.77	0.46	1.00	1.00	1.00
39	1	0.99	0.81	1.00	1.00	1.00	1.00	39	1	0.94	0.98	1.00	1.00	1.00	1.00
50	1	0.98	1.00	0.99	1.00	1.00	1.00	50	1	1.00	0.86	1.00	1.00	1.00	1.00
50	1	1.00	0.99	1.00	1.00	1.00	1.00	50	1	0.98	0.97	1.00	1.00	1.00	1.00
50	1	0.91	0.95	0.96	1.00	1.00	1.00	50	1	0.91	0.91	0.99	1.00	1.00	1.00
100	1	1.00	0.99	0.99	1.00	1.00	1.00	100	1	0.98	0.97	1.00	1.00	1.00	1.00
100	1	0.97	0.99	1.00	1.00	1.00	1.00	100	1	0.96	0.96	0.97	1.00	1.00	1.00
100	1	0.99	0.98	1.00	1.00	1.00	1.00	100	1	1.00	1.00	0.97	1.00	1.00	1.00
150	1	0.98	1.00	1.00	1.00	1.00	1.00	140	1	0.95	0.81	0.99	1.00	1.00	1.00
150	1	0.97	1.00	0.99	1.00	1.00	1.00	140	1	0.96	0.96	1.00	1.00	1.00	1.00
150	1	1.00	0.97	1.00	1.00	1.00	1.00	140	1	1.00	0.97	0.99	1.00	1.00	1.00
170	1	0.97	0.97	0.94	1.00	1.00	1.00	150	1	0.84	0.55	1.00	1.00	1.00	1.00
170	1	0.87	0.64	1.00	1.00	1.00	1.00	150	1	0.99	0.97	1.00	1.00	1.00	1.00
170	1	0.99	0.44	1.00	1.00	1.00	1.00	150	1	0.98	0.96	1.00	1.00	1.00	1.00
180	1	0.98	0.72	1.00	1.00	1.00	1.00	160	1	0.96	0.26	1.00	1.00	1.00	1.00
180	1	1.00	0.49	1.00	1.00	1.00	1.00	160	1	0.85	0.72	1.00	1.00	1.00	1.00
180	1	0.96	0.23	1.00	1.00	1.00	1.00	160	1	0.38	0.23	0.99	1.00	1.00	1.00
190	1	0.93	0.92	1.00	1.00	1.00	1.00	170	1	0.97	0.83	0.99	1.00	1.00	1.00
190	1	0.79	0.90	1.00	1.00	1.00	1.00	170	1	0.26	0.37	0.99	1.00	1.00	1.00
190	1	1.00	0.16	1.00	1.00	1.00	1.00	170	1	0.88	0.77	0.97	1.00	1.00	1.00
200	1	0.98	0.77	0.50	0.84	0.88	0.98	180	1	0.55	0.92	1.00	1.00	1.00	1.00
200	1	0.84	0.76	0.66	0.88	0.94	0.98	180	0	0.09	0.09	1.00	1.00	1.00	1.00
200	1	0.91	0.74	0.92	0.99	0.99	1.00	180	1	0.60	0.75	0.97	1.00	1.00	1.00
210	0	0.03	0.75	0.00	0.00	0.00	0.00	190	0	0.13	0.14	0.98	0.86	1.00	0.99
210	0	0.07	0.53	0.00	0.00	0.00	0.00	190	0	0.98	0.06	0.86	1.00	0.91	1.00
210	0	0.07	0.51	0.00	0.00	0.00	0.00	190	0	0.29	0.05	0.99	0.99	0.99	1.00
220	0	0.21	0.75	0.00	0.00	0.00	0.00	200	0	0.67	0.59	0.00	0.00	0.00	0.00
220	0	0.56	0.68	0.00	0.00	0.00	0.00	200	0	0.09	0.60	0.00	0.00	0.00	0.00
220	0	0.26	0.56	0.00	0.00	0.00	0.00	200	0	0.91	0.71	0.00	0.00	0.00	0.00
250	0	0.00	0.36	0.00	0.00	0.00	0.00	250	0	0.00	0.89	0.00	0.00	0.00	0.00
250	0	0.00	0.11	0.00	0.00	0.00	0.00	250	0	0.00	0.48	0.00	0.00	0.00	0.00
250	0	0.00	0.02	0.00	0.00	0.00	0.00	250	0	0.00	0.08	0.00	0.00	0.00	0.00
300	0	0.00	0.36	0.00	0.00	0.00	0.00	300	0	0.00	0.44	0.00	0.00	0.00	0.00
300	0	0.00	0.05	0.00	0.00	0.00	0.00	300	0	0.00	0.17	0.00	0.00	0.00	0.00
300	0	0.00	0.03	0.00	0.00	0.00	0.00	300	0	0.00	0.63	0.00	0.00	0.00	0.00
350	0	0.00	0.04	0.00	0.00	0.00	0.00	350	0	0.00	0.01	0.00	0.00	0.00	0.00
350	0	0.00	0.02	0.00	0.00	0.00	0.00	350	0	0.00	0.00	0.00	0.00	0.00	0.00
350	0	0.00	0.02	0.00	0.00	0.00	0.00	350	0	0.00	0.00	0.00	0.00	0.00	0.00
400	0	0.00	0.08	0.00	0.00	0.00	0.00	400	0	0.00	0.03	0.00	0.00	0.00	0.00
400	0	0.00	0.05	0.00	0.00	0.00	0.00	400	0	0.00	0.00	0.00	0.00	0.00	0.00
400	0	0.00	0.05	0.00	0.00	0.00	0.00	400	0	0.00	0.06	0.00	0.00	0.00	0.00
450	0	0.00	0.00	0.00	0.00	0.00	0.00	450	0	0.00	0.00	0.00	0.00	0.00	0.00
450	0	0.00	0.00	0.00	0.00	0.00	0.00	450	0	0.00	0.22	0.00	0.00	0.00	0.00
450	0	0.00	0.00	0.00	0.00	0.00	0.00	450	0	0.00	0.00	0.00	0.00	0.00	0.00
500	0	0.00	0.00	0.00	0.00	0.00	0.00	500	0	0.00	0.00	0.00	0.00	0.00	0.00
500	0	0.00	0.00	0.00	0.00	0.00	0.00	500	0	0.00	0.00	0.00	0.00	0.00	0.00
500	0	0.00	0.00	0.00	0.00	0.00	0.00	500	0	0.00	0.00	0.00	0.00	0.00	0.00
550	0	0.00	0.00	0.00	0.00	0.00	0.00	550	0	0.00	0.00	0.00	0.00	0.00	0.00
550	0	0.00	0.00	0.00	0.00	0.00	0.00	550	0	0.00	0.00	0.00	0.00	0.00	0.00
550	0	0.00	0.00	0.00	0.00	0.00	0.00	550	0	0.00	0.00	0.00	0.00	0.00	0.00
600	0	0.00	0.00	0.00	0.00	0.00	0.00	600	0	0.00	0.00	0.00	0.00	0.00	0.00
600	0	0.00	0.00	0.00	0.00	0.00	0.00	600	0	0.00	0.00	0.00	0.00	0.00	0.00
650	0	0.00	0.00	0.00	0.00	0.00	0.00	650	0	0.00	0.00	0.51	0.00	0.00	0.00
650	0	0.00	0.00	0.69	0.00	0.00	0.00	650	0	0.00	0.00	0.16	0.00	0.00	0.00
650	0	0.00	0.00	0.98	0.00	0.00	0.00	650	0	0.00	0.00	0.14	0.00	0.00	0.00
700	0	0.00	0.00	0.11	0.00	0.00	0.00	700	0	0.00	0.00	0.64	0.00	0.00	0.00
700	0	0.00	0.00	0.67	0.00	0.00	0.00	700	0	0.00	0.00	0.64	0.00	0.00	0.00
700	0	0.00	0.00	0.15	0.00	0.00	0.00	700	0	0.00	0.00	0.60	0.00	0.00	0.00
800	0	0.00	0.00	0.02	0.00	0.00	0.00	800	0	0.00	0.00	0.11	0.00	0.00	0.00
800	0	0.00	0.00	0.01	0.00	0.00	0.00	800	0	0.00	0.00	0.11	0.00	0.00	0.00
800	0	0.00	0.00	0.01	0.00	0.00	0.00	800	0	0.00	0.00	0.33	0.00	0.00	0.00
900	0	0.00	0.00	0.05	0.00	0.00	0.00	900	0	0.00	0.00	0.35	0.00	0.00	0.00
900	0	0.00	0.00	0.01	0.00	0.00	0.00	900	0	0.00	0.00	0.15	0.00	0.00	0.00
900	0	0.00	0.00	0.10	0.00	0.00	0.00	900	0	0.00	0.00	0.46	0.00	0.00	0.00
1000	0	0.00	0.00	0.19	0.00	0.00	0.00	1000	0	0.00	0.00	0.41	0.00	0.00	0.00
1000	0	0.00	0.00	0.07	0.00	0.00	0.00	1000	0	0.00	0.00	0.15	0.00	0.00	0.00
1000	0	0.00	0.00	0.04	0.00	0.00	0.00	1000	0	0.00	0.00	0.25	0.00	0.00	0.00

N.B. PCR represents the 'true' result. Grey and black boxes denotes positive or negative classified samples.

Development of New Tools for Forensic Analysis of DNA from Compromised Bone

Table A8.4: Calculated probability of successfully amplifying ≥ 3 STR loci from heated bone samples using different analytical techniques. A: Two hours, B: Four Hours.

A								B							
Temp°C	PCR	APR	SF	Colour	Colour+APR	Colour+SF	Colour+APR+SF	Temp°C	PCR	APR	SF	Colour	Colour+APR	Colour+SF	Colour+APR+SF
39	1	0.95	0.97	1.00	1.00	1.00	1.00	39	1	0.98	0.99	1.00	1.00	1.00	1.00
39	1	0.95	0.94	1.00	1.00	1.00	1.00	39	1	1.00	1.00	1.00	1.00	1.00	1.00
39	1	0.95	0.94	1.00	1.00	1.00	1.00	39	1	0.98	0.96	1.00	1.00	1.00	1.00
39	1	0.93	0.94	1.00	1.00	1.00	1.00	39	1	0.98	0.99	1.00	1.00	1.00	1.00
39	1	0.95	0.93	1.00	1.00	1.00	1.00	39	1	0.97	0.98	1.00	1.00	1.00	1.00
39	1	0.91	0.91	0.97	1.00	1.00	1.00	39	1	0.88	0.81	1.00	1.00	1.00	1.00
39	1	0.97	0.91	1.00	1.00	1.00	1.00	39	1	0.89	0.24	1.00	1.00	1.00	1.00
39	1	0.95	0.81	1.00	1.00	1.00	1.00	39	1	0.74	0.65	0.24	1.00	1.00	1.00
39	1	0.94	0.70	1.00	1.00	1.00	1.00	39	1	0.83	0.96	1.00	1.00	1.00	1.00
50	1	0.92	1.00	0.99	1.00	1.00	1.00	50	1	0.98	0.76	1.00	1.00	1.00	1.00
50	1	0.99	0.98	1.00	1.00	1.00	1.00	50	1	0.94	0.93	1.00	1.00	1.00	1.00
50	1	0.78	0.89	0.98	1.00	1.00	1.00	50	1	0.78	0.83	1.00	1.00	1.00	1.00
100	1	1.00	0.96	0.99	1.00	1.00	1.00	100	1	0.94	0.93	1.00	1.00	1.00	1.00
100	1	0.90	0.96	1.00	1.00	1.00	1.00	100	1	0.87	0.91	0.99	1.00	1.00	1.00
100	1	0.97	0.95	1.00	1.00	1.00	1.00	100	1	0.99	0.98	0.99	1.00	1.00	1.00
150	1	0.93	0.99	1.00	1.00	1.00	1.00	140	1	0.84	0.70	0.99	1.00	1.00	1.00
150	1	0.91	0.98	1.00	1.00	1.00	1.00	140	1	0.87	0.90	1.00	1.00	1.00	1.00
150	1	0.98	0.93	1.00	1.00	1.00	1.00	140	1	0.99	0.93	0.99	1.00	1.00	1.00
170	1	0.89	0.93	0.72	1.00	1.00	1.00	150	1	0.67	0.44	1.00	1.00	1.00	1.00
170	1	0.72	0.53	1.00	1.00	1.00	1.00	150	1	0.95	0.92	1.00	1.00	1.00	1.00
170	1	0.97	0.35	1.00	1.00	1.00	1.00	150	1	0.93	0.90	1.00	1.00	1.00	1.00
180	1	0.93	0.60	1.00	1.00	1.00	1.00	160	1	0.88	0.22	1.00	1.00	1.00	1.00
180	1	0.99	0.40	1.00	1.00	1.00	1.00	160	1	0.68	0.60	1.00	1.00	1.00	1.00
180	1	0.87	0.20	1.00	1.00	1.00	1.00	160	1	0.26	0.20	0.99	1.00	1.00	1.00
190	0	0.81	0.85	0.18	0.77	0.59	0.97	170	1	0.90	0.72	0.98	1.00	1.00	1.00
190	1	0.61	0.81	0.49	0.94	0.92	0.99	170	1	0.18	0.30	0.99	1.00	1.00	1.00
190	1	0.99	0.15	1.00	1.00	1.00	1.00	170	1	0.73	0.65	0.98	1.00	1.00	1.00
200	0	0.93	0.65	0.00	0.00	0.00	0.00	180	0	0.38	0.84	0.96	1.00	1.00	1.00
200	0	0.66	0.64	0.00	0.00	0.00	0.00	180	0	0.07	0.08	0.99	0.99	1.00	1.00
200	0	0.78	0.62	0.00	0.00	0.00	0.00	180	0	0.42	0.64	0.96	1.00	1.00	1.00
210	0	0.03	0.64	0.00	0.00	0.00	0.00	190	0	0.10	0.13	0.00	0.00	0.00	0.00
210	0	0.06	0.43	0.00	0.00	0.00	0.00	190	0	0.93	0.06	0.00	0.00	0.00	0.00
210	0	0.06	0.41	0.00	0.00	0.00	0.00	190	0	0.20	0.05	0.00	0.00	0.00	0.00
220	0	0.15	0.63	0.00	0.00	0.00	0.00	200	0	0.48	0.48	0.00	0.00	0.00	0.00
220	0	0.39	0.56	0.00	0.00	0.00	0.00	200	0	0.07	0.49	0.00	0.00	0.00	0.00
220	0	0.18	0.46	0.00	0.00	0.00	0.00	200	0	0.77	0.60	0.00	0.00	0.00	0.00
250	0	0.00	0.29	0.00	0.00	0.00	0.00	250	0	0.00	0.80	0.00	0.00	0.00	0.00
250	0	0.00	0.10	0.00	0.00	0.00	0.00	250	0	0.00	0.39	0.00	0.00	0.00	0.00
250	0	0.00	0.02	0.00	0.00	0.00	0.00	250	0	0.01	0.08	0.00	0.00	0.00	0.00
300	0	0.00	0.29	0.00	0.00	0.00	0.00	300	0	0.00	0.36	0.00	0.00	0.00	0.00
300	0	0.00	0.05	0.00	0.00	0.00	0.00	300	0	0.00	0.15	0.00	0.00	0.00	0.00
300	0	0.00	0.03	0.00	0.00	0.00	0.00	300	0	0.00	0.51	0.00	0.00	0.00	0.00
350	0	0.00	0.05	0.00	0.00	0.00	0.00	350	0	0.00	0.02	0.00	0.00	0.00	0.00
350	0	0.00	0.03	0.00	0.00	0.00	0.00	350	0	0.00	0.00	0.00	0.00	0.00	0.00
350	0	0.00	0.02	0.00	0.00	0.00	0.00	350	0	0.00	0.00	0.00	0.00	0.00	0.00
400	0	0.00	0.08	0.00	0.00	0.00	0.00	400	0	0.00	0.03	0.00	0.00	0.00	0.00
400	0	0.00	0.05	0.00	0.00	0.00	0.00	400	0	0.00	0.00	0.00	0.00	0.00	0.00
400	0	0.00	0.05	0.00	0.00	0.00	0.00	400	0	0.00	0.06	0.00	0.00	0.00	0.00
450	0	0.00	0.00	0.00	0.00	0.00	0.00	450	0	0.00	0.00	0.00	0.00	0.00	0.00
450	0	0.00	0.00	0.00	0.00	0.00	0.00	450	0	0.00	0.19	0.00	0.00	0.00	0.00
450	0	0.00	0.00	0.00	0.00	0.00	0.00	450	0	0.00	0.00	0.00	0.00	0.00	0.00
500	0	0.00	0.00	0.00	0.00	0.00	0.00	500	0	0.00	0.00	0.00	0.00	0.00	0.00
500	0	0.00	0.00	0.00	0.00	0.00	0.00	500	0	0.00	0.00	0.00	0.00	0.00	0.00
500	0	0.00	0.00	0.00	0.00	0.00	0.00	500	0	0.00	0.00	0.00	0.00	0.00	0.00
550	0	0.00	0.00	0.00	0.00	0.00	0.00	550	0	0.00	0.00	0.00	0.00	0.00	0.00
550	0	0.00	0.00	0.00	0.00	0.00	0.00	550	0	0.00	0.00	0.00	0.00	0.00	0.00
600	0	0.00	0.00	0.00	0.00	0.00	0.00	600	0	0.00	0.00	0.00	0.00	0.00	0.00
600	0	0.00	0.00	0.00	0.00	0.00	0.00	600	0	0.00	0.00	0.00	0.00	0.00	0.00
600	0	0.00	0.00	0.00	0.00	0.00	0.00	600	0	0.00	0.00	0.00	0.00	0.00	0.00
650	0	0.00	0.00	0.00	0.00	0.00	0.00	650	0	0.00	0.00	0.19	0.00	0.00	0.00
650	0	0.00	0.00	0.84	0.00	0.00	0.00	650	0	0.00	0.00	0.02	0.00	0.00	0.00
650	0	0.00	0.00	0.98	0.00	0.00	0.00	650	0	0.00	0.00	0.02	0.00	0.00	0.00
700	0	0.00	0.00	0.01	0.00	0.00	0.00	700	0	0.00	0.00	0.28	0.00	0.00	0.00
700	0	0.00	0.00	0.09	0.00	0.00	0.00	700	0	0.00	0.00	0.25	0.00	0.00	0.00
700	0	0.00	0.00	0.02	0.00	0.00	0.00	700	0	0.00	0.00	0.20	0.00	0.00	0.00
800	0	0.00	0.00	0.00	0.00	0.00	0.00	800	0	0.00	0.00	0.00	0.00	0.00	0.00
800	0	0.00	0.00	0.00	0.00	0.00	0.00	800	0	0.00	0.00	0.00	0.00	0.00	0.00
900	0	0.00	0.00	0.01	0.00	0.00	0.00	900	0	0.00	0.00	0.08	0.00	0.00	0.00
900	0	0.00	0.00	0.00	0.00	0.00	0.00	900	0	0.00	0.00	0.01	0.00	0.00	0.00
900	0	0.00	0.00	0.00	0.00	0.00	0.00	900	0	0.00	0.00	0.12	0.00	0.00	0.00
1000	0	0.00	0.00	0.06	0.00	0.00	0.00	1000	0	0.00	0.00	0.25	0.00	0.00	0.00
1000	0	0.00	0.00	0.02	0.00	0.00	0.00	1000	0	0.00	0.00	0.05	0.00	0.00	0.00
1000	0	0.00	0.00	0.01	0.00	0.00	0.00	1000	0	0.00	0.00	0.12	0.00	0.00	0.00

N.B. PCR represents the 'true' result. Grey and black boxes denotes positive or negative classified samples.

Development of New Tools for Forensic Analysis of DNA from Compromised Bone

Table A8.5: Calculated probability of successfully amplifying ≥ 7 STR loci from heated bone samples using different analytical techniques. A: Two hours, B: Four Hours.

A

Temp. °C	PCR	APR	SF	Colour	Colour+APR	Colour+SF	Colour+APR+SF
39	1	0.94	0.95	1.00	1.00	1.00	1.00
39	1	0.94	0.91	1.00	1.00	1.00	1.00
39	1	0.94	0.91	1.00	1.00	1.00	1.00
39	1	0.92	0.90	1.00	1.00	1.00	1.00
39	1	0.94	0.89	1.00	1.00	1.00	1.00
39	1	0.89	0.86	0.98	1.00	1.00	1.00
39	1	0.97	0.86	1.00	1.00	1.00	1.00
39	1	0.94	0.74	1.00	1.00	1.00	1.00
39	1	0.93	0.63	1.00	1.00	1.00	1.00
50	1	0.90	0.99	1.00	1.00	1.00	1.00
50	1	0.99	0.96	1.00	1.00	1.00	1.00
50	1	0.71	0.84	0.99	1.00	1.00	1.00
100	1	1.00	0.94	1.00	1.00	1.00	1.00
100	1	0.87	0.93	1.00	1.00	1.00	1.00
100	1	0.97	0.91	1.00	1.00	1.00	1.00
150	1	0.92	0.99	1.00	1.00	1.00	1.00
150	1	0.89	0.97	1.00	1.00	1.00	1.00
150	1	0.98	0.89	1.00	1.00	1.00	1.00
170	1	0.86	0.89	0.68	1.00	1.00	1.00
170	1	0.62	0.47	1.00	1.00	1.00	1.00
170	1	0.97	0.32	1.00	1.00	1.00	1.00
180	1	0.91	0.54	0.99	1.00	1.00	1.00
180	1	0.99	0.36	1.00	1.00	1.00	1.00
180	1	0.84	0.19	1.00	1.00	1.00	1.00
190	0	0.75	0.79	0.00	0.00	0.00	0.00
190	0	0.49	0.75	0.06	0.35	0.14	0.32
190	1	0.99	0.14	1.00	1.00	1.00	1.00
200	0	0.91	0.58	0.00	0.00	0.00	0.00
200	0	0.55	0.57	0.00	0.00	0.00	0.00
200	0	0.71	0.56	0.00	0.00	0.00	0.00
210	0	0.01	0.57	0.00	0.00	0.00	0.00
210	0	0.02	0.38	0.00	0.00	0.00	0.00
210	0	0.02	0.37	0.00	0.00	0.00	0.00
220	0	0.07	0.57	0.00	0.00	0.00	0.00
220	0	0.26	0.50	0.00	0.00	0.00	0.00
220	0	0.09	0.41	0.00	0.00	0.00	0.00
250	0	0.00	0.27	0.00	0.00	0.00	0.00
250	0	0.00	0.10	0.00	0.00	0.00	0.00
250	0	0.00	0.03	0.00	0.00	0.00	0.00
300	0	0.00	0.27	0.00	0.00	0.00	0.00
300	0	0.00	0.06	0.00	0.00	0.00	0.00
300	0	0.00	0.04	0.00	0.00	0.00	0.00
350	0	0.00	0.05	0.00	0.00	0.00	0.00
350	0	0.00	0.03	0.00	0.00	0.00	0.00
350	0	0.00	0.03	0.00	0.00	0.00	0.00
400	0	0.00	0.08	0.00	0.00	0.00	0.00
400	0	0.00	0.06	0.00	0.00	0.00	0.00
400	0	0.00	0.06	0.00	0.00	0.00	0.00
450	0	0.00	0.00	0.00	0.00	0.00	0.00
450	0	0.00	0.00	0.00	0.00	0.00	0.00
450	0	0.00	0.00	0.00	0.00	0.00	0.00
500	0	0.00	0.00	0.00	0.00	0.00	0.00
500	0	0.00	0.00	0.00	0.00	0.00	0.00
500	0	0.00	0.00	0.00	0.00	0.00	0.00
550	0	0.00	0.00	0.00	0.00	0.00	0.00
550	0	0.00	0.00	0.00	0.00	0.00	0.00
550	0	0.00	0.00	0.00	0.00	0.00	0.00
600	0	0.00	0.00	0.00	0.00	0.00	0.00
600	0	0.00	0.00	0.00	0.00	0.00	0.00
600	0	0.00	0.00	0.00	0.00	0.00	0.00
650	0	0.00	0.00	0.00	0.00	0.00	0.00
650	0	0.00	0.00	0.76	0.00	0.00	0.00
650	0	0.00	0.00	0.96	0.00	0.00	0.00
700	0	0.00	0.00	0.01	0.00	0.00	0.00
700	0	0.00	0.00	0.04	0.00	0.00	0.00
700	0	0.00	0.00	0.02	0.00	0.00	0.00
800	0	0.00	0.00	0.00	0.00	0.00	0.00
800	0	0.00	0.00	0.00	0.00	0.00	0.00
800	0	0.00	0.00	0.00	0.00	0.00	0.00
900	0	0.00	0.00	0.01	0.00	0.00	0.00
900	0	0.00	0.00	0.00	0.00	0.00	0.00
900	0	0.00	0.00	0.00	0.00	0.00	0.00
1000	0	0.00	0.00	0.06	0.00	0.00	0.00
1000	0	0.00	0.00	0.02	0.00	0.00	0.00
1000	0	0.00	0.00	0.01	0.00	0.00	0.00

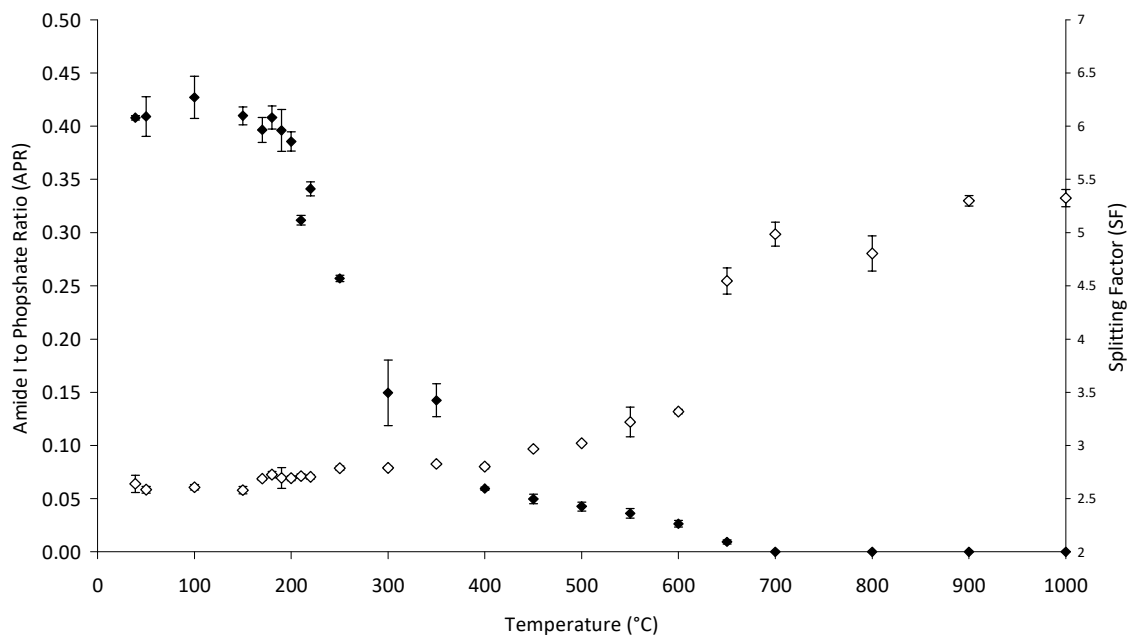
B

Temp. °C	PCR	APR	SF	Colour	Colour+APR	Colour+SF	Colour+APR+SF
39	1	0.97	0.99	1.00	1.00	1.00	1.00
39	1	1.00	1.00	1.00	1.00	1.00	1.00
39	1	0.98	0.93	1.00	1.00	1.00	1.00
39	1	0.98	0.98	1.00	1.00	1.00	1.00
39	1	0.97	0.97	1.00	1.00	1.00	1.00
39	1	0.85	0.74	1.00	1.00	1.00	1.00
39	1	0.87	0.22	1.00	1.00	1.00	1.00
39	1	0.65	0.58	0.21	1.00	1.00	1.00
39	1	0.78	0.93	1.00	1.00	1.00	1.00
50	1	0.98	0.70	1.00	1.00	1.00	1.00
50	1	0.93	0.90	1.00	1.00	1.00	1.00
50	1	0.71	0.77	1.00	1.00	1.00	1.00
100	1	0.93	0.89	1.00	1.00	1.00	1.00
100	1	0.83	0.86	0.99	1.00	1.00	1.00
100	1	1.00	0.97	0.99	1.00	1.00	1.00
140	1	0.79	0.63	0.98	1.00	1.00	1.00
140	1	0.84	0.85	1.00	1.00	1.00	1.00
140	1	0.99	0.90	1.00	1.00	1.00	1.00
150	1	0.56	0.39	1.00	1.00	1.00	1.00
150	1	0.94	0.88	1.00	1.00	1.00	1.00
150	1	0.91	0.85	1.00	1.00	1.00	1.00
160	1	0.84	0.21	1.00	1.00	1.00	1.00
160	1	0.57	0.54	1.00	1.00	1.00	1.00
160	1	0.14	0.18	0.99	1.00	1.00	1.00
170	1	0.87	0.65	0.88	1.00	0.95	1.00
170	1	0.09	0.27	0.96	0.95	1.00	1.00
170	0	0.64	0.59	0.96	1.00	1.00	1.00
180	0	0.24	0.78	0.62	0.89	0.72	0.67
180	0	0.03	0.09	0.94	0.85	1.00	0.99
180	0	0.28	0.57	0.86	0.99	0.99	1.00
190	0	0.04	0.13	0.00	0.00	0.00	0.00
190	0	0.91	0.06	0.00	0.00	0.00	0.00
190	0	0.11	0.06	0.00	0.00	0.00	0.00
200	0	0.34	0.43	0.00	0.00	0.00	0.00
200	0	0.03	0.44	0.00	0.00	0.00	0.00
200	0	0.69	0.53	0.00	0.00	0.00	0.00
250	0	0.00	0.74	0.00	0.00	0.00	0.00
250	0	0.00	0.35	0.00	0.00	0.00	0.00
250	0	0.00	0.08	0.00	0.00	0.00	0.00
300	0	0.00	0.32	0.00	0.00	0.00	0.00
300	0	0.00	0.14	0.00	0.00	0.00	0.00
300	0	0.00	0.46	0.00	0.00	0.00	0.00
350	0	0.00	0.02	0.00	0.00	0.00	0.00
350	0	0.00	0.00	0.00	0.00	0.00	0.00
350	0	0.00	0.00	0.00	0.00	0.00	0.00
400	0	0.00	0.04	0.00	0.00	0.00	0.00
400	0	0.00	0.01	0.00	0.00	0.00	0.00
400	0	0.00	0.06	0.00	0.00	0.00	0.00
450	0	0.00	0.00	0.00	0.00	0.00	0.00
450	0	0.00	0.18	0.00	0.00	0.00	0.00
450	0	0.00	0.00	0.00	0.00	0.00	0.00
500	0	0.00	0.00	0.00	0.00	0.00	0.00
500	0	0.00	0.00	0.00	0.00	0.00	0.00
500	0	0.00	0.00	0.00	0.00	0.00	0.00
550	0	0.00	0.00	0.00	0.00	0.00	0.00
550	0	0.00	0.00	0.00	0.00	0.00	0.00
550	0	0.00	0.00	0.00	0.00	0.00	0.00
600	0	0.00	0.00	0.00	0.00	0.00	0.00
600	0	0.00	0.00	0.00	0.00	0.00	0.00
600	0	0.00	0.00	0.00	0.00	0.00	0.00
650	0	0.00	0.00	0.12	0.00	0.00	0.00
650	0	0.00	0.00	0.02	0.00	0.00	0.00
650	0	0.00	0.00	0.01	0.00	0.00	0.00
700	0	0.00	0.00	0.18	0.00	0.00	0.00
700	0	0.00	0.00	0.16	0.00	0.00	0.00
700	0	0.00	0.00	0.12	0.00	0.00	0.00
800	0	0.00	0.00	0.00	0.00	0.00	0.00
800	0	0.00	0.00	0.00	0.00	0.00	0.00
800	0	0.00	0.00	0.00	0.00	0.00	0.00
900	0	0.00	0.00	0.04	0.00	0.00	0.00
900	0	0.00	0.00	0.01	0.00	0.00	0.00
900	0	0.00	0.00	0.06	0.00	0.00	0.00
1000	0	0.00	0.00	0.13	0.00	0.00	0.00
1000	0	0.00	0.00	0.03	0.00	0.00	0.00
1000	0	0.00	0.00	0.06	0.00	0.00	0.00

N.B. PCR represents the ‘true’ result. Grey and black boxes denotes positive or negative classified samples.

Appendix 9

A



B

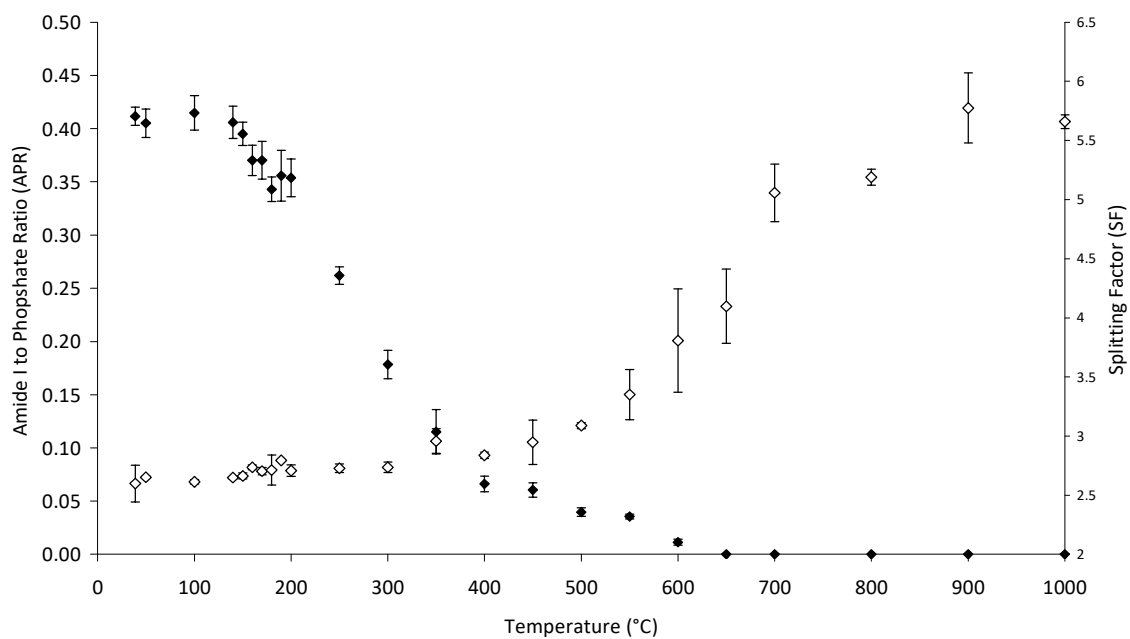
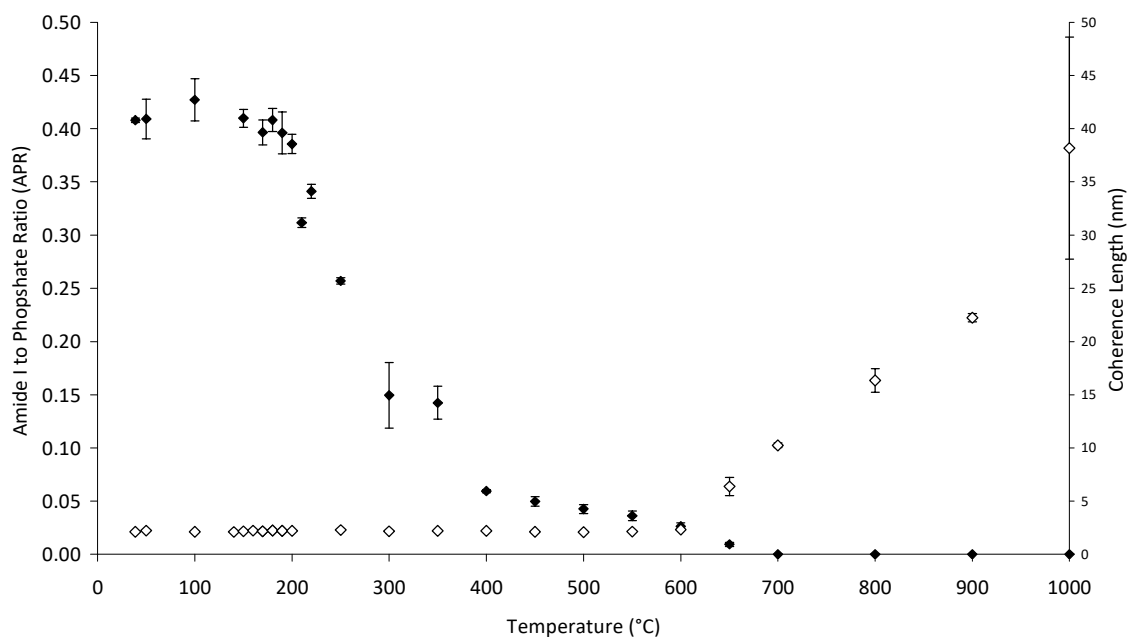


Figure A9.1: Relationship between the APR and SF of bHAP from bone heated at various temperatures. A: two hours, B: four hours. (♦) APR (◇) SF. Errors bars (± 1 standard error).

A



B

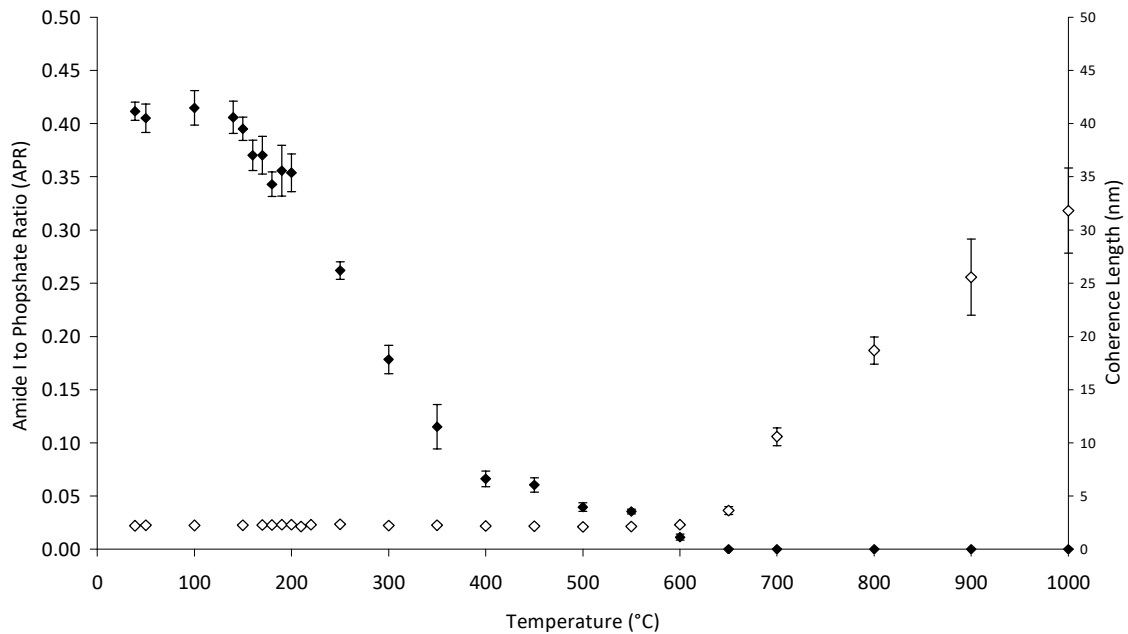
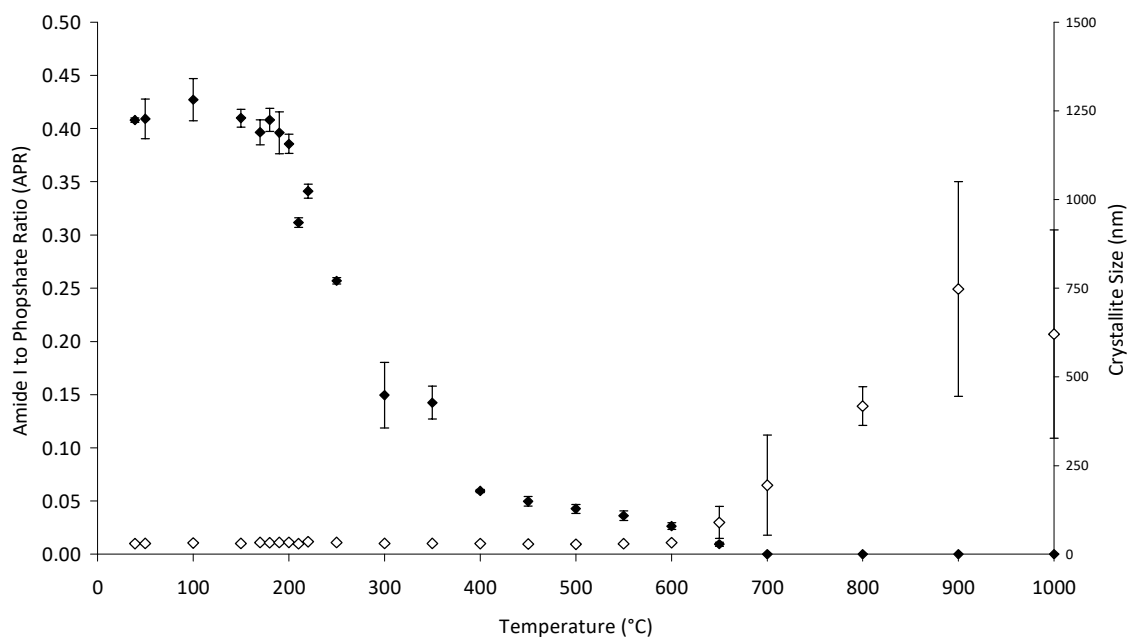


Figure A9.2: Relationship between the APR and CL of bHAP from bone heated at various temperatures. A: Two hours, B: four hours. (◆) APR (◇) CL. Errors bars (± 1 standard error).

A



B

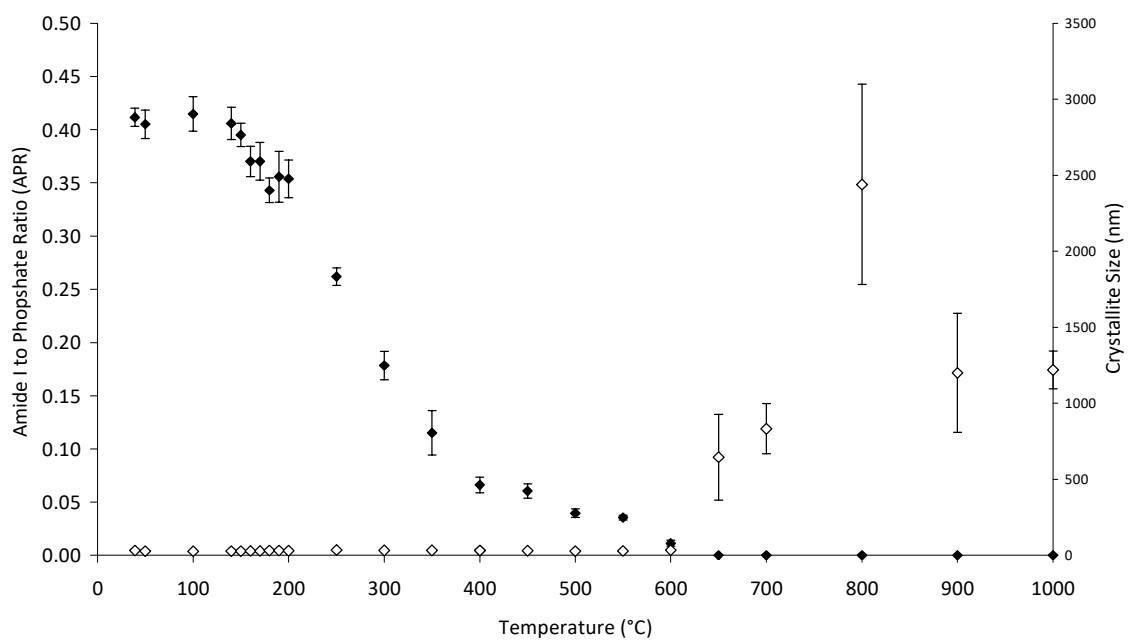


Figure A9.3: Relationship between the APR and Crystallite Size of bHAP from bone heated at various temperatures. A: Two hours, B: four hours. (◆) APR (◇) Crystallite Size. Errors bars (± 1 standard error).

Appendix 10

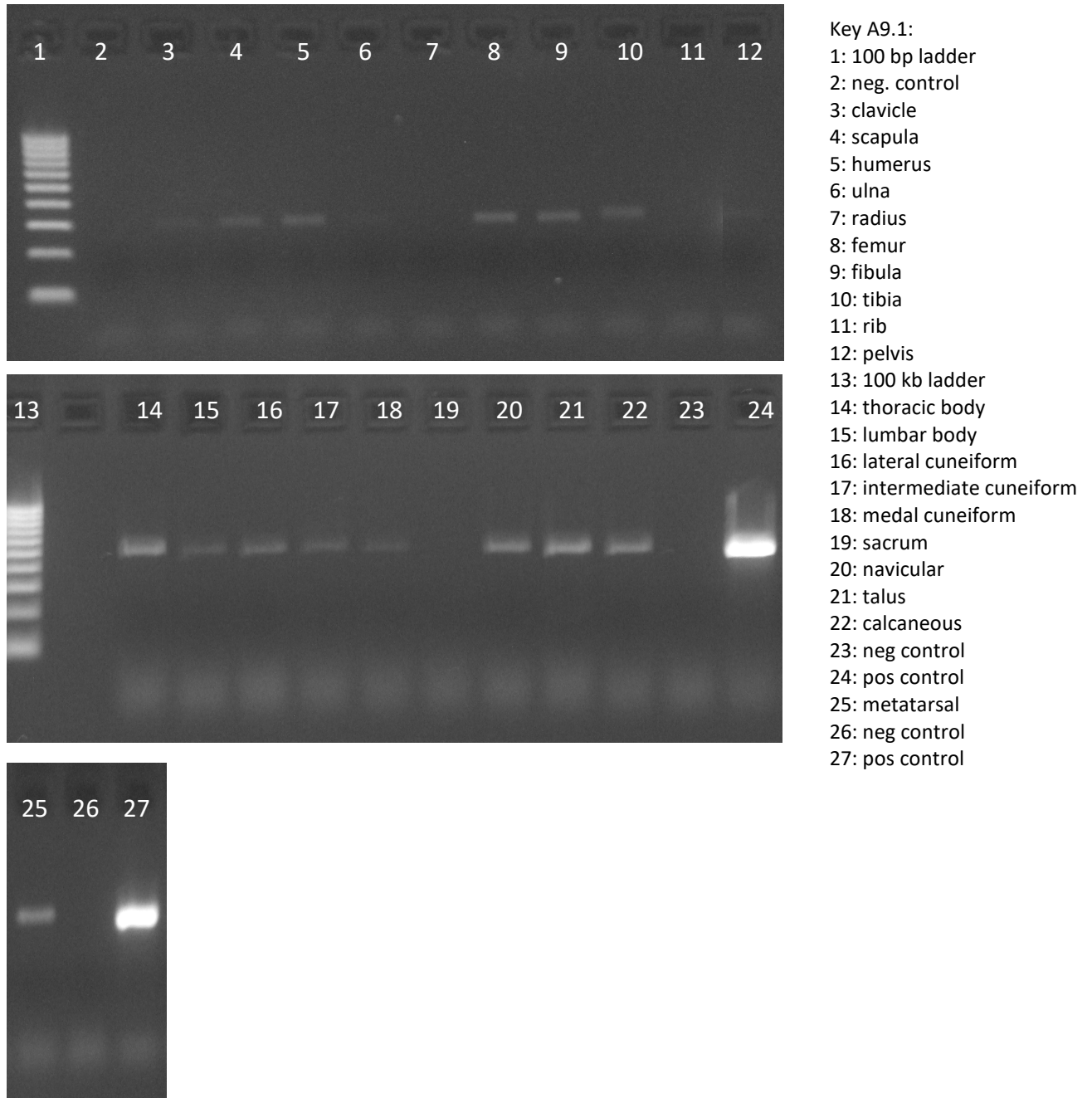


Figure A10.1: Gel electrophoresis images of PCR products (500 bp) amplified from DNA extracted from different bone element recovered from the English Channel (see key).

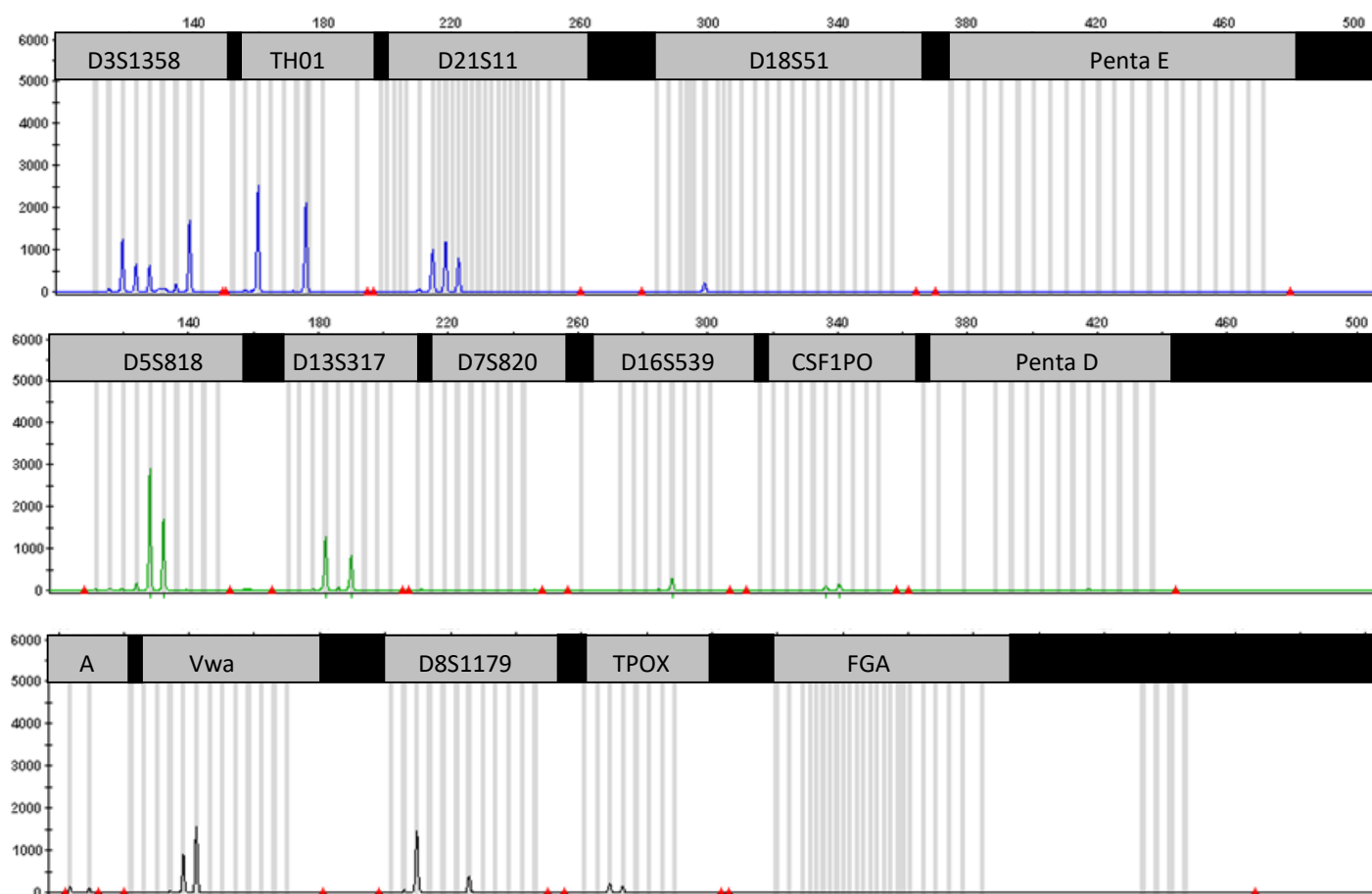


Figure A10.1: DNA profile from the Humerus of the individual recovered from the English Channel.

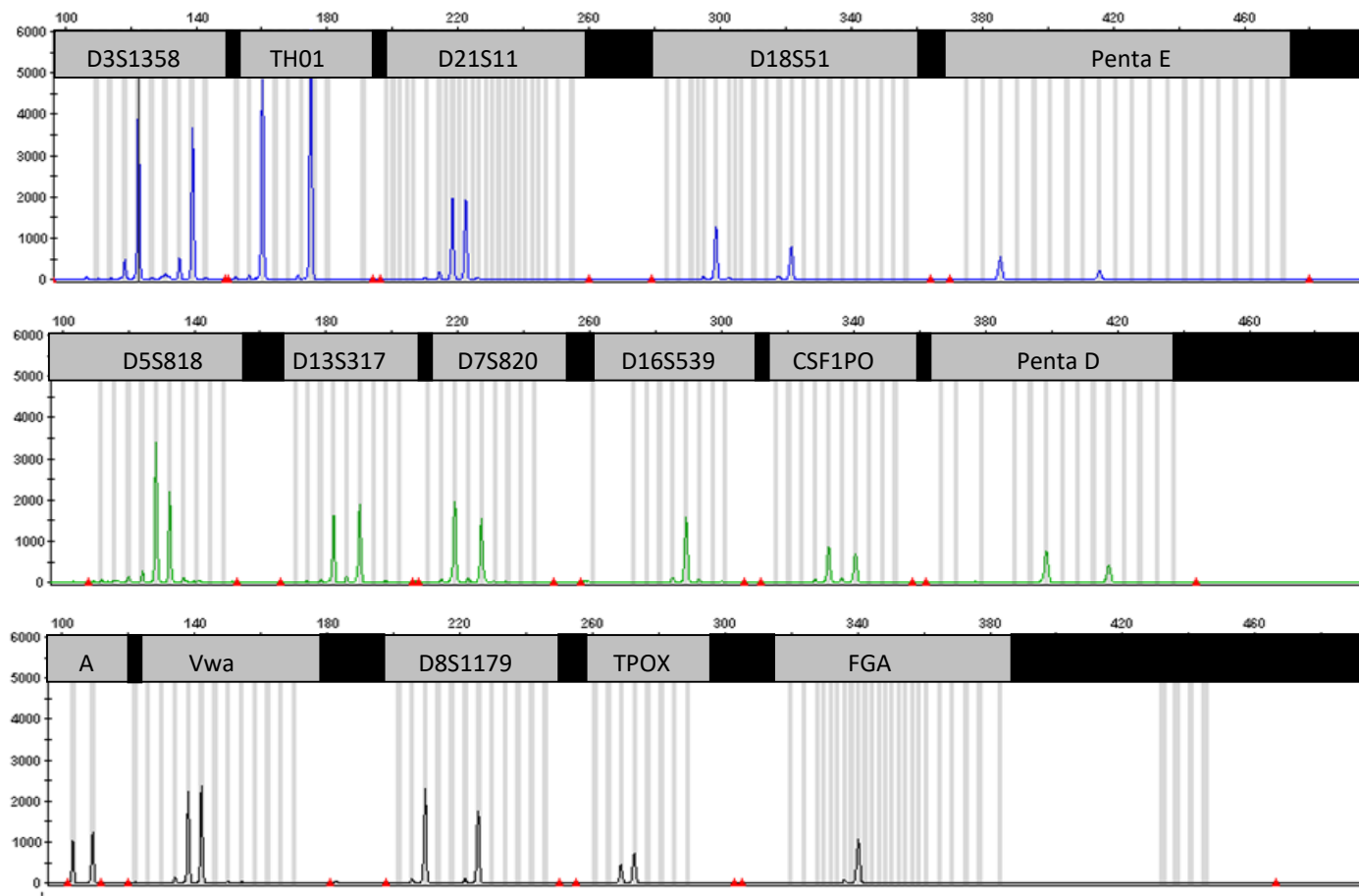


Figure A10.2: DNA profile from the Femur of the individual recovered from the English Channel.

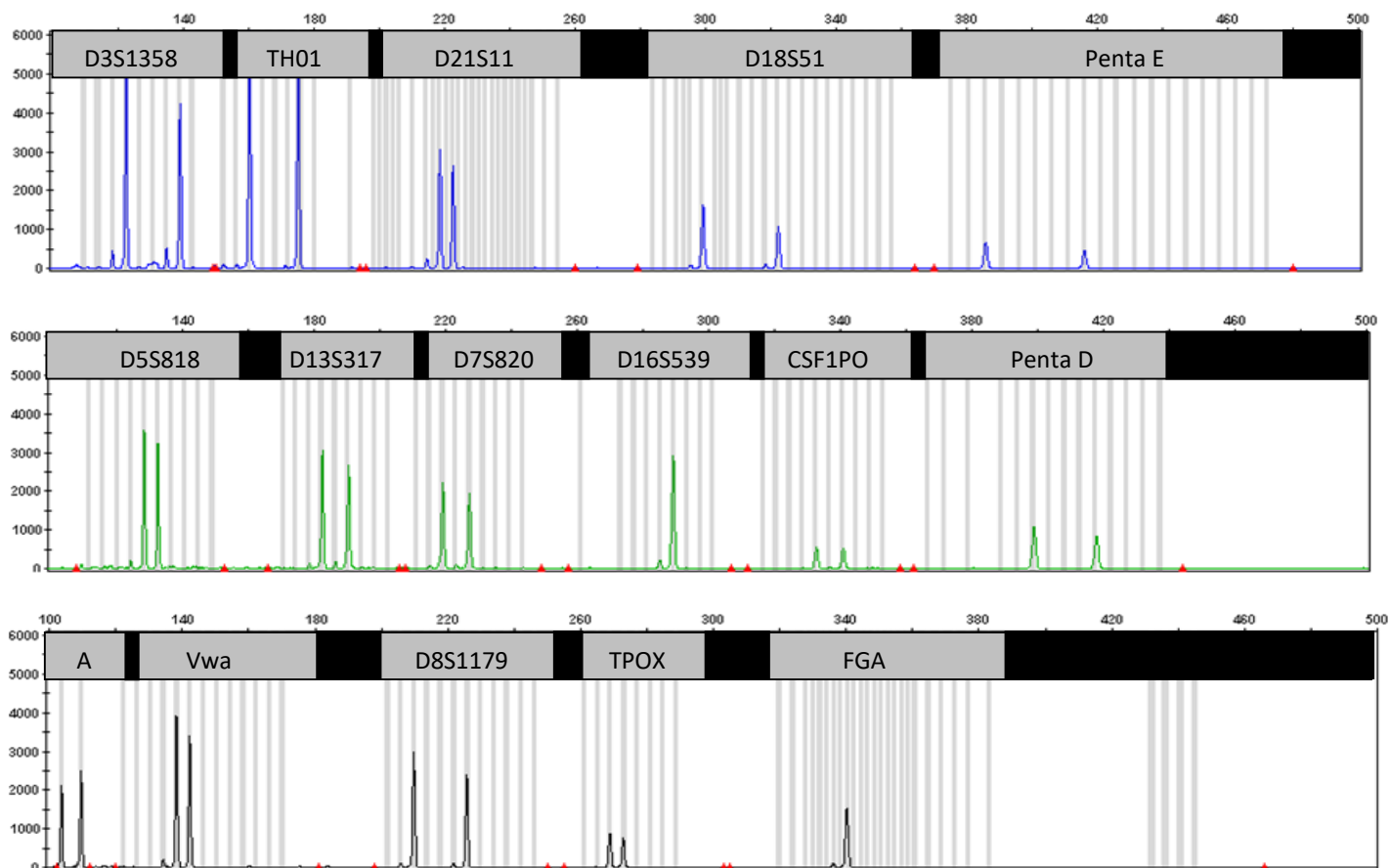


Figure A10.3: DNA profile from Talus of the individual recovered from the English Channel.

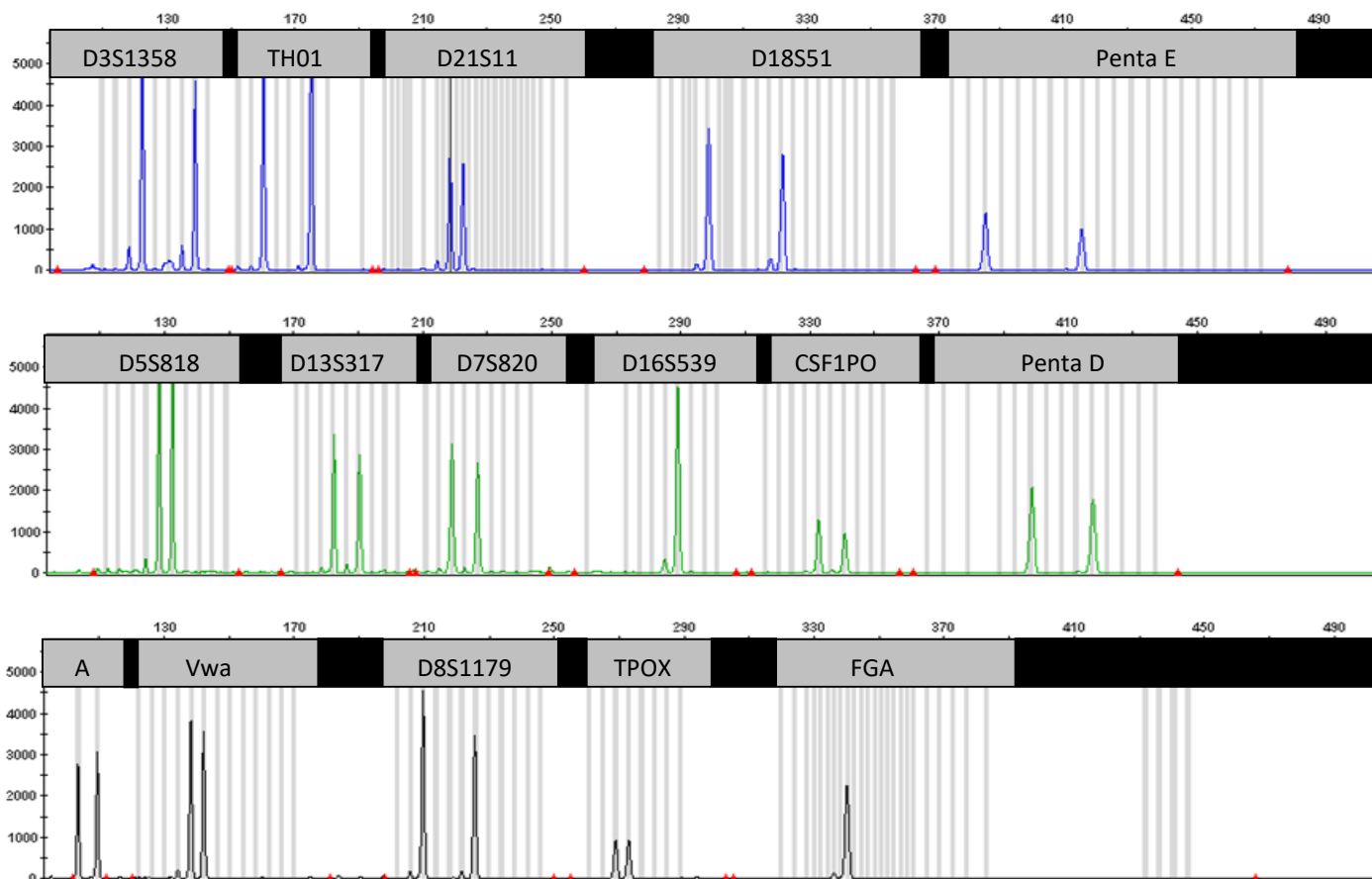


Figure A10.4: DNA profile from the Navicular of the individual recovered from the English Channel.

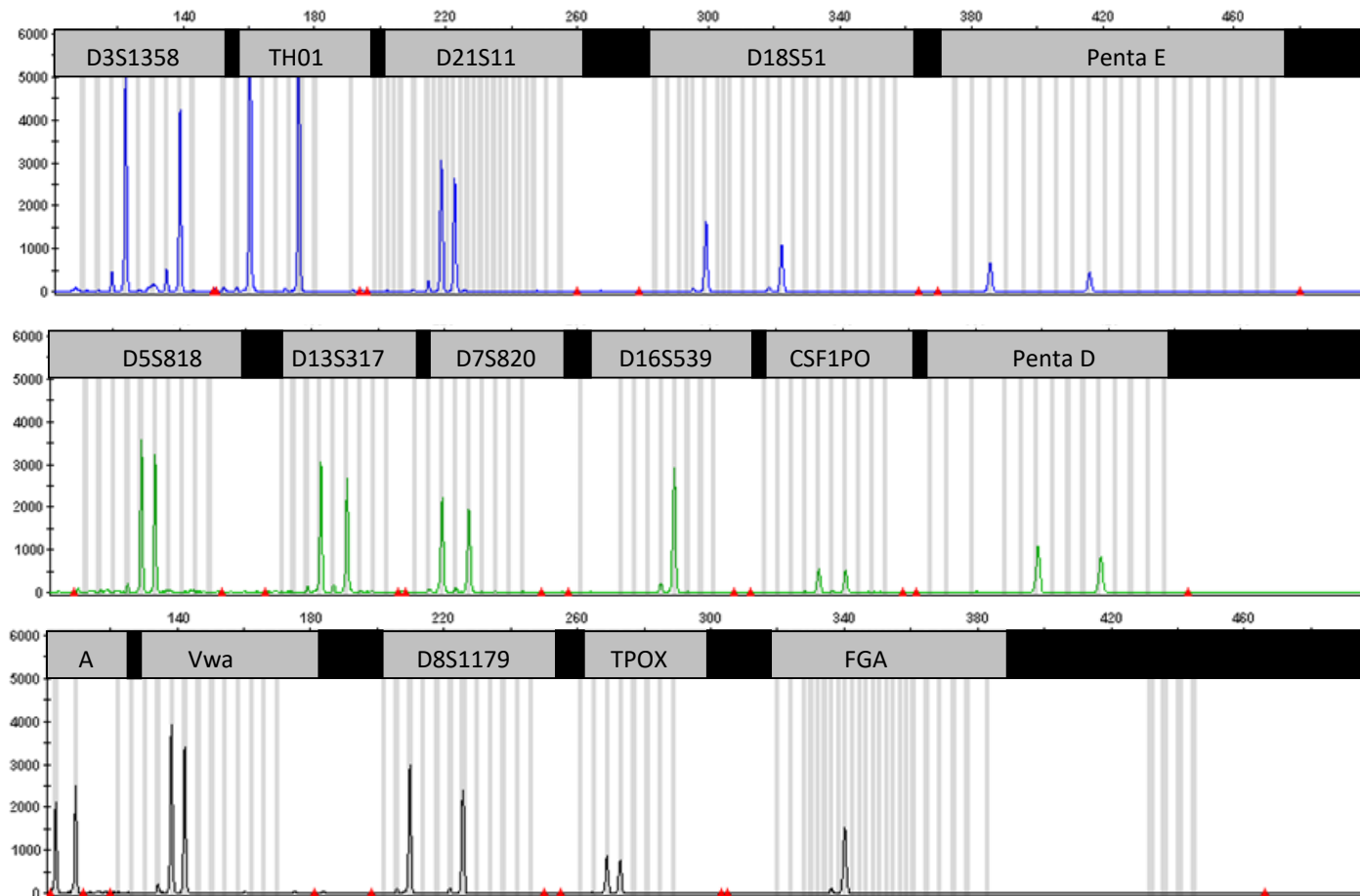


Figure A10.4: DNA profile from the Intermediate cuneiform of the individual recovered from the English Channel.

