Methods for Improving Challenging DNA Profiles and Molecular Preservation of Soft Tissue Samples

by

Lais Vicente Baptista

A thesis submitted in partial fulfilment for the requirements for the degree of
Doctor of Philosophy at the University of Central Lancashire

April 2018

STUDENT DECLARATION FORM

I declare that while registered as a candidate for the research degree, I have not been a registered candidate or enrolled student for another award of the University or other academic or professional institution.

I declare that no material contained in the thesis has been used in any other submission for an academic award and is solely my own work.

No proof-reading service was used in the compilation of this thesis.

Lais Baptista

Lais Vicente Baptista

Type of Award: Doctor of Philosophy

School: School of Forensic and Applied Sciences

Essa cova em que estás,
com palmos medida,
é a cota menor
que tiraste em vida.
— É de bom tamanho,
nem largo nem fundo,
é a parte que te cabe
neste latifúndio.
— Não é cova grande.
é cova medida,
é a terra que querias
ver dividida.
— É uma cova grande
para teu pouco defunto,
mas estarás mais ancho
que estavas no mundo.
— É uma cova grande
para teu defunto parco,
porém mais que no mundo
te sentirás largo.
— É uma cova grande
para tua carne pouca,
mas a terra dada
não se abre a boca.

Morte e Vida Severina,
João Cabral de Melo Neto

The grave you're in
Is measured by hand,
The best bargain you got
In all the land.
– You fit it well,
Not too long or deep,
The part of the latifundio
Which you will keep.
– The grave's not too big,
Nor is it too wide,
It's the land you wanted
To see them divide.
– It's a big grave
For a body so spare,
But you'll be more at ease
Than you ever were.
– You're a skinny corpse
For such a big tomb,
But at least down there
You'll have plenty of room.
– The grave is big
For your skin and bone,
But when land is given,
You can hardly moan.

Death and Life of Severino,
(John Milton translation)

To my grandfather, my example of

loving knowledge, and who always

showed me the joy of learning and

curiosity.

ABSTRACT

Degradation of DNA can lead to either poor quality, imbalanced, or even no profiles. Therefore, appropriate collection and storage methods are critical to minimize its impact. If the DNA is degraded prior to sample collection, then the degradation process can only be arrested and other methods have to be employed to try to improve the quality of the DNA profile. The major aims of this thesis were to assess alternative methods for molecular preservation of muscle tissue samples and to obtain better DNA profiles from degraded samples.

Assessment of DNA degradation was undertaken using an in-house PCR assay which amplifies four amplicons from 70 bp to 384 bp. DNA degradation was evaluated in whole pig carcasses exposed to hot and humid environmental conditions. A full DNA profile could be generated for 24 hours, but some full profiles were obtained from samples taken as late as 72 hours. It was determined that when collecting tissue samples from partially decomposed bodies, those should be preferentially from the surface of the body in touch with the ground, as the results show that DNA persistence is improved.

In order to compare field and laboratory degradation patterns, muscle tissue samples were incubated in the laboratory at 25 °C and 37 °C. The persistence of DNA was increased when compared to field, most likely due to the lack of insect activity and of variations in temperature and humidity. Partially degraded muscle samples were preserved with 96% ethanol, cell lysis solution, or cell lysis solution with 1% sodium azide, which had been stored at room temperature for seven years. Samples were re-extracted to assess the long-term efficacy of these storage solutions. The results show that ethanol and cell lysis solution with 1% sodium azide were successful in preserving DNA for this period.

Fresh muscle tissue samples were stored at 25 °C and 37 °C for up to 42 days using vodka and 37.5% ethanol as preservatives. Complete amplification profiles were obtained up to the last time point from samples that had any preservative solution, while samples left untreated had dropouts after 14 days. It is recommended that the use of drinking ethanol should be considered in situations where the stock of absolute ethanol is limited. The possibility of using vacuum for preservation was tested on fresh muscle tissue samples incubated at 25 °C and 37 °C. The results show that even if there was a limited amount of air inside the storage bag, and not complete vacuum, DNA persistence was enhanced when compared to samples incubated at the same conditions in plastic tubes.

Some approaches were attempted to improve degraded DNA profiles. First, degraded DNA was selectively extracted from agarose gels to manipulate the proportion of longer and smaller DNA fragments present. Despite promising preliminary results, this technique showed no usefulness in improving DNA profiles. Purification columns were used with the same aim, but when comparing the original sample with the processed samples, the best results obtained were of equivalence. As an alternative approach, a protocol of DNA Capture was developed in an attempt to preferentially extract the fragments to be analysed in a degraded DNA sample in equal amounts. Whilst the DNA capture method worked in preliminary experiments, it was not applied to degraded profiles.

The results obtained have allowed recommendations around collection (i.e. how long samples could be viable for DNA analysis) and storage to be refined. Attempts to rebalance already degraded profiles were not successful. Future field experiments planned as a follow up to the work presented involve testing collection methods and the effectiveness of vacuum body bags.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABREVIATIONS

ADD   Accumulated Degree Days

bp   Base pairs

CL   Cell Lysis

DNA   Deoxyribonucleic Acid

DNase I   Deoxyribonuclease I

ETOH   Ethanol

INDEL   Insertion/Deletion Polymorphism

mtDNA   Mitochondrial DNA

MWCO   Molecular Weight Cut-Off

NGS   Next Generation Sequencing

PCR   Polymerase Chain Reaction

PEC   Primer Extension Capture

RAG-1   Recombination Activating Gene 1

RAG-2   Recombination Activating Gene 2

RFU   Relative Fluorescence Unit

RNA   Ribonucleic Acid

SNP   Single Nucleotide Polymorphism

STR   Short Tandem Repeat

TEG   Triethyleneglycol

TRACES   Taphonomic Research in Anthropology: Centre for

               Experimental Studies

FINANCIAL ACKNOWLEDGMENT

ACKNOWLEDGMENTS

This work would not have been possible without the help and support of some people that I need to thank.

First, I want to thank my supervisor Dr. William Goodwin for being a centre of calmness and support through this whole process. I also want to say thanks to the FGG team: Dr. Arati Iyengar, Dr. Judith Smith, Dr. Sibte Hadi, and Christine Woodcock (Chrissy), your advice and general help was indispensable.

To the other PhD students that I met during my time at UCLAN, I want to thank you for sharing this journey. I could not have asked for better comrades. Thank you Dina, Haliza, Tundey, Ali, Erum, Pet-Paul, Hussain, Balnd, Eida, and Hayder for the friendship, talks and support.

To my friends back home, who accepted me being absent of almost everything in the past 4 years, a big hug and thank you. Even physically distant, you were always here with me. Cheesy, but true. Thank you De Sempre, Descongela Mas Não Esquenta, NfBR, NICV, and my Hamiltrash friends.

This work would never be finished and this thesis could not have been written without music. I want to thank Lin-Manuel Miranda, Taylor Swift, Cattle & Cane, Muse, Anitta, Chico Buarque, System of a Down, Kate Nash, Morat, Kendrick Lamar, Residente, Shakira, Murray Gold, Yann Tiersen, John Williams, the original casts of Hamilton, Matilda, Waitress, Natasha, Pierre and the Great Comet of 1812, Dear Evan Hansen, and all the Disney composers for providing the soundtrack I needed.

Last but never least, I wish to thank my family, specially my mum and my cousins Talita and Clara. Thank you for dreaming with me about a Christmas in Preston that never happened, for sharing everyday life even though I was not there and for all the jokes and love that make us the best family in the world.

CHAPTER 1

INTRODUCTION

1.1 Forensic Sciences

Forensic sciences are the application of science to criminal and civil laws, as governed by the legal standards of admissible evidence and criminal procedure in each country. They are believed to have started in the 16th century, when medical practitioners in army and university settings began to gather information on the cause and manner of death. Writings in the late 18th century revealed the first evidence of modern pathology. The first school of forensic science was stablished in France in 1909. Since the development of forensic sciences, they have been used to uncover mysteries, solve crimes, and convict or exonerate suspects of crimes.

The field of forensic sciences includes a number of scientific branches, including physics, chemistry, and biology, and focus on the recognition, identification, and evaluation of physical evidence. It has become an essential part of the judicial system, as it utilizes a broad spectrum of sciences to achieve information relevant to criminal and legal evidence. Constant research is of ultimate importance in order to improve methods and aid police officers, courts and families to solve cases and mysteries (AAFS 2014; Jackson and Jackson 2011; Saferstein 2017).

1.2 Decomposition of Post-Mortem Tissues (Cadaveric Decay)

Decomposition will occur when a cadaver is exposed to the environment, with the soft tissue present in the body starting to decay within a few days (Goff 2009). Cadaveric decay is influenced by two destructive processes, namely autolysis and putrefaction (Dent *et al.* 2004; Janaway *et al.* 2009).

Autolysis is a process of self-digestion, where the tissues and cells are broken down through the action of intrinsic hydrolytic enzymes (Shirley *et al.* 2011). Shortly after death, the oxygen supply to cells ceases and the anaerobic

mechanism of glycolysis serves as an alternative energy source in order to maintain basic cellular metabolic activity. This produces waste products such as carbon dioxide and lactate which cause the cellular pH to drop (Swann *et al.* 2010; Zhou and Byard 2011).

When the cellular membrane can no longer maintain its permeability because of pH difference, it ruptures, releasing cellular contents, including hydrolytic enzymes (Paczkowski and Schuetz 2011). These cellular contents that then act as source of nutrients and energy for the subsequent microbiological activity of the putrefaction process (Zhou and Byard 2011).

Putrefactive breakdown results primarily from the action of bacteria, fungi and protozoa that are already present in the body, mainly in the gastrointestinal tract. These microorganisms digest cellular proteins in local tissue and gain access to the rest of the body via the vascular and lymphatic systems (Dent *et al.* 2004; Paczkowski and Schuetz 2011).

Exogenous factors affect the rate of cadaveric decay, such as the type of environment and the condition of the body at the moment of death. Ambient temperature and insect activity have great effect on decomposition (Campobasso *et al.* 2001; Heaton *et al.* 2014; Ross and Cunningham 2011; Simmons, Adlam *et al.* 2010; Simmons, Cross *et al.* 2010). An increase in temperature will accelerate enzymatic reactions and the rate of decomposition, encouraging more oviposition (which is very limited when the temperatures are below 10 °C) and larval development (Prangnell and McGowan 2009). Oxygen availability is also important, with low oxygen levels restricting the microbial activity to anaerobes, thereby slowing the decomposition process (Dent *et al.* 2004; Statheropoulos *et al.* 2011).

The cause of death can be one of the main aspects in the speed of cadaveric decay. When bodies have open wounds, the rate of decomposition is much faster than that of a body without any penetrative trauma, principally due to the high prevalence of microorganisms and arthropod oviposition within the wound (Cross and Simmons 2010). In addition, if an individual dies as a result of a viral or bacterial infection, putrefactive post mortem changes are accelerated (Janaway *et al.* 2009).

A number of facilities that uses human cadavers in taphonomic research operates in the United States (e.g. the Anthropological Research Facility at the University of Tennessee (Shirley *et al.* 2011)). For ethical and logistical reasons, body farms have not been in use in the UK. While studies using human cadavers might be preferable due to their direct relevance to forensic cases, as a greater number of animals can be available for use at one study, the use of animal models can facilitate larger studies on the variables that influence decomposition (Cross *et al.* 2010).

Pigs have been used extensively as an experimental animal model in taphonomic studies, as they have several features that are similar to humans, such as size, anatomy, physiology and metabolic rate (Cross and Simmons 2010; Dekeirsschieter *et al.* 2009; Myburgh *et al.* 2013; Simmons, Adlam *et al.* 2010; Turner and Wiltshire 1999).

1.3 Accumulated Degree Days

In taphonomic studies, decomposition of tissues is regarded as a process that depends more on accumulated temperature rather than time alone. In essence, Accumulated Degree Days (ADD) constitutes of the accumulation of thermal energy needed for the chemical and biological reactions of decomposition to take

place. By using ADD, the energy input into the system is measured as the accumulation of temperature over time.

This cumulative total of daily average temperatures can be used as a quantitative measurement to estimate post-mortem interval. The formula normally used to calculate ADD is the following:

$$ADD = \frac{Maximum\ Temperature + Minimum\ Temperature}{2} - Minimum\ Threshold$$

Where the minimum threshold is the temperature at which biological process stops. In human decomposition studies, the value of 0 °C is commonly used here, since freezing temperatures inhibit most biological processes (Larkin *et al.* 2010).

The use of ADD allows the comparison of studies across multiple and varied environments. Whenever the same amount of thermal energy (ADD) is put into a carcass, the same amount of reaction (body decomposition) will result (Megyesi *et al.* 2005; Simmons, Adlam *et al.* 2010).

1.4 DNA Degradation

DNA breakage occurs all the time, but the living organism has repairing enzymes in place to fix it. Upon death, cells and tissues become starved of oxygen and at this point these physiological processes cease to work and decay begins (Demple and Harrison 1994). Depending on several internal and external factors, cells would undergo one of two different death patterns: apoptosis or necrosis. One of these factors is the level of intracellular adenosine triphosphate (ATP). Apoptosis features an energy-dependent programmed cell death where intracellular ATP levels remain unaltered until the very end of the process. Alternatively, necrosis is a passive energy-independent degenerative phenomenon.

Apoptosis usually leads to DNA fragments of around 180 base pairs (bp), with the DNA breaks happening between the nucleosomes. While the ladder-like pattern of oligonucleosomal-sized fragments in agarose electrophoresis is produced as a hallmark of apoptosis, the random digestion characteristic of necrosis creates the smear associated with degraded DNA (Alaeddini *et al.* 2010).

In early necrosis, endonuclease-mediated DNA cleavage is characterized by selective generation of 5' overhangs. Endogenous endonucleases will cleave the DNA around the histone structure (the most vulnerable sections) and this generates fragments of 300 kb (rosette structure) or 50 kb (loop structure). After digestion of the chromatin proteins, random digestion by the endonucleases will occur, with the rate of degradation depending on temperature, pH levels, and expression level of the enzymes (Didenko *et al.* 2003; Golenberg *et al.* 1996; Paabo *et al.* 2004). Changes towards necrosis occur faster in higher ambient temperatures and necrosis is typically induced by extremes in the external environmental conditions of the cell (such as hypoxia), or through the action of membrane active toxicants and respiratory poisons (Alaeddini *et al.* 2010).

There are also non-enzymatic processes that are present and responsible for DNA breakdown, which happen much slower, but should not be ignored. Some of them are more likely to happen in muscle tissue than others, but the DNA can be subjected to any of them in an uncontrolled environment.

Denaturation involves the unwinding of the double helix structure, due to breakages in the hydrogen bonds that hold the DNA strands together. Although the nucleotide sequence remains unchanged, denaturation increases the susceptibility to other types of chemical attack (Lindahl and Nyberg 1974). Cross-linking happens when one of the strands of the double helix become chemically

bonded to other molecules. Similar to denaturation, nucleotides are unchanged, but cross-linking can interfere with analyses (Brown 1999).

Hydrolic reactions will attack the glycosidic base–sugar bond and the hydrolysis of DNA is enhanced by the presence of water, resulting in strand breakages, base loss and chemical modifications to the nucleotide units (Lindahl and Karlstro 1973). Chemical modifications can involve the addition, removal or replacement of a chemical group on a nucleotide. These modifications can change the entire nucleotide sequence. Strand breakages occur where there are breaks in the sugar phosphate backbone of the DNA, causing the whole molecule to fragment (Brown 1999).

Oxidative damage can change sugar residues, remove bases and create strand breakages and cross-linkage (Epe *et al.* 1993; Lindahl 1993). Lastly, radiation can create oxidative damage, strand breaks, modification of bases, and formation of dimers (Alaeddini *et al.* 2010; Hoss *et al.* 1996; Overballe-Petersen *et al.* 2012; Paabo *et al.* 2004).

Highly degraded DNA samples will typically display poor amplification of the larger sized alleles when using standard short tandem repeat (STR) multiplex typing kits. Because of how degradation works, the amplification and profiling of shorter amplicons is more successful in degraded templates due to the fact that lower molecular weight loci are more likely to stay intact (Butler *et al.* 2003; Dixon *et al.* 2006; Takahashi *et al.* 1997).

As STR amplicon sizes in commercial multiplex kits used for DNA identification are usually between 100 bp and 450 bp, degraded samples will generate at least an imbalanced DNA profile. In this case, the larger amplicons fall below the detection limits, and a partial genetic profile can be seen (Cotton *et al.* 2000; Greenspoon *et al.* 2006; Krenke *et al.* 2002). A decay curve can be seen in

multiplex kits with a wide range of amplicon sizes, where the peak height is inversely proportional to the amplicon length (Lygo *et al.* 1994; Wallin *et al.* 1998). In more degraded samples, the longer alleles can drop out completely, leaving the profile incomplete (Figure 1.1) (Chung *et al.* 2004; Hughes-Stamm *et al.* 2011; Senge *et al.* 2011; Westen *et al.* 2009).

With increased degradation levels, polymerase chain reaction (PCR) artefacts such as preferential amplification, allele drop out and locus drop out become more common (Bender *et al.* 2004; Foran 2006; Lindahl 1993).
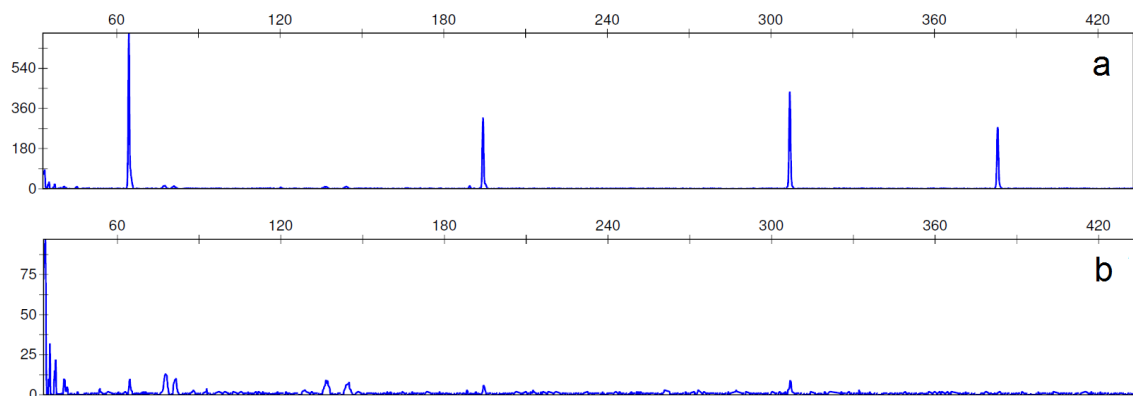
Figure 1.1 – Examples of electropherogram profiles from (a) non-degraded DNA and (b) degraded DNA.

1.5 Tissue Preservation

The International Criminal Police Organization (INTERPOL) recommends friction ridge analysis, dental analysis and DNA analysis as primary methods of identification (INTERPOL 2014). Following mass fatality incidents and in some crime scenes, human corpses are disassembled, burnt or decomposed, making victim identification by means of fingerprinting or odontology extremely difficult. In such situations, DNA analysis can play a crucial role; however, successful DNA analysis from post-mortem samples relies heavily on the appropriate collection and preservation of biological material (Graham *et al.* 2008). The DNA

Commission of the International Society for Forensic Genetics says that storing soft muscle tissue samples in preservative solutions at room temperature can be an alternative to cold storage (Prinz *et al.* 2007).

Tissue type is one of the principal factors affecting DNA preservation. Bone and teeth have resilient structures which slow DNA degradation, especially when compared to soft tissue samples. However, the process of DNA extraction from hard tissues is laborious and time consuming (Budowle *et al.* 2005; Dawson *et al.* 1998).

The rate of DNA degradation is variable amongst soft tissue samples. Bär *et al.* (1988) studied samples from brain cortex, lymphatic node, liver, spleen, psoas muscle, kidney, thyroid gland, and blood with post-mortem ages varying between 6 h and 19 days. Complete DNA degradation was observed in liver tissue after 24 h, while spleen, kidney and thyroid gland showed good DNA stability up to 5 days. Another study by Larkin *et al.* (2010) was able to amplify a fragment of 200 bp from muscle samples collected from pig carcasses exposed for up to 10 days in the winter. Samples of rat brain, kidneys, liver and skeletal muscle of the thigh collected up to 6 weeks after death were analysed with real-time PCR. The results show that the brain had the most DNA persistence, followed by muscle (Itani *et al.* 2011).

In forensic genetics, tissue preservation is usually connected with disaster victim identification (McNevin 2016) and preservation methods should be able to generate a profile using commercial kits. Inefficient preservation methods can lead to the breakdown of intact DNA to such an extent that data are not always available for victim identification (Bing and Bieber 2001). Biological samples have been successfully preserved using a number of physical and chemical

treatments, adjusting temperature, ambient pH and salt concentrations (Dawson *et al.* 1998).

1.5.1 Temperature

Sample storage at -20 °C enhances DNA preservation (Budowle *et al.* 2005). Hara *et al.* (2016) investigated DNA degradation from blood stains over a range of temperatures from -80 °C up to room temperature. Temperatures below -20 °C preserved DNA better and temperatures above 4 °C significantly degraded the DNA when compared to all other temperatures.

The impact of freeze-thaw cycles of whole blood samples on DNA integrity in the sample was previously studied (Bellete *et al.* 2003; Gessoni *et al.* 2004; Krajden *et al.* 1999; Ross *et al.* 1990) and the results showed progressive degradation of the samples as the number of freeze-thaw cycles increased. Freeze-thaw cycles would be expected to be equally detrimental to the stability of DNA in soft tissues, as ice crystals grow upon freezing, which speed up the breakdown of cell membranes, leading to accelerated autolysis.

1.5.2 Preservation Solutions

Cell lysis (CL) solutions have been used to preserve soft muscle tissue samples for different periods of time (Allen-Hall and McNevin 2013). Lysis storage and transportation buffer was used to preserve 5-100 mg of muscle tissue at room temperature and complete STR profiles were obtained for up to 12 months (Graham *et al.* 2008). Lysis buffer was used to preserve liver tissue for up to 2 years. No DNA could be extracted from tissue after 2 years of storage, but high molecular weight DNA was recoverable from the buffer solution (Kilpatrick 2002).

Alcohol storage is considered to be an effective long term tissue storage method that allows for successful DNA extraction from preserved specimens (Penna *et*

al. 2001). Ethanol (ETOH) has been used previously for the preservation of specimens at room temperature (Gillespie *et al.* 2002; Kilpatrick 2002; Michaud and Foran 2011). It is cost-effective and readily obtained, making it an attractive candidate for field applications. King and Porter (2004) investigated the effect of a range of alcohol concentrations from 70-100% on DNA preservation in ant specimens, noting that a concentration of 95-100% was most effective. They reported that ethanol was preferred over other alcohols due to faster penetration of cell membranes and a better ability to deactivate deoxyribonuclease (DNase) activity.

The use of vodka in the preservation of zebra liver samples over several days in the African Bush was reported by Oakenfull (1994). It was found that the DNA extracted after using this method was not as high quality as those samples stored in ethanol or methylated spirits; however, the DNA produced was still satisfactory for amplification.

1.5.3 Vacuum Preservation of Tissues

Vacuum was the first technology applied to packaging methods used commercially in the food industry. It consists of removing the air from the package, thus creating an anoxic environment. Due to the lack of oxygen, bacterial microflora is selected and growth of aerobic microorganisms is hindered, whereas anaerobic and facultative bacteria grow slowly. Vacuum preservation diminishes the growth of the microbial community when compared to aerobic preservation methods (Bellés *et al.* 2017).

Spoilage of raw meat is a combination of biological and chemical activities, but is mainly caused by microbial metabolic activity. The initial microbial community is influenced by storage conditions, such as the temperature and the packaging environment (Casaburi *et al.* 2015; Jääskeläinen *et al.* 2016). Vacuum packaging

reportedly diminishes the spoil of products like fruits, vegetables and various kinds of meat (Araujo *et al.* 2017; Bouletis *et al.* 2017; Costa *et al.* 2016; Hernández, E. J. G. P. *et al.* 2017; Jin *et al.* 2017; Lee *et al.* 2016; Li *et al.* 2017; Lloret *et al.* 2016; Sergio *et al.* 2016; T. Wang *et al.* 2016; Yang *et al.* 2016).

Biobanks and hospitals are also looking at vacuum preservation in order to reduce or cease to use formaldehyde (Condelli *et al.* 2014). In one study Di Novi *et al.* (2010) found that under-vacuum sealing of tissues preserved gross anatomic features better than formalin, improved histological features in solid organs, facilitated gene expression profiling of breast cancer specimens, and preserved RNA, thus permitting tissue banking and gene expression profiling.

Bussolati *et al.* (2008) found that the absence of air decreased autolytic processes, and increased protein and RNA stability. The stability of RNA tends to be shorter than that of DNA, but no studies were found analysing DNA persistence when storing muscle tissue samples with vacuum.

1.6 DNA Extraction

DNA extraction in forensic laboratories is a crucial step in the workflow. Since all following procedures depend on it, then it is important to extract as much DNA as possible (good yield) and it must be of good quality, with no inhibitors. The selection of technique must adapt so that each sample is treated accordingly. Some of the current used protocols are FTA Paper, Chelex 100, organic extraction, and silica-based extraction (Bogas *et al.* 2014).

FTA paper extraction is a simple method for reference samples. The sample is applied to the FTA card, which has chemicals in it that cause cell lysis. The released DNA binds to the paper. Extraction is based on washing steps to remove non-DNA material.

The use of Chelex 100 is quite common in crime samples. The resin protects the DNA during the heating process of the extraction by binding to divalent ions such as Mg^{2+} ions that are a required co-factor to DNases. But besides this technique not being able to remove inhibitors, since Mg^{2+} ions are needed in the amplification reaction, Chelex itself can inhibit following steps (Phillips *et al.* 2012).

Organic extraction produces a cleaner DNA when compared with Chelex 100 extraction, but it requires the use of phenol, which potentially causes health problems because of its toxic nature. The protocol starts with lysing the material, followed by washing steps with a phenol-chloroform mixture and DNA purification (Carpi *et al.* 2011).

Traditional silica-based extraction protocols already showed better results than Chelex and organic extraction (Phillips *et al.* 2012). Combining silica-based techniques with magnetic beads, a silica-coated magnetic bead extraction was created. In this extraction, the samples go through salting-out and the free DNA binds to the beads. Using a magnetic separator and several washing steps, the DNA is cleaned and then released from the beads (Witt *et al.* 2012).

The method of choice for extracting DNA in degraded samples varies depending of the tissue and the decomposition stage. Studies have shown that the low copy number associated with degraded samples may be originated from the low retention rates of small fragments in extraction methods (Barta *et al.* 2014).

1.7 DNA Size Separation of DNA Molecules

Sample purification is included in the routine of most laboratories in order to remove inhibitors. Purification can also be used post-amplification in order to remove excess primers and other reagents. In this research, purification columns

were used as a way to remove small fragments of degraded DNA from samples prior to amplification. As proof of the concept that it is possible to preferentially select DNA fragments of different sizes, agarose gel electrophoresis was used.

1.7.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis is a relatively easy and fast method to assess the quality of DNA. This technique also allows the user to recover DNA intact from a band and individually isolate fragments. Agarose is a polymer that when mixed with an electrophoresis buffer forms a porous matrix that acts like a net for the DNA. When an electric current is applied, smaller fragments pass through the "net" more easily than bigger fragments, which are retained. High molecular weight DNA can be seen as a single band on the top of the gel while degraded DNA appears as a smear (Goodwin *et al.* 2007; Phillips *et al.* 1998).

1.7.2 Purification Columns

Some purification columns were chosen for their application in removal of small molecules and fragments of DNA from samples. They have different size cutouts (Figure 1.2) and utilise different techniques in order to achieve this goal.

Microcon® centrifugal filters can be used for concentration, desalting or buffer exchange of aqueous biological samples. The anisotropic, hydrophilic membranes in Microcon® centrifugal filter devices are characterized by either a molecular weight cut-off (MWCO) or a performance-specific application (e.g., DNA recovery). MWCO is the ability of the membrane to retain molecules above a specified molecular weight, and this retention depends on the molecular size and shape of the solutes. Moreover, solutes with molecular weights close to the MWCO may be only partially retained.

The illustra MicroSpin G-50 Columns purify DNA by the process of gel filtration with the use of Sephadex™ G-50 DNA grade F resin. This column is designed for rapid purification of DNA and it is suitable for recovery of any DNA fragment larger than 20 bp, but will not remove or denature enzymes. The gel filtration process works by penetration of the matrix by the molecules applied. Product retention is inversely relative to molecular size, as the size increases, the retention decreases. Molecules larger than the pores in the Sephadex™ are excluded, and intermediate sized molecules penetrate the matrix to varying extents, depending on size. Small molecules have complete penetration, which retards the process through the column.

The MinElute DNA Cleanup column uses a silica membrane combined with spin-column technology, meaning that it has the selective binding capacities of one along with the convenience of the other. DNA is applied to the silica membrane and absorbed in the presence of high concentrations of salt while contaminants pass through the column. Impurities are washed away and the DNA is eluted. DNA purified with this technique is suitable for any subsequent application.
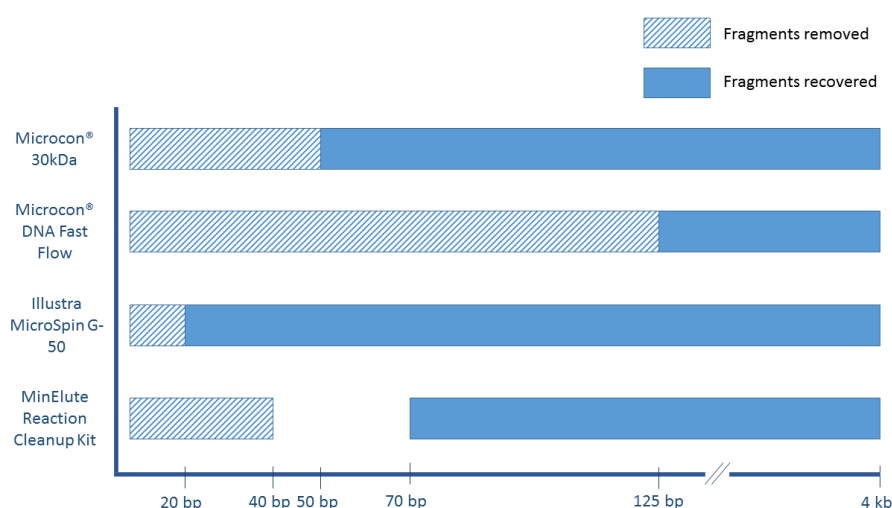
Figure 1.2 – Diagram showing DNA fragment binding-size range of the columns used. Recoveries of DNA fragments in the size ranges between "removed" and "recovered" are variable.

1.8 Magnetic Beads-Based DNA Capture

DNA hybridization capture is a DNA purification method that allows for the separation of the targeted DNA loci from the rest of the DNA in the sample. Magnetic-bead DNA separation is commonly used for total DNA non-specific purification (Wang and McCord 2011). It has been incorporated in automated DNA extraction protocols (Archer *et al.* 2006; Haak *et al.* 2008; Nagy *et al.* 2005; Rittich and Spanova 2013; Witt *et al.* 2012) where the beads bind to non-specific DNA in order to separate it from inhibitors and contaminants.

There are different approaches that can be used for DNA capture and they can be divided into two groups, namely indirect and direct capture (Figure 1.3). The main difference between them is that in the direct capture, hybridization occurs in a solid phase (immobilised), while the indirect capture has its hybridization in solution.
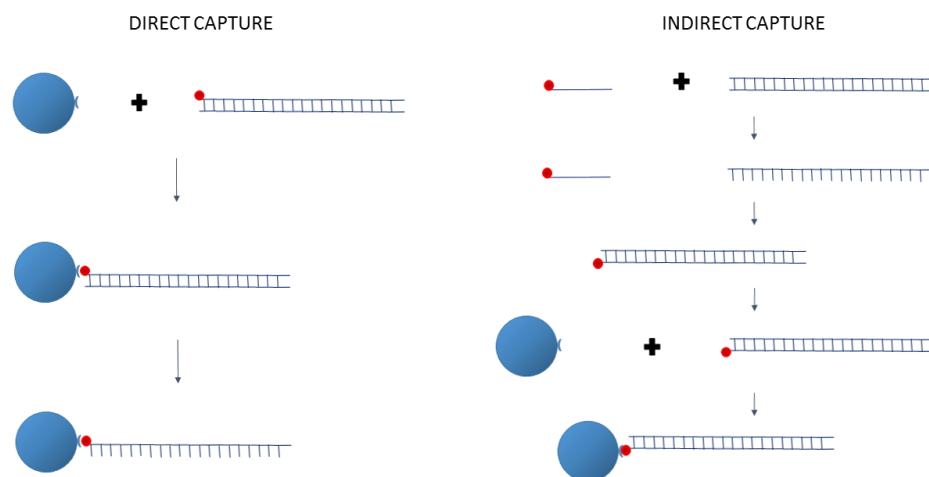
Figure 1.3 – Diagram showing schematic of direct and indirect captures of DNA. In the direct capture, the biotinylated DNA bait is incubated with the magnetic beads and immobilized on them. The bead-bait complex is then incubated with the sample for recovery of the target DNA. In the indirect method, the bait is first incubated with the DNA sample and allowed to hybridise. The magnetic beads are added to the bait-target complex, which is immobilized, allowing separation from the rest of the sample.

The direct capture procedure starts with the immobilization of double-stranded biotinylated DNA fragments onto the beads. The DNA is then denaturised and the single-stranded bead-bound templates are used to capture specific DNA molecules directly from solution.

The indirect capture involves hybridization of the bait with the target-DNA prior to binding to the beads. A biotinylated capture-sequence is incubated with the sample and allowed to hybridize to the targeted DNA molecules in solution. Then the beads are added and the hybridized sequences are immobilized onto them. The DNA-bait can be either RNA or DNA oligos with added biotin, or stretches of biotinylated DNA.

One of the techniques for indirect DNA capture is the Primer Extension Capture (PEC). In it, DNA primers hybridize to the target DNA, are elongated by a polymerase and the whole complex is captured via the attached biotin (Briggs *et al.* 2009; Horn 2011). After retrieval of the target DNA from the rest of the sample, it is possible to separate it from the magnetic bead used for its capture.

Magnetic beads-based DNA capture has been used for building libraries of ancient DNA samples, detecting microorganisms in samples, and detection of cancer-markers and HIV-1 pro-viral DNA in blood samples (Boni *et al.* 2004; Horemans *et al.* 2011; Mangiapan *et al.* 1996; Meyer *et al.* 2007; Miyashiro *et al.* 2001; Opsteegh *et al.* 2010; Parham *et al.* 2007; Shuber *et al.* 2002).

1.9 DNA Profiling

DNA profiling is one of the main techniques used for human identification and is well established. It was first developed in 1985 by Sir Alec Jeffreys, who called it DNA fingerprinting, and started with the typing of minisatellites regions of the DNA (Jeffreys *et al.* 1985). Since then, it has become one of the most important tools

17

in the forensic field helping to solve crimes of various kinds (Decorte 2010; Hochmeister 1995; Thompson *et al.* 2012). It is an important tool in human identification, being used both in forensic casework and disaster situations.

With PCR technology, STR typing quickly became the standard technique for DNA profiling because it meant that only relatively short fragments of DNA were needed. Since then, several kits were developed including loci recommended by forensic scientists and police forces worldwide. STR analysis has the advantage of allowing multiplexing, which was not possible before; and STR regions are highly polymorphic, meaning that the profile has a high discrimination power (Giardina *et al.* 2011; Horsman-Hall *et al.* 2009; Thompson *et al.* 2012; Wiegand and Kleiber 2001)

The methods used for DNA profiling have changed over the past 30 years and continue to grow becoming more powerful, sensitive and fast. Today, the process to obtain a DNA profile usually is comprised of DNA extraction, quantification, PCR amplification, capillary electrophoresis and analysis of data acquired. Since in most cases, a limited amount of sample is available, and it is important to obtain a good profile, then all steps must be most efficient to avoid re-analysis (Thompson *et al.* 2012; van Oorschot *et al.* 2010).

1.10 Challenging DNA Samples

Samples often deviate from the "ideal" for DNA analysis, and many factors can interfere with the profiling; there can be the presence of inhibitors, the DNA in the sample can be in trace amounts, or degraded. All these obstacles can result in either an incomplete profile or no profile (Giardina *et al.* 2011; Thanakiatkrai and Kitpipit 2013; Thompson *et al.* 2012).

Chemical contamination of the DNA sample can result in inhibition of downstream processes, which can prevent, even if partially, the enzymatic reactions needed to generate a profile. Most frequent inhibitors found in forensic samples are heme from blood, calcium from bones, humic acid from soil and indigo from denim. Every inhibitor has its own pathway of inhibition and some inhibit more than one chemical reaction (Burgmann *et al.* 2001; Monroe *et al.* 2013; Phillips *et al.* 2012).

Samples containing small amounts of DNA (<200 pg) are usually classified as low copy number or low template DNA. In order to analyse those samples, laboratories use procedures developed to increase assay sensitivity, such as increased PCR cycles, reducing amplification volumes, concentrating products prior to electrophoresis, and increasing injection times during electrophoresis (Benschop *et al.* 2011; Budowle *et al.* 2009; Gill *et al.* 2000; Gill 2001; Weiler *et al.* 2012).

1.11 Alternative Analysis Methods for Degraded DNA

Despite improvements in the sensitivity, analysis of degraded DNA samples with traditional STR multiplex kits can still be challenging. Some of the strategies for dealing with this are increasing PCR cycles, post-PCR purification prior to capillary electrophoresis, or changing the analysis method (Hofreiter *et al.* 2001; Phengon *et al.* 2008).

The analysis can be improved by also analyzing mitochondrial DNA (mtDNA), the other source of DNA in the cell besides nuclear DNA (Alaeddini *et al.* 2010). Since shorter fragments are more abundant in degraded samples, another alternative is the use of smaller PCR targets, such as miniSTRs, insertion/deletion polymorphisms (INDELs), and single nucleotide polymorphisms (SNPs) (Hughes-Stamm *et al.* 2011; LaRue *et al.* 2012; McCord *et al.* 2011; Senge *et al.*

2011). A relatively new development in the field is multiple parallel sequencing (MPS), also called next generation sequencing (NGS), which can generate a great amount of information from degraded samples. Figure 1.4 shows the different ranges for some of the analysis methods mentioned.
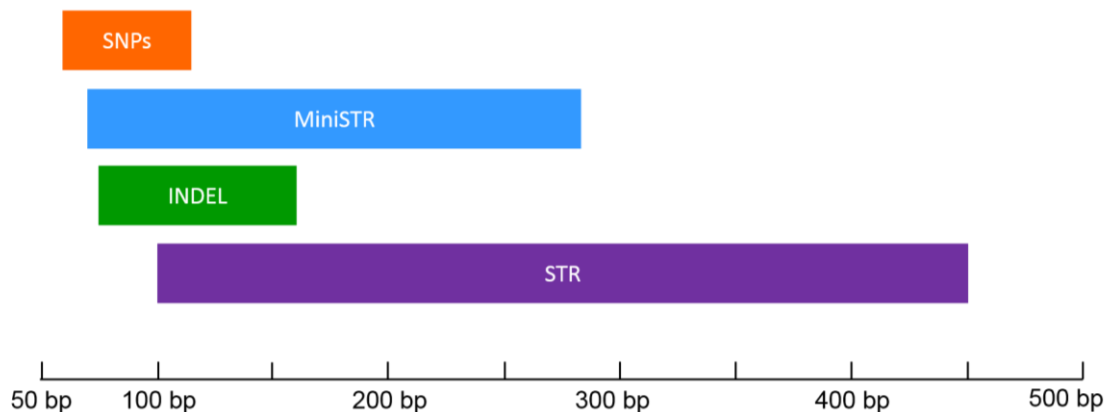
Figure 1.4 – Diagram showing the size ranges for some of the analysis methods used for human DNA profiling. SNPs size range was based on SNPforID kit (Sanchez *et al.* 2006); MiniSTR size range was based on AmpFlSTR® MiniFiler™ (Horsman-Hall *et al.* 2009; Mulero *et al.* 2008); INDEL size range was based on Investigator DIPplex® kit (LaRue *et al.* 2012); and STR size range was obtained in (Senge *et al.* 2011).

The main strategy used in forensic laboratories to analyse degraded DNA is to use amplification kits with primers located closer to the microsatellite site and, thus, smaller amplicon sizes. These reduced sized targets are referred to as miniSTRs. But since the fragment sizes are still up to 200 bp, severely degraded samples will continue to show problems similar to those found in traditional multiplex kits (Butler *et al.* 2003; Mulero *et al.* 2008; Opel *et al.* 2007; Senge *et al.* 2011; Tsukada *et al.* 2002).

Mitochondrial DNA is an alternative because it is present in larger quantities in the cell than nuclear DNA and its structure protects it more from degradation. The problems are that mtDNA analysis is a time-consuming process and the data are

20

less powerful for identification purposes than a STR match with even a few successfully amplified loci (Butler *et al.* 2003; Decorte 2010; Hughes-Stamm *et al.* 2011).

Another important strategy is SNPs analysis. SNPs are generated by substitutions or insertion/deletion of a single nucleotide, and because of this, they can be used even in highly degraded DNA samples or samples containing not enough template for other analysis. What constitutes an issue is that in older post-mortem samples, enzymatic and chemical post-mortem damage can produce alterations at single nucleotides. In addition, the bi-allelic nature of SNPs makes it difficult to analyse mixtures and the analysis is a time-consuming and tiresome process (Dixon *et al.* 2006; Giardina *et al.* 2011; Senge *et al.* 2011; Westen *et al.* 2009).

A relatively new option is INDEL analysis, which has the advantage of being small while being easier to analyse than SNPs and having low mutation rates. They can be used to complement STR analysis, as so far, global panels are still under development and these panels need a large number of markers to compensate the lower informative power (Huang *et al.* 2014; LaRue *et al.* 2014).

Massive parallel sequencing is not used in in routine at present, but it can be a great development for the field. A lot of information can be obtained even from small amounts of DNA and/ or degraded samples. Most of the workflow can be automated, which diminishes the possibilities of human errors and contamination (Borsting and Morling 2015; Iozzi *et al.* 2015; Kidd *et al.* 2015). The main drawbacks of this technique are that the analysis of the vast amount of data is complicated and that its use is still expensive.

1.12 Overview and Aims of the Research

Due to the increased importance in DNA profiling for human identification, research in techniques and methods for collection, preservation and improvement of challenging samples is of extreme importance.

Despite recommendations of use from leading agencies, little empirical evidence of the efficacy of drinking ethanol in preserving muscle tissue was found. The use of vacuum preservation is well documented in the food industry and some attempts have been made to include its use in biobanking. However, no study could be found on the use of vacuum to preserve soft tissue samples. The decision was made to assess if preserving methods for muscle tissue samples could be improved by using drinking ethanol or vacuum.

Some studies have tried to rebalance degraded DNA samples by using DNA Capture technique and got positive results. Since there are different approaches in DNA Capture, it was decided to assess the technique using the in-house developed primers already used in the 4-Plex Multiplex. No studies were found using purification columns for improving profiles of degraded DNA samples. They were included in the study to assess if whether profiles from degraded DNA samples could be improved by size separation techniques. Based on these, the working hypothesis, main aims and specific aims of this study are:

Main aims: The present thesis had two main aims. The first was to assess alternative methods for molecular preservation of muscle tissue samples, and the second was to obtain better DNA profiles from degraded samples.

The specific aims or objectives were:

1. To compare DNA degradation patterns in soft muscle tissue decomposed in a controlled environment and in environmental hot conditions.

Using an in-house developed PCR assay with amplicon sizes between 70 bp and 384 bp, DNA degradation was assessed from muscle tissue incubated at controlled temperatures in the laboratory (25 °C and 37 °C) and exposed in the field in hot and humid environmental conditions.

2. To assess the ability of different preservation solutions to preserve muscle soft tissue.

Ethanol and cell lysis were used to preserve partially decomposed muscle tissue from samples obtained after exposure to high temperatures in the field. Ethanol (96%), cell lysis and cell lysis with 1% sodium azide were used as long-term preservatives for partially degraded muscle tissue. Drinking alcohol (vodka) and ethanol distilled to 37.5% were used as preservative solutions for muscle tissue at 25 °C and 37 °C and undergoing two cycles of thaw-refreeze.

3. To assess the use of vacuum as a strategy to preserve muscle tissue samples before DNA extraction.

Bacteria present inside the body and in the environment perform a part of the decomposition process. The idea was that removing the oxygen supply would inhibit this process and slow down DNA degradation.

4. To assess the possibility of size separation technologies to re-balance the proportion on longer and shorter strands of DNA in degraded samples and enable a more balanced STR profile to be generated.

This concept was tested with the use of agarose gel electrophoresis and DNA being re-extracted from it. This experiment was followed by two different approaches. The first consisted of the use of filtration and purification columns, and the second strategy was to use DNA Capture, with the Primer Extension Capture methodology.

CHAPTER 2

MATERIALS AND METHODS

2.1 Laboratory Overview

The Molecular Biology Suite in the University of Central Lancashire (UCLan) is constituted of five laboratories, with two of these dedicated to forensic genetics research. In order to prevent contamination, these two laboratories are divided between pre-PCR and post-PCR and the flow of samples goes from pre to post, and never back. Prior to use, every work surface was cleaned with 70% ethanol or 5% Decon and all procedures were carried out using laboratory coats and latex gloves. Samples were transferred between laboratories using a hatch and coats used are specific to each laboratory. In order to monitor either potential material or worker originated human DNA contamination, reagent blanks (negative controls) containing everything, except DNA, were included through the entire process every time a reaction was carried out.

2.2 Samples

Throughout this study, domesticated pigs (*Sus scrofa*) were used as model animal. Pigs have been used as a model for human decomposition due to its similarity with humans in terms of body weight, physiology, anatomy and basal metabolic rate (Gunawardane 2009; Larkin *et al.* 2010).

2.2.1 DNA Degradation in Controlled Environments

Soft muscle tissue from freshly killed pig (less than 5 h) was obtained from UCLan's TRACES (Taphonomic Research in Anthropology: Centre for Experimental Studies) facility. The muscle was cut into small pieces of approximately 4 cm^2 and each piece was placed on a suspended platform made of wire inside a 50 mL polypropylene tube. A total of 42 tubes were prepared for incubation at 37 °C, and 45 were prepared for incubation at 25 °C. Samples were collected in triplicate (three tubes) and frozen until DNA extraction.

2.2.2 DNA Persistence in Muscle Tissue in Field High Temperatures

Whole carcasses (approximately 40 kg each) and separate limbs of pig were placed in direct contact with the ground. The carcasses were protected from scavengers by a wire cage and were placed with some distance between them. After collection, samples were stored in 50 mL polypropylene tubes with either cell lysis or ethanol and frozen until extraction.

2.2.3 DNA Persistence in Long-term Storage Using Different Preservation Solutions

In a previous study, pig whole carcasses were placed in direct contact with the ground at UCLAN's TRACES. Carcasses were protected from scavengers by being covered by a wire mesh. Samples of soft muscle tissue were collected at two time points (79 and 210 ADD). In order to serve as positive controls for DNA preservation, samples were also collected at the moment of deposition.

Samples weighing approximately 1 g and 0.5 g were placed into 50 mL polypropylene tubes containing 5 mL of preservative solutions (ethanol, cell lysis solution (with and without sodium azide)). These samples were stored at room temperature until DNA extraction.

In the present study, DNA was extracted from these samples after 7 years of storage at room temperature in order to assess long-term DNA preservation by the preservative solutions used.

2.2.4 DNA Persistence Using Different Preservation Methods

Store bought pig muscle tissue was cut in pieces of about 0.25 g and placed in 1.5 mL tubes. A total of 336 tubes was prepared and set as follow:

- Stored at -20 °C:
    - 21 tubes with muscle preserved in 95% ethanol
    - 21 tubes with muscle preserved in 37.5% ethanol
    - 21 tubes with muscle preserved in vodka (37.5% ethanol)
    - 21 tubes with muscle and no preservation agent
- Incubated at -20 °C and with two cycles of thaw-refreeze (thawed at room temperature for 2 h, frozen again at -20°C):
    - 21 tubes with muscle preserved in 95% ethanol
    - 21 tubes with muscle preserved in 37.5% ethanol
    - 21 tubes with muscle preserved in vodka (37.5% ethanol)
    - 21 tubes with muscle and no preservation agent
- Stored at 25 °C (room temperature):
    - 21 tubes with muscle preserved in 95% ethanol
    - 21 tubes with muscle preserved in 37.5% ethanol
    - 21 tubes with muscle preserved in vodka (37.5% ethanol)
    - 21 tubes with muscle and no preservation agent
- Stored at 37 °C:
    - 21 tubes with muscle preserved in 95% ethanol
    - 21 tubes with muscle preserved in 37.5% ethanol
    - 21 tubes with muscle preserved in vodka (37.5% ethanol)
    - 21 tubes with muscle and no preservation agent

Samples were collected in triplicates (three tubes) and extraction of DNA was initiated immediately after collection.

2.2.5 DNA Persistence in Soft Tissues Using Vacuum Preservation

Fresh pig muscle tissue bought from the local butcher was cut in pieces of about 1 cm^2 and placed in plastic bags to which vacuum was applied. A total of 48 bags

were prepared for incubation at 37 °C and 45 for incubation at 25 °C. The bags were disposed in the incubators avoiding overlapping. A total of six samples were frozen without incubation until DNA extraction.

Control samples were incubated without vacuum parallel to the ones in vacuum. Muscle tissue controls for incubation at 25 °C were placed directly inside an open 1.5 mL tube and incubated in the same incubator as the vacuum bags. Control samples incubated at 37 °C were placed in a closed plastic bag, but without vacuum applied to it. Three control samples were collected alongside the vacuum ones at each time point. Control samples were frozen and then processed in the same way as vacuum samples. Samples were collected in triplicate (three bags) and frozen until DNA extraction.

2.2.6 DNA Persistence in Soft Tissues inside Sealed Bags without Vacuum

Fresh pig muscle tissue bought from the local butcher was cut in pieces of about 1.5 cm^2 and placed in plastic bags that were then sealed. A total of 45 bags of three different sizes were prepared for incubation. Small bags measured 7 x 8.5 cm; medium bags measured 7 x 17 cm; and large bags measured 14 x 17 cm. The volume of air inside each bag was estimated to be around 8 mL in the small bags, 20 mL in the medium bags; and 50 mL in the large ones.

The bags were disposed in the incubators avoiding overlapping. Three samples were frozen without incubation. Samples were incubated at 37 °C for 10 days with collection of one triplicate of each size happening every two days. Samples were immediately frozen until DNA extraction.

2.3 DNA Extraction

DNA extraction was performed using DNeasy® Blood and Tissue kit (Qiagen, UK) according to the manufacturer's instructions. Muscle tissue samples were cut into

small pieces (approximately 0.5 cm x 0.3 cm) using a sterile scalpel (Swann-morton, UK) and one piece was placed into 1.5 mL tubes. Buffer ATL (180 µL) and 20 µL of proteinase K (>318 mAU/mL) (Qiagen, UK) were added to the sample. Samples were mixed thoroughly by vortexing and incubated at 56 °C until the tissues were completely digested (usually 24 h). After incubation, 200 µL of buffer AL were added to the samples and mixed by vortexing. Then a volume of 200 µL ethanol (96%) was added and mixed again thoroughly by vortexing.

The mixture was transferred in to a DNeasy® Mini spin column placed in a 2 mL collection tube, centrifuged at 6000 x g for 1 min. The flow-through was discarded and 500 µL of buffer AW1 were added. Samples were centrifuged at 6000 x g for 1 min; the flow-through was again discarded. Buffer AW2 (500 µL) was added into spin column and the samples were centrifuged at 13200 x g for 3.5 min and flow-through and collection tube was discarded.

The mini spin column was placed in a clean 1.5 mL tube and 200 µL of buffer AE was added. The samples were incubated for 1 min at room temperature and then centrifuged at 6000 x g for 1 min. The samples were labelled and stored at -20 °C until further use.

2.4 Agarose Gel Electrophoresis

In this study, agarose gel electrophoresis was used to assess the quality of the extracted DNA samples. The agarose gel was prepared at 1.5% using the appropriate amount of agarose powder and 1X TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, pH 8.0). Before solidification of the gel, 1 µL of GelRed Nucleic Acid Stain (Biotium, USA) was added to the mixture. For preparation of the samples, a volume of 2 µL of DNA was placed in a tube with 3 µL of 6X ABgene gel loading buffer (Thermo Fisher Scientific, USA) and 5 µL

of distilled water. One positive control with Lambda DNA (Promega, USA), prepared in similar way as the samples, was used per gel.

The electrophoresis was conducted in a 19 cm x 14 cm tray tank. Samples were loaded in to the wells and the gel was run at 100 V for 20 min and visualized with the use of a Bio Doc-It™ imaging system UV transilluminator (UVP, USA).

2.5 DNA Quantification

Samples were quantified using Qubit dsDNA HS Assay Kit (Thermo Fischer, UK) and a Qubit® 3.0 Fluorometer, following the manufacturer's protocol.

In a 15 mL plastic tube, the working solution was prepared by diluting the Qubit® dsDNA HS Reagent 1:200 in Qubit® dsDNA HS Buffer. For each sample or standard, 1 µL of reagent and 199 µL of buffer were added. To each standard tube, 190 µL of the working solution was added to 10 µL of the appropriate standard. Samples tubes contained 199 µL of the working solution and 1 µL of the DNA sample. The tubes were allowed to rest in room temperature for 2 min before reading on the instrument. The DNA concentration for each sample was estimated in ng/µL.

2.6 DNA Degradation with DNase I

Artificial degradation of human DNA was carried out using RQ1 RNase-Free DNase I (Promega, UK) as described by Swango *et al.* (2006) and Asamura *et al.* (2007) at a final volume of 110 µL. Commercial DNA, Human Genomic DNA (Promega, UK) was used to perform this experiment. Prior to the experiment, the enzyme was diluted 100 times from 1 U/µL to 0.01 U/µL. Degradation was assessed with a 1.5% agarose gel electrophoresis using 2 µL of the digested product.

2.7 DNA Re-Extraction from Agarose Gels

Artificially degraded samples were re-extracted from agarose gels in order to evaluate the ability of the method to balance longer and shorter strands of DNA in the sample. Samples were re-extracted using the centrifugation protocol of the E.Z.N.A.® MicroElute® Gel Extraction Kit (Omega Bio-tek, USA), using 2 μL of DNA degraded for 30 min and adding 20 μL of Elution Buffer at the end.

After agarose gel electrophoresis was performed for separation of the DNA, the gel lane was cut around the DNA with the help of a clean scalpel. The slice was weighted in a clean 1.5 mL tube (previously weighted) and it was subtracted the weight of the excised gel fragment. Assuming a density of 1 g/mL, the volume of gel was calculated where a gel slice of mass 0.3 g equalled a volume of 0.3 mL. One volume of Binding Buffer (XP2) was added to the gel fragment. The mixture was incubated at 60 °C for 7 min or until the gel was completely dissolved, with vortexing every 2 or 3 min. A MicroElute® DNA Mini Column was inserted in a 2 mL Collection Tube and up to 700 μL DNA/agarose solution was transferred to the MicroElute® DNA Mini Column. The sample was centrifuged at 10,000 x g for 1 min at room temperature and the filtrate was discarded. This step was repeated until the entire sample had been transferred to the column and 300 μL of Binding Buffer (XP2) was added. The sample was centrifuged at maximum speed (≥13,000 x g) for 30 s at room temperature, the filtrate was discarded and the collection tube reused. SPW Wash Buffer (700 μL) was added to the sample which was again centrifuged at maximum speed for 1 min at room temperature. The filtrate was discarded and the empty MicroElute® DNA Mini Column was centrifuged for 2 min at maximum speed to dry the column matrix. The column was transferred to a clean 1.5 mL tube and 20 μL of Elution Buffer was added directly to the centre of the column membrane. The column was left at room

temperature for 2 min and then centrifuged at maximum speed for 1 min. DNA was stored at -20 °C until further use.

2.8 Purification Columns

Different commercially available columns were used to filter degraded DNA samples in order to balance the proportion of longer and smaller fragments of DNA. The columns used in this study were Microcon® DNA Fast Flow (Merck Chemicals, UK), Microcon® 30kDa (Merck Chemicals, UK), illustra MicroSpin G50 (GE Healthcare Life Sciences, UK), and MinElute Reaction Cleanup Kit (Qiagen, UK). These columns were chosen for their ability to remove small fragments of DNA from the sample, their threshold goes from 20 bp (illustra MicroSpin G50) to 125 bp (Microcon® DNA Fast Flow).

After purification with the columns, samples were quantitated and amplified with the 4-Plex multiplex along with the original sample to assess the improvement in the DNA profile.

2.8.1 Microcon® DNA Fast Flow Protocol

The filter device was allocated in the provided tube and 5 µL of the sample and 100 µL of distilled H_2O were added. The tube was centrifuged at 13500 x g for 10 min and then a volume of 200 µL of distilled H_2O was added. After another round of centrifugation at 13500 x g for 10 min, the filter device was transferred upside down to a new tube, vortexed for approximately 20 s and was spun at 1000 x g for 5 min. The eluate was stored at -20 °C until further use.

2.8.2 Microcon® 30kDa Protocol

The filter device was allocated in the provided tube and 5 µL of the sample and 100 µL of distilled H_2O were added. The tube was centrifuged at 400 x g for

10 min and then distilled 200 µL of H$_2$O were added. After another round of centrifugation at 400 x g for 10 min, the filter device was transferred upside down to a new tube, vortexed for approximately 20 s and was spun at 1000 x g for 5 min. The eluate was stored at -20 °C until further use.

2.8.3 illustra MicroSpin G50 Protocol

First, the resin in the column was re-suspended by briefly vortexing. The cap was loosened with a one-quarter turn and the bottom closure was twisted. The column was placed in the supplied collection tube and the tube was spun at 2000 x g for 1 min. The column was transferred to a 1.5 mL micro-centrifuge tube and 15 µL of the sample was applied carefully to the top-centre of the resin, with the care of not touching it. The tube was centrifuged at 2000 x g for 2 min and the eluate was stored at -20 °C until further use.

2.8.4 MinElute Reaction Cleanup Kit Protocol

In a 1.5 mL micro-centrifuge tube, a volume of 20 µL from the sample and 300 µL of Buffer ERC were added. Subsequently, the mixture was transferred to a column placed in a 2 mL collection tube and the tube was centrifuged for 1 min at maximum speed (16300 x g). The flow-through was discarded, a volume of 750 µL of Buffer PE was added and the centrifugation was repeated. The flow-through was again discarded and the tube was centrifuged one extra time in the same conditions to completely remove ethanol from the column. The MinElute column was then placed in a new 1.5 mL micro-centrifuge tube and a volume of 10 µL of Buffer EB was added to the centre of the membrane. The tube was allowed to stand on the bench for 1 min, and then was centrifuged for 1 min at maximum speed. The eluate was stored at -20 °C until further use.

2.9 DNA Amplification

Amplification process was performed using the 4-Plex multiplex previously developed in the laboratory (Nazir *et al.* 2013). The primers included in the multiplex amplify fragments of 70, 194, 305, and 384 bp located on the Recombination activating gene 1 (RAG-1) (Table 2.1). First, a primer mix was made adding the volumes in Table 2.2 from the 100 µM stock to 28 µL of dH_2O.

Table 2.1 – Table showing the sequence of the primers used in the 4-Plex multiplex

Amplicon Length (bp)	PCR primers Forward and Reverse (5′-3′)	Melting Temperature (°C)
70	CCTCAAAGTCATGGGCAGC	60
	GACTCTCCAGGTCAGTAGG	60
194	GCTGTTTGCTTGGCCATCCG	63
	GTGCTGGAAGACACATTCTTC	60
305	ATGAGGTCTGGCGTTCCAAC	60
	TGGTCATGAGCTTCCTGGCA	60
384	GAGCAATCTCCAGCAGTCCT	60
	GCTAAACTTCCTGTGCATGA	56

Table 2.2 – Table showing the primer volumes added from the 100 µM stock to 28 µL of dH_2O in order to make the primer mix for the 4-Plex multiplex

Amplicon length (bp)	Volume (µL)	Primer concentration in primer mix (µM)	Primer final concentration in PCR (µM)
70 bp	1.0 F 1.0 R	2.5	0.15
194 bp	0.5 F 0.5 R	1.25	0.075
305 bp	1.0 F 1.0 R	2.5	0.15
384 bp	3.5 F 3.5 R	8.75	0.525

The PCR reaction mix was made using 5.0 µL of Platinum Multiplex PCR Master mix (Life Technologies, UK), 0.6 µL of the Primers mix, 1.0 µL of DNA template, and 3.4 µL of dH_2O, with a total reaction volume of 10 µL.

The PCR was performed on an Applied Biosystems 2720 Thermal Cycler (Life Technologies, UK) and the cycle condition was as shown in Table 2.3.

Table 2.3 – Table showing the PCR cycle conditions for the 4-Plex multiplex

Initial incubation		95 °C	5 min
Denaturation		95 °C	1 min
Annealing	30 cycles	60 °C	1 min
Extension		72 °C	1 min
Final Incubation		72 °C	10 min
Hold		4 °C	∞

2.10 Capillary Electrophoresis

Loading mix for capillary electrophoresis was prepared by mixing 8.5 µL of Hi-Di™ formamide (Life Technologies™, UK) and 0.5 µL of GeneScan™ 500 LIZ Size Standard (Life Technologies, UK). Volumes of 9 µL of the mix and 1 µL of the amplified PCR product were added to each well. Prior to loading in the equipment, samples were denatured by heating at 95 °C for 5 min and then cooling in the freezer for 5 min.

Capillary electrophoresis was performed using ABI 3500 Prism Genetic Analyser (Applied Biosystems) in a 50 cm long capillary using POP-6 polymer (Applied Biosystems, UK). Fragment analysis 50_POP6 run module was used with different dye sets DS – 32 (filter set F): 5 – FAM (blue), Joe (green), NED (yellow) and ROX (red). Following parameters of ABI 3500 POP_6: run temperature 60 °C, run voltage 15.0 kVs, pre-run voltage 15 kV, run time 2700, pre-run time 180 s, injection time 10 s, injection voltage 1.6 kVs, run voltage 15 kV.

2.11 Data Analysis

Data obtained from the capillary electrophoresis were analysed using ABI 3500 GeneMapper Software v4.1 (Applied Biosystems). The parameters for the analysis of DNA profiles were kept the same for every run (Table 2.4).

Table 2.4 – Table showing the parameters employed for the analysis of DNA profiles

Parameters	Values
Analysis Range	Full Range
Baseline Window	51 points
Minimum Peak Half Width	2 points
Peak Detection	50 RFU
Peak Window Size	15 points
Polynomial Degree	3 points
Size Call Range	All sizes
Size Calling Method	Local Southern
Slope Threshold for peak start/end	0-0

2.12 DNA Re-Amplification

Some samples that failed to give a complete profile or showed low fluorescence and were re-amplified using another in-house-developed multiplex, the Mini-4-Plex (Hassan 2017). The primers included in this multiplex amplify fragments of 50, 75, 112, and 154 bp (Table 2.5). Firstly, a primer mix was made adding the volumes found in Table 2.6 from the 10 µM stock.

Table 2.5 – Table showing the primers used in the Mini-4-Plex multiplex

Nuclear gene	Amplicon Length (bp)	PCR primers Forward and Reverse (5′-3′)	Melting Temperature (°C)
Recombination activating gene 2 (RAG-2)	50	TGGATTACATGCTGCCCTACT TGGTACCCAAGTGTTGATATCCA	58 59
Recombination activating gene 2 (RAG-2)	70	ACCCAGCCACTTGCACAT T TTTCCCTCCATGGATGATGT	60 59
Recombination activating gene 1 (RAG-1)	112	GAGGGAGCTCAAGCTGCAA GTGCTCATTCCTCGCCCT	60 59
Recombination activating gene 1 (RAG-1)	154	TCGGGGACTCAAGAGGAAGA GCAGTTGGCGATCTTCTTCA	59 58

Table 2.6 – Table showing the primer volumes added from the 10 µM stock solution to make the primer mix for the Mini-4-Plex Multiplex

Amplicon length (bp)	Volume (µL)	Primer concentration in primer mix (µM)	Primer final concentration in PCR (µM)
50 bp	10 F 10 R	1.2 1.2	0.072 0.072
75 bp	11 F 11 R	1.3 1.3	0.078 0.078
112 bp	10 F 10 R	1.2 1.2	0.072 0.072
154 bp	11 F 11 R	1.3 1.3	0.078 0.078

The PCR reaction mix was made using 5.0 µL Platinum Multiplex PCR Master mix (Life Technologies, UK), 0.6 µL of the Primers mix, 1.0 µL DNA template, and 3.4 µL of dH_2O, with a total reaction volume of 10 µL.

The PCR was performed on the Applied Biosystems GeneAmp® PCR System 9700 (Life Technologies, UK) and the cycle conditions can be found in Table 2.7. After amplification, samples were analysed using capillary electrophoresis as described previously in session 2.10.

Table 2.7 – Table showing the PCR cycle conditions for the Mini-4-Plex multiplex

Initial incubation		95 °C	2 min
Denaturation		95 °C	30 s
Annealing	28 cycles	60 °C	1:30 min
Extension		72 °C	30 s
Final Incubation		60 °C	30 min
Hold		4 °C	∞

2.13 DNA Capture

The method of DNA Capture with magnetic bead hybridization was used to attempt to enhance the quality of DNA profiles obtained from degraded samples.

The same primers from the 4-Plex multiplex were used (see table 2.1), with the addition of Biotin-TEG to the 5′ end. Primer Extension Capture modified from the method of Briggs *et al.* (2009) was used in this experiment and was performed as follows.

For the Primer Extension Reaction, 1 µL of DNA (5-50 ng), 48 µL of Platinum Singleplex Mastermix, and 0.5 µL of each biotinylated primer were added in a PCR tube to form a 50 µL reaction. The mixture was then transferred to the Applied Biosystems 2720 Thermal Cycler (Life Technologies, UK) and 2 cycles were performed to bind the biotinylated primers to the target DNA. Cycle conditions were as shown in Table 2.8.

Table 2.8 – Table showing the cycle conditions for the Primer Extension Reaction

Initial incubation		95 °C	5 min
Denaturation		94 °C	2 min
Annealing	2 cycles	60 °C	2 min
Extension		72 °C	2 min

After the primer extension reaction, samples were purified using the MinElute Reaction Cleanup Kit according to manufacturer's instructions. Immediately after the end of the reaction, the Buffer ERC was added to avoid the possibility of non-specific priming as the reaction cools. Samples were re-suspended in 25 µL of Buffer EB at the end of purification.

For the bead capture step, Dynabeads™ MyOne™ Streptavidin C1 (Thermo Fisher Scientific, UK) beads were used. This step was adapted from the manufacturer's suggested protocol. For binding and washing, 2x Binding and Wash (B&W) Buffer (10 mM TRis-HCl (pH 7.5), 1 mM EDTA, and 2 M NaCl) was used as recommended by the beads' manufacturer. For washing of DNA, the buffer was diluted to 1X B&W Buffer with distilled water.

First, a volume of 25 µL of beads was transferred to a clean 1.5 mL microcentrifuge tube and with the help of the magnetic stand, the beads were washed twice in 500 µL of 2X B&W Buffer. Beads were then re-suspended in 25 µL of 2X B&W Buffer and 25 µL of the purified DNA from the primer extension reaction were added. The mixture was incubated at room temperature for 30 min with vortexing at every 2-3 min. Samples were washed five times with 500 µL of 1X B&W Buffer and re-suspended in 30 µL of Buffer EB. For the separation of the beads and the DNA captured, samples were then incubated at 95 °C for 2:30 min. With the help of a magnetic stand, the DNA in solution was collected and transferred to a new tube.

After recovering the DNA with the help of magnetic beads, the capture product was amplified with the use of PCR. For a total volume of 50 µL, 12.5 µL of the capture product was added in a PCR tube with 34.5 µL of Platinum Singleplex MasterMix and 1.5 µL of each non-biotinylated primer. Forward primers used in this reaction were labelled with fluorescein dye at the 5′ end for capillary

electrophoresis analysis (the same used in the 4-Plex multiplex). The conditions of the PCR were the same as used in the 4-Plex Multiplex (Table 2.3).

Samples were then analysed with capillary electrophoresis using an ABI 3500 Prism Genetic Analyser (Applied Biosystems) and ABI 3500 GeneMapper Software v4.1 (Applied Biosystems).

After determining that the primer pairs were working correctly with the protocol developed, multiplex reactions were performed combining two or three primer pairs. The protocol for these reactions was similar to the one used for singleplex reactions, with two main differences. The first was in the Primer Extension Reaction, where the second or third primer pair was added in the same volume as the first and the Platinum Multiplex PCR Mastermix was added in an adjusted volume to reach 50 µL. The second difference was in the amplification step, where some of the reactions were performed using the 4-Plex multiplex.

2.14 Statistical Data Analysis

Samples were analysed in triplicates, when possible (in DNA quantitation results) the data were presented by the average of the triplicates with the standard error of the mean (SEM) present. Calculations of averages and SEM were carried out using Excel 2013.

The experiments in this work were designed in order to provide guidelines on time points and advice to forensic practitioners. Further statistical analysis would not have added useful information to the results.

CHAPTER 3

ESTABLISHMENT OF MOLECULAR TOOLS FOR USE IN THE THESIS

3.1 Introduction

Samples for human remains identification and forensic samples can be degraded, contain inhibitors or a low quantity of DNA. Since repetitive analyses to produce good quality DNA profiles are costly, laboratories are continuously demanding enhanced technologies to process database and casework samples more efficiently and effectively (Alaeddini *et al.* 2010; Golenberg *et al.* 1996). Since pig muscle tissue was used as an experimental model throughout the experiments in this thesis, commercial STR kits could not be used to assess the degradation state of the DNA. As such, it was necessary to employ an alternative method. The aim of this chapter was to introduce the methods used through the thesis for degrading DNA and DNA profiling.

3.1.1 The 4-Plex and The Mini-4-Plex PCR Assays

In order to assess degradation in DNA samples, a multiplex was developed in the Forensic Genetic Research Group at UCLAN. The 4-Plex PCR assay amplifies four regions of the recombination activating gene 1 (RAG-1). It has products between 70 bp and 384 bp that can be detected using conventional capillary electrophoresis (Nazir *et al.* 2011).

The RAG-1 has highly conserved base composition across taxa, without any asymmetry in directional patterns (Bernstein *et al.* 1996; Carlson *et al.* 1991; Schatz *et al.* 1989). This gene was a suitable target due to high levels of similarity in conserved regions between rabbit and human, rabbit and pig and pig and human.

Following developments in techniques that reduced the size of fragments analysed, another in-house multiplex was later developed. The Mini-4-Plex has smaller amplicons of 50 bp, 70 bp from the recombination activating gene 2

(RAG-2) and 112 bp, 154 bp from RAG-1 (Hassan 2017). This new assay was developed to assess the degree of degradation and study the fragments with ranges between 50 bp to 150 bp. This range is useful to assess degradation prior to the use of indels or SNPs.

3.1.2 DNA Degradation Using DNase I

In order to study degradation, it was necessary to choose a technique that allowed for a controlled degradation and produced a degradation curve that was easily reproducible. As such, the study employed degradation with DNase I.

Deoxyribonuclease I (DNase I) is a nonspecific endonuclease that degrades double-stranded DNA, single-stranded DNA, and chromatin. It functions by hydrolysing phosphodiester linkages, producing mono and oligonucleotides with a 5′-phosphate and a 3′-hydroxyl group (Samejima and Earnshaw 2005). DNase I is mainly used in applications where maintaining RNA integrity is critical, but it can be used for a controlled degradation of DNA.

3.2 4-Plex Multiplex Assay

3.2.1 Previous Development and Validation

The 4-Plex multiplex was previously developed and validated in the Forensic Genetics Group at UCLAN by Nazir *et al.* (2011) using the following method.

RAG-1 sequence data for rabbit, pig and human were aligned and conserved regions were selected. The four primer pairs were designed to generate amplicons of 70 bp, 194 bp, 305 bp and 384 bp across the gene (Table 3.1). Each forward primer was labelled with fluorescein dye at the 5′ end.

Table 3.1 – Table showing the primer sequences of amplicons included in the 4-Plex multiplex

Amplicon Length (bp)	PCR primers Forward and Reverse (5′-3′)	PCR Annealing Temperature (°C)
70	CCTCAAAGTCATGGGCAGC	60
	GACTCTCCAGGTCAGTAGG	60
194	GCTGTTTGCTTGGCCATCCG	63
	GTGCTGGAAGACACATTCTTC	60
305	ATGAGGTCTGGCGTTCCAAC	60
	TGGTCATGAGCTTCCTGGCA	60
384	GAGCAATCTCCAGCAGTCCT	60
	GCTAAACTTCCTGTGCATGA	56

Initially the primers were optimised in single PCR reactions and then combined in a multiplex. The four primer pairs were found to function optimally at concentrations of 0.05 µM, 0.1 µM, 0.15 µM and 0.4 µM for 70 bp, 194 bp, 305 bp and 384 bp respectively.

Optimal conditions for the PCR reaction mix were found to be 5.0 µL of Platinum Multiplex PCR Master mix (Life Technologies, UK), 0.6 µL of the primers mix, 1.0 µL of DNA template (with concentration between 0.5-1 ng/µL), and 3.4 µL of dH$_2$O, with a total reaction volume of 10 µL. The PCR cycle conditions employed in this study are depicted in Table 3.2.

Table 3.2 – Table shows the PCR cycle conditions for the 4-Plex multiplex

Initial incubation		95 °C	5 min
Denaturation		95 °C	1 min
Annealing	30 cycles	60 °C	1 min
Extension		72 °C	1 min
Final Incubation		72 °C	10 min
Hold		4 °C	∞

The assay proved to be precise and reproducible by repetitive analyses of positive controls with independent runs on capillary electrophoresis. The standard deviation of the amplicon sizes for multiple runs of the same and different samples was below one base pair for all amplicons.

In order to assess the sensibility of the assay, 4-Plex multiplex PCR reactions were set up using serial dilutions of positive control DNA samples of human, rabbit and pig. The 4-Plex multiplex was found to work efficiently in triplicate samples of all three species down to 0.03 ng of DNA template. The assay was tested for its use with degraded samples using a degradation series and for non-specificity with DNA from various insect species active in carcass decomposition.

3.2.2 Maintenance of Quality

Since this was an in-house assay and not a commercial kit, conditions and parameters had to be re-assessed periodically to ensure that they were working appropriately. Stock primers degrade even when stored at -20 °C and so primer concentration can diminish with time. Due to that, primer volumes to create the primer mix for the PCR needed to be empirically determined in order to generate standard profiles with balanced peaks among amplicons.

The first step was to ensure that primers were working properly by performing single reactions of each primer pair. The first set of reactions was performed with primer stocks of 10 µM that had been previously diluted and did not work. A new

set of reactions was performed with 100 µM stocks, which produced satisfactory

results (Figure 3.1).
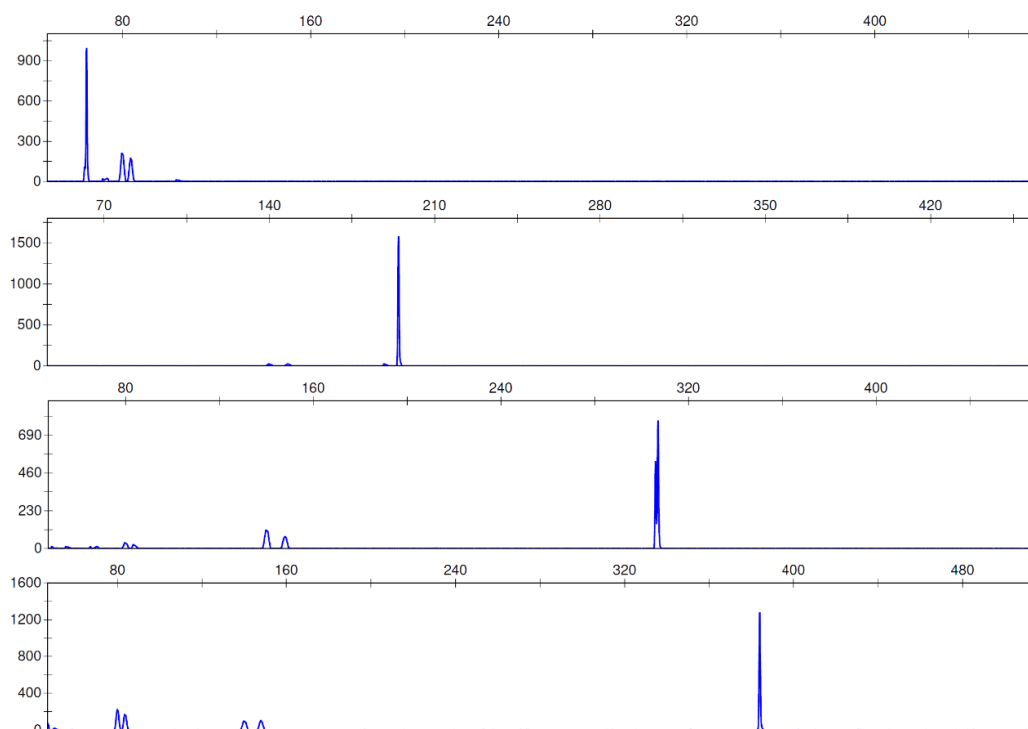
Figure 3.1 – Electropherograms of the singleplex amplifications performed with
100 nM stock primers. Some extra peaks appeared, probably due to primer
excess.

A new 10 µM dilution was made and also resulted in good amplification results

(Figure 3.2). The samples were then joined in a multiplex reaction, first with the

same volume (1 µL, roughly 1.25 µM) for each primer pair. Based on the results

obtained, primers added to the primer mix were adjusted until peak balance was

reached (Figure 3.3). This process was repeated periodically whenever the

reaction controls started showing unbalanced peaks. The primer concentrations

described throughout were based on the final optimisation, but did vary slightly

between experiments.
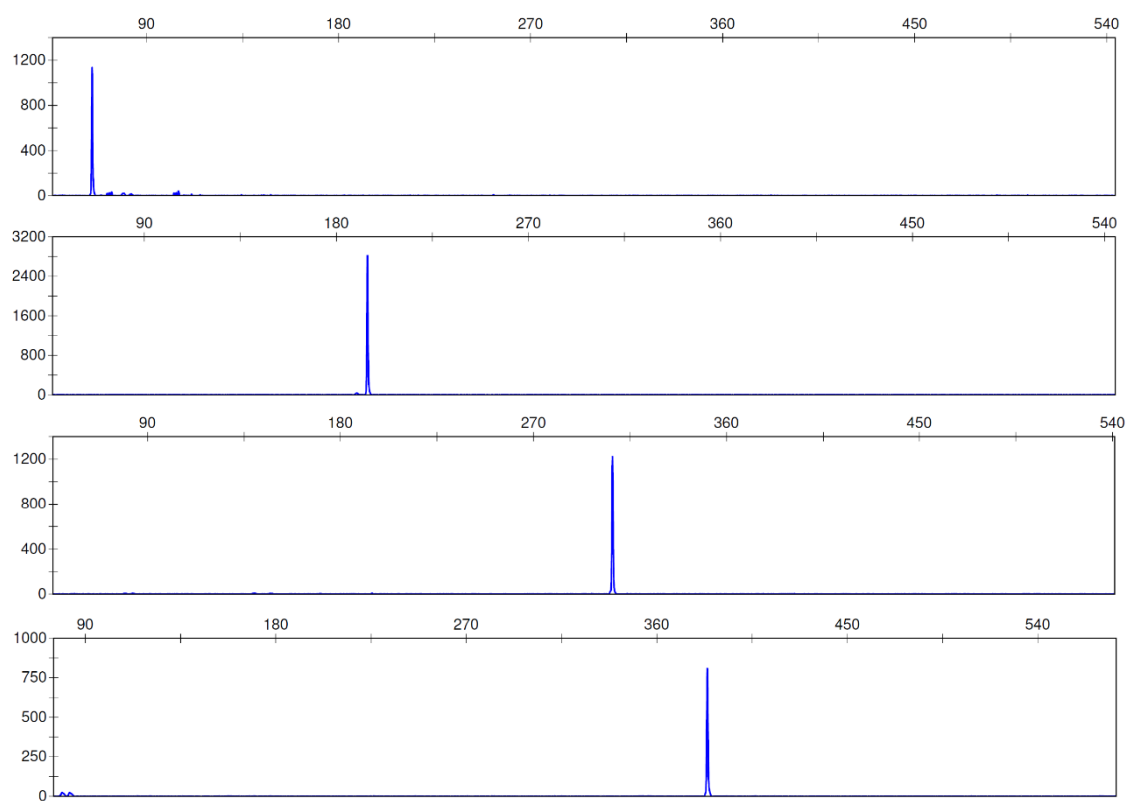
Figure 3.2 – Electropherograms of singleplex amplifications performed with 10 nM stock primers.
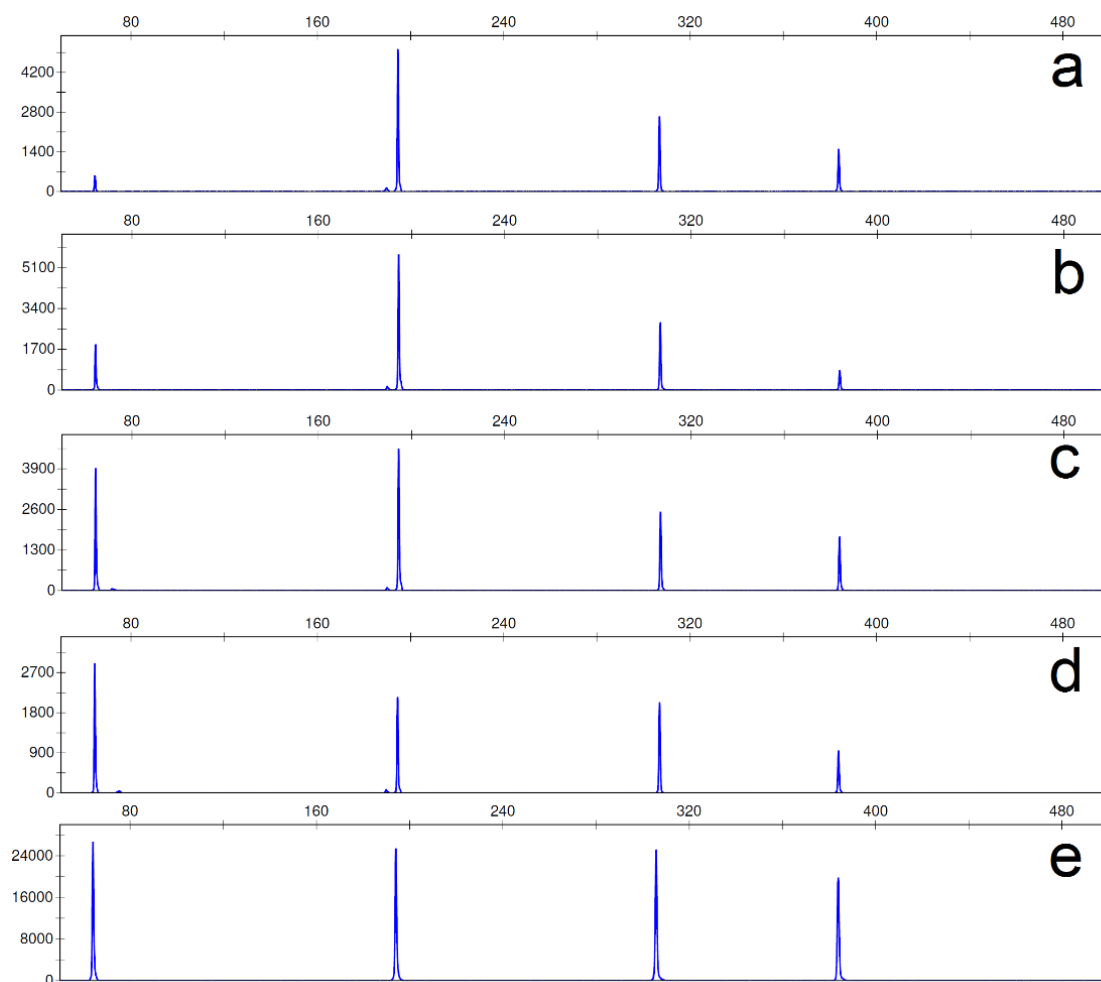
Figure 3.3 – Examples of electropherograms of different primer mixes used while trying to balance the 4-Plex multiplex. (a) is the original primer mix; (b-d) are different mixes with varying volumes of the primers; (e) is the new primer mix used with balanced peaks.

3.3 Mini-4-Plex Multiplex Assay

3.3.1 Previous Development and Validation

Another multiplex PCR tool, the Mini-4-Plex, was also developed in the Forensic Genetic Research Group. This assay is also specific to human and pig DNA, but it has shorter amplicons of 50 bp, 70 bp and 112 bp, 154 bp, and it was meant to assess the degree of DNA degradation for fragments within the range of SNPs and Indels. Development and validation of this new assay were carried out by Balnd Mustafa Albarzinji (current PhD Student, UCLan) and by Hassan (2017). The procedure was as follows.

Four new markers were selected for the new assay, with amplicons lengths of 50 bp and 70 bp from RAG-2 and 112 bp, 154 bp from RAG-1 as these genes are co-expressed together. The sequences and annealing temperature of primers can be found on Table 3.3.

Table 3.3 – Table showing the primer sequences of the Mini-4-Plex amplicons.

Nuclear gene	Amplicon Length (bp)	PCR primers Forward and Reverse (5′-3′)	PCR Annealing Temperature (°C)
RAG-2	50	TGGATTACATGCTGCCCTACT	58
		TGGTACCCAAGTGTTGATATCCA	59
RAG-2	70	ACCCAGCCACTTGCACAT T	60
		TTTCCCTCCATGGATGATGT	59
RAG-1	112	GAGGGAGCTCAAGCTGCAA	60
		GTGCTCATTCCTCGCCCT	59
RAG-1	154	TCGGGGACTCAAGAGGAAGA	59
		GCAGTTGGCGATCTTCTTCA	58

Amplification of the new markers was first optimised in single reactions and amplification as a multiplex was performed using a final reaction volume of 10 μL. Volumes of the PCR mix were the same as the ones used for the 4-Plex, with the cycle conditions shown in Table 3.4.

Table 3.4 – Table showing the PCR cycle conditions for the Mini-4-Plex multiplex

Initial incubation		95 °C	2 min
Denaturation		95 °C	30 s
Annealing	28 cycles	60 °C	1:30 min
Extension		72 °C	30 s
Final Incubation		60 °C	30 min
Hold		4 °C	∞

The sensibility of the assay was assessed by preparing a serial dilution with different concentrations of a control DNA and amplification. The results showed that amplification of the Mini-4-Plex markers was obtained as full profiles until the concentration of 0.03 ng/µL. The Mini-4-Plex multiplex PCR is balanced for 0.5 to 2 ng/µL of template DNA and was also tested with degraded DNA samples.

3.3.2 Maintenance of Quality

Similar to the 4-Plex multiplex, the Mini-4-Plex required periodically re-assessment to ensure quality results. A similar methodology was used to re-balance primer concentrations for the use of the assay in this study.

3.4 DNA Degradation with DNase I

Artificial degradation of human DNA was carried out using RQ1 RNase-Free DNase I (Promega, UK) as described by Swango *et al.* (2006) and Asamura *et al.* (2007) at a final volume of 110 µL. A commercially available high molecular weight DNA, Human Genomic DNA (Promega, UK) was used.

Prior to the experiment, the enzyme was diluted 100 times from 1 U/µL to 0.01 U/µL. One unit of RQ1 RNase-Free DNase is defined as the amount required to completely degrade 1 µg of lambda DNA in 10 min at 37 °C in 50 µL of a buffer containing 40 mM Tris-HCl (pH 7.9 at 25 °C), 10 mM NaCl, 6 mM MgCl$_2$ and 10 mM CaCl$_2$.

The reaction was prepared by mixing 15 µg of DNA, 10X DNase I Reaction Buffer (400 mM Tris-HCl (pH 8.0), 100 mM MgSO$_4$, and 10 mM CaCl$_2$) and sterile water to reach a volume of 110 µL. As a control sample, a volume of 10 µL of the reaction mixture was removed at this point. The diluted DNase I (6 µL) was added to the remaining solution and the mixture was incubated at 37 °C for digestion.

Aliquots of 10 µL were taken from this mixture at (2, 5, 10, 20, 30, 45, 60, 90, 120, and 180) min after incubation started. The reaction was stopped by adding 2 µL of the Stop Solution (20 mM EGTA (pH 8.0)) to each aliquot and incubating at 65 °C for 10 min. The control sample was "stopped" in the same way as the other samples.

Degradation was then assessed with a 2.5% agarose gel electrophoresis using 2 µL of the digested product. Quantification was done using Qubit according to manufacturer's instructions. Both multiplex PCR were performed simultaneously to determine the amplification success of DNA amplicons of different ranges in the samples.

In the agarose gel electrophoresis it was possible to see the control sample and the 2 and 5 min showing large amounts of high molecular weight DNA, but with the incubated samples there also was some degraded DNA present. After 20 min of incubation, there was no high molecular weight band present (Figure 3.4) and later incubation times, as expected, followed this pattern.
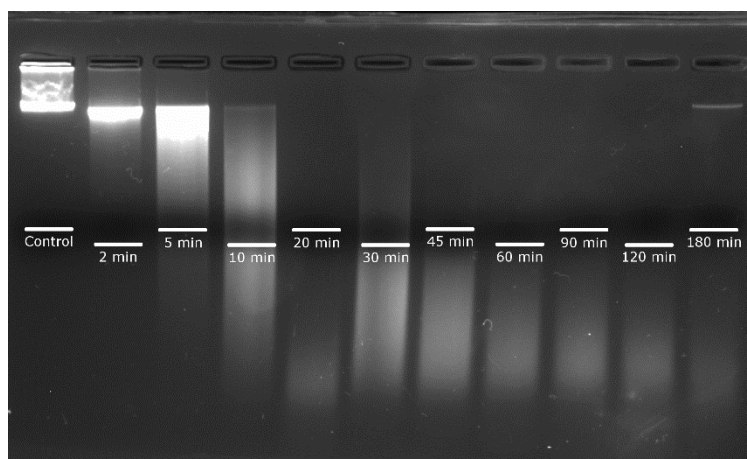
Figure 3.4 – A photograph showing the agarose electrophoresis gel from samples obtained by degrading DNA with DNase I. It is possible to see the smear associated with DNA degradation starting after 2 min of incubation.

After analysis with agarose gel electrophoresis, samples were diluted with 9 parts of dH$_2$O. Amplification with the 4-Plex and Mini-4-Plex multiplexes were performed for all time points using 1 µL of the diluted samples.

The 4-Plex multiplex had full profiles up until 45 min of incubation, with complete absence of amplification in the 180 min samples (Table 3.5). The Mini-4-Plex multiplex only had a drop out of the larger amplicon and after 180 min of incubation (Table 3.6). The RFUs continued to decrease with passing of time in both multiplexes, especially in the larger amplicons (305 and 384 in the 4-Plex and 112 and 154 in the Mini-4-Plex), but ultimately in all (Figures 3.6 and 3.7).

Table 3.5 – Table showing the results of the 4-Plex multiplex amplification for commercial DNA degraded with DNase I.

Time	4-Plex amplification of samples degraded with DNase I			
	70 bp	194 bp	305 bp	384 bp
Control	+++	+++	+++	+++
2 minutes	+++	+++	+++	+++
5 minutes	+++	+++	+++	+++
10 minutes	+++	+++	+++	+++
20 minutes	+++	+++	+++	+++
30 minutes	++-	++-	++-	++-
45 minutes	+++	++-	++-	++-
60 minutes	+++	(+)+-	-(+)-	-+-
90 minutes	++-	---	---	---
120 minutes	+++	---	---	---
180 minutes	---	---	---	---

The "+" denotes the presence and "-" represents the absence of PCR amplicons in samples during the 4-Plex multiplex amplification. (+) represents positive results below threshold of 50 RFU.

Table 3.6 – Table showing the results of the Mini-4-Plex multiplex amplification for commercial DNA degraded with DNase I.

Time	Mini-4-Plex amplification of samples degraded with DNase I			
	50 bp	75 bp	112 bp	154 bp
Control	+++	+++	+++	+++
2 minutes	+++	+++	+++	+++
5 minutes	+++	+++	+++	+++
10 minutes	+++	+++	+++	+++
20 minutes	+++	+++	+++	+++
30 minutes	+++	+++	+++	+++
45 minutes	+++	+++	+++	+++
60 minutes	+++	+++	+++	+++
90 minutes	+++	+++	+++	+++
120 minutes	+++	+++	(+)++	(+)++
180 minutes	+++	+++	(+)++	-++

The + denotes the presence and - represents the absence of PCR amplicons in samples during the 4-Plex multiplex amplification. (+) represents positive results below threshold of 50 RFU.

Figure 3.5 – Examples of electropherograms of 4-Plex multiplex profiles obtained from commercial DNA degraded with DNase I. These samples were collected: (a) after 2 min, (b) after 20 min, and (c) after 180 min of incubation and show a full profile, a profile with larger loci affected by degradation and no profile.
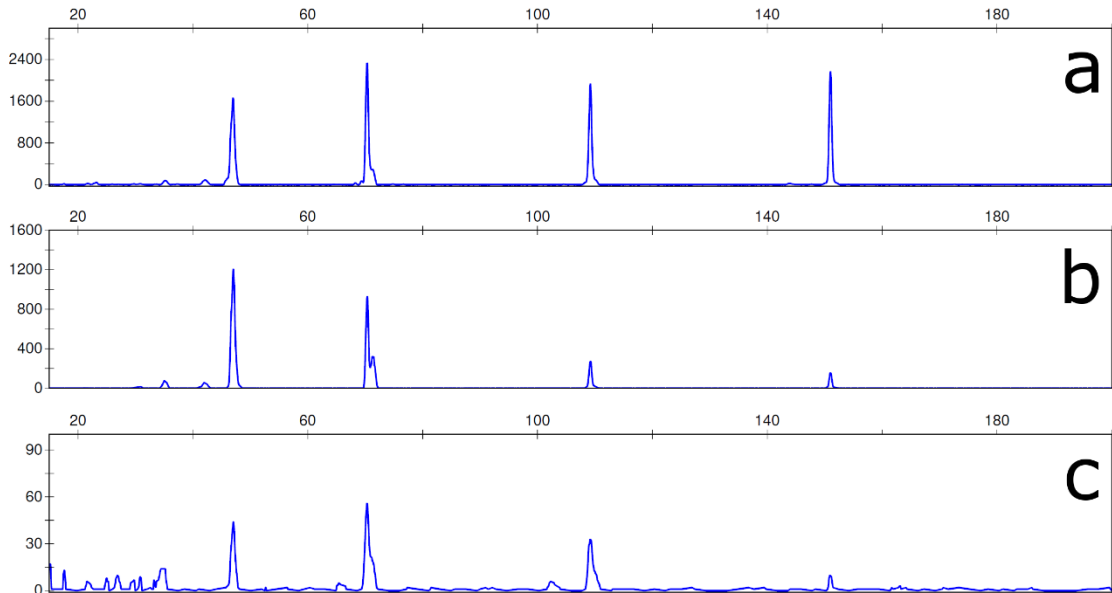
Figure 3.6 – Examples of electropherograms of the Mini-4-Plex multiplex profiles obtained from commercial DNA degraded with DNase I. (a) was degraded for 2 min, and (b) for 60 min. On (c) it is possible to see that some of the amplicons are clear but below the threshold of 50 RFU with 180 min of incubation.

3.5 Discussion

Two multiplexes were previously developed in the laboratory as a forensic biochemical tool to characterize degradation patterns from samples. The larger multiplex (4-Plex) has a range between 70 bp and 384 bp, and the range of the smaller multiplex (Mini-4-Plex) is between 50 bp and 154 bp (Hassan 2017; Nazir *et al.* 2011).

The 4-Plex multiplex requires a very low quantity of DNA template, efficiently amplifies the product from samples that have been exposed to the environment, and produced the expected sized fragment from highly degraded DNA samples. It was found that full 4-Plex profiles for human, pig and rabbit can be obtained using a template amount of 0.3 ng. This multiplex can be used in forensic analysis to assess the DNA persistence in human decomposing bodies and in experimental studies.

The Mini-4-Plex multiplex PCR is sensitive and robust as a tool to study short amplicons in the range of 50 bp to 170 bp. A very small amount of DNA template is needed to obtain a reliable profile. This multiplex can be used to assess the highly degraded DNA from samples that have been exposed to the environment and to differentiate between degradation and PCR inhibition in challenging samples. The use of the Mini-4-Plex allows the further understanding of how degraded DNA fragments are in comparison with the 4-Plex. This shorter multiplex also allows for the visualization of fragments above 100 bp and below 194 bp, an important size range for INDEL analysis.

Both the 4-Plex and the Mini-4-Plex are very useful tools for studying degraded samples and degradation. Having to repeat the optimisation periodically is a drawback, but easily compensated by cost. Being in-house developed makes

them much cheaper than a commercial-developed kit, with the added benefit of having the primers also working with model-species, facilitating research. For the samples used in this study, the 4-Plex assay was more informative than the Mini-4-Plex. The level of degradation created by digesting the DNA with DNase I was not sufficiently severe to need the use of the Mini-4-Plex for discrimination.

A current alternative to study degradation levels in samples is the use of real-time PCR. Some commercial kits available for quantification use two different sizes of markers and compare them both to create a degradation index. However, these commercial kits use primers that are specific to humans and this limits their use. In addition, optimization of real-time PCR reactions are more complicated than that for capillary electrophoresis. Therefore, using an in-house developed protocol would be troublesome.

The choice of using DNA degraded with DNase I in this study was because the technique is relatively easy to perform and it can generate both a controlled and reproducible degradation. It also means that it is possible to choose the samples' degree of degradation for analysis with different techniques.

It was not straightforward to obtain a good degradation pattern with DNase I. Some repetitions were needed to ascertain the correct concentration of the enzyme, since its activity was initially too high for the reaction. After this was established, both agarose gels and electropherogram profiles showed a pattern more consistent with an increasing degradation curve. Degradations done after those generated similar results.

The DNA degraded with DNase I shows a decreased intensity of fluorescence proportional to incubation times. In the 4-Plex multiplex, this decrease is such that the samples incubated for 180 min had no amplification. The Mini-4-Plex multiplex did not reach this extreme, but one of the sets of degraded DNA of the

triplicate had a drop out of the larger alleles after 180 min of incubation – this illustrates the robustness and sensitivity of the Mini-4-Plex.

In conclusion, the results in this chapter have clearly demonstrated that the use of the two multiplexes is a useful way of assessing degradation in samples that are expected to have degraded DNA, therefore proving their usefulness for the experiments of this thesis. DNA degraded with the use of DNase I is a proper forensic biochemical tool to study techniques to improve DNA profiles from degraded samples. It also allows control of the degree of degradation and is easy to repeat and produce more degraded DNA for study.

CHAPTER 4

DNA DEGRADATION PATTERNS IN RESPONSE TO ENVIRONMENTAL INSULTS

4.1 Introduction

In some crime scenes or following mass fatality incidents, human remains can be fragmented, burnt and/or decomposed, making victim identification by means of fingerprinting or odontology extremely difficult (Graham *et al.* 2008). When a human cadaver is exposed to challenging environmental conditions, substantial decomposition will occur, with the soft tissue of the body starting to decay within a few days (Goff 2009).

Decomposition leads to DNA degradation, which makes it more difficult to obtain a complete DNA profile. In addition, artefacts such as preferential amplification, allele drop out and locus drop out become more common (Bender *et al.* 2004; Foran 2006; Lindahl 1993; Senge *et al.* 2011).

Body decomposition is influenced by ambient temperature, humidity, and also by insect activity (Shirley *et al.* 2011; Zhou and Byard 2011). A previous study found that the bodies of pigs exposed to the environment in the UK had different rates of DNA degradation depending on the season of the year when the work was done (Nazir *et al.* 2011). Experiments ran during the summer and subjected to temperatures up to 23 °C, as expected, had faster DNA degradation than experiments that occurred during winter/spring or autumn. It was concluded that to the most significant factor was the difference in maggot mass growth (increased maggot mass results in increased carcass decomposition and ultimately decreased DNA persistence).

The same study also degraded samples in a laboratory controlled environment at the temperatures of 27 °C, 37 °C, and 47 °C in order to understand the effect of different temperatures in DNA persistence. These laboratory incubated samples were compared to the field incubated ones. It was found that DNA

degradation occurred faster in the laboratory samples when comparing similar ADD of field and laboratory incubated samples (Nazir 2012). The limitation of this comparison was that field temperatures during the study (and generally in the UK) were not above 25 °C.

As a follow-up to these previous studies (Nazir *et al.* 2011; Nazir 2012), the experiments in this chapter of the current study were designed to access DNA persistence under field and controlled conditions. Field exposure was undertaken during the summer in Thailand, where the annual average temperature is of 29 °C. Incubation in the laboratory was carried out at room temperature (25 °C) and at the previous temperature of 37 °C.

PART ONE: DNA DEGRADATION IN CONTROLLED ENVIRONMENTS

4.2 Aims and Objectives

The main aim of this part was to compare DNA degradation patterns in soft muscle tissue decomposed in a controlled environment and in environmental conditions. Part one has the following objective:

- To determine the effect of two different controlled temperatures (25 °C and 37 °C) on DNA persistence in pig soft muscle tissue.

4.3 Materials and Methods

Resumed methods were as follows, Chapter 2 contains more details.

4.3.1 Samples

This series of experiments was designed to study DNA persistence in samples incubated in a controlled environment, to enable comparisons with samples degraded in a natural environment.

Muscle tissue from freshly killed (<5 h) pig was cut in pieces of approximately 4 cm^2 and each piece was placed in a suspended platform made of wire inside a 50 mL polypropylene tube (Figure 4.1). Tissue was suspended in order not to be in contact with the liquid expelled during decomposition.

Samples were incubated at both 25 °C and 37 °C. Samples incubated at 25 °C were collected on Days 1, 4, 7, and every three days up to Day 40, roughly at the same hour. Collection of samples incubated at 37 °C was done after 12 h, 24 h, 36 h, 48 h, 60 h, 72 h, and then every 24 h up to 240 h (10 days).
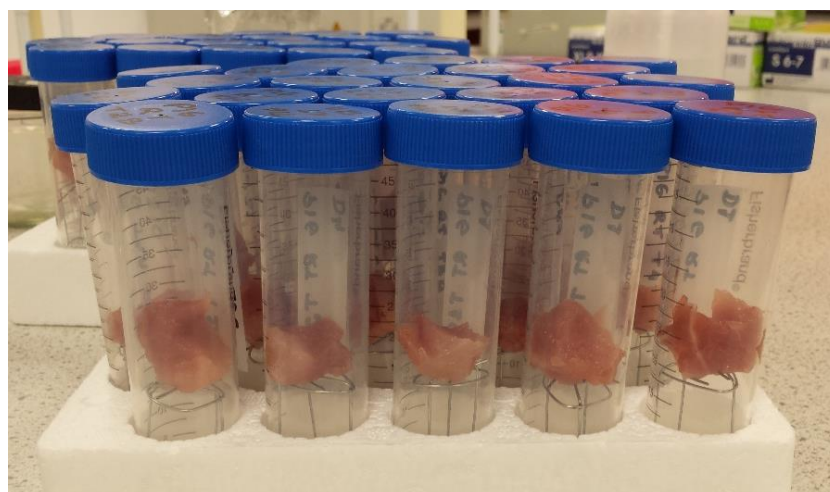
Figure 4.1: A photograph showing the structure of the polypropylene tubes with muscle samples before incubation.

Samples were collected in triplicates (three tubes) and immediately frozen until DNA extraction. One triplicate of samples for each incubation temperature was frozen at the setting up of the experiment to be used as control.

4.3.2 Analysis of Samples

Extraction of DNA was performed using DNeasy® Blood and Tissue kit according to manufacturer's instructions. In order to determine the presence of DNA, a volume of 2 µL of the samples was analysed using agarose gel electrophoresis. Quantification was carried out using Qubit using 1 µL of the samples, according to manufacturer's instructions. The 4-Plex multiplex was performed to determine the amplification success of 70 bp, 194 bp, 305 bp, and 384 bp amplicons in the samples. For some of the samples, another amplification was made using the Mini-4-Plex with amplicon sizes of 50 bp, 70 bp, 112 bp, and 154 bp. Statistical data analysis was performed as described in Chapter 2.

4.4 Results

4.4.1 Incubation at 25 °C

A total of 42 samples was collected from incubation at 25 °C, plus the three controls separated at the set-up moment. The consistency of most samples changed with incubation, with them becoming softer and samples from later collection points being essentially liquefied. Some of the samples had mould on them at the collection moment.

With the use of agarose gel electrophoresis, it was possible to see the smears associated with DNA degradation after 4 days of incubation (Figure 4.2). On the triplicate collected after 7 days of incubation, one of the samples seems to show no presence of high molecular weight DNA. This is repeated in other triplicates afterwards, but the overall presence of high molecular weight DNA seems to be maintained until day 37 of incubation. It is also possible to see a decrease in intensity of the bands with passing incubation time. Some samples presented bands in the lower part of the agarose gel, indicating that low molecular weight DNA was largely present.
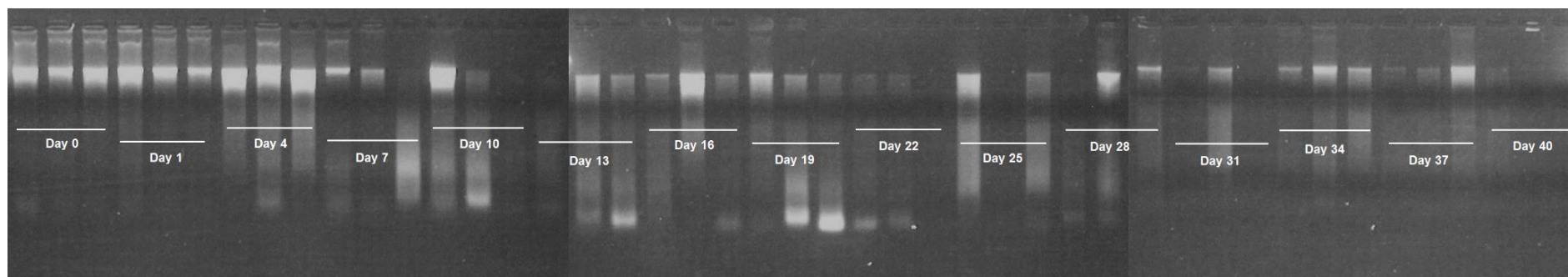
Figure 4.2 – A photograph showing the agarose electrophoresis gels from samples incubated at 25 °C at the laboratory. It is possible to see the smear associated with DNA degradation after only 4 days of incubation. Each lane equals to one sample (n=1) but experiments were done in triplicates.

A similar pattern of results was observed with DNA quantitation (Figure 4.3). All samples that had a clear high molecular weight DNA band on the gel electrophoresis resulted in high DNA quantitation results. However, the opposite was not true, as some of the samples with high DNA concentrations did not present a high molecular weight band. DNA quantification using Qubit quantifies double stranded DNA regardless of the species, thus the DNA detected through this method can come from microbial sources as well as from the muscle tissue. Since samples were not weighted before DNA extraction, quantitation results can only be analysed qualitatively (i.e. for the presence or absence of DNA).
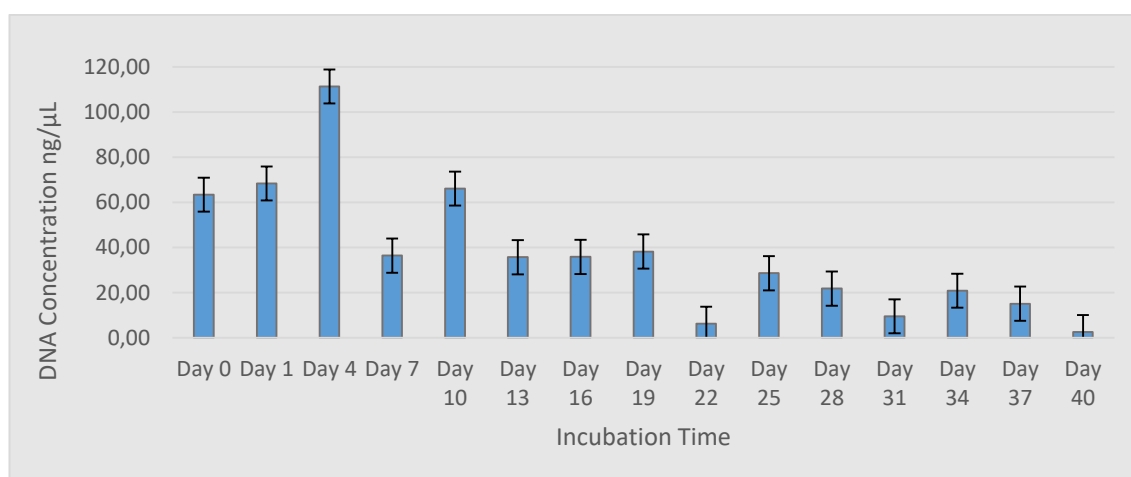
Figure 4.3 – Bar charts showing the average DNA concentrations of samples incubated at 25 °C at the laboratory from Day 0 to Day 40. The results show no change in Day 1, followed by a peak in Day 4 and subsequently continuous time-dependant decrease in quantitation values. All data are presented as mean ± SEM, n=3.

All samples had complete amplification profiles up to 4 days of incubation at 25°C. Two of the samples of the triplicate collected after one week of incubation had no amplification profile, but the last sample to generate a complete profile was from day 13 (Table 4.1). One sample taken out of incubation on day 37 had clear peaks for all four amplicons, but only the 70 bp one was above the threshold of

50 RFU (Figure 4.4). Apart from this sample, there was complete drop out in all samples from day 31 onwards.

Table 4.1 – Table showing the results of the 4-Plex multiplex amplification for samples incubated at 25 °C.

Time	ADD	4-Plex amplification of samples incubated at 25 °C			
		70 bp	194 bp	305 bp	384 bp
Time 0	0	+++	+++	+++	+++
Day 1	25	+++	+++	+++	+++
Day 4	100	+++	+++	+++	+++
Day 7	175	--+	--+	--+	--(+)
Day 10	250	+++	++-	(+)+-	(+)+-
Day 13	325	+++	--+	--+	--+
Day 16	400	+++	---	---	---
Day 19	475	+++	---	---	---
Day 22	550	+++	---	---	---
Day 25	625	+++	--+	---	---
Day 28	700	+(+)-	---	---	---
Day 31	775	---	---	---	---
Day 34	850	---	---	---	---
Day 37	925	+--	(+)--	(+)--	(+)--
Day 40	1000	---	---	---	---

The "+" denotes the presence and "-" the absence of PCR amplicons in samples. The (+) represents clear peaks below threshold of 50 RFU.

Samples that failed to give a full 4-Plex profile were also amplified with the Mini-4-Plex multiplex. Due to the smaller size of the amplicons in this multiplex, drop-outs in samples started only after 16 days of incubation. After this, profile quality varied, but a complete profile was obtained in one of the samples that was incubated for 40 days (Table 4.2). Figure 4.5 shows some examples of electropherograms.
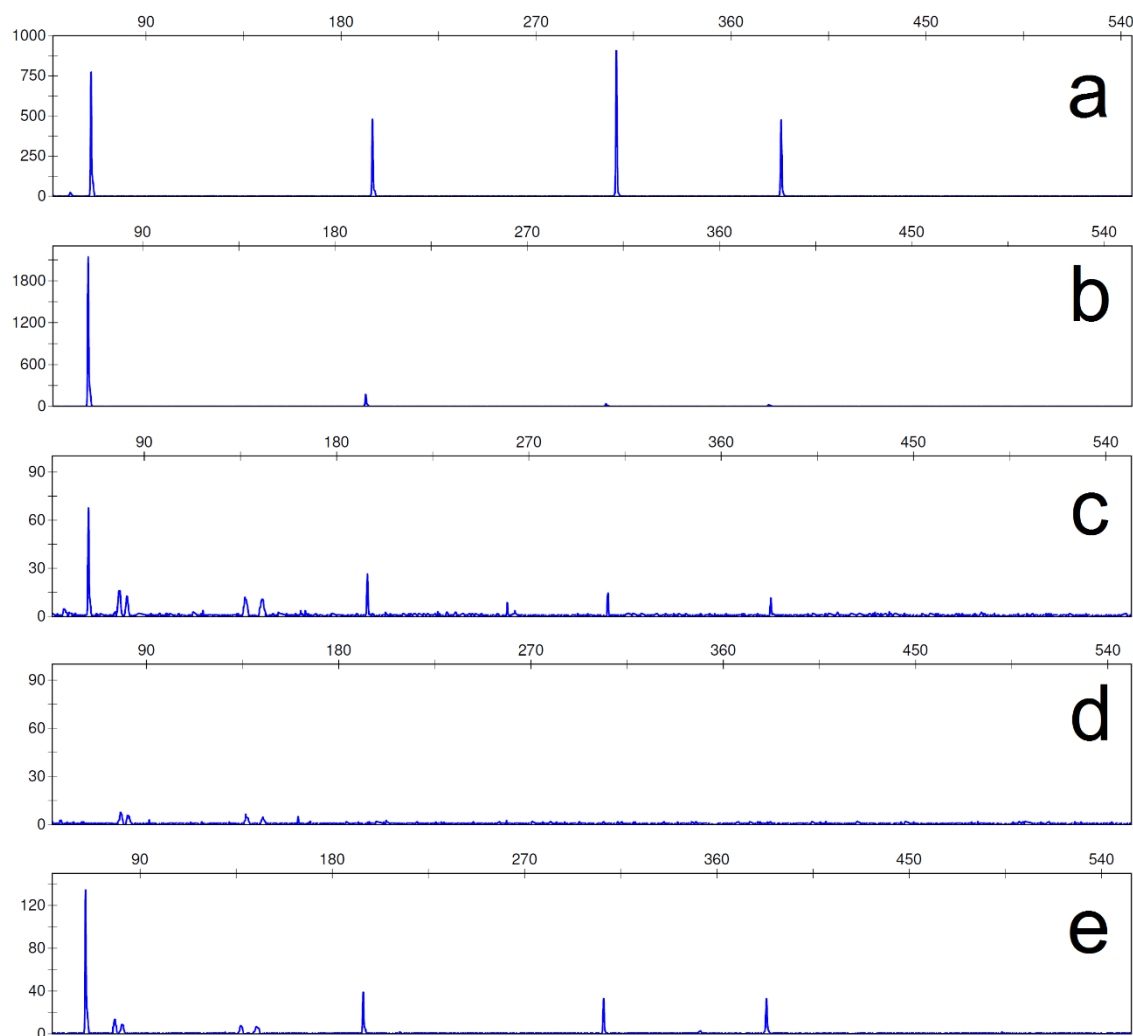
Figure 4.4 – Examples of electropherograms of multiplex profiles obtained from samples incubated at 25 °C for (a) 1 day, (b) 7 days, (c) 25 days, (d) 28 days, and (e) 37 days. The last image is the sample that presented a profile with clear peaks after 37 days of incubation, even after complete failure of amplification of samples collected at previous time points.

Table 4.2 – Table showing the results of the Mini-4-Plex multiplex amplification for samples incubated at 25 °C.

Time	ADD	Mini-4-Plex amplification of samples incubated at 25 °C			
		50 bp	75 bp	112 bp	154 bp
Day 7	175	+++	+++	+++	+++
Day 10	250	+++	+++	+++	+++
Day 13	325	+++	+++	+++	+++
Day 16	400	+-+	+-+	+-+	+-+
Day 19	475	+++	+++	+++	-++
Day 22	550	+++	+++	++-	++-
Day 25	625	+-+	--+	--+	--+
Day 28	700	++-	++-	++-	++-
Day 31	775	-++	-++	-++	-+-
Day 34	850	+++	+++	+++	+++
Day 37	925	++-	++-	++-	+--
Day 40	1000	+-+	+-+	+-+	+--

The "+" denotes the presence and "-" the absence of PCR amplicons in samples.

Figure 4.5 – Electropherograms of some of the samples amplified with the Mini-4-Plex and incubated at 25 °C for (a) 7 days, (b) 22 days, and (c) 37 days. It is possible to see that the profiles get more imbalanced with longer incubation points.

4.4.2 Incubation at 37 °C

A total of 39 samples were incubated at 37 °C, plus the three control samples collected at time zero. Most samples changed consistency during incubation, becoming softer. Samples were frozen after collection until the moment of DNA extraction. After DNA extraction, the extracts were analysed on an agarose gel, quantitated and amplified with the 4-Plex multiplex for analysis. Some of the samples were also amplified with the use of the Mini-4-Plex.

Gel electrophoresis showed a high molecular weight DNA band alongside degradation until the set of samples collected after 72 h of incubation (Figure 4.6). The next set of samples, collected after 96 h, seemed to only have degraded DNA, although samples from later time points showed high molecular weight DNA bands again. Some of the samples had a band in the lower portion of the gel.

The results from DNA quantitation were consistent compared with the data from gel electrophoresis, with samples that had high DNA concentrations having had clearly visible results on the agarose gel (Figure 4.7). The average quantitation of the triplicates declined with the increase in incubation time, with the exception for the last time point. This triplicate had an average which was similar to samples from the third point of incubation (36 h).
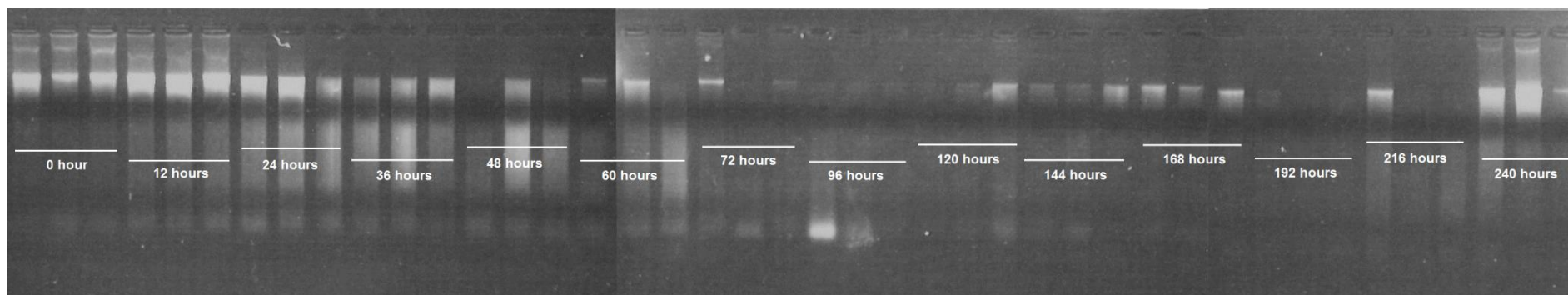
Figure 4.6 – A photograph showing the agarose electrophoresis gels from samples incubated at 37 °C at the laboratory. It is possible to see the smear associated with DNA degradation in samples stored for as little as 12 h. Each lane equals to one sample (n=1) but experiments were done in triplicates.
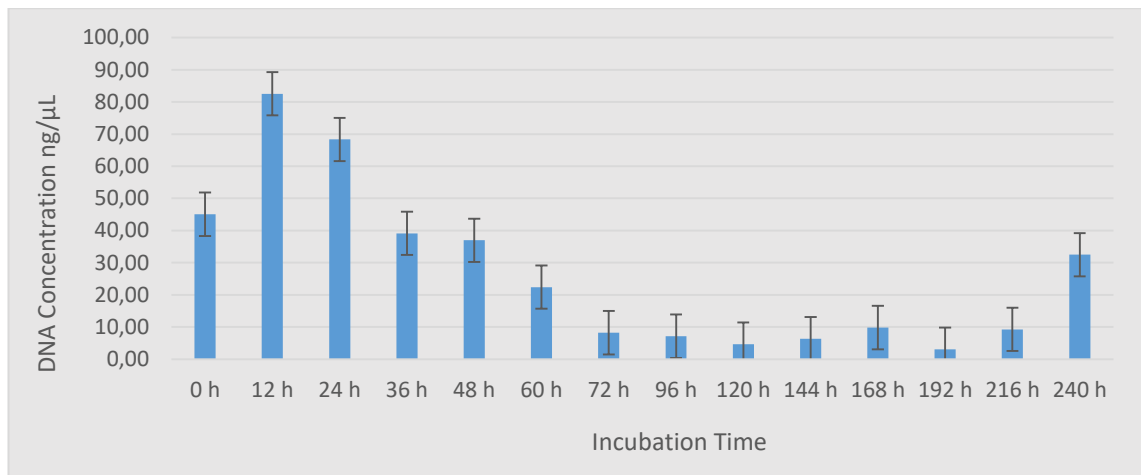
Figure 4.7 – Bar charts showing average DNA concentrations of samples incubated at 37 °C for up to 240 h (10 days). The results show that the quantity of DNA in the samples tended to decrease with time. All data are presented as mean ± SEM, n=3.

Samples incubated at 37 °C gave complete 4-Plex multiplex profiles up to 36 h of incubation. Incubation for 48 h already resulted in drop outs of two amplicons in one sample of the triplicate (Table 4.3). The 70 bp allele was amplified until the last collection point (10 days of incubation), but in only one sample of the triplicate. Interestingly, after 216 h of incubation, one of the samples collected presented almost a full profile, with the larger amplicon having a clear peak, but under the 50 RFU threshold (Figure 4.8).

Table 4.3 – Table showing the results of the 4-Plex multiplex amplification for samples incubated at 37 °C.

Time	ADD	4-Plex amplification of samples incubated at 37 °C			
		70 bp	194 bp	305 bp	384 bp
Time 0	0	+++	+++	+++	+++
12 h	18.5	+++	+++	+++	+++
24 h	37	+++	+++	+++	+++
36 h	55.5	+++	+++	+++	+++
48 h	74	+++	+++	(+)++	(+)+(+)
60 h	92.5	+++	+++	(+)++	(+)++
72 h	111	+++	---	---	---
96 h	148	++-	-(+)-	-(+)-	-(+)-
120 h	185	+++	+--	(+)--	(+)--
144 h	222	+-+	---	---	---
168 h	259	---	---	---	---
192 h	296	+(+)-	---	---	---
216 h	333	-++	--+	--+	--(+)
240 h	370	--+	---	---	---

The "+" denotes the presence and "-" the absence of PCR amplicons in samples. The (+) represents clear peaks below threshold of 50 RFU

Figure 4.8 – Examples of electropherograms showing full and partial profiles obtained from samples incubated at 37 °C. (a) is the control sample, (b) a sample incubated for 36 h, (c) 120 h of incubation, (d) the sample that after 216 h of incubation amplified 3 of the 4 alleles, and (e) a sample incubated for 240 h.

For samples that failed to give a full profile, the Mini-4-Plex multiplex was used. Full profiles were obtained after 10 days of incubation, but samples started to generate partial profiles in earlier times (Table 4.4). Longer incubation times resulted in more unbalanced profiles, even with the smaller amplicon sizes (Figure 4.9).

Table 4.4 – Table showing the results of the Mini-4-Plex multiplex amplification for samples incubated at 37 °C.

Time	ADD	Mini-4-Plex amplification of samples incubated at 37 °C			
		50 bp	75 bp	112 bp	154 bp
60 h	92.5	+++	+++	+++	+++
72 h	111	+++	+++	+++	-++
96 h	148	+++	+++	+++	++-
120 h	185	+++	+++	+++	+++
144 h	222	+-+	+++	--+	+-+
168 h	159	+++	+--	+--	---
192 h	196	+++	-++	+++	-++
216 h	333	+++	-++	+++	-++
240 h	370	+++	+-+	+-+	+-+

The "+" denotes the presence and "-" the absence of PCR amplicons in samples.
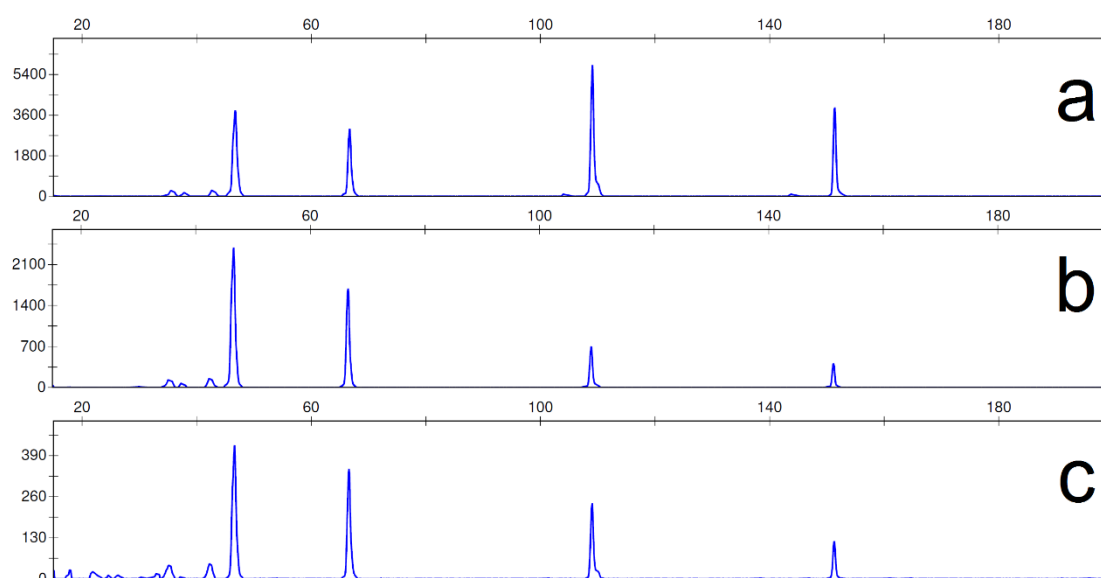
Figure 4.9 – Examples of electropherograms of samples incubated at 37 °C and amplified with the Mini-4-Plex. (a) is a sample incubated for 24 h, (b) was incubated for 96 h, and (c) for 240 h. Total amplification was possible even after 10 days of incubation, but the profile is unbalanced and RFUs are low.

4.5 Discussion

The objective of this series of experiments was to gain a better understanding of DNA degradation patterns by studying DNA persistence in muscle tissue after incubation at two different temperatures (25 °C and 37 °C) in the laboratory, without the influence of insect activity. In order to assess DNA persistence, two multiplexes with ranges of 70-384 bp, and 50-154 bp were used. This series of experiments was performed keeping in view mass disaster or conflict situations where bodies can be exposed to different environmental temperatures.

It has been reported that the quantity of amplified DNA in skeletal muscles of dead rats kept at 20 °C was reduced from 10 ng to 1 ng in 2-4 weeks (Itani *et al.* 2011). Blood and saliva samples were incubated at 37 °C with 100% humidity and total DNA degradation was achieved within 12-16 weeks (Dixon *et al.* 2006).

Incubation in the laboratory showed that muscle tissue incubated at 25 °C maintained complete profiles with 100 ADD (4 days) of incubation and samples incubated at 37 °C were able to give a full profile in all samples up until 74 ADD (48 h) of incubation. The 70 bp allele of the 4-Plex multiplex was obtained at the end of the incubation at 37 °C (370 ADD), but had drop outs in previous time points. The persistence of the 70 bp amplicon and drop out of the larger ones was expected since DNA degradation makes longer fragments less available.

In previous experiments, Nazir (2012) found similar results of DNA persistence in pig muscle after incubation in the laboratory. In these experiments, it was found that muscle tissue from pig incubated at 27 °C had drop-out of the larger fragment after 3 days of incubation. However, samples incubated at 37 °C had complete amplification of the 4-Plex with up to 3 days of incubation, with drop-outs starting after 5 days.

The last complete profile obtained from samples incubated at 25 °C was after 325 ADD, while the last from samples incubated at 37 °C was obtained from samples incubated for 92.5 ADD. Literature states that higher temperatures accelerate autolysis because the hydrolytic actions of the enzymes are enhanced (Zhou and Byard 2011). This can explain why samples incubated at the higher temperature of 37 °C had faster DNA degradation than samples incubated at 25 °C. The enclosed nature of the polypropylene tubes also resulted in a moist environment. Humidity acts as a buffer for temperature and pH levels and it affects chemical reactions that occur during decomposition, and functions as an excellent solvent (Shirley *et al.* 2011), but this would affect both incubation temperatures equally.

The Mini-4-Plex multiplex allowed the analysis of short fragments and it was possible to see that the fragments above 100 bp (112 bp and 154 bp) were present in the incubation at 37 °C even after incubation for 370 ADD (240 h). Previous studies have described how the amplification efficiency increases as PCR target size reduces due to the likelihood that the longer fragments will be degraded with time (Butler *et al.* 2003; Takahashi *et al.* 1997; Utsuno and Minaguchi 2004). In the incubation at 25 °C, they were present until the last point of incubation (1000 ADD / 40 days). In the case of crime scene samples obtained after the dropouts in the 4-Plex multiplex but within the period of incubation of this study, the use of indels or SNP analysis would be more recommendable than that of STRs.

The high molecular weight DNA bands that agarose gel electrophoresis shows after 185 ADD (37 °C) and 175 ADD (25 °C) are probably of microbial DNA, since there is no correspondence with samples presenting the bands and samples that amplified the longer fragments. In fact, it appears that samples presenting high

molecular weight DNA bands at the end of incubation tended to have partial profiles or no profiles at all.

The use of muscle tissue is not recommended by the leading agencies after decomposition begins (ICRC 2009; INTERPOL 2014), but the results from the present study would suggest it could still be used with traditional STR kits for up to 60 h (in temperatures of around 37 °C) or 13 days (in more moderate temperatures of 25 °C).

PART TWO: DNA DEGRADATION IN FIELD HIGH TEMPERATURES

4.6 Aims and Objectives

The aims associated with this part were to compare DNA degradation patterns in soft muscle tissue decomposed in a controlled environment and in environmental conditions of high temperatures and to assess the ability of different preservation solutions to preserve muscle soft tissue.

Part two has the following objectives:

- To determine the effect of field high temperatures on DNA persistence in pig soft muscle tissue.

- To assess the use of ethanol and cell lysis as preservation methods for muscle tissue samples collected in partially decomposed cadavers.

4.7 Background

This series of experiments was based on a series of sampling that took place between 05 June 2014 and 08 June 2014, in Thailand. The experiments were designed as a follow-up of a series of experiments previously performed at TRACES that studied the relation of DNA degradation in muscle tissue with ADD and found seasonal difference (Nazir 2012).

TRACES was established in 2009 by UCLAN and it is the only dedicated taphonomic research facility in the UK, providing new insights into rate and pattern of decomposition under various conditions which has been applied to forensic casework. The site is located at Newton Rigg (54.65 °N, 2.73 °W), and has a 13 acre area of rough grassland surrounded by a native woodland border.

Total woodland on site is around 10 acres (Cross and Simmons 2010; Cross *et al.* 2010).

Temperatures in the United Kingdom are not very high even during summer months (average of 11 °C), so a new setting was required to assess DNA degradation in high temperatures.

The site used in Thailand belongs to the Sawang Ariya Philanthropic Foundation and it is located outside of the town of Nakhon Nayok, which is 116 km from Bangkok (Figure 4.10). The site is attached to a cemetery that is used by the foundation and is not visited frequently by the public. The average temperature during summer is 32 °C, with temperatures during the day reaching 45 °C.
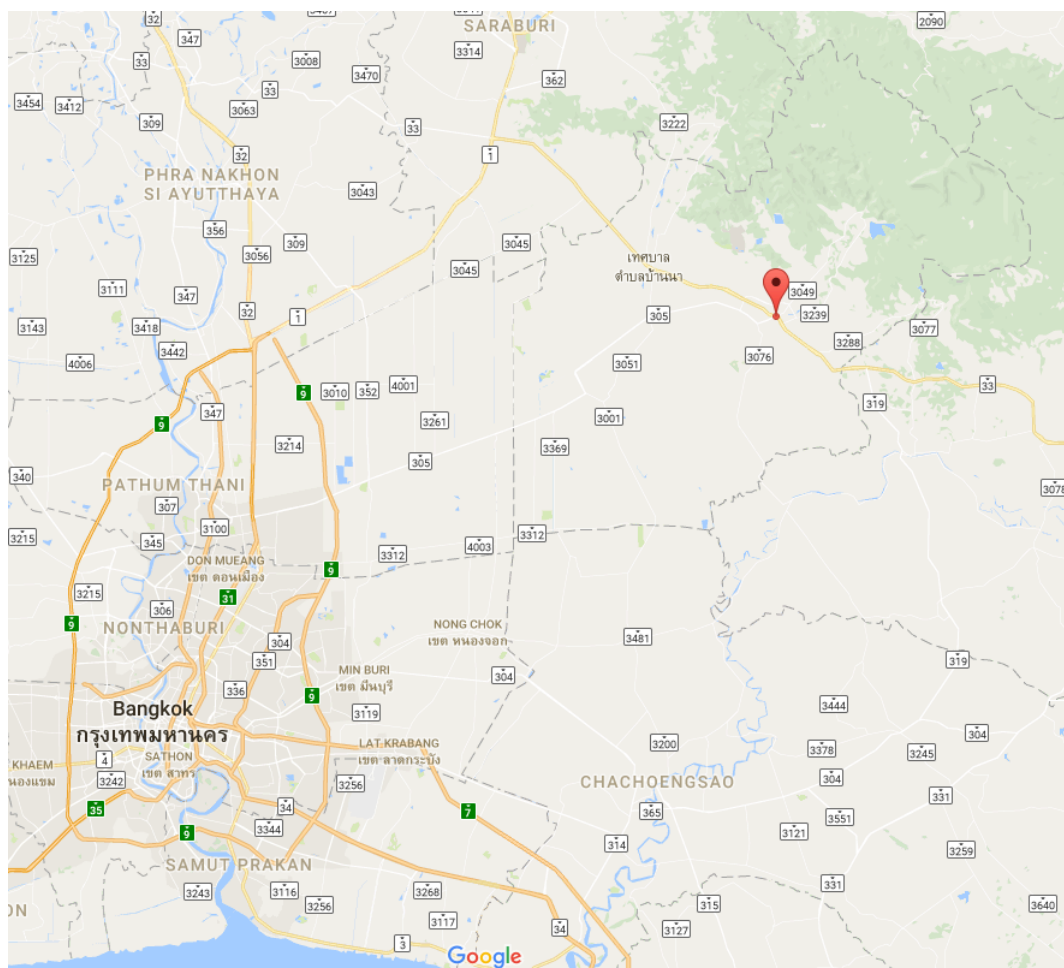
Figure 4.10 – Map showing the location of the city of Nakhon Nayok in relation to Bangkok (116 km apart).

4.8 Materials and Methods

Resumed methods were as follows, Chapter 2 contains more details.

4.8.1 Samples

Whole carcasses and separate limbs of freshly killed pig were placed in an open field in direct contact with the ground (Figure 4.11). The carcasses and limbs were protected from scavenger activity by cages of mesh, thus still allowing insect activity. They were placed with some distance between them. Samples were collected from five different pig carcasses. Control samples were collected at the moment of deposition and collection points were after 12 h, 24 h, 36 h, 48 h, 60 h, and 72 h of exposure. After collection, samples were stored in a plastic tube with either cell lysis or ethanol, frozen as soon as possible and kept frozen until extraction. Samples were collected in groups of six, being one sample collected from the carcass and stored in cell lysis. Three samples collected from the carcass were stored in ethanol, one sample collected from the aerial surface (part facing up) of the separate limb, and one sample collected from the ground surface (part facing down) of the separate limb. Both samples collected from the separated limb were preserved in ethanol.
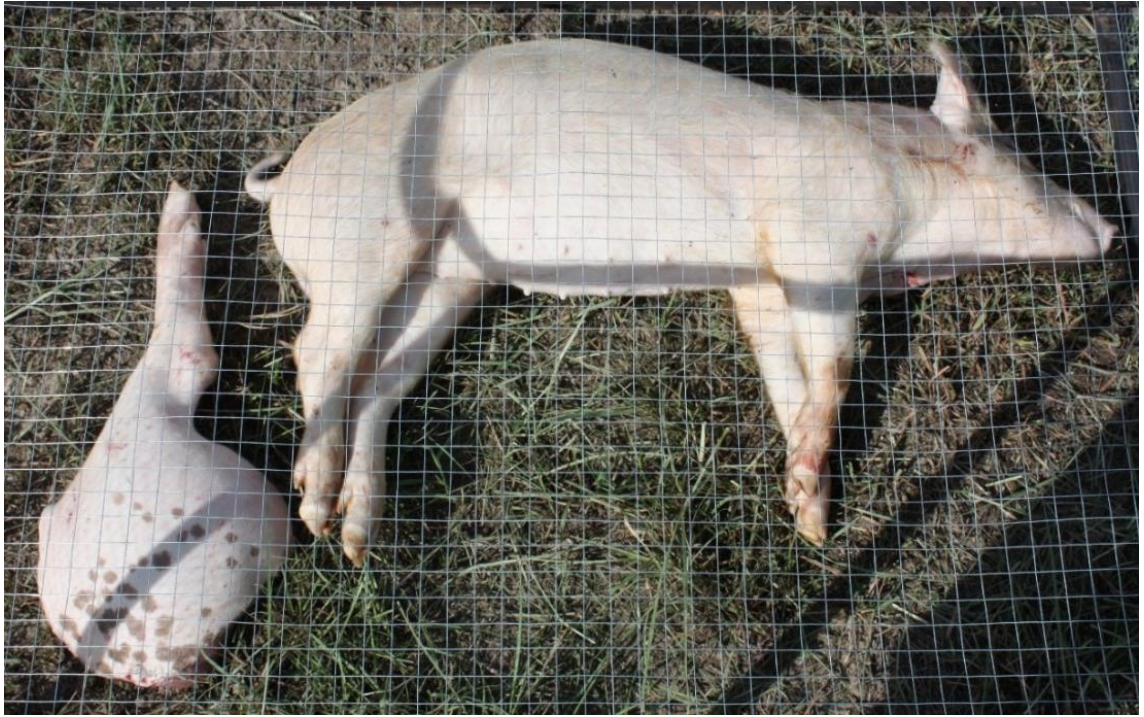
Figure 4.11 – A photograph showing the set-up of the pig carcass and separate limb protected by the mesh cage before the field exposure in Thailand.

4.8.2 Analysis of Samples

Extraction of DNA was performed using DNeasy® Blood and Tissue kit according to manufacturer's instructions. To determine the presence of DNA, a volume of 2 µL of the samples was analysed using agarose gel electrophoresis. Quantification was done with Qubit using 1 µL of the samples according to manufacturer's instructions. The 4-Plex multiplex was performed to determine the amplification success of 70 bp, 194 bp, 305 bp, and 384 bp amplicons in the samples. For some of the samples, another amplification was made using the Mini-4-Plex with amplicon sizes of 50 bp, 70 bp, 112 bp, and 154 bp. Statistical data analysis was performed as described in Chapter 2.

4.9 Results

After exposure of complete pig carcasses and separate limbs, a total of 42 muscle samples were collected across six time points. At the moment of deposition, six muscle samples were collected to serve as controls, with half of them having ethanol added and the other half being preserved with cell lysis. After this, every 12 h, six samples were collected. Four of these samples were collected from the complete carcass, being one sample preserved in cell lysis and the three other samples preserved in ethanol. Two muscle samples were collected from the separate limb, one from the aerial surface and one from the ground surface, and these were also preserved in ethanol. All samples were frozen until DNA extraction. After DNA extraction, the extracts were analysed on an agarose gel, quantitated and amplified with the 4-Plex multiplex for analysis.

Insect activity was already observed in the carcasses at the first time point of collection. With passing incubation time and decomposition and with the access points created by collecting samples, insect activity increased (Figure 4.12). It was also visualized the progression of the decomposition process, with bloating being observed at the first collection time point (12 h). Maggot activity increased after stomach rupture and the composition of the muscle had changed after 48 h of exposure with very little mechanical structure left.

Figure 4.12 – Photograph showing the state of pig carcasses through incubation time points. (a) is a carcass after 12 h of exposure with visible bloating and insect activity on surface; (b) shows insect activity inside previous collection point at 24 h of incubation; (c) to (f) show progressive decomposition and increase of insect activity in carcasses after 36 h, 48 h, 60 h, and 72 h, respectivelly.

Both ambient and internal carcass temperatures were measured during the experiment. Ambient temperatures were collected with the use of five different thermometers placed next to the animals and the exposed carcasses also had their internal temperature monitored. During the three days of the experiment, ambient temperatures varied between 26 °C and 60.3 °C and the carcasses

average internal temperature was between 27.5 °C and 41.2 °C (Figure 4.13). The overall average ambient temperature was of ± 34.37 °C and the internal temperatures average was ± 34.88 °C.
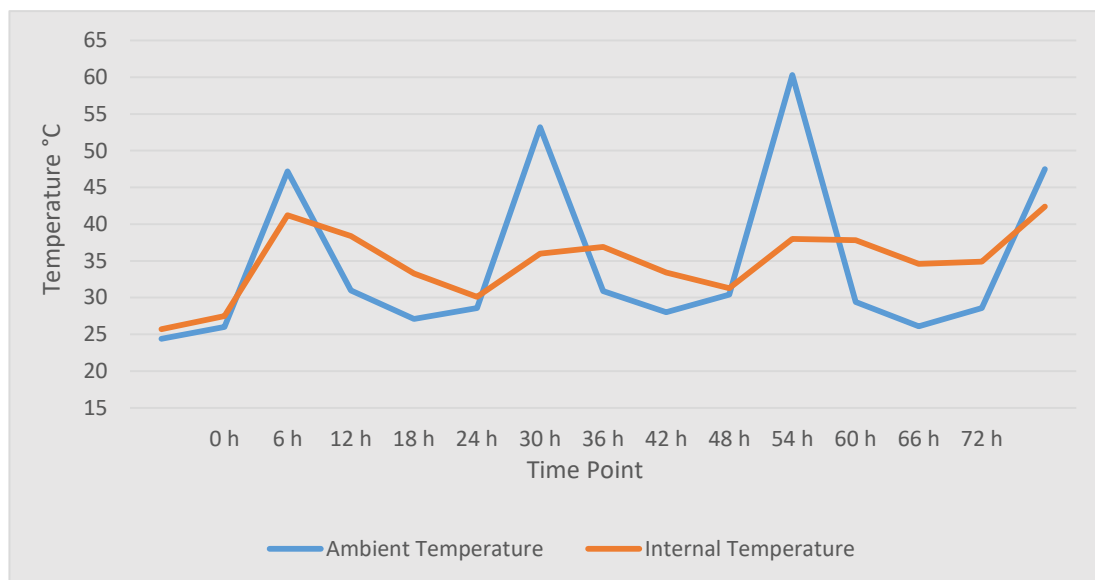
Figure 4.13 – Time course of ambient and internal carcass temperature variations during the three-day-long incubation. The peaks in ambient temperature correspond to noon.

Gel electrophoresis showed the smear associated with DNA degradation, especially in samples stored in ethanol after collection, but also in samples stored in cell lysis (Figure 4.14). Samples collected after 36 h of exposure or later and stored in ethanol showed no bands or smears on the agarose gel. The exception was from samples that were collected from the ground surface of the separate limbs after 36, 60 and 72 h of exposure, which showed in the gel (Figure 4.15).
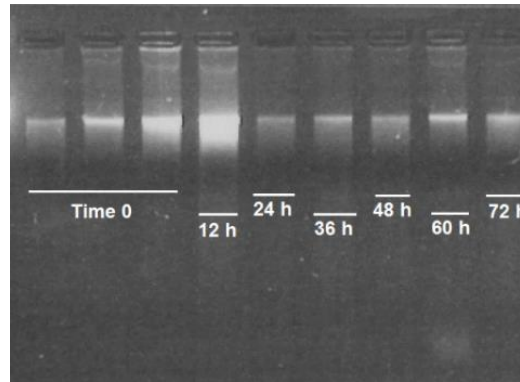
Figure 4.14 – A photograph showing agarose electrophoresis gel from Thailand samples stored in cell lysis. Each lane equals to one sample.
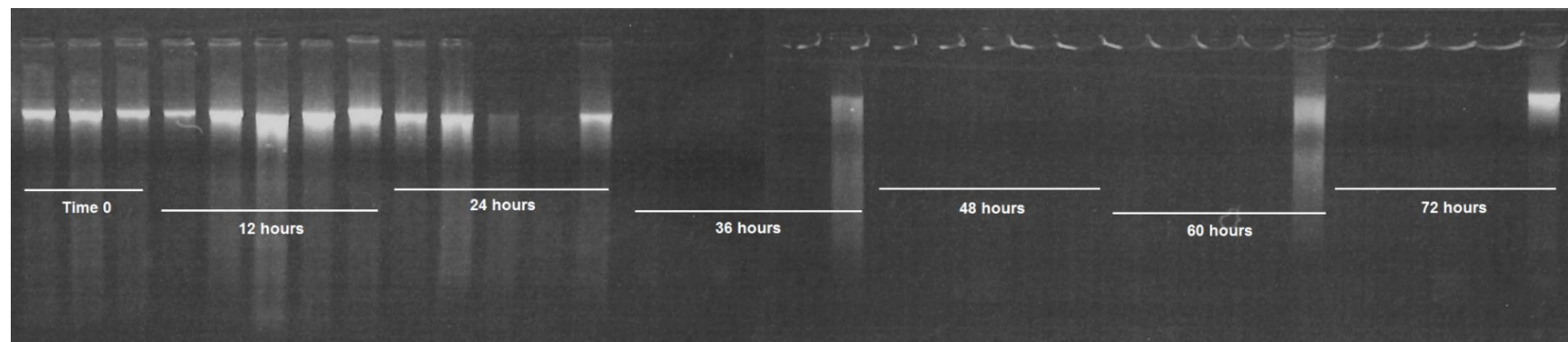
Figure 4.15 – A photograph showing agarose electrophoresis gels from Thailand samples stored in ethanol. Each lane equals to one sample. The three first samples in each set of exposed samples were collected from the carcass, followed by one collected from the aerial surface of the separate limb and one from the ground surface of the limb.

The DNA concentrations obtained after quantitation were consistent with the results observed in the agarose gel electrophoresis. Although all samples stored in cell lysis had the presence of a clear high molecular weight DNA band in the gel electrophoresis, they had relatively low concentration results when compared with their ethanol-preserved counterparts. This was the case even for the three control samples, who had an average concentration of 21 ng/µL while the ethanol-preserved controls had an average of 74 ng/µL (Figure 4.16).
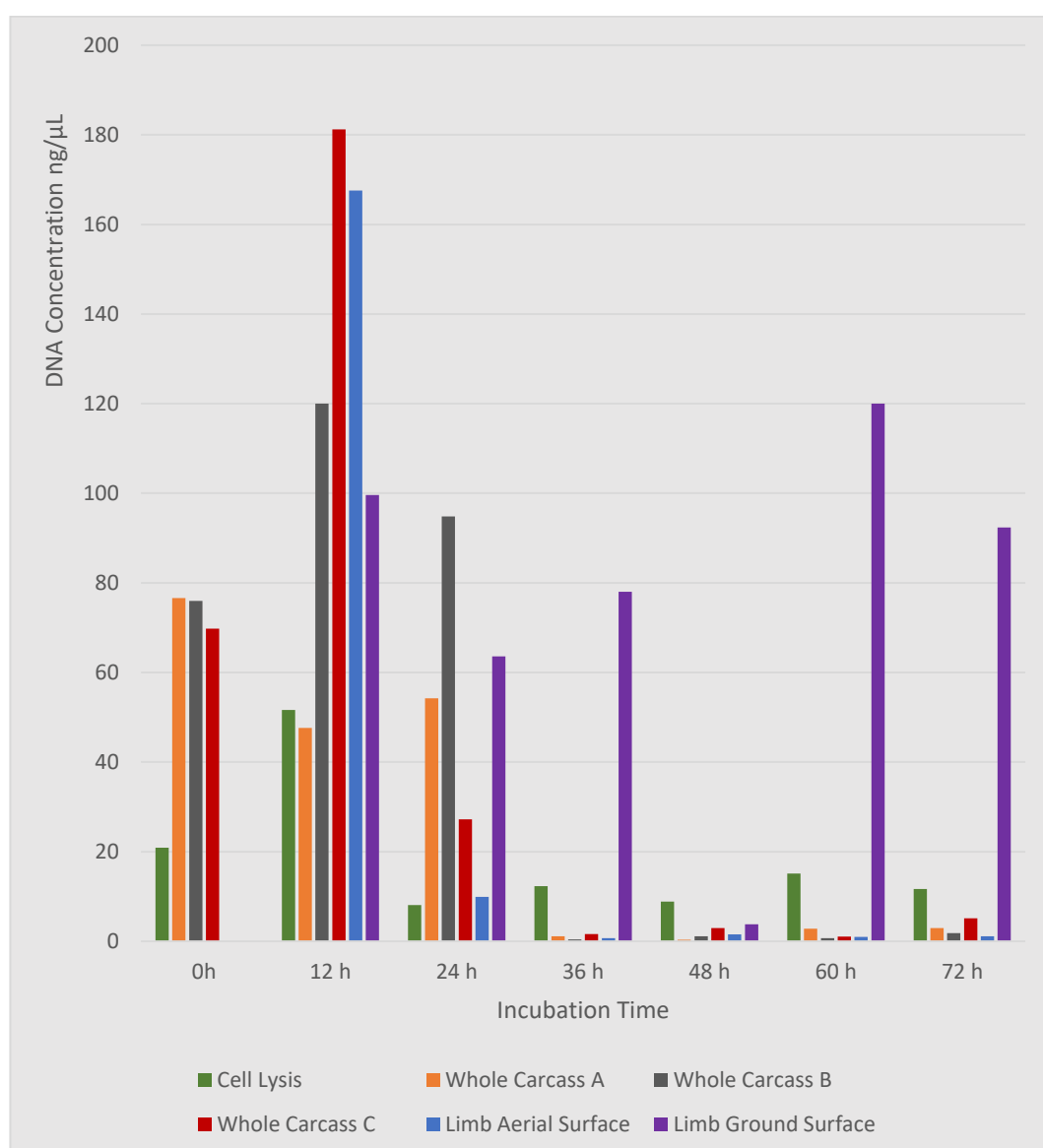
Figure 4.16 – Bar charts showing the DNA concentrations of samples collected in Thailand. Each bar equals to one sample. Some of the samples were not collected in triplicates, so they were not grouped when presenting the results.

There was complete amplification of the 4-Plex multiplex in all samples for the first 24 h. Samples collected from the separate limb presented some drop out after 36 h. For samples stored in CL, dropout was observed after 60 h (Table 4.5), but all samples stored in ETOH had no amplification after 48 h (Table 4.6). It was observed that with longer incubation times profile quality decreased, until no profiles were possible (Figures 4.17 and 4.18). Samples collected from the ground surface of the separate limb showed more DNA persistence than all other types of samples, with complete amplification after 72 h of exposure (Figure 4.19).

Table 4.5 – Table showing the results of the 4-Plex multiplex amplification for samples stored in cell lysis after collection in the field.

Time	ADD	4-Plex amplification of samples stored in cell lysis			
		70 bp	194 bp	305 bp	384 bp
Time 0	0	+++	+++	+++	+++
12 h	16	+	+	+	+
24 h	33.5	+	+	+	+
36 h	51	+	+	+	+
48 h	69.5	+	+	+	+
60 h	88	+	-	-	-
72 h	105	+	-	-	-

The "+" denotes the presence and "-" the absence of PCR amplicons in samples.

Table 4.6 – Table showing the results of the 4-Plex multiplex amplification for samples stored in ethanol after collection in the field.

Time	ADD	4-Plex amplification of samples stored in ethanol							
		70 bp		194 bp		305 bp		384 bp	
		WC	SL	WC	SL	WC	SL	WC	SL
Time 0	0	+++	x	+++	x	+++	x	+++	x
12 h	16	+++	++	+++	++	+++	++	+++	++
24 h	33.5	+++	++	+++	++	+++	++	+++	++
36 h	51	+--	++	+--	+(+)	+--	++	+--	+(+)
48 h	69.5	---	--	---	--	---	--	---	--
60 h	88	---	-+	---	-+	---	-+	---	-+
72 h	105	-+-	-+	---	-+	---	-+	---	-+

WC = whole carcass; SL = separated limb (aerial then ground surface). The "+" denotes the presence and "-" the absence of PCR amplicons in samples. The (+) represents clear peaks below threshold of 50 RFU.
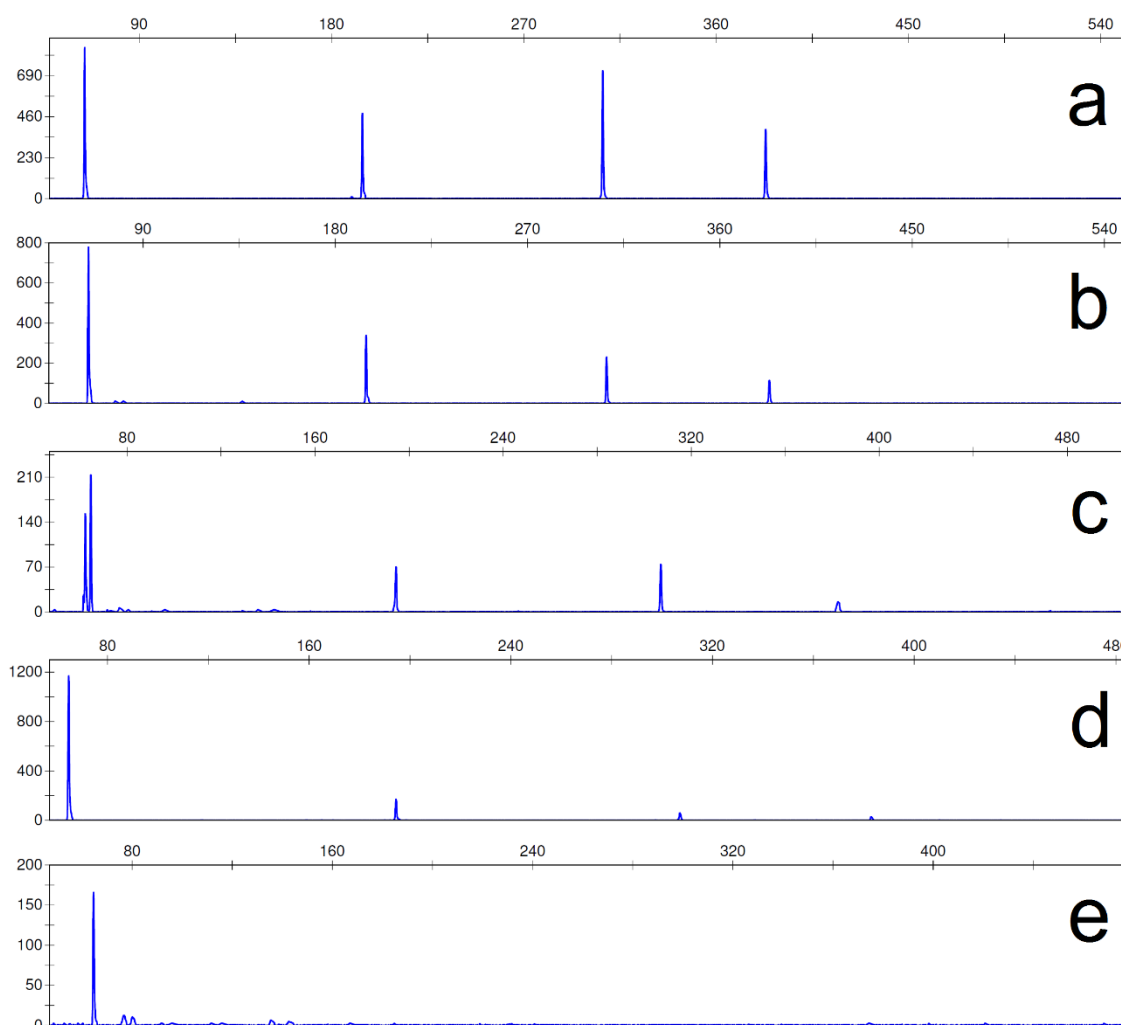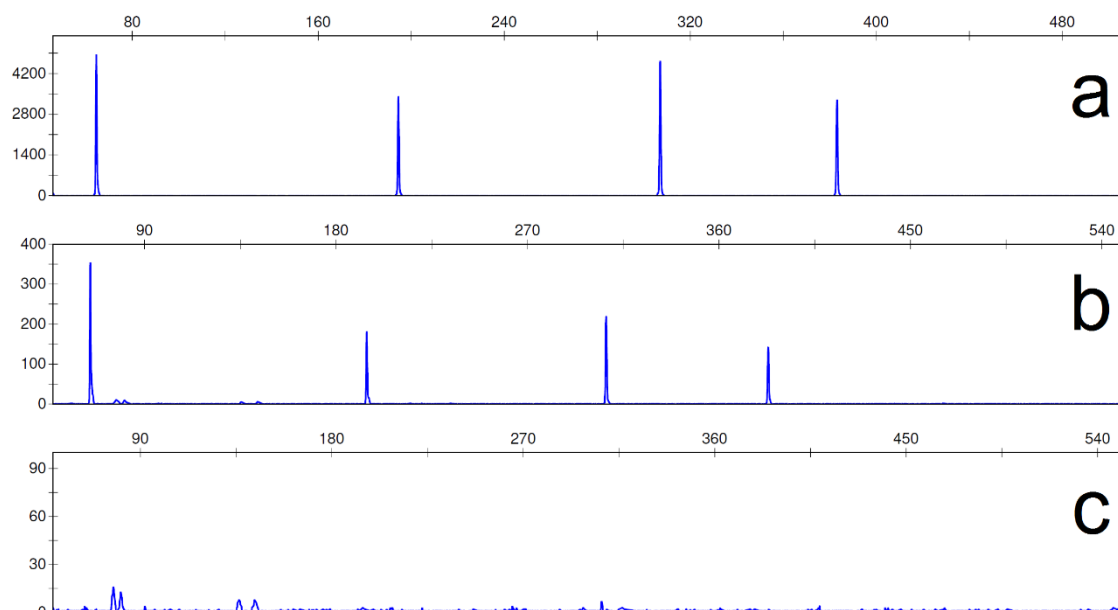
Figure 4.17 – Examples of electropherograms of full multiplex profile and no multiplex profile obtained from pig muscle stored in cell lysis after sample collection. (a) is a control sample, (b) is a sample collected after 48 h of exposure, and (c) was collected after 60 h of exposure.
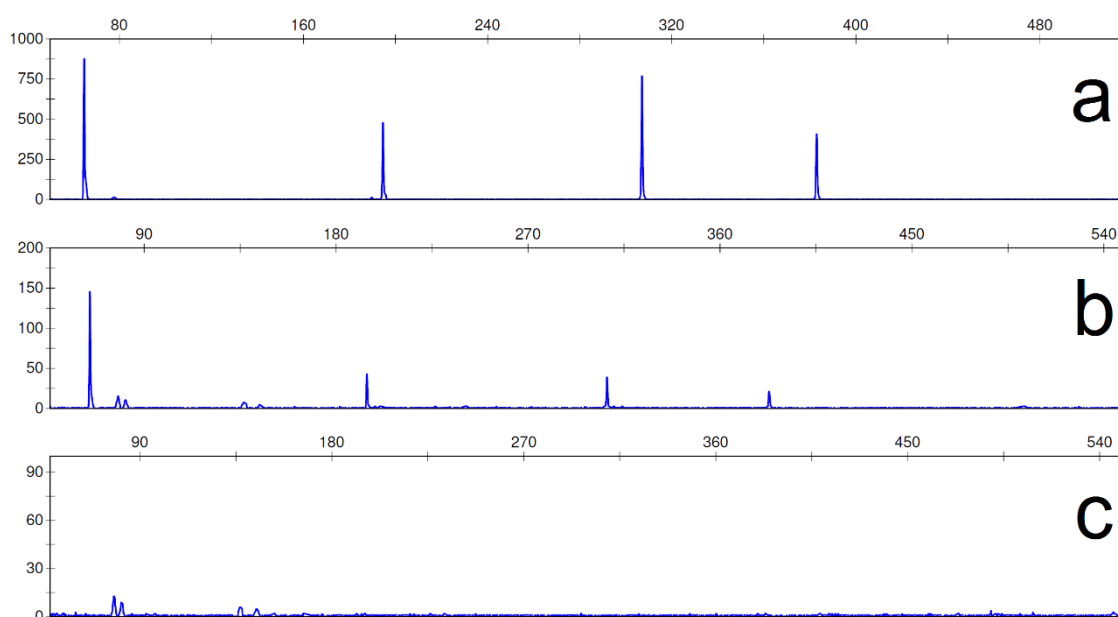
Figure 4.18 – Examples of electropherograms obtained from pig muscle stored in ethanol after collection of sample. It can be observed that the intensity of fluorescence decreased between (a) 12 h and (b) 36 h. (c) was collected after 48 h and had no amplification.
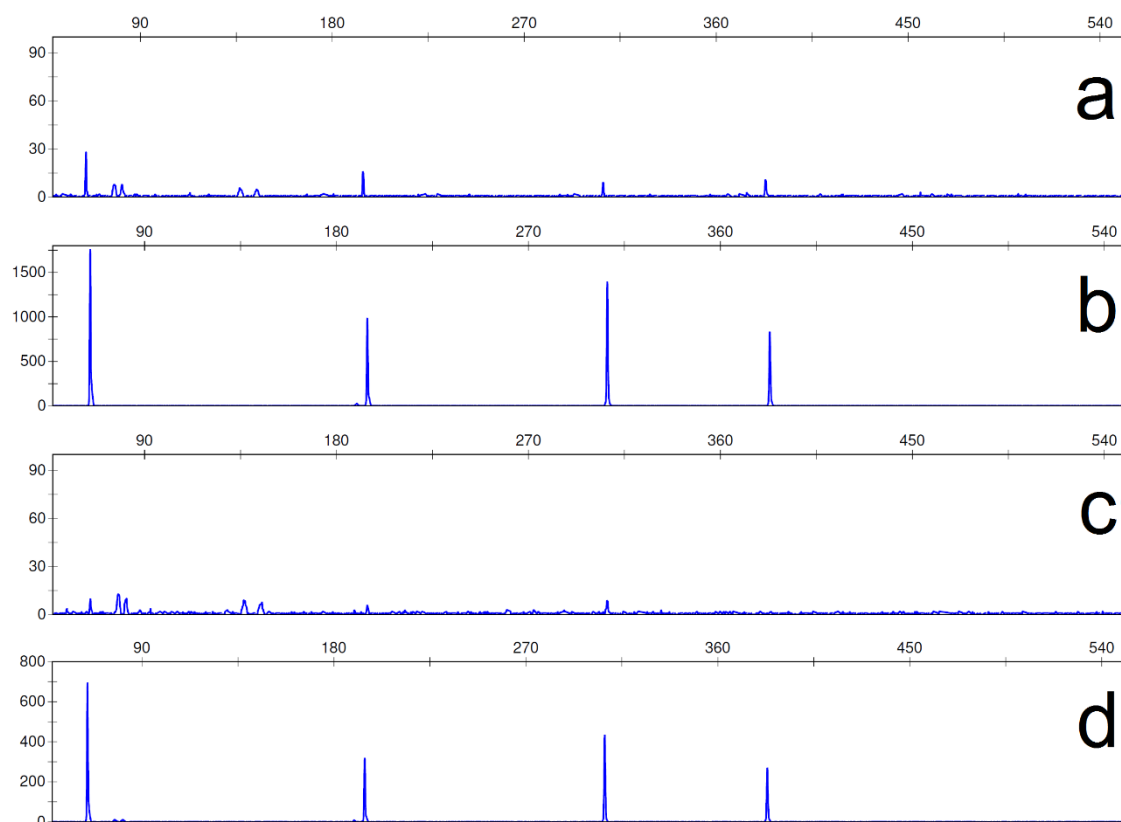
Figure 4.19 – Examples of electropherograms showing that on the two last collection points whilst the samples collected from the aerial surface (a and c) of the limbs had no profile wit RFU levels above 50, the samples collected from the ground surface (b and d) generated complete profiles.

4.10 Discussion

This research was conducted to provide empirical evidence to supplement advice available to the forensic community for the collection of muscle tissue for forensic analysis. A number of samples can be collected from the body for the purpose of DNA identification. Mostly in forensic cases, whole and non-disrupted cadavers are examined at autopsy. In situations where the blood or buccal swabs are not available, especially in the case of highly fragmented remains, alternative biological samples must be collected for analysis. Thus, in fragmented bodies, the preferable tissue type will be muscle tissue rather than bone and teeth because the time and cost associated with the analysis of hard tissues is considerably greater (Graham *et al.* 2008).

It is well known that DNA degradation is influenced by temperature and other environmental insults (Lindahl 1993). This series of experiments was done to assess DNA persistence in pig muscle tissue after exposure to high temperatures in the field.

The results show that DNA degradation was visible in the agarose gel electrophoresis, with the characteristic smears appearing after 16 ADD (12 h of incubation). Samples stored in cell lysis appeared to have less DNA degradation on the gel electrophoresis than samples stored in ethanol.

Samples stored in cell lysis had, on average, lower DNA quantitation results than samples stored in ethanol. In previous studies DNA has been extracted straight from cell lysis and used in following reactions (Graham *et al.* 2008; Nazir 2012). With the breakdown of the muscle tissue, the DNA enters the liquid surrounding it. In this research, some of the samples collected from later time points had no

integrate muscle tissue and the extraction was performed using the cell lysis solution present.

The DNA Commission of the International Society for Forensic Genetics states that storing soft muscle tissue samples in preservative solutions at room temperature can be an alternative to cold storage, if is required (Prinz *et al.* 2007). In this series of experiments, samples were stored in either cell lysis or ethanol for a period before freezing. Both preservative solutions have been used before to store muscle samples at room temperature (Graham *et al.* 2008; Kilpatrick 2002; Michaud and Foran 2011).

Samples stored in cell lysis amplified the 70 bp fragment until the last time point (60 h), while samples stored in ethanol had it drop out after 48 h of exposure. This would suggest that storing samples in cell lysis improves DNA persistence when compared with samples stored in ethanol, but the small number of samples makes it difficult to draw a conclusion on this.

There was no amplification profile in all samples stored in ethanol exposed for 48 h. However, samples collected from the separate limb after that had complete profiles. This could be due to human error during extraction. Depending on the stage of decomposition of tissues, it can get challenging to obtain an acceptable sample of the muscle.

A previous study done in Thailand showed the persistence of a 289 bp amplicon for 4 days for air-dried pig muscle samples (Phengon *et al.* 2008). In this study, samples failed to amplify amplicons bigger than 70 bp after 48 h (69.5 ADD) for samples stored in ethanol, and 60 h (88 ADD) for samples stored in cell lysis. Since the study does not mention the temperature at the time of their experiment, the difference in the findings could be due to that.

Full profiles were obtained from all samples until 33.5 ADD (24 h). After 51 ADD (36 h), samples collected from the separate limbs showed dropouts. This was probably due to a smaller body mass being more affected more by insect activity. It was observed a higher persistence of DNA in samples collected from the ground surface of the limb in contrast with the ones collected from the aerial surface and this could be due to temperature difference between them. The aerial surface of the carcass was directly exposed to the sun and, because of this, subjected to higher temperatures; while the ground surface was protected. Even after 105 ADD (72 h) of exposition, the sample collected from the ground surface of the separate limb had a full profile. These results could be a strategy as to where collect tissue samples from exposed bodies.

In a previous study, Nazir (2012) exposed pig carcasses in different seasons in the UK. They were able to amplify fragments of up to 384 bp for 77 days (494 ADD) in the winter, 24 days (295 ADD) in the spring/summer, and 30 days (342 ADD) in the autumn. It was concluded that increased maggot mass during summer increased carcass decomposition and ultimately decreased DNA persistence. In this current series of experiments, amplification of the 384 bp fragment was not possible after 48 h of incubation (69.5 ADD) for sample stored in ethanol and 60 h (88 ADD) for samples stored in cell lysis.

Body decomposition and ADD can be correlated using a formula based on the $\log_{10}$ of the ADD value. It means that similar ADDs will incur in similar total body decomposition scores (Moffatt *et al.* 2016). It was expected that similar ADD would result in a similar DNA persistence, as it is with body decomposition, but it appears that exposure to higher temperatures accelerate DNA degradation. Besides the increased heat, maggot masses are more intense in warmer

environments, which increase the rate of putrefaction as insect larvae secrete a proteolytic enzyme that enhances tissue destruction (Zhou and Byard 2011).

It is not recommended to sample muscle tissue after the onset of degradation (INTERPOL 2014). However, the results obtained in this series of experiments would suggest that sampling of this kind of tissue could be done for up to 48 h of exposure in hot environments. If this information is converted into practice then the time spent to identify individuals, and returning them to their family could be reduced due to that fact that processing of hard tissues is time-consuming compared to soft muscle tissues (Budowle *et al.* 2005).

Body temperature measurement (*algor mortis*) is one of the criteria used for time of death estimation. The internal temperature of the pig carcasses was measured during this series of experiments. Elevated temperatures are associated with larval masses during decomposition, the combined processes of putrefaction and the metabolic activities of the maggots during the bloating stage cause an increase in the internal temperatures of the body. These temperatures can be significantly above ambient temperature (Goff 2009; Shirley *et al.* 2011).

Ambient temperatures during the study had variation according to the moment of the day (with higher temperatures occurring around noon), while internal body temperatures were more balanced throughout the three days, with the tissue mass acting as a buffer. The internal temperature was higher than the ambient temperature when the sun was lower, even after the stomachs ruptured. These elevated temperatures can accelerate DNA degradation and samples collected from such situations would benefit from immediate cooling down after collection.

Samples incubated in the field seem to degrade more quickly than samples incubated in the laboratory, even though temperatures were similar. Field incubated samples barely gave results after 36 h of incubation (51 ADD) and

101

already had no amplification profiles after 48 h (69.5 ADD). Samples incubated in the laboratory at 37 °C took an extra 24 h to reach both these points (92.5 ADD and 111 ADD) and samples incubated at 25 °C for 4 days (100 ADD) still had good quality profiles. This variation could be because experiments done in laboratories do not take into consideration insect activity and variations in temperature and humidity. Solar radiation also would dry the upper layer of muscle in the body.

Most of the samples were collected from the surface of the animals, which were exposed to more extreme temperature fluctuations. Perhaps increasing the temperature in the ovens to higher temperatures during certain hours to simulate daily fluctuation of temperature would generate more similar results.

Other limitations of this work were the small amount of samples stored in cell lysis and the fact that the temperature of the ground below the carcasses was not measured. The collection of these data might have helped to better elucidate the points studied in this chapter.

In conclusion, these results have provided empirical evidence for advice for collection and preservation of samples in casework. The results show that sampling of muscle tissue should preferentially be from the surface of the body in contact with the ground. Both cell lysis solution and ethanol were able to preserve the DNA in the samples for a period and extraction of DNA from cell lysis was possible even after total disintegration of the tissue.

CHAPTER 5
DNA PRESERVATION
USING SOLUTIONS

5.1 Introduction

In some crime scenes or following mass fatality incidents, human corpses are either disassembled, burnt or decomposed, making victim identification by means of fingerprinting or odontology extremely difficult (Graham *et al.* 2008). When a human cadaver is exposed to environmental conditions, substantial decomposition will occur, with the body's soft tissue starting to decay within a few days (Goff 2009).

In such situations, DNA profiling is essential, however, successful DNA analysis relies heavily on the appropriate collection and preservation of biological material (Graham *et al.* 2008). Inefficient preservation methods can cause degradation of intact DNA to such an extent that data is not always available for victim identification (Bing and Bieber 2001). Storage buffers and tissue types are among the principal factors affecting DNA preservation. The DNA Commission of the International Society for Forensic Genetics states that storing soft muscle tissue samples in preservative solutions at room temperature can be an alternative to cold storage, if required (Prinz *et al.* 2007).

Cell lysis solutions have been used to preserve soft muscle tissue samples for different periods of time (Allen-Hall and McNevin 2013). Lysis storage and transportation buffer was used to preserve muscle tissue at room temperature for up to 12 months (Graham *et al.* 2008). Longmire lysis buffer was used to preserve liver tissue for up to 2 years (Kilpatrick 2002).

Alcohol storage preserves samples, and it does not cause DNA cross-linking. Moreover, it is considered to be an effective long term tissue storage method allowing for successful DNA extraction (Penna *et al.* 2001). Ethanol has been used previously for the preservation of specimens at room temperature (Gillespie

et al. 2002; Kilpatrick 2002; Michaud and Foran 2011) and the use of vodka in the preservation of zebra liver samples over several days in the African Bush was reported by Oakenfull (1994). The INTERPOL Disaster Victim Identification Guide (INTERPOL 2014) recommends different types of ethanol to preserve soft tissue at room temperature, including drinking alcohol.

The impact of freeze-thaw cycles of whole blood samples on DNA integrity in the sample was previously studied (Bellete *et al.* 2003; Gessoni *et al.* 2004; Krajden *et al.* 1999; Ross *et al.* 1990) and the results showed progressive degradation of the samples as the number of freeze-thaw cycles increased.

PART ONE: DNA PERSISTENCE IN LONG-TERM STORAGE USING DIFFERENT PRESERVATION SOLUTIONS

5.2 Aims and Objectives

This main aim of this chapter is to assess the ability of different preservation solutions to preserve muscle soft tissue and part one has the following objective:

- To assess the efficiency of different solutions (96% ethanol, cell lysis and cell lysis with 1% sodium azide) to preserve partially decomposed pig soft muscle tissue samples stored at room temperature for a period of seven years.

5.3 Materials and Methods

Resumed methods were as follows, Chapter 2 contains more details.

5.3.1 Samples

In a previous study, pig whole carcasses were placed in direct contact with the ground at UCLAN's TRACES. Carcasses were protected from scavengers by being covered by a wire mesh. Soft muscle tissue samples were collected at two time points (79 and 210 ADD). In order to serve as positive controls for DNA preservation, samples were also collected at the moment of deposition of carcasses.

Samples weighing approximately 0.5 g and 1 g were placed into 50 mL polypropylene tubes containing 5 mL of preservative solutions (96% ethanol, cell lysis solution (with and without sodium azide)). At day zero soft muscle tissue samples were preserved with 96% ethanol. These samples were set in triplicates (three samples from each time point were stored using the same preservative

solution) and stored at room temperature until DNA extraction. DNA extraction was previously performed after one, six, and 12 months of sample collection in order to analyse short and medium-term DNA preservation by the substances used.

In this study, DNA was extracted from the same samples after 7 years of storage at room temperature in order to assess long-term DNA preservation by the preservative solutions used.

5.3.2 Analysis of Samples

DNA Extraction was performed using DNeasy® Blood and Tissue kit (Qiagen Ltd, UK) according to manufacturer's instructions. To determine the presence of DNA, 2 µL of the samples were analysed using agarose gel electrophoresis. Quantification was done using Qubit (Thermo Fischer, UK) according to manufacturer's instructions and using 1 µL of the samples. The 4-Plex multiplex PCR was performed to determine the amplification success of 70 bp, 194 bp, 305 bp, and 384 bp amplicons in the samples. Statistical data analysis was performed as described in Chapter 2.

5.4 Results

In a previous project, whole pig carcasses were left in the open and six samples (three of 0.5 g and three with 1 g) were collected at the moment of set-up to act as controls. After 79 ADD of incubation, sets of nine samples with 0.5 g and nine samples with 1 g were collected, and to these samples were added 5 mL of either 96% ethanol, cell lysis solution, or cell lysis solution with 1% sodium azide. After 210 ADD, six samples were collected, three with 0.5 g and three with 1 g. To these samples, 96% ethanol was added as preservative. All samples were set in triplicates and were stored at room temperature for approximately seven years.

During the seven-year interval between sample collection and DNA extraction, one of the samples stored in ethanol got lost. Some of the preservative agents dried during this incubation due to cracks in the lids that allowed evaporation, but these samples were still extracted. DNA extraction was done and the extracts were analysed on an agarose gel, quantitated and amplified with the 4-Plex multiplex for analysis.

With the agarose electrophoresis, it was possible to see that after seven years of storage even the control samples showed a little degradation, along with a bright band of high molecular weight DNA. As expected, samples that were collected after a certain incubation time had a smaller band of high molecular weight DNA and the smear associated with degradation was more clearly present. Four of the 0.5 g samples collected after incubation presented only a high molecular weight DNA bands (Figure 5.1). Overall, the set of 1 g samples appeared to have a higher level of degradation than the 0.5 g samples, with less bright bands and more clear degradation smear (Figure 5.2).
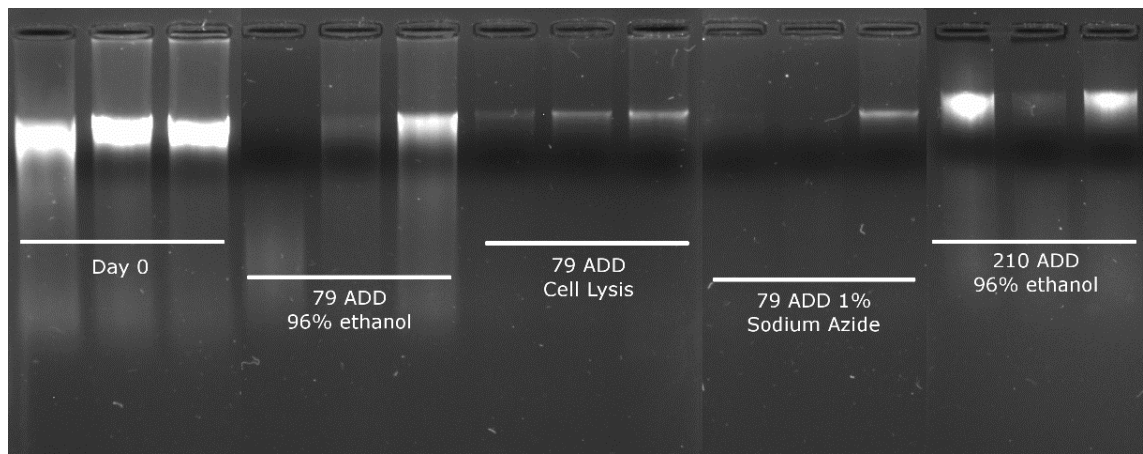
Figure 5.1 – A photograph showing the agarose electrophoresis gels of pig muscle samples of 0.5 g samples of pig muscle stored at room temperature for 7 years with different preservative agents. Each lane has one sample, but each condition had a triplicate.
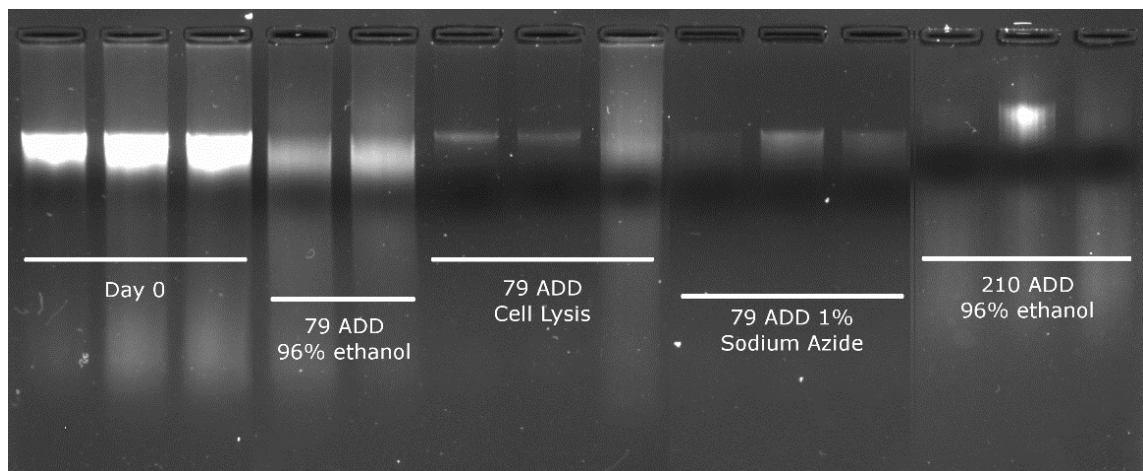
Figure 5.2 – A photograph showing the agarose electrophoresis gels of pig muscle samples of 1 g samples of pig muscle stored at room temperature for 7 years with different preservative agents. Each lane has one sample, but each condition had a triplicate.

Quantitation results matched what was observed earlier in the agarose gels, with samples that had a brighter DNA band resulting in higher DNA concentrations. It is possible to see in Figure 5.3 a drop from the average quantitation readings between the replicates from the control samples to the samples after incubation.

Storing samples in ethanol resulted in better DNA quantitation for both sets of samples collected after 79 ADD of incubation. With the exception of one sample,

all samples stored in cell lysis solution (with or without sodium azide) presented DNA quantitation readings quite similar between themselves.

Samples of 0.5 g collected after 210 ADD of incubation and stored in 96% ethanol had a slightly higher DNA concentration on average than samples collected at 79 ADD.
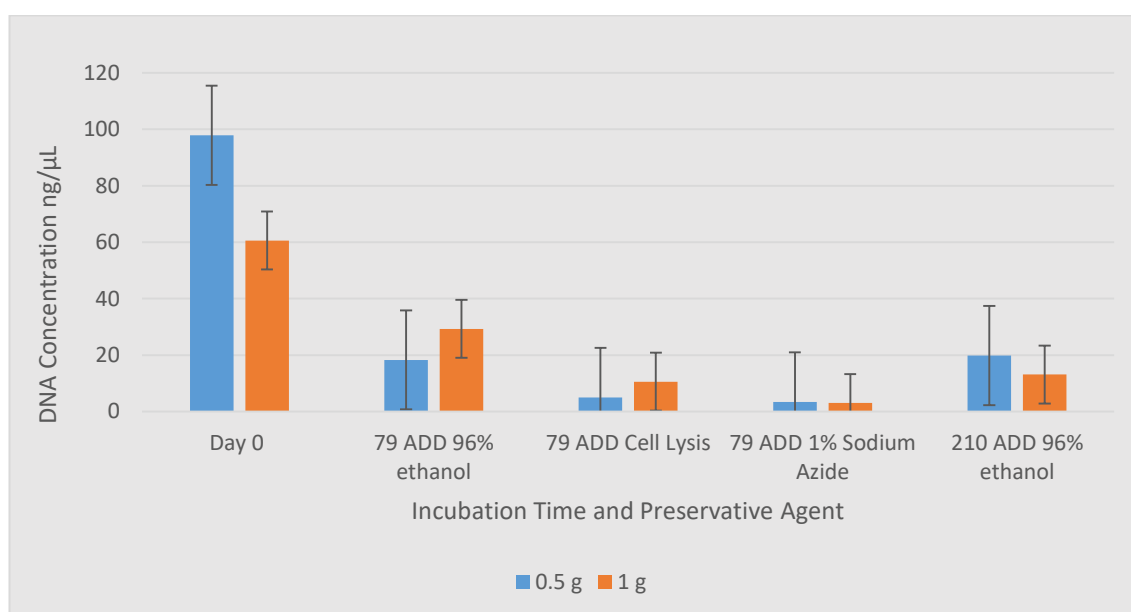
Figure 5.3 – Bar charts showing the average DNA concentrations from samples stored at room temperature for 7 years using different preservative agents. Each bar represents the average of one triplicate of samples. The exception is samples weighting 1 g, incubated for 79 ADD and stored in 96% ethanol, in which the bar represents two samples. Ethanol seems to be more efficient in preserving DNA in the samples. All data are presented as mean ± SEM, n=3.

For samples stored in cell lysis, amplification with the 4-Plex was only possible for the 70 bp amplicon. On the other preservative solutions, only one sample had a drop out of the 384 bp amplicon (Table 5.1). Samples weighting 1 g had slightly better profiles than samples of 0.5 g. Samples stored in ethanol had more balanced profiles when compared to samples collected after the same time of exposure and stored in cell lysis with 1% sodium azide (Figure 5.4).

Table 5.1 – Table showing the results of the 4-Plex multiplex amplification for samples stored at room temperature for 7 years using different preservative agents.

Weight	Time	Preservation Agent	4-Plex amplification of samples incubated at 25 °C for 7 years			
			70 bp	194 bp	305 bp	384 bp
0.5 g	Day 0	96% Ethanol	+++	+++	+++	+++
	79 ADD	96% Ethanol	+++	+++	+++	-++
		Cell Lysis	+++	---	---	---
		1% Sodium Azide	+++	+++	+++	+++
	210 ADD	96% Ethanol	+++	+++	+++	+++
1 g	Day 0	96% Ethanol	+++	+++	+++	+++
	79 ADD	96% Ethanol	++	++	++	++
		Cell Lysis	+++	---	---	---
		1% Sodium Azide	+++	+++	+++	+++
	210 ADD	96% Ethanol	+++	+++	+++	+++

The "+" denotes the presence and "-" the absence of PCR amplicons in samples.
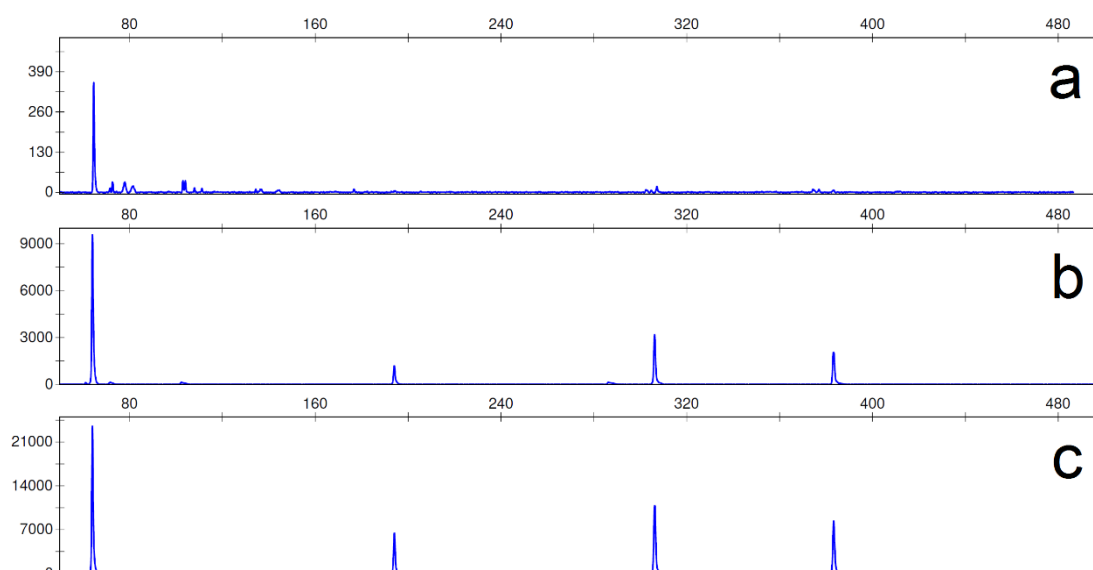
Figure 5.4 – Example of electropherograms from samples of this series of experiments. (a) is a sample store in cell lysis; (b) is a sample stored in cell lysis with 1% sodium azide; and (c) is a sample stored in 96% ethanol. All three samples weighted 0.5 g and were exposed for 79 ADD before collection.

5.5 Discussion

This series of experiments aimed to assess the long-term efficiency of different preservative solutions in preserving partly decomposed muscle tissue. The goal of the experiments was to determine the efficiency of different solutions to preserve partially decomposed soft muscle tissues that would increase the possibility of being able to generate a DNA profile at later time. Special considerations were given to the methods that are less expensive, need little preparation and do not require specialised equipment.

After seven years of incubation at room temperature, almost all samples in this series of experiments had high molecular weight bands present in the agarose gel electrophoresis. Concentration readings suggest that 96% ethanol is a better preservative than cell lysis, either with or without sodium azide. Nazir (2012) obtained complete 4-Plex amplification of pig muscle samples of 1 g and 0.5 g stored with 96% ethanol, cell lysis with and without sodium azide after one year of storage at room temperature.

Samples collected after 79 ADD of exposure and stored in 96% ethanol had almost complete amplification with only one sample of 0.5 g having a drop out of the biggest amplicon (384 bp). Other than that, the results between samples of 0.5 g and samples of 1 g were very similar in terms of peak height and profile balance for samples stored in ethanol.

Ethanol preserves specimens by the inhibition of cellular enzymes and hence the quality of DNA remains intact or undergoes negligible degradation over a period of time (Penna *et al.* 2001; Srinivasan *et al.* 2002). Previous studies for preservation of marine invertebrates and human tissues (prostrate, kidney and

liver) reported ethanol as an effective long-term tissue storage method that allows DNA recovery (Dawson *et al.* 1998; Gillespie *et al.* 2002; Kilpatrick 2002).

The results for ethanol preservation are in contrast with the findings of Michaud and Foran (2011) in which the authors reported that ethanol was not suitable for long term storage (6 months) of partially decomposed pig soft muscle tissues and recommended use of ethanol only for short-term storage. However, another study (Kilpatrick 2002) recovered high molecular weight DNA from ethanol preserved tissues for 2 years at room temperature (20 °C to 25 °C). In this present study, high molecular weight DNA was extracted from the samples after 7 years of storage at room temperature. This indicates that 96% ethanol can be used for long-term preservation of partially degraded muscle samples.

Despite the presence of high molecular weight bands on agarose gel, samples preserved with cell lysis had only successful amplification for the smaller amplicon of 70 bp. These results indicate that an amplification with STR markers after this long-term incubation in cell lysis would not be recommended. Instead, the best approach to analyse these kind of samples would be SNPs or INDELs, which have smaller amplicon sizes.

Samples stored with cell lysis and 1% sodium azide did not present clear high molecular weight DNA bands on the agarose gel, but had results similar to those of samples stored in 96% ethanol, only with smaller peak heights. The results of samples weighting 0.5 g were similar to those of 1 g samples. These two sets of samples had complete 4-Plex Multiplex amplification.

The results found in this study are not in agreement with the previous findings of Graham *et al.* (2008) for the use of commercially available cell lysis solutions to be used under field conditions, at least not for sample storage during extended periods of time.

The results of this study have also shown that ethanol was a better preservative than cell lysis solution in this experiment. Although the use of sodium azide did improve sample quality from the use of cell lysis alone, it is a dangerous compound and its use should be avoided when possible.

Even though sample preservation for long periods is usually not expected, it can be required depending on the case. In a mass disaster situation, it can take a long time before all samples are processed, because infrastructures can be damaged, specialized professionals can be in a small number, and because the number of samples can be great. In a criminal case, samples usually need to be available until conclusion of judgement and sometimes for a certain period afterwards. Room temperature storage is cheaper and requires less infrastructure than freezing samples.

In conclusion, the data clearly demonstrate that it is possible to obtain complete DNA profiles from muscle tissue samples stored at room temperature for 7 years using either 96% ethanol or cell lysis with 1% sodium azide as preservative solutions.

PART TWO: DNA PERSISTENCE USING DIFFERENT

PRESERVATION METHODS

5.6 Aims and Objectives

This main aim of this chapter is to assess the ability of different preservation solutions to preserve muscle soft tissue. Part two has the following objective:

- To assess DNA preservation in pig soft muscle tissue samples stored using different preservative solutions (95% ethanol, 37.5% ethanol, vodka) on different temperatures (-20 °C, 25 °C, and 37 °C) for up to 40 days.

5.7 Materials and Methods

Resumed methods were as follows, Chapter 2 contains more details.

5.7.1 Samples

This series of experiments was designed to evaluate the use of drinking alcohol as an alternative preservative solution for muscle tissue storage, comparing it with other preservative solutions and also storing samples in different temperatures.

Pig muscle bought from local store was cut in pieces of about 0.25 g and placed in 1.5 mL tubes (Figure 5.5). A total of 336 tubes was prepared and set as follow:

- Incubation at -20 °C:
    - 21 tubes with muscle preserved in 95% ethanol
    - 21 tubes with muscle preserved in 37.5% ethanol
    - 21 tubes with muscle preserved in vodka (37.5% ethanol)
    - 21 tubes with muscle and no preservation agent

- Incubation at -20 °C and with two cycles of thaw-refreeze (thawed at room temperature for 2 hours, frozen again at -20°C):
  - 21 tubes with muscle preserved in 95% ethanol
  - 21 tubes with muscle preserved in 37.5% ethanol
  - 21 tubes with muscle preserved in vodka (37.5% ethanol)
  - 21 tubes with muscle and no preservation agent
- Incubation at 25 °C:
  - 21 tubes with muscle preserved in 95% ethanol
  - 21 tubes with muscle preserved in 37.5% ethanol
  - 21 tubes with muscle preserved in vodka (37.5% ethanol)
  - 21 tubes with muscle and no preservation agent
- Incubation at 37 °C:
  - 21 tubes with muscle preserved in 95% ethanol
  - 21 tubes with muscle preserved in 37.5% ethanol
  - 21 tubes with muscle preserved in vodka (37.5% ethanol)
  - 21 tubes with muscle and no preservation agent

Samples were collected in triplicates (three tubes) and collection points were after 1, 3, 7, 14, 21, 35, and 42 days of incubation. Extraction of DNA was initiated immediately after collection.
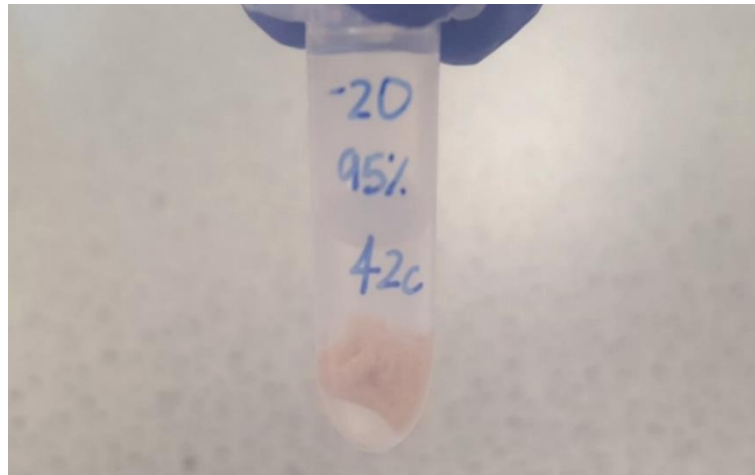
Figure 5.5 – A photograph showing the structure of polypropylene tube with muscle sample and preservative.

5.7.2 Analysis of Samples

DNA Extraction was performed using DNeasy® Blood and Tissue kit (Qiagen Ltd, UK) according to manufacturer's instructions. In order to determine the presence of DNA, a volume 2 µL of the samples was analysed using agarose gel electrophoresis. Quantification was done using Qubit (Thermo Fischer, UK) according to manufacturer's instructions and using 1 µL of the samples. The 4-Plex multiplex PCR was performed to determine the amplification success of 70 bp. 194 bp, 305 bp, and 384 bp amplicons in the samples. Statistical data analysis was performed as described in Chapter 2.

5.8 Results

5.8.1 Incubation at -20 °C

Eighty-four samples in total were incubated at -20 °C, with 12 of them collected at each time point. Of these 12, three were preserved with 95% ethanol, three with 37.5% ethanol, three with vodka, and three were left untreated. Immediately after collection of samples, DNA extraction was started. DNA extracts were analysed with gel electrophoresis, quantitated and then amplified with the 4-Plex to assess DNA degradation.

The analysis of the gel electrophoresis shows degradation after one day of incubation. This is possibly due to the fact that store-bought muscle tissue was used, and therefore it was not fresh. Degradation seems to have stagnated at this level and looks consistent through all time points (Figure 5.5).

Samples stored with 37.5% ethanol had brighter high molecular weight bands than compared to the other preserving agents. However, all of them generated clear high molecular weight DNA bands up to 42 days of incubation.
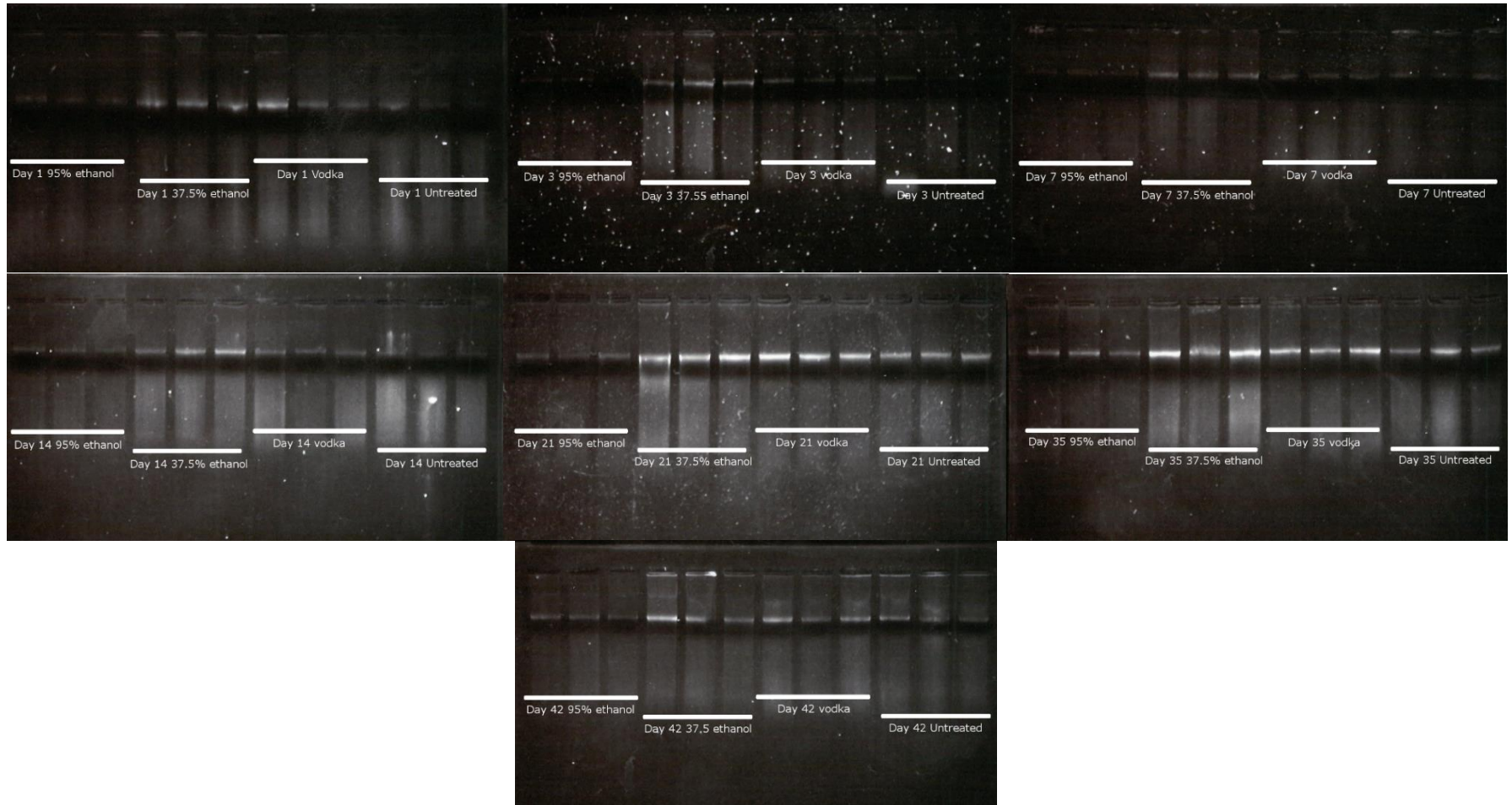
Figure 5.6 – A photograph showing the agarose electrophoresis gels from samples stored at -20 °C. It is possible to see the smear associated with DNA degradation on the first day of incubation. A clear band associated with high molecular weight DNA is visible in all time points of incubation. Each lane has to one sample and every condition was performed in triplicates.

Samples stored in 37.5% ethanol had better DNA concentration averages than samples preserved with any other method, which correlates to what was seen with gel electrophoresis. Storing samples in 95% ethanol resulted in less variation in the DNA concentration than other preservative agents, samples stored with this method had similar concentrations through incubation points (Figure 5.6).
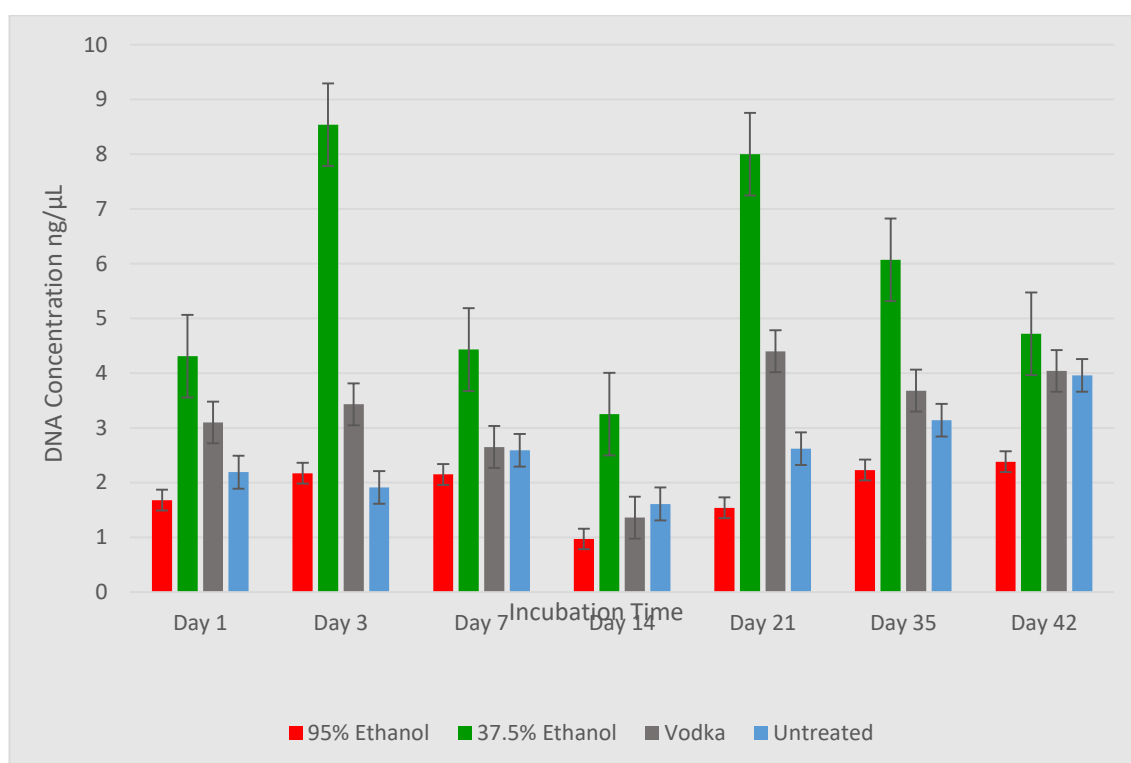
Figure 5.7 – Bar charts showing the average DNA concentrations of samples incubated at -20 °C with different preservation agents. The results indicate that 37.5% ethanol is more efficient in preserving DNA in the samples. All data are presented as mean ± SEM, n=3.

Samples stored at -20 °C, as expected show no signs of degradation after 42 days of incubation, with the gel electrophoresis looking consistent through the collection points and the capillary electrophoresis having all amplicons clearly present (Table 5.2).

In the electropherograms, no big differences could be observed when comparing samples from the first day of incubation with samples from the last day of

incubation (Figure 5.7). All alleles were amplified in all the samples in all time points.

Table 5.2 – Table showing the results of the 4-Plex multiplex amplification for samples stored at -20 °C using different preservation agents

Time	Preservation Agent	4-Plex amplification of samples incubated at -20 °C			
		70 bp	194 bp	305 bp	384 bp
Day 1	95% ethanol	+++	+++	+++	+++
	37.5% ethanol	+++	+++	+++	+++
	Vodka	+++	+++	+++	+++
	Untreated	+++	+++	+++	+++
Day 3	95% ethanol	+++	+++	++(+)	++(+)
	37.5% ethanol	+++	+++	+++	+++
	Vodka	+++	+++	+++	+++
	Untreated	+++	+++	+++	+++
Day 7	95% ethanol	+++	+++	+++	+++
	37.5% ethanol	+++	+++	+++	+++
	Vodka	+++	+++	+++	+++
	Untreated	+++	+++	+++	+++
Day 14	95% ethanol	+++	+++	+++	+++
	37.5% ethanol	+++	+++	+++	+++
	Vodka	+++	+++	+++	+++
	Untreated	+++	+++	+++	+++
Day 21	95% ethanol	+++	+++	+++	+++
	37.5% ethanol	+++	+++	+++	+++
	Vodka	+++	+++	+++	+++
	Untreated	+++	+++	+++	+++
Day 35	95% ethanol	+++	+++	+++	+++
	37.5% ethanol	+++	+++	+++	+++
	Vodka	+++	+++	+++	+++
	Untreated	+++	+++	+++	+++
Day 42	95% ethanol	+++	+++	+++	+++
	37.5% ethanol	+++	+++	+++	+++
	Vodka	+++	+++	(+)++	+++
	Untreated	+++	+++	+++	+++

The "+" denotes the presence and "-" the absence of PCR amplicons in samples. The (+) represents clear peaks below threshold of 50 RFU.

Figure 5.8 – Examples of electropherograms of samples stored at -20 °C, (a) is one sample incubated in 95% ethanol for 1 day; (b) 95% ethanol for 42 days; (c) 37.5% ethanol for 1 day; (d) 37.5% ethanol for 42 days; (e) vodka for 1 day; (f) vodka for 42 days; (g) left untreated for 1 day; (h) left untreated for 42 days. No differences could be observed in the profiles with the increased incubation time, but some degradation is visible at all time points and with all preserving agents.

5.8.2 Incubation at -20 °C and With Two Cycles of Thaw-Refreeze

A total of 84 samples were incubated at -20 °C and suffered two cycles of thaw-refreeze. These cycles were performed by removing the samples from the freezer, leaving them at room temperature until completely thawed for 2 h and them replacing them in the freezer. Samples were processed in the same way as described in session 5.8.1.

The thaw-refreeze cycles before extraction did not appear to cause differences in the results. In a similar way as to samples stored at -20 °C, samples stored in 37.5% ethanol had the brightest bands of high molecular weight DNA and all preserving agents generated clear high molecular weight DNA bands in all incubation points (Figure 5.8).

As with the samples stored at -20 °C, degradation visualised in the agarose gel looks similar in all points of collection.

As in the previous set, samples incubated in the presence of 37.5% ethanol had better concentration average results than other preservation solutions. All samples had the highest concentration averages on the last day of incubation (Figure 5.9).
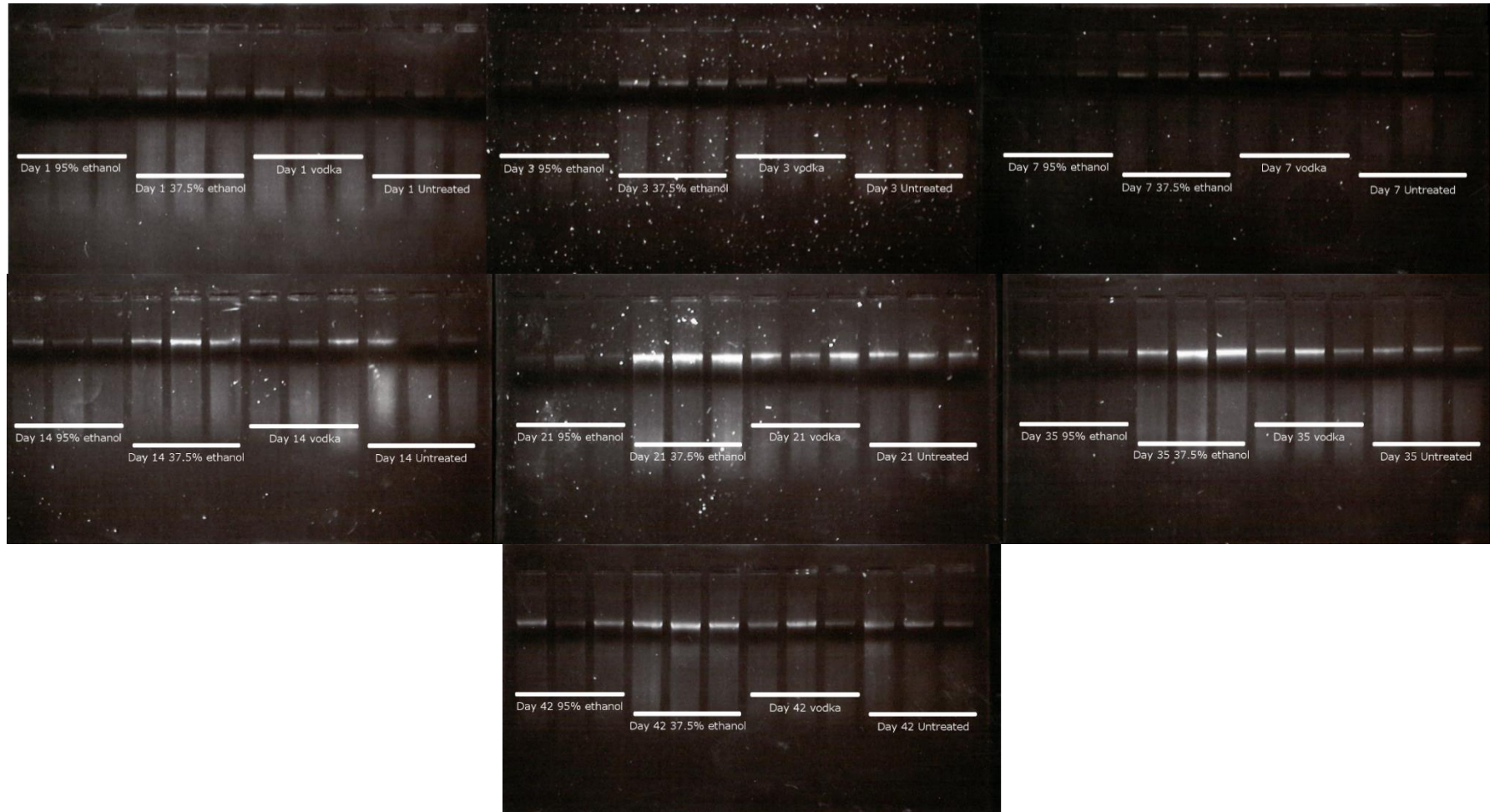
Figure 5.9 – A photograph showing the agarose electrophoresis gels from samples stored at --20 °C and with two cycles of thaw-refreeze. It is possible to see the smear associated with DNA degradation beginning on the first day of incubation. A clear band of high molecular weight DNA is visible in all time points of incubation. Each lane has to one sample and every condition was performed in triplicates.
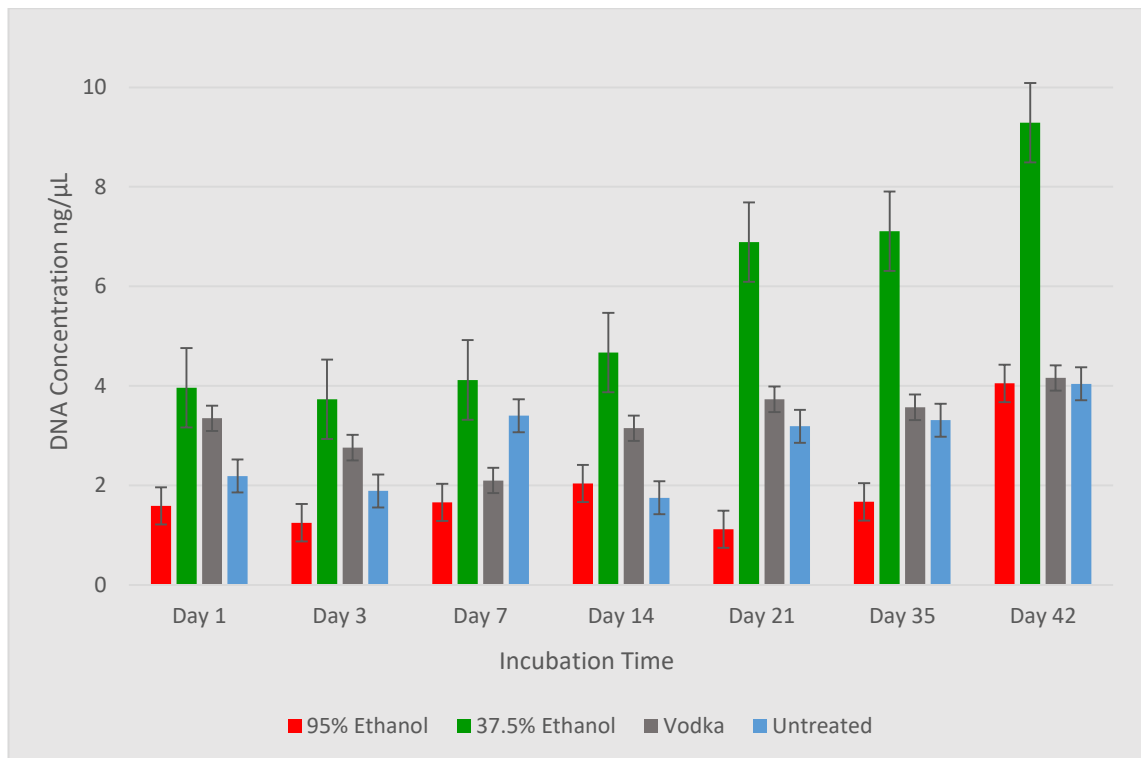
Figure 5.10 – Bar charts showing the average DNA concentrations of samples incubated at -20 °C with different preservation agents and gone through two thaw-freeze cycles. Again, it seems as 37.5% ethanol was the best preservation solution. All data are presented as mean ± SEM, n=3.

All alleles were amplified in all samples in all time points (Table 5.3). In this set of samples no differences could be observed when comparing samples from the first day of incubation with samples from the last day of incubation (Figure 5.10).

Table 5.3 – Table showing the results of the 4-Plex multiplex amplification for samples stored at -20 °C and with two cycles of thaw-refreeze using different preservation agents

Time	Preservation Agent	4-Plex amplification of samples incubated at -20 °C and with two cycles of thaw-refreeze			
		70 bp	194 bp	305 bp	384 bp
Day 1	95% ethanol	+++	+++	+++	+++
	37.5% ethanol	+++	+++	+++	+++
	Vodka	+++	+++	+++	+++
	Untreated	+++	+++	+++	+++
Day 3	95% ethanol	+++	+++	++(+)	++(+)
	37.5% ethanol	+++	+++	+++	+++
	Vodka	+++	+++	+++	+++
	Untreated	+++	++(+)	++(+)	++(+)
Day 7	95% ethanol	+++	+++	+++	+++
	37.5% ethanol	+++	+++	+++	+++
	Vodka	+++	+++	+++	+++
	Untreated	+++	+++	+++	+++
Day 14	95% ethanol	+++	+++	+++	+++
	37.5% ethanol	+++	+++	+++	+++
	Vodka	+++	+++	+++	+++
	Untreated	+++	+++	+(+)+	+++
Day 21	95% ethanol	+++	+++	+++	+++
	37.5% ethanol	+++	+++	+++	+++
	Vodka	+++	+++	+++	+++
	Untreated	+++	+++	+++	+++
Day 35	95% ethanol	+++	+++	+++	+++
	37.5% ethanol	+++	+++	+++	+++
	Vodka	+++	+++	+++	+++
	Untreated	+++	+++	+++	+++
Day 42	95% ethanol	+++	+++	+++	+++
	37.5% ethanol	+++	+++	+++	+++
	Vodka	+++	+++	+++	+++
	Untreated	+++	+++	+++	+++

The "+" denotes the presence and "-" the absence of PCR amplicons in samples. The (+) represents clear peaks below threshold of 50 RFU.

Figure 5.11 – Examples of electropherograms of samples stored at -20 °C and with two cycles of thaw-refreeze. Sample (a) was incubated in 95% ethanol for 1 day; (b) 95% ethanol for 42 days; (c) 37.5% ethanol for 1 day; (d) 37.5% ethanol for 42 days; (e) vodka for 1 day; (f) vodka for 42 days; (g) left untreated for 1 day; (h) left untreated for 42 days. No differences could be observed in the profiles with the increased incubation time.

5.8.3 Incubation at 25°C

Eighty-four samples in total were incubated at 25 °C, with 12 of them collected at each time point. Of these 12, three were preserved with 95% ethanol, three with 37.5% ethanol, three with vodka, and three were left untreated. Samples were processed in the same way as described in session 5.8.1.

In samples stored at 25 °C, it is possible to see an increasing level of degradation with higher incubation points. After 21 days of incubation, the clear high molecular weight DNA bands on the top of the gel that were present at the beginning of incubation are only visible on samples stored in 95% ethanol (Figure 5.11).

Samples stored in 95% ethanol presented defined high molecular weight DNA bands in all time points of this study. Samples incubated in vodka had the second best results in the gel electrophoresis, with not so bright bands, but with high molecular weight DNA still present on the last point of incubation.

Samples left untreated started showing smaller bands of DNA after 14 days of incubation. On days 14, 35, and 42 it is possible to see a pattern of two clearer bands and on days 21 and 35 other samples of the triplicate show a ladder-like pattern.
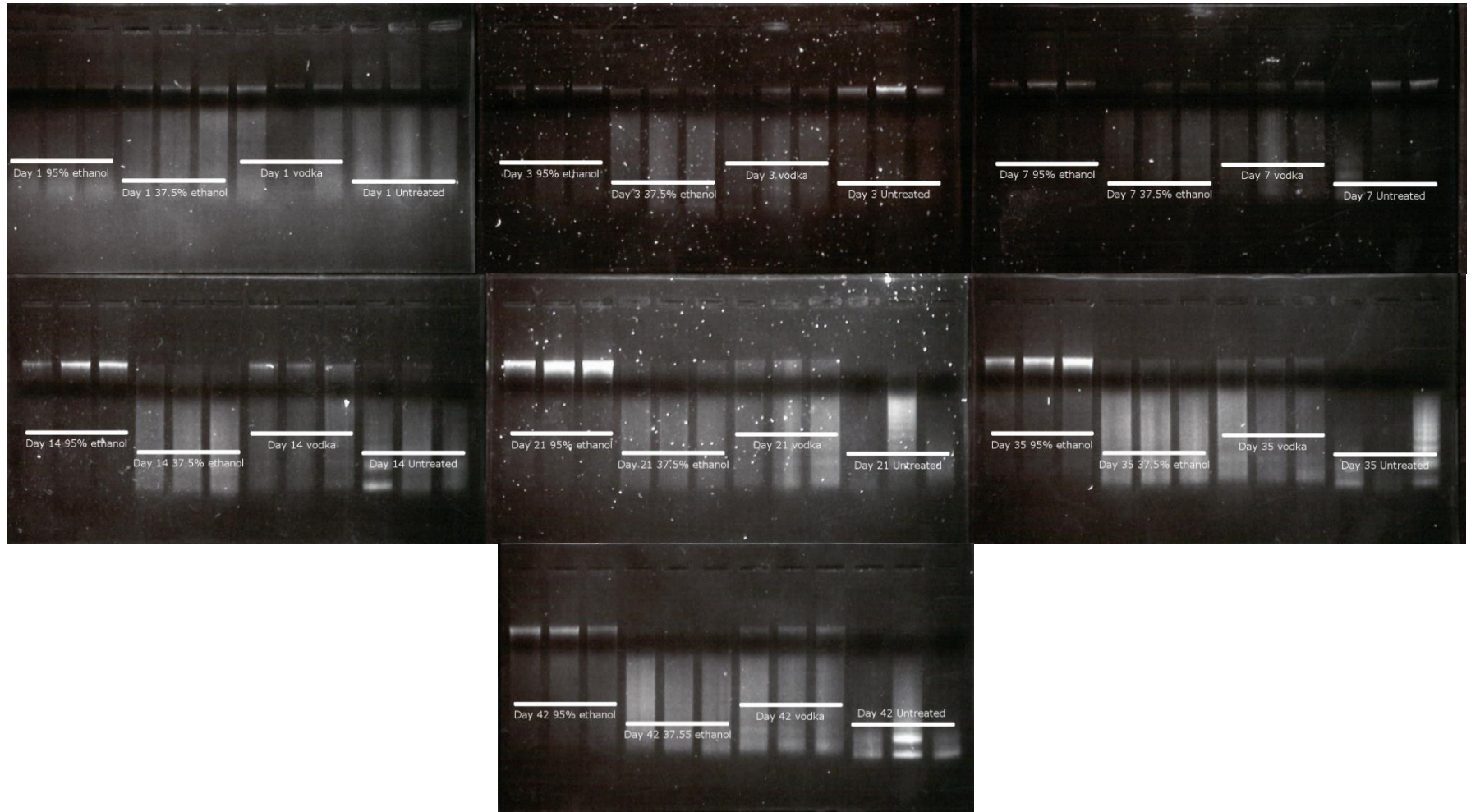
Figure 5.12 – A photograph showing the agarose electrophoresis gels from samples at stored 25 °C. On the last day of the incubation, only half of the samples showed high molecular weight DNA bands on the agarose gel. Each lane has to one sample and every condition was performed in triplicates.

Quantitation readings of samples incubated at 25 °C show an overall decrease in average DNA concentrations from three days of incubation onwards, especially on samples left untreated. It is possible to associate the presence of a high molecular weight band in the agarose gel and higher concentration readings on the triplicates (Figure 5.12).
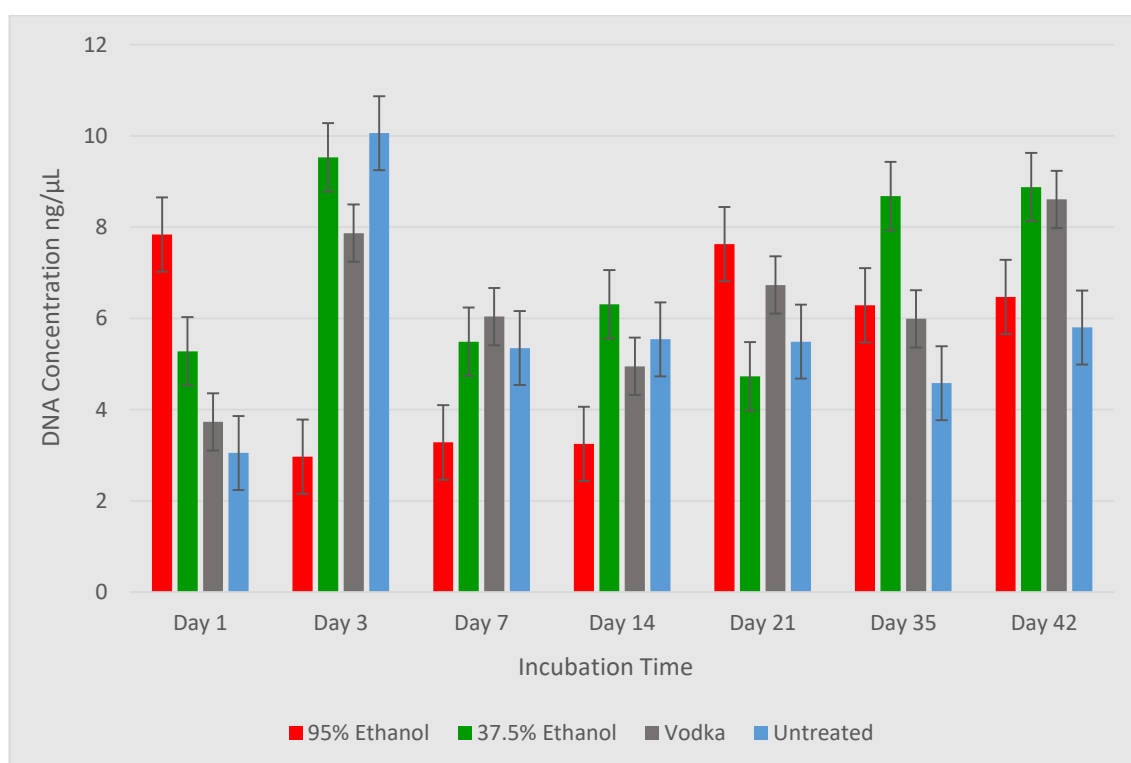
Figure 5.13 – Bar charts showing the average DNA concentrations of samples incubated at 25 °C with different preservation agents. The different conditions seem to have similar DNA concentrations within each time point. All data are presented as mean ± SEM, n=3.

Samples incubated with some kind of preserving agent had complete amplification of the 4-Plex at all time points. However, samples incubated untreated showed drop-outs of alleles after seven days of incubation (Table 5.4).

Untreated samples had peak heights of the larger amplicons decreasing with larger incubation time. While the height of the smaller amplicon (70 bp) seemed to stay relatively constant, the other three amplicons were slowly dropping out (Figure 5.13).

Table 5.4 – Table showing the results of the 4-Plex multiplex amplification for samples stored at 25 °C using different preservation agents

Time	ADD	Preservation Agent	4-Plex amplification of samples incubated at 25 °C			
			70 bp	194 bp	305 bp	384 bp
Day 1	25	95% ethanol	+++	+++	+++	(+)++
		37.5% ethanol	+++	+++	+++	+++
		Vodka	+++	+++	+++	+++
		Untreated	+++	+++	+++	+++
Day 3	75	95% ethanol	+++	+++	+++	+++
		37.5% ethanol	+++	+++	+++	+++
		Vodka	+++	+++	+++	+++
		Untreated	+++	+++	+++	+++
Day 7	175	95% ethanol	+++	+++	+++	+++
		37.5% ethanol	+++	+++	+++	+++
		Vodka	+++	+++	+++	+++
		Untreated	+++	-++	-++	-++
Day 14	360	95% ethanol	+++	+++	+++	+++
		37.5% ethanol	+++	+++	+++	+++
		Vodka	+++	+++	+++	+++
		Untreated	+++	+-+	(+)-(+)	(+)-(+)
Day 21	525	95% ethanol	+++	+++	+++	+++
		37.5% ethanol	+++	+++	+++	+++
		Vodka	+++	+++	+++	+++
		Untreated	+++	-++	-+(+)	-+(+)
Day 35	875	95% ethanol	+++	+++	+++	+++
		37.5% ethanol	+++	+++	+++	+++
		Vodka	+++	+++	+++	+++
		Untreated	-++	--+	--+	--+
Day 42	1050	95% ethanol	+++	+++	+++	+++
		37.5% ethanol	+++	+++	+++	+++
		Vodka	+++	+++	+++	+++
		Untreated	+++	-+-	-+-	-(+)-

The "+" denotes the presence and "-" the absence of PCR amplicons in samples. The (+) represents clear peaks below threshold of 50 RFU.
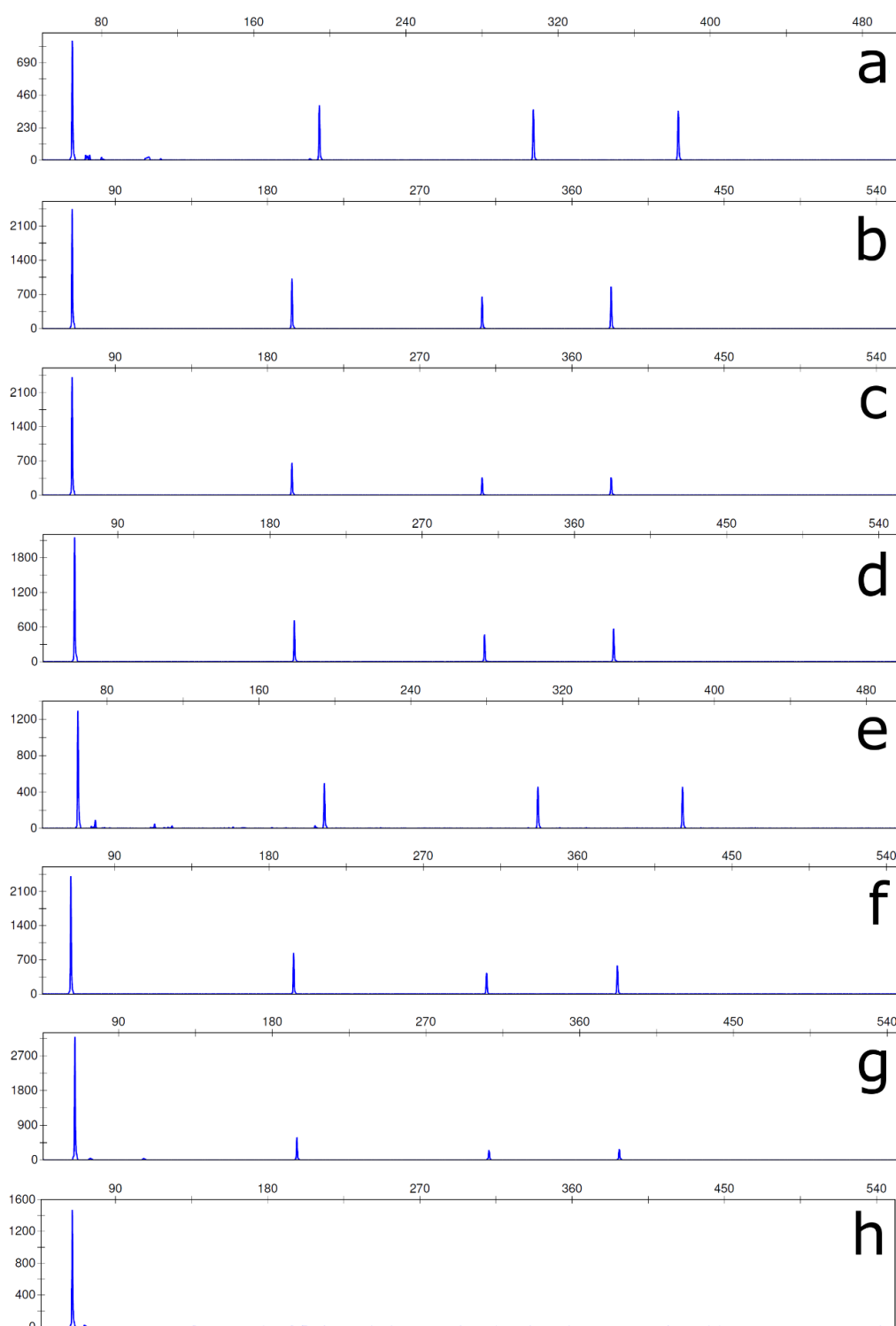
Figure 5.14 – Examples of electropherograms of samples stored at 25 °C. Sample (a) was incubated in 95% ethanol for 1 day; (b) 95 % ethanol for 42 days; (c) 37.5% ethanol for 42 days; (d) vodka for 42 days; (e) left untreated for 1 day; (f) left untreated for seven days; (g) left untreated for 35 days; (h) left untreated for 42 days. In untreated samples, it is possible to see the decrease of the peak heights of larger amplicons with increased incubation time.

5.8.4 Incubation at 37 °C

Eighty-four samples in total were incubated at 37 °C, with 12 of them being collected at each time point. Of these 12, three were preserved with 95% ethanol, three with 37.5% ethanol, three with vodka, and three were left untreated. Samples were processed in the same way as described in session 5.8.1.

In gel electrophoresis, samples stored at 37 °C showed an increasing level of degradation and faint bands of high molecular weight DNA after seven days of incubation. Even samples stored in 95% ethanol presented faint bands on the agarose gel after 42 days of incubation (Figure 5.14).

Untreated samples did not show a high molecular weight band at all after 14 days of incubation. Like samples stored at 25 °C, untreated samples stored at 37 °C also showed the ladder-like pattern (days seven and 14) and the smaller two bands (days three, 14, 21, 35, and 42).
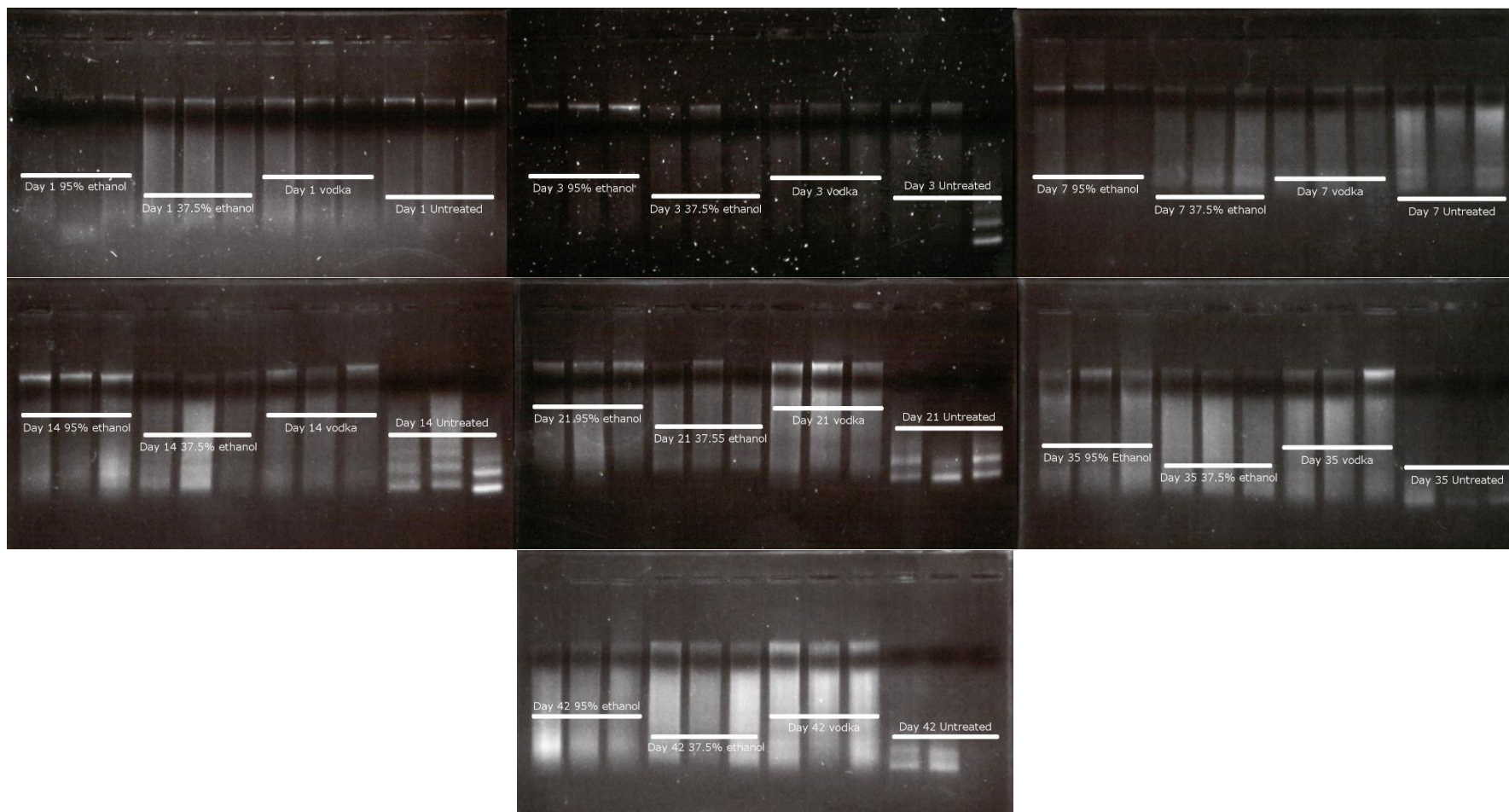
Figure 5.15 – A photograph showing the agarose electrophoresis gels from samples at stored 37 °C. Samples stored with some kind of preservative agent showed high molecular weight DNA bands up until the last day of incubation while untreated samples only had it present until seven days of incubation. Each lane has to one sample and every condition was performed in triplicates.

Samples incubated at 37 °C and left untreated had a decreasing average in DNA concentration through the collection points. Samples incubated with different kinds of preservative solutions had different behaviours, but all of them had higher quantitation readings on the last day of incubation than on the first one. The lowest average concentration of this set of samples was for untreated samples on day 35 (Figure 5.14). This correlates with the agarose gel electrophoresis, where none of the samples on the triplicate had high molecular weight bands.
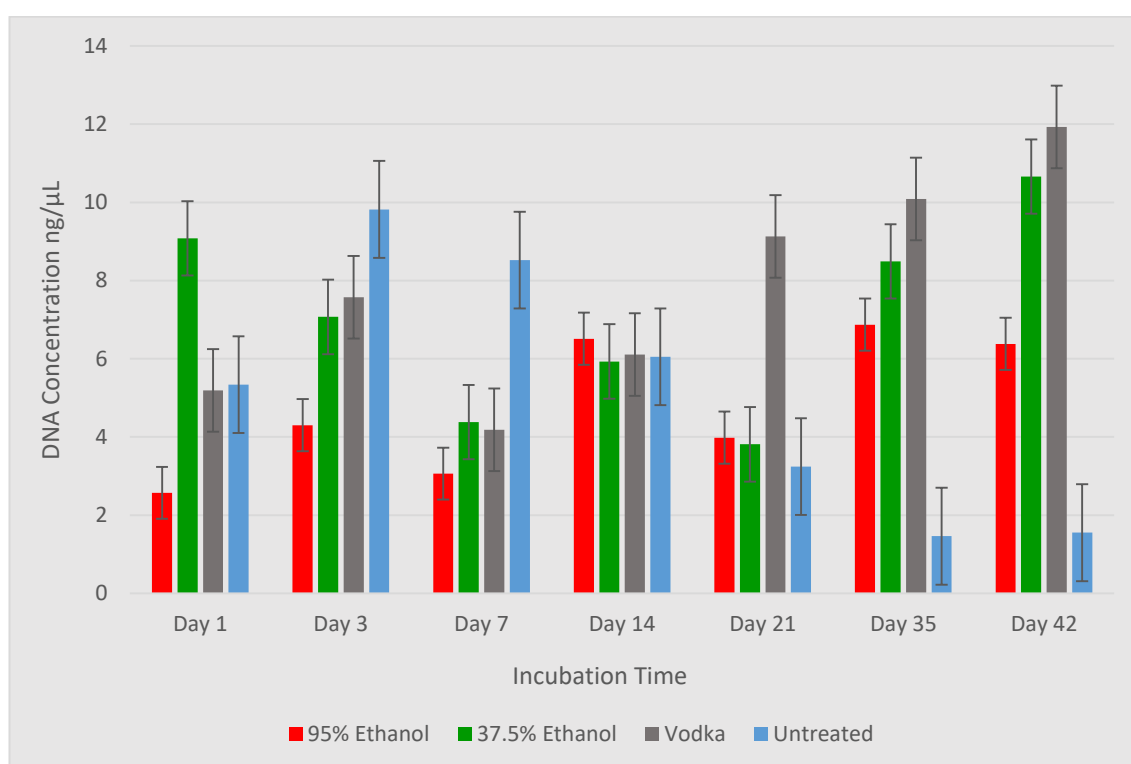
Figure 5.16 – Bar charts showing the average DNA concentrations of samples incubated at 37 °C with different preservation agents. Samples left untreated had a peak in DNA quantity on Day 3 and a decrease after that. All data are presented as mean ± SEM, n=3.

Although dropouts on the capillary electrophoresis started after 14 days of incubation, the same as samples incubated at room temperature, after that point they increased in occurrence faster. After 21 days of incubation, there was a drop-out of the biggest allele (384 bp) in all samples of the untreated triplicate and after 35 days of incubation only the smallest allele (70 bp) was present on the

untreated samples. Samples incubated with a preservative solution had complete

amplification at all time points (Table 5.5).

Untreated samples had peak heights of the larger amplicons decreasing with

larger incubation time. The smaller amplicon (70 bp) seemed to be more

resistant, with its peak height decreasing less than the other amplicons. The other

three amplicons dropped out completely after 35 days of incubation (Figure 5.16).

Table 5.5 – Table showing the results of the 4-Plex multiplex amplification for
samples stored at 37 °C using different preservation agents

Time	ADD	Preservation Agent	4-Plex amplification of samples incubated at 37 °C			
			70 bp	194 bp	305 bp	384 bp
Day 1	37	95% ethanol	+++	+++	+++	+++
		37.5% ethanol	+++	+++	-++	-++
		Vodka	+++	+++	+++	+++
		Untreated	+++	+++	+++	+++
Day 3	111	95% ethanol	+++	+++	+++	+++
		37.5% ethanol	+++	+++	+++	+++
		Vodka	+++	+++	+++	+++
		Untreated	+++	+++	+++	+++
Day 7	259	95% ethanol	+++	+++	+++	+++
		37.5% ethanol	+++	+++	+++	+++
		Vodka	+++	+++	+++	+++
		Untreated	+++	+++	+++	+++
Day 14	518	95% ethanol	+++	+++	+++	+++
		37.5% ethanol	+++	+++	+++	+++
		Vodka	+++	+++	+++	+++
		Untreated	+++	+++	+++	(+)+(+)
Day 21	777	95% ethanol	+++	+++	+++	+++
		37.5% ethanol	+++	+++	+++	+++
		Vodka	+++	+++	+++	+++
		Untreated	+++	(+)-+	(+)-(+)	---
Day 35	1295	95% ethanol	+++	+++	+++	+++
		37.5% ethanol	+++	+++	+++	+++
		Vodka	+++	+++	+++	+++
		Untreated	+++	---	---	---
Day 42	1554	95% ethanol	+++	+++	+++	+++
		37.5% ethanol	+++	+++	+++	+++
		Vodka	+++	+++	+++	+++
		Untreated	++-	---	---	---

The "+" denotes the presence and "-" the absence of PCR amplicons in
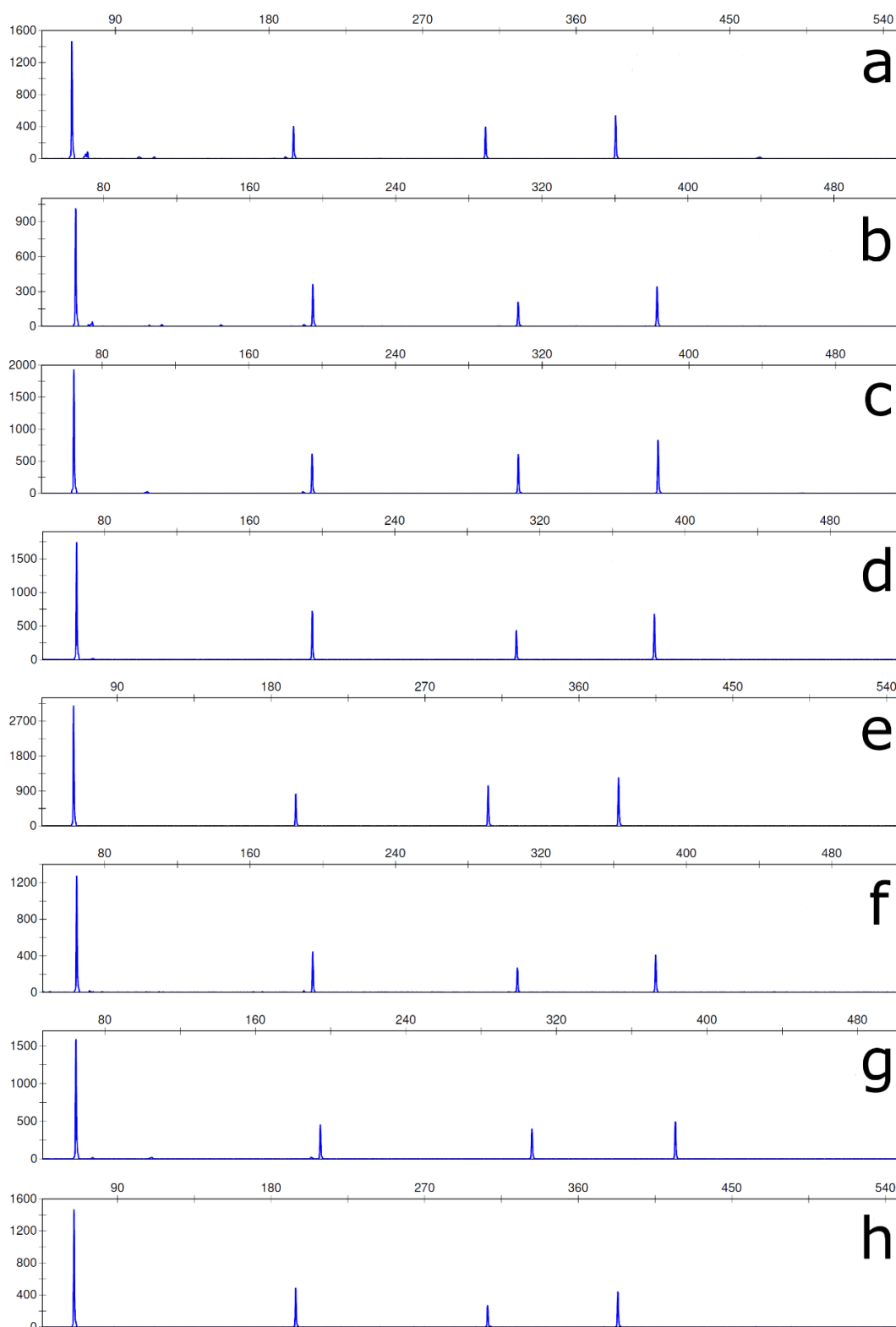samples. The (+) represents clear peaks below threshold of 50 RFU.
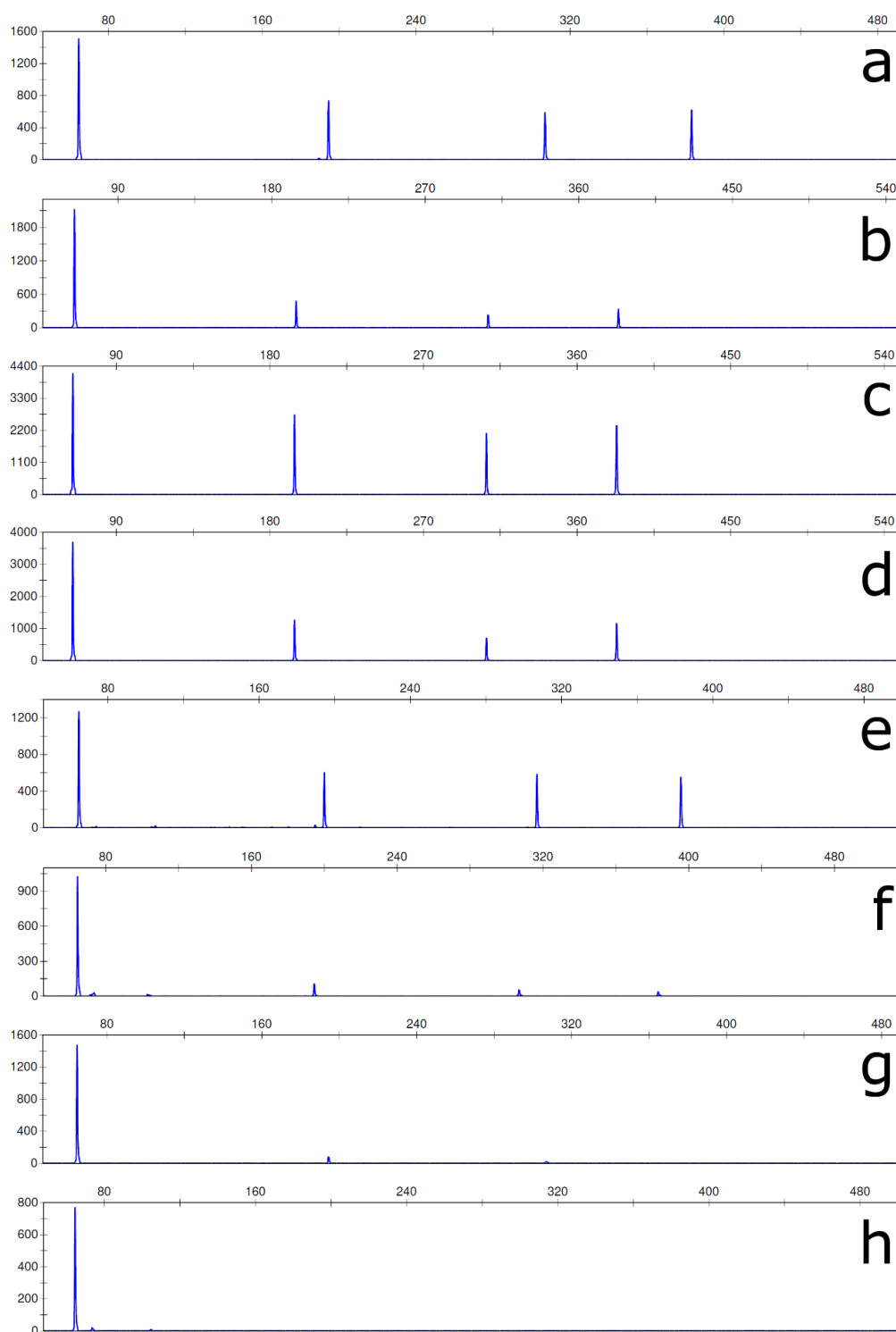
Figure 5.17 – Examples of electropherograms of samples stored at 37 °C.
Sample (a) was incubated in 95% ethanol for 1 day; (b) 95 % ethanol for 42
days; (c) 37.5% ethanol for 42 days; (d) vodka for 42 days; (e) left untreated for
1 day; (f) left untreated for 14 days; (g) left untreated for 21 days; (h) left
untreated for 35 days. It is possible to see the decrease of the peak heights and
drop outs of larger amplicons with a longer incubation time in untreated
samples.

5.9 Discussion

Currently, storing tissue samples in 95% ethanol and/or freezing them is the recommended procedure (Budowle *et al.* 2005; ICRC 2009). As so, in this series of experiments, these samples were used as a control to compare the efficiency of the tested methods. Similarly, samples left untreated were used as the other extreme control.

There are four processes that are generally responsible for DNA breakdown during tissue preservation: denaturation, cross-linking, strand-breakages, and chemical modification (Brown 1999). The use of preservative solutions intends to avoid these processes without the use of low temperatures. Freezing samples is one of the standards because at -20 °C all enzymatic or chemical activity that may degrade genetic material is effectively halted (Pegg 2007).

Different temperatures have previously been studied for sample storage. Cushwa and Medrano (1993) reported that they yielded less DNA from bovine blood samples stored at 37 °C for three days, than from samples stored at either 4 °C or -20 °C. This could be due to microbial digestion, as 37 °C offers optimal conditions for mesophilic bacteria to thrive. Hara *et al.* (2016) stored blood and bloodstains at a range of temperatures for 20 years and found that samples kept at room temperature and at 4 °C were significantly more degraded than fresh blood samples. Storage at -20 °C and -80 °C did not cause this difference.

In a similar way, previous studies have been done in the use of different concentrations of ethanol for preserving samples. King and Porter (2004) assessed a range of alcohol concentrations for the preservation of ant specimens and stated that 95% was the most indicated. Michaud and Foran (2011) tested concentrations of 40%, 70%, and 100% ethanol for preserving fresh and partially

decomposed pig muscle tissue and found no difference between them. Although the Interpol DVI Guide mentions the use of drinking ethanol for preserving soft tissue samples (INTERPOL 2014), limited literature was found on this subject.

The results in this study show that, in general, having any preserving agent is much better than having none. Incubation with 95% ethanol generated better results than with the other preservative agents used. However, the results from samples stored with either vodka or 37.5% ethanol were still good after 42 days of incubation at 37 °C, with high molecular weight DNA present in the agarose gel and clear peaks on the electropherograms. This means that the use of drinking alcohol or ethanol diluted more than the usual should not be discarded in field conditions. In a situation where the stock of ethanol is low or it is not available and it is not possible to freeze samples, these alternatives can be considered as a way to preserve tissue samples.

The impact of freeze-thaw cycles of whole blood samples on DNA integrity in the sample was previously studied (Bellete *et al.* 2003; Gessoni *et al.* 2004; Krajden *et al.* 1999; Ross *et al.* 1990; Shao *et al.* 2012) and the results showed progressive degradation of the samples as the number of freeze-thaw cycles increased. In the present study, no effects were observed on DNA degradation after two freeze-thaw cycles. This could mean that the muscle structure protects DNA integrity more than whole blood samples.

One of the hypothesized mechanisms of nucleic acid damage during freeze-thaw is the formation of ice crystals, which are thought to physically shear the DNA molecules (Roeder *et al.* 2010). The aqueous structure of blood would mean that more ice crystals form during the freezing process. Schaudien *et al.* (2007) provided evidence for this form of damage by observing that the inclusion of cryoprotectants such as glycerol correlates with a better retention of DNA quality

following freeze-thawing, perhaps due to the ability of glycerol to break up the rigidity of pure water ice.

Previous studies that have discovered found freeze-thawing to significantly affect DNA degradation have incorporated a much higher number of cycles, with Ross *et al.* (1990) performing up to 40 freeze-thaw cycles. The low number of freeze-thaw cycles on this experiment could be why no difference was found.

In conclusion, the results have clearly demonstrated that the use of less concentrated ethanol can be encouraged for preserving muscle tissue samples. In mass disaster situations, even though the conditions can be suboptimal, identification of the remains is of extreme importance for both the police and the families. The possibility of using drinking alcohol or a lower concentration of ethanol can increase the number of samples preserved and make the analysis and later identification easier for scientists.

Further research could investigate other easily accessible solutions, such as household products, that could be just as effective at preserving soft tissue DNA as 37.5% ethanol or vodka.

CHAPTER 6

VACUUM PRESERVATION OF MUSCLE TISSUE

6.1 Introduction

Decomposition will occur when a human cadaver is exposed to environmental conditions, with the soft tissue present in the body starting to decay within few days (Goff 2009). Cadaveric decay is influenced by two destructive processes: autolysis and putrefaction (Dent *et al.* 2004; Janaway *et al.* 2009).

Exogenous factors can affect the rate of cadaveric decay, such as the type of environment and the condition of the body at the moment of death. Oxygen availability is also important, with low oxygen levels restricting the microbial activity to anaerobes, thereby slowing the decomposition process (Dent *et al.* 2004; Statheropoulos *et al.* 2011).

Vacuum was the first technology applied to food packaging methods for commercial use. It consists of removing the air from the package, thus creating an anoxic environment. Vacuum preservation diminishes the growth of the microbial community when compared to aerobic preservation methods (Bellés *et al.* 2017).

Biobanks and hospitals are looking at vacuum preservation in order to reduce or cease to use formaldehyde (Condelli *et al.* 2014; Di Novi *et al.* 2010). Bussolati *et al.* (2008) found that the absence of air decreased autolytic processes, and increased protein and RNA stability.

With that in mind, a series of experiments was designed leading to the preservation of complete pig carcasses in vacuum body bags in order to assess the DNA preservation. The study design for whole body preservation with the use of vacuum body bags can be found on Appendix 1.

PART ONE: DNA PERSISTENCE IN SOFT TISSUES USING VACUUM PRESERVATION

6.2 Aims and Objectives

The aim of this chapter was to assess the use of vacuum as a strategy to preserve muscle tissue samples before DNA extraction. Part one has the following objective:

- To determine the effect of two different controlled temperatures (25 °C and 37 °C) on DNA persistence in pig soft muscle tissue in a vacuum condition.

6.3 Materials and Methods

Resumed methods were as follows, Chapter 2 contains full details.

6.3.1 Samples

This series of experiments was designed in order to study DNA persistence in samples incubated in vacuum in a controlled environment to compare them with samples incubated without vacuum.

Muscle tissue from pig obtained from the local butcher was used. The tissue was cut in pieces of approximately 1 cm^2 and each piece was placed in plastic bags to which vacuum was applied (Figure 6.1). Samples were incubated at both 25 °C and 37 °C.

Figure 6.1 – A photograph showing the structure of plastic bag with a piece of pig muscle tissue after vacuum was applied.

Samples incubated at 25 °C were collected on days one, four, seven, and every three days up to day 40, roughly at the same hour. Collection of samples incubated at 37 °C was done after 12 h, 24 h, 36 h, 48 h, 60 h, 72 h, 84 h, 96 h, 108 h, 120 h, and every 24 h up to 240 h (10 days). Samples were collected in triplicate (three bags) and immediately frozen until DNA extraction. One triplicate of samples for each incubation temperature was frozen at the setting up of the experiment. After DNA extraction, the remaining muscle tissue was transferred to a 1.5 mL tube.

Control samples were also incubated without vacuum along with to the ones in vacuum. For incubation at 25 °C, muscle tissue was put inside an open 1.5 mL tube and placed in the same incubator as the vacuum bags. Control samples incubated at 37 °C were placed in a closed plastic bag, but without vacuum applied to it. Three control samples were collected alongside the vacuum ones at each time point. Control samples were immediately frozen and then processed in the same way as vacuum samples.

6.3.2 Analysis

Extraction of DNA was performed using DNeasy® Blood and Tissue kit (Qiagen Ltd, UK) according to manufacturer's instructions. In order to determine the presence of DNA, a volume of 2 µL of the samples was analysed using agarose gel electrophoresis. Quantification was done using Qubit (Thermo Fischer, UK) according to manufacturer's instructions and using 1 µL of the samples. The 4-plex multiplex PCR was performed to determine the amplification success of 70 bp. 194 bp, 305 bp, and 384 bp amplicons in the samples. Statistical data analysis was performed as described in Chapter 2.

6.4 Results

6.4.1 Incubation at 25 °C

Forty two samples was incubated with vacuum at 25 °C, and another 42 control samples were incubated alongside them. Samples incubated with vacuum had little change in muscle structure. They became softer than at the beginning of the incubation, but changes were minimal. Control samples were however, air-dried over time. They became hard, which made them difficult to cut pieces for DNA extraction.

Although DNA was visible at all time points, after 10 days of incubation no high molecular weight DNA was present in gels run from the vacuum samples (Figure 6.2). On later incubation points, it was possible to see two distinct bands of lower molecular weight DNA. Gel electrophoresis also showed an increase in degradation in the control samples, but the presence of high molecular weight DNA was maintained even after 40 days of incubation (Figure 6.3).

The results from DNA quantitation showed that the vacuum samples had a decrease in the average of DNA concentration over time, while control samples had more variable results, with a peak in the middle of the incubation period (Figure 6.4).
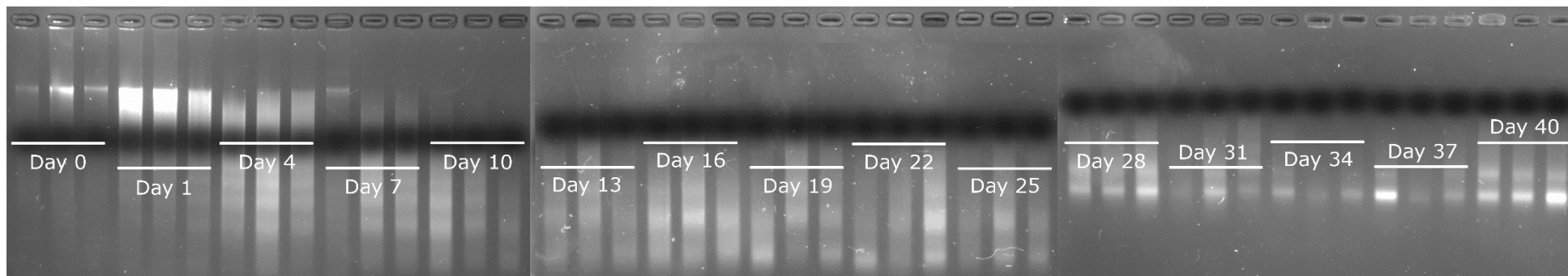
Figure 6.2 – A photograph showing the agarose electrophoresis gels of samples incubated at 25 °C with vacuum at the laboratory. Starting from around day 16 it is possible to see the formation of two clear bands of lower molecular weight DNA. Each lane has one sample, but each condition had a triplicate.
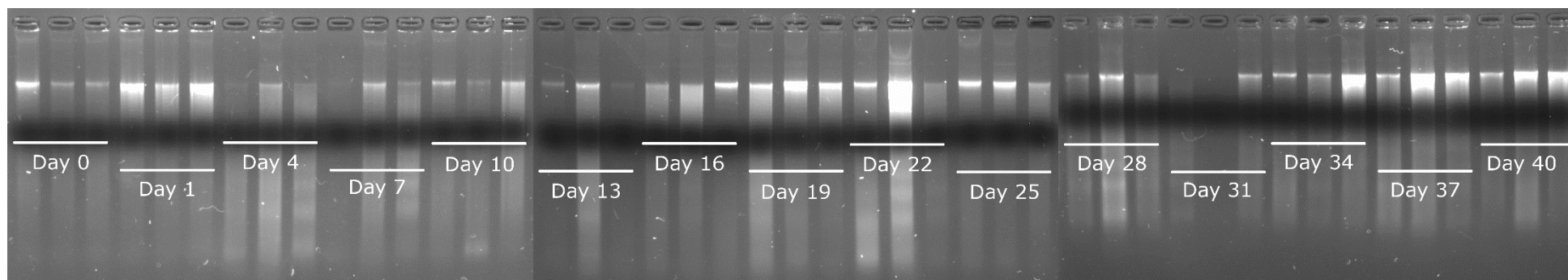
Figure 6.3 – A photograph showing the agarose electrophoresis gels of control samples incubated at 25 °C in an open tube at the laboratory. DNA was preserved with high molecular weight bands present until the last day of incubation. Each lane has one sample, but each condition had a triplicate.
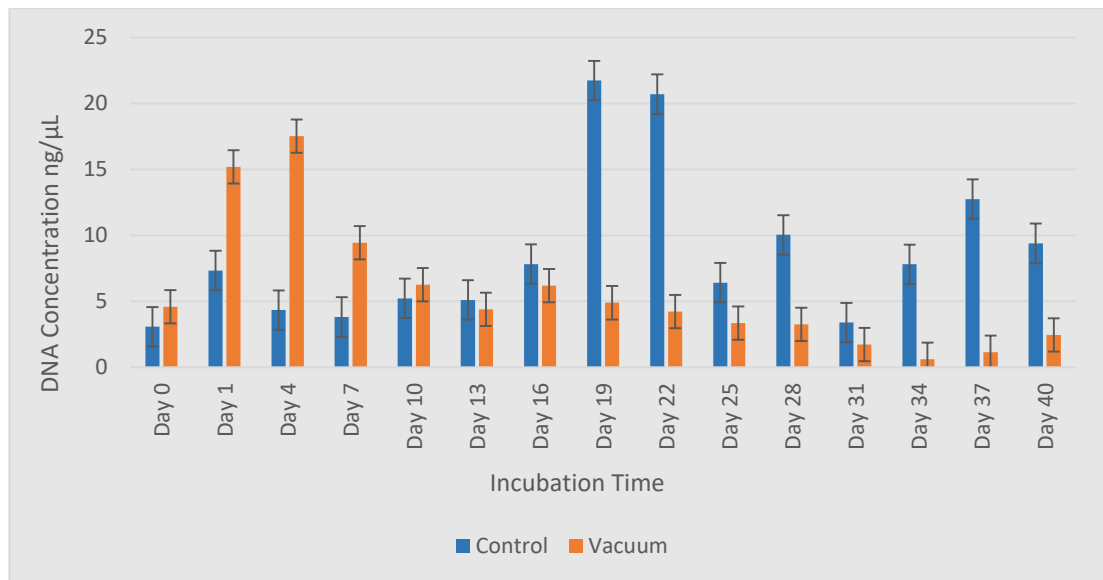
Figure 6.4 – Bar charts showing the average DNA concentrations of samples and controls incubated in the laboratory at 25 °C. Samples stored with vacuum had a continuous decrease in the readings while control samples varied more throughout time points. Each bar represents the average of one triplicate of samples. All data are presented as mean ± SEM, n=3.

Complete amplification of the 4-Plex Multiplex was possible until the last day of incubation both for samples incubated with vacuum and control samples (Tables 6.1 and 6.2), but some samples had dropouts before this point. Although samples incubated with vacuum from the last time point had complete amplification, dropouts in vacuum samples appear to show a pattern of increased degradation being present in the samples. Dropouts in the control samples seem to be sample-related and could have happened because of human error on extraction or due to the way the sample dried. The 70 bp amplicon, which is the most robust one, was not amplified in one of the samples from the last time point. It was amplified on the other two samples of the triplicate, which makes it seem likely the non-amplification is related to a problem with this sample alone.

Table 6.1 – Table showing the results of the 4-Plex multiplex amplification for samples incubated at 25 °C with vacuum.

Time	ADD	4-Plex amplification of samples incubated at 25 °C with vacuum			
		70 bp	194 bp	305 bp	384 bp
Time 0	0	+++	+++	+++	+++
Day 1	25	+++	+++	+++	+++
Day 4	100	+++	+++	+++	+++
Day 7	175	+++	+++	+++	+++
Day 10	250	+++	+++	+++	+++
Day 13	325	+++	+++	+++	+++
Day 16	400	+++	+++	+++	+++
Day 19	475	+++	+++	+++	+++
Day 22	550	+++	+++	+++	+++
Day 25	625	+++	+++	+++	+++
Day 28	700	+++	+++	+++	+++
Day 31	775	+++	-++	-++	-++
Day 34	850	+++	+--	+--	+--
Day 37	925	+++	+-+	+-+	+-+
Day 40	1000	+++	+++	+++	+++

The "+" denotes the presence and "-" the absence of PCR amplicons in samples.

Table 6.2 – Table showing the results of the 4-Plex multiplex amplification for samples incubated at 25 °C without vacuum.

Time	ADD	4-Plex amplification of control samples incubated at 25 °C			
		70 bp	194 bp	305 bp	384 bp
Time 0	0	+++	+++	+++	+++
Day 1	25	+++	+++	+++	+++
Day 4	100	+++	+++	+++	+++
Day 7	175	+++	-++	-++	-++
Day 10	250	++-	-+-	-+-	-+-
Day 13	325	+++	++-	++-	++-
Day 16	400	+++	+++	+++	+++
Day 19	475	+++	+++	+++	+++
Day 22	550	+++	+++	+++	+++
Day 25	625	++-	-+-	-+-	-+-
Day 28	700	+++	+++	+++	+++
Day 31	775	+++	+++	+++	+++
Day 34	850	+++	+++	+++	+++
Day 37	925	+++	+++	+++	+++
Day 40	1000	-++	-+-	-+-	-+-

The "+" denotes the presence and "-" the absence of PCR amplicons in samples.

Figure 6.5 – Examples of electropherograms of samples and controls incubated at 25 °C. (a) is a time zero sample; (b) is a sample incubated for 13 days with vacuum; (c) is a sample incubated for 40 days with vacuum; (d) is a control incubated for 4 days; (e) is a control incubated for 10 days; (f) is a control incubated for 28 days, and (g) is a control incubated for 40 days. It is possible to see that in samples incubated with vacuum the degradation is slow but progressive, whereas control samples that were air-dried did not have a clear pattern of degradation.

6.4.2 Incubation at 37 °C

Forty five samples was incubated with vacuum at 37 °C, and another 45 control samples were incubated alongside them. Samples incubated under a vacuum condition had little change in muscle structure, as with samples incubated at 25 °C, they only became slightly softer than at the beginning of the incubation. Control samples lost more of their physical structure with incubation, becoming softer and some of the samples even became liquefied and had to be scraped out of the bag. Samples were frozen after collection until the moment of DNA extraction.

Gel electrophoresis showed an increase in DNA degradation in the samples, but the presence of high molecular weight DNA was maintained after 240 h of incubation (Figure 6.6). Control samples had more prominent degradation present from earlier incubation times; although DNA was visible at every time point (Figure 6.7).

The results from DNA quantitation showed that control samples had a decrease in the average of DNA concentration over time, while vacuum samples had more variable results (Figure 6.8).
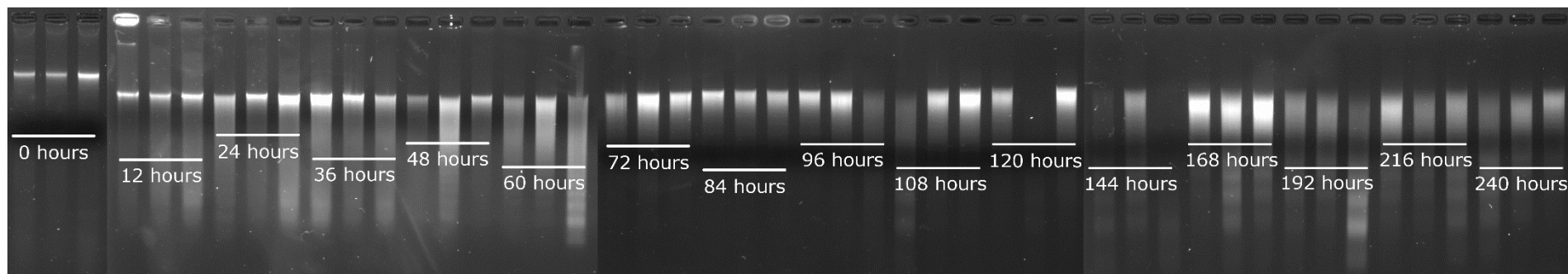
Figure 6.6 – A photograph showing the agarose electrophoresis gels of samples incubated at 37 °C with vacuum at the laboratory. Degradation increases with longer incubation times, but high molecular weight DNA is present until the last point of incubation. Each lane has one sample, but each condition had a triplicate.
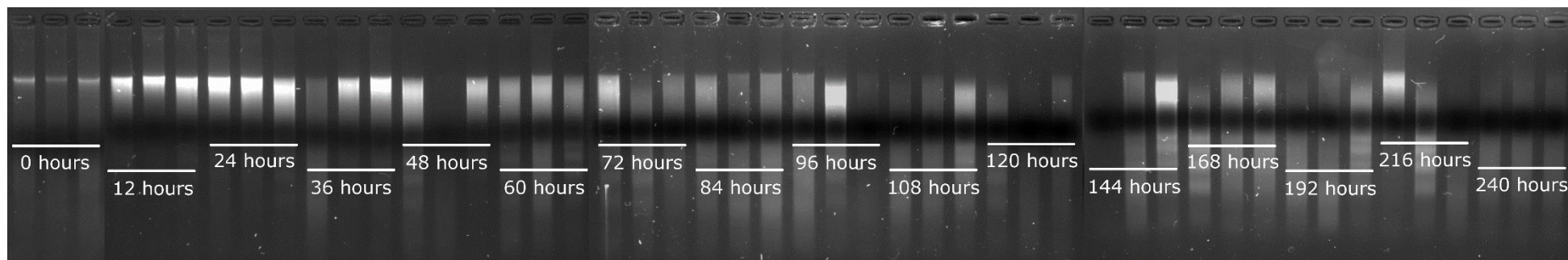
Figure 6.7 – A photograph showing the agarose electrophoresis gels of control samples incubated at 37 °C in a bag without vacuum at the laboratory. DNA was less preserved in this set of samples, with more degradation visible. Each lane has one sample, but each condition had a triplicate.
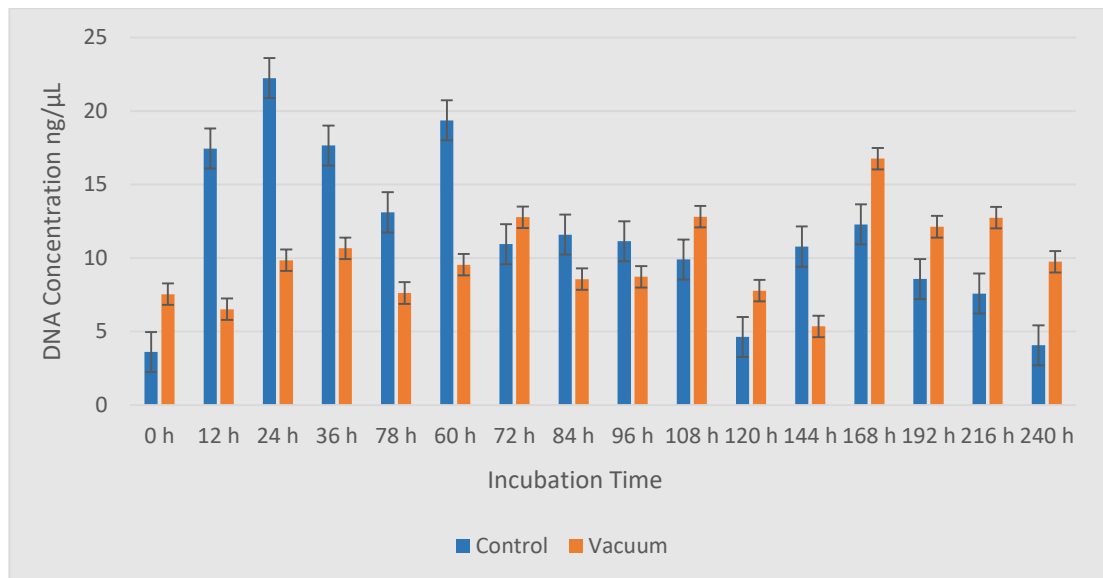
Figure 6.8 – Bar charts showing the average DNA concentrations of samples and controls incubated in the laboratory at 37 °C. Samples incubated under vacuum had less variation in quantitation readings than control samples incubated without vacuum. Each bar represents the average of one triplicate of samples. All data are presented as mean ± SEM, n=3.

Amplification was possible at all time points for vacuum-incubated samples (Table 6.3). The RFUs of the samples' profiles were constantly high and little difference could be seen between samples at the start of the experiment and samples after 10 days of incubation. A couple of samples had smaller RFUs, but it was always just one in the triplicate.

Control samples also had full amplification at all time points in this series of experiments (Table 6.4). They had more variation in the RFUs, but overall profiles were good for all control samples. Some of the samples had profiles that showed increase in degradation, but samples collected after 10 days of incubation had similar profiles as of those from samples at the beginning of the experiment (Figure 6.9).

Table 6.3 – Table showing the results of the 4-Plex multiplex amplification for samples incubated at 37 °C with vacuum.

Time	ADD	4-Plex amplification of samples incubated at 37 °C with vacuum			
		70 bp	194 bp	305 bp	384 bp
Time 0	0	+++	+++	+++	+++
12 h	18.5	+++	+++	+++	+++
24 h	37	+++	+++	+++	+++
36 h	55.5	+++	+++	+++	+++
48 h	74	+++	+++	+++	+++
60 h	92.5	+++	+++	+++	+++
72 h	111	+++	+++	+++	+++
84 h	129.5	+++	+++	+++	+++
96 h	148	+++	+++	+++	+++
108 h	166.5	+++	+++	+++	+++
120 h	185	+++	+++	+++	+++
144 h	222	+++	+++	+++	+++
168 h	259	+++	+++	+++	+++
192 h	296	+++	+++	+++	+++
216 h	333	+++	+++	+++	+++
240 h	370	+++	+++	+++	+++

The "+" denotes the presence and "-" the absence of PCR amplicons in samples.

Table 6.4 – Table showing the results of the 4-Plex multiplex amplification for samples incubated at 37 °C without vacuum.

Time	ADD	4-Plex amplification of control samples incubated at 37 °C			
		70 bp	194 bp	305 bp	384 bp
Time 0	0	+++	+++	+++	+++
12 h	18.5	+++	+++	+++	+++
24 h	37	+++	+++	+++	+++
36 h	55.5	+++	+++	+++	+++
48 h	74	+++	+++	+++	+++
60 h	92.5	+++	+++	+++	+++
72 h	111	+++	+++	+++	+++
84 h	129.5	+++	+++	+++	+++
96 h	148	+++	+++	+++	+++
108 h	166.5	+++	+++	+++	+++
120 h	185	+++	+++	+++	+++
144 h	222	+++	+++	+++	+++
168 h	259	+++	+++	+++	+++
192 h	296	+++	+++	+++	+++
216 h	333	+++	+++	+++	+++
240 h	370	+++	+++	+++	+++

The "+" denotes the presence and "-" the absence of PCR amplicons in samples.

Figure 6.9 – Examples of electropherograms of samples and controls incubated at 37 °C. (a) is a time zero sample; (b) is a sample incubated for 10 days with vacuum; (c) is a control incubated for 12 h; (d) is a control incubated for 48 h; (e) is a control incubated for 144 h; and (f) is a control incubated for 10 days.

6.5 Discussion

Based on the knowledge that muscle decomposition is partially dependent on microbial activity, this series of experiments was designed to assess if preserving soft tissue samples with vacuum would enhance DNA persistence. This was assessed with the use of agarose gel electrophoresis, DNA quantitation and the 4-Plex Multiplex.

The results show that with the application of a vacuum environment, muscle tissue samples were successfully preserved for up to 10 days at 37 °C and 40 days at 25 °C. Control samples also had complete amplification profiles up to the last time points, which was not expected.

The results of agarose gel electrophoresis from samples preserved at 25 °C with vacuum were not as good as those from the control samples. By leaving the 1.5 mL tubes containing the 25 °C control samples open, it allowed these samples to be air-dried. The persistence of DNA in the control samples incubated at 25 °C is due to the fact that air-drying is a preservation technique. This resulted in a situation where the controls were more preserved than the samples, while the incubation at 37 °C had the opposite pattern.

Both vacuum-preserved and control samples incubated at 37 °C showed increased degradation on the gel electrophoresis. Control samples had less DNA persistence and at the last incubation point, almost no DNA is visible. The degradation pattern of samples incubated with vacuum at 37 °C looks similar to the one of samples incubated at 25 °C with vacuum for the same period of time.

A variation in quantitation results from 25 °C control samples was observed and it is probably due to the difficulty of cutting uniform pieces of tissue. Air-dried

samples can become very hard and the process of cutting them in similar-sized pieces was difficult.

Control samples incubated at 25 °C were unintentionally preserved and that fact would explain why it was possible to obtain complete amplification profiles from almost all samples. Because the air-drying was not a controlled and planned process, some of the samples probably degraded, explaining the dropouts observed.

Control samples incubated at 37 °C had complete amplification profiles in all time points. This was not expected because it was assumed that the air inside the bag would allow microorganisms present in the sample to continue the putrefaction process, while not being enough to air-dry the sample. In this study, it was observed was that the samples did not air-dry, and as result, some of them had significant change in structure, losing all structural integrity, but surprisingly, DNA was still present in the samples.

This DNA persistence could be due to the limited air supply running out at some point during incubation. After this moment, microbial activity would be limited to anaerobic, similar to the situation in a vacuum environment. This would lead to a DNA preservation comparable to the one observed in the vacuum samples of this experiment. This hypothesis is corroborated by the fact that it was possible to see some degradation in control samples incubated at 37 °C, especially in samples that have lost physical structure, while such degradation was not observed in vacuum-incubated samples.

The control samples were unintentionally preserved, but a comparison with samples incubated in the same temperatures as described in Chapter 4 is possible. Samples incubated inside a 50 mL polypropylene tube generated full profiles up to 48 h (incubation at 37 °C) and 4 days (incubation at 25 °C). Samples

incubated at 37 °C had no amplification profiles after 168 h of incubation, and this point for samples incubated at 25 °C was after 31 days. In this series of experiments, the use of vacuum allowed for complete amplification at all time points studied. It was found previously that preserving tissue samples in vacuum decreases autolysis and increases RNA stability (Bussolati *et al.* 2008).

In conclusion, the results presented in this chapter show that not only RNA stability is increased, but also DNA. For the periods of incubation studied in this investigation, preserving tissue samples in vacuum can be an alternative way to be explored for forensic purposes.

PART TWO: DNA PERSISTENCE IN SOFT TISSUES INSIDE SEALED BAGS WITHOUT VACUUM

6.6 Aims and Objectives

The aim of this chapter was to assess the use of vacuum as a strategy to preserve bodies and muscle tissue samples before DNA extraction. Part two has the following objective:

- To assess if the volume of air inside a sealed plastic bag influences microbial activity and tissue degradation.

6.7 Materials and Methods

Resumed methods were as follows, Chapter 2 contains more details.

6.7.1 Samples

This series of experiments was designed in order to study DNA persistence in samples incubated in a plastic bag sealed without vacuum. During the previous series of experiments, control samples did not degrade as expected and this study was designed to assess if the volume of air inside the bag could influence microbial activity and tissue degradation. The idea was that the oxygen in a smaller bag could be consumed and stop the activity of aerobic organisms.

Muscle tissue from pig obtained from the local butcher was used. The tissue was cut in pieces of approximately 1.5 cm^2 and each piece was placed in plastic bags that were sealed leaving air inside (Figure 6.10). Three different sizes of bags were prepared: the small ones measured 7 x 8.5 cm; the medium measured 7 x 17 cm; and the large measured 14 x 17 cm. The volume of air inside each bag size was estimated using water, and they were approximately 8 mL in the small

bags, 20 mL in the medium bags; and 50 mL in the large ones. Samples were incubated at 37 °C for 10 days with collection of one triplicate of each size happening every two days. After collection, samples were frozen until DNA extraction. One triplicate of samples was frozen at the setting up of the experiment.
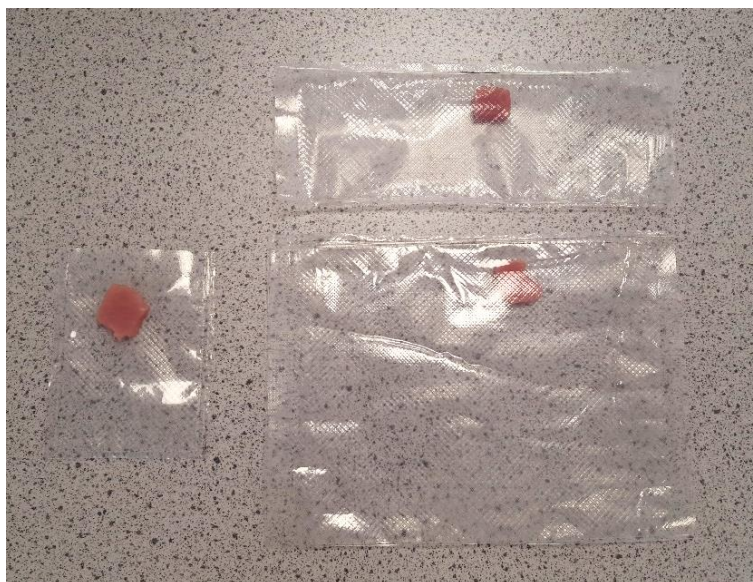
Figure 6.10 – A photograph showing the different sizes of bags with muscle sample inside them. Bags were sealed with air still inside them.

6.7.2 Analysis

Extraction of DNA was performed using DNeasy® Blood and Tissue kit (Qiagen Ltd, UK) according to manufacturer's instructions. To determine the presence of DNA, 2 µL of the samples were analysed using agarose gel electrophoresis. Quantification was done using Qubit (Thermo Fischer, UK) according to manufacturer's instructions and using 1 µL of the samples. The 4-Plex multiplex PCR was performed to determine the amplification success of 70 bp. 194 bp, 305 bp, and 384 bp amplicons in the samples. Statistical data analysis was performed as described in Chapter 2.

6.8 Results

A total of 45 samples were incubated at 37 °C inside plastic bags without vacuum of three different sizes. Samples incubated in the small bags had little change in muscle structure, becoming only slightly softer than at time zero. Samples incubated in medium and large bags dried over time, with this process being faster in samples inside large bags. All samples were frozen immediately after collection until the moment of DNA extraction.

Samples incubated in small bags showed increased degradation in agarose gel electrophoresis, and after six days of incubation there was not a high molecular weight DNA band present (Figure 6.11). However, DNA was still visible until the last point of incubation.
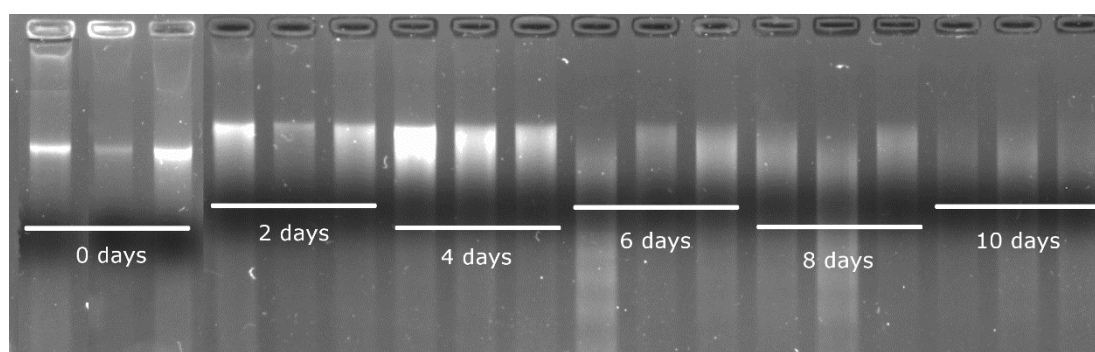
Figure 6.11 – A photograph showing the agarose electrophoresis gels of samples incubated in small bags. Each lane is one sample, but each condition had a triplicate.

Samples incubated in medium and large bags had similar results in the gel electrophoresis, with high molecular weight DNA present in all time points including after 10 days of incubation (Figures 6.12 and 6.13). Samples incubated in large bags have slightly dimmer bands and almost no clear band on the last incubation point.
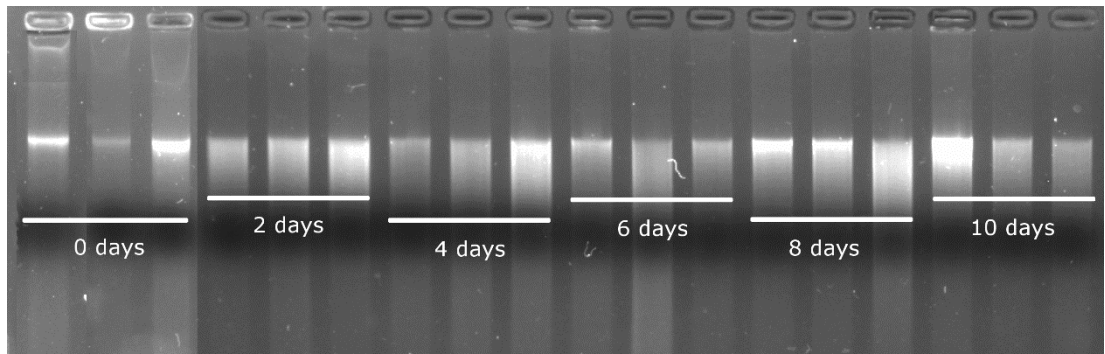
Figure 6.12 – A photograph showing the agarose electrophoresis gels of samples incubated in medium bags. Each lane is one sample, but each condition had a triplicate.
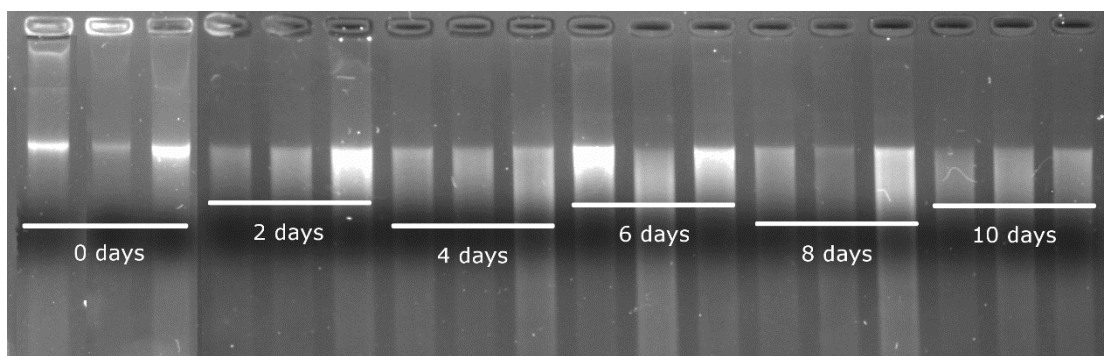
Figure 6.13 – A photograph showing the agarose electrophoresis gels of samples incubated in large bags. Each lane is one sample, but each condition had a triplicate.

All groups of samples had an initial increase in DNA concentration results up to day four. After this, readings from samples incubated in the small bags started to decrease while those from samples incubated in medium and large bags continued to increase. DNA concentrations from samples incubated in small bags did not vary much during the incubation period, but readings from medium and large bags doubled or tripled the day zero DNA concentration value.
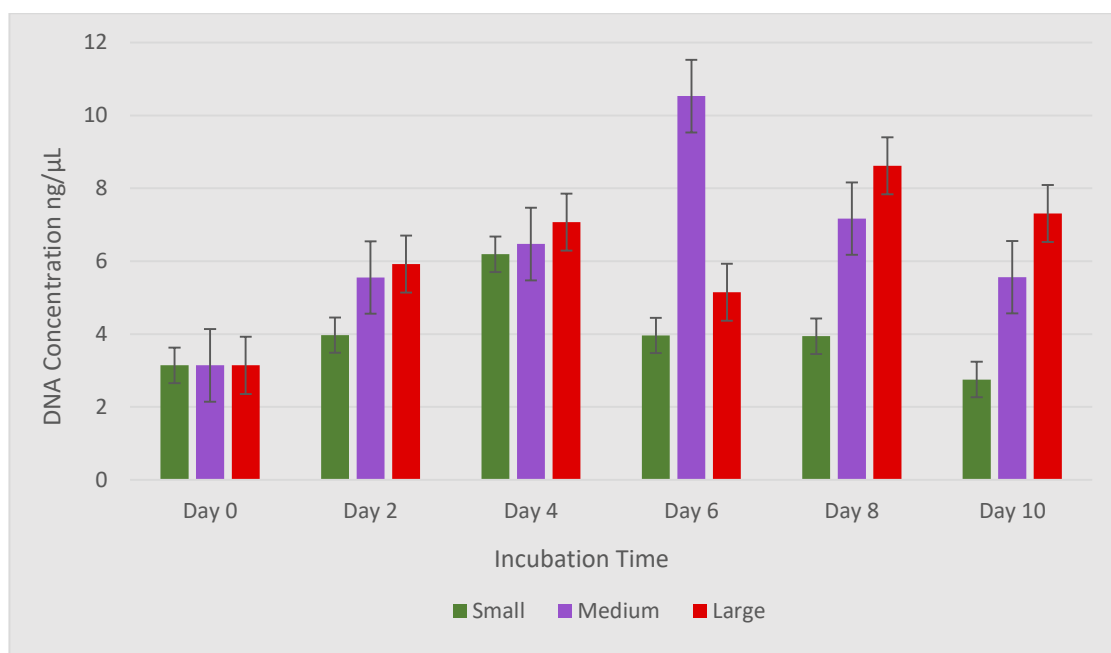
Figure 6.14 – Bar charts showing the DNA quantitation of samples incubated in plastic bags of different sizes at 37 °C. Samples incubated in small bags had less variation in readings than samples incubated in medium and large bags. Each bar represents the average of one triplicate of samples. All data are presented as mean ± SEM, n=3.

Samples incubated in all three sizes of bags generated complete amplification results in all time points studied (Table 6.5). Some degradation could be observed in profiles from the triplicate incubated in a small bag for 10 days, but the difference between the peak heights in the smaller and larger allele was not much different from the one in time zero control samples.

Table 6.5 – Table showing the results of the 4-Plex multiplex amplification for samples incubated at 37 °C inside bags of three different sizes without vacuum.

Time	Size	4-Plex amplification of samples incubated in three sizes of plastic bags without vacuum			
		70 bp	194 bp	305 bp	384 bp
Time 0	-	+++	+++	+++	+++
2 Days	S	+++	+++	+++	+++
	M	+++	+++	+++	+++
	L	+++	+++	+++	+++
4 Days	S	+++	+++	+++	+++
	M	+++	+++	+++	+++
	L	+++	+++	+++	+++
6 Days	S	+++	+++	+++	+++
	M	+++	+++	+++	+++
	L	+++	+++	+++	+++
8 Days	S	+++	+++	+++	+++
	M	+++	+++	+++	+++
	L	+++	+++	+++	+++
10 Days	S	+++	+++	+++	+++
	M	+++	+++	+++	+++
	L	+++	+++	+++	+++

The "+" denotes the presence and "-" the absence of PCR amplicons in samples.
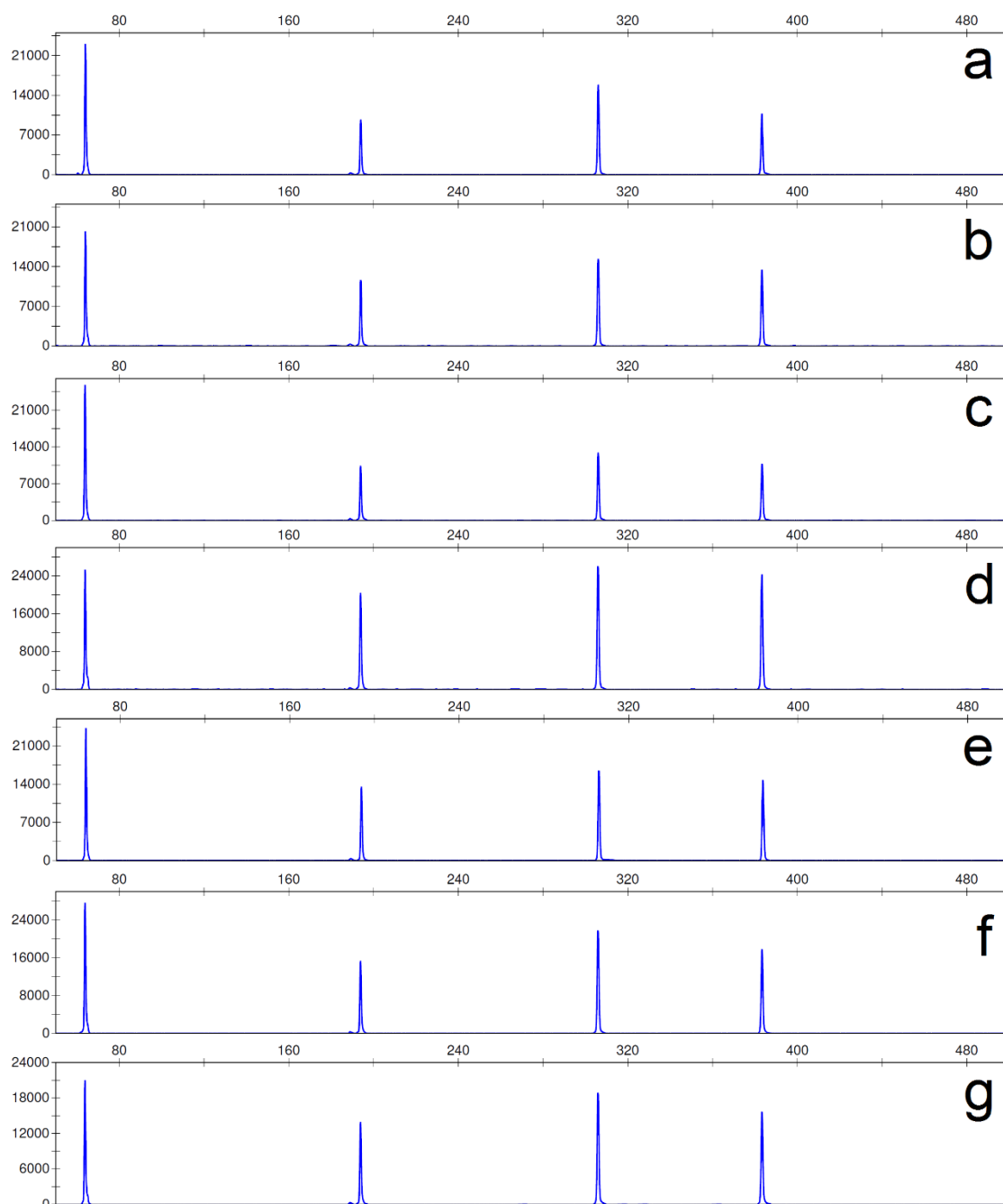
Figure 6.15 – Examples of electropherograms of samples incubated at 37 °C in plastic bags of different sizes. (a) is a time zero sample; (b) is a sample incubated for 2 days in a small bag; (c) is a sample incubated for 10 days in a small bag; (d) is a sample incubated for 2 days in a medium bag; (e) is a sample incubated for 10 days in a medium bag; (f) is a sample incubated for 2 days in a large bag; and (g) is a sample incubated for 10 days in a large bag; n=3..

6.9 Discussion

Based on the results from Part I of this chapter, this series of experiments was designed to see the effect of different sizes of plastic bags in DNA persistence in soft tissue samples preserved without vacuum. This was assessed with the use of agarose gel electrophoresis, DNA quantitation and the 4-Plex Multiplex. It was expected that the different volumes of air would allow for microbial activity, but the closed nature of the bags would prevent the samples from drying.

Agarose gel electrophoresis shows an increase in degradation in samples incubated inside small bags. Samples incubated inside medium and large bags air-dried and it is possible to see in the gel electrophoresis that the DNA was preserved in a better way than in small bags. This was probably due to the fact that the small volume of air in the small bags was not enough for air-drying but allowed microbial activity before being consumed. This microbial activity caused DNA degradation.

An increase of two and three folds in DNA quantitation readings was observed in samples incubated in medium and large bags. This was due to the fact that air-dried samples are hard to cut and different sizes of tissue were used for DNA extraction.

All bag sizes resulted in complete amplification profiles in all time points. The reason behind is the same as observed in Part I of this chapter. In the small bags, which had the same size as bags used in the first part of this chapter, the oxygen inside is consumed and this inhibits microbial activity. In medium and large bags, the volume of air inside was enough to air-dry the samples, with samples inside large bags drying completely faster than samples inside medium bags.

In conclusion, although unexpected, the results in this chapter could potentially be used for preservation of small tissue samples in warm environments. Samples were preserved for up to 10 days at 37 °C and all that was required were plastic bags and a machine capable of sealing them. The seal prevents moisture and oxygen from entering the bag, which either allows the environment inside to run out of oxygen or the sample to air-dry, and both ways proved to enhance DNA persistence.

CHAPTER 7

REBALANCING DEGRADED DNA PROFILES USING SIZE SEPARATION TECHNIQUES

7.1 Introduction

Sample purification is included routinely in most laboratories in order to remove inhibitors. Purification can also be used post-amplification to remove excess primers and other reagents. In this research study, purification columns were used as a way to remove small fragments of degraded DNA from samples prior to amplification.

Agarose gel electrophoresis is a relatively easy and fast method to assess the quality and quantity of DNA. This technique also allows the user to recover DNA intact from a band and individually isolate fragments. Agarose is a polymer that forms a porous matrix that acts like a net for the DNA. When an electric current is applied to the gel, smaller fragments pass through the "net" formed by agarose more easily than bigger fragments, which get retained. High molecular weight DNA can be seen as a single band on the top of the gel while degraded DNA appears as a smear (Goodwin *et al.* 2007; Phillips *et al.* 1998).

Microcon® centrifugal filters can be used for concentration, desalting or buffer exchange of aqueous biological samples. The applications of the Microcon® columns chosen for this experiment include removal of labelled nucleotides, labelled amino acids and primers from amplified DNA.

The illustra MicroSpin G-50 Columns purify DNA by the process of gel filtration with the use of Sephadex™ G-50 DNA grade F resin. Gel filtration process works by penetration of the matrix by the molecules applied. Molecules larger than the pores in the Sephadex™ are excluded, and small molecules have complete penetration, which retards their process through the column. This threshold is around 20 bp.

The MinElute DNA Cleanup column uses a silica membrane combined with spin-column technology. It was designed for isolation of DNA from PCR reactions, agarose gels, or enzymatic reactions and can purify fragments between 70 bp and 4 kb, up to 5 µg DNA in total.

These columns were chosen for their application in removal of small fragments of DNA from samples. They utilise different techniques in order to achieve this goal.

7.2 Aims and Objectives

The aim associated with this chapter was to assess the use of size separation technologies to re-balance the proportion on longer and shorter strands of DNA in degraded samples enabling a more balanced STR profile to be generated.

In order to do that, the following objectives were followed:

- Preferentially re-extract DNA from agarose gels in order to balance the proportion of longer and shorter strands of DNA and generate a better STR profile.
- The use of purification columns could balance the proportion of longer and shorter strands of DNA and generate a better STR profile.

7.3 Materials and Methods

Resumed methods were as follows, Chapter 2 contains more details.

7.3.1 DNA Re-Extraction Following Agarose Electrophoresis Gel

DNA degraded with DNase I for 30 min was re-extracted from agarose gels in order to evaluate the ability of the method to balance longer and shorter strands of DNA in the sample. This artificially degraded sample was assessed with the 4Plex Multiplex before use to ensure that the degradation reaction performed had generated a suitable degradation pattern. Samples degraded for 30 minutes were chosen due to having in the electropherogram profile a good imbalance between the bigger and smaller allele while still having complete amplification (Figure 7.1).
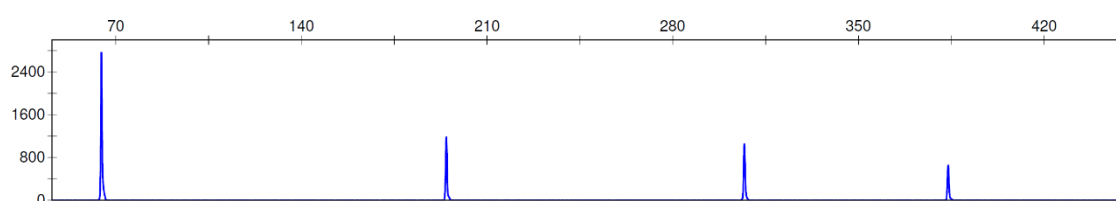
Figure 6.16 – Example of electropherogram from sample degraded for 30 min using DNase I.

Samples were re-extracted using the centrifugation protocol of the E.Z.N.A.® MicroElute® Gel Extraction Kit (Omega Bio-tek, USA), using 2 µL (around 112 ng) of commercial DNA and adding 20 µL of Elution Buffer at the end (full protocol can be found on session 2.7). Gel lanes were cut using two sets, set A with measurements starting from just below the well; and set B with measurements being made around visible DNA in the gel. There were also eight different cuts made (Figure 7.2) to determine if any combination was more efficient. All samples were analysed in triplicates.
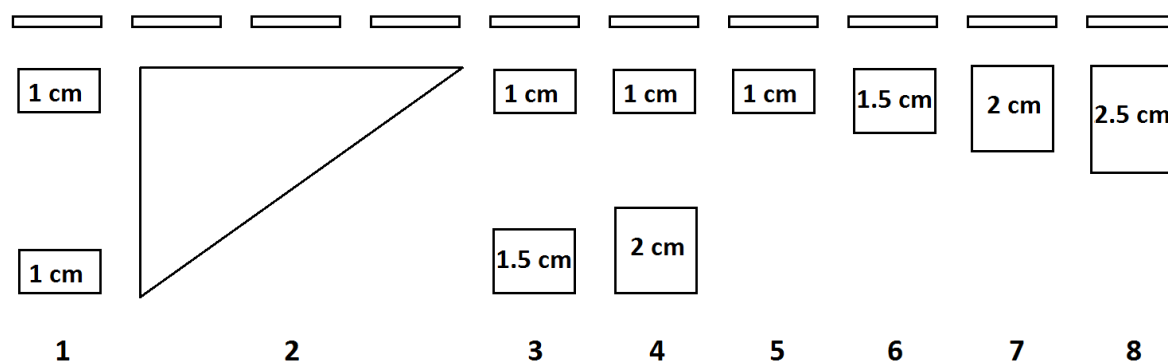
Figure 6.17 – Diagram showing the experimental design of different agarose gel cut methods tried to discover the best yield.

In order to analyse the efficiency of DNA recovery of the different cuts, after extraction of samples from the agarose gel, they were quantitated using Qubit (Thermo Fischer, UK) according to manufacturer's instructions. In order to analyse if the cuts improved the DNA profile from the samples, the 4-Plex was performed and samples after the gel extraction were compared with the original sample.

7.3.2 DNA Rebalancing Using Purification Columns

Different commercially available columns were used to filter degraded DNA samples in order to balance the proportion of longer and smaller fragments of DNA. The columns used were Microcon® DNA Fast Flow Centrifugal Filter Unit with Ultracel Membrane (Merck Chemicals, UK), Microcon® 30kDa Centrifugal Filter Unit with Ultracel-30 membrane (Merck Chemicals, UK), illustra MicroSpin G50 (GE Healthcare Life Sciences, UK), and MinElute Reaction Cleanup Kit (Qiagen, UK). These columns were chosen for their ability to remove small fragments from the sample, their lower threshold goes from 20 bp (illustra MicroSpin G50) to 70 bp (MinElute Reaction Cleanup Kit).

DNA was degraded with DNase I, analysed with gel electrophoresis and quantitated. Samples were then diluted by adding nine times the volume of

distilled water. Subsequently, to the collums different volumes of the samples (5 µL for each Microcon® column, 15 µL for the illustra MicroSpin G50 and 20 µL for MinElute Reaction Cleanup Kit) were added, and columns were processed according to manufacturers' instructions as described in session 2.9.

After purification with the columns, samples were quantitated and amplified with the 4-Plex multiplex along with the original sample to assess DNA recovery and the improvement in the DNA profile. Statistical data analysis was performed as described in Chapter 2.

7.4 Results

7.4.1 DNA Re-Extraction Following Agarose Electrophoresis Gel

Forty-eight DNA samples were re-extracted from the agarose electrophoresis gel using two different sets of cuts each with eight different cut methods. Each combination of set and cut method was performed in triplicate. After re-extraction, samples were quantitated and the 4-Plex multiplex was performed. Samples were compared with the original degraded DNA sample in order to assess the change in DNA concentration and in the DNA profile.

Preliminary experiments were done using just cut 1 and 2. It was observed on capillary electrophoresis an increase in the RFUs of the profiles when compared with the original sample (Figure 7.3) as well as an increase in the peak heights of the longer amplicons of the assay. But it was observed that the new DNA profile had a lower 70 bp peak than what was expected. Based on these results, the other gel cuts were developed and processed in order to see if any of them generated a more balanced profile. The aim was to extract different proportions of small and long fragments. Cuts 1 and 2 were repeated when processing the other cut types.
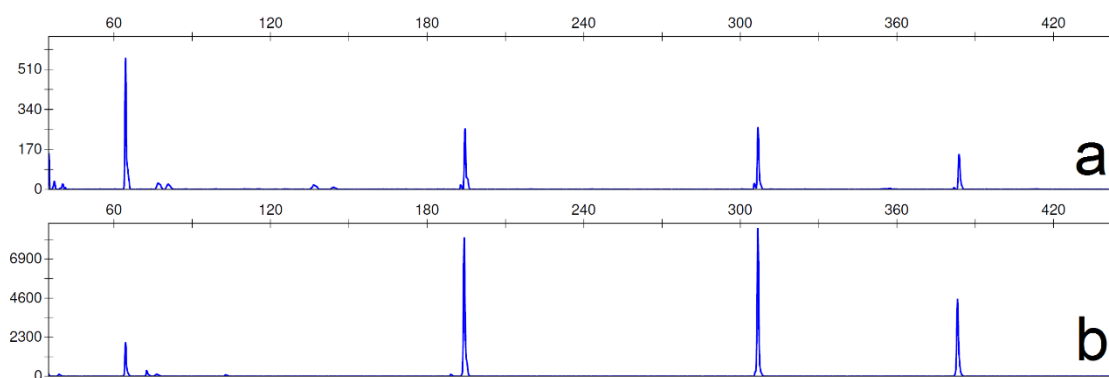
Figure 6.18 – Examples of electrophoresis of (a) the original degraded sample, (b) sample re-extracted after agarose gel electrophoresis.

Based on average recoveries, DNA Quantitation data shows that cut type A5 is the best combination. Samples re-extracted using this cut had an average of 56% of DNA recovery. The worst combination was B1, with 32% of DNA recovery (Figure 7.4). Overall, Set A was better than Set B, but there was not much difference between the sets.
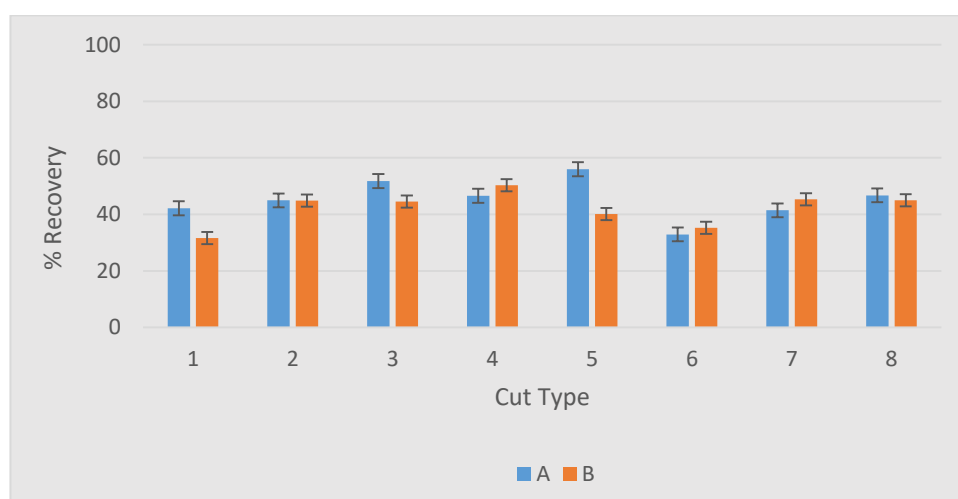
Figure 6.19 – Bar charts showing the average DNA recovery of the different combinations of gel cuts. Set A was cut starting from just below the well; and set B was cut with measurements being made around visible DNA in the gel. All data are presented as mean ± SEM, n=3.

Samples were amplified using the 4-Plex assay and results were compared with the original degraded DNA sample in order to assess the change in quality of the profile.

All cut types were processed in triplicates and it was observed that differently from the preliminary results, the profiles decreased quality after re-extraction from the gel, with RFUs being lower and previously amplified fragments not being present (Figure 7.5). This was true for all cut types in both sets of cuts, with not much difference overall (Figure 7.6).

	1		2		3		4		5		6		7		8	
A																
B																

Figure 6.20 – Figure showing the changes in profile observed after DNA gel agarose separation and re-extraction. Red stands for worsening of the profile, yellow for no change observed and green for improvement of the profile.
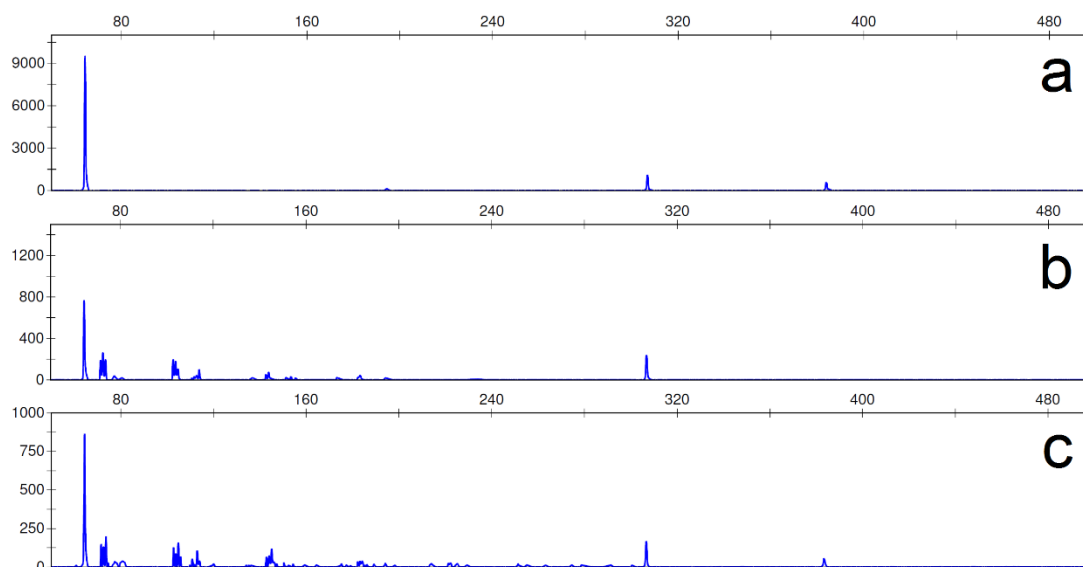
Figure 6.21 – Examples of electropherograms of (a) the original degraded DNA sample; (b) sample re-extracted using cut type A2; and (c) sample re-extracted using gel cut type B4

.

7.4.2 DNA Rebalancing Using Purification Columns

DNA degraded with DNase I was processed with different purification columns (Microcon® DNA Fast Flow Centrifugal Filter Unit, Microcon® 30kDa Centrifugal Filter Unit, illustra MicroSpin G50, and MinElute Reaction Cleanup Kit). All 11 time points of DNA degradation were used with all columns in triplicates, resulting in 132 samples. After processing, samples were quantitated and the 4-Plex was performed. Samples were compared with the originals degraded DNA samples in order to assess the change in DNA concentration and in the DNA profile.

DNA quantitation results showed that the better average recovery was obtained with Microcon® DNA Fast Flow, which recovered 100% of the DNA (Figure 7.7). After this column, the best results were obtained with illustra MicroSpin G50. The worst recovery was with the use of the MinElute Reaction Cleanup Kit, especially with samples incubated for longer, and thus more degraded. This could be due to the fact that the MinElute column had the largest threshold for retention (70 bp). The MinElute kit also had a poor result with the control sample, which was not degraded.
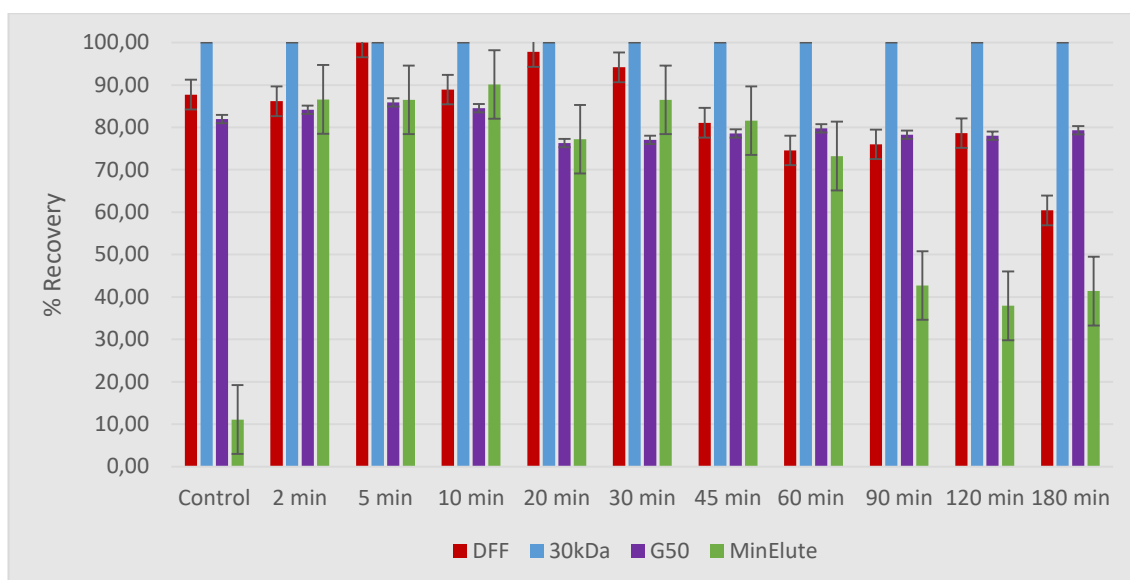
Figure 6.22 – Bar charts showing the average DNA recovery of the filtration columns based on DNA incubation time. All data are presented as mean ± SEM, n=3.

Despite having recovered 100% of the DNA, the Microcon® 30kDa Centrifugal Filter Unit column had the worst amplification results, alongside the Microcon® DNA Fast Flow Centrifugal Filter Unit column (Figure 7.8). The profiles after processing with these two columns were more imbalanced than the ones in the original samples, the peaks of the larger amplicons were dropping out in more degraded samples even when they were present in the original DNA sample.

Figure 6.23 – Figure showing the changes in profile observed the use of the purification columns. Red stands for worsening of the profile, yellow for no change observed and green for improvement of the profile

175

Samples processed with the MinElute Reaction Cleanup Kit had the best amplification results, even though they had the worst average DNA recoveries. For most samples processed with this column, the profile quality remained unaltered, and samples that diverged from this were the control samples and samples with 2 min of degradation. Samples processed with the illustra MicroSpin G50 had results in-between, with most samples having a similar profile with the one of the original sample, but some having decreased quality.

In Figure 7.9, it is possible to see the electropherograms generated after DNA degraded for 30 minutes processed with the different columns.
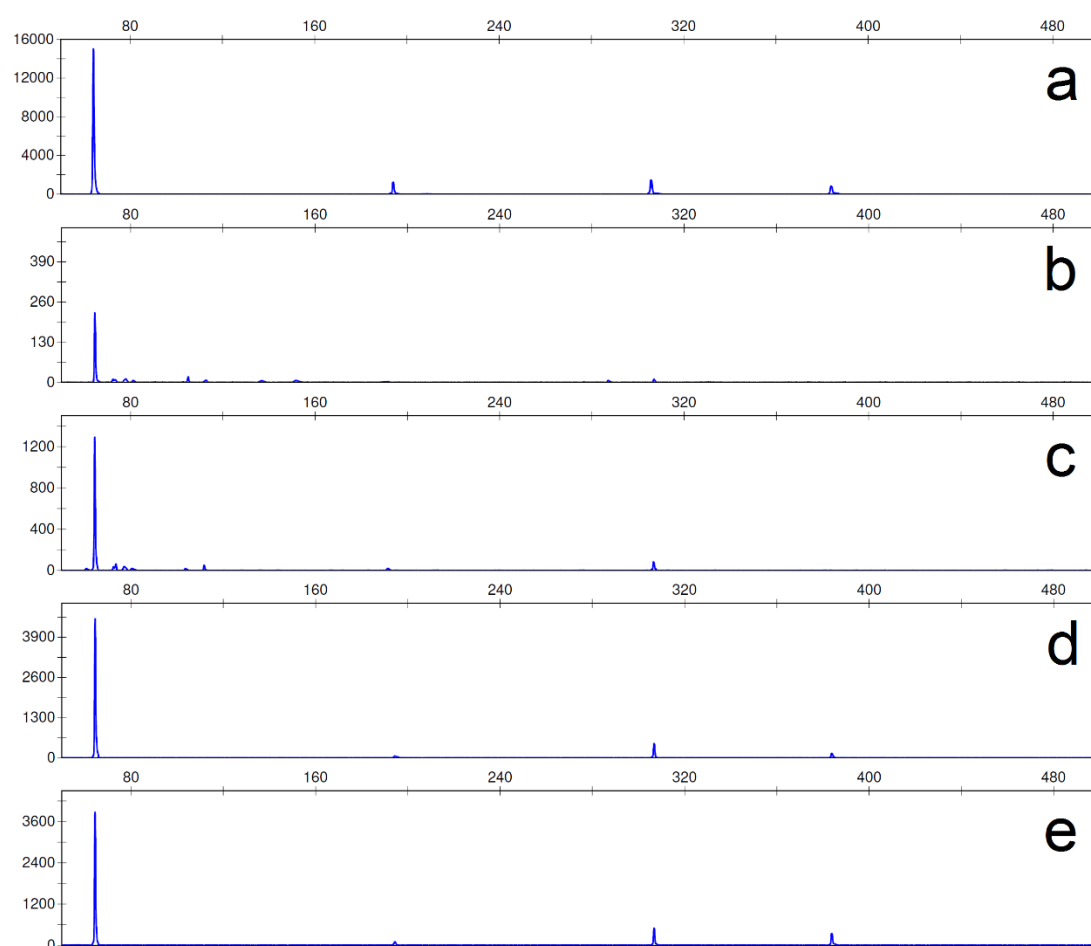
Figure 6.24 Electropherograms of (a) original degraded DNA sample; (b) sample processed with Microcon® DNA Fast Flow Centrifugal Filter Unit column; (c) sample processed with Microcon® 30kDa Centrifugal Filter Unit column; (d) sample processed with illustra MicroSpin G50 column; and (e) sample processed with the MinElute Reaction Cleanup column.

7.5 Discussion

This series of experiments was intended to generate better DNA profiles from degraded samples using either gel agarose electrophoresis or purification columns with cut-out thresholds around 70 bp. The DNA profiles were generated using an in-house-developed multiplex with range similar to most STR kits used in forensic laboratories and analysis were performed by comparing the profiles from the original samples with profiles after manipulation.

The rationale behind this methodology was based on the fact that the greater presence of smaller fragments of DNA in degraded samples causes over-amplification of those fragments (Butler *et al.* 2003; Dixon *et al.* 2006; Takahashi *et al.* 1997). If it were possible to balance the proportion of small and large fragments present in the sample, it would be possible to generate a more balanced DNA profile.

Despite promising preliminary results, re-extracting degraded DNA samples after separation using agarose gel electrophoresis showed not to be useful in improving DNA profiles. Combining set A and set B, eight cut methods were assessed in this research and they resulted a decrease in profile quality for all samples.

The best result obtained with the purification columns used in this study was for samples processed with the MinElute Reaction Cleanup Kit. Nevertheless, in this case the DNA profiles obtained after processing were of only equivalent quality to the original ones. Samples processed with the illustra MicroSpin G50 columns were for the most part able to repeat this result of similar profiles to the original ones. In addition, samples processed with either one of the Microcon® columns generated profiles with worst quality for most of the samples studied.

Kemp *et al.* (2014) used qPCR to estimate the copy count of synthetic standards before and after purification by the MinElute PCR Purification Kit, and documented DNA loss within a pool of 16 different sized fragments ranging from 106–409 bp. The average DNA loss was between 21.75% to 60.56% (mean 39.03%). This was not congruent with the claim of Qiagen that 80% of 70 bp to 4 kb fragments are retained using this product.

Another study tested DNA loss when using Amicon® filters with various molecular weight cutoffs. The study found that all centrifugal filtration devices tested caused substantial DNA loss, affecting low molecular weight DNA (PCR product) somewhat more than high molecular weight DNA (Doran and Foran 2014). Garvin and Fritsch (2013) also reported DNA loss when using Microcon® and Amicon® devices.

This generalised loss of DNA while using purification columns could explain why the profiles did not improve in quality the way it was intended for them to. If both low molecular weight and high molecular weight DNA molecules are lost, the imbalance of the profiles would be kept similar. With increased DNA loss, the profiles would get worst, which was also observed.

With the advancement of technology and the increasing addition of NGS to routine in forensic laboratories, the problems faced with degraded samples might be reduced. Sequencing using NGS is more sensitive than capillary electrophoresis and therefore requires less DNA. Moreover, the way the technology works also decreases the problems faced with CE regarding unbalance between markers in the profiles.

In conclusion, since the idea of this series of experiments was to improve profile quality via size separation techniques, neither the agarose gel electrophoresis separation nor the columns used would be recommended for this application.

CHAPTER 8

USING DNA CAPTURE

TO IMPROVE

DEGRADED DNA

PROFILES

8.1 Introduction

DNA hybridization capture is a DNA purification method that allows the separation of the targeted DNA loci from the rest of the DNA in the sample. Magnetic-bead DNA separation is commonly used for total DNA non-specific purification (Wang and McCord 2011). It has been incorporated in automated DNA extraction protocols where the beads bind to non-specific DNA in order to separate it from inhibitors and contaminants (Archer *et al.* 2006; Haak *et al.* 2008; Nagy *et al.* 2005; Rittich and Spanova 2013; Witt *et al.* 2012).

There are different approaches that can be used for DNA capture and they can be divided into two groups, indirect and direct capture. One of the techniques for indirect DNA capture is the Primer Extension Capture (PEC). In it, DNA primers hybridize to the target DNA, are elongated by a polymerase and the whole complex is captured via the attached biotin (Briggs *et al.* 2009; Horn 2011). After retrieval of the target DNA from the rest of the sample, it is possible to separate it from the magnetic bead used for its capture (Figure 8.1).

PRIMER EXTENSION CAPTURE
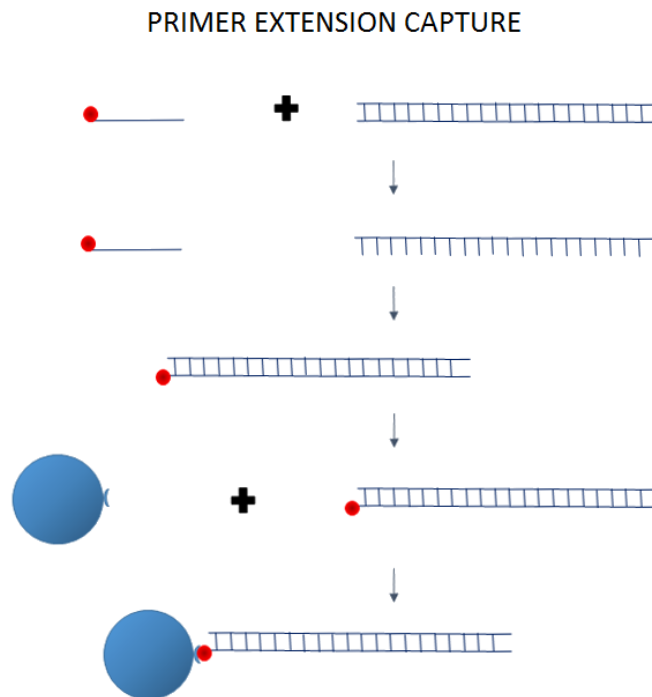
Figure 6.25 – A schematic diagram showing the Primer Extension Capture reaction. On it, the biotinylated primer and the DNA sample are incubated together and allowed to hybridise. The rest of the DNA strand is formed by the polymerase. The product of this reaction is then incubated with the magnetic beads and immobilised. Then the rest of the sample is washed away and the sample is heated in order to free the target DNA from the beads.

8.2 Aims and Objectives

The aim of this chapter of the study was to obtain better DNA profiles from degraded samples by rebalancing the proportion of short and long DNA fragments that are targeted in the 4Plex reaction. To do so, the following objectives were pursued:

- To develop a protocol of DNA hybridization capture using the primers of the 4-Plex Multiplex
- To use the technique of DNA capture in degraded DNA samples in order to generate a better STR profile.

8.3 Materials and Methods

Resumed methods were as follows, Chapter 2 contains more details.

The methodology of Primer Extension Capture modified from Briggs *et al.* (2009) was used in this series of experiments. Three of primers from the 4-Plex multiplex (Table 2.1) were used (70 bp, 194 bp, and 384 bp), with the addition of Biotin-TEG to the 5′ end. These primers were chosen for being the smaller, the largest and being almost in the middle of the range. Every reaction was done in duplicate.

First, a primer extension reaction was performed using two to five PCR-like cycles. After the primer extension reaction, samples were purified using the MinElute Reaction Cleanup Kit according to manufacturer's instructions. Samples were re-suspended in 28 µL of Buffer EB at the end of purification.

For the bead capture step, Dynabeads™ MyOne™ Streptavidin C1 beads were used. This step was adapted from the manufacturer's suggested protocol. The beads were cleaned and 25 µL of the purified DNA from the primer extension reaction were added. The mixture was incubated at room temperature for 30 min with vortexing at every 2-3 min. Samples were then washed and separated from the magnetic beads.

After recovering the DNA with the help of magnetic beads, the capture product was amplified with the use of PCR. Primers used in this step and the conditions of the PCR were the same as used in the 4-Plex Multiplex. Samples were then analysed with capillary electrophoresis. Following the determination that the primer pairs were working correctly with the protocol developed, multiplex reactions were performed combining two or three primer pairs. Statistical data analysis was performed as described in Chapter 2.

8.4 Results

In this study, commercial control DNA was used to optimize the protocol before using degraded DNA to assess the possibility of obtaining better DNA profiles from degraded samples. All reactions were performed in duplicates simultaneously, with a total of 66 reactions. Analysis was done using the quality of the DNA profile generated by capillary electrophoresis.

At first, reactions were optimized as single reactions to ensure that primers were working properly and that the protocol was appropriate. The primer pair used for development of the protocol was the one for the 194 bp amplicon. It was chosen due to its medium size in the 4-Plex assay. The first step was to determine how many cycles were to be performed in the primer extension reaction. First, it was tested the effect of 2, 5, 10, 15, and 20 cycles and a full PCR reaction of 28 cycles (Figure 8.2) and subsequently determined that 2 cycles were sufficient.

Two different half-volume reactions were also tested. The first one reduced all components of the primer extension reaction proportionally to half; while set B kept the DNA and the primers added the same, adjusting the buffer to achieve a 25 µL reaction (Figure 8.3). Both sets used the same PCR on the final step, with all components having being reduced to half. Both reactions worked, with reaction B generating higher RFU, but it was decided to continue with full volume reactions while combining the other primers into the reaction.
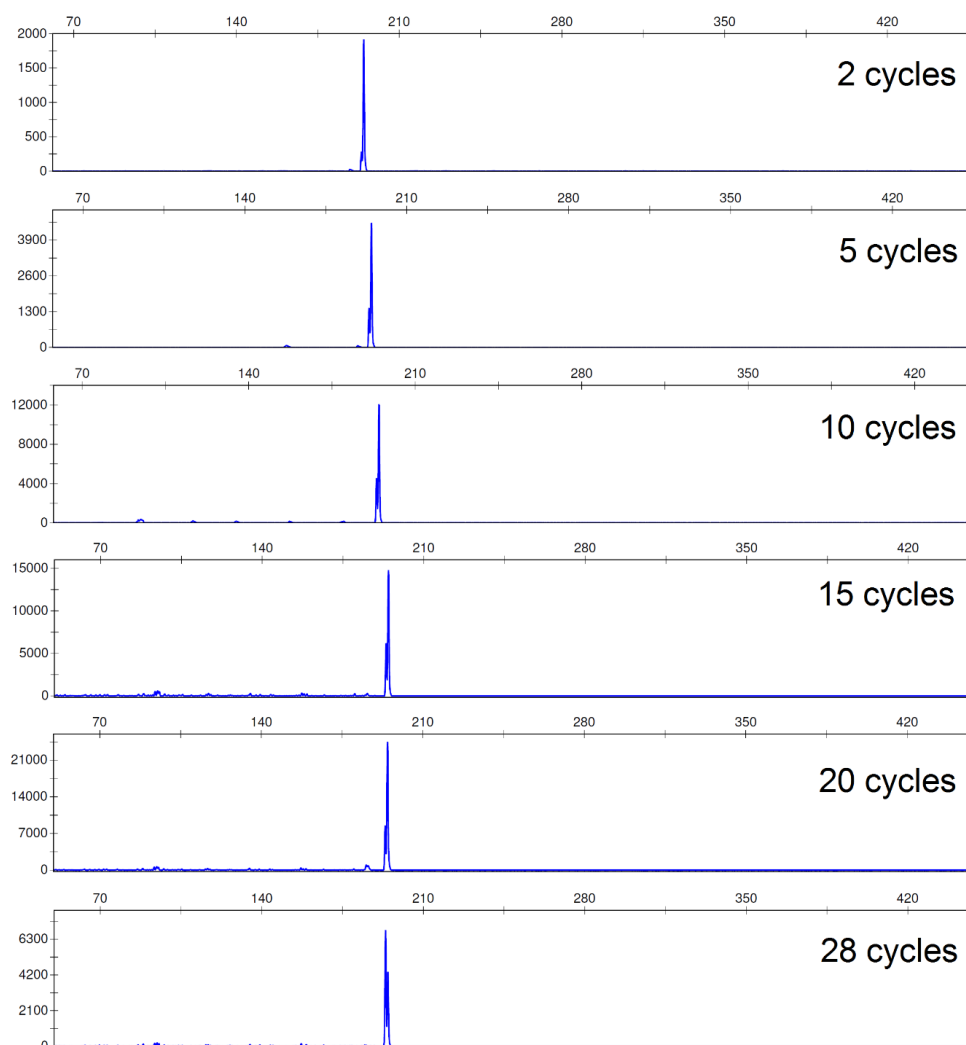
Figure 6.26 – Electropherograms of reactions performed with different number of cycles (2 cycles, 5 cycles, 10, cycles, 15 cycles, 20 cycles and 28 cycles) in the Primer Extension step, followed by bead capture and a complete PCR with 30 cycles.
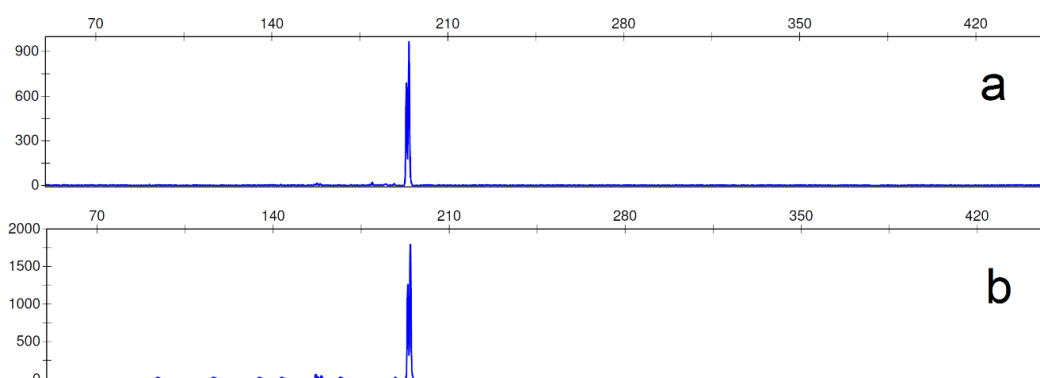
Figure 6.27 – Electropherograms of half-volume reactions performed with two cycles. Set (a) had all components reduced proportionally to half. Set (b) kept the DNA and primers constant and reduced the buffer volume.

Tests were also undertaken to see if DNA was actually being captured and not only carrying over. In order to do that, one reaction was performed without primers in the primer extension step, while the rest of the protocol was followed. The results showed that no amplification was obtained from the duplicate in this experiment (Figure 8.4), providing strong evidence that the DNA amplified in the second reaction had been captured through primer extension and bead-based capture.
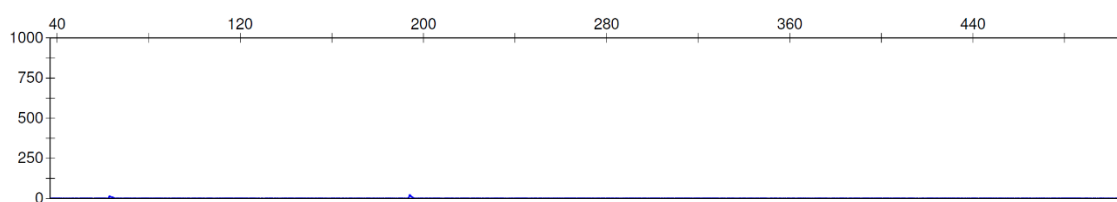
Figure 6.28 – Electropherogram of DNA Capture reaction where the first step (primer extension) was performed without primers. No amplification occurred later.

Once the 194 bp primer pair was working the 70 bp and the 384 bp primers were tested. The reactions were set with two cycles in the primer extension, as determined in the previous settings. The 70 bp primer pair generated a small but clear peak, while the 384 bp had a peak with higher RFU. No other peaks were observed in the electropherograms (Figure 8.5).
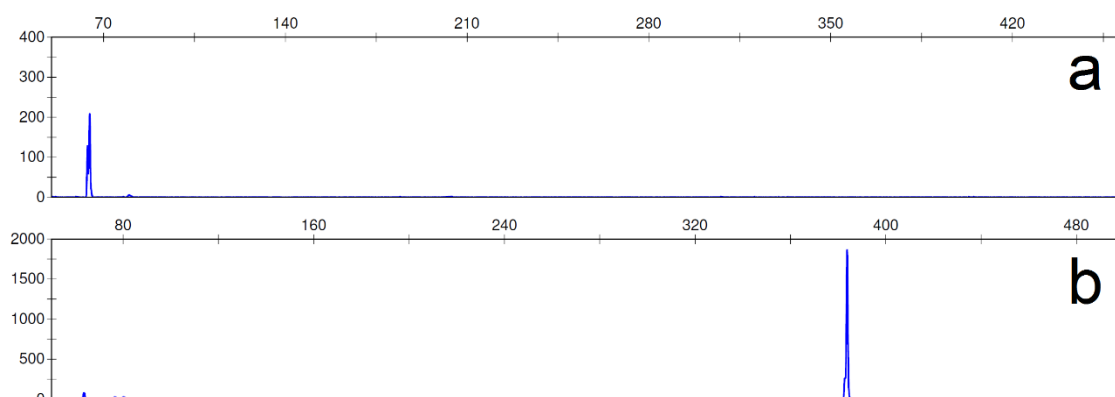
Figure 6.29 – Electropherograms of DNA Capture test runs using (a) the 70 bp and (b) the 384 bp primer pairs.

With the establishment that the three primer pairs were working, the reactions were combined. After combining the 70 bp and the 194 bp primer pairs, the cycle number in the primer extension step was again tested (Figure 8.6). Amplification profiles were similar for the two conditions, with the difference being that, as expected, using five cycles generated higher RFU in the profile.
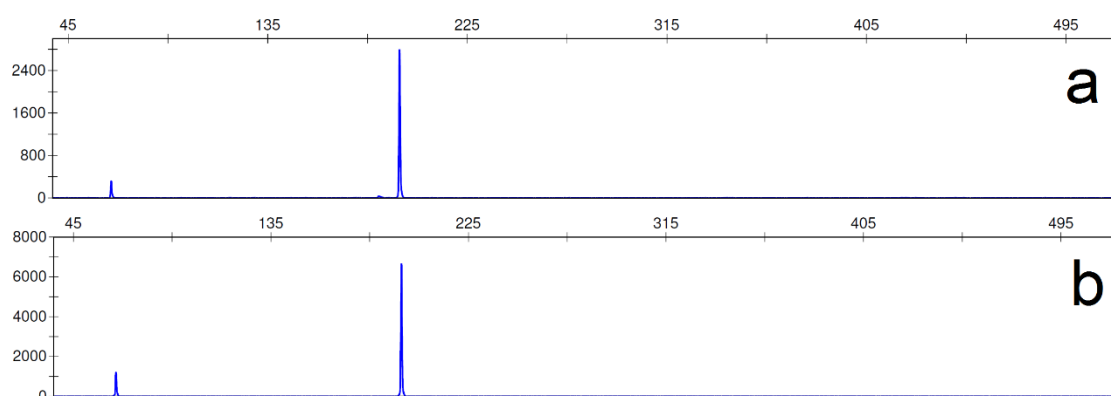
Figure 6.30 – Electropherograms of the test reactions combining two primer pairs (70 bp and 194 bp) using (a) two and (b) five cycles for the primer extension step.

The 70 bp and the 384 bp primer pairs were combined next. When following the protocol with the final amplification with a total volume of 50 µL, the capillary electrophoresis results showed a peak only for the smaller amplicon of 70 bp (Figure 8.7a). It was then tested if using the complete 4-Plex Multiplex as the final step instead of the original protocol PCR would make any difference. What was observed was a complete amplification profile (Figure 8.7b).
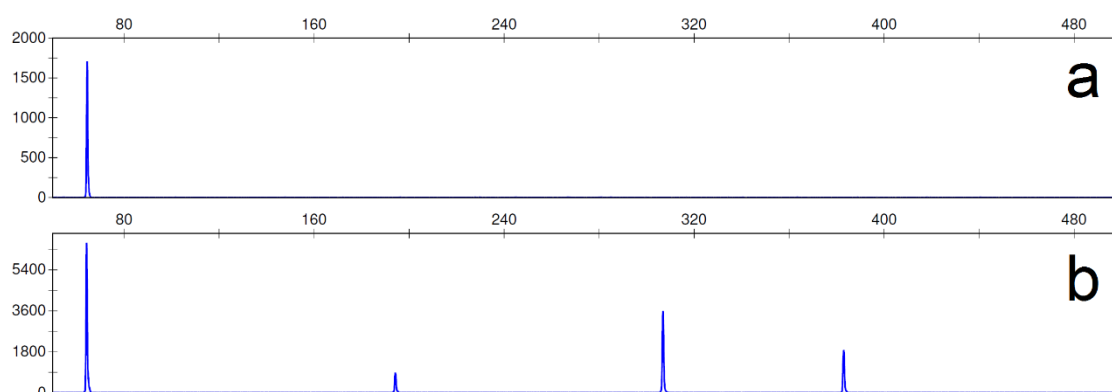
Figure 6.31 – Electropherograms of the same sample with the last step of DNA Capture being (a) the original protocol PCR, showing only the 70 bp amplicon and (b) the 4-Plex Multiplex, showing a complete profile.

It was decided to continue with the 70 bp and 384 bp multiplex reactions due to the fact that these primer pairs are the smallest and biggest in the 4-Plex Multiplex and would represent the limits of the range.

They were combined in a new reaction using 5 cycles in the primer extension step and both sets of PCRs being performed at the end (the original protocol one and the 4-Plex Multiplex). Whether the magnetic beads could be reused for the capture step was also tested, since the manufacturer attested that this was possible. All reactions showed the 70 bp and the 194 bp amplicons and only the reaction using new beads and performing the 4-Plex as the final step had the presence of the 384 bp amplicon (Figure 8.8). The 194 bp amplicon was not expected to be present because the primer pair was not used, yet, it was a clear peak in all profiles.
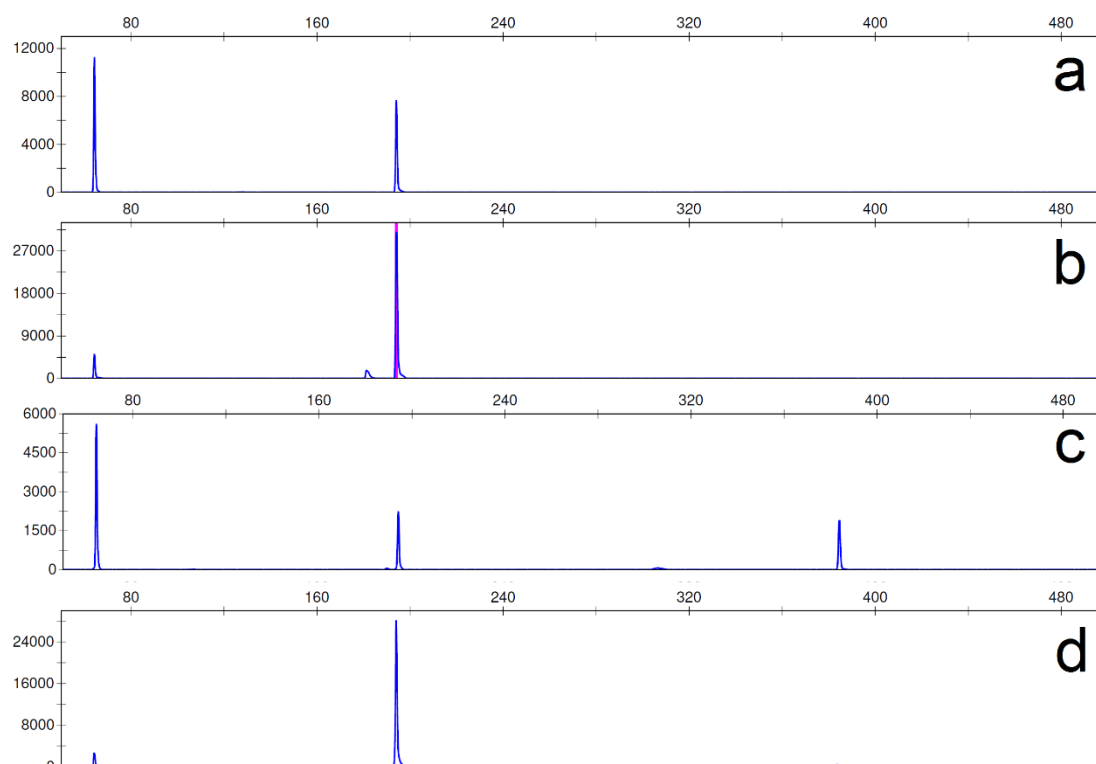
Figure 6.32 – Electropherograms of reactions performed combining the 70 bp and the 384 bp amplicons. (a) was performed with new magnetic beads and the protocol PCR; (b) with reused beads and the protocol PCR; (c) with new magnetic beads and the 4-Plex Multiplex; and (d) with reused beads and the 4-Plex Multiplex.

Another situation that was tested in this study was to determine wether performing the primer extension and capture steps with combined primer pairs was more efficient that performing by separating the primer pairs (i.e. one reaction combining 70 bp and 384 bp primer pairs; or two separate reactions, one with the 70 bp primer pair and the other with the 384 bp, combined only for the final PCR). Thus, the reactions were performed with the 70 bp and the 384 bp primer pairs and were mixed for the PCR in the final step. This amplification was performed using the 4-Plex. Both the 70 bp and the 384 bp amplicons had small peaks, and the 194 bp amplicon was again present. Combining the reactions resulted in a slight improvement for the 384 bp amplicon, but the 70 bp amplicon was lost in noise (Figure 8.9).
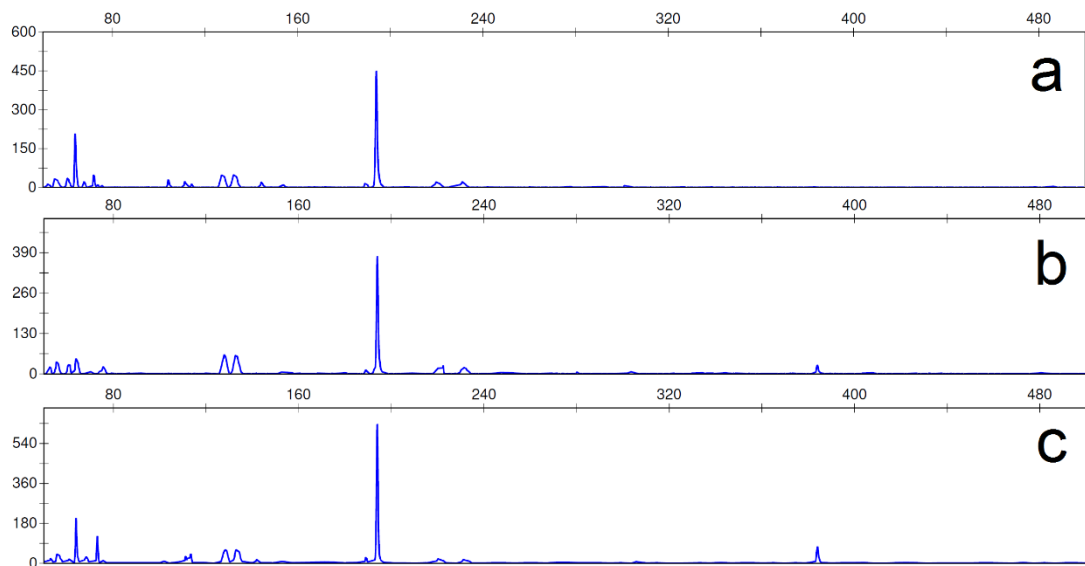
Figure 6.33 – Electropherograms of (a) the 70 bp reaction; (b) the 384 bp reaction, and (c) both reactions combined for the last PCR step.

One more reaction combining the 70 bp and the 384 bp and using 5 cycles in the primer extension step was performed. No 384 bp peak was observed and the 194 bp peak continued to be present (Figure 8.10).
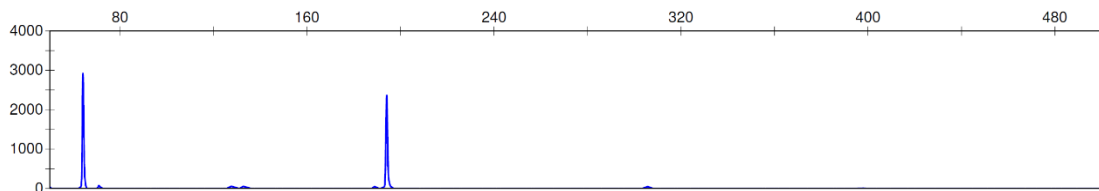
Figure 6.34 – Electropherogram of a reaction using the 70 bp and the 384 bp primer pairs performed after changing all the reagents used but the magnetic beads.

As the 384 bp amplicon was not present in the electropherogram, a new reaction with only the 384 bp primer pair was set up. No amplification profile was observed (Figure 8.11).
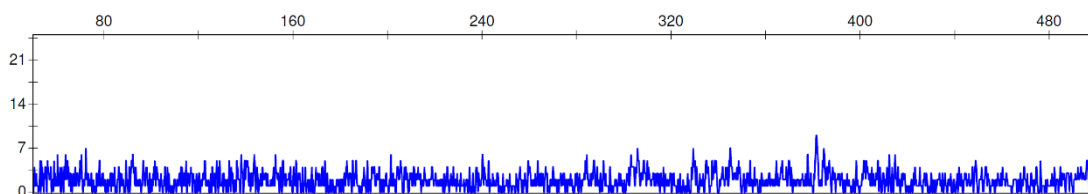
Figure 6.35 – Electropherogram of the second test run of the 384 bp primer pair, which resulted in no amplification.

Another round of single reactions was performed, with 5 cycles of primer extension and the final PCR being the 4-Plex Multiplex. The smallest amplicon (70 bp) was present in the 194 bp amplification, and the 194 bp amplicon was present on the 70 bp reaction. The 384 bp amplicon was not visible, with only the 194 bp peak present (Figure 8.12).
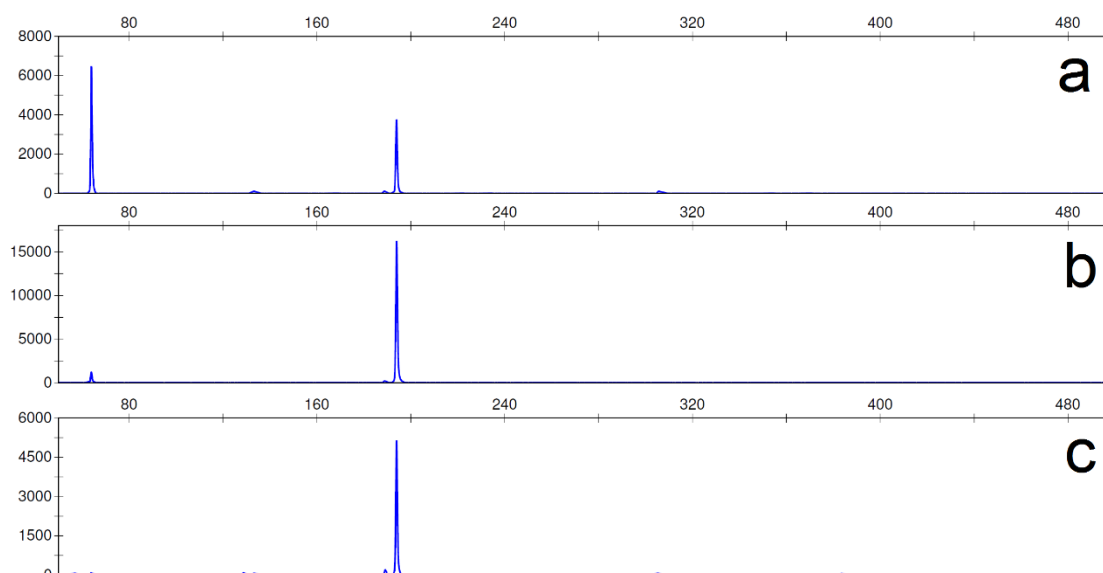
Figure 6.36 – Electropherograms of single reactions performed with (a) the 70 bp primer pair; (b) the 194 bp primer pair; and (c) the 384 bp primer pair.

Due to time constrains, only one reaction was performed with degraded DNA at this stage. The profile obtained after capture from the degraded sample was similar to the profile obtained from the sample using control DNA (Figure 8.13). The 384 bp amplicon was still not present when it was expected.
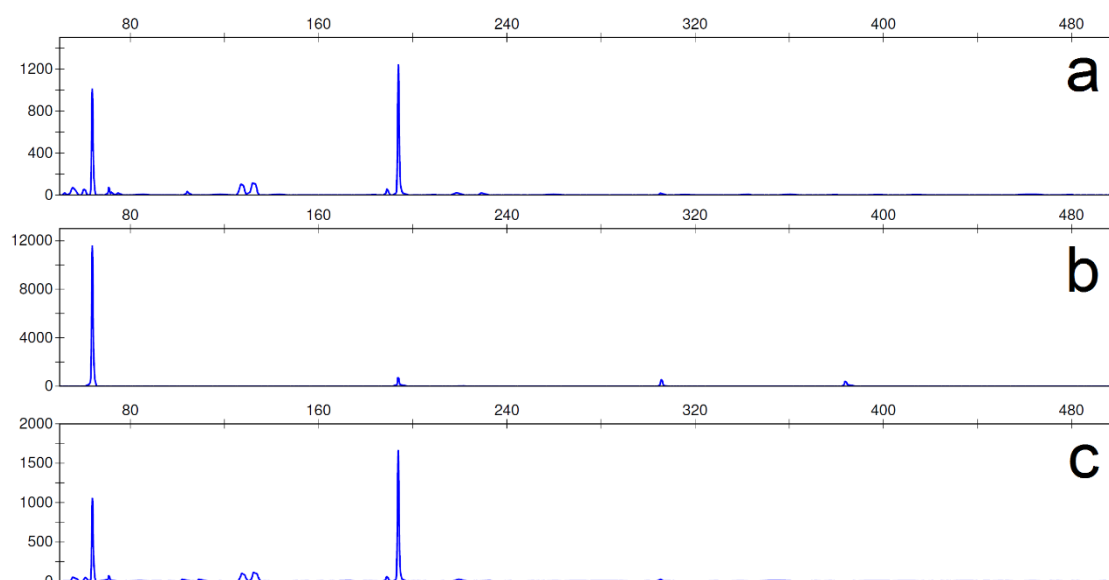
Figure 6.37 – Electropherograms of DNA Capture reactions performed using a combination of the 70 bp and the 384 bp primer pairs (a) with control DNA; (b) is the original degraded DNA sample that was captured in (c).

8.5 Discussion

This series of experiments was designed to re-balance the proportion of DNA fragments in a degraded sample and thus generate a better DNA profile. The principle is that with the DNA capture, an equal amount of the fragments to be studied would be selected and later amplified, resulting in a balanced profile.

Previous studies increased the sensitivity of STR analysis from degraded DNA using pre-amplification with biotin-labelled primers and magnetic beads capture (Ham *et al.* 2016). They differentially amplified longer and smaller loci to combat the fact that longer amplicons are more vulnerable to DNA fragmentation and were able to obtain more information from samples.

Wang and McCord (2011) utilized five biotinylated probes flanking the regions of D3S1358, D13S317, D16S539, FGA, and CSF. After capturing the fragments with magnetic beads, they were able to produce nearly twice the intensity for smaller alleles and a 25% increase in larger alleles in fragmented DNA samples.

In the present study, it was possible to preferentially separate the fragments of 70 bp and 194 bp from the original sample. However, one of the amplicons studied (384 bp) did not perform as expected, with results from it being very inconsistent. The first reaction performed with it had the expected result, but later amplifications had smaller peak heights and then no amplification at all.

Another unexpected result was the presence of the 194 bp peak when the primer pair was not used in the reactions. This was probably due to contamination on the beads, since all the other reagents were changed and re-used beads had higher peak heights of the unwanted amplicon than new beads.

Winters *et al.* (2017) measured DNA in the samples before and after the use of the technique and found that the retention of target DNA molecules (from 109 bp

to 288 bp) averaged just 9.06–3.53% (i.e., loss of 90.94–96.47%) using the DNA capture protocol as originally described. Some improvement was achieved by employing a modified protocol, resulting in average retention of 31.41–12.08% of the same set of targeted molecules. They also noted the lack of efficacy in removing non-target DNA molecules as opposed to targeted molecules. It was also observed that most of the molecules (61.35–69.49%) were "lost" during the essential hybridization step of the protocol, suggesting its suitability for high copy number samples only.

The present study did not measure the DNA loss while using the DNA capture technique, but only non-degraded, high molecular weight DNA was used during the development of the protocol used in this chapter. It is possible that if degraded DNA samples were used, then the results would be limited due to what Winters *et al.* (2017) described.

Alternatives for analysis of degraded DNA include NGS, which needs less amounts of DNA to generate a higher output of data. Next generation sequencing is a great alternative for analysing degraded DNA samples, but it is still being validated and implemented for use in forensic laboratories.

Ideally, this series of experiments could be continued with a new stock of magnetic beads and the primers would be balanced in order to obtain similar peak heights from different-sized amplicons. Testing the use of degraded DNA in the protocol would also be necessary. One of the limitations of the protocol used in this series of experiments is that it involves several manual steps, which can increase the risk for contamination.

Because the protocol was not finalised and problems were found with one of the primers, it was not possible to draw any final conclusions from the results in this chapter. While the preliminary results suggest that the protocol could be used to

preferentially capture the target DNA, it is unclear how it would react with degraded DNA samples. Further experimental work with the conclusion of the protocol using all the primer pairs would be necessary for a complete evaluation of the technique for the intended use in this chapter.

CHAPTER 9

GENERAL DISCUSSION,

CONCLUSIONS

AND FURTHER WORK

9.1 General Discussion

Human identification is a matter of critical importance. In cases of mass disasters, such as armed conflicts, terrorism or natural incidents, victim identification can be the only way whereby the families of the victims will get any form of closure. In case of either armed conflicts or terrorism, identifying the bodies is also one way to distinguish between victims and suspects.

The main methods for post-mortem identification are odontology, fingerprints and DNA analysis, but mass disaster situations are usually more complicated to be dealt with due to number of victims and/or location. Often, the areas where these situations happened are complicated to be reached and the bodies of the victims can be fragmented, burned and/or decomposed. In these cases, identification through fingerprinting becomes more complicated. Odontology depends on previous dental records, and in a situation with many victims or where the infrastructure of the region is damaged, those can be hard to be found and processed.

Successful DNA analysis relies on the appropriate collection, preservation, and processing of biological material and there are many manuals and guides in place to ensure that the professionals involved follow the best practice. Some of those, used in this thesis, can be found in Table 9.1.

The major aims of this thesis were to assess molecular preservation methods for challenging soft tissue samples and to improve the DNA profiles obtained from them in order to provide more information and techniques for the field.

Table 6.6 – International guidelines for human identification

Institution	Name	Reference
DNA Commission of the International Society for Forensic Genetics (ISFG)	Recommendations regarding the role of forensic genetics for disaster victim identification (DVI)	(Prinz *et al.* 2007)
Federal Bureau of Investigation (FBI - US)	Handbook of Forensic Services	(FBI 2013)
International Committee of the Red Cross (ICRC)	Missing People, DNA Analysis And Identification Of Human Remains	(ICRC 2009)
International Criminal Police Organization (INTERPOL)	Disaster Victim Identification Guide	(INTERPOL 2014)
National Institute of Justice (NIJ – US)	Mass Fatality Incidents: A Guide for Human Forensic Identification	(NIJ 2005)

Before assessing preservation methods, degradation patterns in warm environments were studied. Experiments were performed in a controlled situation (inside a laboratory) and in an environmental one (in an open field). These experiments were a continuation of previous experiments done in the research group and aimed to obtain a better understanding of DNA degradation patterns (Nazir *et al.* 2011). Most DNA persistence and body decomposition studies are done in temperate climates, which are not representative of the contexts where a great part of mass disasters and conflict occurs. As such, warm environments were chosen in this study to undertake the investigation.

ADD is used in the taphonomic field for comparing body decomposition studies in different environments and conditions. It has been chosen for that use due to the conclusion that the progress of body decomposition is more dependent on temperature rather than time itself. By using a logarithmical model, it is possible to predict post-mortem interval by the ADD using the body decomposition score (Megyesi *et al.* 2005; Moffatt *et al.* 2016; Myburgh *et al.* 2013). It was speculated

in the research group that ADD could be used for predicting DNA persistence in bodies. The results from Chapter 4 show that despite ADD being a good tool for assessing body decomposition, it might not be as useful when studying molecular degradation. This is the opposite of what Larkin *et al.* (2010) previously found in their study.

The results from the same chapter also allowed to determine that when collecting tissue samples from partially decomposed bodies, those should be preferentially sampled from the ground surface, as the data show that DNA persistence is improved in that area. This is probably due to the lower temperature in that area. While the upper surface of the body can be directly exposed to the sun, the ground surface is protected from such heat and UV radiation. In this study, a lot of temperature data was collected in the field experiment in Thailand, but unfortunately, the temperature of the contact zone between the body and ground was not measured. However, in an expected follow up series of similar experiments, this measurement is planned to be taken.

Samples incubated in the laboratory had higher DNA persistence than samples incubated in the field and the results show that traditional STR kits could still be used to analyse samples incubated for up to 60 h (at 37 °C) or 13 days at a more moderate temperature of 25 °C. Current guidelines do not recommend the sampling of soft tissue after the onset of decomposition (INTERPOL 2014), but the results from this thesis would mean that this window for collection could be longer than currently assumed. Since DNA extraction from soft tissue is much easier than that from hard tissue, using muscle samples can reduce processing time.

Nazir *et al.* (2011) found that the bodies of pigs exposed to the environment in the UK had different rates of DNA degradation depending on the season of the

year. Experiments that ran during the summer had faster DNA degradation than experiments that occurred during winter/spring or autumn. It was also found that DNA degradation occurred faster in the laboratory samples when comparing similar ADD of field and laboratory incubated samples (Nazir 2012).

The series of experiments done in this study was designed as a follow-up to those done previously by Nazir (2012) and some of the results were very similar. The temperatures in the field experiments were higher than the ones studied in the UK and resulted in faster DNA degradation. However, in the set of samples employed in this study the laboratory incubation resulted in higher DNA persistence than field incubation. The samples used in this thesis had faster degradation than the ones from Phengon *et al.* (2008) which were able to amplify a 289 bp amplicon for up to 4 days of exposure in Thailand. In a study by (Itani *et al.* 2011), the quantity of amplified DNA in skeletal muscles of dead rats kept at 20 °C was reduced from 10 ng to 1 ng in 2-4 weeks. The quantitation results in our samples were similar, and also showed a decrease in the DNA present with increased incubation time.

These two series of experiments were designed with mass disaster situations in view. These are usually the situations where the remains are most likely to be found in more unpreserved conditions. Nevertheless, the results from this thesis could form useful advice for forensic scientists located in countries with warmer weathers. Although contamination of soft tissues is easier than that of hard tissues (especially because previous to DNA extraction the hard surfaces of bones and/or teeth are cleaned of contaminants), especially in cases with only one body, this limitation can be minimized by good protocols when collecting and processing the samples. The advantages gained when processing soft instead of hard tissues include easier labour, fewer hours of work and lower cost. The

results from this thesis prove that it is worthwhile to collect muscle tissue samples as long as they are present.

Chapter 5 of this thesis intended to assess long-term storage of partially degraded muscle samples and the possibility of use of drinking alcohol as a preservative solution. The current standard for preserving soft tissue samples is freezing them and/or adding absolute ethanol (Budowle *et al.* 2005; ICRC 2009), but studies disagree on the long-term use of ethanol, with some saying that tissues preserved in ethanol yield primarily highly degraded DNA (Kilpatrick 2002; Michaud and Foran 2011; Nagy 2010). Despite official recommendations to its use, limited literature was found on the use of drinking alcohol for the preservation of tissue samples (Oakenfull 1994).

In Part I of Chapter 5, samples preserved in ethanol and cell lysis (with and without 1% sodium azide) for seven years at room temperature were extracted and analysed using an in-house-developed multiplex, the 4-Plex. The results showed that samples preserved with ethanol and cell lysis with 1% sodium azide had complete amplification profiles, while samples preserved with cell lysis were only able to amplify the smallest amplicon of 70 bp.

The same set of samples had been extracted in a previous experiment which found that all three solutions assessed were efficient in preserving fresh and partially degraded muscle tissue. After one year of storage, all samples preserved in 96% ethanol, cell lysis and cell lysis with 1% sodium azide had high molecular weight DNA present and were able to amplify completely the 4-Plex Multiplex. The present results show that pure cell lysis is not as efficient as the other two solutions for long-term storage.

Usually forensic samples do not require long-term storage time. Nevertheless, it can be necessary depending on the set of circumstances. Forensic laboratories

in locations with fewer resources could take years to process samples from some cases. After mass disasters, laboratories can be overwhelmed with samples and understaffed. A similar situation could happen compared to that which occurred four years after the World Trade Center attack. Up to that date, only 1594 of the 2749 victims had been positively identified (Michaud and Foran 2011). Depending on the laws of the country, samples need to be available through the whole process of judgment and appeals, and sometimes even after that. The possibility of storing tissue samples without refrigeration makes costs lower and logistics of easier storage.

In Part II of chapter 5, the standard methods of preservation (freezing and absolute ethanol) were used as controls for assessing the ability of vodka and 37.5% ethanol to preserve soft tissue samples in warm (25 °C) and moderately hot (37 °C) temperatures. The results show that these alternative preservative solutions were able to successfully conserve the DNA and generate a complete amplification profile for up to 42 days. One previous study preserved zebra liver in vodka for several days before transferring the tissue to methylated spirits, but it did not mention how many days or average daily temperature (Oakenfull 1994).

Despite previous recommendations from INTERPOL (2014), limited empirical evidence could be found on the use of drinking ethanol for preservation of muscle tissue. Drinking alcohol may be more widely available on the field than absolute ethanol and should be considered an alternative in situations where the supply of absolute ethanol is limited. More studies need to be done assessing different types of tissues, temperatures and different liquors, but the results from these experiments provide evidence to support the recommendation, which is especially important for cases with a great number of samples and/or in remote locations.

In Chapter 6 of this study, the possibility of using vacuum as a preservation method for soft tissue samples was studied. Many of the processes involved in body decomposition and DNA degradation are oxygen-dependant, and whether removing access to air would inhibit them and enhance DNA persistence was the main question in this chapter.

When samples were incubated with vacuum, complete amplification of the 4-Plex Multiplex was possible for up to 40 days (incubation at 25 °C) and 10 days (incubation at 37 °C), the maximum amount of time tested. A previous study found that preserving samples in vacuum decreases autolysis (Bussolati *et al.* 2008) and the results from this thesis would confirm that affirmation.

Vacuum preservation increased DNA persistence when compared to the incubations performed in the laboratory at the same temperatures in Chapter 4. While samples incubated in the laboratory without any form of preservation (Chapter 4) had only the smallest allele present after 16 days (at 25 °C) and 72 h (at 37 °C), samples incubated with vacuum had complete profiles at these time-points and even at later times. The quality of profiles was also maintained. The results also showed that a limited amount of air inside the bags is another way of increasing DNA persistence in muscle samples. Samples stored in this condition air-dried and thus became preserved before DNA degradation was possible.

This series of experiments was designed to lead up to an experiment to assess the preservation of whole bodies using vacuum body bags. Unfortunately, it was not possible to perform this series of experiment during the course of the PhD. Whether whole body vacuum can assist with preservation (both molecular and physical) is unclear, but future experiments are planned to address this.

Even with all the care taken by forensic practitioners, because of the time lag between a particular event and the recovery of the body, some of the samples

encountered are going to be degraded. Samples containing degraded DNA are more likely to have failure of amplification, preferential amplification, and other artefacts that make analysis complicated (Alaeddini *et al.* 2010; Hughes-Stamm *et al.* 2011). This can lead to the need of repeating analyses (which is costly to the laboratory) or the use of alternative analysis methods for more informative data (which can be tiresome and time-consuming). Thus, the second major aim of this thesis was to assess techniques for improvement of DNA profiles from degraded samples.

The possibility of using size separation techniques to improve DNA profiles from degraded samples was studied in Chapter 7. The idea was to rebalance the proportion of longer and smaller fragments of DNA present in the samples. Degraded samples have more of smaller fragments of DNA present, and because of that, they are preferentially amplified and the analysis results in an unbalanced profile.

First, samples were preferentially extracted from agarose gels after separation with electrophoresis. Sixteen different cut methods were used for this. Despite preliminary results being encouraging, the average results of the trials (each cut was performed trice) showed that this technique produced profiles of lower quality than the original profiles. Samples were also processed with purification columns before amplification, and four different columns with different cut-out thresholds were used in these experiments. The results showed that processing the DNA samples with these columns could generate a profile of same quality than the original profile.

This series of experiments was designed to provide a low-cost option for degraded DNA analysis on forensic laboratories. Ideally, it would be a fast technique without many manual steps, to minimise the chance of contamination.

It was foreseen that some DNA would be lost, but the results suggest that more than what was expected is lost during processing, as suggested by Kemp *et al.* (2014). Unfortunately, based on the results found in this thesis using either gel electrophoresis, the use of either extraction or purification columns, with the intent or rebalancing degraded DNA samples, is not recommended.

The second technique studied for its ability to improve DNA profiles from degraded samples was DNA Capture on Chapter 8. DNA capture is a technique normally used in the purification during total DNA extraction, and a version of it is used in protocols for next-generation sequencing. The protocol chosen for use in this thesis allows DNA fragments bound with biotinylated probes to be captured by magnetic beads and separated from the rest of the sample. Amplification of this sample after capture in theory would result in more balanced DNA profiles.

Some research groups have used DNA Capture to preferentially select fragments from commonly forensic loci, also focusing on degraded DNA samples (Ham *et al.* 2016; Wang and McCord 2011). Nevertheless, the literature also questions the efficacy of the technique, stating that most of the DNA is lost during the protocol (Winters *et al.* 2017).

Preliminary results using the chosen primers separately showed single amplification peaks after capture, proving that the DNA was being selected. Tests using two primers at a time begun to take place and the first combinations had the results expected (balanced peaks with the expected sizes).

Regrettably, due to time constrains it was not possible for the optimization to be finished, and moreover, there was no time to perform reaction with the three primers chosen in this study. As such, only one reaction was performed with degraded DNA. Thus, the profiles generated after the Primer Extension Capture

could not be compared to the original profiles to assess if there was any improvement.

The results of this thesis clearly demonstrated the beneficial uses, as well as advantages and disadvantages of several techniques to preserve samples for molecular analysis and for processing degraded DNA.

9.2 General Conclusion

Patterns of DNA degradation were compared in samples degraded in a controlled environment and in environmental hot conditions. The results showed that samples degraded in the laboratory had longer DNA persistence than samples exposed to the environment. They have also provided empirical evidence for advice for collection and preservation of samples in casework. The use of muscle tissue is not recommended by the leading agencies after decomposition begins, but the results from the present study would suggest it could still be analysed with traditional STR kits for a longer period. Sampling of muscle tissue should preferentially be from the surface of the body in contact with the ground, as it showed to increase DNA persistence.

Different preservation solutions were assessed for their abilities to preserve DNA in muscle tissue. The first series of experiments analysed muscle tissue preserved for 7 years at room temperature using 96% ethanol, cell lysis and cell lysis with 1% sodium azide. The results from these experiments proved the capability of ethanol and the cell lysis with 1% sodium azide for long-term molecular preservation. These methods are less expensive than refrigeration, need little preparation and do not require specialised equipment. The second series of experiments assessed the use of vodka and more diluted ethanol for molecular preservation. The data have showed that these solutions generate profiles of similar quality to the ones from samples frozen and/or preserved with 95% ethanol. This findings demonstrated that the use of drinking alcohol or less concentrated ethanol can be encouraged for preserving muscle tissue samples.

The use of vacuum for preserving muscle tissue samples for molecular analysis was also assessed. The experiments were designed with the knowledge that muscle decomposition is partially dependent on microbial activity and a vacuum

environment would not provide the oxygen needed. The results show that DNA persistence is increased with either the use of vacuum or reduced oxygen. For the periods of incubation studied in this thesis, preserving tissue samples in vacuum can be an alternative way to be explored for forensic purposes.

The possibility of using size separation technologies to re-balance the proportion on longer and shorter strands of DNA in degraded samples and enable a more balanced STR profile to be generated was studied. The rationale was based on the fact that there is a greater presence of smaller fragments of DNA in degraded samples. If it was possible to balance the proportion of small and large fragments present, then it would be possible to generate a more balanced DNA profile. Two of the methodologies tested for this application (agarose gel electrophoresis and purification columns) would not be recommended for use as the profiles generated after processing were of similar or worse quality than the originals. It was not possible to finalise the DNA Capture protocol and thus no conclusion can be taken, but the preliminary results suggested that the technique could be used to balance degraded DNA profiles.

The results from this thesis showed that regarding preserving muscle tissue: ethanol and cell lysis with 1% sodium azide can be used successfully to store muscle samples at room temperature for 7 years. Moreover, vodka and 37.5% ethanol can be used for molecular preservation of muscle tissues; and vacuum preservation is a promising alternative for sample preservation. As for rebalancing of degraded DNA profiles, neither gel agarose electrophoresis or purification columns should be used with that intent. Nevertheless, the use of a DNA Capture protocol seems like an alternative that needs to be further assessed.

9.3 Problems and Limitations

As with any other research, there are a few limitations in the work done in this thesis. Due to ethical reasons, a model animal was used and although pigs are very similar to humans at metabolic, physiological, and anatomical levels, there are differences that need to be considered in the end. For most of the laboratory experiments, fresh tissue was used. A comparison with partially decomposed tissue could have been done for a more realistic comparison with forensic samples. Due to time limits, the DNA Capture protocol still needs to be finalised and the full body vacuum preservation could not be tested for its efficacy.

9.4 Scope for Future Studies

The results from this thesis show that there are still several alternatives to be studied for preservation of soft tissue samples and improvement of DNA profiles. Still, there are studies that could be performed following these shown here to further the knowledge in the field.

For a complete profile of DNA degradation in different environments, the experiments performed on the field in Chapter 4 would have to be repeated in dry environments. Previous studies started on a humid and temperate environment (UK) and this thesis followed with humid and warm environment (Thailand).

The experiment performed in Chapter 5 using vodka as a preservative solution can be repeated using different liquors and batches, and other easily accessible solutions containing alcohol. In addition, the preservation of partially decomposed tissue should be assessed.

Vacuum preservation should be assessed for total body preservation in a field condition. Besides assessing the actual usefulness of vacuum, the handling of the body bags needs to be tested for feasibility.

The DNA Capture work was not concluded, so future work would involve new batches of the 384 bp primer pair and of the magnetic beads. The protocol optimization needs to be concluded and it needs to be tested with degraded DNA samples.

REFERENCES

AAFS (2014), Choosing a Career [Online]. Available: https://www.aafs.org/wp-content/uploads/FSF_CareerBrochure_2014Edition.pdf. [Accessed on 2018/01/31].

Alaeddini, R., Walsh, S.J. & Abbas, A. (2010). Forensic implications of genetic analyses from degraded DNA-A review. *Forensic Science International-Genetics,* **4(3)**:148-157.

Allen-Hall, A. & McNevin, D. (2013). Non-cryogenic forensic tissue preservation in the field: a review. *Australian Journal of Forensic Sciences,* **45(4)**:450-460.

Araujo, A.C., Oliveira, S.M., Ramos, I.N., Brandao, T.R.S., Monteiro, M.J. & Silva, C.L.M. (2017). Evaluation of Drying and Storage Conditions on Nutritional and Sensory Properties of Dried Galega Kale (*Brassica oleracea L*. var. *Acephala*). *Journal of Food Quality,* **1**:1-9.

Archer, M.J., Lin, B., Wang, Z. & Stenger, D.A. (2006). Magnetic bead-based solid phase for selective extraction of genomic DNA. *Analytical Biochemistry,* **355(2)**:285-297.

Asamura, H., Fujimori, S., Ota, M. & Fukushima, H. (2007). MiniSTR multiplex systems based on non-CODIS loci for analysis of degraded DNA samples. *Forensic Science International,* **173(1)**:7-15.

Bär, W., Kratzer, A., Machler, M. & Schmid, W. (1988). Postmortem Stability of DNA. *Forensic Science International,* **39(1)**:59-70.

Barta, J.L., Monroe, C., Teisberg, J.E., Winters, M., Flanigan, K. & Kemp, B.M. (2014). One of the key characteristics of ancient DNA, low copy number, may be a product of its extraction. *Journal of Archaeological Science,* **46**:281-289.

Bellés, M., Alonso, V., Roncales, P. & Beltran, J.A. (2017). A review of fresh lamb chilling and preservation. *Small Ruminant Research,* **146**:41-47.

Bellete, B., Flori, P., Hafid, J., Raberin, H. & Sung, R. (2003). Influence of the quantity of nonspecific DNA and repeated freezing and thawing of samples on the quantification of DNA by the Light Cycler (R). *Journal of Microbiological Methods,* **55(1)**:213-219.

Bender, K., Farfan, M.J. & Schneider, P.M. (2004). Preparation of degraded human DNA under controlled conditions. *Forensic Science International,* **139(2-3)**:135-140.

Benschop, C.C.G., van der Beek, C.P., Meiland, H.C., van Gorp, A.G.M., Westen, A.A. & Sijen, T. (2011). Low template STR typing: Effect of replicate number and consensus method on genotyping reliability and DNA database search results. *Forensic Science International-Genetics,* **5(4)**:316-328.

Bernstein, R., Schluter, S., Bernstein, H. & Marchalonis, J. (1996). Primordial emergence of the recombination activating gene 1 (RAG1): Sequence of the complete shark gene indicates homology to microbial integrases. *Proceedings of the National Academy of Sciences of the United States of America,* **93(18)**:9454-9459.

Bing, D.H. & Bieber, F.R. (2001). Collecting and Handling Samples for Parentage and Forensics DNA-Based Genetic Testing. *Current Protocols in Human Genetics,* **14**: Unit 14.2.

Bogas, V., Carvalho, M., Anjos, M.J., Pinheiro, M.F. & Real, F.C. (2014). Methods enhancement for improved recovery of human DNA from forensic blood samples on different fabrics using the DNA IQ System. *Australian Journal of Forensic Sciences,* **46(2)**:204-215.

Boni, J., Shah, C., Flepp, M., Luthy, R. & Schupbach, J. (2004). Detection of low copy numbers of HIV-1 proviral DNA in patient PBMCs by a high-input, sequence-capture PCR (Mega-PCR). *Journal of Medical Virology,* **72(1)**:1-9.

Borsting, C. & Morling, N. (2015). Next generation sequencing and its applications in forensic genetics. *Forensic Science International-Genetics,* **18**:78-89.

Bouletis, A.D., Arvanitoyannis, I.S. & Hadjichristodoulou, C. (2017). Application of modified atmosphere packaging on aquacultured fish and fish products: A review. *Critical Reviews in Food Science and Nutrition,* **57(11)**:2263-2285.

Briggs, A.W., Good, J.M., Green, R.E., Krause, J., Maricic, T., Stenzel, U., Lalueza-Fox, C., Rudan, P., Brajkovic, D., Kucan, Z., Gusic, I., Schmitz, R., Doronichev, V.B., Golovanova, L.V., de la Rasilla, M., Fortea, J., Rosas, A. & Paeaebo, S. (2009). Targeted Retrieval and Analysis of Five Neandertal mtDNA Genomes. *Science,* **325(5938)**:318-321.

Brown, T.A. (1999). Genetic Material. In: Care and Conservation of Natural History Collections (eds. Carter D. & Walker,A.). Butterworth-Heinemann, Oxford, pp. 133-138.

Budowle, B., Bieber, F.R. & Eisenberg, A.J. (2005). Forensic aspects of mass disasters: Strategic considerations for DNA-based human identification. *Legal Medicine,* **7(4)**:230-243.

Budowle, B., Eisenberg, A.J. & van Daal, A. (2009). Validity of Low Copy Number Typing and Applications to Forensic Science. *Croatian Medical Journal,* **50(3)**:207-217.

Burgmann, H., Pesaro, M., Widmer, F. & Zeyer, J. (2001). A strategy for optimizing quality and quantity of DNA extracted from soil. *Journal of Microbiological Methods,* **45(1)**:7-20.

Bussolati, G., Chiusa, L., Cimino, A. & D'Armento, G. (2008). Tissue transfer to pathology labs: under vacuum is the safe alternative to formalin. *Virchows Archiv,* **452(2)**:229-231.

Butler, J., Shen, Y. & McCord, B. (2003). The development of reduced size STR amplicons as tools for analysis of degraded DNA. *Journal of Forensic Sciences,* **48(5)**:1054-1064.

Campobasso, C.P., Di Vella, G. & Introna, F. (2001). Factors affecting decomposition and *Diptera* colonization. *Forensic Science International,* **120(1-2)**:18-27.

Carlson, L.M., Oettinger, M.A., Schatz, D.G., Masteller, E.L., Hurley, E.A., Mccormack, W.T., Baltimore, D. & Thompson, C.B. (1991). Selective Expression of Rag-2 in Chicken B-Cells Undergoing Immunoglobulin Gene Conversion. *Cell,* **64(1)**:201-208.

Carpi, F.M., Di Pietro, F., Vincenzetti, S., Mignini, F. & Napolioni, V. (2011). Human DNA extraction methods: patents and applications. *Recent Patents on DNA & Gene Sequences,* **5(1)**:1-7.

Casaburi, A., Piombino, P., Nychas, G., Villani, F. & Ercolini, D. (2015). Bacterial populations and the volatilome associated to meat spoilage. *Food Microbiology,* **45**:83-102.

Chung, D.T., Drabek, J., Opel, K.L., Butler, J.M. & McCord, B.R. (2004). A study on the effects of degradation and template concentration on the amplification efficiency of the STR miniplex primer sets. *Journal of Forensic Sciences,* **49(4)**:733-740.

Condelli, V., Lettini, G., Patitucci, G., D'Auria, F., D'Amico, M., Vita, G., Musto, P., Cuomo, C. & Landriscina, M. (2014). Validation of Vacuum-Based Refrigerated System for Biobanking Tissue Preservation: Analysis of Cellular Morphology, Protein Stability, and RNA Quality. *Biopreservation and Biobanking,* **12(1)**:35-45.

Costa, C., Lucera, A., Lacivita, V., Saccotelli, M.A., Conte, A. & Del Nobile, M.A. (2016). Packaging optimisation for portioned Canestrato di Moliterno cheese. *International Journal of Dairy Technology,* **69(3)**:401-409.

Cotton, E., Allsop, R., Guest, J., Frazier, R., Koumi, P., Callow, I., Seager, A. & Sparkes, R. (2000). Validation of the AMPFISTR (R) SGM Plus (TM) system for use in forensic casework. *Forensic Science international,* **112(2-3)**:151-161.

Cross, P. & Simmons, T. (2010). The Influence of Penetrative Trauma on the Rate of Decomposition. *Journal of Forensic Sciences,* **55(2)**:295-301.

Cross, P., Simmons, T., Cunliffe, R. & Chatfield, L. (2010). Establishing a Taphonomic Research Facility in the United Kingdom. *Forensic Science Policy & Management: An International Journal,* **1(4)**:187-191.

Cushwa, W.T. & Medrano, J.F. (1993). Effects of Blood-Storage Time and Temperature on DNA Yield and Quality. *BioTechniques,* **14(2)**:204.

Dawson, M.N., Raskoff, K.A. & Jacobs, D.K. (1998). Field preservation of marine invertebrate tissue for DNA analyses. *Molecular Marine Biology and Biotechnology,* **7(2)**:145-152.

Decorte, R. (2010). Genetic identification in the 21st century-Current status and future developments. *Forensic Science international,* **201(1-3)**:160-164.

Dekeirsschieter, J., Verheggen, F.J., Gohy, M., Hubrecht, F., Bourguignon, L., Lognay, G. & Haubruge, E. (2009). Cadaveric volatile organic compounds released by decaying pig carcasses (*Sus domesticus L.*) in different biotopes. *Forensic Science international,* **189(1-3)**:46-53.

Demple, B. & Harrison, L. (1994). Repair of Oxidative Damage to DNA - Enzymology and Biology. *Annual Review of Biochemistry,* **63**:915-948.

Dent, B.B., Forbes, S.L. & Stuart, B.H. (2004). Review of human decomposition processes in soil. *Environmental Geology,* **45(4)**:576-585.

Di Novi, C., Minniti, D., Barbaro, S., Zampirolo, M.G., Cimino, A. & Bussolati, G. (2010). Vacuum-based preservation of surgical specimens: An environmentally-safe step towards a formalin-free hospital. *Science of the Total Environment,* **408(16)**:3092-3095.

Didenko, V., Ngo, H. & Baskin, D. (2003). Early necrotic DNA degradation - Presence of blunt-ended DNA breaks, 3 ' and 5 ' overhangs in apoptosis, but only 5 ' overhangs in early necrosis. *American Journal of Pathology,* **162(5)**:1571-1578.

Dixon, L.A., Dobbins, A.E., Pulker, H.K., Butler, J.M., Vallone, P.M., Coble, M.D., Parson, W., Berger, B., Grubwieser, P., Mogensen, H.S., Morling, N., Nielsen, K., Sanchez, J.J., Petkovski, E., Carracedo, A., Sanchez-Diz, P., Ramos-Luis, E., Brion, M., Irwin, J.A., Just, R.S., Loreille, O., Parsons, T.J., Syndercombe-Court, D., Schmitter, H., Stradmann-Bellinghausen, B., Bender, K. & Gill, P. (2006). Analysis of artificially degraded DNA using STRs and SNPs - results of a

collaborative European (EDNAP) exercise. *Forensic Science International,* **164(1)**:33-44.

Doran, A.E. & Foran, D.R. (2014). Assessment and mitigation of DNA loss utilizing centrifugal filtration devices. *Forensic Science International-Genetics,* **13**:187-190.

Epe, B., Haring, M., Ramaiah, D., Stopper, H., Abouelzahab, M.M., Adam, W. & Samaholler, C.R. (1993). DNA-Damage Induced by Furocoumarin Hydroperoxides Plus Uv (360 Nm). *Carcinogenesis,* **14(11)**:2271-2276.

FBI (2013), Handbook of Forensic Services [Online]. Available: https://www.fbi.gov/file-repository/handbook-of-forensic-services-pdf.pdf/view. [Accessed on 2017/03/07].

Foran, D. (2006). Relative degradation of nuclear and mitochondrial DNA: An experimental approach. *Journal of Forensic Sciences,* **51(4)**:766-770.

Garvin, A.M. & Fritsch, A. (2013). Purifying and Concentrating Genomic DNA from Mock Forensic Samples Using Millipore Amicon Filters. *Journal of Forensic Sciences,* **58**:S173-S175.

Gessoni, G., Barin, P., Valverde, S., Giacomini, A., Di Natale, C., Orlandini, E., Arreghini, N., De Fusco, G., Frigato, A., Fezzi, M., Antico, F. & Marchiori, G. (2004). Biological qualification of blood units - Considerations about the effects of sample's handling and storage on stability of nucleic acids. *Transfusion and Apheresis Science,* **30(3)**:197-203.

Giardina, E., Spinella, A. & Novelli, G. (2011). Past, present and future of forensic DNA typing. *Nanomedicine,* **6(2)**:257-270.

Gill, P. (2001). Application of low copy number DNA profiling. *Croatian Medical Journal,* **42(3)**:229-232.

Gill, P., Whitaker, J., Flaxman, C., Brown, N. & Buckleton, J. (2000). An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA. *Forensic Science international,* **112(1)**:17-40.

Gillespie, J.W., Best, C.J.M., Bichsel, V.E., Cole, K.A., Greenhut, S.F., Hewitt, S.M., Ahram, M., Gathright, Y.B., Merino, M.J., Strausberg, R.L., Epstein, J.I., Hamilton, S.R., Gannot, G., Baibakova, G.V., Calvert, V.S., Flaig, M.J., Chuaqui, R.F., Herring, J.C., Pfeifer, J., Petricoin, E.F., Linehan, W.M., Duray, P.H., Bova, G.S. & Emmert-Buck, M.R. (2002). Evaluation of non-formalin tissue fixation for molecular profiling studies. *American Journal of Pathology,* **160(2)**:449-457.

Goff, M.L. (2009). Early post-mortem changes and stages of decomposition in exposed cadavers. *Experimental and Applied Acarology,* **49(1-2)**:21-36.

Golenberg, E., Bickel, A. & Weihs, P. (1996). Effect of highly fragmented DNA on PCR. *Nucleic Acids Research,* **24(24)**:5026-5033.

Goodwin, W., Linacre, A. & Hadi, S. (2007). An Introduction to Forensic Genetics. Wiley, England.

Graham, E.A.M., Turk, E.E. & Rutty, G.N. (2008). Room temperature DNA preservation of soft tissue for rapid DNA extraction: An addition to the disaster victim identification investigators toolkit? *Forensic Science International-Genetics,* **2(1)**:29-34.

Greenspoon, S.A., Sykes, K.L.V., Ban, J.D., Pollard, A., Baisden, M., Farr, M., Graham, N., Collins, B.L., Green, M.M. & Christenson, C.C. (2006). Automated PCR setup for forensic casework samples using the Normalization Wizard and PCR Setup robotic methods. *Forensic Science international,* **164(2-3)**:240-248.

Gunawardane, D.M.D.D.N. (2009). An assessment of the impact of environmental factors on the quality of post-mortem DNA profiling, University of Adelaide.

Haak, B., Porsche, A., Vollack, K., Zimmermann, P. & Pflug, W. (2008). Evaluation of a semi-automated, magnetic bead-based DNA extraction method for genetic fingerprinting of forensic casework samples. *Forensic Science International: Genetics Supplement Series,* **1(1)**:35-36.

Ham, S., Kim, S., Seo, B.Y., Woo, K., Lee, S. & Choi, C.Y. (2016). Differential pre-amplification of STR loci for fragmented forensic DNA profiling. *Electrophoresis,* **37(22)**:3002-3009.

Hara, M., Nakanishi, H., Yoneyama, K., Saito, K. & Takada, A. (2016). Effects of storage conditions on forensic examinations of blood samples and bloodstains stored for 20 years. *Legal Medicine,* **18**:81-84.

Hassan, N.H. (2017). Evaluation of Insertion/Deletion Polymorphisms (INDELs) on Malaysian Population and Forensic Casework, University of Central Lancashire.

Heaton, V., Moffatt, C. & Simmons, T. (2014). Quantifying the Temperature of Maggot Masses and its Relationship to Decomposition. *Journal of Forensic Sciences,* **59(3)**:676-682.

Hernández, E. J. G. P., de Carvalho Jr., R. N., Joele, M. R. S. P., Araújo, C.S. & Lourenço, L.F.H. (2017). Effects of modified atmosphere packing over the shelf life of sous vide from captive pirarucu (*Arapaima gigas*). *Innovative Food Science & Emerging Technologies,* **39**:94-100.

Hochmeister, M.N. (1995). DNA Technology in Forensic Applications. *Molecular Aspects of Medicine,* **16(4)**:315-437.

Hofreiter, M., Serre, D., Poinar, H., Kuch, M. & Paabo, S. (2001). Ancient DNA. *Nature Reviews Genetics,* **2(5)**:353-359.

Horemans, T., Deschacht, M., Clais, S., Van Camp, J., de Rijk, P., Holvoet, J., Van Assche, T., Maes, L. & Cos, P. (2011). An Alternative, Sensitive Method to Detect *Helicobacter pylori* DNA in Feces. *Helicobacter,* **16(2)**:113-118.

Horn, S. (2011). Target Enrichment via DNA Hybridization Capture. In: Ancient DNA - Methods and Protocols (eds. Shapiro B. & Hofreiter,M.). Humana Press, USA, pp. 177-188.

Horsman-Hall, K.M., Orihuela, Y., Karczynski, S.L., Davis, A.L., Ban, J.D. & Greenspoon, S.A. (2009). Development of STR profiles from firearms and fired cartridge cases. *Forensic Science International-Genetics,* **3(4)**:242-250.

Hoss, M., Jaruga, P., Zastawny, T., Dizdaroglu, M. & Paabo, S. (1996). DNA damage and DNA sequence retrieval from ancient tissues. *Nucleic Acids Research,* **24(7)**:1304-1307.

Huang, J., Luo, H., Wei, W. & Hou, Y. (2014). A novel method for the analysis of 20 multi-Indel polymorphisms and its forensic application. *Electrophoresis,* **35(4)**:487-493.

Hughes-Stamm, S.R., Ashton, K.J. & van Daal, A. (2011). Assessment of DNA degradation and the genotyping success of highly degraded samples. *International Journal of Legal Medicine,* **125(3)**:341-348.

ICRC (2009). Missing People, DNA Analysis and Identification of Human Remains: A Guide to Best Practice in Armed Conflicts and Other Situations of Armed Violence. International Committee of the Red Cross, Geneva, Switzerland.

INTERPOL (2014), Interpol Disaster Victim Identification Guide [Online]. Available: http://www.interpol.int/Media/Files/INTERPOL-Expertise/DVI/DVI-Guide-new-version-2013. [Accessed on 2016/08/15].

Iozzi, S., Carboni, I., Contini, E., Pescucci, C., Frusconi, S., Nutini, A.L., Torricelli, F. & Ricci, U. (2015). Forensic genetics in NGS era: New frontiers for massively parallel typing. *Forensic Science International Genetics Supplement Series,* **5**:E418-E419.

Itani, M., Yamamoto, Y., Doi, Y. & Miyaishi, S. (2011). Quantitative Analysis of DNA Degradation in the Dead Body. *Acta Medica Okayama,* **65(5)**:299-306.

Jääskeläinen, E., Hultman, J., Parshintsev, J., Riekkola, M. & Bjorkroth, J. (2016). Development of spoilage bacterial community and volatile compounds in chilled beef under vacuum or high oxygen atmospheres. *International Journal of Food Microbiology,* **223**:25-32.

Jackson, A.R.W. & Jackson, J.M. (2011). Forensic Science. Prentice Hall, New Jersey, USA.

Janaway, R.C., Percival, S.L. & Wilson, A.S. (2009). Decomposition of Human Remains. In: Microbiology and Ageing: Clinical Manifestations (ed. Percival S.). Humana Press Inc., USA, pp. 313-334.

Jeffreys, A.J., Wilson, V. & Thein, S.L. (1985). Individual-Specific Fingerprints of Human DNA. *Nature,* **316(6023)**:76-79.

Jin, T.Z., Huang, M., Niemira, B.A. & Cheng, L. (2017). Microbial Reduction and Sensory Quality Preservation of Fresh Ginseng Roots Using Nonthermal Processing and Antimicrobial Packaging. *Journal of Food Processing and Preservation,* **41(1)**:UNSP e12871.

Kemp, B.M., Winters, M., Monroe, C. & Barta, J.L. (2014). How Much DNA Is Lost? Measuring DNA Loss of Short-Tandem-Repeat Length Fragments Targeted by the PowerPlex 16 (R) System Using the Qiagen MinElute Purification Kit. *Human Biology,* **86(4)**:313-329.

Kidd, K.K., Speed, W.C., Wootton, S., Lagace, R., Langit, R., Haigh, E., Chang, J. & Pakstis, A.J. (2015). Genetic markers for massively parallel sequencing in forensics. *Forensic Science International Genetics Supplement Series,* **5**:E677-E679.

Kilpatrick, C.W. (2002). Noncryogenic preservation of mammalian tissues for DNA extraction: An assessment of storage methods. *Biochemical Genetics,* **40(1-2)**:53-62.

King, J.R. & Porter, S.D. (2004). Recommendations on the use of alcohols for preservation of ant specimens (*Hymenoptera*, *Formicidae*). *Insectes Sociaux,* **51(2)**:197-202.

Krajden, M., Minor, J., Rifkin, O. & Comanor, L. (1999). Effect of multiple freeze-thaw cycles on hepatitis B virus DNA and hepatitis C virus RNA quantification as measured with branched-DNA technology. *Journal of Clinical Microbiology,* **37(6)**:1683-1686.

Krenke, B., Tereba, A., Anderson, S., Buel, E., Culhane, S., Finis, C., Tomsey, C., Zachetti, J., Masibay, A., Rabbach, D., Amiott, E. & Sprecher, C. (2002). Validation of a 16-locus fluorescent multiplex system. *Journal of Forensic Sciences,* **47(4)**:773-785.

Larkin, B., Iaschi, S., Dadour, I. & Tay, G.K. (2010). Using accumulated degree-days to estimate postmortem interval from the DNA yield of porcine skeletal muscle. *Forensic Science Medicine and Pathology,* **6(2)**:83-92.

LaRue, B.L., Ge, J., King, J.L. & Budowle, B. (2012). A validation study of the Qiagen Investigator DIPplex (R) kit; an INDEL-based assay for human identification. *International Journal of Legal Medicine,* **126(4)**:533-540.

LaRue, B.L., Lagace, R., Chang, C., Holt, A., Hennessy, L., Ge, J., King, J.L., Chakraborty, R. & Budowle, B. (2014). Characterization of 114 insertion/deletion (INDEL) polymorphisms, and selection for a global INDEL panel for human identification. *Legal Medicine,* **16(1)**:26-32.

Lee, H.L., An, D.S. & Lee, D.S. (2016). Effect of Initial Gas Flushing or Vacuum Packaging on the Ripening Dynamics and Preference for Kimchi, a Korean Fermented Vegetable. *Packaging Technology and Science,* **29(8-9)**:479-485.

Li, L., Zhang, M., Adhikari, B. & Gao, Z. (2017). Recent advances in pressure modification-based preservation technologies applied to fresh fruits and vegetables. *Food Reviews International,* **33(5)**:538-559.

Lindahl, T. (1993). Instability and Decay of the Primary Structure of DNA. *Nature,* **362(6422)**:709-715.

Lindahl, T. & Karlstro, O. (1973). Heat-Induced Depyrimidination of Deoxyribonucleic Acid in Neutral Solution. *Biochemistry,* **12(25)**:5151-5154.

Lindahl, T. & Nyberg, B. (1974). Heat-Induced Deamination of Cytosine Residues in Deoxyribonucleic-Acid. *Biochemistry,* **13(16)**:3405-3410.

Lloret, E., Fernandez, A., Trbojevich, R., Arnau, J. & Picouet, P.A. (2016). Relevance of nanocomposite packaging on the stability of vacuum-packed dry cured ham. *Meat Science,* **118**:8-14.

Lygo, J.E., Johnson, P.E., Holdaway, D.J., Woodroffe, S., Whitaker, J.P., Clayton, T.M., Kimpton, C.P. & Gill, P. (1994). The Validation of Short Tandem Repeat (STR) Loci for use in Forensic Casework. *International Journal of Legal Medicine,* **107(2)**:77-89.

Mangiapan, G., Vokurka, M., Shouls, L., Cadranel, J., Lecossier, D., vanEmbden, J. & Hance, A. (1996). Sequence capture-PCR improves detection of mycobacterial DNA in clinical specimens. *Journal of Clinical Microbiology,* **34(5)**:1209-1215.

McCord, B., Opel, K., Funes, M., Zoppis, S. & Jantz, L.M. (2011). An Investigation of the Effect of DNA Degradation and Inhibition on PCR Amplification of Single Source and Mixed Forensic Samples. USA.

McNevin, D. (2016). Preservation of and DNA Extraction from Muscle Tissue. In: Forensic DNA Typing Protocols (Methods in Molecular Biology) (ed. Goodwin W.). Humana Press, USA, pp. 43-53.

Megyesi, M., Nawrocki, S. & Haskell, N. (2005). Using accumulated degree-days to estimate the postmortem interval from decomposed human remains. *Journal of Forensic Sciences,* **50(3)**:618-626.

Meyer, Q., Burton, S. & Cowan, D. (2007). Subtractive hybridization magnetic bead capture: A new technique for the recovery of full-length ORFs from the metagenome. *Biotechnology Journal,* **2**:36-40.

Michaud, C.L. & Foran, D.R. (2011). Simplified Field Preservation of Tissues for Subsequent DNA Analyses. *Journal of Forensic Sciences,* **56(4)**:846-852.

Miyashiro, I., Kuo, C., Huynh, K., Iida, A., Morton, D., Bilchik, A., Giuliano, A. & Hoon, D. (2001). Molecular strategy for detecting metastatic cancers with use of multiple tumor-specific MAGE-A genes. *Clinical Chemistry,* **47(3)**:505-512.

Moffatt, C., Simmons, T. & Lynch-Aird, J. (2016). An Improved Equation for TBS and ADD: Establishing a Reliable Postmortem Interval Framework for Casework and Experimental Studies. *Journal of Forensic Sciences,* **61**: S201-S207.

Monroe, C., Grier, C. & Kemp, B.M. (2013). Evaluating the efficacy of various thermo-stable polymerases against co-extracted PCR inhibitors in ancient DNA samples. *Forensic Science International,* **228(1-3)**:142-153.

Mulero, J.J., Chang, C.W., Lagace, R.E., Wang, D.Y., Bas, J.L., McMahon, T.P. & Hennessy, L.K. (2008). Development and validation of the AmpFlSTR (R)

MiniFiler (TM) PCR amplification kit: A MiniSTR multiplex for the analysis of degraded and/or PCR inhibited DNA. *Journal of Forensic Sciences,* **53(4)**:838-852.

Myburgh, J., L'Abbe, E.N., Steyn, M. & Becker, P.J. (2013). Estimating the postmortem interval (PMI) using accumulated degree-days (ADD) in a temperate region of South Africa. *Forensic Science International,* **229(1-3)**: 165.e1.

Nagy, M., Otremba, P., Kruger, C., Bergner-Greiner, S., Anders, P., Henske, B., Prinz, M. & Roewer, L. (2005). Optimization and validation of a fully automated silica-coated magnetic beads purification technology in forensics. *Forensic Science International,* **152(1)**:13-22.

Nagy, Z.T. (2010). A hands-on overview of tissue preservation methods for molecular genetic analyses. *Organisms Diversity & Evolution,* **10(1)**:91-105.

Nazir, M.S., Smith, J.A. & Goodwin, W. (2011). DNA degradation in post-mortem soft muscle tissues in relation to accumulated degree-days (ADD). *Forensic Science International: Genetics Supplement Series,* **3(1)**:e536-e537.

Nazir, M.S. (2012). DNA Persistence and Preservation Following Environmental Insult, University of Central Lancashire.

Nazir, M.S., Iyavoo, S., Alimat, S., Zahra, N., Sanqoor, S.H., Smith, J.A., Moffatt, C. & Goodwin, W. (2013). Development of a multiplex system to assess DNA persistence in taphonomic studies. *Electrophoresis,* **34(24)**:3352-3360.

NIJ (2005), Mass Fatality Incidents: A Guide for Human Forensic Identification [Online]. Available: https://www.ncjrs.gov/pdffiles1/nij/199758.pdf. [Accessed on 2017/03/07].

Oakenfull, E.A. (1994). Vodka, Meths and DNA. *Trends in Ecology & Evolution,* **9(1)**:26-26.

Opel, K.L., Chung, D.T., Drabek, J., Butler, J.M. & McCord, B.R. (2007). Developmental validation of reduced-size STR Miniplex primer sets. *Journal of Forensic Sciences,* **52(6)**:1263-1271.

Opsteegh, M., Langelaar, M., Sprong, H., den Hartog, L., De Craeye, S., Bokken, G., Ajzenberg, D., Kijlstra, A. & van der Giessen, J. (2010). Direct detection and genotyping of *Toxoplasma gondii* in meat samples using magnetic capture and PCR. *International Journal of Food Microbiology,* **139(3)**:193-201.

Overballe-Petersen, S., Orlando, L. & Willerslev, E. (2012). Next-generation sequencing offers new insights into DNA degradation. *Trends in Biotechnology,* **30(7)**:364-368.

Paabo, S., Poinar, H., Serre, D., Jaenicke-Despres, V., Hebler, J., Rohland, N., Kuch, M., Krause, J., Vigilant, L. & Hofreiter, M. (2004). Genetic analyses from ancient DNA. *Annual Review of Genetics,* **38**:645-679.

Paczkowski, S. & Schuetz, S. (2011). Post-mortem volatiles of vertebrate tissue. *Applied Microbiology and Biotechnology,* **91(4)**:917-935.

Parham, N.J., Picard, F.J., Peytavi, R., Gagnon, M., Seyrig, G., Gagne, P., Boissinot, M. & Bergeron, M.G. (2007). Specific magnetic bead-based capture of genomic DNA from clinical samples: Application to the detection of group B streptococci in Vaginal/Anal swabs. *Clinical Chemistry,* **53(9)**:1570-1576.

Pegg, D.E. (2007). Principles of cryopreservation. *Methods in Molecular Biology,* **368**:39-57.

Penna, T.C.V., Mazzola, P.G. & Martins, A.M.S. (2001). The efficacy of chemical agents in cleaning and disinfection programs. *Bmc Infectious Diseases,* **1**:16.

Phengon, P., Wongwiggarn, S. & Panvisavas, N. (2008). Analysis of DNA from degraded tissue. *Forensic Science International: Genetics Supplement Series,* **1(1)**:439-441.

Phillips, C.P., Carracedo, A. & Lareu, M.V. (1998). Manual Electrophoretic Methods for Genotyping Amplified STR Loci. In: Methods in Molecular Biology: Forensic DNA Profilling Protocols (eds. Lincoln P.J. & Thomson,J.). Humana Press, USA, pp. 181-192.

Phillips, K., McCallum, N. & Welch, L. (2012). A comparison of methods for forensic DNA extraction: Chelex-100 (R) and the QIAGEN DNA Investigator Kit (manual and automated). *Forensic Science International-Genetics,* **6(2)**:282-285.

Prangnell, J. & McGowan, G. (2009). Soil temperature calculation for burial site analysis. *Forensic Science International,* **191(1-3)**:104-109.

Prinz, M., Carracedo, A., Mayr, W.R., Morling, N., Parsons, T.J., Sajantila, A., Scheithauer, R., Schmitter, H. & Schneider, P.M. (2007). DNA Commission of the International Society for Forensic Genetics (ISFG): Recommendations regarding the role of forensic genetics for disaster victim identification (DVI). *Forensic Science International-Genetics,* **1(1)**:3-12.

Rittich, B. & Spanova, A. (2013). SPE and purification of DNA using magnetic particles. *Journal of Separation Science,* **36(15)**:2472-2485.

Roeder, B., Fruehwirth, K., Vogl, C., Wagner, M. & Rossmanith, P. (2010). Impact of Long-Term Storage on Stability of Standard DNA for Nucleic Acid-Based Methods. *Journal of Clinical Microbiology,* **48(11)**:4260-4262.

Ross, A.H. & Cunningham, S.L. (2011). Time-since-death and bone weathering in a tropical environment. *Forensic Science International,* **204(1-3)**:126-133.

Ross, K., Haites, N. & Kelly, K. (1990). Repeated Freezing and Thawing of Peripheral-Blood and DNA in Suspension - Effects on DNA Yield and Integrity. *Journal of Medical Genetics,* **27(9)**:569-570.

Saferstein, R. (2017). Criminalistics: an introduction to forensic science. Pearson, New Jersey, USA.

Samejima, K. & Earnshaw, W. (2005). Trashing the genome: The role of nucleases during apoptosis. *Nature Reviews Molecular Cell Biology,* **6(9)**:677-688.

Sanchez, J., Phillips, C., Borsting, C., Balogh, K., Bogus, M., Fondevila, M., Harrison, C., Musgrave-Brown, E., Salas, A., Syndercombe-Court, D., Schneider, P., Carracedo, A. & Morling, N. (2006). A multiplex assay with 52 single

nucleotide polymorphisms for human identification. *Electrophoresis,* **27(9)**:1713-1724.

Schatz, D.G., Oettinger, M.A. & Baltimore, D. (1989). The V(d)j Recombination Activating Gene, Rag-1. *Cell,* **59(6)**:1035-1048.

Schaudien, D., Baumgaertner, W. & Herden, C. (2007). High preservation of DNA standards diluted in 50% glycerol. *Diagnostic Molecular Pathology,* **16(3)**:153-157.

Senge, T., Madea, B., Junge, A., Rothschild, M.A. & Schneider, P.M. (2011). STRs, mini STRs and SNPs - A comparative study for typing degraded DNA. *Legal Medicine,* **13(2)**:68-74.

Sergio, L., Gatto, M.A., Sprernulli, L., Pieralice, M., Linsalata, V. & Di Venere, D. (2016). Packaging and storage conditions to extend the shelf life of semi-dried artichoke hearts. *Lwt-Food Science and Technology,* **72**:277-284.

Shao, W., Khin, S. & Kopp, W.C. (2012). Characterization of Effect of Repeated Freeze and Thaw Cycles on Stability of Genomic DNA Using Pulsed Field Gel Electrophoresis. *Biopreservation and Biobanking,* **10(1)**:4-11.

Shirley, N.R., Wilson, R.J. & Jantz, L.M. (2011). Cadaver Use at the University of Tennessee's Anthropological Research Facility. *Clinical Anatomy,* **24(3)**:372-380.

Shuber, A., Ascano, J., Boynton, K., Mitchell, A., Frierson, H., El-Rifai, W. & Powell, S. (2002). Accurate, noninvasive detection of *Helicobacter pylori* DNA from stool samples: Potential usefulness for monitoring treatment. *Journal of Clinical Microbiology,* **40(1)**:262-264.

Simmons, T., Adlam, R.E. & Moffatt, C. (2010). Debugging Decomposition Data-Comparative Taphonomic Studies and the Influence of Insects and Carcass Size on Decomposition Rate. *Journal of Forensic Sciences,* **55(1)**:8-13.

Simmons, T., Cross, P.A., Adlam, R.E. & Moffatt, C. (2010). The Influence of Insects on Decomposition Rate in Buried and Surface Remains. *Journal of Forensic Sciences,* **55(4)**:889-892.

Srinivasan, M., Sedmak, D. & Jewell, S. (2002). Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *American Journal of Pathology,* **161(6)**:1961-1971.

Statheropoulos, M., Agapiou, A., Zorba, E., Mikedi, K., Karma, S., Pallis, G.C., Eliopoulos, C. & Spiliopoulou, C. (2011). Combined chemical and optical methods for monitoring the early decay stages of surrogate human models. *Forensic Science international,* **210(1-3)**:154-163.

Swango, K., Timken, M., Chong, M. & Buoncristiani, M. (2006). A quantitative PCR assay for the assessment of DNA degradation in forensic samples. *Forensic Science International,* **158(1)**:14-26.

Swann, L.M., Forbes, S.L. & Lewis, S.W. (2010). Analytical separations of mammalian decomposition products for forensic science A review. *Analytica Chimica Acta,* **682(1-2)**:9-22.

Takahashi, M., Kato, Y., Mukoyama, H., Kanaya, H. & Kamiyama, S. (1997). Evaluation of five polymorphic microsatellite markers for typing DNA from decomposed human tissues - Correlation between the size of the alleles and that of the template DNA. *Forensic Science International,* **90(1-2)**:1-9.

Thanakiatkrai, P. & Kitpipit, T. (2013). Current STR-based techniques in forensic science. *Maejo International Journal of Science and Technology,* **7(1)**:1-15.

Thompson, R., Zoppis, S. & McCord, B. (2012). An Overview of DNA Typing Methods for Human Identification: Past, Present, and Future. *DNA Electrophoresis Protocols for Forensic Genetics,* **830**:3-16.

Tsukada, K., Takayanagi, K., Asamura, H., Ota, M. & Fukushima, H. (2002). Multiplex short tandem repeat typing in degraded samples using newly designed primers for the TH01, TPOX, CSF1PO, and vWA loci. *Legal medicine (Tokyo, Japan),* **4(4)**:239-245.

Turner, B. & Wiltshire, P. (1999). Experimental validation of forensic evidence: a study of the decomposition of buried pigs in a heavy clay soil. *Forensic Science International,* **101(2)**:113-122.

Utsuno, H. & Minaguchi, K. (2004). Influence of template DNA degradation on the genotyping of SNPs and STR polymorphisms from forensic materials by PCR. *The Bulletin of Tokyo Dental College,* **45(1)**:33-46.

van Oorschot, R.A., Ballantyne, K.N. & Mitchell, R.J. (2010). Forensic trace DNA: a review. *Investigative Genetics,* **1(1)**:14-14.

Wallin, J., Buoncristiani, M., Lazaruk, K., Fildes, N., Holt, C. & Walsh, P. (1998). TWGDAM validation of the AmpFISTR (TM) Blue PCR amplification kit for forensic casework analysis. *Journal of Forensic Sciences,* **43(4)**:854-870.

Wang, J. & McCord, B. (2011). The application of magnetic bead hybridization for the recovery and STR amplification of degraded and inhibited forensic DNA. *Electrophoresis,* **32(13)**:1631-1638.

Wang, T., Zhao, L., Sun, Y., Ren, F., Chen, S., Zhang, H. & Guo, H. (2016). Changes in the microbiota of lamb packaged in a vacuum and in modified atmospheres during chilled storage analysed by high-throughput sequencing. *Meat Science,* **121**:253-260.

Weiler, N.E.C., Matai, A.S. & Sijen, T. (2012). Extended PCR conditions to reduce drop-out frequencies in low template STR typing including unequal mixtures. *Forensic Science International-Genetics,* **6(1)**:102-107.

Westen, A.A., Matai, A.S., Laros, J.F.J., Meiland, H.C., Jasper, M., de Leeuw, W.J.F., de Knijff, P. & Sijen, T. (2009). Tri-allelic SNP markers enable analysis of mixed and degraded DNA samples. *Forensic Science International-Genetics,* **3(4)**:233-241.

Wiegand, P. & Kleiber, M. (2001). Less is more - length reduction of STR amplicons using redesigned primers. *International Journal of Legal Medicine,* **114(4-5)**:285-287.

Winters, M., Monroe, C., Barta, J.L. & Kemp, B.M. (2017). Are we fishing or catching? Evaluating the efficiency of bait capture of CODIS fragments. *Forensic Science International-Genetics,* **29**:61-70.

Witt, S., Neumann, J., Zierdt, H., Gebel, G. & Roescheisen, C. (2012). Establishing a novel automated magnetic bead-based method for the extraction of DNA from a variety of forensic samples. *Forensic Science International-Genetics,* **6(5)**:539-547.

Yang, X., Zhang, Y., Zhu, L., Han, M., Gao, S. & Luo, X. (2016). Effect of packaging atmospheres on storage quality characteristics of heavily marbled beef longissimus steaks. *Meat Science,* **117**:50-56.

Zhou, C. & Byard, R.W. (2011). Factors and processes causing accelerated decomposition in human cadavers - An overview. *Journal of Forensic and Legal Medicine,* **18(1)**:6-9.

CHAPTER 10

APPENDICES

10.1 Appendix 1

List of experiments which were supposed to be done, but because of time constraint, were not done. They are listed for scope of future studies.

10.1.1 DNA Persistence in Whole Bodies Using Vacuum Preservation

Study Design

This experiment was designed to be performed during the summer of 2017, but due to funding issues, this was not possible.

Two different prototype models of vacuum body bags were designed by a private company. The difference between them is that one of them has a single-layer of reflective material on the outside (Figure 10.1) and the other is double-layered. The reflective material intends to diminish the impact of heat in the bodies inside the bag. Vacuum is applied to the bags with the use of a hand-operated pump. These bags are going to be tested for total body preservation in warm environments without the use of refrigeration.

Two sites will be used for field evaluation of the body bags. The first one is TRACES, UCLAN's 13-acre taphonomic facility, located at Newton Rigg, UK. The second one is located in Thailand, just outside of the town of Nakhon Nayok (roughly 116 km from Bangkok), and belongs to the Sawang Ariya Philanthropic Foundation.
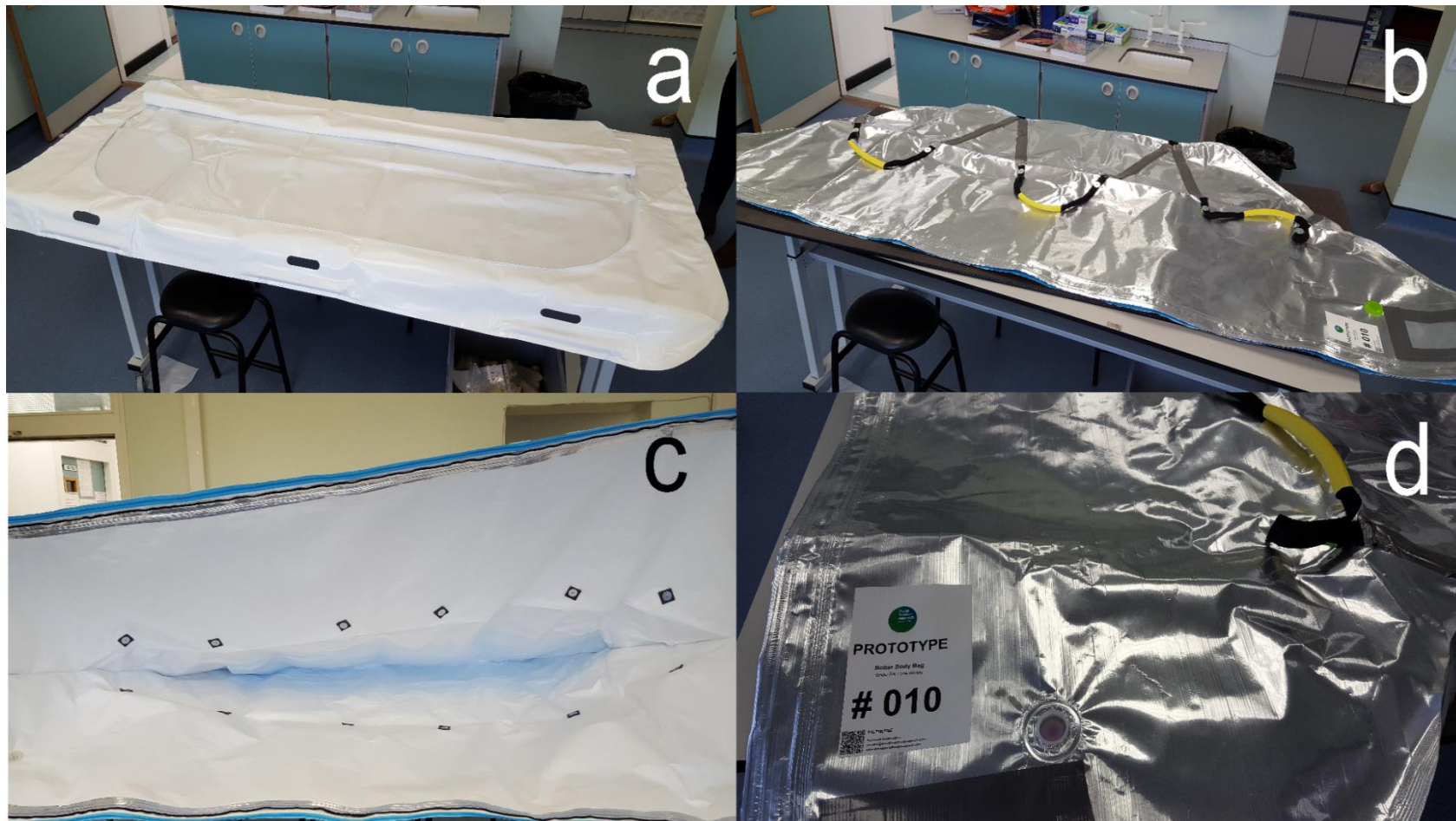
Figure 10.1 – Photographs showing two of the body bags that will be used in the field experiments. (a) is a traditional body bag; (b) is the outside of the single-layered body bag, with handles for carrying; (c) is the interior of the same body bag, with absorbent material; (d) is a close-up of the attachment for the pump.

Four conditions will be tested: one being each of the vacuum body bags, one the traditional body bags, and the last no body bags. Five pig carcasses of freshly killed pig weighting approximately 40 kg will be disposed in the field per condition tested. Temperature will be monitored on the ground next to the carcasses (ambient temperature), inside each carcass (internal temperature) and on the interface of the bodies and the ground (ground temperature). Body decomposition will also be monitored.

In order to assess DNA preservation, samples of muscle tissue will be collected in triplicates every 24 h and stored in either cell lysis or ethanol (three samples each). Samples will be frozen as soon as possible and conserved frozen until DNA extraction. Analysis will be done using agarose gel electrophoresis, DNA Quantitation and the 4-Plex Multiplex. Statistical data analysis was performed as described in Chapter 2.

10.2 Appendix 2

Appendix 2 shows a copy of the ethical approval to undertake the experiments.

8 August 2014

Will Goodwin / Lais Baptista
School of Forensic & Investigative Sciences
University of Central Lancashire

Dear Will / Lais

Re: STEMH Ethics Committee Application
Unique Reference Number: STEMH 220

The STEMH ethics committee has granted approval of your proposal application '**Rebalancing of degraded DNA profiles through preferential extraction**'. This approval is granted on the condition that the trained phlebotomist has a signed risk assessment in place for taking of bloods if undertaken on UCLAN premises. Approval is granted up to the end of project date* or for 5 years from the date of this letter, whichever is the longer.

It is your responsibility to ensure that

- the project is carried out in line with the information provided in the forms you have submitted
- you regularly re-consider the ethical issues that may be raised in generating and analysing your data
- any proposed amendments/changes to the project are raised with, and approved, by Committee
- you notify roffice@uclan.ac.uk if the end date changes or the project does not start
- serious adverse events that occur from the project are reported to Committee
- a closure report is submitted to complete the ethics governance procedures (Existing paperwork can be used for this purposes e.g. funder's end of grant report; abstract for student award or NRES final report. If none of these are available use e-Ethics Closure Report Proforma).

Yours sincerely

Paola Dey
Deputy Vice Chair
STEMH Ethics Committee

* for research degree students this will be the final lapse date

10.3 Appendix 3

Appendix 3 are copies of the journal articles published during the course of the PhD.

DNA persistence in soft tissues exposed to extreme environments

Lais Vicente Baptista[a,b,*], Natnipoon Rattanarungruang[a], Panjai Woharndee[c], William Goodwin[a]

[a] School of Forensic and Investigative Sciences, University of Central Lancashire, Preston, UK
[b] CAPES Foundation, Ministry of Education of Brazil, Brasília DF 70040-020, Brazil
[c] Central Institute of Forensic Science, Ministry of Justice of Thailand, Thailand

ARTICLE INFO

ABSTRACT

After death DNA becomes progressively more fragmented as biological tissue degrades and this results in a decreasing ability to gain a complete STR profile. When extracting and profiling DNA from human remains understanding the likely persistence of DNA in different tissues is important. Studies in the UK have demonstrated that when using pigs as an experimental model, DNA up to 400 bp will persist for up to 3 weeks in the summer. However, it is well known that DNA degradation, especially in muscle tissue that has not become dehydrated, is dependent to a large degree on temperature. To assess DNA persistence in more extreme environmental conditions pig carcasses were exposed to the environment in Thailand during June for 10 days, with samples being collected every 12 h: muscle tissue was present for up to three days post-mortem. Extracted DNA could not be amplified after 36 h exposure unless the muscle was collected from tissue that was in contact with the ground; DNA persisted for up to 72 h in these samples.

1. Introduction

Interpol recommends the use of muscle tissue only in cases of non-decomposed remains. What defines the onset of decomposition is not specified, although agencies typically use 24 h as an approximation [1]. Studies in the UK in the summer have demonstrated that when using pigs as an experimental model, DNA up to 400 bp will persist for up to 3 weeks [2].

Decomposition of tissues is a process that depends more on accumulated temperature rather than time. Accumulated degree days (ADD), the cumulative total of daily average temperatures, can be used as a measure to estimate post-mortem interval and DNA degradation in soft tissues [3].

As it is known that DNA degradation is influenced by temperature, this experiment was done to assess DNA persistence in soft muscle tissue following exposure to high environmental temperatures.

2. Materials and methods

This experiment was carried out during June 2014 in Thailand. Whole pig carcasses (*Sus scrofa*) and separate limbs were placed in direct contact with the ground. Samples were collected every 12 h for 3 days. Samples collected from the separate limbs were collected from both the aerial surface (exposed to the sun) and the ground surface. After collection, samples were stored in ethanol for approximately one week while in the field, and frozen prior to extraction in the laboratory. DNA was extracted using the Qiagen Blood and Tissue Kit and evaluated through agarose gel electrophoresis (AGE) and PCR. A species-specific multiplex that amplified amplicons of 70 bp, 194 bp, 305 bp and 384 bp was used; the PCR were analysed using an ABI 3500.

3. Results and discussion

The average ambient temperatures during the three days of the experiment were 31 °C on the first day, 33.5 °C on the second day, and 35.5 °C on the third day. The average internal temperature of the carcasses was of 33 °C (maximum 35 °C), 31 °C (maximum 35 °C) and 35 °C (maximum 35.9 °C) respectively.

Degradation was visible with AGE, with its characteristic smears that appeared after 16 ADD. Samples collected after 36 h were not detected on the agarose gel, although DNA quantification showed the presence of DNA. The exception was from samples that

* Corresponding author at: School of Forensic & Investigative Sciences, University of Central Lancashire, JB Firth Building, Preston, Lancashire PR1 2HE, UK. Fax: +44 1772 891990.
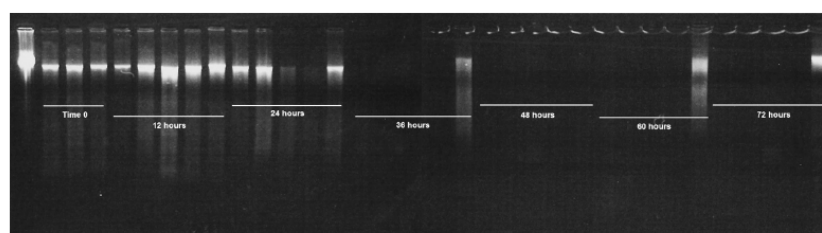E-mail address: LVBaptista@uclan.ac.uk (L.V. Baptista).

Fig. 1. Agarose electrophoresis from DNA samples. The three first samples in each set were collected from the carcass, followed by one collected from the aerial surface of the separate limb and one from the ground surface.

Table 1
Results of the amplification for the 4-plex multiplex.

Time	ADD	70 bp		194 bp		305 bp		384 bp	
		WC	SL	WC	SL	WC	SL	WC	SL
0 h	0	+++	NT	+++	NT	+++	NT	+++	NT
12 h	16	+++	++	+++	++	+++	++	+++	++
24 h	33.5	+++	++	+++	++	+++	++	+++	++
36 h	51	+++	++	+++	–	+++	–	+++	–
48 h	69.5	+++	+–	–	–	–	+–	–	–
60 h	88	+++	(+)+	–+–	(+)+	–+–	(+)+	–+–	(+)+
72 h	105	++–	–+	++–	–+	+–	–+	–	–+

WC: whole carcass; SL: separated limb; NT: sample not taken. The "+" denotes the presence and "–" the absence of PCR amplicons in samples during the 4-plex multiplex amplification; "(+)" represents positive results below threshold of 50 RFU.

were collected from the separate limbs' ground surface with 51, 88, and 105 ADD (Fig. 1).

Full profiles were obtained from all samples until 33.5 ADD. With 51 ADD, samples collected from the separate limbs showed drop-outs, while samples from the whole carcass still had full profiles (Table 1). This could be due to a smaller body mass being more affected by insect activity and offering fewer collection sites. After that, there was a higher persistence in samples collected from the ground surface of the limb in contrast with the ones from the aerial surface, which is very likely due to the difference in temperature. Even after 105 ADD, the sample from the separate limb's ground surface had a full profile. However, samples at 36 h failed to provide high molecular weight DNA, illustrating the challenges of taking samples from highly decomposed bodies.

4. Conclusions

We were able to obtain amplification from all amplicons up to 51 ADD. Samples collected from the ground surface of the limb had drop outs after 51 ADD, but gave complete profiles at 88 and 105 ADD. This could provide strategies for sample collection with bodies exposed to hot environments.

These results also indicate that unlike total body decomposition, DNA degradation is accelerated in warmer climates and similar ADDs do not represent similar DNA persistence in different climates. In colder climates, DNA persistence reach higher ADDs than the ones found in this experiment. Therefore guidelines for sample collection should be adapted according to local knowledge and climate.

In addition, field samples collected in this study were stored in 95% ethanol for up to two weeks and no degradation was seen in the control samples, demonstrating that ethanol is a simple and effective way of preserving tissue.

Conflict of interest

None.

Acknowledgments

References

[1] International Criminal Police Organization, 2014. Interpol Disaster Victim Identification Guide. Available at: http://www.interpol.int/Media/Files/INTERPOL-Expertise/DVI/DVI-Guide-new-version-2013.
[2] M.S. Nazir, S. Iyavoo, S. Alimat, et al., Development of a multiplex system to assess DNA persistence in taphonomic studies, Electrophoresis 34 (24) (2013) 3352–3360.
[3] T. Simmons, R.E. Adlam, C. Moffatt, Debugging decomposition data-comparative taphonomic studies and the influence of insects and carcass size on decomposition rate, J. Forensic Sci. 55 (January (1)) (2010) 8–13.

DNA persistence in soft tissue comparing vodka and absolute ethanol

Lais Vicente Baptista*, William Goodwin

School of Forensic and Applied Sciences, University of Central Lancashire - UCLAN, Preston, UK

ARTICLE INFO

ABSTRACT

Successful DNA analysis of human remains relies on the collection and preservation of biological material. Low temperatures are typically used to preserve soft tissues; however, this is not always available, for example, after mass disasters and conflict, where the infrastructure has been damaged. Lysis buffers and absolute ethanol have also been shown to be effective at preserving material, but again are not always readily available. This study assess the use of drinking alcohol as an alternative preservative solution for muscle tissue storage. Pig muscle was incubated for up to 42 days at different temperatures ($-20\,°C$, $25\,°C$, and $37\,°C$) with 95% ethanol, 37.5% ethanol, vodka (37.5%), and no preservative. Samples were collected weekly and analysis was based on agarose gel electrophoresis, DNA quantitation and amplification success of a multiplex with amplicons between 70 bp and 384 bp. Samples incubated with 37.5% ethanol and vodka had high molecular weight DNA and all samples incubated with preservative solutions generated complete profiles until the last collection point, while samples left untreated had drop-outs after 21 days at $25\,°C$ and $37\,°C$.

1. Introduction

In situations where human remains are fragmented, burnt and/or decomposed, DNA profiling is a valuable tool to contribute to identification; however, successful DNA analysis relies on the appropriate collection and preservation of biological material [1]. Inefficient preservation methods can lead to DNA degradation, which may prevent successful profiling.

Whilst low temperature, typically below $-15\,°C$, is widely used to preserve soft tissue samples, it is not available in all contexts, especially following mass disasters and conflict [2,3]. The DNA Commission of the International Society for Forensic Genetics says that storing soft muscle tissue samples in preservative solutions at room temperature can be an alternative to cold storage [4]. The INTERPOL Disaster Victim Identification Guide also recommends preserving soft tissue in ethanol [5].

Previous work has shown that absolute ethanol is effective at preserving DNA from fresh and partially degraded soft tissues [6,7]. This study intended to assess the use of drinking alcohol as an alternative preservative solution for muscle tissue storage. The rationale being that drinking alcohol is available in many contexts, whereas absolute ethanol is more restricted.

2. Materials and methods

Approximately 0.25 g of pig muscle was incubated in 1.5 ml tubes for up to 42 days at different temperatures ($-20\,°C$, $25\,°C$, and $37\,°C$) with 95% ethanol, 37.5% ethanol, vodka (37.5%), or no preservative. Samples were collected weekly and extraction started immediately; DNA extraction was performed using DNeasy* Blood and Tissue kit and quantification using Qubit. Samples were amplified and the PCR products were analysed using an ABI 3500. Analysis was based on agarose gel electrophoresis (AGE), DNA quantitation and amplification success of a multiplex with amplicons of 70 bp, 194 bp, 305 bp, and 384 bp [8].

3. Results

As expected, incubation at $-20\,°C$ generated clear HMW DNA bands in AGE for all samples up to 42 days of incubation. All samples stored at $25\,°C$ and $37\,°C$ displayed some degradation. Samples incubated with 37.5% ethanol or vodka still had HMW bands after 42 days, similar to samples incubated with 95% ethanol. Untreated samples did not present HMW after 14 days of incubation at $25\,°C$ and $37\,°C$ (data not shown).

236

Samples stored in 37.5% ethanol and vodka had higher DNA concentration than samples stored in absolute ethanol. Samples incubated at −20 °C resulted in the less variation in DNA concentration than other temperatures. Samples incubated at 37 °C and left untreated had a decreasing average in DNA concentration through the collection points (Fig. 1).

In all samples incubated at −20 °C, all alleles were amplified in all time points (Table 1). Samples incubated at 25 °C and 37 °C with any preserving agent also had complete amplification at all time points. Samples incubated untreated showed drop-outs after 14 days. After 21 days at 37 °C, there was a drop-out of the 384 bp allele in all untreated samples and after 35 and 42 days of incubation only the 70 bp allele was present.

4. Discussion

Storing samples in 95% ethanol and/or freezing are established procedures for preserving muscle tissue. In this experiment, these samples were used as controls to compare the efficiency of the tested solutions.

All the preservation solutions used improved DNA preservation. Vodka has been used before to preserve zebra liver over several days in the African bush and the DNA extracted amplified a 1.4 kb gene [9]. This is similar to results in this study where samples incubated for up to 42 days amplified fragments in the range of most STR kits.

5. Conclusion

The use of drinking alcohol or ethanol diluted to lower concentrations can be considered for preserving samples. In a situation where the stock of absolute ethanol is low or it is not available and it is also not possible to freeze samples, these alternatives can be considered in a way to preserve soft tissue samples within the timeframe studied.

Table 1
Amplification results. Samples were amplified with a multiplex with amplicons of 70 bp, 194 bp, 305 bp, and 384 bp.

Time	Preservation Agent	4 Plex amplification		
		−20 °C	25 °C	37 °C
Day 1	95% ethanol	+ + + +	+ + + +	+ + + +
	37.5% ethanol	+ + + +	+ + + +	+ + + +
	Vodka	+ + + +	+ + + +	+ + + +
	Untreated	+ + + +	+ + + +	+ + + +
Day 3	95% ethanol	+ + + +	+ + + +	+ + + +
	37.5% ethanol	+ + + +	+ + + +	+ + + +
	Vodka	+ + + +	+ + + +	+ + + +
	Untreated	+ + + +	+ + + +	+ + + +
Day 7	95% ethanol	+ + + +	+ + + +	+ + + +
	37.5% ethanol	+ + + +	+ + + +	+ + + +
	Vodka	+ + + +	+ + + +	+ + + +
	Untreated	+ + + +	+ + + +	+ + + +
Day 14	95% ethanol	+ + + +	+ + + +	+ + + +
	37.5% ethanol	+ + + +	+ + + +	+ + + +
	Vodka	+ + + +	+ + + +	+ + + +
	Untreated	+ + + +	+ + −	+ + + -
Day 21	95% ethanol	+ + + +	+ + + +	+ + + +
	37.5% ethanol	+ + + +	+ + + +	+ + + +
	Vodka	+ + + +	+ + + +	+ + + +
	Untreated	+ + + +	+ + −	+ − −
Day 35	95% ethanol	+ + + +	+ + + +	+ + + +
	37.5% ethanol	+ + + +	+ + + +	+ + + +
	Vodka	+ + + +	+ + + +	+ + + +
	Untreated	+ + + +	+ − −	+ − −
Day 42	95% ethanol	+ + + +	+ + + +	+ + + +
	37.5% ethanol	+ + + +	+ + + +	+ + + +
	Vodka	+ + + +	+ + + +	+ + + +
	Untreated	+ + + +	+ − −	+ − −

The "+" denotes the presence and "−" the absence of PCR amplicons in samples. Triplicates were grouped and the data presented are the result of at least two of the three samples.

References

[1] E.A.M. Graham, E.E. Turk, G.N. Rutty, Room temperature DNA preservation of soft tissue for rapid DNA extraction: an addition to the disaster victim identification investigators toolkit, Forensic Sci. Int.-Genet. 2 (2008) 29–34.

[2] B. Budowle, F.R. Bieber, A.J. Eisenberg, Forensic aspects of mass disasters: strategic considerations for DNA-basedhuman identification, Leg. Med. 7 (2005) 230–243.

[3] M. Hara, H. Nakanishi, K. Yoneyama, et al., Effects of storage conditions on forensic examinations of blood samples and bloodstains stored for 20 years, Leg. Med. 18 (2016) 81–84.

[4] M. Prinz, A. Carracedo, W.R. Mayr, et al., DNA Commission of the International Society for Forensic Genetics (ISFG): recommendations regarding the role of forensic genetics for disaster victim identification (DVI), Forensic Sci. Int.-Genet. 1 (2007) 3–12.

[5] International Criminal Police Organization, Interpol Disaster Victim Identification Guide, (2014).

[6] A. Allen-Hall, D. McNevin, Human tissue preservation for disaster victim identification (DVI) in tropical climates, Forensic Sci. Int.-Genet. 6 (2012) 653–657.

[7] M.S. Nazir, DNA Persistence and Preservation Following Environmental Insult, (2012).

[8] M.S. Nazir, S. Iyavoo, S. Alimat, et al., Development of a multiplex system to assess DNA persistence in taphonomic studies, Electrophoresis 34 (2013) 3352–3360.

[9] E.A. Oakenfull, Vodka, meths and dna, Trends Ecol. Evol. 9 (1994) 26.

10.4 Appendix 4

Appendix 4 is a list of presentations done during the course of the PhD.

10.4.1 Poster presentation at the 26th ISFG Conference

Event took place on August, 2015.

Abstract

DNA Persistence In Soft Tissues Exposed To Extreme Environments

After death DNA becomes progressively more fragmented as biological tissue degrades and this results in a decreasing ability to gain a complete STR profile. When extracting and profiling DNA from human remains understanding the likely persistence of DNA in different tissues is important. Studies in the UK have demonstrated that when using pigs as an experimental model, DNA up to 400 bp will persist for up to 3 weeks. However, it is well known that DNA degradation, especially in muscle tissue that has not become dehydrated, is dependent to a large degree on temperature. To assess DNA persistence in more extreme environmental conditions pig carcasses were exposed to the environment in Thailand during June for 1 week, with samples being collected every 12 h. In field conditions muscle DNA could not be amplified after 36 h exposure, unless the muscle was collected from tissue that was in contact with the ground; DNA persisted for 72 h in these samples.

DNA PERSISTANCE IN SOFT TISSUES EXPOSED TO EXTREME ENVIRONMENTS

Lais Vicente Baptista [a,b], Natnipoon Rattanarungruang [a], Panjai Woharndeec [c], William Goodwin [a]

[a] University of Central Lancashire – UCLan, UK ; [b] CAPES Foundation, Ministry of Education of Brazil;
[c] Central Institute of Forensic Science, Ministry of Justice of Thailand
lvbaptista@uclan.ac.uk

INTRODUCTION

For identification purposes, Interpol recommends the use of muscle tissue only in cases of non-decomposed remains (International Criminal Police Organization, 2014). What is the onset of decomposition is not specified, although most agencies use 24 h as approximation. Studies in the UK have demonstrated that when using pigs as an experimental model, DNA up to 400 bp will persist for up to 3 weeks, even in the summer (Nazir et al., 2013). However, it is well known that DNA degradation, especially in muscle tissue that has not become dehydrated, is dependent to a large degree on temperature.

In taphonomic studies, decomposition of tissues is regarded as a process that depends more on accumulated temperature rather than time alone. Accumulated Degree Days (ADD), the cumulative total of daily average temperatures, can be used as a quantitative measure to estimate post-mortem interval and to predict DNA degradation in soft tissues (Simmons et al., 2010).

OBJECTIVE

As it is known that DNA degradation is influenced by temperature, this experiment was done to assess DNA persistence in pig muscle tissue after exposure to high temperatures in the field.

MATERIALS AND METHODS

This experiment was carried out during June 2014 in Thailand. Whole pig carcasses (Sus scrofa) and separate limbs were placed in direct contact with the ground (Figure 1). Samples were collected at the moment of deposition, 12 h, 24 h, 36 h, 48 h, 60 h, and 72 hours later. Samples collected from the separate limbs were collected from both the surface exposed to the sun (aerial surface) and from the surface in contact with the ground (ground surface). After collection, samples were stored in a plastic tube with ethanol for about one week and frozen before extraction with the Qiagen Blood and Tissue Kit. The samples were profiled using a species specific multiplex (4-Plex) which simultaneously amplifies genomic DNA amplicons of 70 bp, 194 bp, 305 bp and 384 bp.

DNA extraction → Agarose gel electrophoresis → DNA quantification
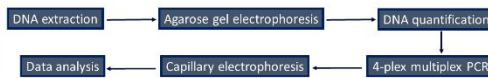Data analysis ← Capillary electrophoresis ← 4-plex multiplex PCR

Figure 1: Images of some of the pigs in the experiment at (a) the moment of deposition; (b and c) after 48 h of exposure; and (d) after 72 h of exposure.

RESULTS AND DISCUSSION

The average ambient temperatures during the three days of the experiment were 31 °C on the first day, 33.5 °C on the second day, and 35.5 °C on the third day. The average internal temperature of the carcasses was of 33 °C (maximum 35 °C), 31 °C (maximum 35 °C) and 35 °C (maximum 35.9 °C) respectively.

Degradation was visible in the agarose gel electrophoresis, with its characteristic smears appearing after 16 ADD (12 hours of incubation). Samples collected after 36 hours of incubation already did not show on the agarose gel, although DNA quantification showed the presence of DNA. The exception was from samples that were collected from the separate limbs' ground surface after 36, 60 and 72 hours of incubation (Figure 2).
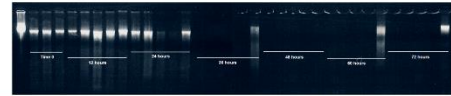
Figure 2: Agarose electrophoresis gels from deposition time to 72 hours. The three first samples in each set were collected from the carcass, followed by one collected from the surface of the separate limb exposed to the sun and one from the surface in contact with the ground.

Full profiles were obtained from all samples until 33.5 ADD (24 h). At 51 ADD (36 h), samples collected from the separate limbs showed drop-outs, while samples from the whole carcass still had full profiles (Tables 1 and 2). This could be due to a smaller body mass being more affected by insect activity and offering fewer collection sites for samples. After that, there was a higher persistence in samples collected from the ground surface of the limb in contrast with the ones collected from the aerial surface, which is very likely due to the difference in temperature. Even after 105 ADD (72 hours) of exposition, the sample collected from the separate limb's ground surface had a full profile (Figure 3). However, samples at 36 h failed to provide high molecular weight DNA, illustrating the challenges of taking samples from highly decomposed bodies.

Table 1: Results of the amplification of carcass samples.

Time	ADD	70 bp	194 bp	305 bp	384 bp
0 h	0	+++	+++	+++	+++
12 h	16	+++	+++	+++	+++
24 h	33.5	+++	+++	+++	+++
36 h	51	+++	+++	+++	+++
48 h	69.5	+++	---	---	---
60 h	88	+++	-+-	-+-	-+-
72 h	105	++-	++-	+--	---

Table 2: Results of the amplification of separate limb samples.

Time	ADD	70 bp		194 bp		305 bp		384 bp	
		A	G	A	G	A	G	A	G
0 h	0	NT	NT	NT	NT	NT	NT	NT	NT
12 h	16	+	+	+	+	+	+	+	+
24 h	33.5	+	+	+	+	+	+	+	+
36 h	51	+	+	-	-	-	-	-	-
48 h	69.5	+	-	-	-	-	+	-	-
60 h	88	(+)	+	(+)	+	(+)	+	(+)	+
72 h	105	-	+	-	+	-	+	-	+

NT = sample not taken. A = aerial surface. G = ground surface. The "+" denotes the presence and "-" the absence of PCR amplicons in samples during the 4-plex multiplex amplification; (+) represents positive results below threshold of 100 RFU.
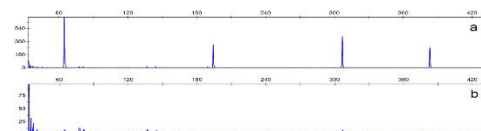
Figure 3: Electropherograms of samples with 105 ADD showing (a) samples collected from the ground surface of the separated limb and (b) samples collected from the aerial surface of the limb.

CONCLUSIONS

We were able to obtain amplification from all amplicons up to 51 ADD. Samples collected from the ground surface of the limb had drop outs after 51 ADD, but gave complete profiles at 88 and 105 ADD. This could provide strategies for sample collection with bodies exposed to hot environments.

These results also indicate that unlike total body decomposition, DNA degradation is accelerated in warmer climates and similar ADDs do not represent similar DNA persistence in different climates. In colder climates, DNA persistence reach higher ADDs than the ones found in this experiment. So guidelines for sample collection should be adapted according to local knowledge and climate.

In this study field samples were stored in 95% ethanol for up to two weeks and no degradation was seen in the control samples, demonstrating that ethanol is a simple and effective way of preserving tissue.

REFERENCES

INTERNATIONAL CRIMINAL POLICE ORGANIZATION, 2014. Interpol Disaster Victim Identification Guide. Available from: http://www.interpol.int/Media/Files/INTERPOL-Expertise/DVI/DVI-Guide-new-version-2013.
NAZIR, M.S., IYAVOO, S., ALIMAT, S., ZAHRA, N., SANQOOR, S.H., SMITH, J.A., MOFFATT, C. and GOODWIN, W., 2013. Development of a multiplex system to assess DNA persistence in taphonomic studies. Electrophoresis, vol. 34, no. 24, pp. 3352-3360 ISSN 1522-2683. DOI 10.1002/elps.201300240.
SIMMONS, T., ADLAM, R.E. and MOFFATT, C., 2010. Debugging Decomposition Data-Comparative Taphonomic Studies and the Influence of Insects and Carcass Size on Decomposition Rate. Journal of Forensic Sciences, JAN, vol. 55, no. 1, pp. 8-13 ISSN 0022-1198. DOI 10.1111/j.1556-4029.2009.01206.x.

10.4.2 Oral presentation at the Postgraduate Research Symposium of the Chartered Society of Forensic Sciences

Event took place on November, 2015.

Abstract

After death DNA becomes progressively more fragmented as tissue degrades resulting in a decreasing ability to gain a complete STR profile. When extracting and profiling DNA from human remains understanding the likely persistence of DNA in different tissues is important. Studies in the UK have demonstrated that when using pigs as an experimental model, DNA up to 400 bp will persist for up to 3 weeks. However, it is well known that DNA degradation is dependent to a large degree on temperature. To assess DNA persistence in more extreme environmental conditions pig carcasses were exposed to the environment in Thailand in summer with samples being collected every 12 h – muscle tissue was only present for three days. Extracted DNA could not be amplified after 36 h exposure unless the muscle was collected from tissue that was in contact with the ground; DNA persisted for 72 h in these samples.

Presentation (partial)

10.4.3 Poster presentation at the 27th ISFG Conference

Event took place on August, 2017.

Abstract

DNA Persistence In Soft Tissue Using Vodka As Preservative Agent (P)

In some crime scenes or following mass fatality incidents, human remains can be fragmented, burnt and/or decomposed, making victim identification by means of fingerprinting or odontology extremely difficult. In such situations DNA profiling is desirable; however, successful DNA analysis relies on the appropriate collection and preservation of biological material. Inefficient preservation methods can cause destruction of intact DNA to such an extent that data is not always available for victim identification. Biological samples have been successfully preserved using a number of physical and chemical treatments, adjusting temperature, ambient pH and salt concentrations. This study intended to assess the use of drinking alcohol as an alternative preservative solution for muscle tissue storage, comparing it with other preservative solutions. Pig muscle was incubated for up to 42 days at different temperatures (-20 °C, 25 °C, and 37 °C) with different preservative solutions: 95% ethanol, 37.5% ethanol, vodka (37.5%), and no preservative. Samples were collected weekly and analysis was based on DNA quantitation and amplification success of an in-house multiplex with amplicons between 70 bp and 384 bp. Samples incubated with 37.5% ethanol and vodka had high molecular weight DNA. All samples incubated with some kind of preservative solution generated complete profiles until the last collection point, while samples left untreated had drop-outs after 21 days of incubation at 25 °C and 37 °C.

DNA Persistence in Soft Tissue Comparing Drinking and Absolute Ethanol

170382

Lais Vicente Baptista, William Goodwin

School of Forensic and Applied Sciences, University of Central Lancashire - UCLAN, Preston, UK
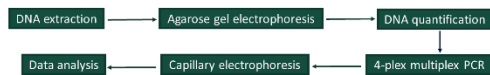
LVBaptista@uclan.ac.uk

INTRODUCTION

In situations where human remains are fragmented, burnt and/or decomposed, DNA profiling is a valuable tool to contribute to identification; however, successful DNA analysis relies on the appropriate collection and preservation of biological material [1]. Inefficient preservation methods can lead to DNA degradation, which may prevent successful profiling.

Whilst low temperature, typically below -15 °C, is widely used to preserve DNA in soft tissue, it is not available in all contexts, especially following mass disasters and conflict [2,3]. The DNA Commission of the International Society for Forensic Genetics advises that storing soft muscle tissue samples in preservative solutions at room temperature can be an alternative to cold storage [4]. The INTERPOL Disaster Victim Identification Guide also recommends preserving soft tissue in ethanol as an option [5].

Previous work has shown that absolute ethanol is effective at preserving DNA from fresh and partially degraded soft tissues [6, 7]. This study intended to assess the use of drinking alcohol as an alternative preservative solution for muscle tissue storage.

MATERIALS AND METHODS

Store-bought pig muscle was placed in a 1.5 mL tube and incubated at different temperatures (-20 °C, 25 °C, and 37 °C) with 95% ethanol, 37.5% ethanol, vodka (37.5%), or no preservative. Samples were collected after 1 day, 3 days, 7 days, 14 days, 21 days, 35 days, and 42 days and frozen until extraction. DNA extraction was performed using DNeasy® Blood and Tissue kit (Qiagen Ltd, UK) and quantification using Qubit (Thermo Fischer, UK). Samples were amplified using a species specific multiplex (4-Plex) which simultaneously amplified genomic amplicons of 70 bp, 194 bp, 305 bp and 384 bp [8]; PCR products were analysed using an ABI 3500. Analysis was based on agarose gel electrophoresis (AGE), DNA quantitation and amplification success of the 4-Plex multiplex.

DNA extraction → Agarose gel electrophoresis → DNA quantification

Data analysis ← Capillary electrophoresis ← 4-plex multiplex PCR

RESULTS

As expected, incubation at -20 °C generated clear HMW DNA bands in AGE for all samples up to 42 days of incubation. All other samples stored at 25 °C and 37 °C displayed some degradation. Samples incubated with 37.5% ethanol or vodka still had HMW bands after 42 days, similar to samples incubated with 95% ethanol. Untreated samples did not present HMW after 14 days of incubation at 25 °C and 37 °C (Figure 1).

Samples stored in 37.5% ethanol and vodka had better DNA concentration than samples stored in absolute ethanol. Samples incubated at 37 °C and left untreated had a decreasing average in DNA concentration through the collection points (Figure 2).
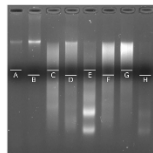
Figure 1 Electrophoresis results of samples incubated for (A) 1 day at -20 °C with 95% ethanol; the other samples were incubated for 42 days (b) at -20 °C untreated; (c) at 25 °C with 37.5% ethanol; (d) at 25 °C with vodka; (e) at 25 °C untreated; (f) at 37 °C with 37.5% ethanol; (g) at 37 °C with vodka; (h) at 37 °C untreated.
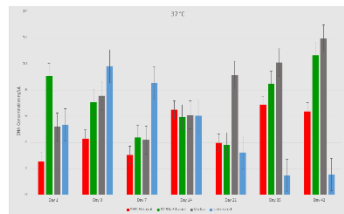
Figure 2 Quantification results of samples incubated at 37 °C. Each bar represents the average of the triplicate.

In all samples incubated at -20 °C, all alleles were amplified in all time points (Table 1). Samples incubated with any preserving agent had complete amplification at all time points, even for incubation at 25 °C and 37 °C. Samples incubated untreated showed drop-outs after 14 days. After 21 days at 37 °C, there was a drop-out of the 384 bp allele in all untreated samples and after 35 and 42 days of incubation only the 70 bp allele was present (Figure 3).

Table 1 Amplification results of a multiplex with amplicons of 70 bp, 194 bp, 305 bp, and 384 bp.

Time	Preservation Agent	4 Plex amplification		
		-20 °C	25 °C	37 °C
Day 1	95% ethanol	++++	++++	++++
	37.5% ethanol	++++	++++	++++
	Vodka	++++	++++	++++
	Untreated	++++	++++	++++
Day 3	95% ethanol	++++	++++	++++
	37.5% ethanol	++++	++++	++++
	Vodka	++++	++++	++++
	Untreated	++++	++++	++++
Day 7	95% ethanol	++++	++++	++++
	37.5% ethanol	++++	++++	++++
	Vodka	++++	++++	++++
	Untreated	++++	++++	++++
Day 14	95% ethanol	++++	++++	++++
	37.5% ethanol	++++	++++	++++
	Vodka	++++	++++	++++
	Untreated	++++	++--	+++-
Day 21	95% ethanol	++++	++++	++++
	37.5% ethanol	++++	++++	++++
	Vodka	++++	++++	++++
	Untreated	++++	++--	+---
Day 35	95% ethanol	++++	++++	++++
	37.5% ethanol	++++	++++	++++
	Vodka	++++	++++	++++
	Untreated	++++	+---	+---
Day 42	95% ethanol	++++	++++	++++
	37.5% ethanol	++++	++++	++++
	Vodka	++++	++++	++++
	Untreated	++++	+---	+---

The "+" denotes the presence and "-" the absence of PCR amplicons in samples. Data presented are the result of at least two of the three samples.

Figure 3 Examples of electropherograms. (a) is a sample incubated for 1 day at -20 °C with 95% ethanol; the other samples were incubated for 42 days (b) at -20 °C untreated; (c) at 25 °C with 37.5% ethanol; (d) at 25 °C with vodka; (e) at 25 °C untreated; (f) at 37 °C with 37.5% ethanol; (g) at 37 °C with vodka; (h) at 37 °C untreated.

DISCUSSION

Storing samples in 95% ethanol and/or freezing them is the recommended procedure for muscle tissue. In this experiment, these samples were used as a control to compare the efficiency of the tested solutions and samples left untreated were used as the other control.

Overall, having any preserving solution improved DNA persistence. Vodka has been used before to preserve zebra liver over several days in the African bush and the DNA extracted amplified a 1.4 kb gene [9]. This is similar to results in this study where samples incubated for up to 42 days amplified fragments in the range of most STR kits. Despite recommendations to the use of more concentrated ethanol, results from samples preserved with vodka and 37.5% ethanol were similar to results from samples using absolute ethanol as a preservative.

CONCLUSION

The use of drinking alcohol or ethanol diluted to lower concentrations can be considered for preserving samples. In a situation where the stock of absolute ethanol is low or it is not available and it is also not possible to freeze samples, these alternatives can be considered in a way to preserve soft tissue samples within the timeframe studied.

ACKNOWLEDGMENTS

REFERENCES

[1] E.A.M. Graham, S.E. Turk, G.N. Rutty, Room temperature DNA preservation of soft tissue for rapid DNA extraction: An addition to the disaster victim identification investigators toolkit? Forensic Science International-Genetics. 2 (2008) 29-34.
[2] B. Budowle, F.R. Bieber, A.J. Eisenberg, Forensic aspects of mass disasters: Strategic considerations for DNA-based human identification, Leg.Med. 7 (2005) 230-243.
[3] M. Hara, H. Nakanishi, K. Yoneyama, K. Saito, A. Takada, Effects of storage conditions on forensic examinations of blood samples and bloodstains stored for 20 years, Leg.Med. 18 (2016) 81-84.
[4] M. Prinz, A. Carracedo, W.R. Mayr, N. Morling, T.J. Parsons, A. Sajantila, et al., DNA Commission of the International Society for Forensic Genetics (ISFG): Recommendations regarding the role of forensic genetics for disaster victim identification (DVI), Forensic Sci.Int.-Genet. 1 (2007) 3-12.
[5] International Criminal Police Organization, Interpol Disaster Victim Identification Guide, (2014).
[6] A. Allen-Hall, D. McNevin, Human tissue preservation for disaster victim identification (DVI) in tropical climates, Forensic Sci.Int.-Genet. 6 (2012) 653-657.
[7] M.S. Nazir, DNA Persistence and Preservation Following Environmental Insult, (2012).
[8] M.S. Nazir, S. Iyavoo, S. Alimat, N. Zahra, S.H. Sanqoor, J.A. Smith, et al., Development of a multiplex system to assess DNA persistence in taphonomic studies, Electrophoresis. 34 (2013) 3352-3360.
[9] E.A. Oakenfull, Vodka, Meths and Dna, Trends Ecol.Evol. 9 (1994) 26-26.

10.5 Appendix 5

Award obtained during the course of the PhD.

INTERNATIONAL SOCIETY FOR FORENSIC GENETICS

Lais Baptista

has been awarded a Travel Bursary
to attend the 27th Congress of the
International Society for Forensic Genetics
in Seoul, Republic of Korea

The President
(Walther Parson)

The Secretary
(Peter M. Schneider)

Seoul, 29th August 2017