Process Improvements for the Extraction of DNA from Skeletonized Human Remains

by

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TABLE OF CONTENTS

ABSTRACT

The extraction of DNA from skeletonized human remains is a challenging issue faced by laboratories world-wide. When confronted with large sets of commingled remains, or even single sets of skeletonized remains, forensic laboratories must make a determination which skeletal element will provide the best opportunity for the recovery of high quality DNA. This thesis outlines the difficulties surrounding the extraction of DNA from skeletonized human remains and provides guidance for both sampling of osseous materials as well as a novel technique by which practitioners may identify PCR-inhibiting materials co-extracting with DNA.

In the first half of the thesis, a large set of skeletal elements was surveyed for success. The remains were recovered from world-wide locations by the Defense POW/MIA Accounting Agency (DPAA) and submitted to the Armed Forces Medical Examine System - Armed Forces DNA Identification Laboratory (AFMES-AFIL) for DNA testing. Samples recovered range from those lost during the Vietnam Conflict (1955-1975), Korean War (1950-1953); and the United States involvement in World War II (1941-1945). Elements surveyed had DNA extracted using four different extraction protocols, two of which involve an organic purification of the DNA removed from the remains. The DNA was tested using five different strategies: Sanger sequencing of mitochondrial DNA (mtDNA); a modified AmpFlSTR® Yfiler™ procotocol; AmpFlSTR® MiniFiler™; PowerPlex® Fusion; and Next Generation Sequencing (NGS).

The goal in the first few chapters was to provide a framework for practitioners to make decisions about both the osseous sampling strategy and the associated DNA testing strategy. By examining a large set of remains gathered from a myriad of conditions, recommendations

can be made that are applicable in a variety of circumstances. In general, a DNA extraction protocol involving the complete demineralization of the skeletal material coupled with an organic purification is optimal for skeletal remains that are chemically compromised or contain a high level of fats. This is also the preferred extraction technique when testing with Sanger sequencing of mtDNA.

For the second half of the thesis, a novel technique is presented for the analysis of skeletal material and the associated DNA. A gas chromatography / mass spectrometry (GC/MS) protocol was developed to ascertain the compounds present in recovered skeletal materials and the associated DNA. The protocol involves the use of a solvent extraction (acetonitrile and dichloromethane) of osseous detritus removed from the surface of a bone during the standard cleaning prior to pulverization for DNA extraction. The material removed is typically discarded as medical waste, but in this new protocol, it provides an insight both into the person from which the skeletal materials originated and the environment in which decomposition occurred.

By comparing the GC/MS profiles generated from the skeletal materials to that of the extracted DNA, it was determined that there is little to no carry-over of compounds from the osseous element to the DNA. The results indicate that the extraction protocols currently in use are highly effective at removing any chemical compounds endogenous to the remains, as carry-over was seen in less than 0.1% of the samples tested.

The outcome of this work is a framework practitioners may use to evaluate groups of skeletonized remains for the osseous element that will provide the best results for the testing strategy being employed. The GC/MS testing performed would seem to indicate that the extraction protocols currently in use are effective at the removal of potential PCR inhibitors

and other endogenous materials. This result should encourage practitioners to revisit DNA extraction protocols to perhaps reduce the stringency of available extraction techniques thereby reducing DNA loss while still maintaining sample purity.

DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Suni M. Edson

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The author received support in the form of a salary from the American Registry of Pathology, LLC until 30 November 2018. At that point, SNA International took charge of the contract for AFMES-AFDIL. The author continued to receive support in the form of a salary.

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DATA ARCHIVING

The data generated during the course of this study has associated casework information from the Armed Forces Medical Examiner System – Armed Forces DNA Identification Laboratory (AFMES-AFDIL). Associated data therefore cannot be released to the public. It will be stored indefinitely on the managed servers of AFMES-AFDIL, located on Dover Air Force Base, Dover, Delaware, US.

ACKNOWLEDGEMENTS

This thesis took a long time to get started. In the winter of 2004, I was sitting with three friends in an Applebee's in Taipei. We were discussing the usefulness of a PhD in the field of forensics. We had just spent the better part of a week touring the country and giving lectures on different aspects of forensic DNA analysis, and I was the only one of the foreign speakers who did not have a PhD. Almost ten years to the day, the setting was another group of colleagues at a beach bar in Honolulu. One of them pronounced to the group, "If you wake up every day wanting to do a particular thing, you should probably go and do it." Six months later, I had applied to Flinders and started collecting samples for analysis.

There are a lot of people who made it possible to perform this research. Firstly, I need to thank my advisor, Adrian Linacre. I deeply appreciate his willingness to take me on as a student and his encouragement. This thesis would not have been completed without his support and his willingness to provide feedback and critical commentary.

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Completion of this thesis involved a lot of wrangling and convincing people it would work. All of those people above believed in me and the research, but thanks should also go to those people who thought it was an impractical idea and would never work. No surer way to get me to do something than to tell me I can't do it.

Lastly, this is for the KB who were there at the start, and Jack is here at the end.

LIST OF FIGURES

generated by the fuel oils. The peak indicated by the arrow is a form of cholestan, a cholesterol derivative.

Figure 6.5. The trace image generated by the GC/MS analysis of sample 5-7 subjected to an acetonitrile extraction and SM#6. The parent osseous sample was recovered from the Tarawa Atoll. The signal noise past 12 minutes is indicative of the solvent front and no detectable materials. All other peaks present are indicative of biological materials that are by-products of human decomposition.

Figure 6.6. The trace image generated by the GC/MS analysis of sample 3-14 subjected to a dichloromethane extraction and SM#8. The peak indicated by the arrow is sulfameter, which is a long acting sulfonamide used to treat infections. All other peaks are by-products of human decomposition or siloxanes.

Figures 6.7a and 6.7b. The trace images generated by GC/MS analysis of two samples subjected to a dichloromethane extraction and SM#9. Sample 2-1 (Figure 6.7a) and Sample 2-5 (Figure 6.7b) were ostensibly recovered from the same location in Laos, and potentially the same individual.

Figure 6.8a. The trace images generated by GC/MS analysis of DNA from osseous sample 3-7. The extracted DNA was diluted with methanol and injected onto the instrument DNA#5. There is a cluster of sugars between 22 and 24 minutes, indicated by the square. The peak indicated by the arrow is phenol, most likely a carry-over from the extraction.

Figure 6.8b. The trace image generated by GC/MS analysis of sample 3-7 subjected to a dichloromethane extraction and SM#9. The series of dominant series of peaks is fuel oil and fats. There is some carry-over of these materials to the DNA.

trace are phenolics derived from either the fuel or putrefaction. Figure 1b is the DNA extracted from that bone sample.

Figure 8.2. An example of phenol carry-over. This the GC/MS trace of a DNA sample extracted from a temporal bone recovered from the USS Oklahoma (Sample 3-2 – See Supplemental Table 1). The two marked peaks are two different types of phenol. The peak at the beginning of the trace that is undefined, was not able to be scored by the instrument and was not called; however, in the corresponding osseous samples, it has been shown to be phenol.

Figure 8.3. Low-level carry-over of fatty acids in a DNA trace extracted using an organic purification. For reference, this is sample 3-40, extracted from a sample recovered from the USS Oklahoma. It is very similar to Figure 2, and indeed the peaks are mostly of the same origin.

Figure 8.4. An example of carry-over from the osseous sample into the DNA extract. This DNA was extracted using an inorganic purification from a cranial fragment recovered from Southeast Asia (sample 2-8).

Figure 8.5. An example of a trace with peaks that are too low in signal strength to be called accurately. This sample is a DNA extract recovered with an inorganic purification from a World War II era case in the Solomon Islands (Sample 1-9). The peaks present do not have a ChemStation station score higher than 12.

Figure 8.6. A comparison between DNA samples extracted by a novice extractor on their first day of extraction and an extractor with over 17 years of experience. Figure 8.6a is a trace of one of four samples extracted by a novice extractor (Sample 7-29). This sample was from the USS Oklahoma and was purified using an organic purification. There is almost no evidence of Tris, and the solvent front is very high. Figure 8.6b is from the same incident but extracted by a more experienced analyst (Sample 4-33). This trace exhibits a high peak of tris at the beginning of

LIST OF TABLES

in mtDNA Sanger sequencing. The low success rate in the fragment category is explained by the non-human nature of approximately 38% of the samples tested.

Table 2.5 Overall success of enhanced AmpFlSTR® Yfiler™ testing for all skeletal samples submitted to AFMES-AFDIL from DPAA between 1990 and 2018. Samples are listed alphabetically. The maxilla and mandible are combined together to form the category of "Jaw". The "Pelvis" category is primarily the os coxa, but also includes ilium, ischium, sacrum, and ramus.

The average number of loci has been rounded to the closest whole number.

There are a limited number of samples tested in any STR platform in the "Extraction Protocol #1". This extraction protocol ceased to be actively used in 2007, just prior to the implementation of STR protocols into active casework.

Table 2.6 Overall success of AmpFlSTR® MiniFiler™ testing for all skeletal samples submitted to AFMES-AFDIL from DPAA between 1990 and 2018. Samples are listed alphabetically. The maxilla and mandible are combined together to form the category of "Jaw". The "Pelvis" category is primarily the os coxa, but also includes ilium, ischium, sacrum, and ramus.

The average number of loci has been rounded to the closest whole number.

Table 2.7 Overall success of PowerPlex® Fusion testing for all skeletal samples submitted to AFMES-AFDIL from DPAA between 1990 and 2018. Samples are listed alphabetically. The maxilla and mandible are combined together to form the category of "Jaw". The "Pelvis" category is primarily the os coxa, but also includes ilium, ischium, sacrum, and ramus.

The average number of loci has been rounded to the closest whole number.

There are a limited number of samples tested in any STR platform in the "Extraction Protocol #1". This extraction protocol ceased to be actively used in 2007, just prior to the implementation of STR protocols into active casework.

Table 2.8 Overall success of Next Generation Sequencing testing for all skeletal samples submitted to AFMES-AFDIL from DPAA between 1990 and 2018. Samples are listed alphabetically. The maxilla and mandible are combined together to form the category of "Jaw". The "Pelvis" category is primarily the os coxa, but also includes ilium, ischium, sacrum, and ramus.

NGS testing involves multiple different extraction protocols. Results are combined from different strategies including EP#2, EP#3, EP#4, and a modified EP#2.

NGS testing produces sequence information for the entire mitochondrial DNA genome; however, only the control region data are reported. All of the samples currently tested in NGS at AFMES-AFDIL are compromised in some fashion. Primarily this is chemical in nature, but can include burning or extreme degradation.

Table 3.1. Cranial elements and teeth tested in Sanger sequencing of Mitochondrial DNA. The "General Crania" category includes samples that were not specified as being a specific cranial element during submission to the DNA testing laboratory. These samples are typically from remains that have been fragmented to such a degree as the anthropologists are only able to determine that the sample originated from the cranium. The total number of teeth may be greater than the sum of the individual teeth as there were some teeth that were not specifically labeled other than "mandible" or "maxillary".

Table 3.2. Cranial elements and tooth samples tested in AmpFlSTR® MiniFiler™. The average number of loci has been rounded to the nearest whole number.

The "General Crania" category includes samples that were not specified as being a specific cranial element during submission to the DNA testing laboratory. These samples are typically from remains that have been fragmented to such a degree as the anthropologists are only able to determine that the sample originated from the cranium. The total number of teeth may be greater than the sum of the individual teeth as there were some teeth that were not specifically labeled other than "mandible" or "maxillary".

Table 3.3. Cranial samples tested in Modified AmpFlSTR® Yfiler™. The average number of loci has been rounded to the nearest whole number.

The "General Crania" category includes samples that were not specified as being a specific cranial element during submission to the DNA testing laboratory. These samples are typically from remains that have been fragmented to such a degree as the anthropologists are only able to determine that the sample originated from the cranium. The total number of teeth may be greater than the sum of the individual teeth as there were some teeth that were not specifically labeled other than "mandible" or "maxillary".

Table 3.4. Cranial elements and tooth samples tested in PowerPlex® Fusion. The average number of loci has been rounded to the nearest whole number.

The "General Crania" category includes samples that were not specified as being a specific cranial element during submission to the DNA testing laboratory. These samples are typically from remains that have been fragmented to such a degree as the anthropologists are only able to determine that the sample originated from the cranium.

Table 3.5. Cranial samples tested in NGS. The average number of bases has been rounded to the nearest whole number.

The "General Crania" category includes samples that were not specified as being a specific cranial element during submission to the DNA testing laboratory. These samples are typically from remains that have been fragmented to such a degree as the anthropologists are only able to determine that the sample originated from the cranium.

Table 3.6. The impact of samples recovered from the USS *Oklahoma*. The USS *Oklahoma* was torpedoed during an attack on the naval base in Pearl Harbor, Hawaii on 7 December 1941. The ship rolled, trapping over 400 U.S. sailors and Marines within the body of the ship. The remains were recovered from the body of the ship in 1945. Initial attempts at identification were made in the 1950's, but the remains were reburied in the National Memorial Cemetery of the Pacific in Hawaii. DNA testing was first attempted in 2003, and modern efforts commenced in 2015. Samples recovered from the USS *Oklahoma* have an elevated rate of success across all samples of 98% in mtDNA Sanger Sequencing. The table shows the most commonly sampled cranial elements extracted in EP#2 and tested in mtDNA Sanger Sequencing, AmpFlSTR® MiniFiler™, and Modified AmpFlSTR® Yfiler™. Frontal and parietal were not tested for the USS *Oklahoma* and are therefore not included.

Table 3.7. Examples of extraction protocols used for skeletal remains. Protocols are arranged by amount of skeletal material used. There is a lack of consistency across the field with regards to protocols for the extraction of DNA from skeletal remains. Each laboratory has an optimal strategy based on their internal validations. Despite the differences, there is some agreement among laboratories for which skeletal elements might be considered choice for DNA analysis.

presumed species based upon anthropological analysis. Animals noted with "[ ]" identify the revised determinations made by faunal experts in cases of discordance between the 12S and DPAA species. The geographic origin is the location in which the specimens were recovered.

*As of 2001, the genus for *Lama pacos* (Alpaca) was changed to *Vicugna* based on genetic evidence that supported the hypothesis that the Alpaca is derived from the Vicuña not the Guanaco (Kadwell et al. 2001). However, the database hits that were 100% homologous with the Sample 32 sequence at the time of the search were noted as *Lama pacos* in the NCBI Nucleotide database even though they were added in 2006. Species of the *Vicugna* and *Lama* genera are closely related and the common non-human taxon, family Camelidae, would be reported.

Table 4.3 The 12S rRNA testing results for each type of skeletal element, and the average weight of each type of element. "Long Bones" and "Bone Fragments" are listed independently of each other as a type of element as the former implies there was enough of the osseous material present to determine element was a long bone, while the latter is a non-specific catch-all for small fragments.

Table 4.4. Summary of animals detected in the 12S rRNA testing and the conflict of origin. The original species designations are indicated and were not adjusted with more recent searches. Oftentimes, the country from which the remains were recovered will give clues as to the animal, even if the 12S results are more general. Some of the results seem unlikely (e.g., the Common House Gecko); however, the sequence was duplicated through either extraction or amplification and confirmed prior to searching in BLAST and being reported. Remains from Southeast Asia were typically recovered from Vietnam, Laos, or Cambodia; those recovered from the Korean War were from the Korean peninsula; and those from World War II were from world-wide locations (e.g., Tarawa Atoll, Germany, Papua New Guinea).

references is a more typical workflow. The "# Reported" is the number of samples meeting the criteria for reporting as defined in the Methods and Materials.

Table 5.5. A comparison of Sanger mitochondrial DNA testing of samples recovered from the USS *Oklahoma*, the Battle of Tarawa, and the Cabanatuan Prison Camps. The only extraction protocol represented is demineralization plus organic purification (EP#2). The "Pelvis" category is largely represented by os coxa, but also includes ilia and ischia. The "Jaw" category includes both the maxilla and the mandible. The "# Reported" is the number of samples meeting the criteria for reporting as defined in the Methods and Materials.

Table 5.6. Mitochondrial DNA testing results from remains recovered from burials at the Cabanatuan Prison Camp and interred at the Manila American War Cemetery. Combined testing indicates that samples were tested under at least two different extraction methods and combined to generate data. Success of the different testing protocols vary widely. The "# Reported" is the number of samples meeting the criteria for reporting as defined in the Methods and Materials.

Table 5.7. NGS testing results. Extraction protocols are not listed for this type of testing as no single protocol was used. There is a published protocol [23]; however, initial testing was performed using a combination of EP#2 and EP#3. The average number of bases is not listed for NGS processing. As this is whole genome sequencing, it is generally 16506bp or zero. The "# Reported" is the number of samples meeting the criteria for reporting as defined in the Methods and Materials. While it may appear that Cabanatuan Prison Camp samples processed by NGS do not work particularly well, it should be noted that these samples were previously reported as inconclusive in Sanger sequencing and were retested using Next Generation Sequencing. Without this protocol in hand those twenty samples would have remained as inconclusive. The low success rate of the Battle of Tarawa samples may be due to an overabundance of DNA being present in the extract.

blue. This is a summary of compounds recovered; therefore, there is some duplication of compounds between the solvents, although this is minimal. In total, 288 compounds were detected. Compounds are listed alphabetically.

Table 7.6. Compounds recovered from skeletal elements sampled from all World War II incidents with the exception of the USS *Oklahoma*. This includes samples that were recovered the Solomon Islands, the Battle of Tarawa, battle locations in the Philippines, Italy, Yugoslavia, and Papua New Guinea. Materials developed from acetonitrile eluates are marked in blue. This is a summary of compounds recovered; therefore, there is some duplication of compounds between the solvents, although this is minimal. In total, 267 compounds were detected. Compounds are listed alphabetically.

Table 8.1. The volumes of DNA and methanol used for GC/MS analysis of DNA extracts. Glass autosampler inserts were used for all of the runs described in this paper; however, the initial proof of concept paper did not use these.

Table 8.2. Compounds detected in organically extracted DNA samples. Compounds with a ChemStation score of less than 20 are not included. Most scores were in the 80's and 90's. The number of injections where the compound was seen is included in the final column. 236 individual DNA extracts generated from osseous materials using an organic purification method were tested. Compounds marked with a "*" were seen in both types of DNA extracts.

Table 8.3. Compounds detected in inorganically extracted DNA samples. Compounds with a ChemStation score of less than 20 are not included. Most scores were in the 80's and 90's. The number of injections where the compound was seen is included in the final column. Compounds marked with a "*" were seen in both types of DNA extracts.

Supplemental Table 8.1. List of DNA Samples Tested. Samples recovered from the same conflict and location are not necessarily from the same incident. The 'location' designator is mostly the region or country from which the remains were recovered, unless the incident was large enough to warrant a specific designator. "Korea" refers to the Korean War and "Southeast Asia" refers to the conflict in Southeast Asia, known colloquially as the "Vietnam War".

If there are two listings for a sample, the sample was loaded twice to verify results from a previous injection. For the duplicated entries, if the volumes of DNA and methanol do not change, the same fraction was re-injected.

Table 9.1. The chemical compounds detected four osseous samples recovered from the USS *Oklahoma*. The samples are the same as those represented in Figures 9.2-9.5. The compounds are arranged in alphabetical order.

Table 10.1. A summary of the Sanger sequencing of mtDNA success for individual skeletal elements tested at AFMES-AFDIL between 1990 and 2016. Only the success percentage of those samples extracted using demineralization coupled with an organic purification is contained in the table. Samples are listed alphabetically.

Table 10.2. The success of individual skeletal elements tested in AmpFlSTR® MiniFiler™. Samples were submitted to AFMES-AFDIL between 1990 and 2018 and are included in the table alphabetically. Extraction Protocol #2 is demineralization coupled with an organic purification. Extraction Protocol #3 is a demineralization coupled with an inorganic purification.

Table 10.3. A summary of mitochondrial DNA Sanger sequencing from a selection of samples recovered from the USS Oklahoma. The data here demonstrate the sampling bias that is inherent in the testing of the samples.

Table A.1. Summary of testing done at AFDIL from 1992 until the spring of 2016. MtDNA Sanger Sequencing testing is of the hypervariable regions I and II (HVI and HVII) of the Control Region. The target to be considered successful is 100bp or more of DNA amplified in duplicate and confirmed to be consistent by two independent analyses. Identifiiler (AmpFLSTR® Identifiler™: Thermo Fisher Scientific), MiniFiler (AmpFLSTR® MiniFiler™ PCR Amplification Kit: Thermo Fisher Scientific), and PowerPlex® Fusion (Promega Corporation, Madison, WI) reactions are unmodified from the manufacturers' recommendations. YFiler (AmpFLSTR® Yfiler®: Thermo Fisher Scientific) is a combination of low copy number (LCN) testing and unmodified. All STR testing platforms are considered 'successful' with the reporting of 4 or more loci that are confirmed through duplicate amplifications. Not all kits and protocols used at AFDIL are included in this table.

LIST OF ABBREVIATIONS

AFDIL	Armed Forces DNA Identification Laboratory
AAFS	American Academy of Forensic Sciences
AFB	Air Force Base
AFMES-AFDIL	Armed Forces Medical Examiner – Armed Forces DNA Identification Laboratory
AFMES	Armed Forces Medical Examiner
AGRS	American Graves Registry Service
aka or a.k.a.	Also known as
ANZFSS	Australia New Zealand Forensic Sciences Symposium
ARP	American Registry of Pathology
AUD	Australian Dollar
auSTR	Autosomal Short Tandem Repeat
bp	Base pair
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
CIL	Central Identification Laboratory
CILHI	Central Identification Laboratory - Hawaii
CIU	Central Identification Unit
DAPCI	Desorption Atmospheric Pressure Chemical Ionization
DART	Direct Analysis in Real Time
DDT	Dichlorodiphenyltrichloroethane (a pesticide)
DESI	Desorption Electrospray Ionization
DiCM	Dichloromethane
DNA	Deoxyribonucleic Acid
dNTPs	Dinucleotide Triphosphates
DoD	Department of Defense
DPRK	Democratic People's Republic of Korea (aka, North Korea)
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
DPAA	Defense POW/MIA Accounting Agency (fka JPAC)
EP#1	Extraction Protocol #1
EP#2	Extraction Protocol #2 – aka Demin1
EP#3	Extraction Protocol #3 – aka Demin2
EP#4	Extraction Protocol #4 – A DNA extraction protocol designed specifically for use with NGS/MPS processing
EtBr	Ethidium Bromide
EtOH	Ethanol
FBI	Federal Bureau of Investigation
FSI	Forensic Science International
FSMP	Forensic Science, Medicine and Pathology (Journal)
fka or f.k.a.	Formerly known as
g	Gram
GC/MS	Gas Chromatography / Mass Spectrometry
HID	Human Identification
HPLC	High-performance Liquid Chromatography
HVI and HVII	Hypervariable Regions One and Two
IBM	Information Business Management
IRB	Internal Review Board
ISFG	International Society of Forensic Genetics
IUCN	International Union for Conservation of Nature
JFS	Journal of Forensic Sciences
JPAC-CIL	Joint POW/MIA Accounting Command – Central Identification Laboratory
JRO	Joint Recovery Operation

K208	Korea 208 – Refers to the set of remains returned to the US from North Korea between 1990 and 1992.
LAESI	Laser Ablation with Electrospray Ionization
LC/MS	Liquid Chromatography / Mass Spectrometry
LC/MS-MS	Liquid Chromatography / Mass Spectrometry – Mass Spectrometry
M	Molar
MALDESI	Matrix-Assisted Laser Desorption Electrospray Ionization
MeOH	Methanol
mg	Milligram
mL	Milliliter
mM	Millimolar
MNI	Minimum Number of Individuals
MPS	Massively Parallel Sequencing (aka NGS)
MS	Mass Spectrometry
mtDNA	Mitochondrial DNA
NCBI	National Center of Biotechnology Information
NCIC	National Crime Information Center
ng	Nanogram
NGS	Next Generation Sequencing (aka MPS)
NIJ	National Institute of Justice
NIST	National Institutes of Standards and Technology
NMCP	National Memorial Cemetery of the Pacific
NMS Labs	National Medical Services Labs
OMB	Office of Management and Budget
PAS	Past Accounting Section
PCIA	Phenol: Chloroform: Isoamyl Alcohol
PCR	Polymerase Chain Reaction
PI	Principal Investigator
POP	Performance optimized polymer
POW	Prisoner of War
PMI	Post-Mortem Interval
PPE	Personal protective equipment
proK	Proteinase K
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
rpm	Revolutions per minute
SAD	Scientific Analysis Division (fka CIL; aka DPAA-Lab)
SAP	Shrimp alkaline Phosphate
SOP	Standard Operating Procedure
STR	Short Tandem Repeat
TOF	Time of Flight
UN	United Nations
US	United States
USD	United States Dollar
USS	United States Ship
UV	Ultra-violet
XSTR	X-chromosomal Short Tandem Repeat
YSTR	Y-chromosomal Short Tandem Repeat
µL	Microliter

PUBLICATIONS

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Genotyping and sequencing of DNA recovered from human skeletal remains using capillary electrophoresis

Jodie Ward, Jeremy Watherston, Irene Kahline, Suni Edson, Timothy McMahon

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GC/MS Analysis of Skeletonized Human Remains: A Tool in the Analysis Osseous Materials

Suni M. Edson

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IN PREPARATION

Extraction Of DNA From Skeletal Remains Buried In Acidic Soils

Suni M. Edson, Ryan Z. Taira, and Gregory E. Berg

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The Presence of Inhibitory Materials in DNA from Skeletal Remains: Is it the Sample or the Extractor?

Suni M. Edson

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PRESENTATIONS

Name in bold indicates presenting author.

Suni M. Edson. Recovery of DNA from Skeletonized Remains: Process Improvements. Oral Presentation at Promega Technology Tour, Bode Annual Meeting, Phoenix, AZ, April 2019.

Suni M. Edson. Skeletonized Human Remains: What's the Story? Oral Presentation at DPAA Human Identification Symposium, AAFS Annual Meeting, Baltimore, MD, February 2019.

Suni M. Edson. Coupling DNA and GC/MS Analysis of Skeletal Remains: A Case Study of the USS *Oklahoma*. Oral Presentation at The American Academy of Forensic Sciences Meeting, Baltimore, MD, February 2019.

Suni M. Edson. Efficient Sampling of Skeletonized Human Crania for DNA Testing. Oral Presentation at The American Academy of Forensic Sciences Meeting, Baltimore, MD, February 2019.

Suni M. Edson, Timothy P. McMahon, and Adrian Linacre. Testing of Skeletonized Human Remains Using GC/MS: Development of a Personal Environmental Profile. Keynote Presentation at the Australia New Zealand Forensic Sciences Symposium, Perth, Western Australia, September 2018.

Suni M. Edson, Timothy P. McMahon, and Adrian Linacre. Testing of Skeletonized Human Remains Using GC/MS: Development of a Personal Environmental Profile. Poster presentation at Gordon Research Conference, Sunday River, Maine, June 2018.

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Jennifer A. O'Rourke and Suni M. Edson. Success of DNA Testing of Skeletonized Human Remains: Not a One Size Fits All Approach. Oral Presentation at the Promega Tech Tour in Hamilton, NJ, September 2017.

Suni M. Edson. Mutation Rates of Y-STR Loci and Incidences of Non-Paternity Among Purported Paternal Relatives. Poster presentation at the International Association of Forensic Sciences, Toronto, Canada, August 2017.

Gina M. Parada and Suni M. Edson. Success of DNA Testing of Skeletonized Human Remains: Not a One Size Fits All Approach. Oral Presentation at the Promega Tech Tour in Pasadena, CA, August 2017.

Kayla R. Sween and Suni M. Edson. Success of DNA Testing of Skeletonized Human Remains: Not a One Size Fits All Approach. Oral Presentation at the Promega Tech Tour in Baton Rouge, LA, June 2017.

Jennifer C. Kappeller and Suni M. Edson. Success Of DNA Testing Of Skeletonized Human Remains: Choose Your Own Adventure Vs. A Cookbook Process To Get The Best Results. Oral Presentation at the Promega Tech Tour in Frankfort, KY, May 2017.

Suni M. Edson. Success of DNA Testing of Skeletonized Human Remains: Efficient Selection of Protocols can Lead to Improved Testing Results. Oral Presentation at Promega Technology Tour, Norfolk, VA, April 2017.

Suni M. Edson and Stephanie Ah Sam. Success of DNA Testing of Skeletonized Human Remains and Comparison of Organic vs. Inorganic Extraction Protocols.

Oral Presentation at The American Academy of Forensic Sciences Meeting, New Orleans, LA, February 2017.

Suni M. Edson and Stephanie Ah Sam. Analysis of Materials Co-Extracting with DNA from Degraded Skeletal Remains. Keynote Presentation at

Australia – New Zealand Forensic Sciences Society 23rd International Symposium on Forensic Sciences, Auckland, New Zealand, September 2016.

Chapter 1

Introduction – Literature Review and Thinking Laterally about DNA Extracted from Skeletonized Materials

1.1 INTRODUCTION

Constant adaptation to new methods and new techniques is a hallmark of science. In the last few decades, there have been enormous advancements in the area of DNA science and human identification. It was almost 100 years from the theories of inheritance developed by Gregor Mendel to the description of the physical structure of DNA by Watson and Crick in the 1950s (Watson and Crick, 1953). It was another 32 years before Sir Alec Jeffreys, Peter Gill, and associates released two seminal papers that revolutionized human identification (Jeffreys et al., 1985; Gill et al., 1985). From there, techniques and technologies available have changed rapidly: Kary Mullis and polymerase chain reaction (Mullis and Faloona 1987); the descriptions of various microsatellites in nuclear DNA for human identification (Hammond et al., 1994; Urquhart et al., 1994); and the standardization of nomenclature of short tandem repeat (STR) analysis (Bar et al., 1994). Not to be ignored is using the Sanger sequencing method to decode mitochondrial DNA (mtDNA) (Anderson et al., 1981; Holland et al., 1993; Holland and Parsons, 1999; Sanger et al., 1977) and STR analysis of the Y-chromosome (Butler, 2003).

As a field, forensic DNA analysis for human identification has continued to grow and expand and is largely unrecognizable from the early years of manual manipulation of samples during PCR analysis. No longer are tubes manually transferred from water baths or hot blocks of specific temperatures in order to amplify DNA. Thermal cyclers do it with little human input other than to push a button. Southern blot analysis of hypervariable regions is mostly a thing of the past, and indeed, most college students in forensic DNA analysis or biochemistry courses today would be puzzled to realize that such analysis took days rather than 8 hours or less. With the advent of rapid DNA analysis equipment (among many: Bienvenue et al., 2010; Hopwood et al., 2010; Tan et al., 2013), the “insta-science” of CSI has become a reality.

While the new technologies are exciting and will continue to change the 'face' of human identification, it is important for the practitioner to remember that many of these new technologies are rooted in the original fundamentals of the science. STRs would probably not have been discovered so rapidly without the initial work of Jeffreys. New techniques exist because of the history of the field. It is beneficial to keep an eye on the past in order to continue to move forward.

1.2 STATEMENT OF THE PROBLEM: EXTRACTION OF DNA FROM SKELETONIZED REMAINS

Dried skeletal specimens and teeth are the typical sample types that the Past Accounting Section at the Armed Forces DNA Identification Laboratory (AFDIL) receives from the Defense POW/MIA Accounting Agency (DPAA) Laboratory, formerly Joint POW/MIA Accounting Command – Central Identification Laboratory (JPAC-CIL). AFDIL assists the DPAA Scientific Analysis Division (more commonly called DPAA-Lab) in identifying service members from past military conflicts such as World War II, Korean War, Southeast Asia conflict, the Cold War, and other incidents by processing the DNA analysis from the remains. From its inception in 1992, AFDIL used an organic extraction method in the extraction of total genomic DNA from skeletonized remains (Extraction Protocol #1, aka EP#1). This protocol, described in Edson et al., (2004), typically used 2.5 g of pulverized osseous material dissolved overnight at 56°C in an extraction buffer (10 mM Tris, pH 8.0; 100 mM NaCl; 50 mM EDTA, pH 8.0; 0.5% SDS) and proteinase K, followed by purification with 25:24:1 phenol:chloroform:isoamyl alcohol and, the now obsolete, Centricon-100® centrifugal filters (Millipore). At the time, the only DNA platform testing used was Sanger sequencing of mitochondrial DNA (mtDNA). In a survey of skeletal samples tested from 1992 to 2003, success was found to be somewhat predictable: femora were the most successful element for mtDNA testing and should be sampled preferentially.

In 2006, AFDIL validated a new demineralization technique ("Demin1", aka Extraction Protocol #2 or EP#2: Loreille et al., 2007; Edson and McMahon, 2016) that reduced the input of skeletal material from 2.5 g to 0.25 g. The extraction buffer itself was modified to be primarily EDTA (0.5 M EDTA, pH 8.0; 1% *N*-Lauroylsarcosine), but otherwise the protocol did not change significantly. Purification of the extract still occurred using PCIA and a purification filter, now Amicon® Ultra-4 Centrifugal Filter Units (EMD Millipore, Germany). The fundamentals of the procedure itself remained largely unchanged, even with the reduction in input of the sample. However, the success rates for mitochondrial DNA testing increased markedly. Gone was the preferential selection of the femur or other compact bones. Any skeletal sample selected would tend to give a reportable mtDNA sequence (Edson et al., 2011).

At the same time, AFDIL was expanding testing to include STR analysis. Modified PowerPlex® 16 (Promega Corporation, Madison, WI) or AmpFlSTR® Yfiler® (Thermo Fisher Scientific, Waltham, MA) protocols were used successfully to identify the remains of soldiers from the Vietnam War, Korean War, and World War II (Irwin, et al., 2007a,b). While useful, these modified protocols were not broadly incorporated into casework use at the time. It took another change in the extraction protocol for STR analysis to be fully implemented for use on a daily basis.

In 2011, the Past Accounting Section of AFDIL adopted a modification of the inorganic purification protocol ("Demin#2", aka Extraction Protocol #3 or EP#3: Edson and McMahon 2016) that was already in use by the Current Accounting Section for use on fresh skeletal remains and other agencies and laboratories, such as the ICMP, on aged remains (Amory et al., 2012; Davoren et al., 2007; Lee et al., 2010; Rohland and Hofreiter 2007). The AFDIL protocol remains the same as the demineralization technique adopted in 2006, with the

introduction of silica column purification step using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA) and the elimination of any PCIA purification step. In theory, this protocol would be faster, more efficient, more successful, and less harmful to the staff. While the last is certainly true, the remaining points did not necessarily turn out as expected.

Examination of the mtDNA success rates for all samples showed that the inorganic purification technique, known as "Demin2" in-house, gave an 80% success rate across all skeletal samples tested. This is understandably disappointing after the almost 90% success for Demin1. In addition, the overall quality of the data being reported was decreased. The target for reporting in the Control Region of mtDNA is 611 - 705 bases. Demin2 generated an average of 543 bases reported. While not as low as the average for the original extraction method (459 bases), it is still rather disappointing. What was markedly more successful was STR testing. Demin2 proved to provide a marked improvement in almost all STR platforms tested over either Demin1 or the original extraction protocol.

As with many labs, AFDIL is increasing the output of degraded skeletal remains tested with STR kits. Demin2 would seem to be a relatively decent fit to the workflow of the laboratory: success with STR analysis is needed; and mtDNA analysis is becoming less dominant, despite the make-up of the family reference database. However, some samples have been exposed to environmental conditions immediately antemortem, perimortem, or postmortem that may inhibit PCR processing should the materials co-extract with the DNA.

In 2015, the DPAA disinterred 45 graves from the National Memorial Cemetery of the Pacific (NMCP) containing the highly commingled remains of sailors and Marines who died on the USS *Oklahoma* 7 December 1941. Since 1941, the remains had undergone a series of burials and disinterments, including an extended period within the hull of the breached ship. During

this time, the fuel from the ship had leaked into the water and the hull and extensively contaminated the remains. Even with time and cleaning, skeletal samples sent to AFDIL for DNA testing retain the scent of fuel.

The first set of skeletal samples sent to AFDIL was extracted twice according to our standard SOP; however, Demin1 was used for the first extraction and Demin2 for the second. The goal was to determine which of the extraction protocols would work consistently better for mtDNA and STR testing on this particular set of samples. Given the presence of fuel, it should not be surprising that the Demin2 extraction protocol did not work as well as could be expected for this specific set of samples. The fuel could bind to the silica column and prevent the DNA from binding during the wash steps, thus increasing the amount of DNA lost. Previous work on other cases that have been exposed to inhibitory materials has shown that Demin1 tends to work better overall when the remains are chemically compromised. As with those cases, the samples from the USS *Oklahoma* tended to work better overall with an organic purification.

Given the in-house results observed, the evaluation of what is present on surface and interior of the remains seems to be a warranted study. Materials that the remains are exposed to antemortem, perimortem, or postmortem may have a deleterious effect on not only downstream processing, but the extraction itself, causing a reduction in the quality and quantity of DNA recovered.

With the continued build-up of unidentified skeletonized remains in medical examiner laboratories across the United States and world-wide, as well as large and long scale mass fatality events, it is increasingly paramount that quality DNA be recovered from these samples with the aim of entering the profiles into searchable databases. In many sets of

remains, the DNA present is not only damaged or degraded, but also inhibited in downstream processes by materials present in the environment or on the remains themselves (Alaeddini, 2014; Kreader, 1996). There have been a number of studies that evaluate both how inhibitors effect post-extraction processing of DNA and the large scale removal of such inhibitors from skeletal materials (e.g., Eilert and Foran 2009; Kemp et al., 2006), yet there have been few that address samples taken from a wide variety of real-world situations.

Previous studies, specifically on inhibition and bone density, have used animal bone (Salmonid vertebrae: Kemp et al., 2014 & Monroe et al., 2013; seal ribs: Barta et al., 2014a; bovine: Antinick and Foran, 2015) and synthetic bone (Barta et al., 2014b) as substitutes for human remains. Even when studies have used *in situ* human remains, they are typically of small sample size (Yang et al., 1998); recovered from a single location (Keyser-Tracqui et al., 2003; Misner et al., 2009) or a single event (Mundorff et al., 2008; Mundorff et al., 2009); or staged (Mundorff and Davoren, 2014). There is limited variability among the samples, and while this may allow for a development of a model for that specific time and place, extrapolation to other events is limited. By studying real world samples across a variety of environments and circumstances, the veracity of these studies can be evaluated within that framework.

To have a clear understanding of what is present in the samples to begin with would be optimal. Each extraction method currently in use has its own issues. Organic extractions are typically considered 'dangerous' and present certain health hazards. Phenol may also carry-over in the extraction and cause denaturation of *Taq* (Katcher and Schwartz, 1994). Inorganic extractions may provide a cleaner extract overall, but also have a tendency to have reduced yield, as seen in the literature (e.g., Petersen and Kaplan 2011; Stray et al., 2013) and in practice. By evaluating what is present in the samples, we may be able to produce a more

efficient extraction protocol that specifically targets the remove of materials that cause inhibition without sacrificing either yield or quality of the DNA.

1.3 USE OF MASS SPECTROMETRY TO DETERMINE PRESENCE OF INHIBITORS IN OSSEOUS MATERIALS AND ASSOCIATED DNA

In order to evaluate the materials on the surface of skeletonized remains and what is co-extracting with the DNA, mass spectrometry seems to be the optimal tool to be used. Mass spectrometry (MS or mass spec) has been shown to be useful in a wide range of applications. It is commonly used in forensics to evaluate trace materials in fires and explosions (e.g., Maurer, et al., 2010; Dhabbah, et al., 2014), toxicology (e.g., Skender, et al., 2002; Strano-Rossi, et al., 2010), and ink composition (e.g., Yao, et al., 2009; Koenig, et al., 2015), to name a few. Mass spec is also widely used outside of forensics for food safety (e.g., Gilbert-López, et al., 2010; Jaffrès, et al., 2011), pharmaceuticals (e.g., Meyer, et al., 2013; van den Broek, et al., 2015), and health studies (e.g., Farré, et al., 2007; Manning, et al., 2015). Given the overall sensitivity of MS to detect and characterize intact proteins in biological fluids (Huang et al., 2006), it is somewhat curious that forensics has no more fully embraced this technique. With detection limits to femtomoles per milliliter or less, it would seem to be ideal. However, it appears that this is not a straight-forward as one would think.

There are two main forms of mass spec: GC/MS and LC/MS. These two formats differ in how the materials to be tested are volatilized and require different equipment. The same sample can be tested on both platforms with differing results. The analytes to be detected may not respond well to the differing forms of ionization and the preparation required. This is not necessarily a problem for targeted analysis, such as pharmacology or toxicology or even food safety. When the product to be detected is known, the settings on the equipment can be easily set based on the composition of the target.

In this particular study, the detection of all materials within a sample, a broad-based screening needed to be developed. Certain analytes were not detected using either GC/MS or LC/MS and it may be optimal to use both types of equipment. Preliminary work done on a GC/MS (Agilent 7890B) vs. an LC/MS-MS (Thermo Fisher Q-Exactive Plus) showed varying detection of materials within the same sample. Running the machinery in positive or negative mode may also change the materials that are detected. For example, some explosives can be detected in both positive and negative mode (Ifa et al., 2009), but others are not.

Preparation of the materials was also called into question. Given the complex matrix of the bone material, it was difficult to prepare it for analysis. Trace analysis of accelerants and fuels generally requires a specialized set-up involving the ability to concentrate the materials to be detected in an absorptive substrate, which is itself then exposed to the ionization source, rather than the contaminated material. During the planning stages of this project, it was suggested that the materials within the skeletal materials would be too low to detect under normal circumstances and may need to be subjected to such specialized treatment (J. Butler, pers. comm.). Rather than seek out a specialized technique for detection of materials, a standard chemical protocol for extracting and concentrating the materials within the samples was examined, as there was a need to keep the protocol rather simple. The bone powder was exposed to a solvent, concentrated, and then resuspended in the same or a different solvent prior to being run on the mass spectrometer.

Selection of a single solvent was also considered a technical challenge, as some materials to be detected cannot be solubilized in what might otherwise be a preferred solvent. Methanol is a commonly used solvent for much MS analysis; however, cholesterol cannot be readily ionized using this solvent and water (Ifa et al., 2009). This reaction was observed in initial

work with the USS *Oklahoma* samples that became irreversibly cloudy upon addition of methanol and water (in a "dilute and shoot" protocol recommended by Thermo Fisher demo chemist, R. Doyle, pers. comm.). Multiple solvents were needed for a more accurate description of what was present in the remains. However, some materials, such as polycyclic aromatics may not be detectible in any solvent-based ionization method (Domin et al., 1997). Selection of a single solvent was not considered to be effective.

An ionization method that is not dependent on solvents is mass spec using an ambient ionization source. These methods expose the sample itself to the ionization source and do not involve any lengthy sample preparation. Small molecules, such as those that may be found in both the skeletal remains and in the DNA itself, have been detected by such methods as DART (Direct Analysis in Real Time: Cody et al., 2005; Pierce et al., 2007), DESI (Desorption Electrospray Ionization: Takáts et al., 2004), DAPCI (Desorption Atmospheric Pressure Chemical Ionization: Takáts et al., 2005), MALDESI (Matrix-Assisted Laser Desorption Electrospray Ionization: Sampson et al., 2006), and LAESI (Laser Ablation with Electrospray Ionization: Nemes and Vertes 2007). These ionization methods can be dependent on the type of sample to be tested. DESI has been used successfully on dehydrated samples (Huang et al., 2006) and for individuation of overlapping fingerprints (Ifa et al., 2008). LAESI has been successfully used for detection of large molecules such as peptides and has been used on animal and plant tissues; however, it requires a water rich target.

DART seems to be most promising of these methods, not only for this study, but for potential implementation into the laboratory for future usage. Moreno and McCord (2016) successfully used DART to determine the presence of inhibitors in DNA extracted from blood. The researchers used a DART ion source coupled with a JEOL AccuTOF (JEOL, Peabody, MA, USA) set in negative mode to successfully determine amount of indigo,

phenol, bile salts, tannic acid, and EDTA remaining in blood samples spiked with known quantities of the inhibitors. DART has also been in non-targeted studies (beer: Cajka et al., 2011; olive oil: Vaclavik et al., 2009), in a manner similar to what is being attempted in this study. However, DART has been shown to break down bonds in some metabolites, preventing detection of anything other than the parent material (Yu et al., 2009). Detection of only a parent compound may lead to a faulty assignment of the source of the material.

It seemed a simple solution to use an ambient ionization source rather than a solvent based technique. However, ambient ionization has many drawbacks, some of which are noted in the above paragraphs. Some of the most efficient methods are also considered destructive in that a small amount of material is removed from the tested substrate. It is also possible that the osseous material may prove to be too complex of a matrix for any ambient technique to appropriately ionize.

Determination of a single method by which bone powder and the DNA can be tested using MS may be next to impossible due to the need to survey the bone powder for all possible materials present. The testing was not for a single item or class of items, such as pesticides. Inhibitors of DNA are not a single class of detectable materials, although it is certainly a category. Different solvents and different methods for ionization were examined in order to ascertain which may provide the most detailed amount of information about the sample. This same method was used to examine which of the materials co-extracted with the DNA and may be causing inhibition with the downstream processing, or even with the DNA extraction itself.

1.4 POTENTIAL OUTCOMES

Once the materials co-extracting with the DNA can be determined, extraction protocols can be optimized. As noted above, each form of extraction (inorganic vs. organic) primarily in use today in forensic labs comes with a panoply of benefits and drawbacks. Some laboratories have implemented an inorganic extraction coupled with a single PCIA wash (D. Peters, pers. comm.), which they find to be quite efficient for both mtDNA and STR testing. There are a wide-variety of different extraction techniques that have been examined or are in use, including, but not limited to: a full demineralization paired with an automated extraction (Pajnič et al., 2016; Vlahović and Kubat 2012); soil kits for human osseous materials (Hebda and Foran 2015); or even a kit manufactured specifically for DNA extractions (e.g., NucleoSpin® DNA Trace Kits: Piglionica et al., 2012). It is improbable that all methods will be examined fully; however, a set selection or combination of protocols can be examined depending on the materials detected in the samples.

Besides qualifying the presence of inhibitors in osseous materials and improving DNA extraction protocols, detection of materials in human skeletal remains may allow for the creation of a predictive model for recovery location. Zhang et al., (2011) were able to detect geographical differences in tea grown in China using MS. It follows that MS could be used on osseous remains in a similar manner. However, a small grouping of samples from known recovery locations would have to be used to test if such a predictive model could be generated, and it would only be applicable to that general region/country. It would be useful to expand this type of analysis to a much larger area; however, the degree of complexity involved in testing of skeletal remains is mostly likely too great to allow for such a model to be feasible.

In the initial stages of this project development, it seemed that the project would be fairly simple and straight-forward: determine the materials present in a wide-range of skeletal materials, determine which of these materials co-extracts with DNA and impacts down-stream processing, and optimize DNA extraction techniques to remove the confounding materials. As the project continued, it appeared that optimizing the mass spectrometry protocol may prove to be the most difficult of the tasks to be done. While not impossible to do, it is certainly more complex than initially anticipated. However, the results of this testing will prove to be valuable to those performing DNA extractions from ossified human remains through optimization of techniques, and to the human identification community as a whole by providing a method by which additional information can be garnered from the skeletonized remains of deceased persons.

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1.6 PUBLICATION

The extraction protocols in use at AFDIL were described in a book chapter. The chapter includes not only the protocol, but tips for the implementation of the techniques.

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1.7 STATEMENT OF AUTHOR CONTRIBUTIONS

Ms. Edson wrote the body of the text and the suggestions for casework processing.

Dr. McMahon is responsible for the introduction.

1.8 TITLE PAGE

Extraction of DNA from Skeletal Remains

Suni M. Edson and Timothy P. McMahon

1.8.1 DISCLAIMER

The opinions expressed herein are those of the authors and not those of the U.S. Government, Department of the Navy, Department of the Army, the U.S. Army Medical and Development Command (MRDC), the Armed Forces Medical Examiner System (AFMES), the American Registry of Pathology (ARP), or the Armed Forces DNA Identification Laboratory (AFDIL). Mention of any product is merely a statement of use and should not be construed as an endorsement.

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1.8.3 SUMMARY

Acquisition of DNA from skeletal remains can be a delicate process. With the advent of improved extraction buffers that provide complete demineralization of the osseous materials, extraction of total genomic DNA from nearly any skeletal element is possible. This chapter describes both traditional organic and more newly developed inorganic extraction methods for fresh and dried skeletal remains.

1.8.4 KEY WORDS: DNA from skeletal remains; organic extraction; inorganic extraction; PCIA

1.9 INTRODUCTION

Considering the potentially reactive chemical groups that comprise the molecule, double stranded DNA is a stable, inert chemical. Buried within the helix, reactive groups are steadied by hydrogen bonds. The bases that make up the DNA itself are protected from the outside by a casing of phosphates and sugars that is reinforced through strong internal stacking forces. DNA's robust packaging protects it from most intracellular decomposition processes, which makes it ideal for use in criminal forensics and human identification.

Although chemically stable, the DNA molecule itself is physically unstable and subject to hydrodynamic shearing forces. DNA in an aqueous medium is a condensed supercoiled molecule that is stabilized by stacking interactions between the individual base pairs, and negative charge repulsions between the phosphate molecules in the DNA backbone. The flow of liquid across the DNA molecule due to pipetting, vortexing, or stirring creates flow resistance across the DNA double strands with enough energy to break the DNA. The longer the DNA molecule, the lower the amount of force needed to break the DNA into smaller fragments. On average any DNA molecule greater than 200 bp is readily susceptible to flow force breakage. However, in criminal and human identity DNA forensic testing, DNA shearing has little to no impact since most Short Tandem Repeat and Mitochondrial sequencing methods test for DNA fragments between 100-500 bp.

The greatest impact on the success of forensic human identity testing is degradation of the DNA molecule (DNases, bacteria, body decomposition, etc.) and environmental insults (acidic soil, temperature, humidity, etc.). To combat degradation and environmental factors associated with different samples, the scientist can optimize sample selection. Once sample selections have been optimized, the laboratory can increase success rates through the enhancement of extraction methods to guarantee complete cell lysis and amplification methods to combat degradation and inhibition.

To extract DNA from cells, four primary extraction techniques are available to DNA forensic laboratories. These include organic, Chelex®, FTA®, and solid phase (inorganic) methods. The extraction method chosen will depend upon the biological sample being examined as well as what environmental or chemical insults the sample may have been exposed to. Laboratories can chose to have a combination of different extraction methods validated for use to insure the greatest chance of success. The scope of this chapter will deal with organic and solid phase extractions.

Organic extraction, commonly referred to as Phenol-Chloroform or PCIA extraction, has been used for DNA purifications since the late 1950s. Although time consuming, with many transfer steps and the requirement to use harmful chemicals, organic extractions are still the gold standard to which all new extraction methods are compared. Organic extractions start with the addition of a lysis buffer that contains a buffer agent (commonly Tris), a detergent (SDS or *N*-Lauroylsarcosine), Proteinase K, and a chelating agent (EDTA or EGTA). The detergent and Proteinase K are used to solubilize the cellular membrane and denature the proteins that protect the DNA in the nucleus. Proteinase K, which is necessary for efficient protein denaturation is optimally active in 0.5 to 1.0% detergent and 56 °C.

Once released, the DNA is susceptible to DNase activity. The addition of EDTA inhibits DNase activities by binding divalent cations like Mg^{++}. After digestion, Phenol:Chloroform:Isoamyl-Alcohol (24:24:1) is added at 50:50 ratio to extraction buffer. Phenol is an organic solvent that has a specific gravity of 1.07 and forms the lower organic phase when mixed with an aqueous solution and acts as a protein solvent. Chloroform is an organic solvent that acts as a protein and RNA solvent, while isoamyl alcohol functions as a foam reducing agent. The lysate/PCIA mixture is vortexed until an emulsion is formed and then separated into an aqueous (top layer) and organic (bottom layer) phase by centrifugation. The denatured proteins and cellular debris are pulled into the organic layer, the lipids will

accumulate at the interface between the aqueous and organic layer, and the DNA will accumulate in the aqueous phase. Due to the chemical properties of PCIA, it is essential to denature the protein away from the DNA and to buffer the solution to a pH >7.8, in order to prevent DNA from accumulating in the organic layer. After several PCIA washes, the aqueous phase can be extracted with chloroform or n-butanol to remove any residual traces of phenol, a potent inhibitor of downstream amplification process. Then the DNA can be purified and concentrated by either ethanol precipitation or centrifugal filter units / ultrafiltration concentrators.

However, recent advances in solid phase (inorganic) methods and ultrafiltration concentrators have allowed DNA to be purified from lysate without the need for the PCIA purification steps, which decreases processing times, limits the number of transfer steps, and removes interactions with hazardous chemicals.

It is the authors' desire to outline procedures for the organic and inorganic extractions of dried and aged skeletal material and a modified inorganic procedure for fresh skeletal material. The procedures outlined below use Extraction (demineralization) buffer (0.5 M EDTA, pH8.0; 1% *N*-Lauroylsarcosine). The high amount of EDTA serves two functions, one to inhibit DNase activity, and two, to completely dissolve the CA^{++} rich bone matrix and free any and all DNA that maybe contained in challenged bone samples (1). However, the demineralization extraction buffer and procedures outlined below can be used on any biological specimens to obtain higher yields of DNA, when compared quick lysis and purification methods such as Chelex® or FTA®.

1.10 MATERIALS

All materials may be stored at room temperature unless otherwise noted.

Recommendation: Those objects that can be ultraviolet (UV) irradiated should be prior to initiating the protocol. Irradiation time, which will vary depending on the equipment used, should be set to deliver 6.0 J/cm^2. These include such items as the Waring blender cups, 50 mL and 15 mL conical tubes and 1.7 mL microcentrifuge tubes. Pipettes should never be UV irradiated, as repeated exposure to UV light will cause the plastic to decay; however, sterilization by wiping down the outside with 8.5% bleach (v/v) (70 mM NaOCl) or other DNA removal solution is recommended. Certain extraction reagents whether purchased externally or made internally may be UV irradiated, but the contents of the QIAquick and MinElute kits should never be UV irradiated.

To reduce the chance of contamination, the surface of all hoods (laminar flow, chemical fume, and PCR), and bleach tolerant equipment should be wiped down with 8.5% (v/v) commercial bleach followed by a 95% ethanol wipe to reduce the corrosive impact of the bleach. Additionally, staff participating in the extraction, to include all individuals entering the lab, should, at a minimum, wear the following personal protective equipment (PPE): non-permeable, disposable laboratory coat; one layer of latex or nitrile gloves; non-permeable, disposable sleeves; goggles; and a face mask. Gloves and sleeves should be changed between samples during cleaning in order to reduce contamination. During the preparation of the dried skeletal sample, the wearing of two pairs of gloves is recommended, as it is fairly common to damage the outer pair of gloves during cleaning. Individuals with longer hair should pull hair back away from the face. Hair nets/caps are not required, but can be used. Coats should be discarded at the end of the day, or sooner, if the scientist feels that the coat has been compromised.

1.10.1 Skeletal Sample Preparation and Cleaning (Dried)

1. Dremel® rotary tool (Bosch, Stuttgart, Germany)
2. Fit for use aluminum oxide sanding bits (size dependent on need) and emery grinding wheels compatible with the Dremel® rotary tool.
3. Surgical/dental mallet and osteotome/periodontal chisel
4. Parafilm® M Barrier Film
5. 50 mL polypropylene conical tubes (BD, Franklin, NJ, USA)
6. Absolute ethanol – 99.8% (Pharmco-AAPER, Brookfield, CT, USA)
7. Waring blender 700S/700G with appropriately sized mini container (MC1, MC2, or MC3) (Waring, Torrington, CT, USA)
8. 15 mL polypropylene conical tubes (Sarstedt, Newton, NC, USA)
9. Liquinox (Alconox, White Plains, NY, USA)

1.10.2 Intact Tooth Preparation and Cleaning (Dried)

1. Absolute ethanol
2. 50 mL polypropylene conical tubes
3. Small sonicating water bath
4. Dental Handpiece (Forza L50K Lab Micromotor, Brasseler USA, Savannah, GA, USA)
5. #2, #4, and #6 dental burs
6. Periodontal chisel
7. Forceps or spoon excavator
8. 15 mL polypropylene conical tubes
9. 4 x 4 cm sterile pad

1.10.3 Organic Extraction of Powdered Skeletal Samples or Teeth (Dried)

1. 15 mL polypropylene conical tubes (*see* **Note 1**).
2. Extraction (demineralization) buffer: 0.5 M EDTA, pH8.0; 1% *N*-Lauroylsarcosine
3. Proteinase K (20mg/ml)
4. Incubator shaker capable of maintaining 56 °C
5. Phenol: Chloroform: Isoamyl Alcohol (25:24:1), pH8.0 ± 0.2
6. Centrifuge
7. N-Butanol
8. Amicon® Ultra-4/30K centrifugal filter units (Millipore, Billerica, MA, USA)
9. TE Buffer (10 mM Tris, 1 mM EDTA, pH 7.5) (aka TE^{-4})
10. Costar® 1.7 mL polypropylene microcentrifuge tubes (Corning, Ithaca, NY, USA)

1.10.4 Non-Organic Extraction for both Skeletal Samples and Teeth (Dried)

1. 15 mL polypropylene conical tubes
2. Extraction (demineralization) buffer: 0.5 M EDTA, pH8.0; 1% *N*-Lauroylsarcosine
3. Proteinase K (20 mg/mL)
4. Parafilm® M Barrier Film
5. Incubator shaker capable of maintaining 56 °C
6. Amicon® Ultra-4/30K centrifugal filter units
7. Costar® 1.7 mL polypropylene microcentrifuge tubes
8. QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany)
    a. PB Buffer
    b. PE Buffer
    c. EB Buffer (provided, but not used)
    d. QIAquick spin columns
9. Microcentrifuge
10. 95-100% ethanol

11. TLE (10 mM Tris, 0.01 mM EDTA; pH 7.5)

1.10.5 Skeletal Sample Preparation and Cleaning (Fresh)

1. 15 mL polypropylene conical tubes
2. Scalpels
3. 95% ethanol
4. Mortar
5. Surgical/dental mallet and osteotome/periodontal chisel
6. Waring blender 700S/700G with appropriately sized mini container (MC1, MC2, or MC3)

1.10.6 Intact Fresh Tooth Preparation and Cleaning (Fresh)

1. 15 mL polypropylene conical tubes
2. Scalpels
3. 95% ethanol
4. Mixer/Mill MM 200 (Retsch, Haan, Germany)

1.10.7 Non-Organic Extraction for both Skeletal Samples and Teeth (Fresh)

1. 15 mL polypropylene conical tubes
2. Extraction (demineralization) buffer: 0.5 M EDTA, pH8.0; 1% *N*-Lauroylsarcosine
3. Proteinase K (20 mg/mL)
4. Incubator shaker capable of maintaining 56 °C
5. Amicon® Ultra-4/50K centrifugal filter units (Millipore, Billerica, MA, USA)
6. MinElute PCR Purification Kit (QIAGEN, Hilden, Germany)
    a. PB Buffer

b. PE Buffer

c. EB buffer

d. MinElute columns

7. Costar® 1.7 mL polypropylene microcentrifuge tubes
8. Microcentrifuge
9. 95-100% Ethanol

1.11 METHODS

All steps may be carried out at room temperature unless otherwise noted.

1.11.1 Sample Selection

Prior to extraction, selection of the optimal bone sample for testing will increase the odds of obtaining reportable results. If possible, collaboration with an anthropologist is recommended prior to cutting a sample for extraction from the intact bone. In general, compact bones with a dense physical structure, such as the femur, tibia, and humerus, tend to provide greater yield of DNA (**2-4**). However, modified extraction protocols can negate the impact of the sample type itself, and almost any skeletal element should provide sufficient quality DNA (**1, 5**).

Dental elements should be selected with care. Compromised external structure may introduce bacteria or other factors of decay following death of an individual. Pre-mortem dental work or disease may likewise compromise the internal integrity of the tooth structure and eliminate or reduce the recovery of DNA (**6-7**).

1.11.2 Skeletal Sample Preparation and Cleaning (Dried)

This description is for any dried osseous element other than teeth. For tooth preparation, see below.

The following steps should take place in a bone sanding hood or other laminar flow hood with sufficient ventilation (*see* **Note 2**).

1. Sand the exterior surface of the bone (*see* **Note 3**) using a clean aluminum oxide sanding bit fitted into a Dremel® rotary tool. All visible surfaces of the bone need to be sanded so as to remove any dirt, vegetative materials, or other exogenous contaminants. All trabecular (spongy) bone should be sanded away. Spongy bone can harbor detritus that may either contaminate or inhibit the extraction. In instances where visible trabecular bone is present between layers of compact bone (such as the cranial vault), it is useful to use a cutting wheel to separate the layers of compact bone in order to remove the spongy bone prior to proceeding. Retention of trabecular bone that may have been exposed to the environment for an extended period of time will increase the chance of introducing unwanted materials to the extraction.
2. Remove approximately 0.2-0.5 g of bone specimen from the larger, now sanded sample, using either a sanding wheel or a mallet and chisel of appropriate size. The remaining portion of bone sample can be repackaged by wrapping in Parafilm or placed in a sterile conical tube and retained for further processing, return to the submitting agency, or placed in long-term storage.

The following steps should take place in a laminar flow hood (*see* **Note 2**).

3. Place the bone fragment to be extracted in a 50 mL conical tube containing approximately 25 ml sterile deionized water (diH_2O). By hand, shake the tube vigorously back and forth several times.

4. Decant the water into a waste container.
5. Repeat steps 4 and 5 until the water is no longer cloudy (*see* **Note 4**).
6. Cover the bone fragment in the conical tube with absolute EtOH. Shake the container back and forth vigorously several times and decant liquid into a waste container.
7. Decant bone sample into a cleaned weigh boat or other non-porous, wide-mouthed container, and allow the sample to completely dry prior to proceeding. Depending on the density of the bone, this may take from one to two hours.
8. Once the sample is dry, place within a small sterilized blender cup (*see* **Note 5)**. Seal lid prior to removing from the hood and placing on the blender base.
9. "Blend" bone sample until a fine powder is generated (*see* **Note 6**).
10. Return the blender cup to the hood prior to removing the lid. Pour powdered bone into a cleaned weigh boat, and then transfer ~0.2 g to a sterile 15 mL conical tube. Any remaining powder should be transferred to a separate 15 mL conical tube for storage (*see* **Note 7**).

The procedure can be paused at this point and the bone powder stored at -20°C.

1.11.3 Intact Tooth Preparation and Cleaning (Dried)

This description is for intact dried teeth. Powdered teeth may be extracted using either of the following methods with no other preparation involved. Ideally, a laboratory would collect enough tooth powder to perform duplicate extractions.

1. Examine the exterior of the tooth. If the tooth is whole and undamaged, proceed to step 3.
2. If the tooth is cracked, damaged, or has untreated caries, the exterior of the tooth should be cleaned using a 4x4 cm sterile gauze pad moistened with 8.5% (v/v) commercial bleach. When the exterior surface is clean, immediately remove any

remaining bleach using a 4x4 cm sterile gauze pad moistened with absolute EtOH. Proceed to step 5.

3. Place the whole, undamaged tooth in a 50 ml sterile conical tube and cover with 25 ml of 8.5% (v/v) commercial bleach. Place sealed tube in a sonicating water bath for 5 min.
4. Remove tooth from bleach wash and wipe down with first a 4x4 cm sterile gauze pad moistened with 8.5% (v/v) commercial bleach and then a second sterile gauze pad moistened with absolute EtOH.
5. Place cleaned tooth in a cleaned weigh boat and allow to dry under UV irradiation in a laminar flow hood for approximately 15 min. If the tooth is not dry at the end of 15 min, turn off the UV light and allow to dry completely before proceeding.

The following steps should take place in a bone sanding hood or other laminar flow hood with sufficient ventilation (*see* **Note 2**).

6. Using a #2 or #4 dental bur, slowly cut around the base of the crown of the tooth. The intact tooth can be held with a hemostat if necessary; however, holding the tooth by hand allows for easier manipulation during cutting. Avoid cutting the enamel or cutting so low on the roots that the roots cannot be removed in a single piece. Cut around the crown until approximately 1 mm is left intact. This cutting needs to be performed over a clean weigh boat. The powder recovered from the external cut should be saved in a sterile 15 mL conical tube, though not used for extractions unless necessary.
7. Place a small, periodontal chisel between the crown and the roots and twist very gently to remove the crown from the root (*see* **Note 8**).
8. Should there be visible pulp, remove it to a clean 15 ml conical tube using a spoon excavator or forceps.

9. Using a #4 or #6 dental bur, carefully drill the dentin from the interior of the crown and root, catching it in sterile weigh boat (*see* **Note 9**). Remove as much dentin as possible, being careful to not puncture either the root or crown.
10. As drilling progresses, regularly remove the powder generated to a UV irradiated 15 mL conical tube. When approximately 0.2 g of powder has been collected, switch to a second UV irradiated 15 mL conical tube and collect another 0.2 g of powder, or until no dentin remains. If the tooth is too small, as may be the case for incisors, only 0.2 g total dentin powder may be recovered.
11. Store the remaining tooth structure in a UV irradiated 15 mL conical tube to prevent further damage.

The procedure can be paused at this point and the bone powder stored at -20°C.

1.11.4 Organic Extraction for both Dried Skeletal Samples and Teeth (Dried)

The following steps should take place in a laminar flow hood.

1. Start with approximately 0.2 g of powdered osseous material or dentin. A reagent blank should be initiated at this step and carried through the remainder of the procedure.
    a. If starting with 0.3-0.5 g of either, the volume of some reagents will need to be adjusted accordingly.
    b. If starting with less than 0.2 g, the procedure may proceed as indicated with a potential reduction in the final volume recovered.
2. Add 3 ml of extraction buffer and 100 µL of proteinase K (proK) to each tube. The buffer may be pre-warmed to 56 °C if desired.
    a. If starting with 0.3-0.5 g of powder, maintain the same volume of extraction buffer, but increase the proK to 200 µL.)

3. Shake or invert the tubes gently to completely saturate the bone powder. Continue to gently shake until no dry spots are visible in the powder.
4. Place the tubes into an incubator/shaker set to 56 °C. The tubes should be set to an angle of approximately 45 degrees and gently agitated overnight. Ensure that the liquid does not touch the cap of the tube (*see* **Note 10**).

The following steps should take place in a chemical fume hood:

5. Add 3 ml of phenol: chloroform: isoamyl alcohol (PCIA) to each tube.
6. Mix vigorously until a complete emulsion is formed.
7. Centrifuge tubes for 3 min at 4000 x *g*. There should be a clear delineation between the layers.
8. Transfer the aqueous (top) layer of each sample to clean 15 mL conical tubes (*see* **Note 11**).
9. Repeat steps 5-8 until the interface is clean (or a minimum of two times) (*see* **Note 12**).
10. Add 3 mL of n-Butanol to each tube.
11. Mix thoroughly.
12. Centrifuge tubes for 3 min at 4000x*g*. Again, there should be a clear delineation between the layers.
13. Remove most of the upper layer to a waste container. This will aid in cleanly removing the desired aqueous (bottom) layer.
14. Remove the bottom layer of each sample to clean Ultra-4/30K centrifugal filters. Take care not to transfer any remaining butanol along with the sample/reagent blank. Butanol may cause holes to form in the filter membranes, and encourage loss of DNA.
15. Spin the filters for 40-50 min at 2000x*g* (*see* **Note 13**). There should be approximately 200 µL of sample remaining at this time. If there is markedly more

volume left, the filters should be spun for additional time until this volume is reached (*see* **Note 14**).

16. Discard the filtrate.

The following steps should take place in a hood. No specific requirements for type of hood.

17. Add 2 ml of sterile TE^{-4} Buffer to each filter unit.
18. Spin all filter units for 10-15 min at 2000x*g*. The volume of the retentate should again be approximately 200 µL. If it is not, return the filter units to the centrifuge for an additional time until such volume is reached.
19. Discard the filtrate.
20. Repeat steps 17-19 once.
21. Recover the retentate and transfer to clean 1.7 mL tubes. The tip of the P-100 pipette may not fit all the way to the bottom of the filter unit. If so, recover the majority of the sample with a P-100 and the remaining sample with a P-10.
22. Measure the final volume and bring to 200 µL with TE buffer as needed. If the bone/tooth sample started at a significantly lower powder weight (less than 0.1 g) and is deemed to be of poor quality, it is recommended that the final volume be brought only to 100 µL.
23. Sample is now ready for processing.

1.11.5 Non-Organic Extraction for both Skeletal Samples and Teeth (Dried)

The following steps should take place in a laminar flow hood.

1. Start with approximately 0.2 g of powdered osseous material or dentin. If starting with 0.3-0.5 g of either, the volume of some reagents will need to be adjusted accordingly. If starting with less than 0.2 g, the procedure may proceed as indicated

with a potential reduction in the final volume recovered. A reagent blank should be initiated at this step and carried through the remainder of the procedure.

2. If extracting 0.26 g of bone/tooth powder or less, add 4 ml of extraction buffer to the sample and the reagent blank. If extracting more than 0.26 g of bone/tooth powder, add 7.5ml of extraction buffer to the sample and the reagent blank. The buffer may be pre-warmed to 56 °C if desired.
3. Add 200 µL proteinase K to each tube.
4. Shake or invert the tubes gently to completely saturate the bone powder. Continue to gently shake until no dry spots are visible in the powder.
5. Place the tubes into an incubator/shaker set to 56 °C. The tubes should be set to an angle of approximately 45 degrees and gently agitated overnight. Ensure that the liquid does not touch the cap of the tube (*see* **Note 15**).
6. Centrifuge tubes for 3 min at 4000 x *g*. This will bring any remaining bone powder to the bottom of the tube.

The following steps should take place in a laminar flow hood.

7. Transfer up to 4 mL of supernatant to at Ultra-4/30K centrifugal filter unit.
8. Spin the filter unit for 40-60 min at 2000 x *g* (*see* **Note 13**).
9. Discard filtrate.
10. If starting with more than 4ml of supernatant, add the remaining volume to the appropriate filter unit and spin for an additional 40-60 min at 2000x*g*.
11. Repeat steps 7-10 until all of the supernatant has been added to the filter unit.
12. The final retentate volume should be approximately 120 µL (*see* **Note 16**). If this is not so, the filter unit may be spun for additional time at 2000x*g*, until a final volume of 250 µL or less is reached.
13. Remove the retentate from the filter unit directly to a 1.7 mL microcentrifuge tube. The tip of the P-100 pipette may not fit all the way to the bottom of the filter unit. If

so, recover the majority of the sample with a P-100 and the final volume with a P-10 (*see* **Note 17**).

14. Measure and record the recovered volume.
15. Add 5 volumes of PB Buffer to 1 volume of sample. For example, if the recovered volume of the sample was 100 µL, 500 µL of PB Buffer would be added.
16. Mix well and tap down to remove any liquid from the lid of the tube.
17. Assemble the appropriate number of QIAquick spin columns in the provided 2 mL collection tubes.
18. Aliquot up to 750 µL of the buffer/sample mixture into each QIAquick spin column.
19. Spin columns for 30 s at 17,900x*g* in a microcentrifuge. If there is still visible liquid on the membrane after this step, spin columns for an additional 30 s at 17,900x*g*.
20. Discard waste (*see* **Note 18**).
21. Repeat steps 18-20 until all of the sample has been added to the spin column.
22. Add 750 µL PE buffer to each spin column (*see* **Note 19**).
23. Spin columns for 30 s at 17,900x*g* in a microcentrifuge. If there is still visible liquid on the membrane after this step, spin columns for an additional 30 s at 17,900 x*g*.
24. Discard waste.
25. Centrifuge the spin columns for an additional 60 s at 17,900 x*g*.
26. Place spin column in new, clean 1.7ml microcentrifuge tubes.
27. Add 100 µL of sterile TLE to the center of the column
28. Let stand for at least one min.
29. Centrifuge the column for 1 min at 17,900x*g* (*see* **Note 20**).
30. If needed, transfer the eluate to a new 1.7 mL microcentrifuge tube and discard the spin column.
31. Add 500 µl of PB Buffer to the 100 µL of eluate.
32. Repeat steps 16-26.
33. Add 50-200 µL of TLE to the center of the column and allow to stand for at least one min (*see* **Note 21**).

34. Spin the columns for 1 min at 17,900 x*g*.
35. Transfer eluate to a new, clean microcentrifuge tube as needed and discard column.
36. Sample is now ready for processing.

Extracts may be held at -20 °C or -80 °C for extended storage. In the short term, 4 °C is adequate. It is best to minimize freeze/thaw cycles as freezing may damage the DNA.

1.11.6 Skeletal Sample Preparation and Cleaning (Fresh)

Unless the bone specimen is suspected of being exposed to chemical or other agents, there is no need to perform the following steps in a hood. However, a Biological Safety Cabinet (BSC) hood may be used at the discretion of the scientist.

1. Remove any tissue or debris that might be adhering to the sample using a scalpel. Depending on the source or the needs of the laboratory, the removed tissue may be stored in a 15ml conical tube for evidence or extraction.
2. Place the sample in a 50 mL conical tube and add enough 95% EtOH to cover the sample.
3. Shake the tube vigorously.
4. Decant off the ethanol.
5. Repeat steps 2-4 twice.
6. Place sample in a dry, clean weigh boat and allow to dry in a laminar flow hood. Sample must be completely dry before proceeding. This should take approximately one hour.
7. Place sample in a clean mortar and cover the mortar with Parafilm (*see* **Note 22**).
8. Using a surgical mallet and an osteotome, punch a small hole in the Parafilm, and split the bone sample into fragments. Collect approximately 1.0 g of bone fragments.

The remainder of the bone, if there is any, can be placed in a 50 mL conical tube for storage.

9. Place 1.0 g of bone fragments into a Waring blender cup and seal the lid carefully (*see* **Note 5**).
10. Place the blender cup on a blender base and "blend" the bone sample until a fine powder is generated (*see* **Note 6**).
11. Pour the powder into a clean weigh boat and the transfer to a 15 mL conical tube (*see* **Note 23**).

The procedure can be paused at this point and the bone powder stored at -20°C.

1.11.7 Intact Fresh Tooth Preparation and Cleaning (Fresh)

Unless the tooth specimen is suspected of being exposed to chemical or other agents, there is no need to perform the following steps in a hood. However, a Biological Safety Cabinet (BSC) hood may be used at the discretion of the scientist.

1. Place an intact tooth in a clean weigh boat.
2. Cover the tooth with 95% EtOH.
3. Using a scalpel, remove any adhering dirt or tissue.
4. Wipe down the exterior of the tooth thoroughly with a 4x4 cm sterile gauze pad moistened with 8.5% (v/v) commercial bleach, followed by sterile gauze pad moistened with EtOH.
5. Place clean tooth in a cleaned weigh boat and allow to dry for at least 30 min or until completely dry.
6. Place the entire tooth in the Mixer/Mill jar along with the ball. Seal the lid in place and follow manufacturer's instructions for setting the jar in place in the Mixer/Mill itself (*see* **Note 24**).

7. Turn the Mixer/Mill to the following settings: Frequency – 1/S = 25.0; Time = 30-45 seconds.
8. Pulverize the tooth.
9. Pour the tooth powder into a clean weigh boat and the transfer to a clean 15 mL conical tube.
10. If significantly more than 1.0 g of powder is recovered, the remaining powder should be transferred to a second 15 ml conical tube for storage.

The procedure can be paused at this point and the powder stored at -20 °C.

1.11.8 Non-Organic Extraction for both Skeletal Samples and Teeth (Fresh)

1. Start with approximately 1.0 g of bone or tooth powder.
2. Add 3.0 mL pre-warmed extraction buffer and 100 µL proteinase K (*see* **Note 25**).
3. Mix thoroughly by shaking the tubes until there are no dry patches of powder in the tube, particularly the very bottom.
4. Place the tubes into an incubator/shaker set to 56 °C. The tubes should be set to an angle of approximately 45 degrees and gently agitated overnight (*see* **Note 26**). Ensure that the liquid does not touch the cap of the tube.
5. Centrifuge tubes for 3 min at 4000x*g*. This will bring any remaining bone powder to the bottom of the tube.

The following steps should take place in a laminar flow hood.

6. Transfer the supernatant to clean Ultra-4/50K centrifugal filters. Take care to not aliquot any bone powder as this will tend to clog the filter unit.
7. Spin the centrifugal filters in a centrifuge at 2700x*g* for approximately 60 min (*see* **Note 13**). The final volume should be 120 µL or less (*see* **Note 14**).

8. Remove the retentate from the filter unit directly to 1.7 mL microcentrifuge tubes. The tip of the P-100 pipet may not fit all the way to the bottom of the filter unit. If so, recover the majority of the sample with a P-100 and the final volume with a P-10.
9. Measure and record the recovered volume (*see* **Note 17**).
10. Add 5 volumes of PB Buffer to 1 volume of sample. For example, if the recovered volume of the sample was 100 µL, 500 µL of PB Buffer should be added.
11. Mix well and tap down to remove any liquid from the lid of the tube.
12. Place clean MinElute columns into the 2.0 mL collection tubes.
13. Transfer the DNA/PB Buffer solution to the columns.
14. Centrifuge columns at 13,000 rpm for 1 min.
15. Discard filtrate and return column to the same tube (*see* **Note 18**).
16. Add 750 µL PE Buffer to each column and incubate at room temperature for 5 min (*see* **Note 19**).
17. Spin columns at 13,000 rpm for 1 min.
18. Discard filtrate and return MinElute column to the same collection tube.
19. Spin columns at 13,000 rpm for 1 min.
20. Place column in a clean 1.7 mL microcentrifuge tube (*see* **Note 20**).
21. Add half of the final eluate volume of EB Buffer to the columns and incubate for 1 min at room temperature. The final target volume is typically 50 µL; however, it can be as little as 10 µL.
22. Centrifuge columns for 1 min at 13,000 rpm.
23. Repeat steps 21 and 22.
24. Samples are now ready for quantification and/or amplification.

Extracts may be held at -20 °C or -80 °C for extended storage. In the short term, 4 °C is adequate. It is best to minimize freeze/thaw cycles as freezing may damage the DNA.

1.12 NOTES

1. While the brand and size of 15 mL conical tube used for PCIA extraction can be chosen by the laboratory, the tube must be composed of polypropylene. Other materials, such as polystyrene, will dissolve when exposed to phenol.
2. Sanding of the fragment should occur within a bone sanding hood or laminar flow hood. It is critical for the powder generated from the sanding of the bone to be captured within the hood or removed by a ducted vacuum system. The powder generated by sanding is very fine and there is the potential for cross contamination of samples within the laboratory.
3. The element to be sampled is usually chosen by an anthropologist or a medical examiner prior to extraction. It is unusual for the DNA bench scientist to be allowed the choice of element to be processed for DNA. It is optimal for the osseous fragment submitted for DNA processing to be at least 0.5 g. Fragments smaller than these are difficult to hold during the cleaning process.
4. The purpose of the washing step is to remove any dirt and debris that may still be remaining on the sample as well as any bone powder that may be on the bone. Washing, along with the sanding procedure, reduces the possibility of recovering exogenous DNA during the extraction procedure. Exogenous DNA can cause mixtures with the endogenous DNA or even overwhelm the authentic profile (**8**).
5. Equipment used to pulverize the bone sample should be cleaned thoroughly between uses. It is recommended that the blender cup be cleaned with at least one wash of each of the following liquids in order: 1% liquinox with water, 8.5% (v/v) commercial bleach, water, and 95% EtOH; and then exposed to UV irradiation. Cups should be completely dry before grinding of the samples.
6. Rather than removing the blender cup to the hood to determine the degree of pulverization, place a gloved hand on top of the blender cup while the motor is running. Larger pieces of bone may be felt as they bounce against the rubber lid.

Take care not to "over blend" the sample. Excessive heat may damage the endogenous DNA. If you believe the bone powder is becoming hot, you may turn off the blender, let it cool, and turn the blender back on. It is also possible for the bone sample to become lodged under the blades of the blender cup. If this occurs, stop the blender and attempt to remove the sample from under the blades by tapping on the counter or rotating the blades from below. If this is insufficient to dislodge the sample, remove the lid of the blender cup (in a hood if dealing with a dried specimen) and manually dislodge the bone using either a periodontal chisel or forceps.

7. When using a Waring blender, the entirety of the powder will not be extremely fine. Another process or tool, such as a Freezer/Mill (SPEX, Metuchen, NJ, USA) or Mixer/Mill, may be used for powdering. When transferring the powder to use in the 15 mL conical tube, you may decant the larger pieces into a different conical tube for storage and then transfer the finer powder into a tube for extraction. It is also possible to use more than 0.2 g of bone powder for extraction. Up to 0.5 g of powdered bone may be used. More than 0.5 g of bone is not recommended as there will be a marked decrease in the dissolution of the powder in the demineralization buffer and an increase in inhibition.
8. The point in having a small notch in the tooth crown and root is to enable correct reassembly of the tooth. While the cementum of the roots contains perhaps the best source of DNA in the tooth (**7, 9**), acquiring this tissue requires destruction of the tooth root. The method described herein is designed to minimize external tooth damage so as to be able to return an intact tooth structure to a family member of a missing person. (After removal of the dentin, the tooth structure can be glued back together.) If structural integrity of the tooth is not an issue, skip steps 9 and 10. The root can be placed into the finger of a latex or nitrile glove and crushed with a hammer or pulverized using a Mixer/Mill (as described in the fresh tooth preparation) or equivalent. The crown can be stored as in step 11 and then proceed as normal.

9. There is a great deal of static electricity generated during the drilling of the tooth. It is a challenge to dissipate this energy without losing some of the powder being drilled. To ameliorate this issue, a large beaker of water can be set to steam within the laboratory, preferably within a few feet of the processing hood. Keeping a utility wipe, such as a Kimwipe (Kimberly-Clark, Neenah, WI, USA) moistened with 8.5% (v/v) bleach solution nearby to regularly moisten the fingertips and prevent charge build-up is also helpful; however, care should be taken to not introduce bleach to the powder.
10. The 0.2 g bone/tooth powder will dissolve in approximately 8 hours. If time is of the essence, observe the tubes during the incubation process. Once the powder has been completely dissolved, you may proceed to the organic extraction steps of this protocol. However, it should be noted that it is not an infrequent occurrence that some bone powder will remain in the solution even after an overnight incubation.
11. It is crucial that the PCIA solution be maintained at pH > 7.8. Depending on the source of the PCIA being used, a separate buffer will be supplied to equilibrate the solution pH. In a high volume laboratory, PCIA stored under normal conditions will usually be exhausted before any significant oxidation occurs, which will change the pH of the solution. However, low volume labs may use a single bottle of PCIA for several months and there is the potential for oxidation to occur. PCIA that has been oxidized beyond usefulness will appear yellow or red and in these instances if the pH <5. If this occurs the DNA will accumulate in the organic phase and not the aqueous phase during purification.
12. When drawing off the aqueous layer after each PCIA wash, take care not to collect any of the lipid-protein interface or organic phase. This is especially true in the final wash, as the proteins and other waste at the interface can inhibit downstream amplification processes. Some DNA is lost each time that a PCIA wash is performed and therefore it is prudent to minimize the number of PCIA washes if possible.

13. When placing the centrifugal filter units/ultrafiltration concentrators in the centrifuge, take care to align the largest parts of the unit with the center column of the centrifuge and the outer wall. This will allow of a more efficient flow through of the wash waste. If the filtrate seems to flow through faster or more completely than is to be expected, there is a chance that there is a hole in the filter membrane. In this case, recover the filtrate and transfer it to a new, clean filter unit and continue with the centrifugation steps.
14. In some instances it will take significantly longer than the indicated times for the waste product to flow through the filter. This is due to either an excessive amount of high quality DNA being present in the solution or extra waste product. It is possible for the filter unit to become completely clogged and no TE will flow through. If this occurs, the retentate should be transferred to a second clean filter unit for the additional washes.
15. With the larger volume of liquid, there may be concern that there will be leakage from the cap of the tube. Bone or tooth powder trapped in the threads of the cap will increase this possibility. Therefore, to prevent loss of extract, Parafilm may be wrapped around the top of the tube after capping. Scissors will most likely be needed to remove the wrap after incubation.
16. The maximum input of extract into a QIAquick column is 250 µL. It is optimal to reach a volume of retentate that is equal to or less than that during steps 7-12. However, it is possible to proceed if the retentate volume is greater than that. Additional QIAquick columns will need to be used for any volume over 250 µL and the final product recovered pooled.
17. It should be noted that the retentate will be somewhat viscous. It is rather difficult to pipette without some level of bubbling. Do not over handle the liquid as the downstream steps require a reasonably accurate measurement of retentate volume. If too many bubbles form, they can be brought down by gently tapping the tube on the counter or popping them with a P-10 tip.

18. It is critical that the flow-through is discarded after spin. Otherwise the liquid will not pass through the column during the following spin.
19. PE Buffer is received in the kit as a concentrate. The buffer will need to be prepared in advance of processing as indicated in the manufacturer's instructions using 96-100% EtOH.
20. When centrifuging the columns in the 1.7 ml microcentrifuge tubes, it is likely that the caps of the microcentrifuge tubes will be ripped off by the centrifuge. It is best to have several clean 1.7 mL microcentrifuge tubes available for downstream processing and storage. If you are spinning this particular set of tubes more than once, it is not necessary to transfer the eluate to a new microcentrifuge tube until the process is complete.
21. The volume of TLE added at this step will depend on the downstream processing of the sample. Samples being processed for mitochondrial DNA testing only should be brought to 200 µl. Samples being processed for nuclear DNA only may be brought up to a lesser volume depending on the history of the case and/or the quality of the sample.
22. While it may initially seem unnecessary to cover the mortar and bone fragment with Parafilm, the reasons why will become abundantly clear upon striking the bone with the osteotome. The addition of the Parafilm prevents the bone fragments and splinters from departing the mortar.
23. Depending on how fresh the remains are, the bone powder will tend to be rather sticky. It may not pour out of the blender cup as easily as dried remains. It may be necessary to scrape the powder from the blender cup using a scoopula (a spatula like scoop) or a small spatula.
24. The Mixer/Mill must be balanced and have two jars loaded at the same time. If pulverizing only one sample, place a jar containing only the ball in the other position.
25. The extraction buffer should be pre-warmed to 56 °C. This can be achieved by placing the needed aliquots of buffer in the incubator shaker for approximately 45

min. This is an option when working with dried skeletal remains, but is recommended when working with fresh skeletal remains.

26. Unlike in the protocol for dried skeletal remains, the overnight incubation time is largely a necessity. An entire gram of bone powder will not sufficiently dissolve in an 8-hour period.

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Chapter 2

Extraction of DNA from Skeletonized Post-Cranial Remains: A Discussion of Protocols and Testing Modalities

2.1 INTRODUCTION

Extraction of DNA from skeletonized remains is considered to be a somewhat difficult task. In the field of human identification, there is a great deal of debate as to the optimal skeletal element from which to recover DNA (Mundorff and Davoren, 2014; Edson, et al., 2004), whether it is the weight-bearing bones or smaller elements (Mundorff and Davoren, 2014) or even the location on the element from which a DNA sample should be taken (Antinick and Foran, 2015). The focus of this chapter was to provide analysis of a broad set of samples, recovered from a wide variety of locations world-wide. From this dataset, it was hoped that a single set of recommendations could be provided to practitioners to follow when working with skeletonized human remains.

The anthropologists from the Defense POW/MIA Accounting Agency (DPAA) primarily recovered the set of remains examined. The remains exhibited a post-mortem interval (PMI) of approximately 47 – 77 years. Samples recovered were from individuals lost during battles occurring during the Vietnam Conflict (1955-1975), Korean War (1950-1953); and the United States involvement in World War II (1941-1945). Some recoveries were made outside of these time ranges, but did not contribute significantly to the samples examined. The anthropological remains were examined at the laboratory at DPAA and submitted to the Armed Forces Medical Examiner – Armed Forces DNA Identification Laboratory (AFMES-AFDIL, aka AFDIL) for DNA testing.

Samples submitted and tested between 1990 and the first quarter of 2018 were considered for this retrospective study. During this time, four different DNA extraction protocols were used. Multiple different testing modalities were also used; however, only the primary testing strategies are considered here. They are as follows:

- Sanger sequencing of mitochondrial DNA (mtDNA)

- Modified AmpFlSTR® Yfiler™
- AmpFlSTR® MiniFiler™
- PowerPlex® Fusion
- Next Generation Sequencing (NGS), aka Massively Parallel Sequencing (MPS)

The decision was made to examine post-cranial and cranial remains separately. Sampling strategies for cranial remains are different than those of post-cranial elements, although in some regards this is a sociological consideration, as destruction of the cranial elements for DNA testing can be concerning for family members of the deceased.

This chapter is meant to provide foundational information on the need to improve the extraction of DNA from skeletal elements. There are marked differences between not only the elements themselves, but the extraction protocols employed.

2.1.1 INTRODUCTION REFERENCES

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2.2 PUBLICATION

The results of this study were published in The Journal of Forensic Sciences.

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2.3 TITLE PAGE

Extraction of DNA from Skeletonized Post-Cranial Remains: A Discussion of Protocols and Testing Modalities.

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The work was presented in part at the 2017 American Academy of Forensic Sciences Annual meeting in New Orleans, Louisiana.

2.3.1 DISCLAIMER

The opinions or assertions presented are the private views of the author and should not be construed as official or as reflecting the views of the Department of Defense; the Defense Health Agency; the Armed Forces Medical Examiner System; or the Defense POW/MIA Accounting Agency.

2.3.2 FINANCIAL DISCLOSURE

The author received no monies in the completion of this work other than a salary as an employee.

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2.4 ABSTRACT

This paper provides a retrospective of the DNA analysis performed by the Armed Forces Medical Examiner – Armed Forces DNA Identification Laboratory between 1990 and 2018. Over 13000 post-cranial osseous materials, comprised of wartime losses from World War II, the Korean War, and Southeast Asia, were examined by: mitochondrial DNA sequencing, a modified AmpFlSTR® Yfiler™, AmpFlSTR® MiniFiler™, PowerPlex® Fusion, or NGS. Four different DNA extraction protocols were used: incomplete demineralization coupled with an organic purification; complete demineralization with an organic purification; complete demineralization with an inorganic purification using QIAquick PCR purification Kit; and a protocol designed specifically for use with Next Generation Sequencing. In general, complete demineralization coupled with an organic purification was the optimal extraction protocol for sequencing of mitochondrial DNA, regardless of the osseous element tested. For STR testing, demineralization paired with an inorganic purification provided optimum results, regardless of kit used or osseous element tested.

2.5 KEY WORDS

Forensic Science; forensic DNA analysis; DNA typing; forensic anthropology; skeletonized human remains; STR analysis; mitochondrial DNA; Sanger sequencing; next generation sequencing

2.6 ARTICLE INTRODUCTION

Extraction of DNA from skeletonized human remains continues to be a challenging aspect of human identification, particularly in sets of aged and/or commingled remains. Methods have improved; however, there is a lingering debate on which skeletal elements provide the best overall results for DNA testing. Mundorff and Davoren (1) contend that smaller elements, such as metatarsals and metacarpals, provide some of the greatest success for STR analysis. This finding is supported by Andronowski, et al.'s (2) work with a cyclotron, which determined there is tissue remaining within the highly porous structure of such samples. Barta, et al. (3) also suggests non-weight bearing bones may provide improved results for mitochondrial DNA (mtDNA) testing, recommending ribs, as they are simple to remove; however, this work was largely based on non-human remains (i.e., seals). However, other laboratories suggest that the smaller elements or non-weight bearing bones may not be optimal for DNA recovery. The International Committee for Missing Persons (ICMP) recommends an ordered sampling strategy to begin with the femur and proceeding through the long bones (4) as has the Armed Forces Medical Examiner System – Armed Forces DNA Identification Laboratory (AFMES-AFDIL) (5). More recent studies recommend the removal of the petrous portion of the cranium (6,7), believed to be the densest bone in the body.

One drawback to most of these studies is the emphasis on remains of recently skeletonized individuals. Modern remains may not have been subjected to the same insults of environment and time that older remains may have. In addition, studies rarely examine more than a single platform of DNA testing. Recommendations to the at large community for the sampling of skeletonized remains should be widely applicable and not limited to either Sanger sequencing of mitochondrial DNA (mtDNA: 5,6,8) or any of the Short Tandem Repeat (STR) platforms (1,4,9). Multiple modalities are frequently used in human identification; therefore, sample recommendations should address the most commonly used.

This paper will provide a summary of testing performed on post-cranial skeletonized human remains at the AFMES-AFDIL between 1990 and mid-2018. Cranial elements and teeth have more complex considerations and will be addressed in future publications. The summary encompasses four different extraction techniques and application to: Sanger sequencing of mtDNA; AmpFlSTR® MiniFiler™ (Life Technologies, Gaithersburg, MD); Modified AmpFlSTR® Yfiler™ (Life Technologies); PowerPlex® Fusion (Promega, Madison, WI); and Next Generation Sequencing (NGS, aka MPS) of the whole mitochondrial DNA genome. This publication seeks to provide general recommendations for effective DNA recovery from skeletal remains across a wide variety of burial circumstances and post-mortem interval (PMI).

2.7 METHODS AND MATERIALS

All samples processed at AFMES-AFDIL between 1990 and the summer of 2018 were compiled and compared across four extraction types and five platforms tested. Additional platforms have been used in regular casework; however, they are limited in number and were not tabulated for this study. Techniques have changed over time. In cases where a listing of the different techniques would be prohibitive, the current technology is described.

2.7.1 Samples Collected

Samples selected were processed during the course of regular casework between the Defense POW/MIA Accounting Agency (DPAA) and AFMES-AFDIL between 1990 and 2018. This is an ongoing process and involves remains from all past United States military conflicts (i.e., Southeast Asia, Korean War, World War II, World War I) and non-conflict incidents (e.g., training accidents). Skeletal elements have a post mortem interval of 40-100 years and were recovered from a variety of different environments. All elements were fully skeletonized and retained no soft tissue.

Remains are fully examined by anthropologists at DPAA prior to selection of samples for DNA testing. Typically, elements that are most forensically relevant are selected, and a small window of bone is removed. Size of the bone sample sent depends on the size of the original bone as well as the requirements of the extraction protocol. When the sample being examined is smaller than the minimum input for the DNA extraction protocol being used, the entire sample is submitted for DNA testing.

2.7.2 Sample Preparation

The exterior of the sample is removed using a Dremel® sanding tool (Bosch, Stuttgart, Germany) and washed using sequential washes of diH_2O and 100% ethanol (5,10). A fragment of the cleaned, dried osseous sample is pulverized using a Waring® 1.0/1.2L laboratory blender motor and a MC2 blender cup (Waring, Torrington, CT).

Table 2.1 Summary of Extraction Protocols Used at AFMES-AFDIL. Protocols #2-4 are actively in use at AFMES-AFDIL. Overnight incubation should result in a complete demineralization of the osseous material present. Minimum incubation time is eight hours. Extraction Protocol #2 (EP#2) is a modification of the protocol described in Loreille, et al. (11). Upon implementation into casework, it was determined that the initial volume of osseous material should be reduced from 0.5 g to 0.2 g. Extraction Protocol #3 (EP#3) is a modification of the protocol described in Loreille, et al (25). For Protocols #3 and 4, the concentration step is performed prior to purification. In all other protocols, purification occurs first.

Extraction Protocol #4 (EP#4) continues to be modified. NGS/MPS processing has been used for casework at AFMES-AFDIL since the winter of 2016-2017. The protocol listed above was the initial protocol used for processing; however, the protocol is a modified organic extraction with 1.0 g of sample input.

	Extraction Protocol #1: Original (EP#1)	**Extraction Protocol #2: Demin 1 (EP#2)**	**Extraction Protocol #3: Demin 2 (EP#3)**	**Extraction Protocol #4: NGS specific (EP#4)**
Citation	Edson, et al. (5)	Edson and McMahon (10); Loreille, et al. (11)	Edson and McMahon (10); Loreille, et al. (25);	Marshall, et al. (15)
Amt. of osseous material required	2.5 g	0.2 - 0.5 g*	0.2 - 0.5 g*	1.0 g*
Volume of Extraction Buffer	3.0 mL	3.0 mL	4.0 mL	4.0 mL
Composition of Extraction Buffer	10mM Tris, pH 8.0 100mM NaCl 50mM EDTA, pH 8.0 0.5% SDS	0.5M EDTA, pH 8.0 1% N-Lauroylsarcosine	0.5M EDTA, pH 8.0 1% N-Lauroylsarcosine	0.5M EDTA, pH 8.0 1% N-Lauroylsarcosine
Proteinase K (200mg/mL)	100µL	100µL	200µL	200µL
Incubation	Overnight at 56°C	Overnight at 56°C	Overnight at 56°C	Overnight at 56°C
Purification	2-3 washes with Phenol:Chloroform:Isoamyl Alcohol (25:24:1) followed by a single wash using n-Butanol	2-3 washes with Phenol:Chloroform:Isoamyl Alcohol (25:24:1) followed by a single wash using n-Butanol	QIAquick PCR purification Kit (QIAGEN, Hilden, Germany)	Qiagen MinElute® Kit
Concentration	Centricon-100 centrifugal filter units (Millipore, Billerica, MA)	Amicon Ultra-4/30K centrifugal filter units (Millipore)	Amicon Ultra-4/30K centrifugal filter units (Millipore)	Amicon Ultra-4/30K centrifugal filter units (Millipore)
TE	TE-4 (10mM Tris, 0.1mM EDTA; pH 7.5)	TE-4 (10mM Tris, 0.1mM EDTA; pH 7.5)	TE-4 (10mM Tris, 0.1mM EDTA; pH 7.5)	TE-4 (10mM Tris, 0.1mM EDTA; pH 7.5)
Final Volume	50-100 µL	100-200 µL	50-200 µL	70 µL
*Notes	Active protocol 1990-2006. No longer in use.	If extracting 0.3 g-0.5 g osseous material, increase proteinase K used to 200 µL	If extracting 0.26-0.5 g osseous material, increase volume of extraction buffer to 7.5 mL	The target input is 1.0 g; however, up to 2.0 g may be input as can smaller volumes. Extraction buffer volume should be adjusted as necessary.

2.7.3 DNA Extraction

There are four extraction protocols that have been used at AFMES-AFDIL, all of which are described in Table 2.1. Extraction Protocol #1 (EP#1) was used 1990-2006 and has been retired. Extraction Protocol #2 (EP#2) and Extraction Protocol #3 (EP#3) involve complete demineralization of the osseous material. EP#1 used a target input of 2.5 g, while both EP#2 and EP#3 have a target input of 0.25 g, although a larger volume may be used. Extraction Protocol #4 (EP#4) was implemented in 2016 and is used specifically for samples designated for the Next Generation Sequencing (NGS) workflow.

A reagent blank is initiated as a control for each extraction set performed. An extraction set may contain one to four osseous samples. The reagent blank is carried through the downstream processes in conjunction with the associated samples.

2.7.4 Quantification

Extracted DNA is not quantified prior to mtDNA amplification. Samples processed for any STR platform are quantified using commercial kits and equipment. These data are not presented here, as the kits have varied markedly and will not provide a standardized result.

2.7.5 Mitochondrial DNA Amplification and Sanger Sequencing

Amplification and subsequent Sanger sequencing of mitochondrial DNA has largely remained unchanged at AFMES-AFDIL since 1998 (5). At that time, mini-primer sets were implemented into casework (12), allowing for DNA of reduced fragment size to be amplified. DNA extracted from dried skeletal remains is typically amplified with primer sets, resulting in an amplicon of 187-231 bp in length. Mini-primer sets reduce the amplicon size to 79-115 bp (Figure 2.1). All mtDNA primers used at AFMES-AFDIL are made in-house. For

samples suspected of being non-human in origin, a 12S rRNA amplification can be performed for confirmation and possible species identification.

For amplification, a PCR Master Mix containing 10x PCR Buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM $MgCl_2$); 2.5 mM dNTPs; 6.25 µg/µL NA-BSA; 10 µM each of paired primers; 5 units/µL AmpliTaq Gold® DNA polymerase; and sterile diH_2O is used. The targeted input of DNA is 10-1000 pg. Both DNA input and Taq polymerase volumes may be adjusted as needed, depending on the perceived quality of the sample. For each reaction, two negatives and a positive (HL60, 200 pg/10 µL, Life Technologies) are amplified and carried through the remainder of the downstream processing. Amplification takes places in GeneAmp® PCR Systems 9700 (Life Technologies) using in-house designed and validated programs. The program most commonly used for the larger primer sets is: a 10 minute soak at 96°C, followed by 38 cycles of: 20 seconds at 94°C, 20 seconds at 56°C, and 30 seconds at 72°C, and ending with a hold of 4°C upon completion. Modifications to this program for the mini-primer sets and other regions can be found in Gabriel, et al. (12).

Amplified product is run on 2% agarose gels stained with ethidium bromide (5 mg/mL). If the amplification is successful (i.e., generates a visible band on the gel), input of amplified product into a sequencing reaction is determined by comparison to a mass ladder (DNA Ladder II; APExBIO Research, Houston, TX).

Two techniques have been primarily used for purification of amplified product prior to sequencing. Prior to discontinuation of the product in 2006, Centricon-100 and Centricon-30 centrifugal filter units (Millipore, Billerica, MA) were used in concert with diH_2O to purify the amplified product. Currently, an enzymatic PCR with ExoSAP-IT® (Life Technologies), is used. For each set of samples processed, a master mix is created using 1.5 µL ExoSAP-IT®

and 18.5 µL SAP dilution buffer per sample. Prior to placing the samples in an unheated thermal cycler, 20 µL of the master mix is added to each sample. The reaction for purification is as follows: 30 minute hold at 37°C, 15 minute hold at 85°C, and an indefinite hold at 4°C.

Sequencing reactions are currently processed in a 96-well plate format. Samples and all associated amplification controls (two negatives, one reagent blank, and one positive) are assigned two wells on the plate, one for a forward primer and one a reverse. The same primers are used for sequencing are the same as for amplification, with a few exceptions of primers internal to the original primer binding sites. At present, a one half reaction set up is used with the following volumes: 1.0 µL sequencing primer (10 µM); 3.6 µL BigDye® Terminator v1.1 (Life Technologies); 0.4 µL dGTP BigDye® (Life Technologies); 4.0 µL sequencing dilution buffer (400 mM Tris; 10 mM $MgCl_2$, pH 8.0); 1.0-7.0 µL DNA; and *q.s.* to 20 µL with diH_2O. Plates are sealed one column at a time with strip caps to eliminate well-to-well contamination.

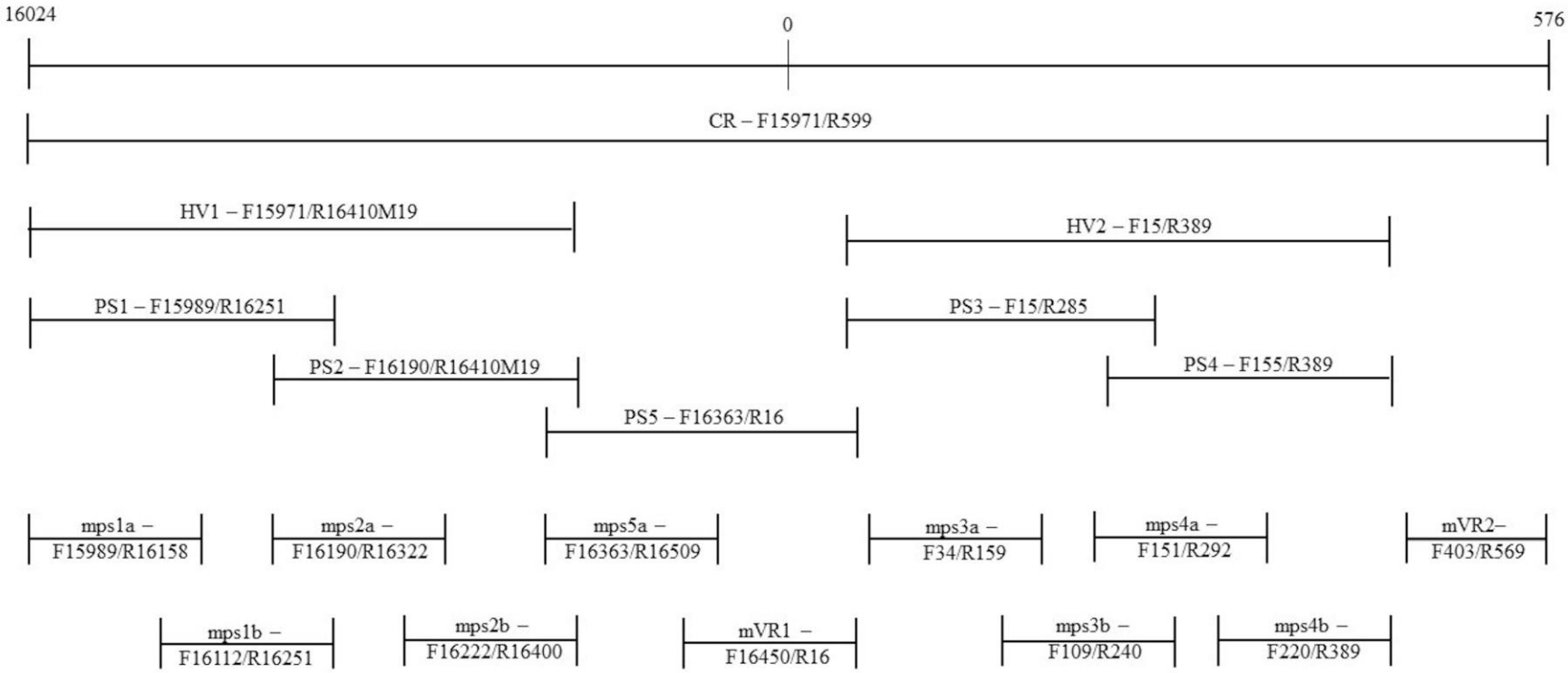

Figure 2.1 The overlapping primer pairs available for amplification of mitochondrial DNA at AFMES-AFDIL. The analyst may move from largest to smallest to gain sufficient coverage. Typically, amplification begins with PS2. Should that amplification fail, the analyst may repeat the amplification and vary the inputs of both DNA template and *Taq* Gold or move to the mini-primer sets (mps) as desired.

The sequencing reactions take place in GeneAmp® PCR Systems 9700 with the following parameters: 25 cycles of 96°C for 15 seconds; 50°C for 5 seconds; and 60°C for 2 minutes, with an indefinite hold at 4°C. Upon completion of the sequencing reaction, samples are stored at 4°C until purification. To purify the sequencing product, samples are placed into Performa DTR 96-well Ultra gel filtration block (Edge BioSystems, Gaithersburg, MD) and centrifuged at 850 x g for 5 minutes. Purified product is dried in a Labconco CentriVap Concentrator (Labconco, Kansas City, MO) and the plate sealed and stored at -20°C until such time as fragment separation can be done.

2.7.6 STR Amplification

Multiple different kits have been used over the course of this study. The following kits are in current, day-to-day use:

1. A Modified AmpFlSTR® Yfiler™ protocol. The modifications include a doubling of the volume AmpliTaq Gold® DNA polymerase from the recommended 0.8 µL per sample to 1.6 µL per sample and an increase in the number of program cycles from 28 to 36 (13,14).
2. AmpFlSTR® MiniFiler™ used as per manufacturer recommendations.
3. PowerPlex® Fusion used as per manufacturer recommendations.

While other commercial kits have been used, they do not have a significant number of results and are not currently in use at AFMES-AFDIL; therefore, they will not be described in this publication.

2.7.7 Fragment Separation

Prior to 2003, all fragment separation was performed on slab gels using ABI PRISM 377® DNA Sequencers (Applied Biosystems, Gaithersburg, MD). Use of the AB 377 instrument is

only relevant to mtDNA Sanger sequencing, as STRs were not validated for use until 2004. Between 2003 and 2016, the AB 3100 and 3130*xL* Genetic Analyzers were the primary instruments in use. The instruments were outfitted with 36 cm capillary arrays and Performance Optimized Polymer 4 (POP-4™; 4% dimethylacrylimide, 8M Urea, 5% 2-pyrrolidinone) for STR analysis or a 50 cm capillary array and POP-6™ (6% dimethylacrylimide, 8M Urea, 5% 2-pyrrolidinone) for mtDNA.

Since 2015, the laboratory has gradually shifted to using the AB 3500*xL* Genetic Analyzer. The POP and capillary arrays used remain the same as those used on the 3130*xL*. Prior to loading, samples are resuspended in the appropriate loading buffers as listed in Table 2.2, and injected with the indicated parameters.

Table 2.2 Summary of parameters in use for loading on the 3500*xL* Genetic Analyzer.

	mtDNA Sanger Sequencing	**AmpFlSTR® MiniFiler™**	**Modified AmpFlSTR® Yfiler™**	**PowerPlex® Fusion**
Resuspension prior to loading (per sample)	10 µL Hi-Di™ Formamide	0.5 µL GeneScan™ 600 LIZ® v 2 8.5 µL Hi-Di™ Formamide	0.5 µL GeneScan™ 600 LIZ® v 2 8.5 µL Hi-Di™ Formamide	0.5 µL WEN ILS 500 8.5 µL Hi-Di™ Formamide
Allelic Ladder	None	Kit specific	Kit specific	Kit specific
Positive Control	HL60	DNA 007	2800M	2800M
Polymer	POP-6™	POP-4™	POP-4™	POP-4™
Capillary Length	50 cm	36 cm	36 cm	36 cm
Injection Times	8 seconds (Primer sets only) 16 seconds (primer sets and Mini-primer sets)	7 seconds (default) 15 seconds	7 seconds (default) 15 seconds	7 seconds (default) 15 seconds

2.7.8 NGS Processing

Next Generation Sequencing is performed on Illumina platforms (Illumina, San Diego, CA) with an in-house validated protocol described in Marshall, et al. (15).

2.7.9 Mitochondrial DNA Data Analysis

Mitochondrial DNA that has been Sanger sequenced is analyzed by two independent scientists using GeneCodes Sequencher Plus (Ann Arbor, MI). Polymorphisms reported are the differences from the sequences generated to the revised Cambridge Reference Sequence (rCRS: 16,17).

Whole mitochondrial genome data, generated via the NGS process, are analyzed using the CLC Genomics Workbench Software, version 7.5 or higher (QIAGENBioinformatics, Gaithersburg, MD). Data generated from compromised skeletal remains require a read depth coverage of at least 10 reads to determine a base call within analytical range. A variant must be seen in at least four reads to be called and at least 10% of the total reads in order to be reported.

2.7.10 STR Data Analysis.

STR data analysis is currently performed using GeneMapper® *ID-X* v. 1.3 (Life Technologies). Analysis thresholds are described in Table 2.3.

Table 2.3 Reporting criteria for primary STR kits used at AFMES-AFDIL.

	AmpFlSTR® MiniFiler™	Modified AmpFlSTR® Yfiler™	PowerPlex® Fusion
Positive Control	DNA 007	2800M	2800M
Analytical Threshold	40 RFU	100 RFU	70 RFU
Stochastic Threshold (Heterozygotes)	75 RFU	100 RFU	70 RFU
Stochastic Threshold (Homozygotes)	150 RFU	100 RFU	400 RFU

2.7.11 Reporting Criteria:

In order for a Sanger mtDNA profile to be reported, the data must be duplicated, by either amplifications of two extractions or two amplifications of a single extraction. Mitochondrial DNA data generated by NGS must meet the criteria indicated above. Sequences generated from samples tested using the 12S rRNA assay are searched against the National Center of Biotechnology Information (NCBI) database using the Basic Local Alignment and Search Tool (BLAST) available online (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the species of origin reported (18).

Reporting criteria for STR analysis is similar in that multiple extractions or amplifications must exist in order for a sample to be reported. However, loci must be replicated at least twice, above stochastic threshold, in order to be reported. Analytical and stochastic thresholds are the same for low and high quality samples. For Modified AmpFlSTR® Yfiler™, amplifications being used for confirmation must contain at least four above-threshold loci.

For all platforms, a reported profile must be concordant between two analysts. This profile is screened against all staff at AFMES-AFDIL and DPAA. Data undergoes both a technical and an administrative review before being reported to the requesting agency.

2.7.12 Data Tabulation

For the purposes of this study, a 'successful' mtDNA sequence is 100 confirmed base pairs or more. All STR profiles were considered successful if four or more reportable loci were duplicated. Percent success was calculated by dividing the number of successful samples by the number of samples tested. The average loci or the average number of bases reported were calculated from the samples that generated reportable data, even if it was less than criteria set for success.

Skeletal elements were divided by element type. The category of "Pelvis" is primarily the os coxa, and also includes the ilium, ischium, sacrum, and ramus. The maxilla and mandible are reported as a single category of "Jaw". Metacarpals and metatarsals are not subdivided by number but are maintained as distinct categories.

2.8 RESULTS

2.8.1 Sanger Sequencing of Mitochondrial DNA

In total, 13609 skeletal elements were examined for Sanger sequencing of mitochondrial DNA across three different extraction protocols (Table 2.4). Extraction Protocol #2 (EP #2: complete demineralization with organic purification) proved to be most successful for any type of bone sample selected for processing. Of the 7110 samples tested, 6528 (92%) generated reportable results with an average of 682 base pairs reported. In general, there appears to be no trend in success across osseous elements, with almost all having a 90% or greater success rate. The exceptions to this are the talus (15 sampled; 80% success); fragments (492 sampled; 55% success); and trapezium (2 sampled; 50%). Of the 270 fragments failing to produce human mtDNA, 182 were tested using the 12S rRNA assay and 115 (63%) were found to be non-human in origin.

Extraction Protocols #1 and #3 were much less successful (77% and 79%, respectively). EP#1 was the primary protocol in use at AFDIL from 1990 until 2007 and was used to test 4526 osseous samples. The femur (974 tested; 90% successful) and tibia (641 tested; 87% successful) were most likely to produce results when tested in mtDNA Sanger sequencing. In general, other weight bearing bones (fibula: 75%) or long bones (humerus: 79%; ulna: 72%) also tended to be successful. However, the relatively high success rate of the ribs (69 tested; 77%) was unexpected. Nineteen (36%) of those ribs that were successful in mtDNA analysis were recovered from a single location in the Gio Linh district of the Quàng Tri Province of Vietnam.

Table 2.4 Overall success of mtDNA Sanger sequencing for all skeletal samples submitted to AFMES-AFDIL from DPAA between 1990 and 2018. Samples are listed alphabetically. The maxilla and mandible are combined together to form the category of "Jaw". The "pelvis" category is primarily the os coxa, but also includes ilium, ischium, sacrum, and ramus.

The increase in the number of 'fragments' submitted for processing coincides with a change in the extraction protocol. Extraction Protocol #2 (demineralization plus organic purification) typically provided a 90% success rate for any element tested in mtDNA Sanger sequencing. The low success rate in the fragment category is explained by the non-human nature of approximately 38% of the samples tested.

	Extraction Protocol #1				Extraction Protocol #2				Extraction Protocol #3			
	# Tested	# Success	% Success	Avg. # Bases	# Tested	# Success	% Success	Avg. # Bases	# Tested	# Success	% Success	Avg. # Bases
All	4526	3479	77	614	7110	6528	92	692	1973	1568	79	684
Acetabulum	2	1	50	608								
Calcaneus	7	3	43	500	26	24	92	677	4	3	75	703
Capitate									2	2	100	680
Clavicle	187	101	54	548	344	328	95	669	70	61	87	675
Cuboid					1	0	0	0				
Cuneiform									4	3	75	694
Femur	974	875	90	638	805	749	93	701	236	189	80	696
Fibula	238	179	75	612	536	524	98	695	110	103	94	693
Fragments	240	138	58	616	492	269	55	665	280	109	39	663
Hallux	1	1	100	611								
Humerus	768	606	79	613	876	824	94	699	173	152	88	690
Jaw	134	94	70	596	154	140	91	682	59	45	76	663
Manubrium	1	1	100	681								
Metacarpal	31	16	52	537	64	58	91	682	98	92	94	680
Metatarsal	104	64	62	542	161	154	96	678	103	101	98	696
Navicular					1	1	100	800	8	8	100	634
Patella					14	14	100	661	41	40	98	710
Pelvis	231	168	73	590	566	549	97	694	81	76	94	678
Phalanx	10	4	40	532	13	12	92	607	18	16	89	687
Radius	289	201	70	587	464	443	95	692	112	97	87	693

	Extraction Protocol #1				Extraction Protocol #2				Extraction Protocol #3			
	# Tested	# Success	% Success	Avg. # Bases	# Tested	# Success	% Success	Avg. # Bases	# Tested	# Success	% Success	Avg. # Bases
Rib	69	53	77	607	433	397	92	679	75	52	69	674
Scapula	140	94	67	566	454	437	96	691	58	49	84	689
Sphenoid					2	2	100	686				
Sternum	1	0	0	0	1	1	100	704	2	2	100	703
Talus	11	8	73	605	15	12	80	665	19	17	89	694
Tibia	641	555	87	631	746	704	94	699	205	174	85	689
Trapezium					2	1	50	534				
Ulna	363	260	72	612	519	493	95	697	104	87	84	675
Vertebra	61	44	72	618	390	375	96	687	92	79	86	687
Zygomatic					10	9	90	667	12	11	92	675

Extraction Protocol #3 (EP#3: complete demineralization with inorganic purification) does not show the same trend towards long bones being more successful. Femora are one of the least successful samples tested (236; 80%). Although the fibula (110; 94%) was still successful for this extraction protocol, smaller bones appeared to be more successful as a whole. The navicular, a bone in the foot, was successful 100% of the time; however, only eight were sampled. Patellae also produced results 98% of the time for EP#3 and 100% for EP#2 (14 sampled). Metatarsals and metacarpals (94% and 98%, respectively) were among the most successful for EP#3, and were also successful 91% and 96% of the time in EP#2.

2.8.2 Modified AmpFlSTR® Yfiler™ Testing

Of the 1780 samples evaluated for Modified Yfiler™ testing (Table 2.5), 885 were tested in EP#3, with an overall success of 62%. The femur and the tibia were the most successful at 90% and 91%, respectively. The metacarpal and metatarsal were only 30% and 39% successful, making them a less desirable choice for Modified Yfiler™ testing.

Extraction protocols #1 and #2 were markedly less successful than EP#3 (40% and 33%, respectively). The femur (69%, 55%), fibula (53%, 47%), and tibia (66%, 68%) were the most successful for both extraction protocols. Metacarpals and metatarsals both had a 0% success rate for both EP#1 and EP#2, although this result is not of import for EP#1 as only one of each type of element was tested.

2.8.3 AmpFlSTR® MiniFiler™ Testing

Extraction Protocol #3 is most successful of the extraction protocols for MiniFiler™ testing (Table 2.6). Of the 628 samples tested in EP#3, 52% presented at least four reportable loci. The femur (86%) and tibia (84%) were among the most successful elements. However, the

general jaw category, consisting of both the maxilla and mandible, was the most successful (90%; 20 sampled). The metacarpals and metatarsals were generally unsuccessful at 10% and 33%, respectively.

Extraction Protocols #1 and #2 were less successful than EP#3 (37% and 34%, respectively). The fibula (83%) was the most successful element for EP#1 with the femur being the second most successful (65%). The femur was the best element for EP#2 (61%).

Table 2.5 Overall success of enhanced AmpFlSTR® Yfiler™ testing for all skeletal samples submitted to AFMES-AFDIL from DPAA between 1990 and 2018. Samples are listed alphabetically. The maxilla and mandible are combined together to form the category of "Jaw". The "Pelvis" category is primarily the os coxa, but also includes ilium, ischium, sacrum, and ramus.

The average number of loci has been rounded to the closest whole number.

There are a limited number of samples tested in any STR platform in the "Extraction Protocol #1". This extraction protocol ceased to be actively used in 2007, just prior to the implementation of STR protocols into active casework.

	Extraction Protocol #1				**Extraction Protocol #2**				**Extraction Protocol #3**			
	# Tested	# Success	% Success	Avg. # Loci	# Tested	# Success	% Success	Avg. # Loci	# Tested	# Success	% Success	Avg. # Loci
All	160	64	40	9	735	244	33	8	885	551	62	10
Calcaneus					1	0	0	0	3	1	33	8
Clavicle	3	0	0	0					45	3	7	3
Femur	36	25	69	8	109	60	55	9	157	141	90	13
Fibula	15	8	53	7	59	28	47	10	58	46	79	13
Fragments					13	2	15	6	26	19	73	10
Humerus	20	6	30	7	93	28	30	6	126	94	75	9
Jaw	2	0	0	0	39	14	36	7	24	21	88	12
Metacarpal	1	0	0	0	6	0	0	2	10	3	30	5
Metatarsal	1	0	0	0	18	0	0	1	23	9	39	8
Pelvis	10	1	10	3	30	0	0	2	62	19	31	6
Phalanx									1	1	100	9
Radius	19	2	11	7	58	8	14	4	70	24	34	7
Rib					50	8	16	4	18	8	44	5
Scapula					35	1	3	4	54	14	26	5
Talus									1	1	100	17
Tibia	32	21	66	12	90	61	68	11	111	101	91	13
Ulna	19	1	5	4	64	12	19	5	68	34	50	6
Vertebra					41	21	51	9	26	11	42	8
Zygomatic					1	0	0	0				

Table 2.6. Overall success of AmpFlSTR® MiniFiler™ testing for all skeletal samples submitted to AFMES-AFDIL from DPAA between 1990 and 2018. Samples are listed alphabetically. The maxilla and mandible are combined together to form the category of "Jaw". The "Pelvis" category is primarily the os coxa, but also includes ilium, ischium, sacrum, and ramus.

The average number of loci has been rounded to the closest whole number.

There are a limited number of samples tested in any STR platform in the 'Extraction Protocol 1' extraction category. This extraction protocol ceased to be actively used in 2007, just prior to the implementation of STR protocols into active casework.

	Extraction Protocol #1				Extraction Protocol #2				Extraction Protocol #3			
	# Tested	# Success	% Success	Avg. # Loci	# Tested	# Success	% Success	Avg. # Loci	# Tested	# Success	% Success	Avg. # Loci
All	95	35	37	6	637	216	34	5	628	324	52	6
Clavicle	3	1	33	7					40	1	3	2
Femur	20	13	65	7	85	52	61	6	91	78	86	8
Fibula	6	5	83	8	51	28	55	6	38	28	74	7
Fragments					18	7	39	6	22	10	45	5
Humerus	21	4	19	6	87	30	34	4	88	51	58	6
Jaw	3	1	33	9	26	10	38	5	20	18	90	7
Metacarpal					2	0	0	0	10	1	10	5
Metatarsal	2	0	0	0	13	0	0	1	12	4	33	5
Pelvis	2	0	0	0	28	0	0	2	53	20	38	5
Phalanx									2	1	50	6
Radius	10	2	20	4	57	6	11	3	56	18	32	4
Rib					44	4	9	3	11	4	36	5
Scapula					21	2	10	3	35	7	20	3
Talus									1	1	100	9
Tibia	16	7	44	6	94	55	59	7	75	63	84	8
Ulna	12	1	8	5	58	5	9	3	52	20	38	4
Vertebra					38	17	45	6	19	9	47	5

2.8.4 PowerPlex® Fusion Testing

PowerPlex® Fusion is the most successful of the STR kits tested (Table 2.7). Of the 68 samples extracted using EP#2, 74% generated successful results. For EP#3, 207 samples were tested with a 92% success rate. In general, the long bones again performed among the best, with the femur (95%), fibula (92%) and tibia (95%) all having over a 90% success rate. The "jaw" was also highly successful using the combination of EP#3 and PowerPlex® Fusion (100%), as was the pelvis (92%).

2.8.5 NGS Testing

The overall success of NGS was 43% across the 550 samples tested (Table 2.8). Samples are not divided by extraction type as some initial testing contained one extraction using EP#2 and one extraction using EP#3. Successful generation of genetic data may appear to be somewhat low; however, it must be considered that samples selected for NGS testing have been subjected to severe chemical or environmental insult. Some elements have been tested multiple times in mtDNA Sanger sequencing using all three extraction protocols with no reportable results.

Table 2.7. Overall success of PowerPlex® Fusion testing for all skeletal samples submitted to AFMES-AFDIL from DPAA between 1990 and 2018. Samples are listed alphabetically. The maxilla and mandible are combined together to form the category of "Jaw". The "Pelvis" category is primarily the os coxa, but also includes ilium, ischium, sacrum, and ramus.

The average number of loci has been rounded to the closest whole number.

There are a limited number of samples tested in any STR platform in the "Extraction Protocol #1". This extraction protocol ceased to be actively used in 2007, just prior to the implementation of STR protocols into active casework.

	Extraction Protocol #1				**Extraction Protocol #2**				**Extraction Protocol #3**			
	# Tested	# Success	% Success	Avg. # Loci	# Tested	# Success	% Success	Avg. # Loci	# Tested	# Success	% Success	Avg. # Loci
All	2	2	100	13	68	50	74	9	207	192	92	12
Calcaneus									1	1	100	14
Clavicle									3	0	0	2
Femur					18	11	61	8	44	42	95	15
Fibula					11	9	82	13	25	23	92	16
Fragments									6	4	67	5
Humerus	1	1	100	14	7	4	57	7	25	21	84	10
Jaw					1	1	100	6	11	11	100	12
Metatarsal									2	2	100	12
Pelvis									13	12	92	7
Phalanx									1	1	100	7
Radius	1	1	100	11					16	14	88	7
Rib									2	0	0	3
Scapula									2	2	100	7
Tibia					22	19	86	10	37	35	95	18
Ulna					1	0	0	2	16	12	75	6
Vertebra					8	7	88	9	3	3	100	11

Table 2.8. Overall success of Next Generation Sequencing testing for all skeletal samples submitted to AFMES-AFDIL from DPAA between 1990 and 2018. Samples are listed alphabetically. The maxilla and mandible are combined together to form the category of "Jaw". The "Pelvis" category is primarily the os coxa, but also includes ilium, ischium, sacrum, and ramus.

NGS testing involves multiple different extraction protocols. Results are combined from different strategies including EP#2, EP#3, EP#4, and a modified EP#2.

NGS testing produces sequence information for the entire mitochondrial DNA genome; however, only the control region data are reported. All of the samples currently tested in NGS at AFMES-AFDIL are compromised in some fashion. Primarily this is chemical in nature, but can include burning or extreme degradation.

	NGS testing			
	# Tested	# Success	% Success	Avg. # Bases
All	550	237	43	16447
Calcaneus	2	0	0	0
Clavicle	16	2	13	16506
Femur	103	63	61	16481
Fibula	16	7	44	16502
Fragments	5	3	60	16508
Humerus	111	53	48	16461
Jaw	2	0	0	0
Metacarpal	6	4	67	16316
Metatarsal	8	5	63	16506
Pelvis	17	5	29	16304
Radius	34	8	24	16492
Scapula	8	1	13	16506
Tibia	176	71	40	16400
Ulna	36	14	39	16440
Vertebra	10	1	10	16143

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2.9 DISCUSSION

2.9.1 Extraction Protocol

In the field of human identification, skeletonized human remains are often presented as one of the greatest challenges. It has long been recommended that practitioners need a specific sampling strategy when faced with an assemblage of remains. This is especially true for commingled remains found in mass fatality incidents or mass graves. There are multiple papers expounding the ideal element to sample from remains (1,4,5). Recent publications have even specified from where on an element a DNA sample should be taken (19). However, what is evident from the presented results is that it is a combination of the extraction protocol and the modality from which results are desired that is the most important consideration when performing DNA testing. An organic purification of a completely demineralized sample (EP#2), regardless of the element tested, is the best choice if Sanger sequencing of mitochondrial DNA is being performed, with a 91% success achieved across all elements. For any STR platform, an inorganic purification of a completely demineralized sample (EP#3) is the best choice, which was also seen by Amory, et al. (20). The type of inorganic purification may prove to be irrelevant, although it certainly bears further study. For example, this study uses a Qiagen kit designed for amplification purification, Amory, et al. (20) uses an automated purification system (QIAcube robotic platform), and Hong, et al. (21) uses Chemagic MSM I (PerkinElmer, Waltham, MA).

Exposure of remains to chemical contamination is an aspect of DNA testing of skeletonized human remains that will be more fully addressed in another paper (Edson *in press*); however, it does bear discussing here. In-house results have indicated that organic purification of chemically compromised skeletal remains is more successful than an inorganic purification. EP#2 was originally developed at AFDIL during an attempt to ameliorate the effects of formalin saturation of remains recovered from the Korean War. Remains of US soldiers

passing through overseas port mortuaries during the Korean War were soaked in liquid formalin and then packed in a hardening compound for transportation. Attempts to extract DNA from the osseous materials associated with these remains routinely failed using EP#1. While results for these remains is slightly improved with EP#2 (data not shown), they are currently being processed using an NGS workflow. While NGS has been in active casework use since the beginning of 2016, the extraction protocol that most efficiently generates the best DNA for the downstream processing is still being determined. A protocol specifically designed for use with NGS cases was initially implemented (15); however, continued use has indicated that a modified Extraction Protocol #2 (EP#2) with an elevated input of osseous material (1.0 g vs 0.2 g) is better suited.

The largest set of chemically compromised remains that have been processed at AFDIL comprise over 5000 osseous elements submitted from the recovery of the USS *Oklahoma*. The remains saponified during decomposition due to submersion in an anaerobic environment comprised of salt water and fuel oil. Despite efforts to clean the remains, the odor of oil is still present and GC/MS testing of the osseous materials indicates the presence of fuel (22). Upon receipt of the cases to AFDIL, a subset of the samples was extracted in both EP#2 and EP#3. Those extracted using EP#2 were able to be amplified using the larger primer sets and less input volume of DNA than those extracted using the inorganic purification method. The long chain hydrocarbons present in the fuel bind to the materials within the QIAquick column and prevent the binding of a proportion of the DNA fragments, allowing them to be removed in subsequent washes. Given the results of this test, organic purifications are routinely used as the first pass extraction method for any osseous materials determined to be chemically compromised.

The interaction of larger molecules with the QIAquick columns gives an insight into why EP#3 is not as successful with the extraction of mitochondrial DNA as organic extractions (EP#2). It is possible that the larger molecules of DNA are binding with the column, which prevents smaller fragments from binding as well as they should. The subsequent DNA extract recovered off of the column after purification is very 'clean', yet fails to contain many of the target fragments of mitochondrial DNA. Samples extracted using EP#3 clearly do not fail more often than not in Sanger sequencing of mtDNA. They are simply less successful than would be desired should the primary modality of the laboratory be mtDNA analysis.

As shown in the data, not all organic extractions provide successful results for DNA analysis. Organic purification using an elevated amount of skeletal material (EP#1) is suboptimal for either Sanger sequencing of mtDNA or STR analysis. The buffer used in this extraction protocol contains much less EDTA (50 mM vs. 0.5 M) than the extraction buffer currently in use. It is also more similar to Queen's lysis buffer (10 mM Tris-HCl; 10 mM NaCl; 10 mM EDTA; 1.0% *N*-lauroylsarcosine; pH 8.0) (23), which is commonly used to lyse blood and tissue cells and stabilize DNA. While it is completely serviceable as a buffer with which to preserve DNA, it is not optimal for the task of extracting DNA from bone. Another reason why EP#1 may not have worked so well as EP#2 or EP#3 is the volume of bone being extracted. The 2.5 g of bone powder being extracted in EP#1 always failed to completely demineralize. As has been determined internally, the ratio of bone powder to extraction buffer was not correct. The volume of bone to be extracted simply could not be demineralized in 3.0 mL of extraction buffer. Increasing the amount of available buffer may have increased success rates. During the development of EP#4, larger volumes of extraction buffer were used for the extraction of up to 2.0 g of powdered osseous material to some success. Were samples originally tested in EP#1 tested using present protocols (e.g., EP#2 or EP#3), there is an expectation that the sample success would be more reflective of the updated protocol rather than the original.

2.9.2 Element Selection

Most of the publications on the subject fail to completely agree on which element is the best from which to sample for DNA testing. For the most part, the conventional wisdom recommends sampling from a long bone or any bone that is very compact in structure (4,5,24), and this is common practice in many forensic laboratories. Long bones provide a large mass from which to remove a sample for DNA testing. In addition, they tend to articulate to other elements or can be pair-matched via anthropological techniques, allowing for an identification of a greater number of elements to an individual without having to resort to expensive DNA testing for all of the samples. Concurrence between the anthropological and DNA testing results are necessary, and communication between the anthropologists and DNA analysts during testing will improve the quality of results, as better choices of skeletal samples and extraction protocols can be made if both groups are aware of the circumstances surrounding the event and the DNA testing protocols available.

For this study, the femur, tibia, and fibula were generally the most successful for any extraction protocol and modality tested. The exception to this is Next Generation Sequencing, in which only the femur was considered successful (61%). While other elements are sometimes successful, they are not consistently successful across all platforms. Metacarpals and metatarsals are shown to have a high rate of success in mtDNA Sanger sequencing for both full demineralization protocols (EP#2 and EP#3). However, they fail to be successful in either Minifiler (EP#2: 0% for both; EP#3: 10% and 33%, respectively) or the modified Yfiler protocol (EP#2: 0% for both; EP#3: 30% and 39% respectively). Although other studies have shown that metacarpals and metatarsals were successful for DNA testing, this result is somewhat unexpected. Mundorff and Davoren (1) found these two elements were useful for STR analysis of modern skeletal remains and did not examine Sanger sequencing of mtDNA. It has been hypothesized that the success of metacarpals and metatarsals is due to the high degree of trabecular bone within the elements. Andronowski, et

al. (2) found that tissue accretes within the porous bone during decomposition, thus providing additional biological material from which DNA may be extracted. It is possible that the material is retained during cleaning. It is difficult to fully remove trabecular bone from smaller fragments, as to do so could reduce the sample tested to a thin shell of dense bone.

Selection of smaller elements such as metatarsals, metacarpals, patellae and tali can be very useful when the purpose is to identify largely intact skeletons. These elements can easily be removed in the field and sent to the DNA testing lab before the remains have even been removed from the gravesite. However, caution should be taken should a laboratory chose this path of analysis. It should be recommended that a complete skeletal survey be undertaken, including a determination of the minimum number of individuals, prior to the removal of elements for DNA testing. Preferentially choosing to sample smaller elements will also only provide evidence of the presence of an individual, and may not provide sufficient evidence for an identification as these elements tend to not be anthropologically relevant.

With the implementation of EP#2, it should be noted that there was an almost three-fold increase in bone fragments submitted from 2007 until present versus those submitted between 1992 and 2006 (772 vs. 240). This is due to the reduction in size of fragment required for the DNA extraction protocols. EP#1 required an input of 2.5 g, while both EP#2 and EP#3 allow for a smaller fragment size of 0.2 g (Figure 2.2). This reduction in size of required input encouraged DPAA scientists to reevaluate cases and sample materials previously considered untestable, the smallest fragment tested being 0.05 g. Concurrent with the implementation of EP#2 was a 12S rRNA survey, which has proven to be very useful in the course of regular casework (18). Elements submitted as non-specific “long bones” or “fragments” are typically tested using the 12S rRNA assay as the first amplification after extraction. Samples

confirmed to be non-human at this early stage are removed from the workflow, thus saving time and expense.

There is an expectation that there will be marked differences between elements recovered from different conflicts (e.g., Korean War, Southeast Asia, World War II), and even different events within the same conflict (e.g., USS *Oklahoma*, Battle of Tarawa). These differences are not explored within the context of this paper, as they are beyond the scope of the analysis. Studies of each conflict and certain incidents within each conflict are currently underway.

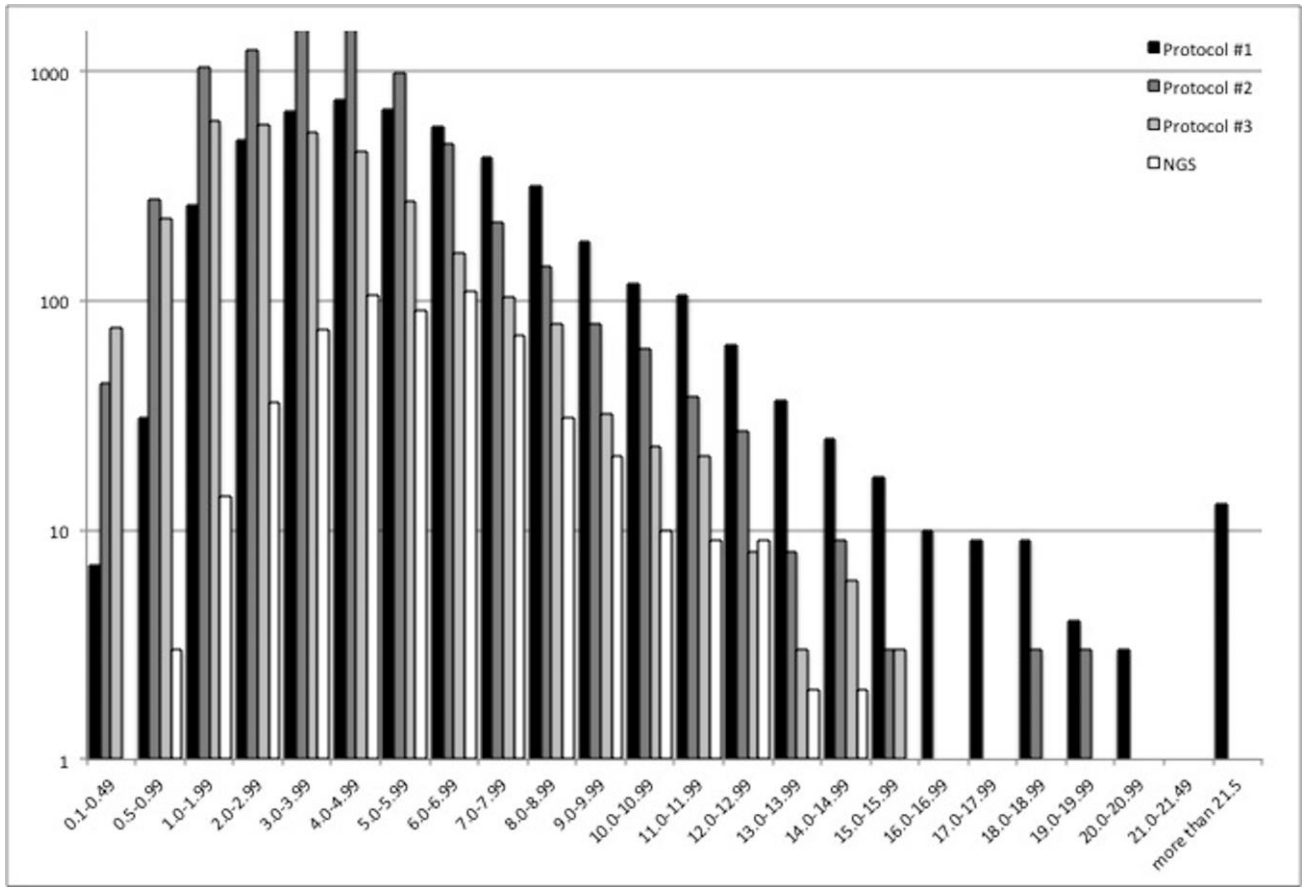

Figure 2.2. Distribution of size of samples submitted for testing. Original weights in grams of samples submitted for testing are listed across the horizontal axis. The numbers of samples are distributed on a logarithmic scale for normalization. Extraction Protocol #1 has a wider distribution of samples sizes. Extraction Protocols #2 and #3 trend towards reduced sample weights due to the small input (0.2 g) required. NGS testing is trending towards larger sample sizes. While 1.0 g is the initial sample required, multiple extractions may occur due to the typically compromised nature of the skeletal remains.

2.9.3 PowerPlex® Fusion

When examining the results of PowerPlex® Fusion results versus the results of both AmpFlSTR® MiniFiler™ and the modified AmpFlSTR® Yfiler™, it should be noted that Fusion performed better than either of the other two kits. MiniFiler and modified Yfiler are designed specifically to work with degraded skeletal remains, yet only have 52% and 62% success rates, respectively, when used with DNA extracted using EP#3. Conversely, Fusion has a 92% success rate with similarly extracted DNA. This success may be artificially inflated due to the workflow within the laboratory. Samples are typically tested initially in Minifiler, and a partial profile is generated prior to the sample being pushed to a Fusion workflow. There are rare incidents in which no profile is generated Minifiler, yet the sample proceeds to be amplified in Fusion. Should samples be tested in Fusion without first being triaged, it could be expected that the Fusion success rates would be more similar to those seen in MiniFiler.

2.10 CONCLUSIONS

The efficiency of the extraction process determines the overall success of downstream DNA processing, regardless of whether the testing is direct Sanger sequencing of mitochondrial DNA, STR analysis, or NGS/MPS. Effective removal of inhibitors without a concurrent loss of DNA is the hallmark of a 'good' extraction protocol. Improvements of extraction protocols need to be considered. While laboratories can continue to use the existing protocols, as they are certainly effective, a reduction in stringency that targets only known to be present inhibitors/chemicals should cause an increase in the retained DNA.

What can be clear from the results is that it is not necessarily the bone type that it the deciding factor in whether the sample is successful. One must first take into consideration the platform to be tested. For example, selecting Extraction Protocol #2 (complete demineralization with

organic purification) may not be the best choice for any STR platform, regardless of what skeletal element is to be tested. The practitioner must first consider the circumstances surrounding the event (i.e., whether the remains are highly commingled or in discrete burials), and then the end goal for DNA testing. If it is enough to know that a person was simply present, smaller, easily accessible bones such as metacarpals, metatarsals, and patellae, are sufficient. However, if anthropological reassociation or extensive sorting is necessary, elements that rearticulate or provide some form of anthropological benchmark (e.g., height) would be preferred. Selection of a skeletal element for DNA testing is more complex that simply what is accessible. Many times, it is not the DNA scientist who is making these decisions, but rather the anthropologist or medical examiners. Education across all disciplines involved in human identification is necessary for rapid, effective use of DNA testing on skeletonized remains.

DNA testing of skeletonized human remains cannot, and should not, be a one size fits all solution. Selection of a skeletal element for DNA testing is a matrix of choices rather than a simple yes or no.

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2.12 SUPPLEMENTAL DIAGRAMS

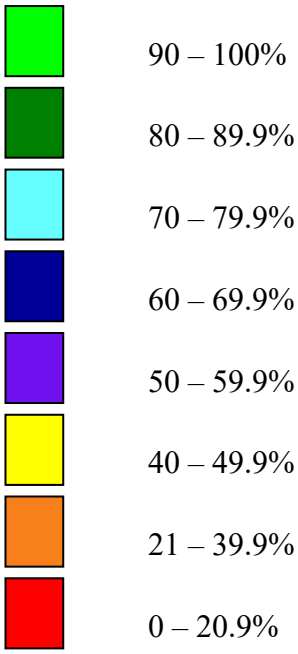
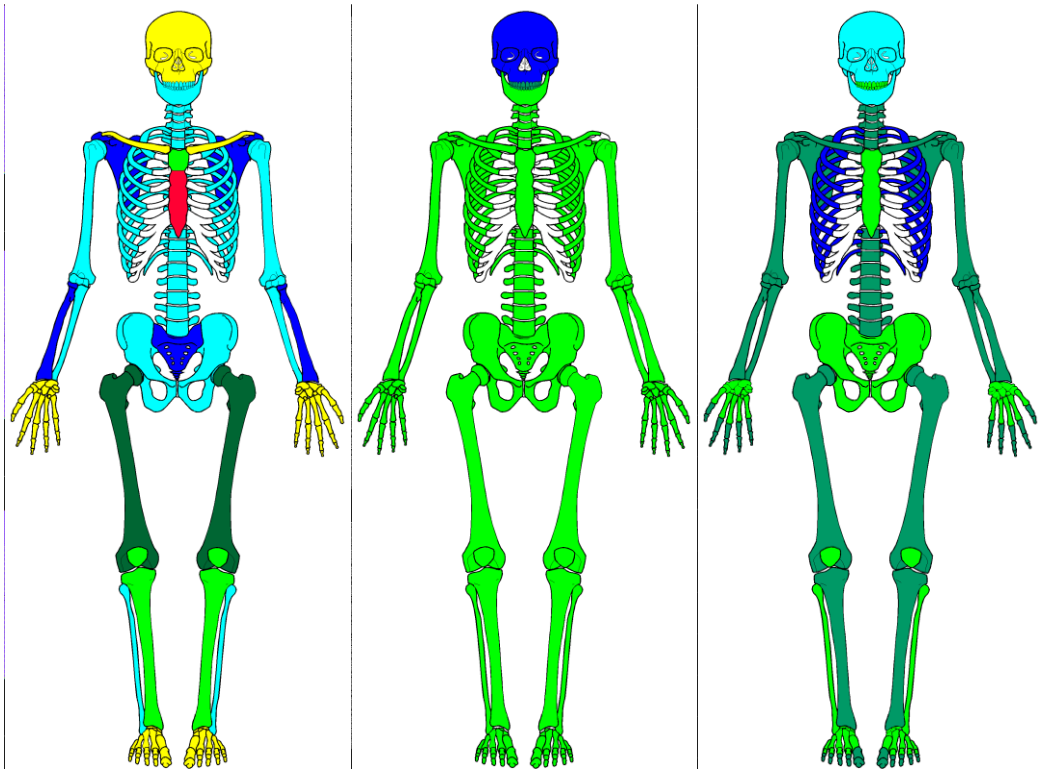
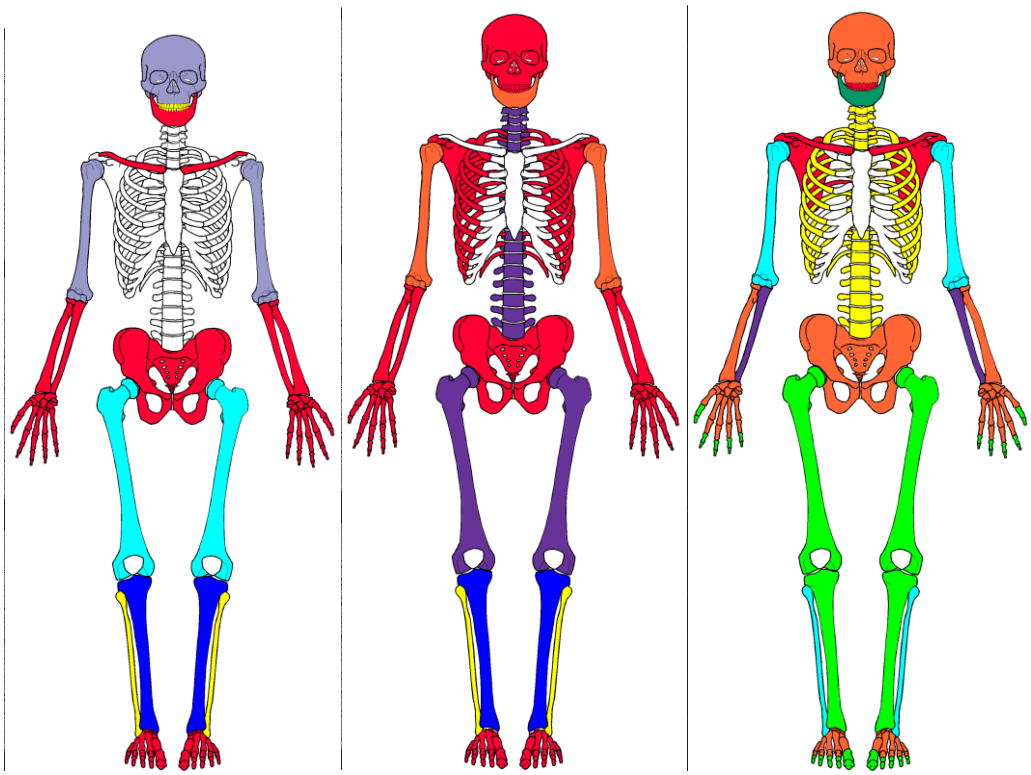


Figure 2.3 The key for the following diagrams.



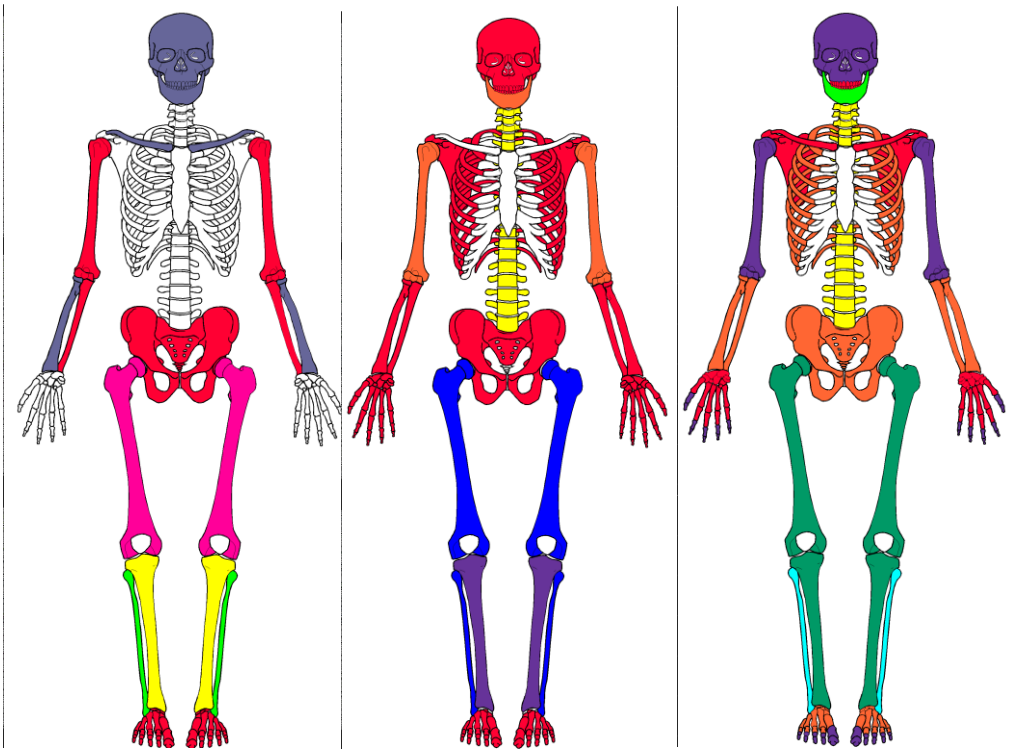
EP#1 EP#2 EP#3

Figure 2.4 Mitochondrial DNA using Sanger Sequencing testing by skeletal element. The individual skeletal elements are labeled based on the overall success for that element. Each extraction protocol, 1 – 3, is accounted for. This is a graphic representation of the data shown in Table 2.4.



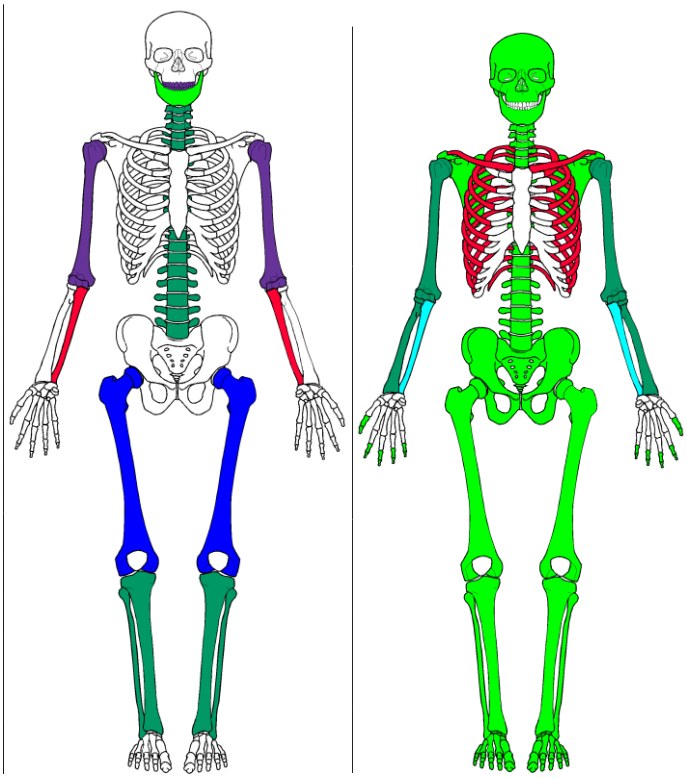
EP#1 EP#2 EP#3

Figure 2.5 Modified AmpFlSTR® Yfiler™ testing success by skeletal element. The individual skeletal elements are labeled based on the overall success for that element. Each extraction protocol, 1 – 3, is accounted for. This is a graphic representation of the data shown in Table 2.5.



EP#1 EP#2 EP#3

Figure 2.6 AmpFlSTR® MiniFiler™testing success by skeletal element. The individual skeletal elements are labeled based on the overall success for that element. Each extraction protocol, 1 – 3, is accounted for. This is a graphic representation of the data shown in Table 2.6.



EP#2 EP#3

Figure 2.7 PowerPlex Fusion® testing success by skeletal element. The individual skeletal elements are labeled based on the overall success for that element. No samples were tested in PowerPlex Fusion using EP#1. This is a graphic representation of the data shown in Table 2.7.

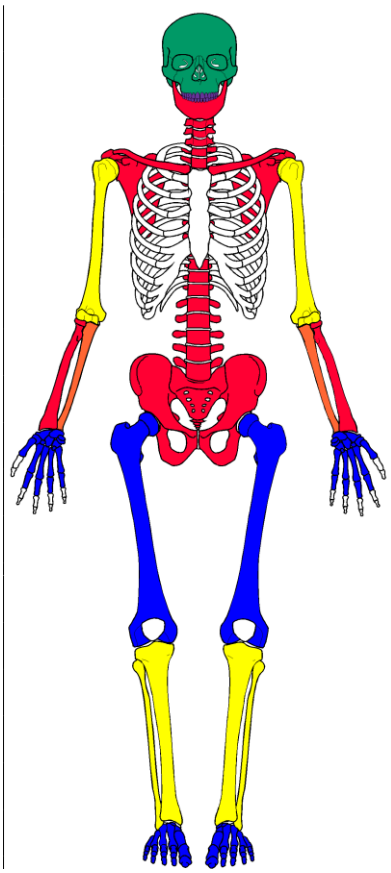


Figure 2.8 Next Generation Sequencing (NGS) testing success by skeletal element. The individual skeletal elements are labeled based on the overall success for that element. Only EP#4 is considered for this protocol. This is a graphic representation of the data shown in Table 2.8.

2.13 PRESENTATIONS

A presentation entitled “Success of DNA Testing of Skeletonized Human Remains and Comparison of Organic vs. Inorganic Extraction Protocols” was presented at the annual meeting of the American Academy of Forensic Sciences Meeting in New Orleans, LA (February 2017). The presentation encompassed skeletal extraction data from 1990-2016. The presentation can be found on the following pages.

A variation on this presentation was given by Ms. Edson at the Promega Corporation Tech Tour in Norfolk, VA (April 2017). In addition, a similar presentation was given by other analysts at AFDIL with Ms. Edson listed as a co-author. The additional presentations are as follows:

JC Kappeller and SM Edson. Success Of DNA Testing Of Skeletonized Human Remains: Choose Your Own Adventure Vs. A Cookbook Process To Get The Best Results. Presented at the Promega Tech Tour in Frankfort, KY, May 2017.

KR Sween and SM Edson. Success of DNA Testing of Skeletonized Human Remains: Not a One Size Fits All Approach. Presented at the Promega Tech Tour in Baton Rouge, LA, June 2017.

GM Parada and SM Edson. Success of DNA Testing of Skeletonized Human Remains: Not a One Size Fits All Approach. Presented at the Promega Tech Tour in Pasadena, CA, August 2017.

JA O’Rourke and SM Edson. Success of DNA Testing of Skeletonized Human Remains: Not a One Size Fits All Approach. Presented at the Promega Tech Tour in Hamilton, NJ, September 2017.

Success of DNA Testing of Skeletonized Human Remains and Comparison of Organic vs. Inorganic Extraction Protocols

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Disclaimer

The opinions or assertions presented hereafter are the private views of the speaker and should not be construed as official or as reflecting the views of the Department of Defense; the Defense Health Agency; the Armed Forces Medical Examiner System; ARP Sciences, LLC; or the Defense POW/MIA Accounting Agency.

Commercial Products

Commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible, and does not imply that any of the commercial products identified are necessarily the best available for the purpose.

Financial Disclaimer

- The authors are both employees of their respective agencies.
- Ms. Edson is a student a Flinders University and has a scholarship.
- No other financial remuneration was received by either author in exchange for this work.

Outline

- Introduction to the Mission
- Extraction Processes
  - Modifications – 3 different techniques
  - Variations in Success for mitochondrial DNA and STR analysis
- Skeletal sampling strategy
- What does this all mean?

Armed Forces DNA Identification Laboratory (AFDIL)

- Established in 1991 as the DoD DNA Registry
  - With the primary purpose of identifying the remains of missing US service members.
- A subdivision of the Armed Forces Medical Examiner System (AFMES)
- Mission Partner with Defense POW/ MIA Accounting Agency (DPAA)

Defense POW/MIA Accounting Agency -- Scientific Analysis Division

- Largest laboratory of Forensic Anthropologists in the United States
- Formed for the purpose of identifying the remains of missing US service members

The primary mission of the AFMES DNA Identification Laboratory Past Accounting Section & DPAA

is the identification of missing US service members from past military conflicts.

Extraction of DNA from Skeletal Materials

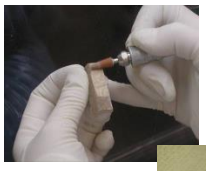
Extraction of DNA from Skeletal Materials

- Since 1992, AFDIL has reported 17,280 analyses of skeletonized remains.
- Encompasses:
  - Three different extraction protocols
  - Sanger sequencing of mitochondrial DNA
  - STR analysis

Extraction of DNA from Skeletal Materials

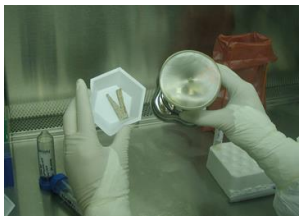
- Results from all conflicts are combined in this presentation for normalization.
- Focusing only on a single location or samples from only one conflict could skew the results and prevent developing a strategy that could be effective across a broad set of circumstances.

Cleaning



Cleaning samples is common to all of the procedures regardless of downstream processing.

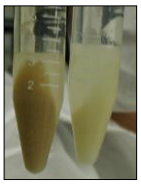
Powdering



All bones are powdered in a Waring blender regardless of protocol

Extractions

	Original Extraction
Weight of Bone	2.0-2.5g
Extraction Buffer Composition	10mM Tris, pH8.0 50mM EDTA, pH8.0 0.5% SDS Proteinase K
Incubation Time	Overnight at 56°C
Purification	PCIA Wash with Butanol
Concentration	Centrifugal filters



Extractions

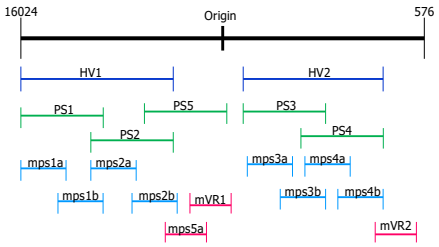
	Original Extraction	Demineralization 1
Weight of Bone	2.0-2.5g	0.2-0.25g
Extraction Buffer Composition	10mM Tris, pH8.0 50mM EDTA, pH8.0 0.5% SDS Proteinase K	0.5M EDTA, pH8.0 1% lauroyl-sarcosinate Proteinase K
Incubation Time	Overnight at 56°C	Overnight at 56°C
Purification	PCIA Wash with Butanol	PCIA Wash with Butanol
Concentration	Centrifugal filters	Centrifugal filters

Extractions

	Original Extraction	Demineralization 1	Demineralization 2
Weight of Bone	2.0-2.5g	0.2-0.25g	0.2-0.25g
Extraction Buffer Composition	10mM Tris, pH8.0 50mM EDTA, pH8.0 0.5% SDS Proteinase K	0.5M EDTA, pH8.0 1% lauroyl-scarcosinate Proteinase K	0.5M EDTA, pH8.0 1% lauroyl-scarcosinate Proteinase K
Incubation Time	Overnight at 56°C	Overnight at 56°C	Overnight at 56°C
Purification	PCIA Wash with Butanol	PCIA Wash with Butanol	QiaQuick Column/Wash
Concentration	Centrifugal filters	Centrifugal filters	Centrifugal Filters

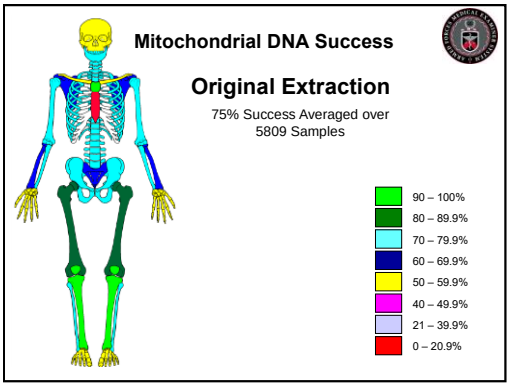
Mitochondrial DNA Testing

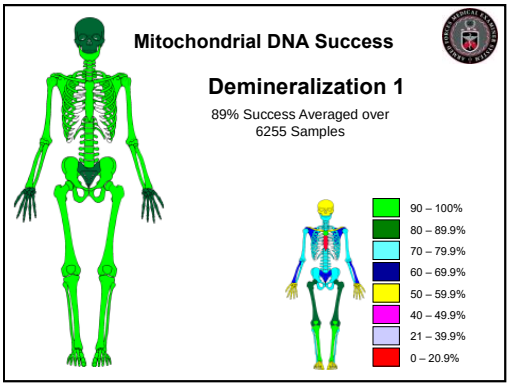
- Sanger Sequencing of the mitochondrial DNA control region.

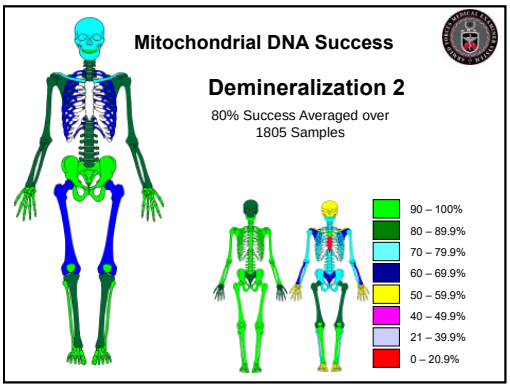


Success Rates

	Original Extraction	
	Number tested	%Success
Mitochondrial DNA	5809	75%
Minifiler		
Modified Y-Filer		
Identifiler Plus		
PowerPlex Fusion		







Success Rates

	Original Extraction		Demineralization 1 (Organic)		Demineralization 2 (Inorganic)	
	Number tested	%Success	Number tested	%Success	Number tested	%Success
Mitochondrial DNA	5809	75%	6256	89%	1805	80%
Minifiler						
Modified Y-Filer						
Identifiler Plus						
PowerPlex Fusion						

Is an inorganic extraction an issue for DNA analysis?

Not Really.

STR Strategies

- Minifiler
- Modified Y-Filer
- Identifiler Plus
- PowerPlex Fusion

To be considered 'reportable' the alleles at a locus must be replicated in at least two amplifications AND at least four loci must be present.

Success Rates

	Original Extraction		Demineralization 1 (Organic)		Demineralization 2 (Inorganic)	
	Number tested	%Success	Number tested	%Success	Number tested	%Success
Mitochondrial DNA	5809	75%	6256	89%	1805	80%
Minifiler	103	32%	839	29%	411	48%
Modified Y-Filer						
Identifiler Plus						
PowerPlex Fusion						

Success Rates

	Original Extraction		Demineralization 1 (Organic)		Demineralization 2 (Inorganic)	
	Number tested	%Success	Number tested	%Success	Number tested	%Success
Mitochondrial DNA	5809	75%	6256	89%	1805	80%
Minifiler	103	32%	839	29%	411	48%
Modified Y-Filer	173	40%	988	31%	634	57%
Identifiler Plus						
PowerPlex Fusion						

Success Rates

	Original Extraction		Demineralization 1 (Organic)		Demineralization 2 (Inorganic)	
	Number tested	%Success	Number tested	%Success	Number tested	%Success
Mitochondrial DNA	5809	75%	6256	89%	1805	80%
Minifiler	103	32%	839	29%	411	48%
Modified Y-Filer	173	40%	988	31%	634	57%
Identifiler Plus	1	0%	25	60%	30	60%
PowerPlex Fusion						

Success Rates

	Original Extraction		Demineralization 1 (Organic)		Demineralization 2 (Inorganic)	
	Number tested	%Success	Number tested	%Success	Number tested	%Success
Mitochondrial DNA	5809	75%	6256	89%	1805	80%
Minifiler	103	32%	839	29%	411	48%
Modified Y-Filer	173	40%	988	31%	634	57%
Identifiler Plus	1	0%	25	60%	30	60%
PowerPlex Fusion			81	77%	50	96%

Success Rates

	Demineralization 1 (Organic)			Demineralization 2 (Inorganic)		
	Number tested	%Success	Average quality	Number tested	%Success	Average quality
Mitochondrial DNA	6256	89%	611	1805	80%	543
Minifiler	839	29%	2.4	411	48%	3.9
Modified Y-Filer	988	31%	2.8	634	57%	6.7
Identifiler Plus	25	60%	5.8	30	60%	7
PowerPlex Fusion	81	77%	8.9	50	96%	15.8

But that's not the whole story…

- Samples that have been compromised by oils and other preservatives, do not work with inorganic protocols.
  - The oils preferentially bind to the column and DNA is lost.

So which extraction is better?

It depends on what you want to do and the quality of the remains.

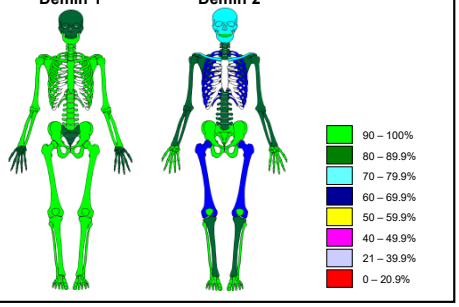
But it also depends on what element you're testing.

Skeletal Sampling Strategy

Skeletal Sampling Strategy

- A lot of research has been done to determine a hierarchy of skeletal elements for DNA testing.
  - Conventional wisdom says large, weight-bearing bones are the best.
  - Some papers have indicated that smaller bones such as metatarsals, metacarpals, and patellae are better.

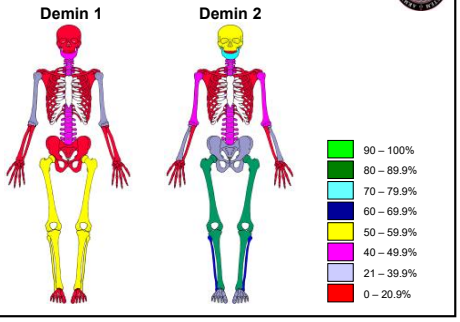
Mitochondrial DNA Success



Mitochondrial DNA Success

	Demineralization 1 (Organic)					Demineralization 2 (Inorganic)				
	Femur	Hum.	MC (Hand)	MT (Foot)	Patella	Femur	Hum.	MC (Hand)	MT (Foot)	Patella
mtDNA	90%	92%	89%	96%	100%	68%	82%	97%	98%	97%
Minifiler										
Modified Y-Filer										
Identifiler Plus										
PowerPlex Fusion										

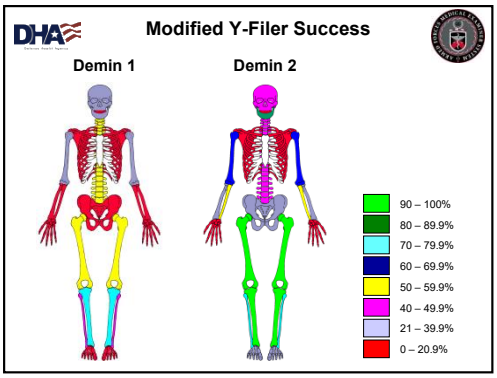
Minifiler Success



Minifiler Success

	Demineralization 1 (Organic)					Demineralization 2 (Inorganic)				
	Femur	Hum.	MC (Hand)	MT (Foot)	Patella	Femur	Hum.	MC (Hand)	MT (Foot)	Patella
mtDNA	90%	92%	89%	96%	100%	68%	82%	97%	98%	97%
Minifiler	58%	32%	0%	0%		84%	83%	0%	17%	
Modified Y-Filer										
Identifiler Plus										
PowerPlex Fusion										

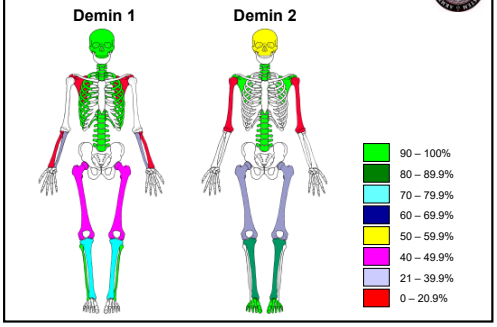
Modified Y-Filer Success



Modified Y-Filer Success

	Demineralization 1 (Organic)					Demineralization 2 (Inorganic)				
	Femur	Hum.	MC (Hand)	MT (Foot)	Patella	Femur	Hum.	MC (Hand)	MT (Foot)	Patella
mtDNA	90%	92%	89%	96%	100%	68%	82%	97%	98%	97%
Minifiler	58%	32%	0%	0%		84%	83%	0%	17%	
Modified Y-Filer	55%	31%	0%	0%		90%	63%	0%	38%	
Identifiler Plus										
PowerPlex Fusion										

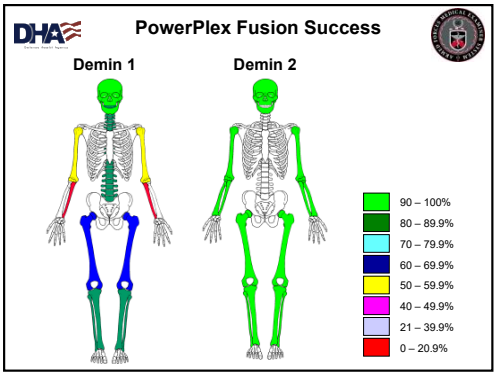
Identifiler Plus Success



Identifiler Plus Success

	Demineralization 1 (Organic)					Demineralization 2 (Inorganic)				
	Femur	Hum.	MC (Hand)	MT (Foot)	Patella	Femur	Hum.	MC (Hand)	MT (Foot)	Patella
mtDNA	90%	92%	89%	96%	100%	68%	82%	97%	98%	97%
Minifiler	58%	32%	0%	0%		84%	83%	0%	17%	
Modified Y-Filer	55%	31%	0%	0%		90%	63%	0%	38%	
Identifiler Plus	43%					25%	0%		100%	
PowerPlex Fusion										

PowerPlex Fusion Success



PowerPlex Fusion Success

	Demineralization 1 (Organic)					Demineralization 2 (Inorganic)				
	Femur	Hum.	MC (Hand)	MT (Foot)	Patella	Femur	Hum.	MC (Hand)	MT (Foot)	Patella
mtDNA	90%	92%	89%	96%	100%	68%	82%	97%	98%	97%
Minifiler	58%	32%	0%	0%		84%	83%	0%	17%	
Modified Y-Filer	55%	31%	0%	0%		90%	63%	0%	38%	
Identifiler Plus	43%					25%	0%		100%	
PowerPlex Fusion	65%	50%				92%	100%			

Consistency

	Demineralization 1 (Organic)					Demineralization 2 (Inorganic)				
	Femur	Hum.	MC (Hand)	MT (Foot)	Patella	Femur	Hum.	MC (Hand)	MT (Foot)	Patella
mtDNA	90%	92%	89%	96%	100%	68%	82%	97%	98%	97%
Minifiler	58%	32%	0%	0%		84%	83%	0%	17%	
Modified Y-Filer	55%	31%	0%	0%		90%	63%	0%	38%	
Identifiler Plus	43%					25%	0%		100%	
PowerPlex Fusion	65%	50%				92%	100%			

Skeletal Sampling Strategy

- It is difficult to give a one size fits all strategy for sampling of skeletal remains.
- Small bones are convenient, but they're not anthropologically useful (generally).
- You need to pick a strategy that works for your specific situation.

Conclusions

- Use the extraction technique that is best for your specific incident and testing requirements.
  - Chemically treated samples work best with organic extractions.
- Choose a skeletal element based on the circumstances, not a checklist.

This is science, not IKEA instructions!

Continuing Work

- Evaluation of 2000 additional reactions completed in the last year.
- Incorporation of quantification values into the quality metrics.
- Evaluation of materials on the skeletal elements and what may be co-extracting with the DNA.
- Site-specific variation in DNA success.

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- Dr. Adrian Linacre
- Dr. Duncan Taylor
- Dr. Mike Coble
- Ms. Audrey Meehan

The AFMES, AFDIL, and DPAA Staff

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AFMES Additional Information: http://www.health.mil/afmes

DPAA Additional Information: http://www.dpaa.mil/

Chapter 3

Getting Ahead: Extraction of DNA from Skeletonized Cranial Material and Teeth

3.1 INTRODUCTION

Sampling of the cranium for the purposes of testing the osseous materials for DNA analysis can be considered more difficult that sampling of post-cranial remains. While it is certainly true that it is physically trickier to remove an adequate bone fragment from an intact skull rather than a femur, the difficulty lies in preserving the quality of the skull for the family of the decedent. In many cultures, the skull and/or face is considered to the determinant of 'self' (Paterson, 2010; Overholter and Argueta 2018; Ciliberti, et al., 2018) and disruption of that portion of the remains may cause the family undue distress. It is in the best interests of the practitioner to not only remove the portion of the skull for DNA testing that will provide the greatest chance of success, but also to preserve the outward appearance of the skull for repatriation.

This is a continuation of the work done in Chapter 2. Selection of the optimal bone within the crania that couples the high probability of DNA success with minimal destruction is examined by comparing all cranial elements across four different DNA extraction protocols as well as five DNA testing modalities. Included in this study is an examination of DNA testing on the teeth. Due to a high degree of mineralization as compared to osseous material, an extraction protocol using an inorganic purification method provides an improved DNA yield for teeth. In the supplemental materials, the DNA testing success of each individual tooth in each of three modalities (Sanger sequencing of mtDNA, AmpFlSTR® MiniFiler™; and a modified AmpFlSTR® Yfiler™) are examined. Teeth with a larger amount of dentine (e.g., molars) tend to work better than all other teeth for Sanger sequencing, although there is an unexplained decrease in success for those teeth in the lower right quadrant.

This chapter provides a discussion of a selection of the DNA extraction protocols used in different forensic laboratories world-wide. Despite the continued desire within the field to

improve downstream DNA testing protocols, there has been a limited focus on the front end of the workflow. We, as a community, fail to agree on how to best extract DNA from osseous materials. Other than a general acceptance that the DNA extract be free of inhibitors, the actual protocols on how to achieve this are widely disparate (Table 3.7).

This chapter will present some suggested testing strategies for the DNA testing of cranial elements and teeth. While there is some sampling bias caused by the selection of a large number of occipitals from a single mass casualty event, this is addressed and the corrected data provided.

3.1.1 INTRODUCTION REFERENCES

Ciliberti R, F Monza, F De Stefano, M Licata. The trial of the skull studied by the founder of Criminal Anthropology: The war of the Lombroso Museum. Journal of Forensic and Legal Medicine 59:13-15.

Overholtzer L, JR Argueta. 2018. Letting skeletons out of the closet: the ethics of displaying ancient Mexican human remains. International Journal of Heritage Studies 24(5):508-530.

Paterson, RK. 2010. Heading Home: French Law Enables Return of Maori Heads to New Zealand. International Journal of Cultural Property 17(4):643-652.

3.2 PUBLICATION

This paper is accepted for publication in the Journal of Forensic Sciences. It is due to be included in the November 2019 print edition and published on-line July 2019.

SM Edson. Getting Ahead: Extraction of DNA from Skeletonized Cranial Material and Teeth.

Journal Impact Factor: 1.438

3.3 TITLE PAGE

Getting Ahead: Extraction of DNA from Skeletonized Cranial Material and Teeth

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The work was presented in part at the 2019 American Academy of Forensic Sciences Annual meeting in Baltimore, Maryland.

3.3.1 DISCLAIMER

The opinions or assertions presented are the private views of the author and should not be construed as official or as reflecting the views of the Department of Defense; the Defense Health Agency; the Armed Forces Medical Examiner System; or the Defense POW/MIA Accounting Agency.

3.3.2 FINANCIAL DISCLOSURE

The author received no monies in the completion of this work other than a salary as an employee.

3.3.3 ACKNOWLEDGEMENTS

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3.4 ABSTRACT

Between 1990 and 2018, the Defense POW/MIA Accounting Agency submitted 2177 cranial elements and 1565 teeth to the Armed Forces Medical Examiner System – Armed Forces DNA Identification Laboratory for DNA testing. In an effort to identify missing United States service members, materials were recovered from wartime losses inclusive of World War II, the Korean War, and Southeast Asia. Using four different DNA extraction protocols, DNA testing was performed using mitochondrial DNA Sanger sequencing, modified AmpFlSTR® Yfiler™, AmpFlSTR® MiniFiler™, PowerPlex® Fusion, or Next Generation Sequencing (NGS). This paper aims to provide optimal strategies for the DNA testing of skeletonized cranial materials. Cranial elements produced the most consistent results in Sanger sequencing using an organic purification; however, teeth were most successful for the same platform with an inorganic purification. The inverse is true for STR testing of cranial bones. Of all of the cranial elements, the temporal provided the most consistent results.

3.5 KEY WORDS

Forensic Science; forensic DNA analysis; DNA typing; forensic anthropology; skeletonized human remains; STR analysis; mitochondrial DNA; teeth; cranial bones; temporal

3.6 ARTICLE INTRODUCTION

In cases of mass fatalities, a number of metrics are part of forming an identification of the deceased. DNA, anthropology, pathology, archaeology, and fingerprints can be a few of these metrics, and may not all be needed. As cases of human identification become more complex, due to fragmentation or commingling, a greater reliance is placed on DNA as the lead to the identification process. When the remains are skeletonized and DNA testing is required, recommendations have been made to select the femur first and then other weight bearing bones, as these tend to have improved success rates for all forms of DNA testing (1,2,3,4). When fragmentation or commingling is limited, smaller bones such as metacarpals, phalanges, or patellae may be chosen as they also have the potential to generate genetic data for associations to be made, although their anthropological value is minimal (5,6,7).

With large sets of commingled, skeletonized human remains, it may be necessary to sample the cranium or teeth for DNA in order to reassociate the crania with post-cranial remains. Dentition may provide positive evidence for the identity of the skull itself; however, the lack of points of articulation with the post-crania prevents reassociation without DNA analyses. A number of studies have indicated that the petrous portion of the temporal is the optimal element of the skull for DNA testing regardless of extraction protocol or testing platform used (8,9,10,11,12). The petrous is often considered the densest bone in the body (13), as it has a 'woven' structure that protects the DNA present and limits microbial activity post-mortem (14).

Teeth are similar to the petrous in that they are likewise self-contained and have an exterior structure that reduces the amount of microbial activity, which protects the DNA present. Studies of teeth pulled from modern mass fatality events typically use the fresh pulp (15,16,17,18). However, in dried remains, the pulp has typically decomposed or desiccated,

requiring the use of either the dentine or the cementum for DNA testing. Teeth are considered an optimal source of DNA (4,19), yet they may not always be available for testing and are similar to small bones in lacking in anthropological value when they are not articulated within the jaw.

Since 1990, the Defense POW/MIA Accounting Agency (DPAA) has submitted 2177 cranial fragments to the Armed Forces Medical Examiner System – Armed Forces DNA Identification Laboratory (AFMES-AFDIL, aka AFDIL) for DNA processing. These include the vault (23 samples); frontal (79); occipital (686); parietal (223); and temporal (372). Teeth were considered separately from the osseous materials. DPAA has submitted 1565 teeth to AFDIL since 1990. These include canine (359 samples); incisor (190); premolar (321); and molar (676). In addition, a non-specified "general cranium" category was submitted (371 samples). These samples may be too small or too damaged to be categorized as a specific portion of the skull. The zygomatic, maxilla, and mandible are also included in the analysis of the crania. This publication seeks to evaluate the testing of the cranial elements in regular casework over four different extraction protocols and five DNA testing platforms: mitochondrial DNA (mtDNA) Sanger sequencing; AmpFlSTR® MiniFiler™ (Life Technologies, Gaithersburg, MD); PowerPlex® Fusion (Promega, Madison, WI); a modified AmpFlSTR® Yfiler™ (Life Technologies); and Next Generation Sequencing (NGS), aka Massively Parallel Sequencing (MPS). The goal being to present recommendations for DNA testing of cranial elements that could be used in a variety of mass fatality events.

3.7 METHODS AND MATERIALS

Except where relevant, the most current protocol in use at AFDIL is described.

3.7.1 Sample Selection

Recovery sites may seem straight-forward but may vary widely in the condition of the remains. Ambient temperatures, humidity, and acidity of the soil are variant and unable to be controlled. The post-mortem intervals of the recovered remains included in this study are 40 to 75 years. These include remains of putative United States servicemembers lost during World War II (1939-1945), the Korean War (1950-1953), and Vietnam War (1959-1973). A small number of samples (four) from the United States Civil War (1861-1865) were tested but were not included in this survey.

The recovery sites themselves also varied widely. *In situ* burials were those in which the remains were deposited at time of death. Field burials could have been a by-product of the incident (e.g., plane crash, sinking of a boat or other watercraft) or performed by individuals such as locals or other service members. Other recovery sites were disinterments of unknowns from curated cemeteries. Samples from all locations were evaluated together in order to provide a singular recommendation for the processing of skeletonized cranial material for DNA analysis.

Anthropologists and odontologists at DPAA evaluated the recovered skeletal elements and selected materials for DNA testing once the appropriate measurements and metrics were taken. Tooth samples selected were preferred to have no restorations, no caries, and limited or no damage. The dentine is preferentially removed from the teeth and restorations or fillings often destroy the dentine or introduce bacteria to the interior of the tooth. Damage to the tooth post-mortem also introduces bacteria or debris to the tooth interior, which also causes degradation to the dentine and cementum. For cranial elements, a small window was cut and sent to AFDIL for DNA testing. The size and weight of the cut fragment varied depending on the available skeletal material and the requirements of the DNA extraction protocol. Cranial samples ranged in size from 0.36 to 34.87 g. The maxilla and mandible

were included in the bones of the cranium for the purposes of this study (Figure 3.1). Representative examples of the fragments and teeth tested are shown in Figures 3.2 and 3.3.

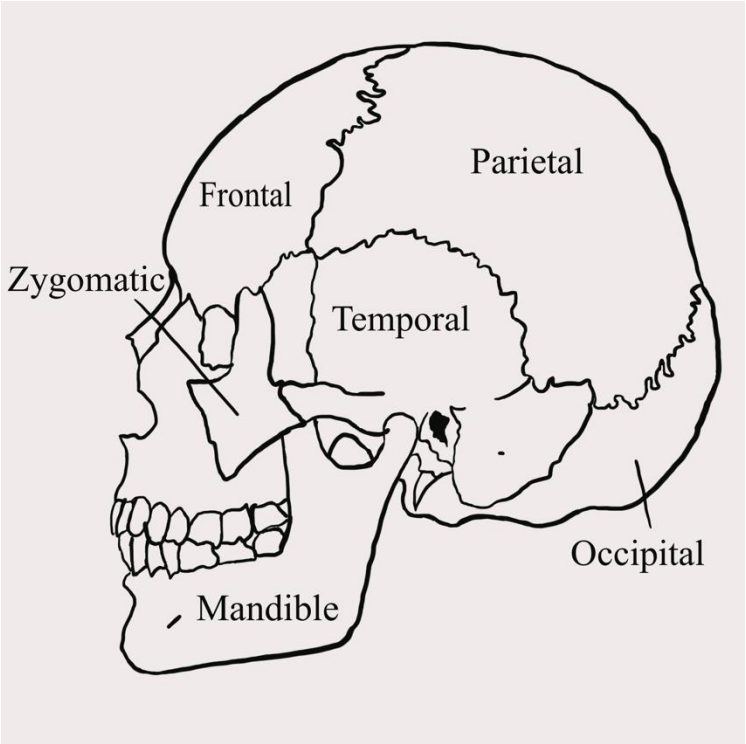

Figure 3.1. Labeled diagram of the skull.

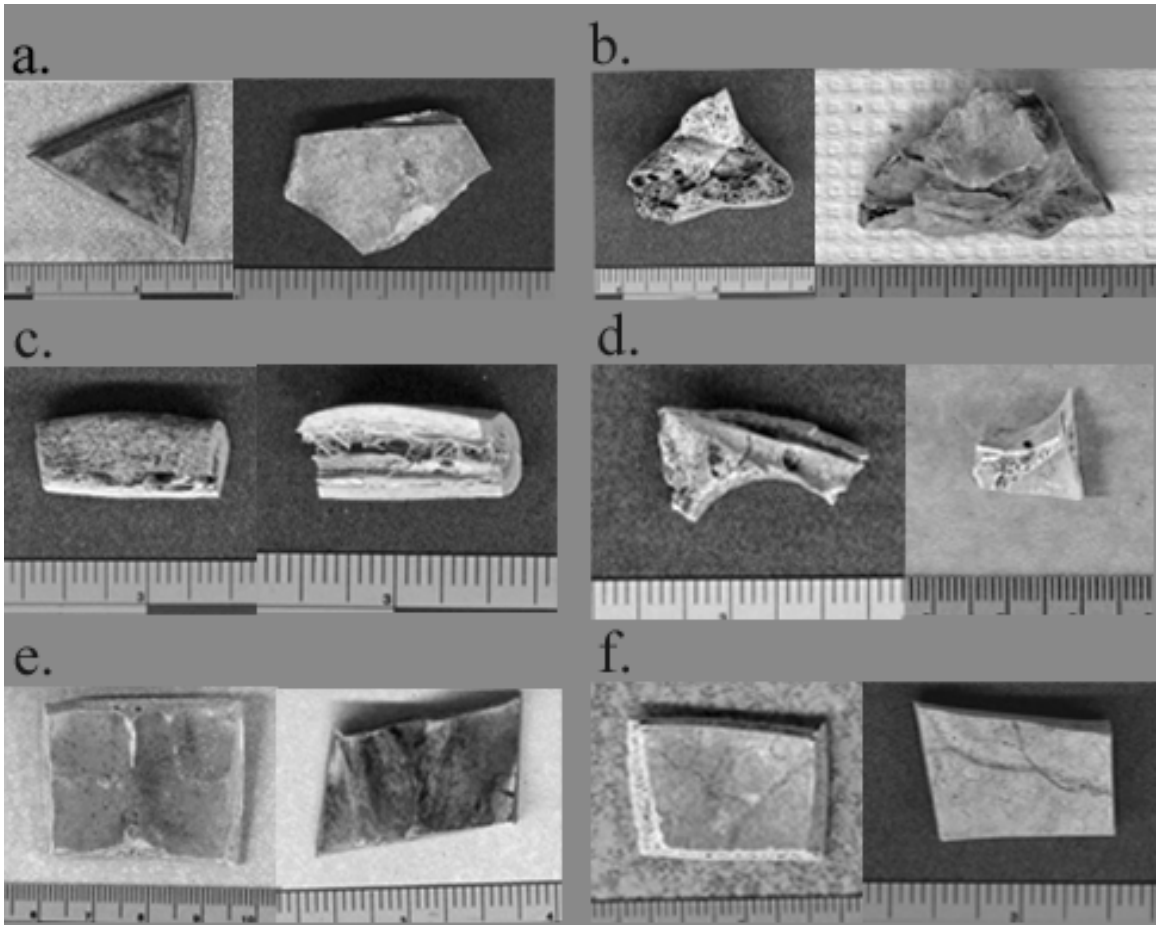

Figure 3.2 Examples of cranial samples submitted to AFDIL for DNA testing. Samples are removed from partial or intact crania and may have been exposed to the elements prior to removal. Two examples are presented for each element. Where possible, there is a dorsal and a ventral view. The elements represented are as follows: a: frontal; b: temporal; c: mandible; d: zygomatic; e: occipital; and f: parietal.

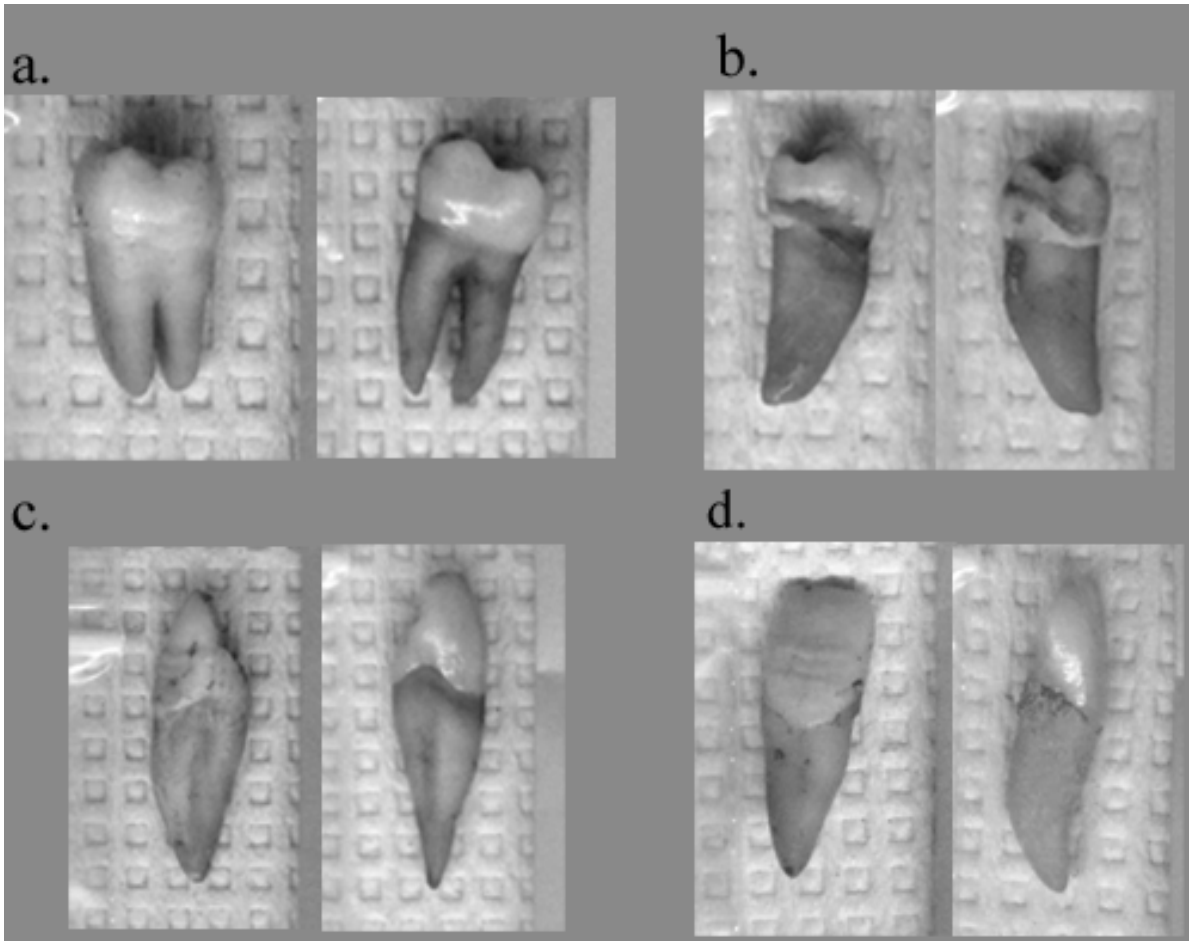

Figure 3.3 Examples of teeth submitted to AFDIL for DNA testing. These are examples of the optimal teeth for testing. Those teeth with caries, cracking, or other damage are not recommended, as damage can introduce bacteria to the interior of the tooth. Samples with restorations are also not recommended as dental work removes or damages dentine. Two views of the same tooth are presented for each example, with the exception of the molar. The molars are examples of nearly fused roots and a normal tooth. The teeth represented are as follows: a: molar; b: premolar; c: canine; and d: incisor.

3.7.2 *Preparation of Samples*

3.7.2.1 Osseous Materials

Prior to pulverization for extraction of DNA, the exterior of the sample was removed using a foot-pedal operated Dremel® tool (Bosch, Stuttgart, Germany) fitted with aluminum oxide sanding bits. For all cranial bones, with the exception of the temporal, the two layers of compact bone were split apart using a disposable Dremel cut-off wheel attached to a #402 rotary tool mandrel. Once split apart, the layer of trabecular bone within was removed. If

the temporal bone was represented by the petrous portion of that element, the exterior of the bone was cleaned and only the trabecular bone that was reachable from the exposed lacunae was removed.

Once sanding was completed, a smaller portion of the bone was removed using either a mortar and pestle or a cut-off wheel. The size of the fragment removed for pulverization depended on the extraction protocol to be used (0.25 – 2.5 g). This portion was placed within a 50 mL Falcon™ conical polypropylene tube (Corning, Corning, NY) with sufficient deionized H_2O (diH_2O) to cover the sample. The sample was vigorously agitated and the liquid poured off. This step was repeated until the water appeared clear, at which point the sample was covered with 100% (v/v) ethanol (Pharmco, Greenfield Global, Brookfield, CT) and the step repeated. The sample was removed from the conical tube, placed in a cleaned weigh boat, and allowed to air dry within a hood for 1 – 2 hours or until dry. Once the sample appeared completely dry, it was placed within a MC2 Waring® blender cup and pulverized (Waring, Torrington, CT). Additional tips and suggestions for the cleaning of skeletal elements can be found in Edson and McMahon (20).

3.7.2.2 Teeth

Prior to 2006, the tooth cleaning and drilling was performed by the DPAA odontologists (21). Submitted powder weight ranged from 0.022 – 1.0 g, which is markedly less than the input required for the extraction protocol in use at the time. Since 2006, intact teeth have been submitted to AFDIL and the teeth drilled by the DNA analysts. The procedure was largely the same between the laboratories with the exception of the cleaning strategy. What follows is the current protocol in use.

Intact teeth were cleaned by placing the tooth in a 50 mL Falcon™ conical polypropylene tube with 25 mL 8.5% (v/v) bleach and submerging in an ultrasonic water bath for 5 minutes. Upon removal from the wash tube, the tooth was wiped clean with a 4 x 4 cm gauze pad moistened with 8.5% (v/v) bleach followed by an additional wiping with a 4 x 4 cm gauze pad moistened with 100% (v/v) ethanol (Pharmco). Should the tooth be cracked or the surface otherwise compromised, the sonication step was omitted and the tooth was cleaned with the moistened gauze pads as mentioned. After cleaning, the tooth was allowed to air dry within a hood and under a UV light for approximately 15 minutes. Should the tooth not be dry after that time, the UV light was turned off and the tooth allowed to stay within the hood until dry.

Using a dental drill hand-piece fitted with a #2 or #4 round bur and attached to a high-performance brushless motor (Brasseler USA, Savannah, GA), the crown of the tooth was removed by bisecting the tooth along the enamel/cementum line. The dentine of both the crown and the root were removed using a straight #4 bur or a round #6 bur. Care was taken to not perforate the crown and introduce additional enamel to the material to be extracted. The amount of dentine removed was 0.1 - 0.2 g of material. The hollowed-out tooth was returned to the submitting laboratory to be reassembled for possible return to the family in the event of identification.

3.7.3 Extraction of DNA

There are three DNA extraction protocols currently in use at AFDIL, all of which involve complete demineralization of the osseous material in an extraction buffer (0.5 M EDTA, pH 8.0; 1% *N*-Lauroylsarcosine). A fourth DNA extraction protocol was in use from 1990-2006; however, this protocol has since been retired. Data from this protocol will be included in this

paper as it is relevant to a comparison of results to a previous publication (8). All four protocols are described in Edson (7) and are briefly summarized here.

Extraction Protocol #1 (EP#1) was retired from active use in 2006 (3). This protocol involved the partial dissolution of ~2.5 g powdered osseous material using a different extraction buffer than the one listed above (10 mM Tris, pH 8.0; 100 mM NaCl; 50 mM EDTA, pH 8.0; 0.5% SDS) and 100 µL Proteinase K (200 mg/mL). Samples were incubated overnight at 56°C with agitation. Purification was undertaken with two to three washes of phenol:chloroform:isoamyl alcohol (25:24:1) followed by a single wash using *n*-Butanol. The purified extract was concentrated using Centricon-100 centrifugal filter units (Millipore, Billerica, MA) and brought to a final volume of 50-100 µL with TE^{-4} (10 mM Tris, 0.1 mM EDTA; pH 7.5).

Extraction Protocols #2 and #3 involved a complete demineralization of 0.2 – 0.5 g powdered osseous material in a demineralization buffer (0.5 M EDTA, pH 8.0; 1% *N*-Lauroylsarcosine) and 100-200 µL Proteinase K (200 mg/mL). Samples were incubated overnight at 56°C with agitation. Extraction Protocol #2 (EP#2) used an organic purification of the samples in the same manner as EP#1 and concentration using Amicon Ultra-4/30K centrifugal filter units (Millipore). The extract was brought to a final volume of 100-200 µL with TE^{-4} (20,22).

Extraction Protocol #3 (EP#3) used an inorganic purification method of the QIAquick PCR purification Kit (QIAGEN, Hilden, Germany). Samples were concentrated using the Amicon Ultra-4/30K centrifugal filter units prior to purification. The final volume of the extract was brought to 50-200 µL with TE^{-4} (20,23).

The final extraction protocol (EP#4) currently in use is designed specifically for samples designated for the Next Generation Sequencing (NGS) workflow. This protocol used 1.0 - 2.0 g powdered osseous material incubated in the demineralization buffer overnight at 56°C with 200 µL Proteinase K (200 mg/mL). The protocol originally described in Marshall, et al. (24) used a Qiagen MinElute® Kit for extract purification. As NGS use is further expanded in use at the laboratory, EP#4 continues to be modified and may involve an organic purification. The final volume of the DNA extracted from EP#4 is 70 µL.

3.7.4 Quantification

Samples designated for a Sanger sequencing workflow were not quantified prior to amplification. If a sample was marked for any testing in an STR platform, quantification occurred using commercially available kits. Currently, the Plexor® HY Kit (Promega, Madison, WI) was used in concert with the Applied Biosystems™ 7500 Real-Time PCR Instrument (Thermo Fisher Scientific, Waltham, MA).

3.7.5 Mitochondrial DNA Amplification and Sanger Sequencing

Amplification of the mitochondrial DNA (mtDNA) Control Region used overlapping primer sets (3,7). Four 'large' primer pairs were used, two of which amplify Hypervariable Region One (HVI) and two for Hypervariable Region Two (HVII). Each of those large primer pairs used for DNA recovered from degraded skeletal remains generates an amplicon of 187 - 231 bp in length. Smaller primer pairs, referred to as mini-primer sets, reduce the amplicon to a size of 79 - 115 bp (25). There were four mini-primer sets for each of the hypervariable regions.

Amplification was conducted using PCR Master Mix containing 10x PCR Buffer (100 mM Tris-HCl, pH8.3; 500 mM KCl; 15 mM $MgCl_2$); 2.5 mM dNTPs; 6.25 µg/µL NA-BSA; 10 µM each of paired primers; 5 units/µL AmpliTaq Gold® DNA polymerase; and sterile diH_2O. The primers used were made in-house. The targeted input of DNA was 10 - 1000 pg, which translated to between 1 - 10 µL of extract. Each amplification set-up contained two negative controls, a positive control, and a reagent blank initiated at the DNA extraction step. The positive control used was HL-60, DNA recovered from a promyelocytic cell line.

PCR amplification took place in GeneAmp® PCR Systems 9700 (Life Technologies) using in-house designed and validated programs. The large primer pairs were amplified under the following conditions: a 10 minute soak at 96°C, followed by 38 cycles of 20 seconds at 94°C, 20 seconds at 56°C, and 30 seconds at 72°C, and a final hold at 4°C. The smaller mini-primer sets programs each have validated modifications to this program and are further described in Gabriel, et al. (25). Success or failure of the amplification was determined by running the product on a 2% agarose gel stained with ethidium bromide (5 mg/mL). If the amplification was successful, a sequencing reaction input volume of either 1 µL or 7 µL was determined from comparison to a mass ladder (DNA Ladder II; APExBIO Research, Houston, TX).

Purification of the amplified product took place using an enzymatic PCR with ExoSAP-IT® (Life Technologies). For each set of samples and all associated controls, a master mix was created using 1.5 µL ExoSAP-IT® and 18.5 µL SAP dilution buffer per sample. Prior to placing the samples in an unheated thermal cycler, 20 µL of the master mix was added to each sample. The reaction for purification was as follows: 30 minute hold at 37°C, 15 minute hold at 85°C, and an indefinite hold at 4°C. Prior to implementation of this protocol in 2006, amplified product was purified using diH_2O and Centricon-100 or Centricon-30 centrifugal filter units (Millipore, Billerica, MA).

Sanger sequencing of the purified product was performed using the same primers as used for amplification. Sequencing reactions were set-up in 96-well plates, with two wells for each sample, one for each primer. All controls were carried through from amplification to sequencing.

3.7.6 STR Amplification

STR amplification was performed following the manufacturers' recommendations for AmpFlSTR® MiniFiler™ (Life Technologies, Gaithersburg, MD) and PowerPlex® Fusion (Promega, Madison, WI). A modified AmpFlSTR® Yfiler™ (Life Technologies), described in Sturk, et al. (26) and Irwin, et al. (27) was used. The modified protocol involved a volume increase for AmpliTaq Gold® DNA polymerase from the recommended 0.8 µL per sample to 1.6 µL per sample and an increase in the number of program cycles from 28 to 36. Amplification took place in GeneAmp® PCR Systems 9700.

As with mtDNA analysis, a positive control, reagent blank, and at least one negative were amplified concurrently with the relevant samples. AmpFlSTR® Positive Control DNA – Human Male 007 (0.1 ng / µL) was used for MiniFiler™ amplifications. For both the modified Yfiler™ and Fusion amplifications, the single-source male 2800M Control DNA (0.75 ng / µL) (Promega) was used.

Other STR kits have been used, including AmpFlSTR® Identifiler™, AmpFlSTR® Identifiler Plus™ (Life Techologies), and PowerPlex®16 (Promega), but the data are not presented in this publication due to limited results.

3.7.7 Separation of Fragments

Currently Sanger sequencing and STR fragment separation occurs on the AB 3500*xL* Genetic Analyzer. From 1990 – 2003, the ABI PRISM 377® DNA Sequencers were used. This is a slab gel technology and is only relevant to Sanger sequencing results during that time frame, as STRs were not validated for use until 2006.

The AB 3100 and 3130*xL* Genetic Analyzers were used from 2003 until 2018. From 2015 to 2018, the 3130*xL*s were used in concert with the AB 3500*xL* Genetic Analyzers and were fully retired from casework at the end of 2018. Both instruments were outfitted with 36 cm capillary arrays and Performance Optimized Polymer 4 (POP-4™; 4% dimethylacrylimide, 8 M Urea, 5% 2-pyrrolidinone) for STR analysis or a 50 cm capillary array and POP-6™ (6% dimethylacrylimide, 8 M Urea, 5% 2-pyrrolidinone) for mtDNA Sanger sequencing.

Injection times were specific for the different platforms tested. MtDNA Sanger sequencing had two different injection times: 16 seconds for smaller amplicons (i.e., primer sets or mini primer sets) and 24 seconds for larger (i.e., full control or hypervariable regions). The default injection time for all STR kits was 15 seconds, but this could be reduced to seven seconds as needed.

3.7.8 Next Generation Sequencing

Next Generation Sequencing (NGS) was performed using an in-house developed capture protocol (24,28). Samples were run on the Illumina MiSeq using the v2 kit (Illumina, San Diego, CA).

3.7.9 Data analysis

Two separate analysts performed all data analysis independently from one another. DNA profiles must be consistent between both analysts prior to reporting.

3.7.9.1 Mitochondrial DNA Analysis

Sanger sequencing data for mtDNA was assembled and analyzed using GeneCodes Sequencher Plus (Ann Arbor, MI). Polymorphisms reported are the differences from the sequences generated to the revised Cambridge Reference Sequence (rCRS: 29,30). MtDNA data generated by NGS were analyzed using analyzed the CLC Genomics Workbench Software, version 7.5 or higher (QIAGENBioinformatics, Gaithersburg, MD).

3.7.9.2 STR Analysis

STR data analysis was undertaken using GeneMapper® *ID-X* v. 1.3 (Life Technologies). Analytical and stochastic thresholds were determined during the validation process (7).

3.7.10 Reporting

For all platforms, two analysts independently analyzed the data and concurred on the generated profile. Sample data underwent a technical and administrative review, and were searched against a database of AFDIL, AFMES, DPAA staff and visitors before being considered authentic and reported to the requesting agency.

For the purposes of this study, at least 100 bp of mitochondrial DNA data were confirmed in order for a sample to be considered successful. All STR platforms required four confirmed loci to be reported and considered successful. Samples processed using NGS required a read

depth of at least 10 reads and a variant must have been seen in at least four reads to be called. The average number of bases for both Sanger sequencing and NGS were calculated from the samples for each element type that were considered successful.

3.8 RESULTS

3.8.1 Sanger Sequencing of Mitochondrial DNA

3.8.1.1 Cranial Elements

In total, 1886 cranial elements were subjected to one of the three different DNA extraction protocols from which extracts were used to generate data by Sanger sequencing of mitochondrial DNA (Table 3.1). When evaluating the cranial elements, extraction Protocol #2 (EP#2: complete demineralization coupled with an organic purification) proved to be the most successful of the extraction protocols compared, with an overall success of 87%. Of the 978 elements tested, 853 had 100 bp or more of reportable data. The most successful of the cranial elements were: the mandible (153 sampled; 91% successful); occipital (436 sampled; 91%); and parietal (46 sampled; 91%). The zygomatic (10 sampled; 90%) and temporal (189 sampled; 87%) also generated mtDNA data.

Extraction Protocols #1 and #3 were generally less successful than EP#2 (62% and 75%, respectively). EP#1 was in use at AFDIL from 1990 until 2007 and was used in the Sanger sequencing testing of 562 cranial elements. The most successful of the elements with this extraction protocol was the temporal (77 sampled; 92%). All other cranial elements tested had a success of 77% or less, with the least successful element being the parietal (132 sampled; 42%).

Extraction Protocol #3 (EP#3: complete demineralization with an inorganic purification) showed an overall decrease in cranial element success as compared to the other protocols. The unspecified cranial element was the most commonly sampled element at 125 tested. The zygomatic, which is thinner and lighter than most other cranial elements, was the most successful tested (12 sampled; 92%).

3.8.1.2 Teeth

Of the 1533 teeth tested in mtDNA Sanger sequencing, EP#3 was the most successful at 100% (79 sampled). EP#2 was also highly successful (686 sampled; 90%), with the incisor being the most successful (79 sampled; 96%) followed by the molar (308 sampled; 93%). EP#1 was less successful from the other two extraction protocols, although teeth extracted in this manner are more successful than any cranial element, with the exception of the temporal.

3.8.2 AmpFlSTR® MiniFiler™ Testing

Using AmpFlSTR® MiniFiler™ according to manufacturer recommendations, 267 cranial elements were tested with an overall success of 37% (Table 3.2). When breaking results down by extraction protocol, EP#2 was less successful than EP#3 (25% vs. 55%). Teeth were less successful in both EP#2 (70 sampled; 23%) and EP#3 (9 sampled; 22%) than the cranial elements, but there was little difference between the two extraction protocols for teeth overall. EP#1 was used minimally for STR testing, as it was discontinued in 2007 prior to the validation of STR protocols at the laboratory.

Table 3.1. Cranial elements and teeth tested in Sanger sequencing of Mitochondrial DNA. The "General Crania" category includes samples that were not specified as being a specific cranial element during submission to the DNA testing laboratory. These samples are typically from remains that have been fragmented to such a degree as the anthropologists are only able to determine that the sample originated from the cranium. The total number of teeth may be greater than the sum of the individual teeth as there were some teeth that were not specifically labeled other than "mandible" or "maxillary".

	Extraction Protocol #1				Extraction Protocol #2				Extraction Protocol #3			
Element	# Tested	# Successful	% Successful	Avg. # of bases	# Tested	# Successful	% Successful	Avg. # of bases	# Tested	# Successful	% Successful	Avg. # of bases
Total Cranial Elements	562	349	62%	321	978	853	87%	557	346	258	75%	485
Frontal	23	13	57%	302	32	26	81%	525	11	6	55%	322
Mandible	133	94	71%	421	153	139	91%	620	61	48	79%	509
Maxilla	1	0	0%	0	4	3	75%	498				
Occipital	67	41	61%	348	436	398	91%	661	58	47	81%	527
Parietal	132	56	42%	238	46	42	91%	537	28	21	75%	498
Temporal	77	71	92%	608	189	165	87%	603	51	38	75%	506
Vault	23	14	61%	320								
Zygomatic					10	9	90%	600	12	11	92%	619
General Crania	106	60	57%	338	108	71	66%	412	125	87	70%	471
Total Teeth	768	571	74%	621	686	618	90%	690	79	79	100%	692
Canine	178	135	76%	619	153	132	86%	694	23	23	100%	692
Incisor	94	62	66%	632	79	76	96%	694	15	15	100%	694
Premolar	158	108	68%	618	137	117	85%	681	19	19	100%	690
Molar	328	259	79%	630	308	287	93%	691	22	22	100%	691

Table 3.2. Cranial elements and tooth samples tested in AmpFlSTR® MiniFiler™. The average number of loci has been rounded to the nearest whole number.

The "General Crania" category includes samples that were not specified as being a specific cranial element during submission to the DNA testing laboratory. These samples are typically from remains that have been fragmented to such a degree as the anthropologists are only able to determine that the sample originated from the cranium. The total number of teeth may be greater than the sum of the individual teeth as there were some teeth that were not specifically labeled other than "mandible" or "maxillary".

	Extraction Protocol #1				Extraction Protocol #2				Extraction Protocol #3			
Element	# Tested	# Successful	% Successful	Avg. # of Loci	# Tested	# Successful	% Successful	Avg. # of Loci	# Tested	# Successful	% Successful	Avg. # of Loci
Total	5	2	40%	5	153	38	25%	2	109	60	55%	4
Frontal					4	0	0%	0	6	1	17%	1
Mandible					26	10	38%	3	21	19	90%	7
Occipital	4	1	25%	1	95	17	18%	1	40	12	30%	2
Parietal					7	2	29%	3	6	2	33%	4
Temporal	1	1	100%	9	13	9	69%	6	22	19	86%	8
General Crania					8	0	0%	0	14	7	50%	4
Total Teeth	5	0	0%	0	70	16	23%	4	9	2	22%	9
Canine	4	0	0%	0	16	2	13%	4	4	1	25%	9
Incisor					11	1	10%	4	3	1	33%	9
Premolar					6	2	33%	4	4	1	25%	9
Molar	1	0	0%	0	36	11	31%	5	2	0	0%	0

Using EP#2, the temporal bone was the most successful cranial element tested (13 tested: 69%). However, the occipital was the most commonly tested (95 sampled: 18%); note that this is due to a policy decision at DPAA to specifically select the occipital for sampling of the USS *Oklahoma*. Ninety-two of the occipital bones tested were recovered from this specific incident. EP#2 was, and remains, the extraction protocol of choice for this incident, as the contaminating fuel oil and adipocere are more easily removed with an organic extraction.

Extraction Protocol #3 is currently used more frequently for samples designated for STR testing. Of the 109 cranial elements tested, 55% generated a MiniFiler profile of four or more loci. The mandible was the most successful (21 tested; 90%) and the temporal the second best (22 tested; 86%). Extraction Protocol #2 was the most commonly used protocol for teeth solely because of the need to test the tooth in mtDNA Sanger sequencing first. There is little to no material remaining from a tooth for the sample to be re-extracted, requiring the use of the existing extract or the submission of a different tooth.

3.8.3 Modified AmpFlSTR® Yfiler™ Testing

Samples extracted using Extraction Protocol #3 were most successful when using a modified Yfiler protocol (Table 3.3). Of the 197 cranial elements extracted with EP#3, 51% generated a reportable profile of four or more loci. Teeth have a 31% success, but had a limited number tested (13 sampled). The mandible (25 tested) and temporal (38 tested) were the most successful cranial elements, at 88% and 87%, respectively. The occipital fragments were the most commonly sampled (89), but among the least successful (38%).

Extraction Protocols #1 and #2 were equally less successful in modified Yfiler (31% overall success for both) for cranial elements. EP#2 was the most commonly used extraction

protocol for teeth (84 sampled), but was less successful than EP#3 (23% vs. 31%). Molars were the most commonly tested tooth (46) and were also the most successful (30%). EP#1 had a very limited number of cranial elements (13) and teeth (2) tested. The temporal and parietal were the most commonly tested using this extraction protocol at four each. The temporal elements were successful 100% of the time and the parietal failed every time.

Table 3.3. Cranial samples tested in Modified AmpFlSTR® Yfiler™. The average number of loci has been rounded to the nearest whole number.

The "General Crania" category includes samples that were not specified as being a specific cranial element during submission to the DNA testing laboratory. These samples are typically from remains that have been fragmented to such a degree as the anthropologists are only able to determine that the sample originated from the cranium. The total number of teeth may be greater than the sum of the individual teeth as there were some teeth that were not specifically labeled other than "mandible" or "maxillary".

	Extraction Protocol #1				Extraction Protocol #2				Extraction Protocol #3			
Element	# Tested	# Successful	% Successful	Avg. # of Loci	# Tested	# Successful	% Successful	Avg. # of Loci	# Tested	# Successful	% Successful	Avg. # of Loci
Total Crania	13	4	31%	4	176	55	31%	3	197	101	51%	6
Frontal									9	1	11%	1
Mandible					38	14	37%	4	25	22	88%	11
Occipital	2	0	0%	0	98	23	23%	2	89	34	38%	4
Parietal	4	0	0%	0	8	2	25%	3	8	1	13%	2
Temporal	4	4	100%	15	22	14	64%	9	38	33	87%	13
Zygomatic					1	0	0%	0				
General Crania	3	0	0%	0	9	2	22%	2	28	10	36%	5
Total Teeth	2	1	50%	6	84	19	23%	6	13	4	31%	13
Canine	1	1	100%	6	24	2	8%	8	6	2	33%	11
Incisor					5	1	20%	12	3	1	33%	15
Premolar					8	2	25%	3	2	1	50%	4
Molar	1	0	0%	0	46	14	30%	7	2	0	0%	0

3.8.4 PowerPlex® Fusion Testing

A limited number of cranial elements (36) and teeth (8) were tested in PowerPlex® Fusion (Table 3.4). Extraction Protocol #2 was the most successful for cranial elements and teeth; however, only eleven samples were extracted using this protocol (three cranial bones and eight teeth). The occipital was the least successful in PowerPlex® Fusion when using EP#3 (8 tested; 63% successful). All other cranial samples tested produced a reportable profile of four or more loci 100% of the time. Teeth were successful 50% of the time, with the premolar being the most successful; however, only one was tested.

Table 3.4. Cranial elements and tooth samples tested in PowerPlex® Fusion. The average number of loci has been rounded to the nearest whole number.

The "General Crania" category includes samples that were not specified as being a specific cranial element during submission to the DNA testing laboratory. These samples are typically from remains that have been fragmented to such a degree as the anthropologists are only able to determine that the sample originated from the cranium.

	Extraction Protocol #2				Extraction Protocol #3			
Element	# Tested	# Successful	% Successful	Avg. # of Loci	# Tested	# Successful	% Successful	Avg. # of Loci
Total	3	3	100%	10	33	30	91%	15
Mandible	1	1	100%	6	12	12	100%	12
Occipital					8	5	63%	6
Parietal					1	1	100%	18
Temporal	2	2	100%	13	10	10	100%	19
General Crania					2	2	100%	18
Total Teeth	8	4	50%	7				
Incisor	2	1	50%	3				
Premolar	1	1	100%	7				
Molar	5	2	40%	10				

3.8.5 NGS Testing

Next Generation Sequencing (NGS) testing was implemented into use in 2016 and was typically used for samples that were unsuccessful in Sanger sequencing. The extraction protocol used was not a single SOP, but varied based on the sample available or the historical context. Forty-five cranial elements were tested using the NGS protocol with a success rate of 53% (Table 3.5). The occipital (24 samples) and temporal (14 samples) were the most commonly sampled, with success of 54% and 50%, respectively. The majority of samples tested came not from a single site, but rather a single conflict. Thirty-eight of the 45 cranial samples tested were recovered world-wide from World War II era sites. No teeth were tested in NGS.

Table 3.5. Cranial samples tested in NGS. The average number of bases has been rounded to the nearest whole number.

The "General Crania" category includes samples that were not specified as being a specific cranial element during submission to the DNA testing laboratory. These samples are typically from remains that have been fragmented to such a degree as the anthropologists are only able to determine that the sample originated from the cranium.

	Extraction Protocol #4			
Cranial element	# Tested	# Successful	% Successful	Avg. # of Bases
Total	45	24	53%	8813
Frontal	1	0	0%	0
Occipital	24	13	54%	8902
Parietal	1	1	100%	16510
Temporal	14	7	50%	8883
General Crania	5	3	60%	9771

3.9 DISCUSSION

3.9.1 Confounding Effect

One of the confounding effects of a large-scale study that examines real-world casework is the inability to control for the samples chosen for analysis. The laboratory is reliant on mass fatality events or other circumstances surrounding the submission of samples for DNA

testing. This sampling bias is evident in a survey of post-cranial skeletal elements from the same group of submissions. The rib samples extracted using EP#1 show an elevated success of 77%, which was unexpected (7). Ribs have a very thin layer of cortical bone, and are an outlier to reported results of weight-bearing bones producing better results. However, thirty-six percent of those sampled were recovered from a single location, and all generated reportable mtDNA Sanger sequencing results.

The same can be found in this data set of cranial elements. As mentioned in the Methods and Materials, the DPAA made a decision to preferentially sample from the occipital in the case of the USS *Oklahoma*. The 347 occipital elements account for 16% of the cranial elements submitted for testing and 80% of the occipital samples tested for mtDNA Sanger sequencing. Given that the samples from the USS *Oklahoma* have a 98% success for mtDNA across all skeletal elements, this skews the results for the occipitals to 91% success. Removing the samples recovered from the USS *Oklahoma* from the samples tested, drops the occipital to a 78% success for mtDNA Sanger sequencing (Table 3.6). The overall success for AmpFlSTR® MiniFiler™ and Modified AmpFlSTR® Yfiler™ did not change for the occipital with the USS *Oklahoma* samples removed. All other samples maintain nearly the same success rate, as do the post-cranial remains presented in Edson (7).

Table 3.6: The impact of samples recovered from the USS *Oklahoma*. The USS *Oklahoma* was torpedoed during an attack on the naval base in Pearl Harbor, Hawaii on 7 December 1941. The ship rolled, trapping over 400 U.S. sailors and Marines within the body of the ship. The remains were recovered from the body of the ship in 1945. Initial attempts at identification were made in the 1950's, but the remains were reburied in the National Memorial Cemetery of the Pacific in Hawaii. DNA testing was first attempted in 2003, and modern efforts commenced in 2015. Samples recovered from the USS *Oklahoma* have an elevated rate of success across all samples of 98% in mtDNA Sanger Sequencing. The table shows the most commonly sampled cranial elements extracted in EP#2 and tested in mtDNA Sanger Sequencing, AmpFlSTR® MiniFiler™, and Modified AmpFlSTR® Yfiler™. Frontal and parietal were not tested for the USS *Oklahoma* and are therefore not included.

		mtDNA Sanger sequencing			AmpFlSTR® MiniFiler™			Modified AmpFlSTR® Yfiler™		
Sample type and Recovery Location		# tested	# successful	% successful	# tested	# successful	% successful	# tested	# successful	% successful
Mandible	All samples	153	139	91%	26	10	38%	38	14	37%
	USS *Oklahoma* only	32	32	100%	6	4	67%	10	7	70%
	All samples minus USS *Oklahoma*	121	107	88%	20	6	30%	28	7	25%
Temporal	All samples	189	165	87%	13	9	69%	22	14	64%
	USS *Oklahoma* only	21	19	90%	1	0	0%	2	1	50%
	All samples minus USS *Oklahoma*	168	146	87%	12	9	75%	20	13	65%
Occipital	All samples	436	398	91%	95	17	18%	98	23	23%
	USS *Oklahoma* only	347	329	95%	91	16	18%	93	22	24%
	All samples minus USS *Oklahoma*	89	69	78%	4	1	25%	5	1	20%
Teeth	All samples	686	618	90%	70	16	23%	84	19	23%
	USS *Oklahoma* only	36	35	97%	11	2	18%	17	1	6%
	All samples minus USS *Oklahoma*	650	583	90%	59	14	24%	67	18	27%

3.9.2 Element Specific Discussion

3.9.2.1 Frontal, Parietal, Occipital, and Mandible

With the removal of the USS *Oklahoma* samples from the totals of bones, the success for Sanger sequencing testing of the occipital is more similar to that of the frontal (78% vs. 81%); however, the parietal has a 91% success when coupled with EP#2. The parietal fails to have success with any other modality or extraction protocol and the occipital remains the best of the three from which to extract DNA.

Dense bones, such as weight-bearing bones, tend to be best for the recovery of DNA from osseous materials (7). The occipital has a nuchal crest or line where the musculature of the back of the neck forms an attachment. Likewise, the mandible has multiple attachment points for musculature, with the largest being along the mandibular angle. As with long bones, there is a region of density where the muscles attach, which should provide an optimal source of DNA. This is shown not to be so for the occipital with this survey. Even with the USS *Oklahoma* samples included, the occipital has only a 30% success in MiniFiler and 38% in modified Yfiler with EP#3, compared to the 86% and 87% success, respectively for the temporal. However, the mandible is comparable to the temporal in overall success.

The generally low success of the frontal, parietal, and occipital can be attributed to the overall structure of the three bones. Each is a thin layer of diploë sandwiched between two thin layers of cortical bone. This cancellous bone is removed during the preparation of the bone for DNA extraction. The remaining bone is simply a thin shell of osseous material, more akin to that of the rib or other non-weight bearing bone.

The diploë itself also provides an avenue for the introduction of exogenous materials into the cranial bone. Any cracks or fissures in the outer (or inner surface) allows for the introduction of bacteria, soil, or plant material into the diploë and fill up the spaces creating microfractures that will speed up the decay of both the bone and the DNA present within (31,32). Other elements, such as the metatarsals and phalanges, are also comprised of thin bone filled with cancellous. However, these elements have the benefit of being covered with an increased density of tissue post-mortem, thus protecting the DNA within. The occipital, parietal, and frontal have only a thin layer of tissue and hair, which affords little protection to the osseous materials and increases break-down of the tissue and DNA making these less than optimal for DNA extraction.

3.9.2.2 Zygomatic

The increase in the number of zygomatic samples tested in Sanger sequencing and extracted in EP#2 (10) and EP#3 (12) versus EP#1 (0) is associated with the decrease in the size of the sample needed for EP#2 and EP#3 (i.e., 0.25 g vs. 2.5 g). The zygomatic is relatively small and fragile compared to the other bones of the cranium (33) and thus may be crushed peri-mortem or after skeletonization. The average weight of a zygomatic sample submitted for mtDNA analysis is 0.95 g, which would not have been acceptable prior to the change in extraction protocols from EP#1 to EP#2.

The overall high degree of success for the zygomatic seems unlikely, especially due to its small size and overall fragility. The success however seems akin to that of the metatarsals and metacarpals (7), which are likewise small and fragile. Mundorff and Davoren (6) and Andronowski, et al. (34) have indicated that the metatarsals and metacarpals may be more successful in generating genetic data due to the high amount of trabecular bone within those elements accreting blood and tissue fragments during decomposition. While the zygomatic is

more compact than those elements, several arteries do pass through that bone, including the zygomaticofacial and infraorbital arteries. It is the blood supply to this element that accounts for the high degree of success when it is used for DNA analysis. There were a limited number of zygomatics tested (22 in total) for Sanger sequencing and it may yet be determined that they are not consistently successful.

3.9.2.3 Temporal

The original survey of mtDNA Sanger sequencing with EP#1 (8) showed that the temporal had a statistically significant success rate over all other cranial elements, as well as the femur. The decreased success in EP#2 for mtDNA Sanger sequencing is puzzling. There was no sampling bias in terms of location or conflict from which the temporal elements were recovered. Despite the decrease in success for mtDNA analysis, the temporal remains consistently successful generating genetic data and should be chosen over other cranial elements.

One of the difficulties in selecting the temporal for DNA testing is the removal of the element from the skull. Removal of the temporal from an intact skull requires creating either a large hole in the bottom or side of the skull or removing the skull-cap and sectioning the base of the skull (35). Both methods cause obvious destruction of the materials of the skull that cannot be easily hidden. In addition, removal of the petrous/temporal damages anthropological landmarks. If the skull is fragmented, the temporal may be removed and the skull reassembled to obscure the loss.

Recommendations have recently been made for the anthropologist to remove the otic capsule during initial examination and submitting the powder to the DNA laboratory (12,36,37). The

otic capsule is ossified during gestation, and is considered the densest part of the petrous (38,39). In order to remove the otic capsule without removing the temporal, the base of the skull just over the otic capsule may be shaved or ground away until the capsule can be seen. The capsule may be sanded with a rasp and the powder collected through the foramen magnum (36) or ground out with a drill bit (37).

The samples sent from the anthropology lab are intact petrous portions, which contain little to none of the surrounding cortical bone. It is the DNA analyst that makes the final selection of the portion of the petrous from which to remove the sample to be extracted. Research by Antinick and Foran (40) indicated that the portion of the bone from which a DNA sample is removed factors into the success, and indeed, density is not uniform throughout the petrous (10). Due to the difficulty in cleaning the petrous, analysts may tend to sample from the apex. While still successful, this portion of the petrous has been shown to contain less DNA that the denser portion, or the otic capsule itself (10,37).

3.9.2.4 Teeth

For Sanger sequencing of mtDNA, teeth are among the best for generating genetic data when using EP#2 (90%; 686 sampled) and the best element for EP#3 (100%; 79 sampled). An organic purification is unnecessary for teeth as they contain very little fats and proteins. EP#3 provides a more efficient DNA extraction for dental material as the inorganic protocol is optimized for the removal of salts and enzymes and more effectively handle the high mineral content of teeth.

However, with a few exceptions, teeth are collectively the least successful cranial element from which to sample DNA for STR testing. This runs contrary to other studies indicating

that teeth should be preferentially sampled from skeletonized remains (19,41,42,43). It should be noted that these studies did not use the same portion of the tooth for DNA extraction, nor samples of comparative age. Miloš, et al., (19) pulverized the entire tooth, including the crown; Adler, et al. (42) crushed the entire root of the tooth; Corrêa, et al. (43) used the bottom 5 mm of the tooth roots; and Smith, et al. (41) and this study drilled the dentine out of the interior of the tooth.

3.9.3 *Extraction Specific Comments*

The extraction protocols tested generated varying success, not only between the cranial elements themselves, but the different testing modalities used. EP#2, a complete demineralization followed by an organic purification, was more successful for mtDNA Sanger sequencing than any STR platform. EP#3, a complete demineralization followed by an inorganic purification, was more successful for any STR platform tested. However, teeth are more successful for mtDNA Sanger sequencing using an inorganic purification, with 100% of the samples tested producing a reportable profile. Silica purification kits are designed to remove mineral inhibitors and bind high quality DNA. Teeth have a lesser amount of proteins and fats than skeletal elements, thus these materials are not competing with the DNA for binding in the column.

The results for the testing of the osseous materials reflect the results found for post-cranial samples (7). As with that study, STR platforms performed better with EP#3 due to the loss of smaller fragments during the silica column washes (44). It is probable that smaller fragments do not bind efficiently to the silica column and are lost during the wash steps, thus reducing the success of mtDNA Sanger sequencing. Despite the high binding of DNA to silica columns (45) and an assertion by Qiagen (44) that MinElute columns retain 80% of DNA fragments, other research has found a significant loss of smaller DNA fragments during

extraction. Kemp, et al. (46) used synthetic standards of a known DNA input to calculate a DNA loss of 21.75 – 60.56 % with MinElute columns. Dabney, et al. (47) found that MinElute columns retained 95% of fragments down to 35 bp in size. It should be noted that what applies to the MinElute may not be applicable to the QIAquick PCR purification kit; however, this may be applicable across many silica column purification protocols. Davoren, et al. (45) also compared an organic purification to a Qiagen kit. Using the Qiagen Blood Maxi kit and amplifying with PowerPlex 16, the researchers found that the skeletal material extracted with the Blood Maxi kit contained nearly three times the DNA of those samples extracted with a PCIA purification.

What is marked about each citation listed is that there is no uniform input of bone or tooth material or demineralization buffer. Each protocol uses a range of input (0.08 – 9.8 g) and demineralization buffers that rely on different volumes of EDTA and detergents (Table 3.7). Even the incubation period and temperature vary between protocols. Two tenths of a gram of bone powder will completely demineralize in approximately 9 hours when incubated in 4.0 mL of buffer at 56°C with agitation (data not shown). While larger volumes of osseous material and longer incubation periods do work, efficiencies should be evaluated by each laboratory and the protocol selected that best fits the workflow.

3.10 CONCLUSIONS

Evaluation of large collections of samples must be checked for clusters of testing that may skew the results. Although a similar study was performed on post-cranial remains (7), the USS *Oklahoma* samples were not found to modify the success rates significantly as the samples were taken from a wide variety of post-cranial elements. Here, the majority of occipital samples tested were recovered from that single incident, generating a fairly significant bias to the results, which was corrected with the removal of those elements.

Strategic sampling of cranial elements is warranted. The petrous portion of the temporal is the most consistent of the cranial elements tested, even though it is not always the best. Teeth, on the other hand, provided somewhat lackluster results for STR analysis, despite having near 100% success using Sanger sequencing of mtDNA for both extraction techniques. The other 'vault' elements of the crania, occipital, parietal, and frontal, are generally not considered optimal for DNA testing, although the zygomatic was surprisingly successful.

The chemistry in the kits used has a clear impact on the success of the DNA testing. Inorganic kits are designed to remove inhibitors, including the minerals present in the osseous or tooth materials. The matrix of teeth is largely mineral in nature, with a lack of fats and proteins, thus making an inorganic purification preferred when working with teeth. Organic purifications are primarily designed to remove fats and proteins, making it a more efficient purification for bone materials. Should laboratories have the option, maintaining both protocols in active standard operating procedures could be considered a best practice.

Table 3.7. Examples of extraction protocols used for skeletal remains. Protocols are arranged by amount of skeletal material used. There is a lack of consistency across the field with regards to protocols for the extraction of DNA from skeletal remains. Each laboratory has an optimal strategy based on their internal validations. Despite the differences, there is some agreement among laboratories for which skeletal elements might be considered choice for DNA analysis.

Citation	Amount of Osseous Powder (g)	Extraction Buffer	Incubation Temp (°C)	Length of Incubation	Final Volume (µL)	Purification "Kit"	Purification Method
Balayan, et al. (48)	Unspecified	0.5 M or 0.25 M EDTA	37	7 days with daily changes of buffer	Not noted	PCIA	Organic
Hansen, et al. (11)	Not specified	EDTA; TE; N-Lauroylsarcosine; phenolred	37	24 hours	Not noted	Silica in solution plus a buffer described by Allentoft, et al., 2015	Inorganic
Dabney ,et al. (47)	0.08 – 0.12	0.45 M EDTA, pH 8.0	37	18 hours	25	5 M guanidine hydrochloride, 40% (v/v) isopropanol; 0.05% Tween-20; 90 mM NaOAc; Plus QIAamp MinElute	Inorganic
Pinhasi, et al. (10)	0.15	1 M TrisHCl; SDS (10%); 0.5 M EDTA	55 and 37	24 hours at 55◦C and 24 hours at 37◦C	100	QIAamp MinElute (Qiagen)	Inorganic
Edson and McMahon (20)	0.2 - 0.25	0.5 M EDTA, pH 8.0; 1% N-Lauroylsarcosine	56	Less than 24 hours	100 – 200	PCIA	Organic
Edson and McMahon (20)	0.2 - 0.25	0.5 M EDTA, pH 8.0; 1% N-Lauroylsarcosine	56	Less than 24 hours	100	QIAquick PCR Purification (Qiagen)	Inorganic
Kulstein, et al. (36)	0.3	0.5 M EDTA, pH 8.0; N-Lauroylsarcosinate; ATL Buffer (Qiagen); DTT	56	20 hours	50	Maxwell® RSC Blood DNA Kit (Promega)	Inorganic
Pilli, et al. (12) (followed 47)	0.5	0.45 M EDTA, pH 8.0	37	18 hours	40	5 M guanidine hydrochloride, 40% (v/v) isopropanol; 0.05% Tween-20; 90 mM NaOAc; Plus QIAamp MinElute	Inorganic
Carvalho, et al. (49)	1.0 – 5.0	QIAamp DNA Investigator Kit (Qiagen)	Room Temp.	Less than 30 minutes	20 – 100	QIAamp MinElute (Qiagen)	Inorganic
Davoren, et al. (45)	5.6-9.8 g	Qiagen Blood Maxi Kit	56	18 hours, with a 2nd digestion at 70◦C for 1 hour	100	Qiagen Blood Maxi Kit	Inorganic
Davoren, et al. (45)	5.6-9.8 g	50 mM Tris-HCl; 100 mM NaCl; 50 mM EDTA, 0.5% SDS, pH 8.0	55	18-24 hours	100	PCIA	Organic

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3.12 SUPPLEMENTAL CRANIAL DIAGRAMS

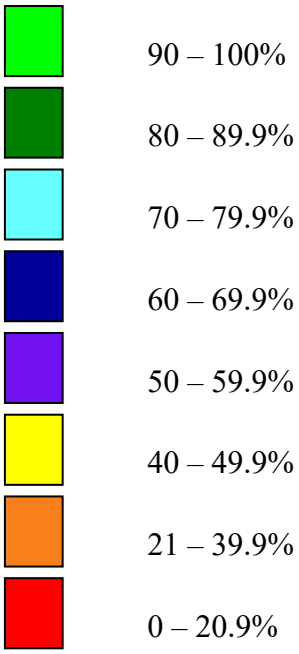
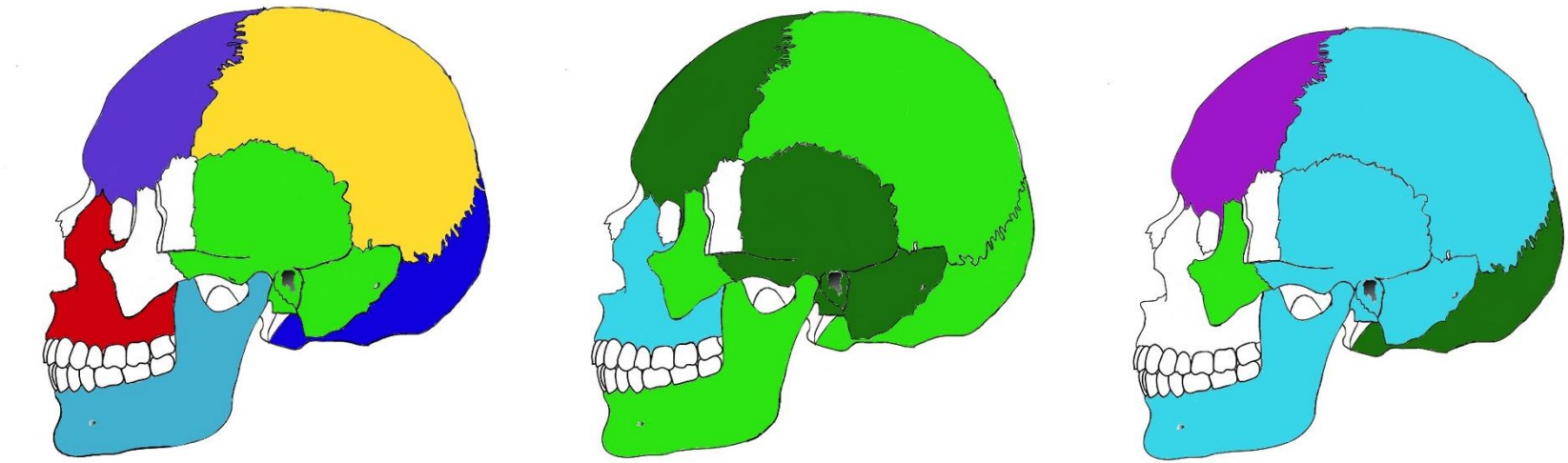
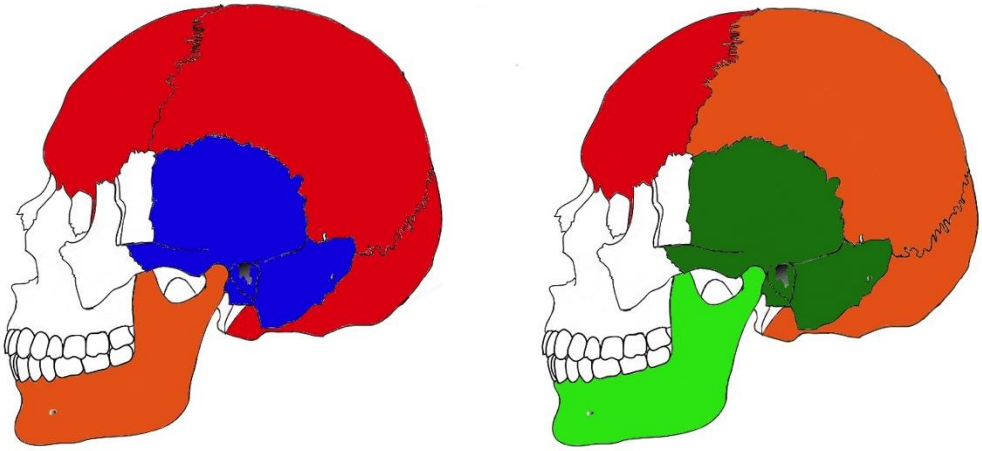


Figure 3.4 The key for the following diagrams.



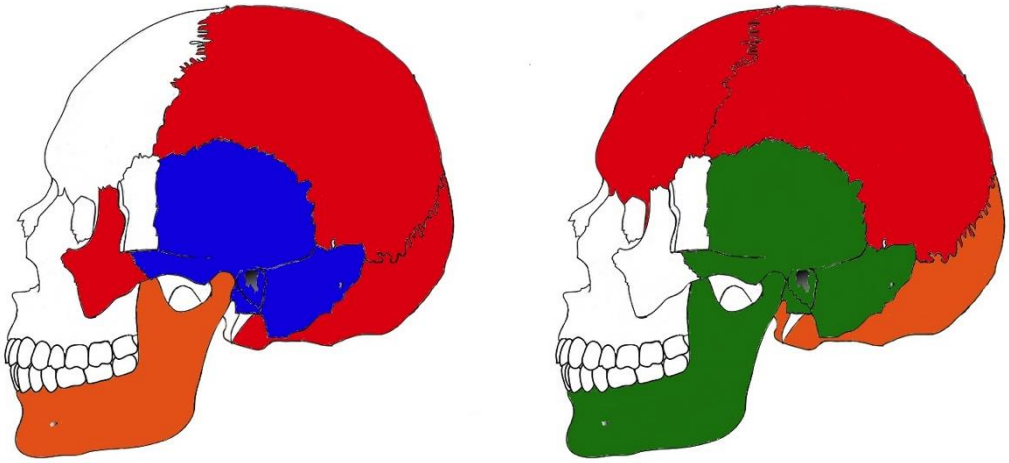
EP#1 EP#2 EP#3

Figure 3.5. Mitochondrial DNA using Sanger Sequencing testing by cranial element. The individual cranial elements are labeled based on the overall success for that element. Each extraction protocol, 1 – 3, is accounted for. This is a graphic representation of the data presented in Table 3.1.



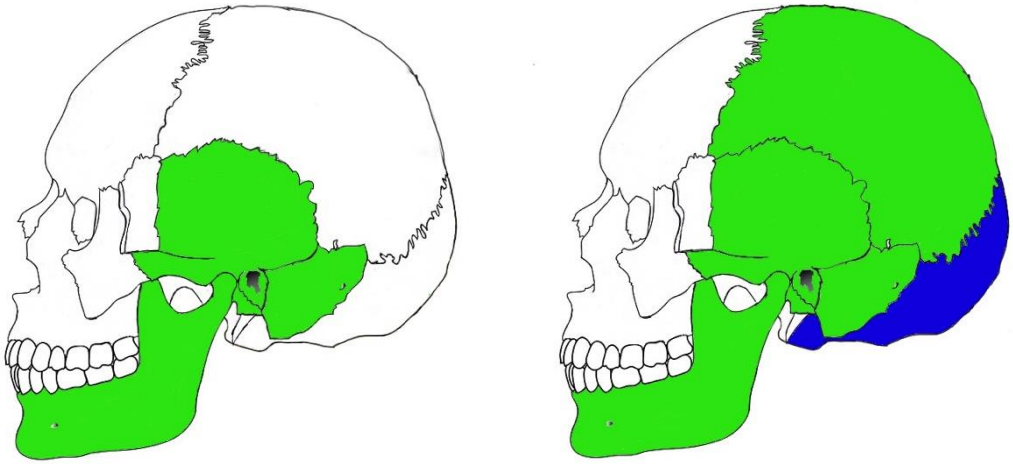
EP#2 EP#3

Figure 3.6. AmpFlSTR® MiniFiler™testing success by cranial element. The individual cranial elements are labeled based on the overall success for that element. This is a graphic representation of the data presented in Table 3.2.



EP#2 EP#3

Figure 3.7 Modified AmpFlSTR® Yfiler™ testing success by cranial element. The individual cranial elements are labeled based on the overall success for that element. This is a graphic representation of the data presented in Table 3.3.



EP#2 EP#3

Figure 3.8 PowerPlex® Fusion testing success by cranial element. The individual cranial elements are labeled based on the overall success for that element. This is a graphic representation of the data presented in Table 3.4.

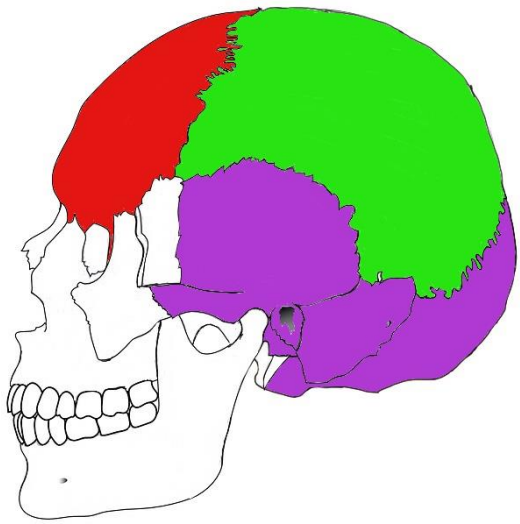


Figure 3.9. Next Generation Sequencing (NGS) testing success by cranial element. The individual cranial elements are labeled based on the overall success for that element. Only EP#4 is considered for this protocol. This is a graphic representation of the data presented in Table 3.5.

3.13 SUPPLEMENTAL TOOTH TABLES

Table 3.8. Success of Individual Teeth in Sanger sequencing of Mitochondrial DNA. Teeth are organized by type of tooth. The numbering system is the Universal system and is demonstrated in the tooth diagrams. Percent success and average number of bases are rounded to the next whole number. Only teeth that were numbered by the forensic odontologists are included.

		Extraction Protocol #1				Extraction Protocol #2				Extraction Protocol #3			
	Tooth #	# Tested	# Successful	% Success	Avg Bases	# Tested	# Successful	% Success	Avg Bases	# Tested	# Successful	% Success	Avg Bases
Molars	1	21	17	81	640	22	21	95	680				
	2	17	12	71	657	16	16	100	683				
	3	19	16	84	644	20	18	90	692				
	14	24	18	75	654	23	23	100	695				
	15	16	12	75	651	22	21	95	681				
	16	14	12	86	564	12	11	92	684				
	17	22	20	91	613	14	12	86	680				
	18	38	31	82	652	16	12	75	707				
	19	22	19	86	638	15	13	87	687				
	30	16	14	88	598	14	13	93	702				
	31	33	28	85	627	23	22	97	705				
	32	25	19	76	613	19	18	95	684				
Premolars	4	11	7	64	587	9	9	100	705				
	5	12	8	67	675	9	8	89	641	1	1	100	681
	12	14	8	57	599	16	15	94	673	2	2	100	700
	13	11	10	91	622	19	13	68	700	2	2	100	704
	20	20	16	80	624	15	12	80	686	1	1	100	705
	21	19	14	74	618	16	13	81	673	2	2	100	646
	28	24	15	63	610	9	9	100	688	3	3	100	685
	29	23	17	67	621	17	13	76	705	1	1	100	681
Canines	6	37	32	86	614	40	36	90	695	4	4	100	699
	11	42	35	83	652	41	38	93	696	2	2	100	704
	22	37	28	76	643	42	35	83	684	8	8	100	682
	27	48	32	86	609	28	24	86	703	7	7	100	694
Incisors	7	10	8	80	576	8	8	100	655	3	3	100	688
	8	18	16	89	643	14	14	100	699	1	1	100	705
	9	16	13	81	742	20	19	95	644				
	10	13	8	62	685	10	10	100	709	2	2	100	692
	23	6	3	50	606								
	24	2	1	50	419								
	25	2	0	0	0								
	26	4	2	50	612	3	3	100	690				

Table 3.9. Success of Individual Teeth in AmpFlSTR® MiniFiler™. Teeth are organized by type of tooth. The numbering system is the Universal system and is demonstrated in the tooth diagrams. Percent success and average number of bases are rounded to the next whole number. Only teeth that were numbered by the forensic odontologists are included.

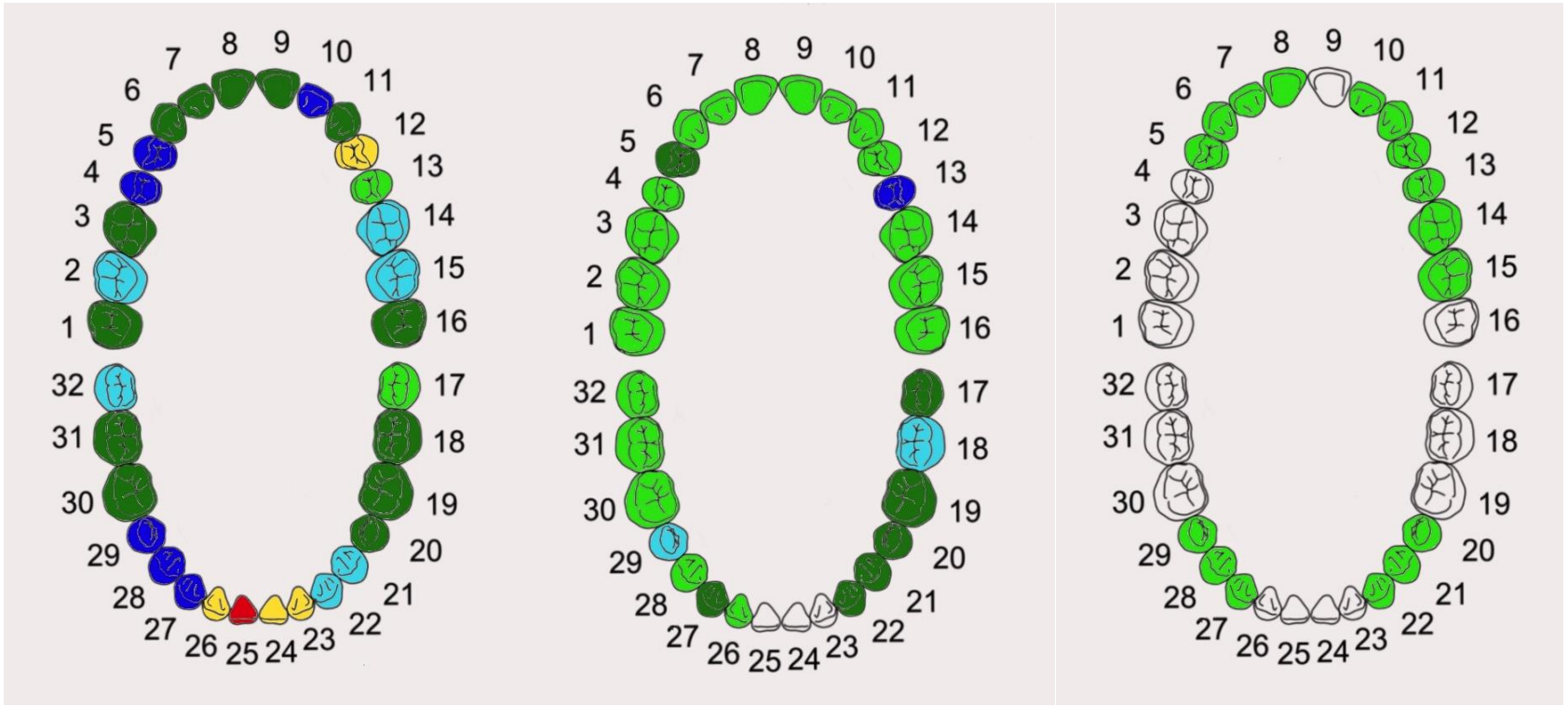
		Extraction Protocol #1				Extraction Protocol #2				Extraction Protocol #3			
	Tooth #	# Tested	# Successful	% Success	Avg Loci	# Tested	# Successful	% Success	Avg Loci	# Tested	# Successful	% Success	Avg Loci
Molars	1					1	0	0	0				
	2					4	3	75	6				
	3					3	1	33	5				
	14					3	0	0	2	1	0	0	0
	15					2	1	50	4				
	16					2	0	0	0				
	17												
	18					1	0	0	0				
	19												
	30												
	31					1	0	0	3				
	32					4	1	25	9				
Premolars	4					1	0	0	3				
	5												
	12					1	0	0	0				
	13					1	0	0	2				
	20												
	21												
	28												
	29					1	1	100	4				
Canines	6	1	0	0	0	2	0	0	0	1	0	0	0
	11	1	0	0	0	3	0	0	3	1	1	100	9
	22	1	0	0	0	10	2	20	4	1	0	0	0
	27	1	0	0	1	1	0	0	0	1	0	0	0
Incisors	7									1	0	0	0
	8					4	1	25	6				
	9					1	0	0	3	1	0	0	0
	10					10	0	0	2	1	1	100	9
	23												
	24												
	25												
	26												

Table 3.10. Success of Individual Teeth in Modified AmpFlSTR® Yfiler™. Teeth are organized by type of tooth. The numbering system is the Universal system and is demonstrated in the tooth diagrams. Percent success and average number of bases are rounded to the next whole number. Only teeth that were numbered by the forensic odontologists are included.

		Extraction Protocol #1				Extraction Protocol #2				Extraction Protocol #3			
	Tooth #	# Tested	# Successful	% Success	Avg Bases	# Tested	# Successful	% Success	Avg Bases	# Tested	# Successful	% Success	Avg Bases
Molars	1					1	0	0	0				
	2					3	2	67	10				
	3					4	2	50	9				
	14					4	3	75	6				
	15					1	0	0	0				
	16					2	1	50	7				
	17					2	0	0	1				
	18					3	0	0	2				
	19					1	0	0	0				
	30					2	0	0	0				
	31					5	1	20	3				
	32					3	0	0	0				
Premolars	4					2	0	0	1				
	5												
	12					2	0	0	2				
	13					2	1	50	5	1	0	0	0
	20												
	21												
	28									1	1	100	4
	29					1	1	100	5				
Canines	6					1	0	0	0	2	1	50	14
	11					5	1	20	4	1	1	100	17
	22					14	1	7	8	1	0	0	0
	27	1	1	100	6	4	0	0	0	2	0	0	2
Incisors	7									1	0	0	0
	8					3	1	33	12				
	9									1	0	0	0
	10									1	1	100	15
	23												
	24												
	25												
	26												

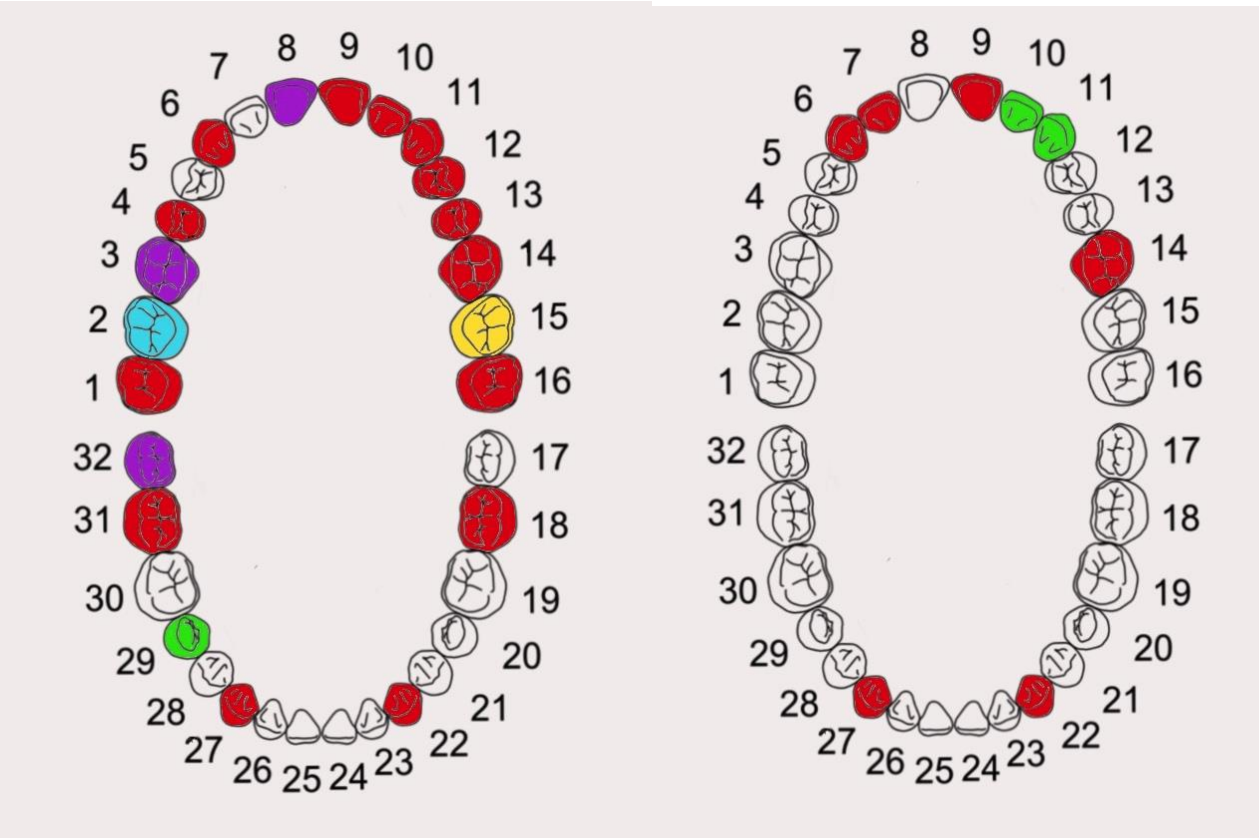
3.14 SUPPLEMENTAL TOOTH DIAGRAMS

The color key for these diagrams can be seen in Figure 3.4.



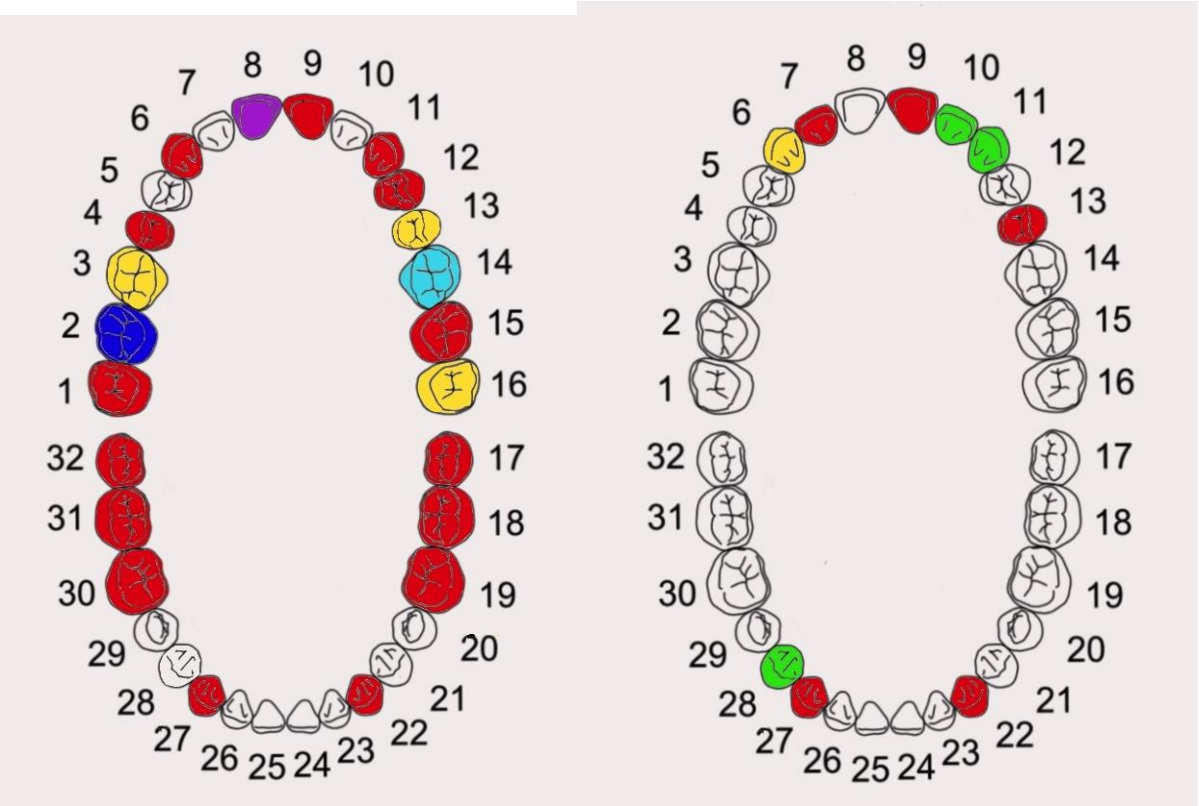
EP#1 EP#2 EP#3

Figure 3.10. Mitochondrial DNA using Sanger Sequencing testing success by individual tooth. The individual teeth are labeled based on the overall success for that tooth. Teeth are numbered based on the Universal numbering system. Only teeth numbered by the forensic odontologists are included. This is a graphic representation of the data in Table 3.8.



EP#2 EP#3

Figure 3.11. AmpFlSTR® MiniFiler™testing success by individual tooth. The individual teeth are labeled based on the overall success for that tooth. Teeth are numbered based on the Universal numbering system. Only teeth numbered by the forensic odontologists are included. This is a graphic representation of the data in Table 3.9.



EP#2 EP#3

Figure 3.12. AmpFlSTR® MiniFiler™testing success by individual tooth. The individual teeth are labeled based on the overall success for that tooth. Teeth are numbered based on the Universal numbering system. Only teeth numbered by the forensic odontologists are included. This is a graphic representation of the data in Table 3.10.

3.15 PRESENTATION

This presentation was given at the annual American Academy of Forensic Sciences meeting held in Baltimore, MD, 21 February 2019.

Efficient Sampling of Skeletonized Human Crania for DNA Testing

Suni M. Edson[1,2]

AAFS Annual Meeting
Baltimore, MD
21 February 2019

[1]Armed Forces DNA Identification Laboratory, Armed Forces Medical Examiner System, Dover AFB, DE, USA
[2]Flinders University, College of Science and Engineering, Adelaide, South Australia

Disclaimer

The opinions or assertions presented hereafter are the private views of the speaker and should not be construed as official or as reflecting the views of the Department of Defense; the Defense Health Agency; the Armed Forces Medical Examiner System; or the Defense POW/MIA Accounting Agency.

Commercial Products

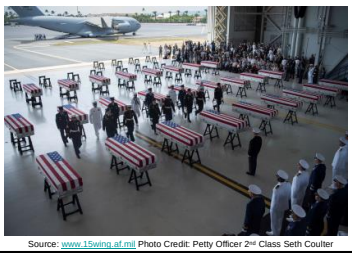
Commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible, and does not imply that any of the commercial products identified are necessarily the best available for the purpose.

Outline

- Introduction to the Mission
- DNA Extraction Techniques
  - Testing modalities
- Crania sampling success
- What does this mean?

Armed Forces Medical Examiner System – Armed Forces DNA Identification Laboratory (AFMES-AFDIL)

Our express purpose is to aid in the identification of the remains of US service members



Source: www.15wing.af.mil Photo Credit: Petty Officer 2nd Class Seth Coulter

Armed Forces Medical Examiner System – Armed Forces DNA Identification Laboratory (AFMES-AFDIL)

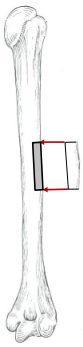
- A subdivision of the Armed Forces Medical Examiner System (AFMES)
- Established in 1990 as the DoD DNA Registry
- Mission Partner with Defense POW/ MIA Accounting Agency (DPAA)

Sample Collection

- Samples are recovered by scientists from DPAA or partner agencies.
- Samples are typically 40-70 years post-mortem
- Elements may be found under a variety of conditions:
  - Buried in soil
  - Surface
  - Unilateral turnovers
  - Submerged in water

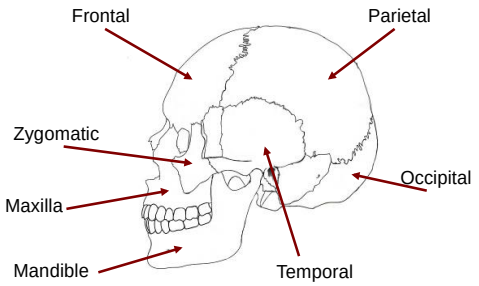
DPAA website. Photo credit: SSgt Erik Cardenas

Sample Collection

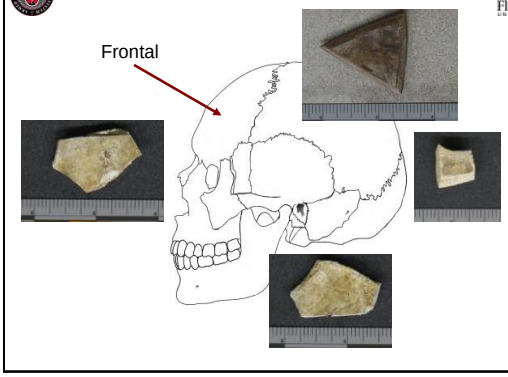


- Samples are returned to the lab.
- Elements are sorted and nominated by an anthropologist
- A small fragment is removed for DNA testing
  - Samples are typically 2.0-5.0g
  - Smallest sample tested was 0.06g

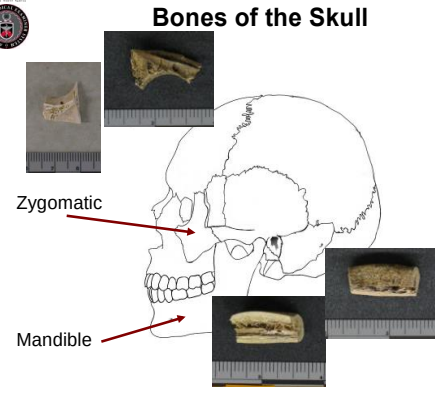
Bones of the Skull



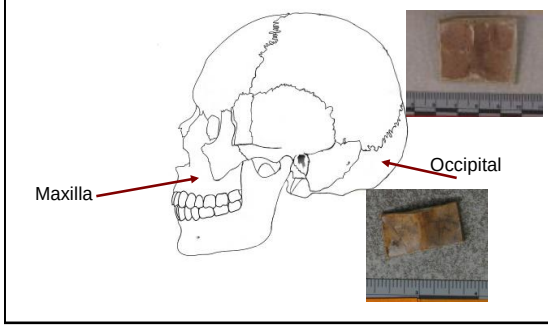
Bones of the Skull



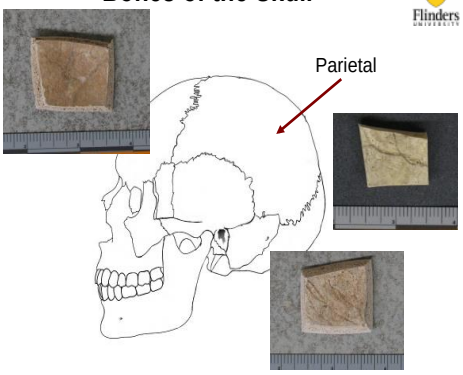
Bones of the Skull



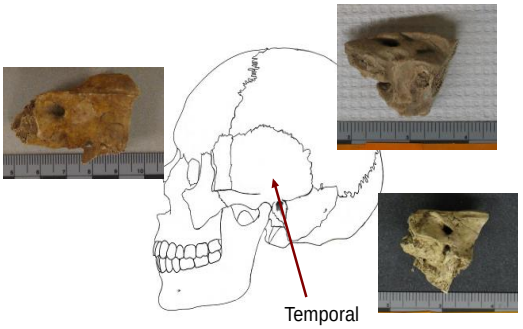
Bones of the Skull



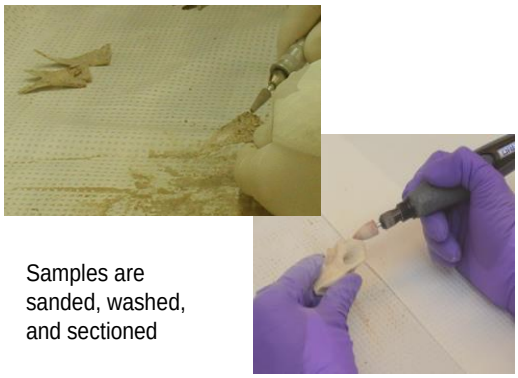
Bones of the Skull



Bones of the Skull



Sample Preparation



Samples are sanded, washed, and sectioned

Sample Preparation

- Bone sample is powdered and then incubated in buffer overnight.
- Complete demineralization/dissolution of the sample is typically achieved.
- Purification is performed using using either organic or inorganic purification methods.

Sample Processing

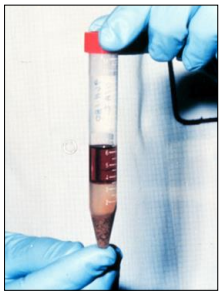
- Downstream processing includes:
  - mtDNA Sanger sequencing
  - auSTR (Minifiler and/ or Fusion)
  - LCN YSTR (Yfiler)
  - NGS/MPS whole genome sequencing

Samples Tested

- 1908 – Sanger sequencing of mitochondrial DNA
- 267 – Minifiler
- 386 – Modified Yfiler
- 36 – PowerPlex Fusion
- 45 – NGS

"Original" Extraction

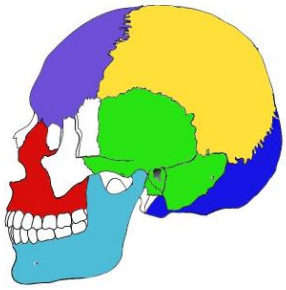
Original 1990-2006
2.0-2.5g bone powder
Extraction Buffer: 10mM Tris, pH 8.0 50mM EDTA, pH 8.0 0.5% SDS +Proteinase K
Phenol:Chloroform Purification



"Original" Extraction mtDNA Sanger Sequencing

Original 1990-2006
2.0-2.5g bone powder
Extraction Buffer: 10mM Tris, pH 8.0 50mM EDTA, pH 8.0 0.5% SDS +Proteinase K
Phenol:Chloroform Purification

90-100
80-89.9
70-79.9
60-69.9
50-59.9
40-49.9
30-39.9
0-29.9



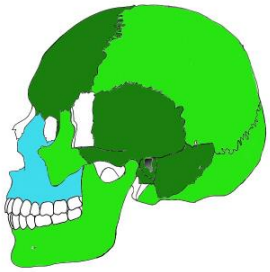
"Demin 1" Extraction

"Demin 1" 2006-present
0.2-0.25g bone powder
Extraction Buffer: 0.5M EDTA, pH 8.0 1% N-lauroyl sarcosinate +Proteinase K
Phenol:Chloroform Purification

"Demin 1" Extraction mtDNA Sanger Sequencing

"Demin 1" 2006-present
0.2-0.25g bone powder
Extraction Buffer: 0.5M EDTA, pH 8.0 1% N-lauroyl sarcosinate +Proteinase K
Phenol:Chloroform Purification

90-100
80-89.9
70-79.9
60-69.9
50-59.9
40-49.9
30-39.9
0-29.9



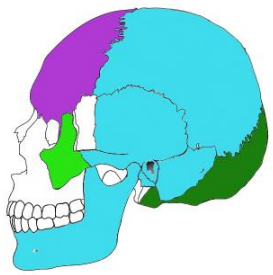
"Demin 2" Extraction

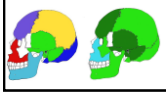
"Demin 2" 2012-present
0.2-0.25g bone powder
Extraction Buffer: 0.5M EDTA, pH 8.0 1% N-lauroyl sarcosinate +Proteinase K
QIAquick PCR Purification

"Demin 2" Extraction mtDNA Sanger Sequencing

"Demin 2" 2012-present
0.2-0.25g bone powder
Extraction Buffer: 0.5M EDTA, pH 8.0 1% N-lauroyl sarcosinate +Proteinase K
QIAquick PCR Purification

90-100
80-89.9
70-79.9
60-69.9
50-59.9
40-49.9
30-39.9
0-29.9

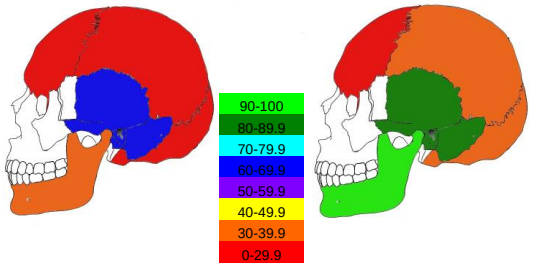




Minifiler

Demin 1
Organic

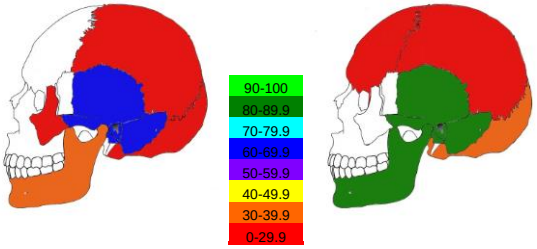
Demin 2
Inorganic



Modified YFiler

Demin 1
Organic

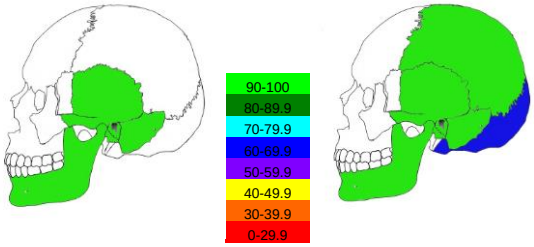
Demin 2
Inorganic



Fusion

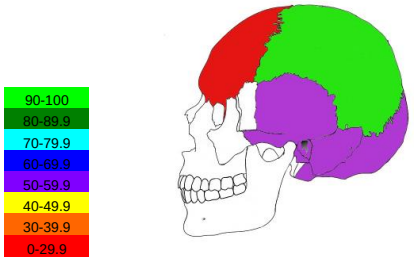
Demin 1
Organic

Demin 2
Inorganic



NGS

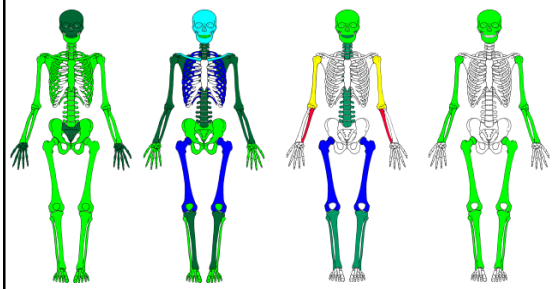
Modified Demin 1
1.0g of bone powder



Conclusions

- These results mirror those of post-cranial skeletal elements.
  - Inorganic extractions work better for STR
  - Organic extractions work better for Sanger

Post-Cranial Success



Demin 1
Sanger

Demin 2
Sanger

Demin 1
Fusion

Demin 2
Fusion

Conclusions

- These results mirror those of post-cranial skeletal elements.
  - Inorganic extractions work better for STR
  - Organic extractions work better for Sanger
- Inorganic extractions may retain larger pieces of DNA and wash away smaller fragments.
  - Sanger works, just not as well as it could.
- Fusion may be artificially high in success due to being screened in Minifiler first.

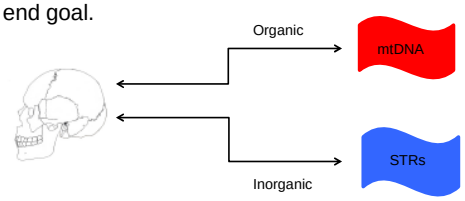
What protocol should I use?

It depends.

What protocol should I use?

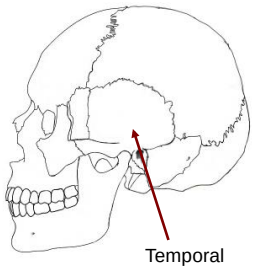
It depends.

If you know what modalities need to be tested, you can choose an extraction based on your end goal.



What Element is Best?

- The temporal.
  - It's not always the best, but it is consistently good regardless of the protocol or modality used.



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- Administrative Support – James Canik, Dr. Mike Coble

Any Questions?

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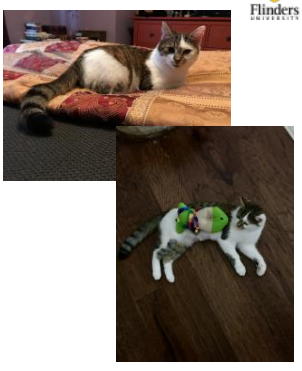
AFMES Additional Information:
http://www.health.mil/afmes

DPAA Additional Information:
http://www.dpaa.mil/



Chapter 4

Why Extracted DNA Fails to Produce a Result: The Samples are Non-Human

4.1 INTRODUCTION

DNA testing from ossified humans is not a simple process as indicated in the previous chapters. In cases of mass fatality samples need to be processed quickly and efficiently. When samples do not provide a positive DNA result, analysts need to determine why this is so. The targeting of possible issues allows them to keep the workflow moving rapidly and eliminates impediments to the identification process. This chapter and the next will focus on possible issues that may prevent identification of osseous human remains.

As indicated in the previous chapters, some samples may not be human in nature and therefore, need to be removed from the DNA testing workflow as soon as possible, so as not to impede the testing progress. If a sample is suspected to be non-human, a simple test is the evaluation of the 12S ribosomal RNA subunit within the mitochondrial DNA. Testing of the 12S rRNA region is not very technical, but rather requires a simple amplification using the same testing strategies as employed in regular casework. Analysts and technicians do not require special training, and can incorporate 12S testing into regular casework. The region is amplified using non-species specific primers and processed along with normal casework samples for sequencing and fragment separation on CE instrumentation.

The validation and blind study presented in this chapter were not designed as part of the thesis work; however, the implementation of this strategy into casework and the results presented came out of the evaluation of the data sets described in Chapters 2 and 3. Chapter 4 presents the technical notes regarding the validation as well as the use in day-to-day casework. At present, the non-human results the Southeast Asia region are being further evaluated and mapped against the recovery locations in an effort to determine if there are relevant biogeographical data.

4.2 PUBLICATION

The results of this study are to be published as Chapter 3 *in* From Field to Laboratory: A Memorial Volume in Honor of Robert J. Baker. Edited by Robert D. Bradley, Hugh H. Genoways, David J. Schmidly and Lisa C. Bradley. Special Publications of Museum of Texas Tech University, Number 71. Target Publication Date: August 2019.

The Use of Mitochondrial 12S rRNA Gene Sequencing in a Human Identification Laboratory for Species Determination of Compromised Skeletal Remains

Suni M. Edson, Kimberley Sturk-Andreaggi, Alexander F. Christensen, Suzanne Barritt-Ross.

4.2.1 ATTRIBUTION OF TASKS

The casework portions of the paper were collected and written by Ms. Edson. In addition, Ms. Edson collated the portions written by the other contributors and merged it into a cohesive document.

The validation was designed by Ms. Sturk-Andreaggi with input from Ms. Suzanne Barritt-Ross. Ms. Sturk-Andreaggi wrote the sections of this chapter dealing with the validation and blind study.

The blind study was designed by Dr. Christensen and Ms. Sturk-Andreaggi.

The validation and the blind study were not undertaken as part of this thesis and have been completed for a number of years; however, the application to casework and the survey of data associated with casework was developed out of the analysis of data presented in Chapters 2 and 3.

4.2.2 PEER REVIEW

The paper was peer reviewed by three blinded reviewers and the primary editor of the volume Dr. Robert Bradley.

4.3 TITLE PAGE

The Use of Mitochondrial 12S rRNA Gene Sequencing in a Human Identification Laboratory for Species Determination of Compromised Skeletal Remains

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4.3.1 DISCLAIMER

The opinions or assertions presented are the private views of the authors and should not be construed as official or as reflecting the views of the Department of Defense; the Defense Health Agency; the Armed Forces Medical Examiner System; or the Defense POW/MIA Accounting Agency.

4.3.2 ACKNOWLEDGMENTS

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4.4 ABSTRACT

In the field of human identification, remains may be skeletonized and highly fragmented. This damage to remains often precludes identification as to the species of origin. PCR-based amplification of a portion of the mitochondrial 12S rRNA gene is a quick, inexpensive method for determination of a species. This chapter describes the development of such an assay at the Armed Forces Medical Examiner System – Armed Forces DNA Identification Laboratory (AFMES-AFDIL) and the subsequent implementation of the protocol into regular casework. The species identified from 605 samples tested are described, along with the impact of this protocol on the streamlining of testing osseous materials in a human identification laboratory.

4.5 KEY WORDS

12S rRNA, BLAST, DNA, human identification, protocol development, skeletal remains, species identification

4.6 ARTICLE INTRODUCTION

In modern mass fatality events, the remains presented for analysis are typically intact, and are, at the least, visually identifiable as human. Remains from past events, however, can be fully skeletonized and may be found in fragments or in a highly damaged state in which the species of origin is not readily apparent. Fully skeletonized remains may be subjected to fragmentation post-mortem from human impact (e.g., farming, industrial activities, road building) or simply age. Additionally, remains may undergo fragmentation at time of death, particularly in events that involve plane crashes or explosions. This can be particularly true in times of conflict, when high-energy events are more common.

The specimens submitted to the Armed Forces Medical Examiner System - Armed Forces DNA Identification Laboratory (AFMES-AFDIL, a.k.a. AFDIL) by the Defense POW/MIA Accounting Agency (DPAA) include remains excavated from decades-old events world-wide. Some locations, particularly those in Southeast Asia, experience an annual variability in soil temperature and moisture combined with high soil acidity that rapidly erodes any skeletal fragments. In many cases, the only conclusion that can be drawn from osteological analysis of these remains is that they are consistent with, but not exclusive to, human in origin.

In 2007, AFDIL implemented into casework a complete demineralization protocol for the extraction of DNA from osseous materials (Loreille et al. 2007). This protocol involves a complete dissolution of the skeletal materials, and a more efficient extraction of DNA than presented in Edson, et al. (2004). This protocol allowed for the reduction in size of samples submitted to AFDIL by the DPAA Laboratory. Prior to 2007, the requested size of the element sampled was 5.0 g or greater, as the required input for DNA extraction was 2.0–2.5 g of material (Edson et al. 2004). With complete demineralization, coupled with an organic purification, the input size was reduced to 0.2 g, allowing the DPAA to re-evaluate cases

previously thought to be untestable due to the small size of fragments recovered. Excluding teeth, the average size of a sample submitted to AFDIL decreased from 7.66 g to 4.11 g with the implementation of complete demineralization. The trend has continued with implementation of an inorganic purification coupled with the complete demineralization (Loreille et al. 2010; Edson and McMahon 2016), to an average sample size of 3.59 g. Therefore the size of the samples submitted to the AFDIL decreased by approximately 50% and, although DPAA anthropologists examine the remains prior to submission, accurate determination of species origin is often precluded by the small and severely compromised condition of the elements.

Since the implementation of the demineralization protocol, AFDIL has successfully reported mtDNA sequence data for 86% of the more than 9,000 samples tested. When reportable sequence data cannot be produced, one possible explanation is that the endogenous DNA is either too fragmented or too limited in quantity to be recovered with currently validated assays. Another possibility is that the fragments are not of human origin. When preliminary attempts to obtain mtDNA are unsuccessful, modifications are made to the amplification reaction to accommodate DNA fragmentation, inhibition, and low quantities of DNA. Additionally, standard practice at DPAA has been, when possible, to resample those skeletal elements that did not yield reportable sequence data so that AFDIL can attempt to successfully obtain a mtDNA profile. These processes are time-consuming and costly, and may also continue to be unsuccessful if the specimen is not of human origin. To prevent needless DNA testing, and to provide critical information to DPAA, it is important to determine if the failure to produce conclusive data is due purely to sample degradation, and thus low quantity/quality DNA, or is instead due to the non-human origin of the skeletal element. This is extremely vital in cases for which small fragments of uncertain origin are the only biological remains recovered for a particular incident.

Human identification efforts may not seem to have that much in common with wildlife biology; however, the DNA analysis tools that can be used are very similar. Although advanced methods exist for precise species identification of biological materials (e.g., melt curve analysis: Kitpipit et al. 2016; cytochrome b: Tobe and Linacre 2010; Ciavaglia et al. 2015; Linacre and Lee 2016), AFDIL uses the amplification of the 12S ribosomal (rRNA) gene as a rapid screen to determine if smaller skeletal elements are human in origin. In 2005, primers that amplified the cytochrome *b* gene on the mitochondrial genome were evaluated for use in casework (Freeman, internal validation). While cytochrome *b* has been found to be successful for determination in forensics settings (Branicki et al. 2003), the size of the amplicon (300 bp) is too large for usage with degraded skeletal remains, leading to the evaluation of the 12S rRNA gene. Polymerase chain reaction (PCR) primers developed by Balitzki-Korte et al. (2005) target this gene within mtDNA. These primers bind to a small, highly conserved region across a range of species and amplify a short (146 bp), yet variable portion of the mitochondrial 12S rRNA gene, allowing for the development of a species identification assay tailored for highly compromised remains. The size of this amplicon is comparable to the primer sets commonly used on the most degraded DNA that target small fragments (typically 150 bp or less) of the human mtDNA control region (Gabriel et al. 2001). Although small, this portion of the 12S rRNA gene has been shown to provide information sufficient to differentiate taxa at the species level (Balitzki-Korte et al. 2005; Melton and Holland 2007). The following text provides a description of the protocol development and a summary of the usage of the technique in casework.

4.7 PROTOCOL DEVELOPMENT

4.7.1 Morphological and histological determination of human vs. non-human origin

Prior to DNA testing, anthropologists assess human versus non-human origin of skeletal remains based on macro- and microscopic morphological characteristics. Larger elements

can generally be characterized as either human or non-human based upon morphological features. However, when small bone fragments are encountered, size may preclude a human or non-human designation based upon bone morphology. In these cases, a thin section cut from the fragment may be examined microscopically, and qualitative analyses, which include determination of different types of micromorphology such as plexiform bone or osteon banding, are used to determine whether or not the bone is consistent with non-human origin (Mulhern and Ubelaker 2001; Benedix 2004; Hillier and Bell 2007). Although the presence of plexiform bone or osteon banding definitively classifies a bone as non-human, the absence of this bone type does not automatically indicate human origin. According to DPAA procedures utilized during this study, histological analysis results in a judgment of either "match to non-human" or "inconclusive." If the osseous material cannot be conclusively identified as non-human based upon microscopic analyses or the sample is not large enough to examine its histology, a fragment is submitted to AFDIL for 12S mtDNA testing.

4.7.2 Extraction of DNA from the Bone

Upon arrival at AFDIL from DPAA, osseous fragments are cleaned using a Dremel® tool (Dremel, Racine, WI), washed with sterile deionized water (diH_2O) and 100% (v/v) ethanol (Pharmco-Aaper, Brookfield, CT), and allowed to air dry. After cleaning, the osseous sample is sectioned for pulverization. Samples submitted to AFDIL are typically 2.0 – 5.0 g, but the desired input for the extraction protocol is 0.25 – 0.5 g. Pulverization is performed using a Waring blender with a professional base (MC2 cup; Waring, Stamfield, CT).

Samples in this study used two different extraction protocols: complete demineralization coupled with an organic purification, and complete demineralization coupled with an inorganic purification (Edson and McMahon 2016; Edson 2019). For both protocols, the pulverized bone material is incubated overnight at 56°C using an extraction buffer (0.5 M

EDTA, pH 8.0; 1% *N*-Lauroylsarcosine) and Proteinase K (200 mg/mL; Ambion™, Thermo Fisher, Gaithersburg, MD). Purification follows with either an organic purification using 25:24:1 phenol:chloroform:isoamyl alcohol (Sigma-Aldrich, St. Louis, MO) followed by a wash with *n*-Butanol (Sigma-Aldrich) or an inorganic purification with the QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Samples are concentrated using Amicon Ultra-4/30K centrifugal filter units (Millipore, Billerica, MA) and the extracted DNA is brought to a final volume of 100 – 200 µL with TE (10 mM Tris, 0.1 M EDTA; pH 8.5).

4.7.3 12S Amplification, Sequencing, and Data Analysis

PCR was conducted using primers that target a 146-bp region of the 12S rRNA gene described in Balitzki-Korte et al. (2005). Amplification of 1–3 µL DNA extract was performed in a 50 µL reaction containing 10 units AmpliTaq® Gold DNA polymerase (Life Technologies, Gaithersburg, MD); 1X GeneAmp® PCR Buffer I (Life Technologies); 200 µM dNTPs (Life Technologies); and 0.4 µM of each primer. Non-acetylated bovine serum albumin (BSA; 0.025 mg/mL, Sigma-Aldrich, St. Louis, MO) was eliminated from the amplification after it was found that there was cross-reactivity with the primers, giving false results of *Bos taurus* DNA. In accordance with in-house quality control standards, appropriate extraction and amplification controls were included. Thermal cycling for both amplification and sequencing reactions was carried out in a GeneAmp® 9700 (Life Technologies) using the 9600 emulation mode. The optimized cycling conditions for amplification were 96˚C for ten minutes followed by 38 cycles of 94˚C for 30 seconds, 50˚C for 30 seconds, and 72˚C for one minute with a final extension step of 72˚C for seven minutes. The PCR products were confirmed using a 2% agarose gel stained with Ethidium bromide (5 mg/mL). If a positive result was obtained, purification was performed using 1.5 µL Exo-SAP-IT® (Affymetrix, Santa Clara, CA) and 17.5 µL dilution buffer (50 mM Tris; pH 8.0).

Cycle sequencing was conducted in 20 µL reactions with 3.6 µL BigDye® Terminator v1.1 Cycle Sequencing Kit (Life Technologies), 0.4 µL dGTP BigDye® Terminator v1.0 (Life Technologies), 4 µL dilution buffer (400 mM Tris, 10 mM $MgCl_2$; pH 9.0), and 0.5 µM of sequencing primer. Both amplification primers were utilized to generate sequence data from both strands for each sample. Input volume of purified product was either 1 µL or 7 µL depending on band intensity observed on the agarose gel. Sequencing products were purified with Performa® DTR V3 Short or Ultra 96-Well Plates (Edge Biosystems, Gaithersburg, MD) and dried down in an evaporator/concentrator centrifuge. Samples were resuspended with 10 µL Hi-Di™ Formamide (Life Technologies) prior to separation on an Applied Biosystems 3130*xl* and/or 3500 Genetic Analyzer (Life Technologies).

Sequences were aligned using Sequencher™ version 4.1 or higher (GeneCodes, Ann Arbor, MI) and a consensus sequence of approximately 109 bases, depending on species origin, was generated for each sample. Once the 12S consensus sequence was established, the Basic Local Alignment and Search Tool (BLAST) available online (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990; Zhang et al. 2000) was used to search the National Center of Biotechnology Information (NCBI) database. The consensus sequence string, which excludes the primers, was entered into the nucleotide-nucleotide BLAST (BLASTN) program and searched against the "Nucleotide collection (nr/nt)" (NCBI Nucleotide) database using the default search parameters. The BLASTN search results were then reviewed to assess sample origin. For each sequence returned from the database search, BLAST generates statistics that reflect the similarly of the alignment (bit score, or "Max Score"), the statistical significance (Expect value, or "E-value") of the database hit, as well as the percentage of identical ("Max Ident") and covered ("Query Coverage") bases (Madden 2002). The sequence homology (reported as the "Max Ident" in BLASTN search results) was used to establish the thresholds described in Table 4.1. A 12S sequence of 75 or more bases

can be reported as “human”, “non-human” or “inconclusive”. All mixed sequences are reported as “inconclusive” due to the inability to definitively determine human or non-human origin, and sequences less than 75 bases are reported as “insufficient data”. Samples determined to be of human origin are reported as “consistent with” or “presumed to be” human depending on the sequence homology with the *Homo sapiens* sequence (100% and ≥90%, respectively). “Non-human” sequences are reported similarly, and are classified as the common taxon (e.g. genus, family) opposed to a specific species when more than one non-human species is homologous with the searched sequence. These BLASTN interpretation guidelines, though developed primarily to distinguish between human and non-human origin, also permit more specific classifications to be made by the analyst. Further, any identification as “human” using this assay is considered with caution as exogenous modern human DNA has the potential to contaminate lower quality specimens.

Table 4.1. Classification guidelines implemented at AFDIL for the 12S species identification assay. The sequence homology refers to the maximum identity (“Max Ident”) reported for each alignment generated by the BLASTN query. Regardless of the sequence homology, a sample is classified as “Inconclusive” if *both* human and non-human species are present in the search results, or also if no human or animal species are homologous with the queried sequence.

Classification	**Sequence Homology**	**BLASTN Search Results**
Human	100%	*Homo sapiens* (and *Homo neanderthalensis*) ONLY
Presumed to be Human	≥90%	*Homo sapiens* (and *Homo neanderthalensis*) ONLY
Non-Human	100%	One or more taxa (other than *Homo sapiens*)
Presumed to be Non-Human	≥90%	One or more taxa (other than *Homo sapiens*)
Inconclusive	-	*Homo sapiens* and other animal taxa
	-	Neither animal or *Homo sapiens* e.g. bacteria
	<90%	One or more taxa
	N/A	"No significant similarity found"
	Mixed sequence	
Insufficient Data	Sequence less than 75 bases	

4.8 BLIND STUDY

Thirty-eight skeletal fragments were selected by DPAA anthropologists and submitted blindly to AFDIL for species identification using the 12S assay. The samples varied by species, age, preservation, and geographic origin. Of the 38 samples submitted for this study, 37 (97%) produced sequence data resulting in an unambiguous match in the NCBI Nucleotide database (Table 4.2). In 76% (29) of cases, 12S testing produced sequence data consistent with the species determination made via anthropological analysis. Eight of the remaining samples (4, 8, 16, 23, 25, 26, 29 and 33) generated reproducible 12S sequence data and were successfully associated with a species in the NCBI Nucleotide database, but were inconsistent with the species assignment made by the DPAA anthropologists. DPAA anthropologists subsequently performed more extensive physical examinations and determined that six of the eight samples were consistent with the genera, if not the species, indicated by the 12S assay. The osteological reassessment of the other two samples (8 and 23) indicated that they were consistent with the 12S determinations. Regardless, all eight samples were non-human according to both the 12S and anthropological taxonomic classifications, which is the foremost purpose of the assay.

Of particular interest were the species identification results for Sample 9. Replicate amplifications produced the same 12S sequence for which the BLASTN search produced a best match inconsistent with the geographical location of the recovery site. *Acinonyx jubatus* (Cheetah) was the top hit but there were three mismatches (94% identity) between the queried and database sequences. A subsequent BLASTN query performed less than a year later resulted in a 100% match to *Viverricula indica* (Small Indian Civet), a more likely origin based on the sample metadata and consistent with the anthropological classification. Nevertheless, the sequence data clearly indicated a non-human source and would have been initially reported as “Non-Primate” based on the previously stated guidelines (Table 4.1).

Sample 19 was the only blind study bone specimen that remained unresolved at the conclusion of this study. Initial 12S testing classified the bone fragment as human. However, the skeletal element was a complete right radius from a medium-sized canid and had been unequivocally identified as non-human by DPAA anthropologists. Extraction of a re-sampled fragment revealed the presence of a mixture between two species, *Homo sapiens* and *Canis lupus familiaris* (Domestic Dog). The major contributing sequence of the 12S mixture was human and assumed to be a contaminant that dominated the endogenous canid DNA. In these situations, when a 12S "inconclusive" classification results from a human:non-human mixture, case-specific details would be examined in order to establish the best course of action for the sample and to determine which component of the mixture is the contaminating species. Possible strategies include 12S testing of a new cutting of the same bone or re-extraction of the original sample if additional material is available.

Table 4.2. Species identification results from the blind study samples. Taxonomic classifications are shown for the 12S assay as the top BLASTN species and the classification determined by AFDIL guidelines. For the DPAA species identification, the human/non-human determination is provided as well as the presumed species based upon anthropological analysis. Animals noted with "[ ]" identify the revised determinations made by faunal experts in cases of discordance between the 12S and DPAA species. The geographic origin is the location in which the specimens were recovered.

*As of 2001, the genus for *Lama pacos* (Alpaca) was changed to *Vicugna* based on genetic evidence that supported the hypothesis that the Alpaca is derived from the Vicuña not the Guanaco (Kadwell et al. 2001). However, the database hits that were 100% homologous with the Sample 32 sequence at the time of the search were noted as *Lama pacos* in the NCBI Nucleotide database even though they were added in 2006. Species of the *Vicugna* and *Lama* genera are closely related and the common non-human taxon, family Camelidae, would be reported.

Sample	12S Taxonomic Classification		DPAA Taxonomic Classification		Geographic Origin
	Top BLASTN Match	AFDIL Guidelines	Species	Human/Non-Human	
1	*Cervus elaphus*	Red Deer	Elk	Non-human	Contiguous United States
2	*Castor canadensis*	Beaver	Beaver	Non-human	Contiguous United States
3	*Odocoileus hemionus*	Mule Deer	Deer	Non-human	Contiguous United States
4	*Canis lupus familiaris*	Dog	Civet [Canid]	Non-human	Vietnam
5	*Sus scrofa*	Pig	Pig	Non-human	Vietnam
6	*Homo sapiens*	Human	-	Human	Vietnam
7	*Bos taurus*	Cow	Cow/Buffalo	Non-human	Papua New Guinea
8	*Muntiacus muntjak*	Indian Muntjac	Sheep/Goat	Non-human	Vietnam
9	*Viverricula indica*	Small Indian Civet	Civet	Non-human	Vietnam
10	*Bos taurus*	Cow	Cow/Buffalo	Non-human	Hawaii
11	*Bos taurus*	Cow	Cow/Buffalo	Non-human	Laos
12	*Bos taurus*	Cow	Cow/Buffalo	Non-human	Laos
13	*Homo sapiens*	Human	-	Human	Vietnam
14	*Sus scrofa*	Pig	Pig	Non-human	Luxemburg
15	*Bos taurus*	Cow	Cow	Non-human	Luxemburg
16	*Ovis aries* or *ammon*	Sheep	Pig [Sheep/Goat]	Non-human	Solomon Islands
17	*Bos taurus*	Cow	Cow/Buffalo	Non-human	China
18	*Homo sapiens*	Human	-	Human	Vietnam

19	Mixture	Inconclusive	Dog	Non-human	Hawaii
20	*Odocoileus hemionus*	Mule Deer	Deer	Non-human	Contiguous United States
21	*Equus grevyi*	Zebra	Zebra	Non-human	Kenya
22	*Cervus elaphus*	Red Deer	Elk	Non-human	Contiguous United States
23	*Sus scrofa*	Pig	Sheep/Goat	Non-human	Laos
24	*Bos taurus*	Cow	Cow	Non-human	Hungary
25	*Cervus elaphus*	Red Deer	Cow [Deer]	Non-human	Hungary
26	*Vulpes vulpes*	Fox	Dog [Fox]	Non-human	Hungary
27	*Capra hircus*	Goat	Sheep/Goat	Non-human	Hungary
28	*Felis catus*	Cat	Cat	Non-human	Hungary
29	*Capreolus capreolus*	Western Roe Deer	Dog [Deer]	Non-human	Hungary
30	*Capreolus capreolus*	Western Roe Deer	Deer	Non-human	Hungary
31	*Homo sapiens*	Human	-	Human	Thailand
32	*Lama pacos, glama* or *guanicoe**	Camelid	Llama	Non-human	Bolivia
33	*Felis catus*	Cat	Sheep/Goat [Cat]	Non-human	Kwajalein Island
34	*Homo sapiens*	Human	-	Human	Thailand
35	*Bos taurus*	Cow	Cow/Buffalo	Non-human	Kwajalein Island
36	*Bubalus bubalis*	Asian Water Buffalo	Cow/Buffalo	Non-human	Laos
37	*Homo sapiens*	Human	-	Human	Thailand
38	*Ovis aries* or *ammon*	Sheep	Sheep/Goat	Non-human	Contiguous United States

4.9 USE IN CASEWORK

Since the validation of this testing protocol in 2010 and through the spring of 2018, 605 samples have been tested using the 12S assay. Of these, 254 (42%) were reported as inconclusive and 95 (16%) were determined to be human. Those shown to be human continued though the regular casework processing of mtDNA Sanger sequencing. The remaining 256 (42%) were determined to be non-human. It is most common for non-specific elements (i.e., long bones or bone fragments) to be found to be non-human (Table 4.3). There appears to be little or no correlation between size of the fragment submitted and whether it is non-human in origin.

Samples recovered from Southeast Asia are most commonly tested using the 12S amplification strategy and also are more likely to be non-human (Table 4.4). Remains recovered from Southeast Asia often can be highly fragmented due to the circumstances surrounding the loss and/or the acidic nature of the soil, which can break down osseous material. It is more difficult to accurately ascribe smaller fragments as human or non-human. In addition, very small fragments may be all that is recovered and it becomes a choice as to whether to use the entire sample for microscopic or DNA analysis. In two different cases, the results were a mixture of human and pig (*Sus scrofa*). The low-quality mtDNA profiles generated from the samples were determined to be consistent with the profiles of members of the field recovery team (Edson and Christensen 2013). Other mixtures of animal and human, or animal and animal, are thought to have occurred via excessive handling or gnawing. While not a validated protocol, the observed mixtures can be visually separated by an analyst and searched in BLAST if so desired. This is a fairly simple process since the human sequence is known.

One of the difficulties with using BLAST is that the NCBI database it accesses is self-curated and continuously being updated. The assignment of 'non-human' will not change; however, the species assignment may be different. This is particularly notable for rare species that may not be commonly added to the database. For example, a recent search of the NCBI database for the purposes of this study revealed that thirteen of fifteen samples are now classified as a more specific species (e.g., original search result Family Cervidae and new search result *Rusa unicolor*, Sambar Deer) and one sample did not change (Genus *Muntiacus*, muntjacs). However, one sample changed to a more general category. This sample was previously determined in 2014 to be a Wattle-necked Softshell Turtle (*Palea steindachneri*). Re-running the search in 2019 resulted in a 100% match to not only *P. steindachneri*, but also the Asiatic Softshell Turtle (*Amyda cartilaginea*), an IUCN threatened species. By the calling criteria of AFDIL, the sample would now be reported to DPAA as being Family Trionychidae, rather than a specific species.

In addition, those who upload sequences are on their own to provide accurate information on the taxa to which the sequence belongs. The standards put in place as part of the AFDIL validation tend to eliminate incorrect 'matches'. However, there are cryptids that are part of the NCBI database that occasionally match to samples submitted by DPAA. The most common 'match' is to the Kting Voar (*Pseudonovibos spiralis*), also known as the Snake-eating Cow or the Spiral-horned Ox. Although the designation is subject to controversy (Olson and Hassanin 2003), testing has indicated that the specimens are most likely from Domestic Cows (Hassanin et al. 2001) or Water Buffalo (Kuznetsov et al. 2001) rather than a mythical beast. Nonetheless, the sequences are still present in the NCBI database (e.g., GenBank Accession No. AF231029). Matches to the Kting Voar include other Bovids and are usually attributed to the Family Bovidae.

Table 4.3 The 12S rRNA testing results for each type of skeletal element, and the average weight of each type of element. "Long Bones" and "Bone Fragments" are listed independently of each other as a type of element as the former implies there was enough of the osseous material present to determine element was a long bone, while the latter is a non-specific catch-all for small fragments.

	Human		Inconclusive		Non-Human	
	Number Tested	Avg. Weight (g)	Number Tested	Avg. Weight (g)	Number Tested	Avg. Weight (g)
Bone Fragment	47	0.94	78	1.23	107	1.30
Calcaneus			2	3.45		
Clavicle			2	1.0		
Cranium (general)	3	1.31	31	1.92	4	1.91
Cuneiform			1	1.9		
Femur	2	4.39	13	3.94	2	2.7
Fibula			2	1.89		
Frontal			4	1.67		
Humerus	1	3.10	10	2.99	4	1.46
Long Bone	26	1.28	58	1.77	101	1.75
Mandible	2	2.25	4	2.05	3	2.19
Metacarpal	1	0.40	2	0.80	3	0.54
Metatarsal	1	0.99	2	1.65	1	0.60
Occipital			1	4.2		
Os coxa			3	3.09	2	1.87
Parietal			3	2.46		
Phalanx			2	0.44		
Radius	1	2.50	7	2.36	1	3.00
Rib	5	0.83	6	1.07	23	1.08
Scapula	1	2.45	1	1.4	1	1.35
Talus			3	2.33		
Temporal			4	4.04	2	0.86
Tibia	1	1.50	4	3.64	2	1.98
Tooth (Molar)			2	n/r		
Ulna			6	2.22		
Vertebra (Any)	3	1.86	1	4.5		
Zygomatic			1	0.78		

Table 4.4. Summary of animals detected in the 12S rRNA testing and the conflict of origin. The original species designations are indicated and were not adjusted with more recent searches. Oftentimes, the country from which the remains were recovered will give clues as to the animal, even if the 12S results are more general. Some of the results seem unlikely (e.g., the Common House Gecko); however, the sequence was duplicated through either extraction or amplification and confirmed prior to searching in BLAST and being reported. Remains from Southeast Asia were typically recovered from Vietnam, Laos, or Cambodia; those recovered from the Korean War were from the Korean peninsula; and those from World War II were from world-wide locations (e.g., Tarawa Atoll, Germany, Papua New Guinea).

	WWII	Southeast Asia	Korean War
Human (*Homo sapiens*)	21	70	4
Arctic cod (*Arctogadus glacialis*)		1	
Order Artiodactyla (non-specific)		2	
Asian Black Bear (*Ursus thibetanus*)		1	
Family Bovidae		7	
Family Cercopithecidae		1	
Family Cervidae		5	
Chicken (*Gallus gallus*)		1	
Cow (*Bos taurus*)	30	90	
Deer (non-specific)	2	9	
Order Diprotodontia	1		
Dog (*Canis lupus familiaris*)		2	
Dolphin (non-specific)		1	
Giant Grouper (*Epinephalus lanceolatus*)		1	
Goat (*Capra hircus*)		2	
Common house gecko (*Hemidactylus frenatus*)		1	
Horse (Genus *Equus*)	1		1
Edward's Giant Rat (*Leopoldamys edwardsi*)		1	
Macaque (Genus *Macaca*)		5	
Muntjac (Genus *Muntiacus*)		2	
Family Phasianidae	1		
Pig / Wild Boar (*Sus scrofa*)	23	35	1
Rat (Genus *Rattus*)	1		
Sea Turtle (Superfamily Chelonioidea)		1	
Softshell Turtle (*Palea steindachneri*)		1	
Sheep (*Ovis aries*)	4		
Water Buffalo (*Bubalus bubalis*)		18	
Non-human (non-specific)		2	
Inconclusive	82	169	3

4.10 DISCUSSION

The use of highly sensitive methods, such as those employed at AFDIL including the 12S assay, is a necessity in cases involving decades-old skeletal remains. Unfortunately, with this type of testing, modern contaminating DNA is always a concern (Malmstrom et al. 2005; Gilbert et al. 2006; Pilli et al. 2013) despite precautions taken to minimize contamination during remains recovery and laboratory processing (Edson et al. 2004; Kemp and Smith 2005; Barta et al. 2013; Edson and Christensen 2013; Edson and McMahon 2016). Consequently, an identification of human should be considered in the context of other case information and molecular data including any human mtDNA testing since exogenous modern human DNA may mask the authentic DNA from the non-human species, which is likely only present at low levels in poor quality specimens. The classification guidelines established at AFDIL for the interpretation of the 12S data aim to ensure the greatest level of confidence in the resulting species identification. However, all information must be considered if contamination from an exogenous source, human or non-human, is suspected.

The comparison between 12S and osteological taxonomic assignment of the blind study samples demonstrates how difficult it can be for anthropologists to accurately differentiate between various non-human species in situations involving small, severely compromised skeletal fragments. Although immunological and histological analyses have been shown to facilitate the determination of human or non-human origin (Cattaneo et al. 1999; Ubelaker et al. 2004; Lowenstein et al. 2006; Hillier and Bell 2007), reliable species identification based solely on these analyses may still be limited. Morphological determination of species is dependent on the experience and knowledge of the anthropologist in addition to the size of the fragment. The reproducibility of the 12S result is not reliant on the analyst but rather on the BLAST alignment algorithm and composition of the NCBI Nucleotide database. Therefore, the sequence data generated by the 12S assay enables an unbiased determination of taxonomic origin, and in particular whether a sample is or is not human.

Species identification using this 12S assay, though superior to osteological analyses, is limited by the composition of the NCBI Nucleotide database and, depending on the application, the inter-species variation of the targeted mtDNA region. As evidenced by the initial BLASTN search for Sample 9 of the blind study, a 100% homologous sequence may not be returned by the search if the exact taxon has not been captured in the database. In these situations, the most closely related species represented in the database will be returned as the most significant alignment. This was the case for Sample 9 in which the *Viverricula indica* sequence was not present in the database at the time of the initial query (May 2010) and was added approximately seven months later (December 2010). Although the database continues to grow, no doubt facilitating sequence identifications at the species level over time, current designations using this 12S assay should be weighted heavily on sequence homology. This consideration is reflected in the classification guidelines employed at AFMES-AFDIL (Table 4.1) in which 100% homology is required in order to report a specific species. With that being said, identical queried and searched sequences may not definitively identify the exact taxon since this small region of the 12S rRNA gene could potentially be conserved among closely related species.

Because DPAA recovery missions take place across the globe, often in areas with indigenous primate populations, AFDIL may receive skeletal fragments from other primates commingled with human remains. In fact, the 12S sequence generated from several samples in a case from the Vietnamese province of Quang Binh was classified as genus *Macaca* (macaque), as it was 100% consistent with two macaque species. Macaques, though primates, are members of the Cercopithecidae family. Humans are much more similar to other apes within the Hominidae family. Minimal differences and large regions of homologous bases are also observed between the sequences of *Homo, Gorilla,* and *Pongo* genera. Based on the similarity of 12S sequences among hominids, AFDIL guidelines require at least 75 bases of sequence and 100% homology to conclusively classify a sample as having originated from a human.

4.11 CONCLUSIONS

Based on the results obtained from the application of this protocol to the blind study as well as routine case samples, the 12S assay described here is a robust and reliable method for the species identification of degraded bone fragments. This protocol could easily be implemented into any forensic laboratory already performing standard mtDNA sequence analysis. The 12S assay remains a low-cost, low-tech process by which species of origin may be determined. This species identification assay has become an invaluable tool for human identification efforts at AFDIL due to its ability to determine the species origin of severely compromised skeletal specimens and thereby allow laboratory resources to be focused on samples that are human in origin.

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Chapter 5

The Effect of Chemical Compromise on the Recovery of DNA from Skeletonized Human Remains: A Study of Three World War II Era Incidents Recovered from Tropical Locations

5.1 INTRODUCTION

From the previous chapters, it is clear that there are differences in success of DNA recovered from different skeletal elements, as well as differences in yield of DNA from different extraction protocols. In Chapter 3, it was noted that there was sampling bias from the oversampling of one particular incident, that of the USS *Oklahoma,* a World War II era loss in Pearl Harbor, Hawaii. As indicated, the remains from this incident went through the initial stages of decomposition while entombed within the belly of the inverted ship. Despite being exposed to less than ideal conditions for DNA survival, salt water, elevated levels of fuel oil, and an elevated ambient temperature, the success rates for this particular incident are nearly 100% for mtDNA Sanger sequencing. These results would run counter to the prevailing wisdom that burial in tropical environments or elevated temperatures during decomposition tend to cause DNA to degrade more rapidly (Smith, et al., 2003; Prinz, et al., 2007; Hofreiter, et al., 2014; Nieves-Colón, et al., 2018).

Given the success of the USS *Oklahoma* samples, two other groups of World War II era fatalities were considered for comparison: the Battle of Tarawa and the prison camps of Cabanatuan in the Philippines. All three losses were exposed to a tropical environment since time of death; however, each set was exposed to a different set of chemicals or none at all. The USS *Oklahoma* remains were incidentally treated with a coal based fuel oil and the Cabanatuan loses were deliberately treated with a drying compound comprised of plaster of Paris, naphthalene, and other compounds (468 Graves Registration Service, 1948). The remains recovered from the Battle of Tarawa were considered as untreated controls.

The concept behind evaluating these particular groups of remains was to evaluate the effects of chemical treatment on the DNA contained within human skeletal remains. This would

allow for foundational data for the subsequent chapters analyzing the presence of potentially deleterious compounds on skeletonized materials and whether they carry forward into the extracted DNA. Should it be found that samples known to be treated with chemicals still have effective DNA recovery, there is credence to the ideas that DNA is not damaged by exposure to such compounds and there is little to no carry-over of said compounds to the extracted DNA.

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5.2 PUBLICATION

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5.3 TITLE PAGE

The Effect of Chemical Compromise on the Recovery of DNA from Skeletonized Human Remains: A Study of Three World War II Era Incidents Recovered from Tropical Locations

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The author is an employee of their agency and otherwise received no monetary support for this project.

5.3.2 DISCLAIMER

The opinions or assertions presented are the private views of the author and should not be construed as official or as reflecting the views of the Department of Defense; the Defense Health Agency; the Armed Forces Medical Examiner System; or the Defense POW/MIA Accounting Agency.

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5.3.4 CONFLICT OF INTEREST

The author declares that they have no conflict of interest.

5.3.5 ETHICAL APPROVAL

While human materials were used in this study, the analysis presented herein is a reporting of the results of active casework. No personally identifiable information or genetic data are presented. Approval to report the testing results was granted by the author's agency.

5.4 ABSTRACT

The use of DNA extracted from skeletonized human remains is a common challenge for those working in human identification. Thermal age and chemical compromise should be considered prior to performing DNA testing on skeletonized remains. Both heat and chemical contamination may cause damage to the DNA present in the osseous materials and a subsequent increase in both the difficulty and expense of DNA testing. For this study, three World War II era mass fatality events involving the US military, the USS *Oklahoma*, the Battle of Tarawa, and the Cabanatuan Prison Camps, were examined for the overall success of DNA testing using five DNA modalities: Sanger sequencing of mitochondrial DNA, AmpFlSTR® MiniFiler™; PowerPlex® Fusion; a modified AmpFlSTR® Yfiler™; and a Next Generation Sequencing (NGS) protocol. The three incidents chosen were buried in tropical environments and had the same approximate postmortem interval of 75 years. The incidents vary by chemical compromise. The remains from the USS *Oklahoma* were soaked in fuel oil and saltwater immediately post-mortem; the Cabanatuan Prison Camp remains were treated with a 'hardening' compound; and those from the Battle of Tarawa were not treated. Skeletal elements from each incident were compared across the modalities for success. The chemical insult to the skeletal materials appears to have the greatest impact on every modality of DNA testing examined.

5.5 KEY WORDS

Skeletonized human remains; human identification; DNA testing; thermal age; chemical compromise

5.6 KEY POINTS

- Skeletal materials exposed to a tropical environment during long-term burial do not exhibit marked DNA degradation.
- Organic purification during DNA extraction provides improved results when extracting DNA from remains exposed to petroleum products.
- Chemical insult to skeletal remains has a marked impact on downstream DNA processing.
- DNA extraction technique should be chosen based on the condition of the skeletal materials and the required downstream testing.

5.7 ARTICLE INTRODUCTION

The recovery of DNA from skeletonized human remains is a difficult task due to the complex, rigid matrix of the skeletal material [1], and is often compounded by age, heat, and chemical insult. As the remains age, DNA breaks down, leaving nuclear DNA in fragments and the smaller, more robust mitochondrial DNA (mtDNA) largely intact. Heat and chemical contamination speed up the degradation of the DNA [2]. Elevated post-mortem ambient temperature, often referred to as thermal age, causes a break-down of DNA in the remains as decomposition progresses [3,4,5]. Heat is of such concern to DNA analysts that it is often recommended to not use a mechanized sander to clean the exterior surface of the remains, as even this small increase in temperature could lead to extensive DNA damage [6]. Some laboratories preferentially use chilled fragmentation chambers (e.g., freezer mills) to pulverize skeletal elements so as to not raise the temperature of the osseous materials [7,8].

Chemical or environmental contamination is not as easily controlled for in the laboratory. Remains may be contaminated at the time of death, due to the circumstances of the incident [e.g., World Trade Center: 9,10], the burial itself [humic acid: 11], or during analysis [bone glue: 12]. In times of conflict or other mass fatality events, remains can be deliberately treated with materials that damage or inhibit DNA processes: such as lye, formalin, or plaster of Paris. The intention was preserve the remains for transportation or to reduce the odor when remains are unable to be properly buried, not to damage the DNA, which is often the unintended consequence.

To examine the cumulative effect of age and chemicals, three separate incidents that occurred during World War II and involved large clusters of skeletonized remains buried in tropical environments, were examined for success of recovery of intact DNA that could be analyzed using both mitochondrial and autosomal protocols. Remains were recovered from the USS

Oklahoma in Pearl Harbor, Hawaii; the Battle of Tarawa, Tarawa Atoll, Republic of Kiribati; and the Cabanatuan Prison Camp, Manila, Philippines. Each of these locations is considered to be a tropical climate, and as all losses occurred during World War II, the post-mortem interval (PMI) was approximately 75 years. Losses on Tarawa Atoll experienced no chemical treatment, and individuals were buried at time of death. This incident serves as a 'normal' loss location. The other two incidents involve extensive commingling, repeated burials and disinterments, and saturation with chemical materials: fuel oil in the case of the USS *Oklahoma* and a hardening compound in that of the Cabanatuan Prison Camp. In addition, both the USS *Oklahoma* and Cabanatuan remains experienced water damage.

Comparisons of DNA testing results between the three events will lend an additional perspective to the analysis of aged and compromised skeletonized remains. Each set of remains tested was also divided into individual skeletal elements to determine if the DNA of a specific element might more successfully survive the insults of ambient temperature and chemicals. Five DNA modalities were compared, including Sanger sequencing of mitochondrial DNA (mtDNA); AmpFlSTR® MiniFiler™; PowerPlex® Fusion; a modified AmpFlSTR® Yfiler™; and an in-house developed Next Generation Sequencing (NGS) protocol.

5.8 HISTORICAL BACKGROUND

5.8.1 USS Oklahoma – Pearl Harbor, Hawaii -- 7 December 1941

On 7 December 1941, the Imperial Japanese Navy launched an attack on the United States Naval Base in Pearl Harbor, Hawaii Territory. During the battle, the USS *Oklahoma* (Figure 5.1) was struck with torpedoes, causing the ship to list heavily to the side. The ship rolled until the masts of the ship hit the bottom of the harbor, leaving one side of the ship filled with

air and above the surface of the water. Of the sailors and Marines who were defending the ship, 429 would be trapped within the vessel and perish.

Rather than retain the USS *Oklahoma* as a memorial or ossuary, the ship was righted in 1943. The remains were recovered from the ship and interred in a series of graves in the Halawa and Nu'uanu cemeteries on the island of Oahu. An attempt at identification was made in 1947, when the Central Identification Laboratory (CIL) disinterred all of the remains. The skeletal materials were cleaned and an effort was made to sort the elements into discrete skeletons and provide identifications. The US Navy rejected the initial identifications and the project was abandoned. The remains were reburied in 65 caskets and 45 graves in the National Memorial Cemetery of the Pacific (NMCP) within the Punchbowl Crater on the island of Oahu.

In 2003, a single casket was disinterred. Research from a civilian historian indicated that five individuals should be found within the casket. The Armed Forces Medical Examiner – Armed Forces DNA Identification Laboratory (AFMES-AFDIL, aka AFDIL) performed mitochondrial DNA (mtDNA) testing on 177 elements removed from this original casket. MtDNA testing results indicated that the minimum number of individuals (MNI) present in that single casket was 95. Given that mtDNA profiles are shared down the maternal line, it is possible that additional individuals were present. Five sailors were identified from the cranial remains coupled with DNA testing. In 2015, the Defense POW/MIA Accounting Agency (DPAA) exhumed the remaining 64 caskets and, in partnership with AFDIL, began the task of identifying the remaining individuals.

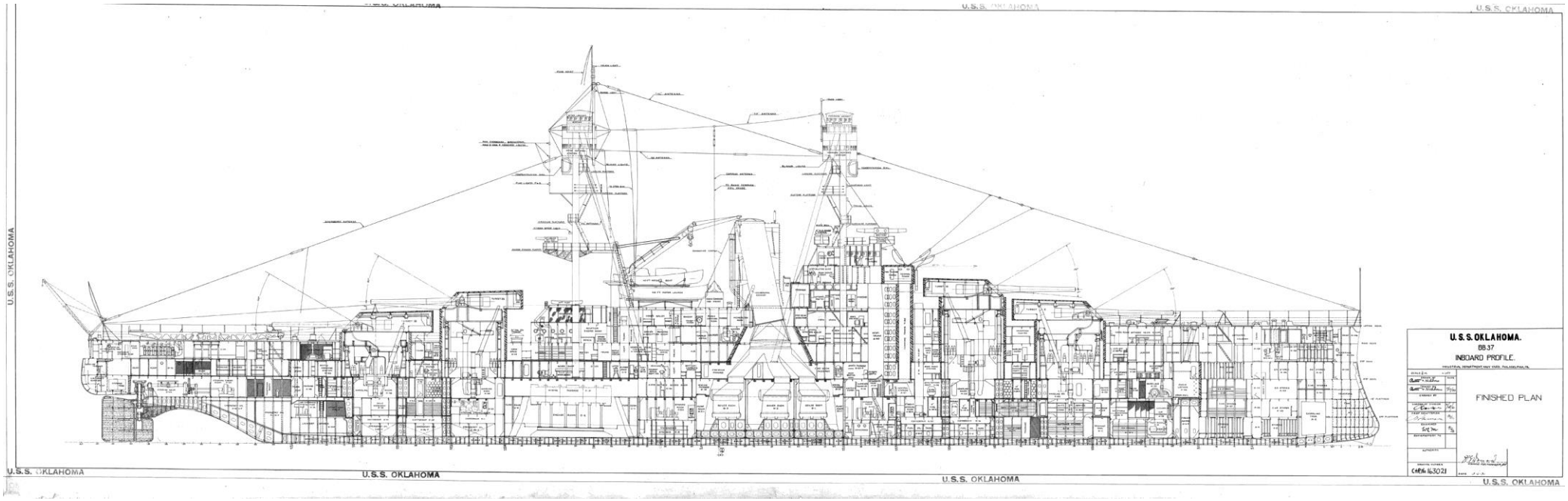

Figure 5.1. The inboard profile of the USS *Oklahoma* [13]. The USS *Oklahoma*, also known as BB-37, was a *Nevada*-class battleship that ran fully on liquid fuel. Much of the very bottom of the ship was dedicated to storage of fuel oil.

5.8.2 *Battle of Tarawa – Tarawa Atoll, Gilbert Islands – 20-23 November 1943*

The Battle of Tarawa took place 20-23 November 1943 at the Tarawa Atoll in the Gilbert Islands (Figure 5.2). Of the 12,000 United States Marines committed to the battle, 3,166 died. Of the island defenders, 3,619 Japanese soldiers and 1,071 Korean laborers were casualties. Remains from both sides were recovered by US forces and buried in hastily dug cemeteries. The cemeteries were comprised of individual graves or discrete burials within trenches. Minimal records were kept of who was placed within each.

In 1946, Graves Registration searched the island for any of these cemeteries. What they found were consolidated to a single location, the Lone Palm Cemetery. In 1947, these remains were disinterred and transferred to the Central Identification Laboratory (CIL, now DPAA) in Hawaii. Analysis at the time resulted in 465 identifications, with the remainder being buried as individual unknowns in the NMCP.

Over the following years, it became clear that Graves Registration was not complete in the identification and recovery of burials associated with the Battle of Tarawa. The DPAA and its predecessors have received unilateral turn-overs from locals and have also performed recovery of remains uncovered by construction projects. In 2008, a private contractor located potential burial sites. Since that time, multiple excavations, performed by both scientists from the DPAA and private contractors, have occurred and recovery operations are currently ongoing.

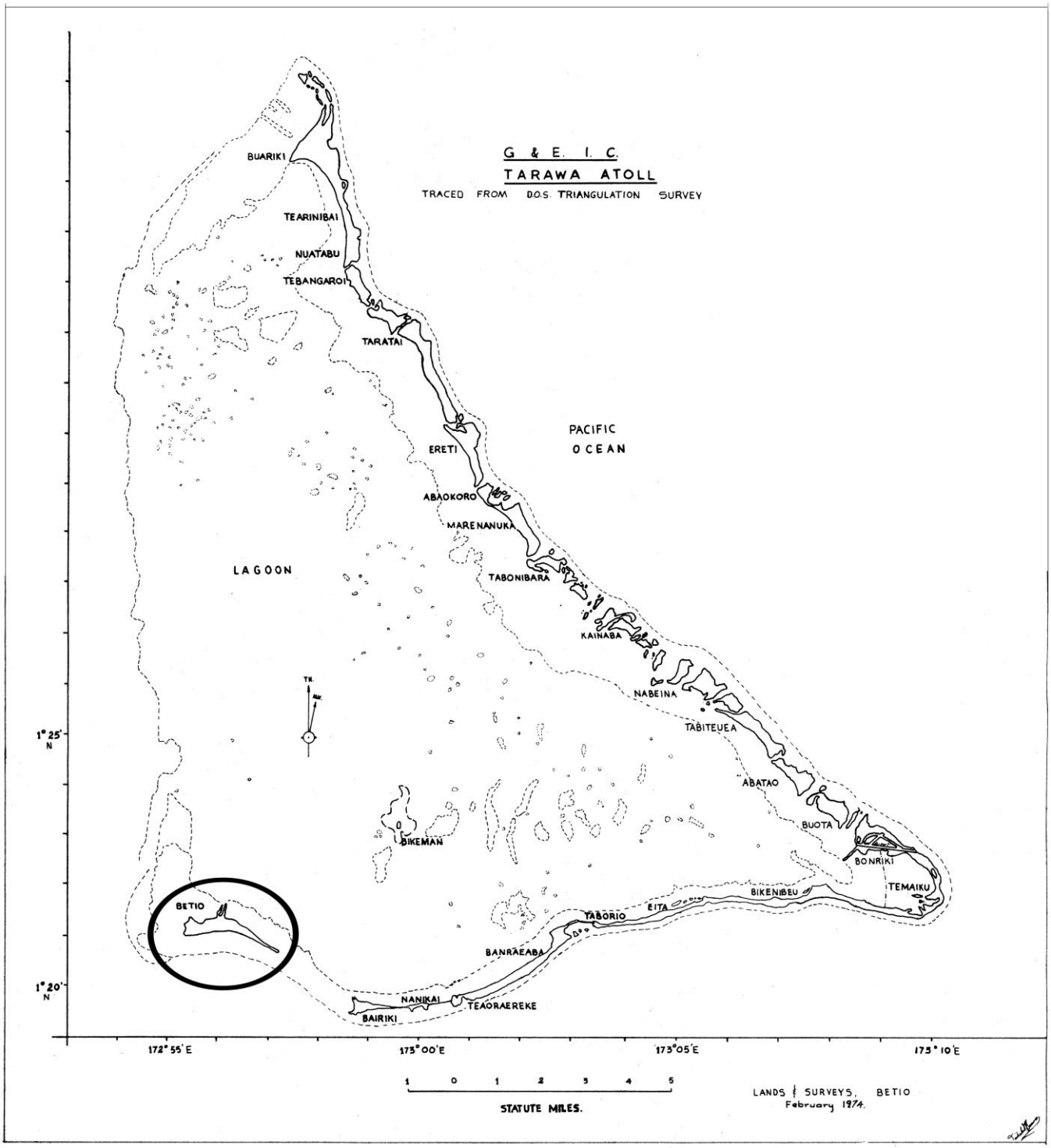

Figure 5.2. A map of Tarawa Atoll [14]. The islet of Betio, where most of the Battle of Tarawa took place, is indicated by the black circle.

5.8.3 *Cabanatuan Prison Camp (Camp #1) – Manila, Philippines – June 1942 – January 1945*

The Cabanatuan Prison Camp (aka Camp Pangatian) was run by the Japanese forces occupying the Philippines. Prisoners of the camp were mostly US and other Allied soldiers held there following the Battles of Bataan and Corregidor in 1942. When US forces liberated the camp in 1945, 2,764 prisoners of war had died. Records of deaths and burials were kept by a Camp Graves Registration officer, self-appointed from among the prisoners. These were often incomplete due to the volume of deceased individuals and the lack of cooperation of the jailers. All of those persons who died on one day were buried in a single mass grave that had been dug the previous evening [15]. An attempt was made to organize the gravesites; however, this was often not possible due to the numbers of individuals perishing (Figure 5.3).

Throughout 1945 and 1946, the Quartermaster Graves Registration Platoon disinterred a number of the graves and transferred the remains to the US Armed Forces Manila #2 Cemetery. Names were attributed where possible and otherwise assigned numbers for tracking purposes. Between 1947 and 1948, remains were removed from Manila #2 Cemetery to the American Graves Registry Service (AGRS) Mausoleum at Nichols Field in Manila. Remains were treated with a hardening compound comprised of: 80% sawdust; 5% quick soluble powdered ammonium Alum; 5% slow soluble granular ammonium alum; and 10% flake naphthalene for preservation [17]. The hardening compound was locally produced; therefore, there are no records of the source of the materials. While a number of identifications were made and the remains were returned to families, the remainder was interred in the Manila American Cemetery in 1951. Current work on the Cabanatuan remains was started in 2013 at the request of a family member of one of the unidentified. The testing reported here mostly involves remains recovered from that initially recovered gravesite; however, work is ongoing and multiple other graves have since been recovered.

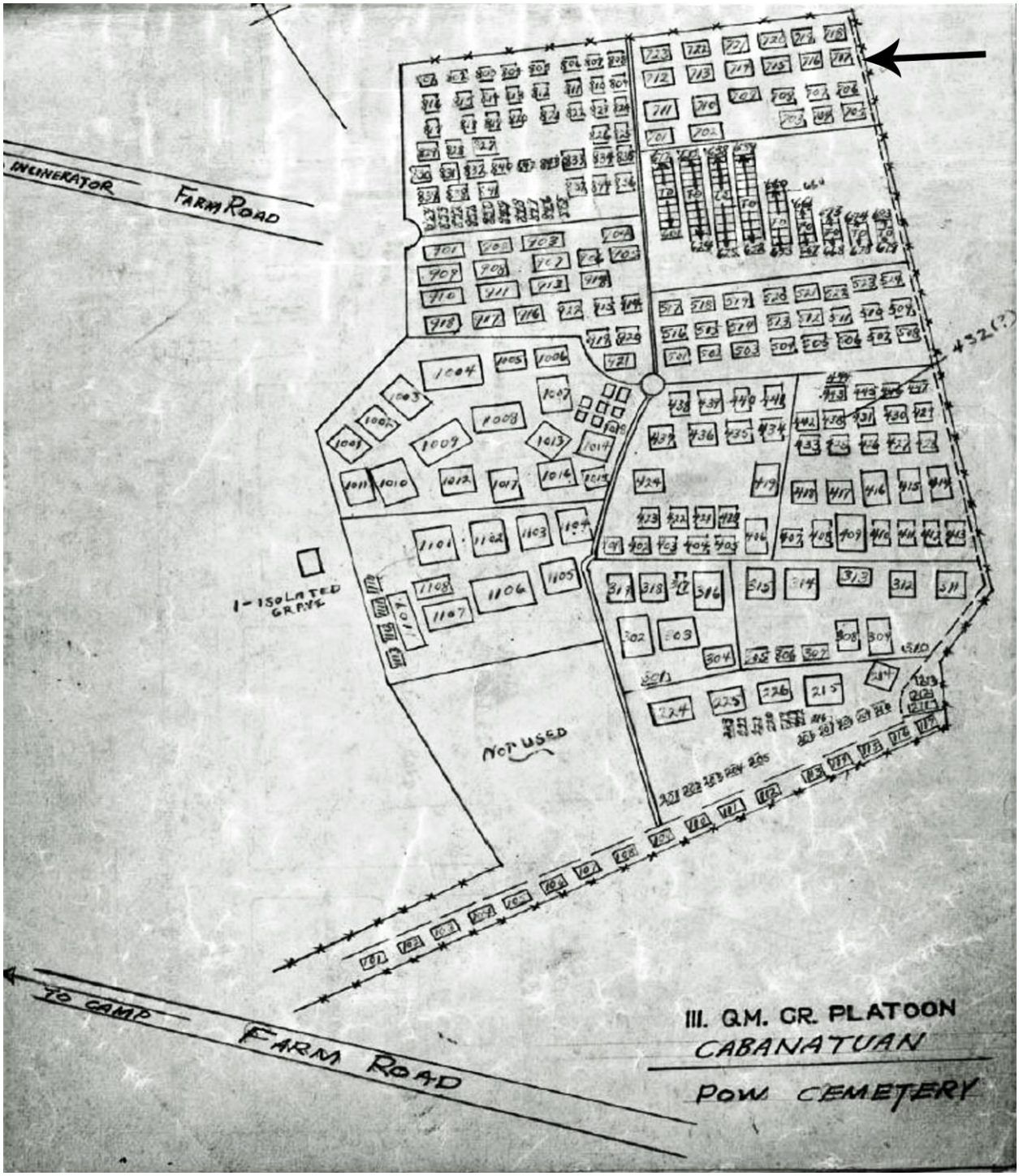

Figure 5.3. A map of the Cabanatuan Prison Camp cemetery [16]. The grave being discussed is in the upper right-hand corner of the cemetery and is indicated with the black arrow. The graves are numbered in groups, starting with #101, which is nearest the Farm Road. Numbering continues in a more-or-less counterclockwise fashion, and ends with grave #1113 on the far left hand side of the map.

5.9 METHODS AND MATERIALS

5.9.1 Recovery of Remains

Skeletal remains believed to be those of United States service members are recovered by anthropologists from the DPAA or partner agencies from battlefields or cemeteries world-wide. The remains in this study were disinterred from: the National Memorial Cemetery of the Pacific (NMCP) in Hawaii (USS *Oklahoma* and Battle of Tarawa); the Manila American Cemetery (Cabanatuan Prison Camp); or battle-field cemeteries (Battle of Tarawa). Remains were returned to the DPAA laboratories for examination and sorting. For some of the Battle of Tarawa samples, a partner agency removed specific elements for testing (e.g., patellae, metatarsals, or metacarpals) according to their standard operating procedures. This led to a bias in sampling of those elements for that event. Otherwise, elements were nominated for DNA typing by DPAA anthropologists. A small window of bone was removed from the nominated sample using a Dremel® tool (Dremel, Racine, WI) and a 545 diamond cutting wheel. The weight of bone removed varied depending on the size of the original element, but typically ranged from 0.5 - 3.0 g. Burial conditions were noted and provided to AFDIL to assist in trouble-shooting of DNA testing.

5.9.2 Preparation of Elements for DNA Testing

5.9.2.1 Osseous Elements

Upon receipt of samples at AFDIL, fragments were cleaned using sanding bits and a Dremel® tool. The exterior of the fragment and any materials that may have been adhering were removed during sanding. Elements with an elevated level of adipocere were scraped clean using a scalpel prior to sanding. If not removed, the adipocere melted during sanding and prevented the bit from performing adequately.

After sanding, a 0.2 g fragment was cut from the sanded piece. Prior to 2006, a 2.5 g fragment of osseous material was used. Since that time, the extraction protocols have been modified to reduce the amount of skeletal material required; however, the cleaning protocols have remained the same. This fragment removed from the submitted sample was further cleaned using 2 - 3 washes of deionized H_2O (diH_2O) and a single wash of 100% (v/v) ethanol (Pharmco-Aaper, Brookfield, CT) and allowed to air dry. Once the fragment was dry, it was pulverized using a Waring blender professional base (MC2 cup: Waring, Stamfield, CT).

5.9.2.2 Teeth

Teeth were not completely pulverized. After examination by a forensic odontologist at DPAA, intact teeth were submitted to AFDIL. If a tooth had no sign of caries or cracking, it was placed in a 50 mL conical tube with 25 mL 8.5% (v/v) bleach and placed in an ultrasonic water bath for five minutes. The cleaned tooth was removed from the bath and wiped thoroughly with a 4 x 4 cm gauze pad moistened with 8.5% (v/v) bleach, followed by a wipe down with a similar gauze pad moistened with absolute ethanol. If the tooth showed significant caries or cracking, agitation in the water bath was eliminated and the tooth was cleaned manually as above. After cleaning, the tooth was placed under a UV light for approximately 15 minutes for drying.

After cleaning, the crown of the tooth was removed using a dental hand-piece attached to a high performance brushless motor (Brasseler USA, Savannah, GA). Using a #2 or #4 round bur attached to the hand-piece, the tooth was bisected along the enamel/cementum line. A small notch was added to allow for the crown to be reattached to the root once processing was completed. Using a #4 straight bur or #6 round bur, the dentin in both the crown and roots was removed.

5.9.3 DNA Extraction

The pulverized bone or tooth was fully demineralized in an overnight incubation at 56°C using an extraction buffer (0.5M EDTA, pH 8.0; 1% *N*-Lauroylsarcosine) and Proteinase K (200 mg/mL) (EP#2 and EP#3). Prior to 2006, the extraction buffer was comprised of 10 mM Tris, pH 8.0; 100 mM NaCl; 50mM EDTA, pH 8.0; and 0.5% SDS (EP#1) [18]. DNA from the demineralized bone was purified using either an organic purification (EP#1 and EP#2) with 25:24:1 phenol:chloroform:isoamyl alcohol (Sigma-Aldrich, St. Louis, MO) followed by a wash with *n*-Butanol (Sigma-Aldrich) and subsequent concentration with Amicon Ultra-4/30K centrifugal filter units (Millipore, Billerica, MA) or a concentration using Amicon Ultra-4/30K centrifugal filter units and an inorganic purification (EP#3) with the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) [19,20,21]. The extracted DNA was brought to a final volume of 100 - 200 µL with TE^{-4} (10mM Tris, 0.1M EDTA; pH 8.5).

For simplicity, the extraction protocols will be described as follows in the remaining text:

- Extraction Protocol #1 (EP#1) – Non-demineralization plus organic purification (2.5 g osseous material input)
- Extraction Protocol #2 (EP#2) – Demineralization plus organic purification (0.2 g input)
- Extraction Protocol #3 (EP#3) – Demineralization plus inorganic purification (0.2 g input)

For the Cabanatuan Prison Camp samples, both EP#2 and EP#3 were used in concert to generate data on some samples. These are indicated in the results as a "Combined" category. Table 5.1 provides a summary of samples tested.

Table 5.1. All samples tested during the course of this study. The "Comb." category refers to using two extracts for a sample to generate results. In this particular instance, one extraction was done with EP#2 and one with EP#3.

	USS *Oklahoma*				Battle of Tarawa				Cabanatuan Prison Camp			
	EP#1	EP#2	EP#3	Comb.	EP#1	EP#2	EP#3	Comb.	EP#1	EP#2	EP#3	Comb.
Sanger Sequencing (mtDNA)	34	3272	18		13	307	380			34	47	83
MiniFiler		437	39				21			2	10	3
Modified YFiler		508	128				24			11	39	26
PowerPlex Fusion		71					5					
NGS								255				51

5.9.4 Quantification

If samples were designated for STR testing, quantification was performed using Quantifiler™ Human DNA Quantification Kit (Thermo Fisher Scientific, Foster City, CA) or Plexor® HY (Promega, Madison, WI). This was only applied to the samples submitted since 2007, as STR testing was not validated for usage in the laboratory prior to that date.

5.9.5 Mitochondrial DNA Testing.

Mitochondrial DNA testing was undertaken using PCR amplification and subsequent Sanger sequencing with in-house developed primers. The primers are described in Edson, et al. [18] and Gabriel, et al. [22]. The current protocols are presented in Edson [23].

5.9.6 STR Testing

Autosomal STR testing was performed using the following kits and manufacturer recommendations: AmpFlSTR® Minifiler™ (Thermo Fisher, Gaithersburg, MD) and PowerPlex® Fusion (Promega, Madison, WI).

A modified AmpFlSTR® Yfiler protocol was also used. Modified Yfiler involved an increase in the number of PCR cycles from 28 to 36 and a doubling of the volume of AmpliTaq Gold® DNA polymerase from the recommended 0.8 µL per sample to 1.6 µL per sample [24,25]. All skeletal elements tested were presumed to be male.

5.9.7 Fragment Separation

Between 2004 and 2016, fragment separation primarily occurred on the AB 3100 and 3130*xL* Genetic Analyzers (Applied Biosystems, Gaithersburg, MD). The instruments used a 50 cm

capillary array and Performance Optimized Polymer 6 (POP-6™: 6% dimethylacrylimide, 8M urea, 5% 2-pyrrolidinone: Applied Biosystems, Gaithersburg, MD) for mtDNA or a 36 cm capillary array and POP-4™ (4% dimethylacrylimide, 8M Urea, 5% 2-pyrrolidinone) for STR analysis.

The transition to the AB 3500*xL* Genetic Analyzer occurred in 2015 and was completed in 2018. The POP and capillary arrays remained the same as for the 3130*xL*. Injection times for mtDNA Sanger sequencing were 8 seconds for longer fragments and 16 seconds for shorter. The default injection time for all STR platforms was 7 seconds.

5.9.8 Next Generation Sequencing

Next Generation Sequencing (NGS), also known as Massively Parallel Sequencing (MPS), was performed using an in-house designed protocol described in Marshall, et al. [26]. Illumina platforms (Illumina, San Diego, CA) were used. At AFMES-AFDIL, NGS testing is used on samples that had been chemically or environmentally compromised or in cases where there is a high degree of commingling and common mtDNA profiles. NGS was implemented into active casework in 2016. At that time, it had already been determined that the USS *Oklahoma* samples would not require extreme measures to generate data despite being heavily contaminated with fuel oil. However, NGS testing was performed on samples associated with the Cabanatuan Prison Camp and the Battle of Tarawa. Battle of Tarawa samples were tested if the mtDNA profile generated from Sanger processing was determined to be common or shared between multiple individuals. Whole mitochondrial genome data can be useful for separation of individuals sharing a common Control Region mitotype.

5.9.9 Data Analysis

Sanger sequenced mtDNA was analyzed by two independent scientists using Gene Codes Sequencher Plus (Ann Arbor, MI). The reported profiles are polymorphisms from the generated data to the revised Cambridge Reference Sequence [rCRS: 27,28]. Mitochondrial DNA data generated from NGS were in whole genome format. This was analyzed using CLC Genomics Workbench Software, version 7.5 or higher (QIAGENBioinformatics, Gaithersburg, MD).

STR data analyses were performed using GeneMapper® *ID-X* v. 1.3 (Life Technologies). The stochastic and analytical thresholds were set from in-house validations.

5.9.10 Reporting

For all platforms, data generated must be duplicated by either extraction or amplification. The data were independently analyzed by two scientists, who must concur on the results. Failure to agree pushes the sample back into the workflow for additional work to resolve the area of non-concurrence or the sample is reported as being "inconclusive". Data were further subjected to technical and administrative review prior to reporting of the results to the requesting agency.

For the purposes of this study, a mitochondrial DNA sample was considered successful with 100 bp or more of data being reported. All STR platforms require a minimum of four loci in order to be designated as successful. All reporting thresholds were determined by in-house validations and are consistent with previous studies [18,29]. The percentage of samples considered successful was determined by dividing the number of samples considered successful by the number of samples tested for any given platform. The average number of

loci or bases reported was generated only from those that produced reportable data. Elements having no samples tested were not included.

5.10 RESULTS

5.10.1 USS Oklahoma -- Sanger sequencing of Mitochondrial DNA

A total of 3,272 bone and tooth samples from the USS *Oklahoma* were tested using complete demineralization with organic purification (EP#2) and Sanger sequencing of mitochondrial DNA (Table 5.2). Overall, 98% of the samples produced 100 bp or more of reportable data. All (100%) of the samples submitted for the following elements produced mtDNA data: clavicle (100 tested); fibula (279); and tibia (356). The temporal bones tested (21) provided the least success at 90%, despite the preferential sampling of the petrous portion of the element.

Table 5.2. Testing results for USS *Oklahoma* samples. Skeletal elements are listed in alphabetical order. The "Jaw" category includes mandibles and maxilla. Only samples extracted using complete demineralization with an organic purification are included. Samples tested in other extraction protocols are limited to 34 for the EP#1 (non-demineralization plus organic purification) and 18 for EP#3. The "# Reported" is the number of samples meeting the criteria for reporting as defined in the Methods and Materials.

	# Tested	# Reported	% Successful	Avg. # of Bases	Avg. quant value (ng/μL)
Total	3272	3216	98.2	712	
Clavicle	100	100	100	706	0.001457
Femur	326	324	99.3	709	0.007626
Fibula	279	279	100	688	0.015819
Humerus	389	387	99.4	706	0.002939
Jaw	33	32	96.9	700	0.007484
Occipital	347	330	95.1	701	0.001222
Os Coxa	288	287	99.6	704	0.002902
Radius	213	212	99.5	706	0.001854
Rib	163	155	95.0	675	0.001742
Scapula	186	182	97.8	698	0.003118
Teeth	220	206	93.6	700	0.004158
Temporal	21	19	90.4	656	0.122018
Tibia	356	356	100	710	0.016806
Ulna	227	225	99.1	708	0.002231
Vertebra	124	122	98.3	704	0.013675

5.10.2 USS Oklahoma -- STR Analysis

Samples tested in any STR platform were less successful (Table 5.3), despite having quantifiable DNA present (Table 5.2). Using the modified Yfiler protocol, 508 elements were tested with 38.3% providing 4 or more reportable loci. The femur (39 tested) was the most successful (87.1%), while the ribs (35 tested) and radii (29 tested) were the least successful (17.2%). MiniFiler showed a similar lower success rate of 31.5% across all samples tested. The femur (34 tested) was again the most successful at 79.4%.

Table 5.3. STR analysis success for USS *Oklahoma* samples. Only those samples extracted using EP#2 are listed here. The other extraction protocols had minimal samples tested. As before, the "Jaw" category contains the maxilla and mandible only. Teeth are a separate category. Average loci are rounded to the closest whole number and the "# Reported" is the number of samples meeting the criteria for reporting as defined in the Methods and Materials.

	Modified Yfiler				MiniFiler				PowerPlex Fusion			
	# Tested	# Reported	% Success	Avg. # of Loci	# Tested	# Reported	% Success	Avg. # of Loci	# Tested	# Reported	% Success	Avg. # of Loci
All	508	195	38.3	8	437	138	31.5	5	71	51	71.8	9
Femur	39	34	87.1	11	34	27	79.4	6	18	11	61.1	7
Fibula	22	13	59.0	14	19	12	63.1	8	12	10	83.3	13
Humerus	47	15	31.9	10	46	16	34.7	5	5	2	40.0	6
Jaw	11	7	63.6	8	6	4	66.6	6	1	1	100%	6
Occipital	94	23	24.4	5	92	17	18.4	4				
Radius	29	5	17.2	4	25	2	8.00	3				
Rib	35	6	17.1	3	29	3	10.3	3				
Temporal	2	1	50.0	9	1	0	0	3				
Teeth	123	24	19.5	7	89	7	7.86	3	3	1	33.0	23
Tibia	46	39	84.7	12	44	32	72.7	7	23	19	82.6	10
Ulna	34	9	26.4	5	29	3	10.3	3	1	0	0	2
Vertebra	26	19	73.0	9	23	15	65.2	6	8	7	87.5	9

Powerplex Fusion was the most successful of the STR kits tested, 71.8% of the 71 samples tested providing 4 or more loci. The average number of loci reported across all samples was nine. The femur (18 tested; 61.1%) was not the most successful element tested, but rather the vertebra (8 tested; 87.5%); fibula (12; 83.3%); and tibia (23; 82.6%).

5.10.3 Battle of Tarawa -- Sanger Sequencing of Mitochondrial DNA

A total of 380 samples (Table 5.4) from the Battle of Tarawa were tested in Sanger sequencing of mtDNA using demineralization and inorganic purification (EP#3). An additional 307 samples were tested in mtDNA using EP#2 (Table 5.5). There is minimal difference between the two extraction techniques: 94.7% of EP#3 tested samples generating 100 bp or more of reportable data and 91.5% of EP#2 tested. Ribs and parietal bones were the least successful of elements tested in either EP#2 or EP#3.

5.10.4 Battle of Tarawa -- STR Analysis

A total of 24 samples were tested using EP#3 and the modified Yfiler protocol (Table 5.4). The overall success rate is 58.3% for this modality. MiniFiler testing results were similar, with 21 samples tested at 61.9% success. Fusion success was improved at 80%; however, the significance of this number is relative given only five samples were tested in this platform. The limited number of samples tested in STR analysis is due to the types of family references available for comparisons, which are mostly limited to matrilineal.

5.10.5 Cabanatuan Prison Camps -- Sanger Sequencing of Mitochondrial DNA

A total of 164 samples from Cabanatuan Prison Camps were tested in Sanger sequencing of mtDNA (Table 5.6). Three different extraction strategies were used: EP #2 (34 samples); EP#3 (47 samples); and combined (83 samples). The combined category used both EP#2 and

EP#3 extractions to generate results (i.e., a fraction of the sample tested was extracted once with EP#2 and once with EP#3. The data from the two extracts was combined to generate a profile). The combined category produced the worst results at 24% success. EP#3 generated the best results at 76.5%.

Table 5.4. EP#3 analysis success for Battle of Tarawa samples. Only those samples extracted using EP#3 are listed here. The other extraction protocols had minimal samples tested for STR analysis. As before, the "Pelvis" category includes os coxa, ilium, ischium, and other regions of the pelvis. The "Jaw" category contains the maxilla and mandible only. Teeth are a separate category. Average loci are rounded to the closest whole number. A limited number of samples are tested in STR protocols due to the limited number of available STR family references. Whole genome testing and comparison to available mitochondrial DNA family references is a more typical workflow. The "# Reported" is the number of samples meeting the criteria for reporting as defined in the Methods and Materials.

	Sanger Mitochondrial DNA				**Modified Yfiler**				**MiniFiler**				**PowerPlex Fusion**			
	# Tested	# Reported	% Success	Avg. # of Bases	# Tested	# Reported	% Success	Avg. # of Loci	# Tested	# Reported	% Success	Avg. # of Loci	# Tested	# Reported	% Success	Avg. # of Loci
All	380	360	94.7	651	24	14	58.3	9	21	13	61.9	5	5	4	80.0	12
Clavicle	11	11	100	734												
Cranium – General	10	8	80.0	655					2	0	0	2				
Femur	19	19	100	719	2	2	100	11								
Fibula	12	11	91.6	705												
Fragments	2	0	0	0												
Frontal	1	1	100	697												
Humerus	23	23	100	714	2	1	50.0	9	1	0	0	0				
Jaw	2	2	100	581	1	1	100	12	1	1	100	7	1	1	100	12
Metacarpal	73	70	95.8	688												
Metatarsal	61	61	100	701	6	1	16.6	3								
Occipital	4	4	100	546												
Parietal	2	1	50.0	695												
Patella	35	35	100	711												
Pelvis	4	4	100	684	1	0	0	0								

	Sanger Mitochondrial DNA				Modified Yfiler				MiniFiler				PowerPlex Fusion			
	# Tested	# Reported	% Success	Avg. # of Bases	# Tested	# Reported	% Success	Avg. # of Loci	# Tested	# Reported	% Success	Avg. # of Loci	# Tested	# Reported	% Success	Avg. # of Loci
Radius	12	12	100	696	2	0	0	3	1	0	0	0				
Rib	12	7	58.3	684												
Scapula	3	3	100	653												
Talus					1	1	100	17	1	1	100	9				
Temporal	6	4	66.6	726	1	1	100	16	1	1	100	9	1	1	100	12
Tibia	51	50	98.0	708	8	7	87.5	12	14	10	71.4	7	3	2	66.6	12
Ulna	16	16	100	693												
Vertebra	21	18	85.7	687												

Table 5.5. A comparison of Sanger mitochondrial DNA testing of samples recovered from the USS Oklahoma, the Battle of Tarawa, and the Cabanatuan Prison Camps. The only extraction protocol represented is demineralization plus organic purification (EP#2). The "Pelvis" category is largely represented by os coxa, but also includes ilia and ischia. The "Jaw" category includes both the maxilla and the mandible. The "# Reported" is the number of samples meeting the criteria for reporting as defined in the Methods and Materials.

	USS *Oklahoma*				**Battle of Tarawa**				**Cabanatuan**			
	# Tested	# Reported	% Success	Avg. # of Bases	# Tested	# Reported	% Success	Avg. # of Bases	# Tested	# Reported	% Success	Avg. # of Bases
Total	3272	3008	91.9	712	307	281	91.5	612	34	16	47.0	378
Clavicle	100	100	100	706	10	10	100	714				
Femur	326	324	99.3	709	16	15	93.7	714	3	2	66.6	503
Fibula	279	279	100	688	5	4	80.0	677				
Frontal					2	1	50.0	588				
Humerus	389	387	99.4	706	21	16	76.1	693	1	0	0	0
Jaw	33	32	97	700	7	7	100	685				
Metacarpal					7	7	100	754				
Metatarsal					12	12	100	718				
Occipital	347	330	95.1	701	3	3	100	683				
Parietal					1	0	0	0				
Pelvis	288	287	99.6	704	4	4	100	666				
Radius	213	212	99.5	706	10	9	90.0	699	2	0	0	0
Rib	163	155	95.0	675	3	0	0	0				
Scapula	186	182	97.8	698	8	8	100	690	1	0	0	0
Teeth	220	206	93.6	700	164	151	92.0	652	24	11	45.8	723
Temporal	21	19	90.4	656	4	4	100	669	1	1	100	592
Tibia	356	356	100	710	17	17	100	693	2	2	100	828
Ulna	227	225	99.1	708	11	11	100	708				
Vertebra	124	122	98.3	704	2	2	100	680				

Table 5.6. Mitochondrial DNA testing results from remains recovered from burials at the Cabanatuan Prison Camp and interred at the Manila American War Cemetery. Combined testing indicates that samples were tested under at least two different extraction methods and combined to generate data. Success of the different testing protocols vary widely. The "# Reported" is the number of samples meeting the criteria for reporting as defined in the Methods and Materials.

	Combined				**Extraction Protocol #2**				**Extraction Protocol #3**			
	# Tested	# Reported	% Success	Avg. # of Bases	# Tested	# Reported	% Success	Avg. # of Bases	# Tested	# Reported	% Success	Avg. # of Bases
All	83	20	24.0	512	34	16	47.0	378	47	36	76.5	605
Clavicle	13	2	15.3	496					4	1	25.0	658
Femur	2	2	100	570	3	2	66.6	503	8	8	100	678
Fibula	8	4	50.0	574					5	3	60.0	715
Fragments	1	0	0	0								
Humerus	9	1	11.1	663	1	0	0	0	6	4	66.6	647
Occipital	2	1	50.0	672					3	2	66.6	669
Pelvis	8	3	37.5	552					4	4	100	594
Radius	14	1	7.14	518	2	0	0	0	1	0	0	0
Scapula	8	2	25.0	619	1	0	0	0	4	2	50.0	713
Temporal	1	0	0	0	1	1	100	592	3	3	100	729
Teeth					24	11	45.8	723				
Tibia	6	3	50	743	2	2	100	828	7	7	100	554
Ulna	11	1	9.09	747					2	2	100	702

5.10.6 Cabanatuan Prison Camps -- STR Analysis

STR testing was performed on this set of samples with limited success. Of the 15 samples tested in MiniFiler, none produced reportable data. Modified Yfiler was performed on 76 samples, with 22 (28.9%) generating a profile of more than 4 loci. No samples were tested using Powerplex Fusion.

5.10.7 Sanger sequencing – USS Oklahoma, Battle of Tarawa, Cabanatuan Prison Camps

The Sanger mitochondrial DNA testing success from USS *Oklahoma* and Battle of Tarawa samples extracted with complete demineralization plus organic purification (Extraction Protocol #2) is 91.9% and 91.5%, respectively (Table 5.5). While these are similar, the overall quality of the data reported are different. The average number of bases reported for the same set of samples for the USS *Oklahoma* is 712 bp, versus the 612 bp for the Battle of Tarawa.

The samples from Cabanatuan produced a Sanger sequencing result only 47.0% of the time when extracted using EP#2. Unlike other incidents, especially those involving chemically compromised remains, Cabanatuan samples produced the most successful Sanger sequencing results using EP#3, which uses an inorganic purification.

5.10.8 NGS testing – Battle of Tarawa and Cabanatuan Prison Camps

A total of 51 samples from the Cabanatuan Prison Camp and 255 from the Battle of Tarawa were tested using NGS (Table 5.7). The success rates were comparable, 39.2% for Cabanatuan and 36.8% for Tarawa. NGS testing was the primary protocol used for Cabanatuan Prison Camps once the degree of chemical contamination had been determined. While it seems that the success rate of EP#3 and Sanger sequencing of these samples would

give a better overall success rate (76.5% vs. 39.2%), it is much less expensive and time-consuming to perform NGS testing. In addition, those samples being NGS tested for Cabanatuan had already been tested using Sanger sequencing and failed to produce reportable data.

Table 5.7. NGS testing results. Extraction protocols are not listed for this type of testing as no single protocol was used. There is a published protocol [23]; however, initial testing was performed using a combination of EP#2 and EP#3. The average number of bases is not listed for NGS processing. As this is whole genome sequencing, it is generally 16506bp or zero. The "# Reported" is the number of samples meeting the criteria for reporting as defined in the Methods and Materials. While it may appear that Cabanatuan Prison Camp samples processed by NGS do not work particularly well, it should be noted that these samples were previously reported as inconclusive in Sanger sequencing and were retested using Next Generation Sequencing. Without this protocol in hand those twenty samples would have remained as inconclusive. The low success rate of the Battle of Tarawa samples may be due to an overabundance of DNA being present in the extract.

	Cabanatuan Prison Camps NGS			**Battle of Tarawa NGS**		
	# Tested	# Reported	% Success	# Tested	# Reported	% Success
All	51	20	39.2	255	94	36.8
Clavicle	2	1	50.0	5	1	20.0
Femur	8	3	37.5	17	9	52.9
Fibula	3	0	0			
Fragments	1	0	0			
Humerus	7	6	85.7	45	15	33.3
Metacarpal				1	0	0
Metatarsal				4	2	50.0
Occipital	3	2	66.6	4	3	75.0
Parietal				1	1	100
Pelvis	3	0	0	4	1	25.0
Radius	7	3	42.8	12	2	16.6
Scapula	4	0	0	3	1	33.3
Temporal				2	0	0
Teeth	5	4	80.0	43	22	51.1
Tibia	3	0	0	100	31	31.0
Ulna	5	1	20.0	13	6	46.1
Vertebra				1	0	0

5.11 DISCUSSION

5.11.1 Temperature at Burial

Separating large assemblages of commingled skeletonized human remains is an issue faced world-wide by agencies performing human identification. Compounding the difficulty of many sets of remains are the conditions of the recovery locations. While heat of the surrounding environment at deposition and decomposition is considered key to the survival of the DNA present in the remains [29], studies have shown that thermal conditions had only a minor effect [30]. However, it is of note that Pinhasi, et al. [30] also stated that DNA extracted from skeletal materials buried in hot regions always performed worse than DNA recovered from remains buried in temperate regions. Large-scale studies of DNA recovered from remains buried at elevated temperatures for extended periods of time are perhaps warranted [31].

It was the intent of this study to present at least the beginnings of a foundational study on the effects of thermal age on modern remains buried for an extended period of time. All three events involved remains buried almost immediately post-mortem under tropical conditions. Should thermal age be the driving factor in the quality of DNA recovered from such remains, there should be little to no difference in the quality of the DNA between the sites. Unfortunately, the chemical treatment for each set of remains may have overwhelmed any impact of thermal age, thus largely negating the temperature aspect of the study.

5.11.2 Chemical Treatment

Skeletal remains recovered from the USS *Oklahoma* are a primary example of how age and perceived treatment of skeletal remains may not be an indicator of the quality of the DNA recovered. During the initial attack on the ship, the fuel tanks were ruptured, filling the

interior of the ship with fuel oil. The remains were soaked not only in saltwater, but the fuel as well. Upon disinterment, the osseous materials retained the odor of oil and were darkly colored. In 2004, scientists working on the first casket expected the extracted DNA would be highly inhibited in downstream processing or be so damaged as to be unable to PCR amplify. However, the oil acted with the saltwater and the fat within the decomposing body to form a layer of adipocere that protected the DNA from destruction [32]. The multiple disinterments and cleaning activities in the 1940s and 1950s failed to fully remove this protective layer.

This is converse to the remains that were treated by AGRS with protective compounds thought to prevent or reduce decomposition during transit. Remains from the mass graves at the Cabanatuan prison camps were treated with lye by the prison guards and were further treated with a hardening compound upon exhumation [17]. In this effort to protect the remains, the DNA was severely fragmented. Formalin, a component of the hardening compound and widely used in pathology and museology as a preservative, forms cross-links of histones and DNA structure that cannot easily be undone [33].

It is of note that the both the 'normal' remains of the Battle of Tarawa and the chemically compromised remains of the Cabanatuan Prison Camp tended to work better in Sanger sequencing when extracted with EP#3 (demineralization plus inorganic purification). In general, EP#3 does not produce the best mtDNA testing results, but is rather produces more successful results when testing with STR platforms [23].

5.11.3 Next Generation Sequencing

The NGS results are unexpected in that the "normal" remains from the Battle of Tarawa were comparable in success to those recovered from the highly compromised remains of the

Cabanatuan Prison Camps. Both sets of remains had DNA extracted in the same fashion: a combination of EP#2 and EP#3; however, they were tested for different reasons. Samples from Cabanatuan failed in Sanger sequencing and were being processed via NGS in an attempt to generate any genetic data for comparison. Those samples from Tarawa were often successful in Sanger sequencing, yet had been pushed to the NGS workflow as the mtDNA sequencing reported was shared between references for multiple servicemembers. Whole mitochondrial genome processing can provide separation of unrelated individuals who share an mtDNA profile. This is typically performed when there are no family reference samples available for STR comparison (either YSTR or autosomal STR). After examination of the individual cases by the lead analyst, it was thought that the low success rate of the Tarawa samples (37%) may be attributed to an excess of DNA present in the samples that overwhelms the protocol designed for very low quality samples (quantification data not shown).

5.11.4 DNA Extraction and Strategic Sampling of Remains

Samples recovered from the USS *Oklahoma* were distinctly different from other sets of remains recovered from WWII. Samples were highly preserved and retained a large quantity of high quality DNA, despite the presence of fuel oil, which led to a change in extraction protocol. EP#3 was actively in use at the laboratory in 2015 and was the primary method by which DNA was extracted from skeletal material. At the initiation of the modern USS *Oklahoma* project in 2015, a comparative analysis was done on a small group of samples, in which elements were extracted in both EP#2 and EP#3 and analyzed independently. Those tested in EP#3 did not amplify as well as those extracted in EP#2. Fuel oil tends to bind with the silica column used in EP#3, causing an increased loss of the smaller fragments of DNA during the extraction procedure [23]. This determination, along with supporting evidence from other compromised remains (e.g., Korean War samples treated with formalin), has led to

an implementation of using an organic extraction protocol (i.e., EP#2) on all samples that appear to have been chemically compromised.

With the remains recovered from the Battle of Tarawa, there is a marked increase in the number of patellae (35 samples), metacarpals (80 samples), and metatarsals (73 samples) as compared to the USS *Oklahoma* (zero for those three element types). The increase in smaller samples may be the result of papers that recommended the sampling of the patella, metatarsals, or metacarpals, which are more easily removed from modern gravesites [10,34]. Many of these elements were recovered by and submitted to the lab by a partner agency that designed their protocols based on these papers. These bone samples may be more easily removed from intact remains; however, they are not particularly useful for the rearticulation of skeletonized remains. The remains from the Battle of Tarawa are largely intact and not commingled, which allows for the sampling of less anthropologically desirable skeletal elements. The DNA testing strategy for this incident was to provide a confirmation through DNA of the identification that may have already been completed through anthropology and archaeology, rather than a novel identification.

5.12 CONCLUSIONS

The circumstances of the loss must be considered in conjunction with the actual burial conditions when deciding upon a DNA testing strategy. Dried, skeletonized remains are generally expected to perform poorly the longer the post-mortem interval (PMI) and the higher the average temperature of the burial conditions. However, this study of samples suggests that thermal age is not an indicator of the success of DNA testing. All three events examined had a PMI of 75 years and were buried in locations considered to have a tropical environment and thus an average daily air temperature of 18°C. All other conditions being

the same, chemical modification appears to be the greatest indicator of success for any platform tested, with the exception of NGS.

However, chemical modification alone does not determine the quality or quantity of DNA recovered from skeletal elements. It is the interaction of the chemicals with the surrounding post-mortem environment that may have the greatest impact on quality DNA recovery. Samples from the USS *Oklahoma* experienced a high degree of chemical contamination. The fuel oil permeated the osseous remains to such an extent that the fragments yet retain an odor of fuel. Initial decomposition occurred in a highly anaerobic environment, fuel oil and stagnant saltwater, allowing for the build-up of a protective layer of adipocere around the remains that reduced the typical breakdown of DNA. Conversely, Cabanatuan Prison Camp remains, treated with preservatives, have a limited amount of quality DNA, which was most successfully recovered through NGS. However, NGS protocols designed for low-quality DNA samples may be confounded when the input DNA is of better quality, as can be seen in the comparison of skeletal material recovered from Cabanatuan to those from the Battle of Tarawa.

There are limited large-scale studies of DNA extracted from skeletonized remains recovered from tropical environments. This study provides some guidance to laboratories indicating that long-term burial at elevated ambient temperatures should not be the only factor to considered when attempting DNA testing on these elements. Rather the treatment of the remains peri- or post-mortem should be considered when selecting a both a DNA extraction protocol and the appropriate DNA testing modality.

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Chapter 6

Development of a GC/MS Protocol for the Detection of Possible Inhibitory Materials in Osseous Remains and the Associated DNA

6.1 INTRODUCTION

There is a general consensus in forensic DNA analysis that there are materials that carry-over from the biological material into the extracted DNA (e.g., Matheson, et al., 2010; Kemp et al., 2014b; Moreno and McCord, 2016;). Compounds found in the environment or on the person will be present on skeletal material and be unable to be removed either through physical cleaning or purification of the DNA extract. Along with recovery of DNA, extraction protocols have been specifically designed to remove as many inhibitors as possible. The downside to the high degree of stringency is a correlated loss of 20 - 60% of the DNA present (Qiagen, 2008; Kemp, et al., 2014a). An increase in the knowledge of the presence of inhibitors and other compounds would allow for a modification to extraction protocols, which would perhaps increase the amount of DNA recovered.

Chapter 5 demonstrated that immersion in fuel failed to inhibit downstream DNA testing on remains recovered from the USS *Oklahoma*. While there might be an expectation that the oil would have dissipated over time, visual and olfactory assessment of the skeletal remains provided analysts with the knowledge that oil was still present, even 75 years post-mortem. The DNA extraction protocol used, a complete demineralization of the osseous material coupled with a phenol:chloroform purification, is effective at the removal of fairly extreme contamination of osseous elements by exogenous compounds, which would seem to indicate that it would be too stringent for use on samples that have no chemical exposure or lower amounts of endogenous DNA.

The purpose of this chapter is to present the development of the gas chromatography / mass spectrometry (GC/MS) protocol for the testing of skeletal materials and the associated extracted DNA. The protocol should allow analysts to examine the compounds present in

both, and eventually develop a DNA extraction pathway that is appropriate for specific compounds.

6.1.1 INTRODUCTION REFERENCES

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6.2 PUBLICATION

The results of this study have been submitted for publication to Forensic Science International – Synergy. The paper has been accepted with edits as of July 2019.

Determination of Materials Present in Skeletonized Human Remains and the Associated DNA: Development of a GC/MS Protocol

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6.2.1 ATTRIBUTION OF TASKS

Ms. Edson developed the concept for this project, provided the samples, performed the testing in the laboratory and analyzed the data. In addition, she wrote the publication.

Dr. Roberts provided the laboratory space and aided in the development of the testing parameters.

6.3 TITLE PAGE

Determination of Materials Present in Skeletonized Human Remains and the Associated DNA: Development of a GC/MS Protocol

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6.3.2 DISCLAIMER

The opinions or assertions presented are the private views of the authors and should not be construed as official or as reflecting the views of the Department of Defense; the Defense Health Agency; the Armed Forces Medical Examiner System; John Jay College of Criminal Justice; or the Defense POW/MIA Accounting Agency.

6.4 ABSTRACT

DNA testing of skeletonized human remains is often considered to be challenging, especially in incidents where the remains are highly fragmented or have been exposed to inhibitory materials during decomposition. Inhibitors affect the processing of DNA, either by preventing efficient extraction (i.e., formalin) or interfering with down-stream PCR-based processes. Limited studies have been performed on real-world samples that have potentially been exposed to such inhibitors peri- or post-mortem. This paper presents the development of a gas chromatography / mass spectrometry (GC/MS) protocol for the evaluation of materials present, some of which may be inhibitory for subsequent analyses, in skeletonized human remains recovered from the field, as well as the DNA extracted from the same materials. Twenty-one bone samples and seventeen DNA extracts were evaluated across three solvents and multiple GC/MS parameters to determine the optimal conditions for the recovery of trace materials present. The aim of this work is to provide a technique that can easily determine the presence of inhibitors prior to DNA extraction, allowing the analyst to modify the extraction protocol for the optimal removal of inhibitory materials.

6.5 KEY WORDS

Forensic science; GC/MS; DNA; skeletonized human remains; DNA extraction

6.6 INTRODUCTION.

Retrieval of DNA from osseous materials continues to be a challenging aspect of the process of human identification. In cases of mass fatality, where there is a high degree of commingling, efficiency in DNA extraction is key to a timely resolution of the identification process. Downstream processing of the DNA recovered from skeletonized remains has continued to increase in sensitivity. STR kits have been optimized for smaller sized loci (i.e., MiniFiler) and increased sensitivity (Modified Y-filer: Sturk, et al., 2009; Irwin, et al., 2007); and current Next Generation Sequencing (NGS, aka MPS) protocols are able to recover DNA from chemically compromised and aged remains that were previously considered untestable (Marshall, et al., 2017; Ring, et al., 2017). Efforts have been made to improve DNA extraction protocols; however, these have largely focused on improvements in the release of DNA from the complex skeletal matrix. Complete demineralization of bone in extraction protocols (Loreille, et al, 2007; Loreille, et al 2010; Amory, et al., 2012; Edson and McMahon 2016) has reduced the required volume of skeletal materials, and increased overall success of testing (Edson 2019).

Concurrent with the need to increase DNA yield from skeletal materials, is a need to remove inhibitors that may be present in the bone. Full demineralization releases a large amount of calcium and other minerals into the extraction buffer that need to be removed or there is a risk that downstream processing will be inhibited. Other inhibitory materials are commonly found in the soil and the environment surrounding the remains during decomposition. Humic acid (Sutlović, et al., 2005; Matheson et al., 2010), heme (Akane, et al., 1994), and indigo (Del Rio, et al., 1996) are among the most common chemicals encountered; however, in mass fatality events other compounds may be encountered, such as fuel oil (Guo, et al., 1997; Fortin, et al., 2004) or metals (Combs, et al., 2015). Remains being transported or preserved for long term storage may also be treated with compounds, such as formalin, that prevent efficient recovery of DNA from biological materials (Tokuda, et al., 1990).

In the optimization of extraction protocols, efforts have been made to not only recover as much DNA as possible but to strip away any inhibitors that might carry-over to the purified DNA and thus impede downstream processes. Unfortunately, by making a broad-scale attempt to remove any and all inhibitors, the loss of associated DNA can be extensive. When performing a DNA extraction using an organic purification, a large amount of DNA is lost during the post-purification washes (Doran and Foran, 2014). The same can be said for protocols using inorganic purifications, in which 20 – 60 % of the DNA present may be lost during the purification process (Qiagen 2008; Kemp, et al. 2014a).

Optimization of DNA extraction protocols to remove inhibitors known to be present in the skeletal materials would be desirable as this could decrease wash steps and increase the amount of DNA recovered in an extraction event. There is no need to remove every potential inhibitor if a specific subset could be targeted. For example, if a set of remains is known to have been buried in soil with an elevated presence of humic acid, an extraction pathway could be chosen that removes humic acid and not necessarily all other possible inhibitors. In effect, this is similar to the difference in choosing an organic purification versus an inorganic purification when the remains have been subject to saponification. Organic purification methods are more effective at the removal of fats and proteins than an inorganic purification (Allard, et al., 1990; Chaturvedi, et al., 2008; Edson 2019).

The first stage of developing an inhibitor-specific extraction protocol is to determine the materials present in the remains. For modern mass disasters, it may be easily apparent what chemicals the remains have been exposed to. With remains recovered with an unknown provenience, assumptions can be made about inhibitor exposure based on the soil or historical accounts of the event, which may or may not prove to be correct. Many studies have focused on animal bones (seal ribs: Barta, et al., 2014a; salmonid bones: Kemp, et al., 2014b) and

synthetic bones spiked with known quantities of inhibitory materials (Barta, et al., 2014b). There have been some small scale real-world studies of human remains (Yang, et al., 1998) as well as staged sets of remains (Mundorff and Davoren 2014); however, few studies have sought to analyze remains recovered across a broad range of burial conditions and environments.

Gas chromatography / mass spectrometry (GC/MS) is widely used in forensics for the detection of trace amounts of materials, particularly in toxicology (López-Guarnido, et al., 2012) and arson investigations (Pert, et al., 2006). GC/MS relies on the solubility of desired materials in organic solvents. Molecules of compounds may be detected with optimized instrument parameters. Moreno and McCord (2016) recently used DART (Direct Analysis in Real Time) ionization with mass spectrometry to analyze DNA recovered from blood samples spiked with multiple known PCR inhibitors. DART relies on direct ionization of a solid phase. As inhibitory materials may be found in low-concentrations throughout a skeletal element, and therefore require concentration before detection is possible, DART was not selected for use in this study. GC/MS workflow provides the ability to remove materials from a substrate through exposure to solvents and a subsequent concentration by volatilization prior to loading on the instrument. This paper describes the development of a protocol that can be used to detect both the presence of inhibitory compounds within osseous materials, as well as those that may carry through into the extracted DNA.

6.7 METHODS AND MATERIALS

6.7.1 Collection of Skeletal Materials

Osseous materials were submitted to the Armed Forces Medical Examiner – Armed Forces DNA Identification Laboratory (AFMES-AFDIL, aka AFDIL) from the Defense POW/MIA Accounting Agency (DPAA) as part of regular casework submissions. Samples used in this

study were chosen randomly from casework samples submitted between May 2016 and August 2016. Skeletal materials have post-mortem intervals of 45 – 80 years, and have been recovered from a variety of burial conditions (e.g., surface, shallow, curated, coffin, preserved). The peri-mortem conditions were also widely variant and included ground losses, high-impact plane crashes, and sunken ships.

During standard casework processing, the bone is cleaned by removing the exterior removed using a Dremel® tool (Bosch, Stuttgart, Germany) fitted with an aluminum oxide sanding bit. The detritus generated during this process is typically discarded as medical waste. However, for the purposes of this study, the 'powder' was collected and stored in 15 mL polypropylene tubes (Sarstedt, Nűmbrect, Germany) at -20°C until needed. A total of 439 samples were collected and anonymized with a code number so as to eliminate bias or expectations during analysis. The code number was randomly assigned. The first number represents the two-week period during which the sample was collected and the second number being the order in which the sample was randomly pulled from that grouping. For example sample 3-1 was the first sample pulled from the third collection set.

6.7.2 Extraction of DNA

The DNA extraction protocol used is described at length in Edson and McMahon 2016. It is briefly summarized here. DNA was extracted from the cleaned bones using a complete demineralization protocol coupled with an organic phenol:chloroform:isoamyl alcohol purification or a complete demineralization coupled with an inorganic purification using QIAquick PCR purification Kit (QIAGEN, Hilden, Germany). In both instances, samples are further concentrated using Amicon Ultra-4/30K centrifugal filter units (Millipore, Billerica, MA) and brought to a final volume of 50 - 200 µL with TE^{-4} (10 mM Tris, 0.1 mM EDTA; pH 7.5).

6.7.3 Selection of Samples for GC/MS Testing

For the purposes of this proof-of-concept study, 21 osseous material samples were chosen from the 439 collected (Table 6.1). These samples were selected based on having approximately 1.0 g of powder or more available, which would allow for a variety of solvents to be tested. The samples also had to be completed through regular casework processing so the associated DNA extracts could be used without the possibility of being needed for additional testing. DNA extracts were chosen from 17 of the associated samples (Table 6.2).

Table 6.1. Samples used for testing. An attempt was made to select samples from a variety of locations; however, the general deciding factor for sample selection was whether an adequate amount of osseous detritus had been generated during the cleaning process.

Sample	Conflict	Location Recovered	Approximate PMI (years)	Element
1-1	Southeast Asia	Cambodia	47	Thoracic Vertebra
1-2	Southeast Asia	Cambodia	47	Temporal
2-1	Southeast Asia	Laos	47	Femur
2-2	WWII	Philippines	75	Occipital
2-5	Southeast Asia	Laos	47	Frontal
2-12	WWII	Papua New Guinea	75	Lumbar Vertebra
3-1	Korean War	South Korea	67	Temporal
3-7	WWII	Hawaii	77	Lumbar Vertebra
3-8	Southeast Asia	Vietnam	47	Cranium
3-9	Southeast Asia	Vietnam	47	Cranium
3-12	WWII	Solomon Islands	75	Os Coxa
3-13	Korean War	South Korea	67	Ulna
3-14	Korean War	South Korea	67	Humerus
4-2	Southeast Asia	Laos	47	Tibia
4-3	Korean War	Namjong-gu	67	Temporal
5-2	WWII	Solomon Islands	75	Patella
5-7	WWII	Tarawa	75	Occipital
6-1	WWII	Hawaii	77	Occipital
10-5	WWII	Hawaii	77	Vertebra
10-6	WWII	Hawaii	77	Tibia
10-9	WWII	Hawaii	77	Ulna

Five of these proof-of-concept samples were taken from remains recovered from the USS *Oklahoma*. These were specifically selected due to the known fuel contamination immediately post-mortem. The osseous elements themselves retained an odor of fuel and the surface materials removed were black and somewhat sticky (Figure 6.1a). There was an

expectation that these samples would provide a result with almost any solvent, which would allow for a possible benchmark from which other testing could be based. Other samples, such as those from the Korean War (Figure 6.1b), were very powdery and lacking in coloration.

Table 6.2. DNA Samples used in the testing strategies. DNA extracts were generated during the course of regular casework. Samples selected for the GC/MS testing had been completely through the casework process and limited extract was available; therefore, there are skeletal samples tested that do not have associated DNA.

Sample	Conflict	Location Recovered	Extraction Protocol
1-1	Southeast Asia	Cambodia	Inorganic
1-2	Southeast Asia	Cambodia	Inorganic
2-1	Southeast Asia	Laos	Inorganic
2-2	WWII	Philippines	Inorganic
2-4	Southeast Asia	Vietnam	Inorganic
2-5	Southeast Asia	Laos	Inorganic
2-6	WWII	Kiribati	Inorganic
2-12	WWII	Papua New Guinea	Inorganic
3-1	Korean War	South Korea	Inorganic
3-7	WWII	Hawaii	Organic
3-8	Southeast Asia	Vietnam	Inorganic
3-9	Southeast Asia	Vietnam	Inorganic
3-12	WWII	Solomon Islands	Inorganic
3-13	Korean War	South Korea	Inorganic
3-14	Korean War	South Korea	Inorganic
4-3	Korean War	Namjong-gu	Inorganic
10-5	WWII	Hawaii	Organic

Figure 6.1a. Surface materials removed from a lumbar vertebra recovered from the USS *Oklahoma.* The outer surface of the osseous samples clumps upon removal and can form a waxy coating on the sanding bit.

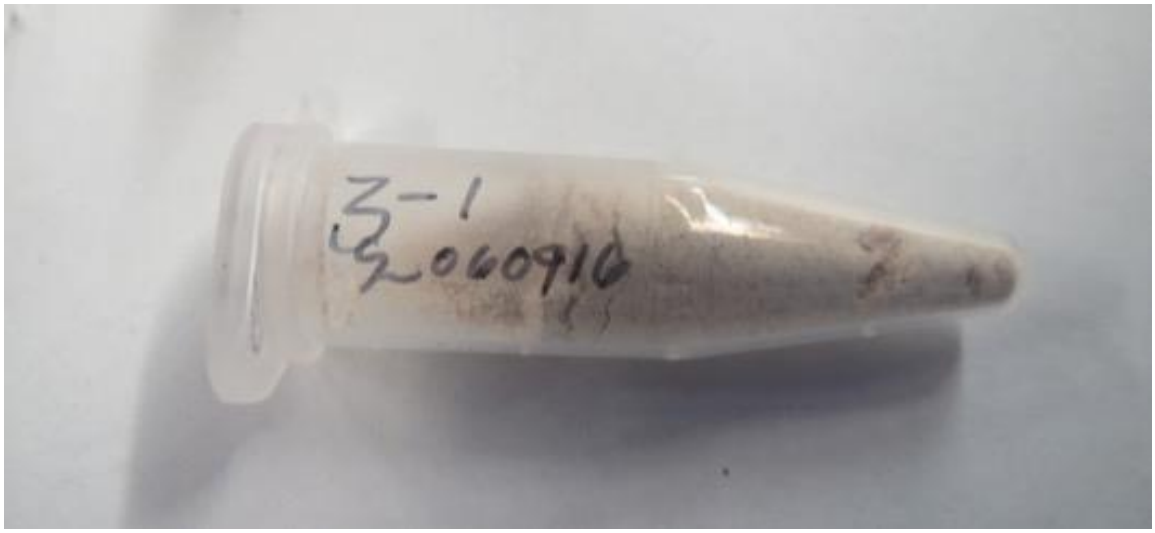


Figure 6.1b. Surface materials removed from a temporal bone recovered from South Korea. The materials removed were very powdery and talc-like.

6.7.4 Testing Parameters for Osseous Materials

Three different solvents were used to extract materials from the osseous detritus: methanol, acetonitrile, and dichloromethane (HPLC, LC/MS grade). Variations in the solubility of the various compounds in these solvents will affect the chromatography of the samples. Eleven

different GC/MS and solvent combinations were used. These are summarized in Table 6.3, but described in more detail below. Some samples were tested multiple times simply due to the quantities available. The testing strategies progressed systematically towards increasing sensitivity and generating readable data.

Unless otherwise indicated, 1.5 mL of the solvent being evaluated was added to approximately 0.1 g of bone powder contained within a 2.0 mL polypropylene tube. Samples were vortexed and placed on a rotating shaker for 1 – 17 hours at room temperature. There are modifications to this strategy as noted in the more detailed descriptions below.

For preparation of each sample, 9 mm glass vials with screw caps (Thermo Fisher, Walther, MA, USA) were used. Analysis was done using an Agilent 7890A/5875C GC/MS System with a 20 m column (Agilent, Santa Clara, CA) with a full scan and no subtraction of possible known elements. This was deliberately done, as it was unknown as to what would be detected, if anything, from the osseous detritus. All injections were split except where noted.

Analysis was performed using ChemStation (Agilent) and comparison to the NIST 2005 Spectral Library. In the event that peaks were not automatically called by the software, analysis was performed by the analyst with a comparison of the spectra generated to those determined to be the closest possible matches by the software.

Table 6.3. Parameters Tested. Description of tests performed on skeletal material (SM) in order. All injections were split, with the exception of SM#9 and SM#9a. "SM" is the abbreviation of "Sample Method".

Method	Solvent	# of samples	Treatment	GC/MS Parameters
SM #1	Methanol	1	Incubation in solvent & direct injection	200°C oven. Hold for 20min.
SM #2	Methanol	2	Incubation in solvent & direct injection	200°C oven. Ramp to 300°C at 20°C / min. Hold for 20min.
SM #3	Methanol	1	Incubation in solvent, allowed to volatize, resuspended in MeOH for injection	200°C oven. Ramp to 300°C at 20°C / min. Hold for 20min.
SM #4	Methanol	7	Incubation in solvent, allowed to volatize, resuspended in MeOH for injection	150°C oven. Ramp to 300°C at 20°C / min. Hold for 20min.
SM #5a	Methanol	4	Incubation in solvent, allowed to volatize, resuspended in MeOH for injection	150°C oven. Ramp to 300°C at 20°C / min. Hold for 30min.
SM #5bT	Acetonitrile	4	Incubation in solvent, allowed to volatize, resuspended in MeOH for injection	150°C oven. Ramp to 300°C at 20°C / min. Hold for 30min.
SM #6	Dichloromethane	3	Incubation in solvent, allowed to volatize, resuspended in MeOH for injection	150°C oven. Ramp to 300°C at 20°C / min. Hold for 30min.
SM #7	Dichloromethane	3	Same portions as tested in SM#6	150°C oven with a hold for 20min. Ramp to 250°C at 20°C / min. Hold for 30min.
SM #8	Dichloromethane	5	Incubation in solvent, allowed to volatize, resuspended in MeOH for injection	150°C oven with a hold for 20min. Ramp to 250°C at 20°C / min. Hold for 30min.
SM #9	Dichloromethane	5	Same fractions as from SM #8	150°C oven with a hold for 20min. Ramp to 250°C at 20°C / min. Hold for 30min. Splitless injection.
SM #9a	Dichloromethane	1	Fraction of sample 3-7 was concentrated overnight and resuspended in meOH	150°C oven with a hold for 20min. Ramp to 250°C at 20°C / min. Hold for 30min. Splitless injection.

6.7.4.1 Sample Method #1 (SM#1)

Sample 10-5 was selected for testing. Approximately 0.1 g of detritus was placed in a 2.0 mL polypropylene tube with 1.5 mL methanol (≥99.9%, HPLC grade, Sigma-Aldrich, St. Louis, MO, USA). Sample was vortexed and incubated for 2 hours with periodic agitation. At the end of incubation, the sample was spun down to reduce the particulates and 550 µL was removed to the loading vial. The oven temperature was set at 200°C for a run of 20 minutes.

6.7.4.2 Sample Method #2 (SM#2)

Samples 3-13 and 10-5 were used for testing. Approximately 0.1 g of detritus from 3-13 was placed in a 2.0 mL polypropylene tube with 1.5 mL methanol. Sample was vortexed and incubated overnight at room temperature. At the completion of incubation, each sample was spun down to pellet the particulate and 700 µL placed in the glass loading vial. A fraction of the 10-5 sample that had been prepared for SM#1 was removed to a glass loading vial. The loading program for the instrument was modified to a starting oven temperature of 200°C, with ramp to 300°C at a rate of 20°C / min, followed by a hold at the final temperature for 20 minutes.

6.7.4.3 Sample Method #3 (SM#3)

The remaining methanol fraction of 10-5 from the initial preparation was removed from the detritus and placed in a clean glass beaker. The beaker was placed in a chemical fume hood and the sample was allowed to volatilize overnight at room temperature. The concentrated material was resuspended in 700 µL of methanol, of which 500 µL was placed in a glass loading vial. The program on the instrument was the same as in SM #2.

6.7.4.4 Sample Method #4 (SM#4)

Seven new samples were selected for testing (1-1, 1-2, 2-2, 2-12, 3-13, 4-2, 10-6). For each sample, approximately 0.1 g of osseous material was placed into each of three 2.0 mL tubes. Methanol (1.5 mL) was added to each tube. Samples were vortexed and placed on a nutator at room temperature for one hour. Samples were spun down to pellet particles. The solvent extract was removed to clean watch glasses. The three solvent extracts for each sample were combined on a single watch glass. Samples were allowed to dry completely before being recovered off the watch glass with 1.0 mL methanol. The loading program of the instrument was modified to a starting temperature of 150°C. The run progressed with a ramp to 300°C at a rate of 20°C / min, followed by a hold at the final temperature for 20 minutes.

6.7.4.5 Sample Method #5 (SM#5a and SM#5b)

A new solvent was added in this method. Four samples (3-8, 4-3, 5-2, 10-9) were selected for incubation in methanol and four (3-1, 3-9, 5-7, 6-1) were selected for incubation in acetonitrile (Sigma-Aldrich). As with SM#4, 0.1 g of sample was placed into each of three 2.0 mL tubes. To each tube was added 1.5 mL of the designated solvent. Samples were placed on the nutator and allowed to incubate for an hour at room temperature. After incubation, the tubes were spun down to pellet the materials and the solvent poured off into a clean watch glass. The three aliquots for each sample were placed in a single watch glass. Volatilization continued until the samples were dry and the dried material was resuspended in 1.0 mL of the respective solvent. The instrument protocol started with an oven temperature of 150°C, followed with a ramp to 300°C at a rate of 20°C / min, and a hold at the final temperature for 30 minutes.

6.7.4.6 Sample Method #6 (SM#6)

A new solvent was used in this method. Three samples (3-1, 5-7, 6-1) were incubated in dichloromethane (HPLC grade; Pharmco AAPER, Brookfield, CT, USA). Approximately 0.1 g of osseous material was incubated in 1.6 mL solvent for one hour on a nutator. Multiple tubes of substrate were not available for this reaction, as the detritus had been exhausted by previous testes. After one hour, the tubes were spun down and the solvent removed to individual watch glasses for volatilization. Once the samples were completely dry, they were resuspended in 550 µL of methanol and placed in the glass loading vials. The program was not modified from SM#5.

6.7.4.7 Sample Method #7 (SM#7)

The same fractions of solvent extract generated in SM#6 were used in this testing strategy. Modifications were made to the injection program. The starting oven temperature remained at 150°C, but remained at that temperature for a 20 minute hold before ramping at 20°C / min to 250°C for an additional 30 minute hold.

6.7.4.8 Sample Method #8 (SM#8)

A new set of five samples was selected for testing using SM#7. Approximately 0.1 g of osseous detritus was incubated in 1.5 mL dichloromethane on a nutator for one hour at room temperature. Samples were centrifuged to pellet any particulates, and the solvent fraction removed to a watch glass. Complete volatilization occurred in approximately one hour. Samples were resuspended in 500 µL of methanol and placed in the glass loading vials. The run parameters were the same as described in SM#7.

6.7.4.9 Sample Methods #9 and #9a (SM#9 and SM#9a)

The five samples used for SM#9 were the same as those used in SM#8. Rather than modify the solvent, the instrument parameters were adjusted to a splitless injection. Otherwise, the run module remained the same as in SM#7.

SM#9a contained only one sample: a concentrated version of sample 3-7. The watch glass containing the concentrated solvent fraction had remained at room temperature overnight. The concentrated residue was black and tarry. Using 1.85 mL of methanol, the sample was recovered from the watch glass for injection as SM#9a.

6.7.5 Testing Parameters for Extracted DNA

Three different solvents were used to suspend DNA extracted from skeletal materials received in the course of regular casework. Samples were extracted using either an organic inorganic purification method (Table 6.2) and suspended in TE^{-4} (10mM Tris, 0.1mM EDTA; pH 7.5). DNA extracts had been stored in 1.7 mL polypropylene tubes (Costar, Corning, NY, USA). The demineralization buffer used to decalcify the osseous materials contains 1% *N*-Lauroylsarcosine, a detergent, so there was some initial concern that this might cause bubbles during injection on the instrument. As with the osseous materials, an aliquot of DNA was combined with the indicated solvent in 9 mm glass vials with screw caps. Testing strategies are summarized in Table 6.4 and described in more detail below.

Table 6.4. Parameters Tested for DNA Extractions. Description of tests performed on DNA extracts in order. All injections were split with the exception of DNA#5.

Method	Solvent	# of samples	Treatment	GC/MS Parameters
DNA #1	Methanol	3	10 µL DNA added to 500 µL MeOH	200°C oven. Ramp to 300°C at 20°C / min. Hold for 20min.
DNA #2	Methanol	5	10 µL DNA added to 500 µL MeOH	150°C oven. Ramp to 300°C at 20°C / min. Hold for 20min.
DNA #3	Acetonitrile	4	10 µL DNA added to 500 µL Acetonitrile	150°C oven. Ramp to 300°C at 20°C / min. Hold for 30min.
DNA #4	Dichloromethane	5	10 µL DNA added to 500 µL Dichloromethane	150°C oven with a hold for 20min. Ramp to 250°C at 20°C / min. Hold for 30min.
DNA #5	Dichloromethane	5	Same fraction as from DNA #4	150°C oven with a hold for 20min. Ramp to 250°C at 20°C / min. Hold for 30min. Splitless Injection.

6.7.5.1 DNA Method #1 (DNA #1)

Ten microliters of three DNA extracts (2-12, 3-13, 10-5) were added to 500 µL of methanol in the glass loading vials. Samples were run at the same parameters as SM#3.

6.7.5.2 DNA Method #2 (DNA #2)

Ten microliters of five DNA extracts (1-1, 1-2, 2-2, 2-4, 2-6) were added to 500 µL of methanol in the glass loading vials. Samples were run at the same parameters as SM#4.

6.7.5.3 DNA Method #3 (DNA #3)

Ten microliters of four DNA extracts (3-1, 3-8, 3-9, 4-3) were added to 500 µL of acetonitrile in the glass loading vials. Samples were run at the same parameters as SM#5.

6.7.5.4 DNA Method #4 (DNA #4)

Ten microliters of five DNA extracts (2-1, 2-5, 3-7, 3-12, 3-14) were added to 550 µL of dicholoromethane in the glass loading vials. Samples were injected onto the instrument with the same parameters as SM#7.

6.7.5.5 DNA Method #5 (DNA #5)

The same fractions used in DNA #4 were used. The run parameters from SM#7 were used on the instrument, with the exception of the injection being splitless.

6.8 RESULTS

The testing strategies had varying degrees of success. They are summarized briefly in Tables 6.5 and 6.6 and described more fully below.

6.8.1 Sample Method Results

6.8.1.1 Sample Methods #1 and #2

No detectable peaks were generated.

Table 6.5. Summary of Skeletal Materials Tested. Samples were randomly assigned a number based on the date of testing in order to prevent cognitive bias during analysis. Some samples were tested multiple times under different parameters, due to the large amount of detritus available for testing. Compounds detected are summarized. Most peaks were not over the analytical threshold set by the instrumentation; however, they were well defined and manually analyzed. Only the analysis of the primary peaks detected is listed. Refer to Table 6.3 for the testing parameters. Samples are listed in the order in which they were tested.

Sample	Conflict	Location Recovered	SM Test	# Peaks Detected	Compounds Detected
10-5	WWII	Hawaii	1	None	None
3-13	Korean War	South Korea	2	None	None
10-5	WWII	Hawaii	2	None	None
10-5	WWII	Hawaii	3	1	Cocaine
1-1	Southeast Asia	Cambodia	4	4	Phthalic acid; a broad-leaf herbicide; an analgesic
1-2	Southeast Asia	Cambodia	4	3	Phthalic acid; by-products of decomposition
2-2	WWII	Philippines	4	3	Cyclopentane; siloxane; variant of a compound used in a broad spectrum sunscreen.
2-12	WWII	Papua New Guinea	4	2	Fatty acids; quinine or a derivative
3-13	Korean War	South Korea	4	1	Glycerol
4-2	Southeast Asia	Laos	4	2	Glycerol; an alkaloid associated with plant materials
10-6	WWII	Hawaii	4	3	Fatty acids
3-1	Korean War	South Korea	5b	1	By-product of decomposition
3-8	Southeast Asia	Vietnam	5a	None	None
3-9	Southeast Asia	Vietnam	5b	None	None
4-3	Korean War	Namjong-gu	5a	None	None
5-2	WWII	Solomon Islands	5a	None	None
5-7	WWII	Tarawa	5b	None	None
6-1	WWII	Hawaii	5b	Numerous	Anthracene; aromatic hydrocarbons; cholestan
10-9	WWII	Hawaii	5a	Numerous	Plastic precursors; fatty acids; cyclohexane
3-1	Korean War	South Korea	6	Numerous	Broad-spectrum fungicide; benzoic acid; by-products of decomposition
5-7	WWII	Tarawa	6	Numerous	By-products of decomposition; benzoic acid
6-1	WWII	Hawaii	6	Numerous	Accelerant cluster; by-products of decomposition; benzoic acid
3-1	Korean War	South Korea	7	3	By-product of decomposition; benzoic acid; Allylamine
5-7	WWII	Tarawa	7	2	Benzoic acid
6-1	WWII	Hawaii	7	Numerous	By-product of decomposition; benzoic acid; traces of non-specific fuels

Sample	Conflict	Location Recovered	SM Test	# Peaks Detected	Compounds Detected
2-1	Southeast Asia	Laos	8	Numerous	Fatty acids; Tetraoxane; phthalic acid;
2-5	Southeast Asia	Laos	8	Numerous	Benzoic acid; phthalic acid; Benzamide; by-products of decomposition
3-7	WWII	Hawaii	8	Numerous	Accelerants; Boric acid; by-products of decomposition
3-12	WWII	Solomon Islands	8	2	By-products of decomposition; Mevalonic acid
3-14	Korean War	South Korea	8	Numerous	Sulfameter; by-products of decay; preservatives; herbicide
2-1	Southeast Asia	Laos	9	Numerous	Siloxane; phthalic acid; methyl palmate; possible fuel additive
2-5	Southeast Asia	Laos	9	7	Siloxane; phthalic acid; methyl palmate; possible fuel additive
3-7	WWII	Hawaii	9	Numerous	Accelerant cluster; dodecane; triphenylene
3-7	WWII	Hawaii	9a	Numerous	Accelerant cluster (less resolution than SM #9)
3-12	WWII	Solomon Islands	9	Numerous	By-products of decomposition; sugars; medication
3-14	Korean War	South Korea	9	Numerous	By-products of decomposition; plastics precursor

Table 6.6. Summary of DNA Extracts Tested. Sample numbering corresponds to the skeletal sample tested. Some samples were tested more than once. In most cases, the same fraction was used.

Sample	Conflict	Location Recovered	DNA Test Number	# Peaks Detected	Compounds Detected
2-12	WWII	Papua New Guinea	1	None	None
3-13	Korean War	South Korea	1	None	None
10-5	WWII	Hawaii	1	None	None
1-1	Southeast Asia	Cambodia	2	None	None
1-2	Southeast Asia	Cambodia	2	None	None
2-2	WWII	Philippines	2	None	None
2-4	Southeast Asia	Vietnam	2	None	None
2-6	WWII	Kiribati	2	None	None
3-1	Korean War	South Korea	3	None	None
3-8	Southeast Asia	Vietnam	3	None	None
3-9	Southeast Asia	Vietnam	3	None	None
4-3	Korean War	Namjong-gu	3	None	None
2-1	Southeast Asia	Laos	4	2	Dipeptides
2-5	Southeast Asia	Laos	4	2	Benzene or Oxazine
3-7	WWII	Hawaii	4	None	None
3-12	WWII	Solomon Islands	4	None	None
3-14	Korean War	South Korea	4	1	Benzene or Oxazine
3-7	WWII	Hawaii	5	Numerous	Sugars; accelerant complex; by-products of decomposition
2-1	Southeast Asia	Laos	5	Numerous	Siloxane
2-5	Southeast Asia	Laos	5	Numerous	Siloxane; by-products of decomposition
3-12	WWII	Solomon Islands	5	Numerous	Sugars; by-products of decomposition
3-14	Korean War	South Korea	5	Numerous	Sugars; by-products of decomposition

6.8.1.2 Sample Method #3

A single peak was detected (Figure 6.2). This peak was called by the Mass Hunter software as cocaine. A fraction of the sample was re-run to confirm, but no detectable peaks were recovered.

6.8.1.3 Sample Method #4

All samples generated at least one readable peak. Samples 1-2, 2-12, 3-13, 4-2, and 10-6 showed evidence of fatty acids and metabolic materials (e.g., glycerol). Sample 4-2 had a peak consistent with a plant alkaloid, possibly nantenine. Sample 1-1 contained a peak consistent with Isoproturon, a broad-leaf herbicide (Figure 6.3). Samples 1-1 and 1-2, both recovered from sites in Southeast Asia, contained phthalic acid, which can be derived from naphthalene.

6.8.1.4 Sample Methods #5a and #5b

Five of the eight samples tested using these parameters failed to generate any readable peaks. Sample 3-1 (Korea), incubated in acetonitrile, showed evidence of by-products of decomposition. Two other samples, 6-1 and 10-9, both recovered from the USS *Oklahoma*, showed evidence of possible accelerants and fats. Sample 10-9 (Figure 6.4a), incubated in methanol, mainly showed peaks of fatty acids and sugars with a peak that is characteristic of flammable materials, but lacking an accelerant arc. This arc is present in sample 6-1 (Figure 6.4b), which was incubated in acetonitrile.

6.8.1.5 Sample Method #6

All three samples produced detectable results. Sample 3-1 (Korea) showed data similar to that recovered previously with products of decomposition. However, there was also possible evidence of a broad-spectrum fungicide. Sample 5-7 (Tarawa), which had previously generated no results under SM#5b, now showed a series of peaks mostly related to materials of human decomposition (Figure 6.5). Sample 6-1 (USS *Oklahoma*) showed an accelerant arc that is difficult to characterize, due to the interaction between the fats and the fuel present.

6.8.1.6 Sample Method #7

The number of peaks generated reduced in samples 3-1 and 5-7. Both retained some of the original compounds, but at different retention times due to the change in protocols. Sample 6-1 maintained a profile showing an accelerant trace and by-products of decomposition.

6.8.1.7 Sample Method #8

Sample 3-12 (WWII) produced the least number of callable peaks, both of which appear to be by-products of metabolic pathways. Sample 3-7 (USS *Oklahoma*) produced an accelerant arc, as well as some evidence of by-products of decomposition. Sample 3-14 (Korea) produced peaks consistent with by-products of decomposition, but also evidence of sulfameter, which is a long acting sulfonamide (Figure 6.6).

6.8.1.8 Sample Methods #9 and #9a

All samples generated callable peaks. Samples 3-12 and 3-13 both generated peaks consistent with sugars and by-products of decomposition. Samples 2-1 and 2-5, both recovered from Laos, showed a similar series of peaks (Figures 6.7a & b), containing fats and a possible fuel

additive. Sample 3-7 produced the now expected accelerant cluster. The concentrated version of Sample 3-7 generated a similar cluster, but the peaks lacked resolution and the overall trace image lacked resolution.

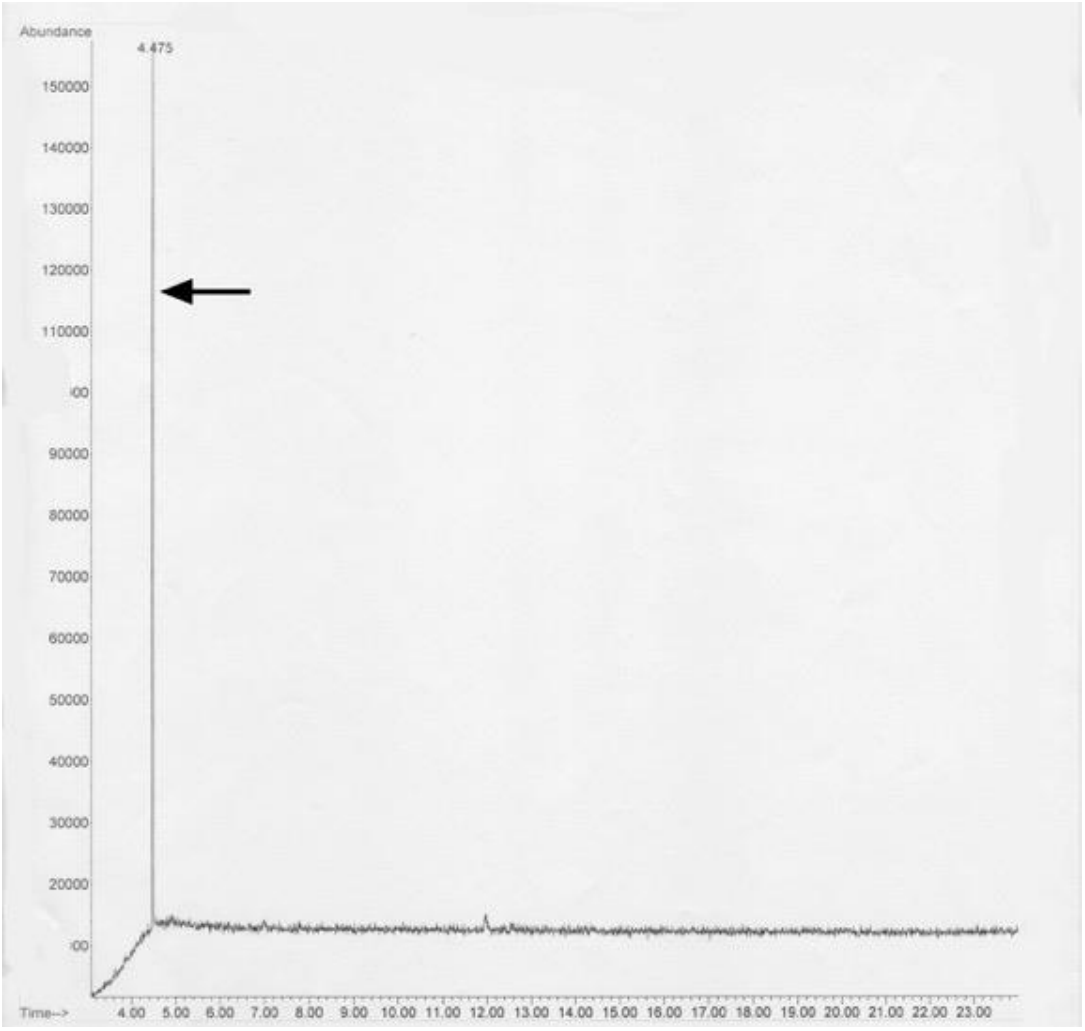


Figure 6.2. The trace image generated by the GC/MS analysis of sample 10-5 subjected to a methanol extraction and SM#3. The peak indicated by the arrow was called by the instrument software as cocaine. The parent osseous element was recovered from the USS *Oklahoma.*

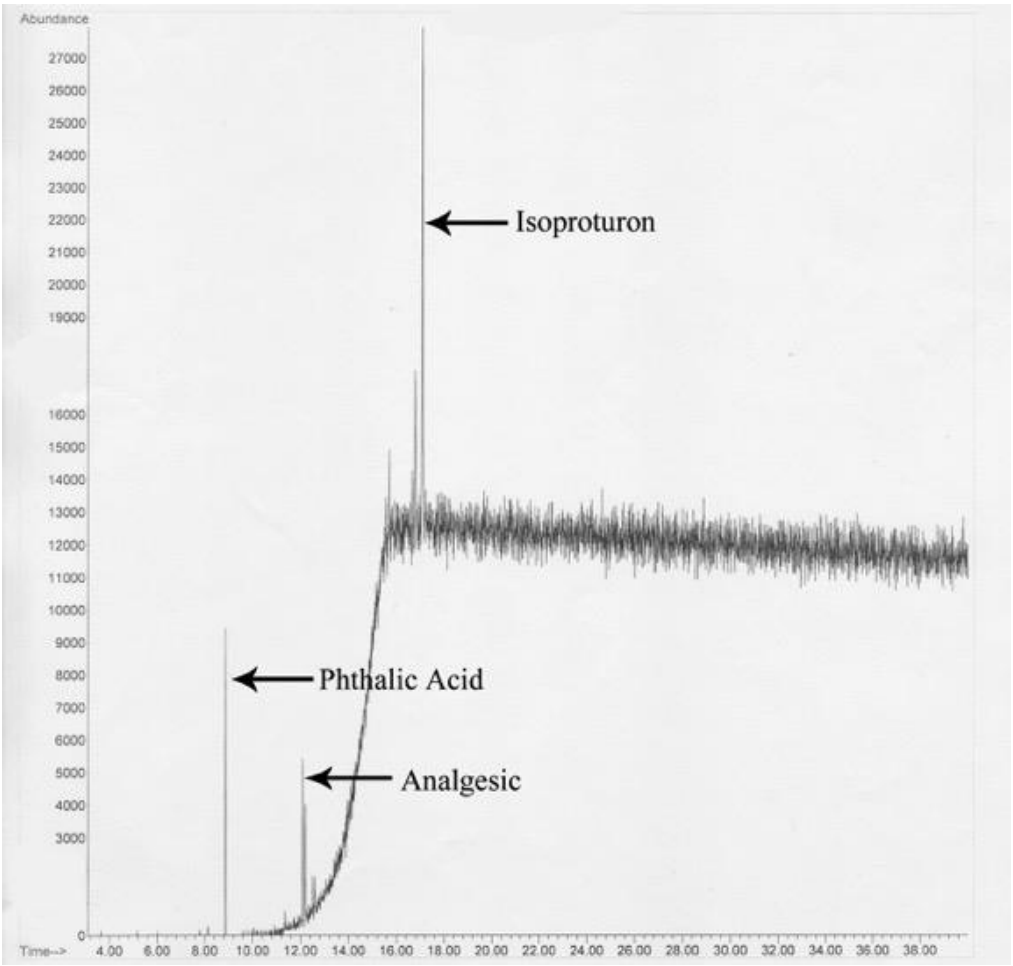


Figure 6.3. The trace image generated by the GC/MS analysis of sample 1-1 subjected to a methanol extraction and SM#4. Sample 1-1 was recovered from Cambodia and was deposited during the US conflict in Southeast Asia. While the trace shows some compression, there are callable peaks. The three most distinctive peaks are indicated by arrows and labeled according to the most likely material as indicated by Mass Hunter. The analgesic was determined to most likely be phenacetin, which was banned in the United States in 1983.

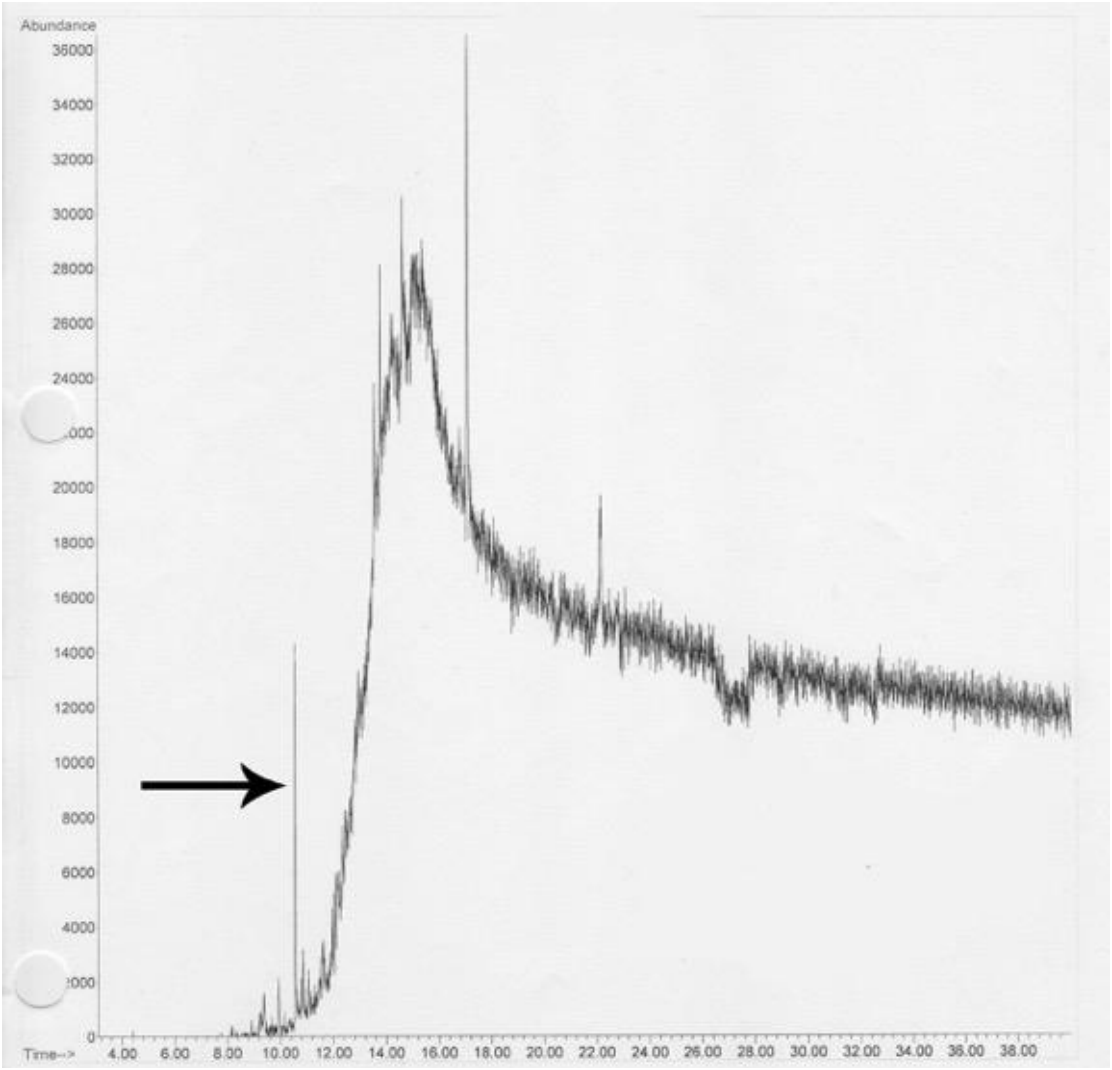


Figure 6.4a. The trace image generated by the GC/MS analysis of sample 10-9 subjected to a methanol extraction and SM#5. While the osseous sample was recovered from the USS *Oklahoma*, the visible peaks are mainly those of fatty acids. The peak indicated by the arrow is from a flammable liquid.

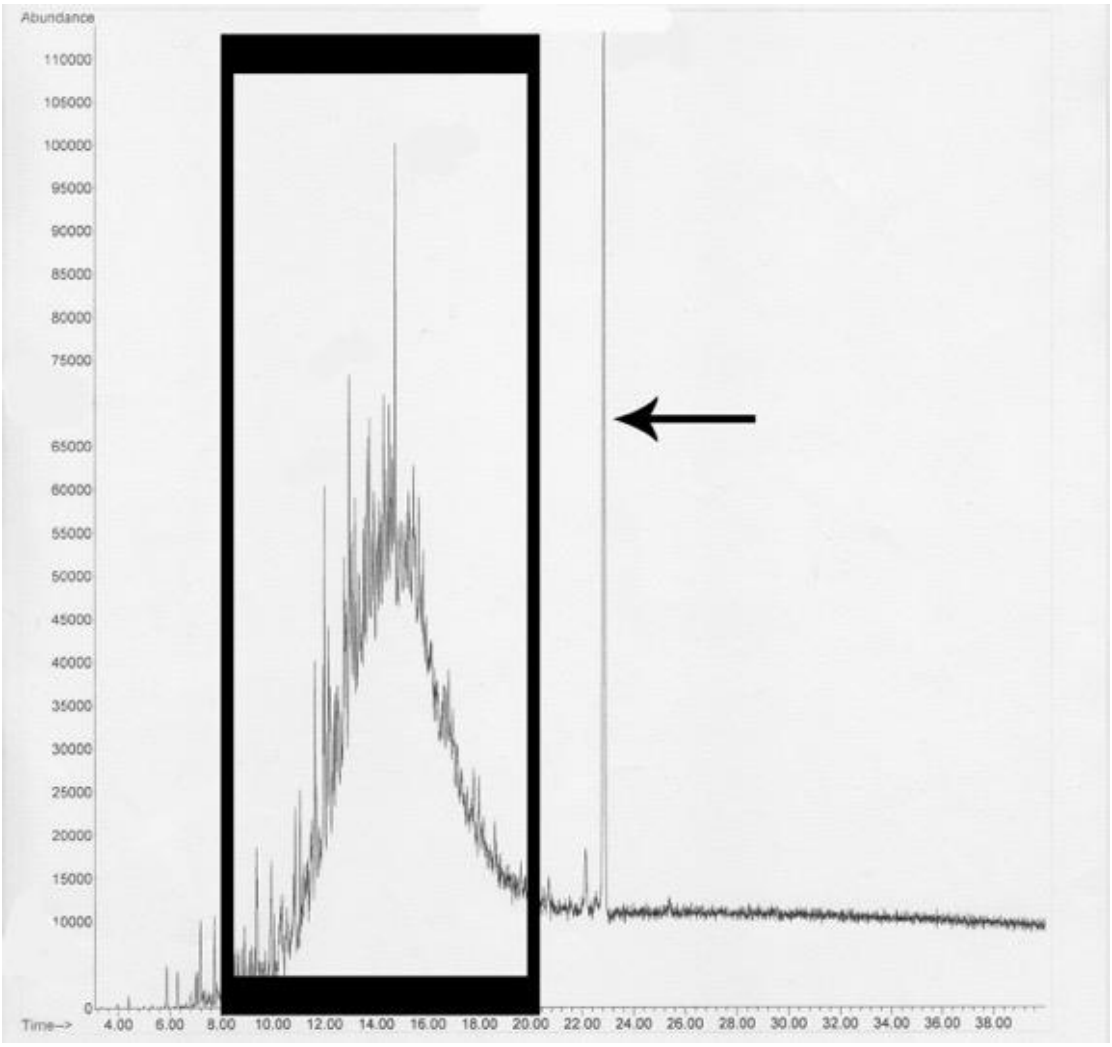


Figure 6.4b. The trace image generated by the GC/MS analysis of sample 6-1 subjected to an acetonitrile extraction and SM#5. The area surrounded by the rectangle is a series of peaks characteristic of an accelerant cluster. Even though the fuel is known to have come from the USS *Oklahoma*, the fuel cannot be accurately characterized using GC/MS as the presence of lipids is obscuring the profile generated by the fuel oils. The peak indicated by the arrow is a form of cholestan, a cholesterol derivative.

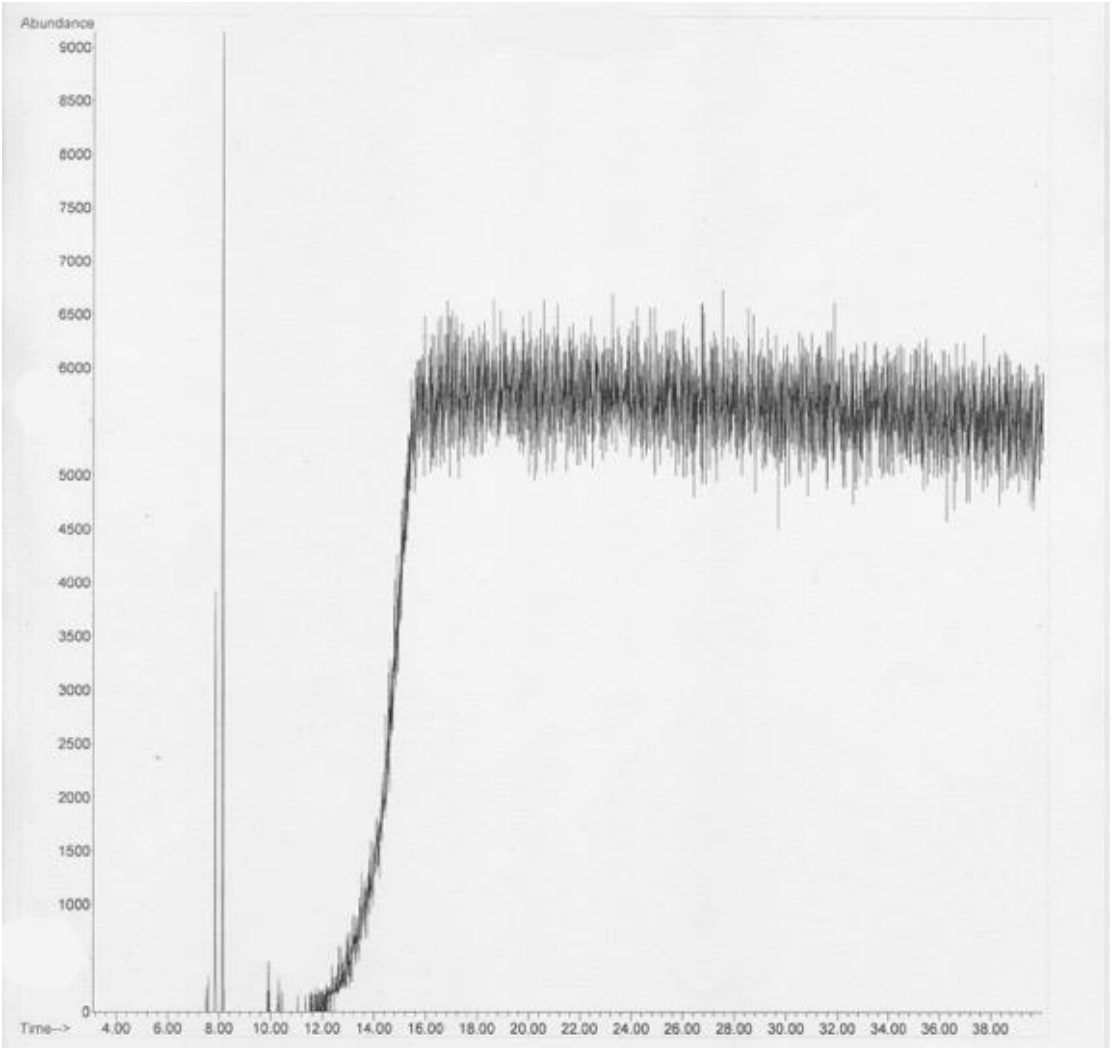


Figure 6.5. The trace image generated by the GC/MS analysis of sample 5-7 subjected to an acetonitrile extraction and SM#6. The parent osseous sample was recovered from the Tarawa Atoll. The signal noise past 12 minutes is indicative of the solvent front and no detectable materials. All other peaks present are indicative of biological materials that are by-products of human decomposition.

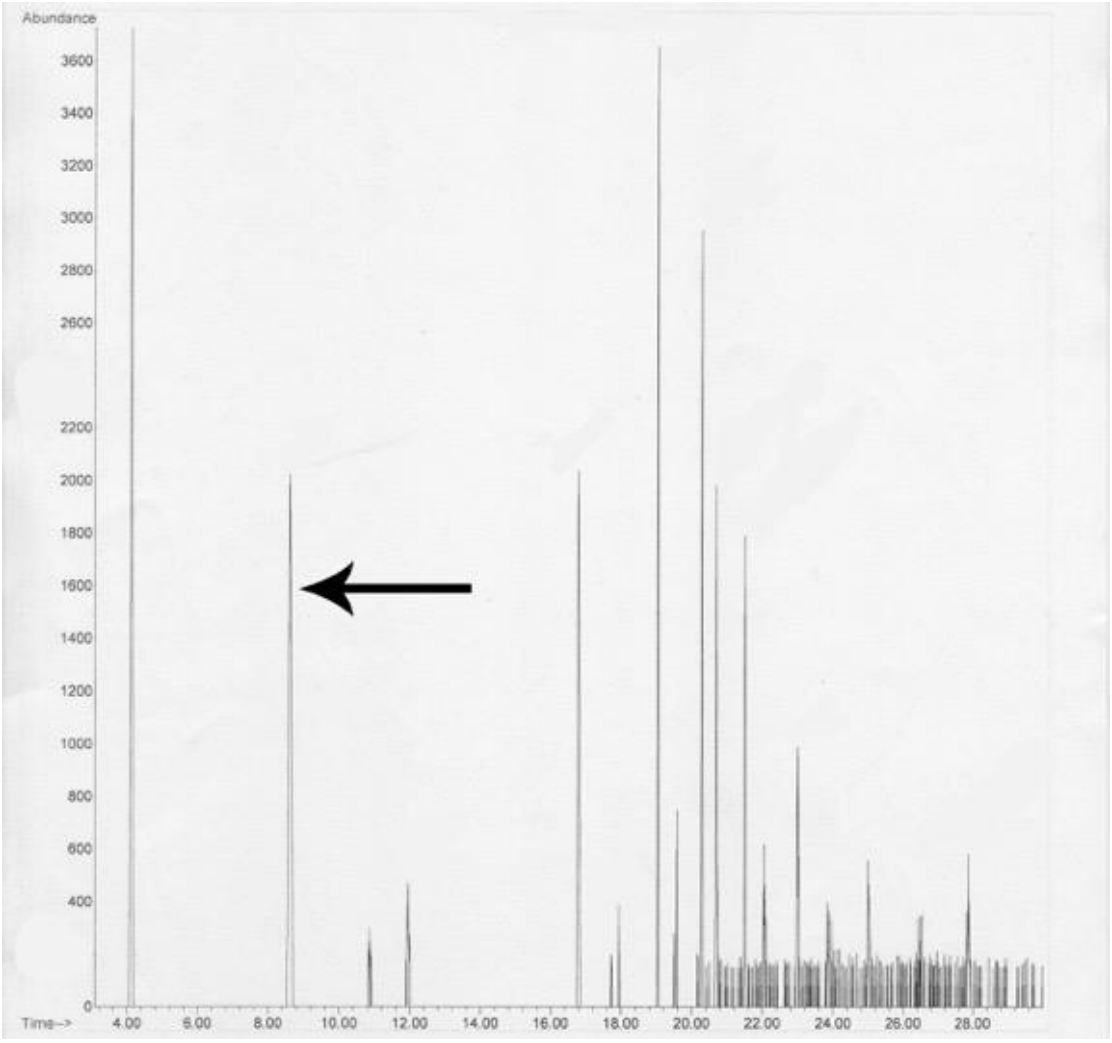
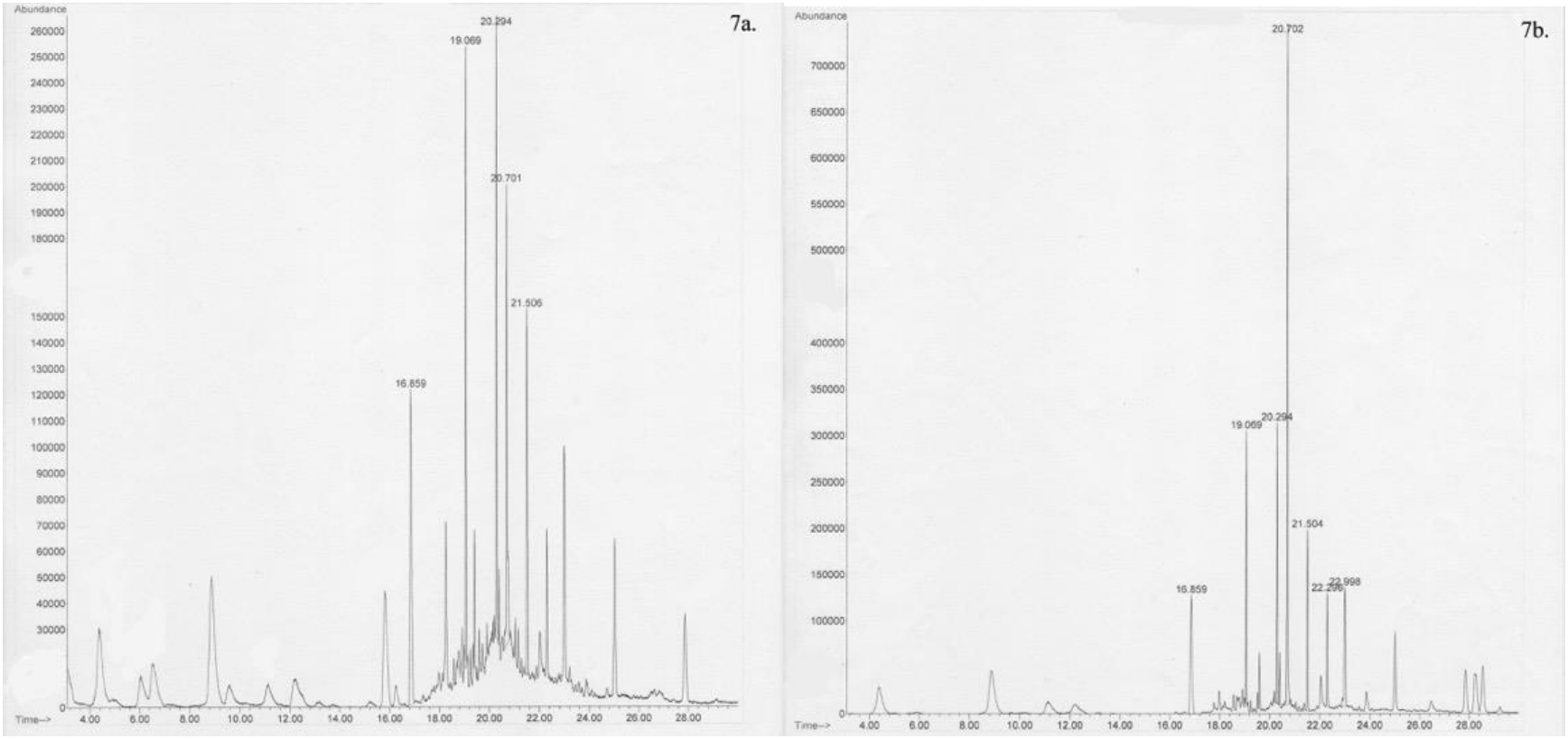


Figure 6.6. The trace image generated by the GC/MS analysis of sample 3-14 subjected to a dichloromethane extraction and SM#8. The peak indicated by the arrow is sulfameter, which is a long acting sulfonamide used to treat infections. All other peaks are by-products of human decomposition or siloxanes.



Figures 6.7a and 6.7b. The trace images generated by GC/MS analysis of two samples subjected to a dichloromethane extraction and SM#9. Sample 2-1 (Figure 6.7a) and Sample 2-5 (Figure 6.7b) were ostensibly recovered from the same location in Laos, and potentially the same individual.

6.8.2 DNA Method Results

6.8.2.1 DNA Methods #1, #2, and #3

No detectable peaks were generated.

6.8.2.2 DNA Method #4

Samples 3-7 and 3-12 generated no detectable peaks. All three showed a peak that was characterized by the Mass Hunter as callable, but unresolvable as a specific compound. The most likely result was determined to be a benzene or oxazine (which can be derived from benzene).

6.8.2.3 DNA Method #5

All five samples generated callable peaks. Each sample appeared to contain some degree of by-products of decomposition, including sugars. There is some detection of siloxane, which may be from the column itself. Sample 3-7 generated a profile most similar to that of the associated skeletal material (Figures 6.8a & b), and showed a limited accelerant trace. The 3-7 DNA was immediately after the blank, so carry-over from a previous run was not possible.

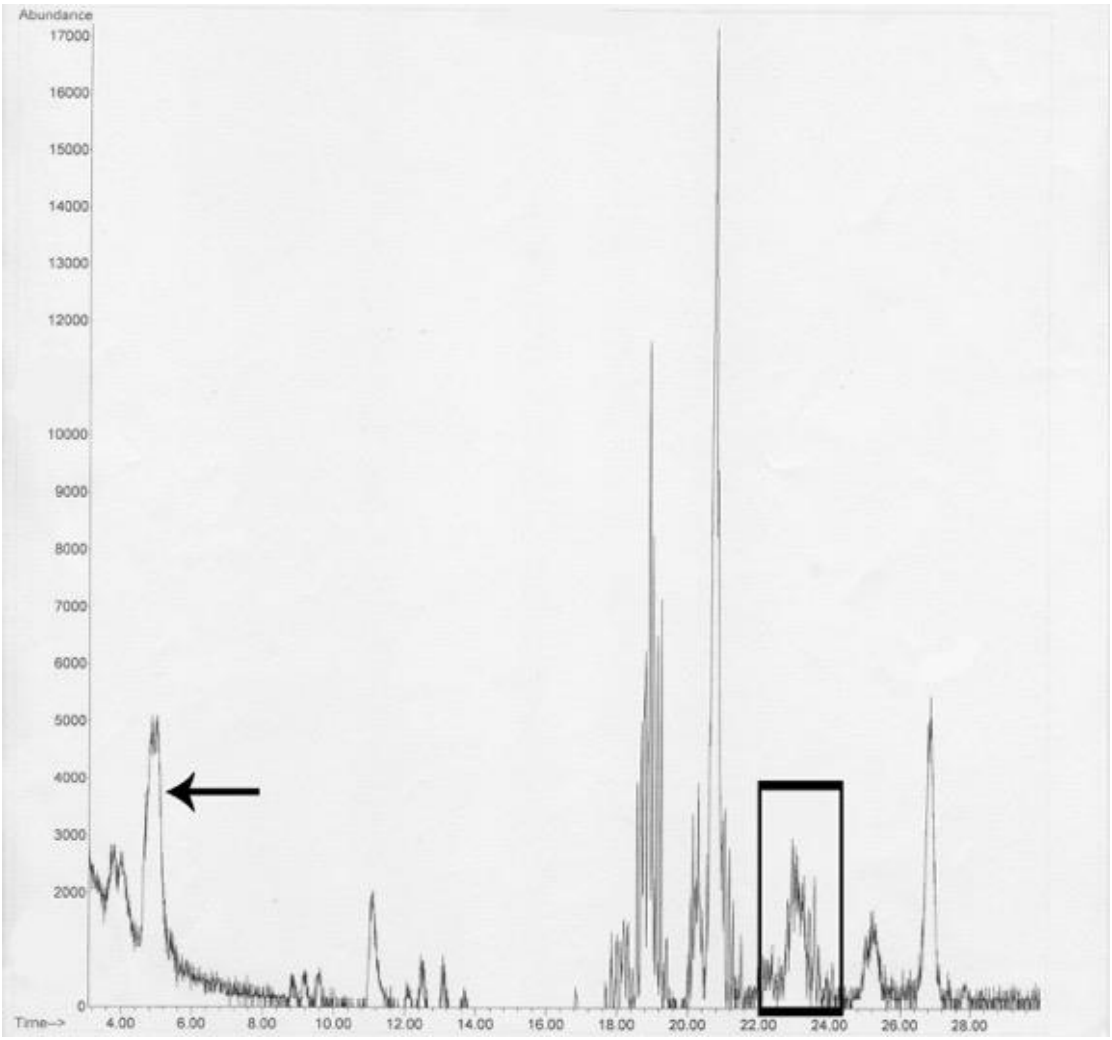


Figure 6.8a. The trace images generated by GC/MS analysis of DNA from osseous sample 3-7. The extracted DNA was diluted with methanol and injected onto the instrument DNA#5. There is a cluster of sugars between 22 and 24 minutes, indicated by the square. The peak indicated by the arrow is phenol, most likely a carry-over from the extraction.

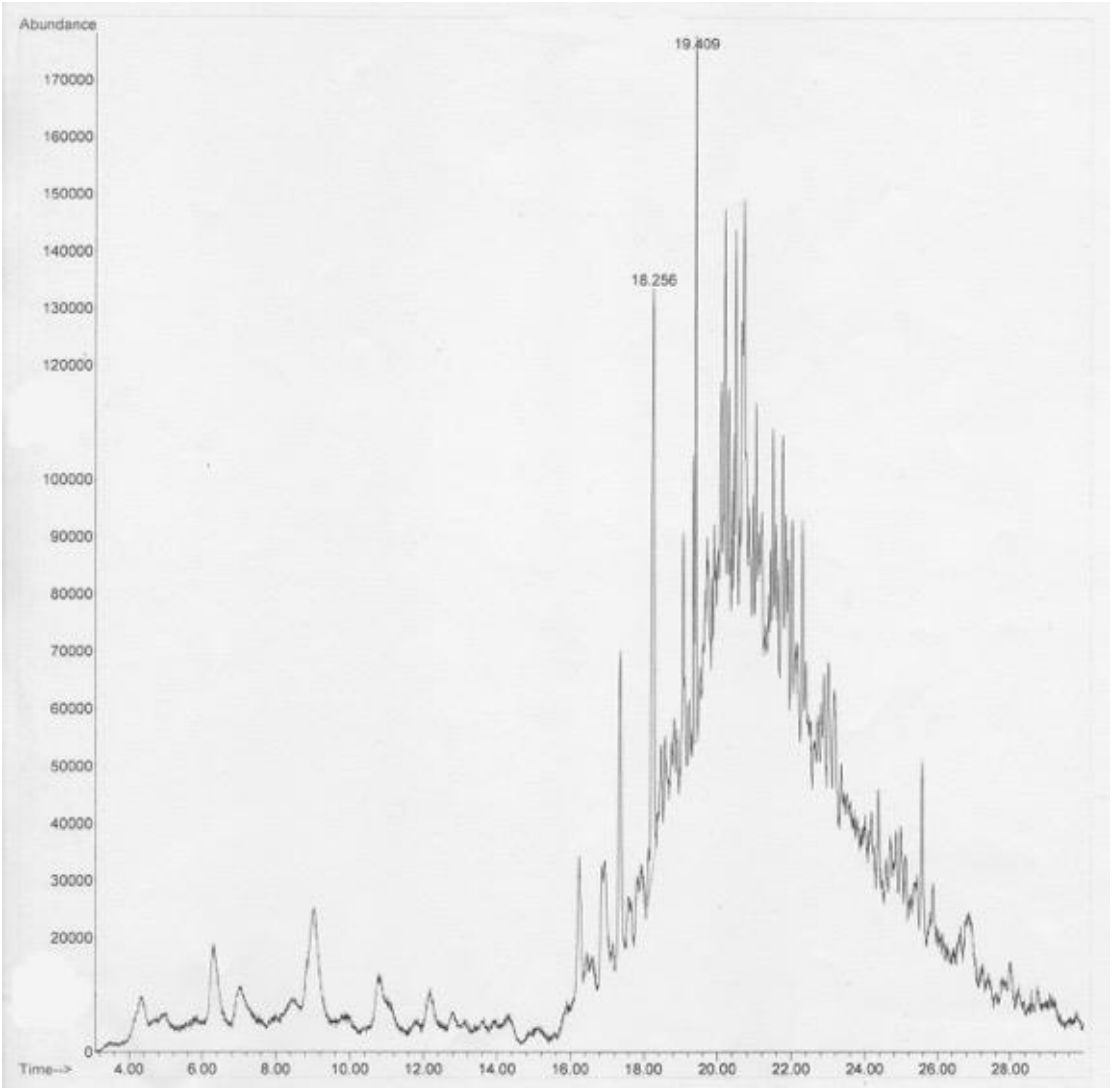


Figure 6.8b. The trace image generated by GC/MS analysis of sample 3-7 subjected to a dichloromethane extraction and SM#9. The series of dominant series of peaks is fuel oil and fats. There is some carry-over of these materials to the DNA.

6.9. DISCUSSION

6.9.1 Detection of Materials in the Osseous Samples

A large variety of materials were detected in the osseous samples tested. The use of a less stringent solvent (methanol), failed to generate a great deal of data, and it was initially expected that the amount of data that could be recovered from skeletal materials would be very low. Prior to the start of this testing series, it was thought that samples would need to be extensively concentrated, using strategies similar to those used in arson investigations. This

has been shown not be true. Using more stringent solvents, such as acetonitrile and dichloromethane, data can be generated from samples at least as old as 77 years post mortem.

The results appear to be slightly inconsistent in terms of specific items, such as the fungicide present in sample 3-1 for SM#6 and not in SM#7. However, as the parameters for the injections were constantly being changed, this was an expected result. As further work is done and more replicates of data are gathered using the same set of solvents and parameters, the results should be repeatable.

6.9.2 Detection of Modern Materials

A possible confounding factor of this testing strategy is the detection of modern materials. Sample 2-2, tested using SM#4, showed a peak representative of a component found in a broad-spectrum sunscreen. This sunscreen is thought not to be authentic to the skeletal materials themselves, as it is unlikely both that the compound would persist for the over 70 years post-mortem and that the particular sunscreen was manufactured during World War II. It is known that field teams often handle the skeletal materials without gloves; therefore, modern compounds may be transferred to the skeletal elements. Testing of additional samples from the same recovery site would be necessary to determine if the compound was conveyed throughout the remainder of the samples.

In addition, there was some detection of plastics and plastic precursors in some of the samples. These could be conveyed to the samples themselves from the polypropylene tubes the samples were stored in prior to testing. While this is a possibility, a result of "plastics" was not consistent between samples.

6.9.3 Detection of Materials in the DNA

Unlike Moreno and McCord (2016), very little carry over from the extraction procedure to the DNA was found. No phenol or guanidinium was detected in the recovered DNA. Rather, expected materials were recovered, such as sugars or other products of human decomposition. In only one instance was there marked carry-over from the bone sample to the DNA extract (sample 3-7, DNA Method #5). This DNA extract was from a USS *Oklahoma* sample that generated a similar series of peaks related to accelerants. One other sample from the same incident was also tested in this series of experiments, yet failed to generate any evidence of carry-over (10-5, DNA Method #1). It is tempting to make a conclusion that this DNA extract was free from many impurities; however, DNA sample 3-7 also failed to show evidence of carry-over when tested with different parameters (DNA Method #4). It is most likely that the sensitivity of the testing was not sufficient until the final test series, at which point, the DNA extracts had been exhausted.

6.9.4 Sample 10-5 and the Unexpected Result.

The detection of cocaine for sample 10-5 in SM#3 was wholly unexpected. A fraction of the DNA sample plus solvent was run through a spectrophotometer. The spectrum indicated the presence of DNA, in addition to environmental materials. It is not uncommon that the parent peak of a chemical would be detected without any of the associated metabolite peaks; however, given the previous results of (i.e., no detectable peaks), it seemed unlikely that only a drug peak would be present. This particular sample was taken from osseous materials recovered from the USS *Oklahoma*, which had been soaked in fuel oil within the ship for approximately two years prior to being recovered and buried in a cemetery on the island of Oahu. It would be more likely that components of fuel and fats would be detected, and indeed this is what was seen in other USS *Oklahoma* samples that were tested (6-1, 10-9, and 3-7). The source of the cocaine was not determined during trouble-shooting. The laboratory

in which the experiments were run does not have a license for the handling or testing of Schedule II controlled substances.

6.10 CONCLUSIONS

GC/MS has been shown to be potentially a very useful tool of the identification of biological and environmental compounds present in osseous remains. This is particularly useful when remains have been stored for extended periods of time and the provenience not known. Typically extraction protocols in a forensics laboratory are designated as a single pathway; however, detection of certain materials might allow for the analyst to consider alternative methods prior to extraction. The presence of fats or waxes in skeletal samples could point the DNA analyst to using an extraction protocol that would be more efficient in the removal of such materials. Detection of fuels or accelerants might indicate a different extraction pathway would be necessary.

It is clear that there are a plethora of biological and chemical materials that would need to be removed from the skeletal material during an efficient extraction of DNA. Additional studies are being undertaken to determine if the DNA extraction procedure is efficient at producing a purified extract, free from potential PCR inhibitors.

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6.12 PRESENTATION

Analysis of Materials Co-Extracting with DNA from Degraded Skeletal Remains

Keynote Presentation at Australia – New Zealand Forensic Sciences Society 23rd International Symposium on Forensic Sciences, Auckland, New Zealand, September 2016.

Analysis of Materials Co-Extracting With DNA from Degraded Skeletal Remains

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Disclaimer

The opinions or assertions presented hereafter are the private views of the speaker and should not be construed as official or as reflecting the views of the Department of Defense, its branches, the Defense POW/MIA Accounting Agency, the US Army Medical Research and Material Command, the Armed Forces Medical Examiner System, or the American Registry of Pathology.

Commercial Products

Commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible, and does not imply that any of the commercial products identified are necessarily the best available for the purpose.

Outline

- Introduction to the Mission
- Extraction Processes
  - Modifications
  - Variations in Success
- Mass Spec Analysis
  - Trial and Error
  - What was Detected
- What does this all mean?

Armed Forces DNA Identification Laboratory

- Established in 1991
  - With the primary purpose of identifying the remains of missing US service members.
- Part of the Armed Forces Medical Examiner System (AFMES)
- Partner with Defense POW/MIA Accounting Agency – Scientific Analysis Division

Defense POW/MIA Accounting Agency -- Scientific Analysis Division

- Largest laboratory of Forensic Anthropologists in the United States
- Formed for the purpose of identifying the remains of missing US service members

The primary mission of the AFDIL Past Accounting Section & DPAA

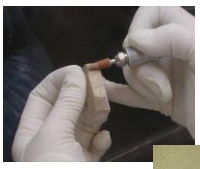
is the identification of missing US service members from past military conflicts.

This mission provides us with an unprecedented wealth of information with regards to DNA and skeletonized remains.

Extraction of DNA from Skeletal Materials

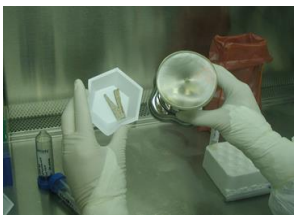
Progress and Modifications

Cleaning



Cleaning samples is common to all of the procedures regardless of downstream processing.

Powdering



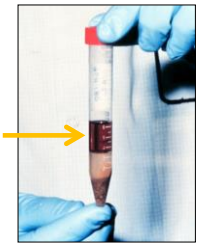
All bones are powdered in a Waring blender regardless of protocol

Original Extraction

- 2.0-2.5g of pulverized bone
- Extraction buffer:
  - 10mM Tris, pH 8.0
  - 50mM EDTA, pH 8.0
  - 0.5% SDS
  - Proteinase K

Original Extraction

- Overnight incubation is followed by:
  - a series of Phenol:Chloroform: Isoamyl Alcohol and Butanol washes.
  - Purification with filters



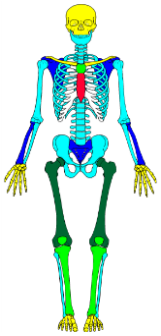
Original Extraction

- Turns out there were a lot of different color changes upon phenol addition.
  - Burgundy for a good extract.
  - Blue/Green for copper exposure.
  - Dark yellow for exposure to Tetracycline.

This was pretty cool, but so what?

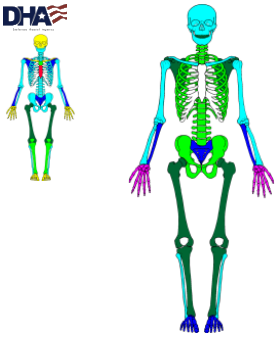
Did it impact how well the downstream processing of the DNA worked?

Original Extraction



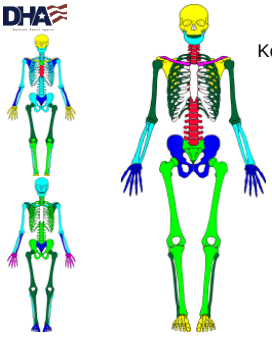
Mitochondrial DNA Success

- 90 – 100%
- 80 – 89.9%
- 70 – 79.9%
- 60 – 69.9%
- 50 – 59.9%
- 40 – 49.9%
- 30 – 39.9%
- 0 – 20%



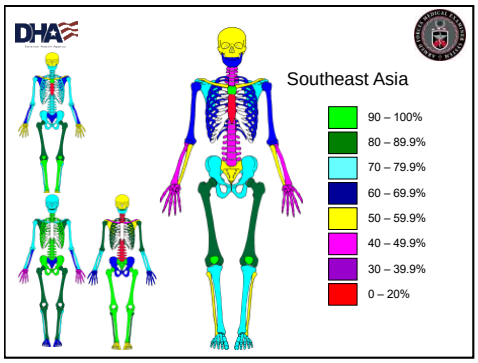
World War II

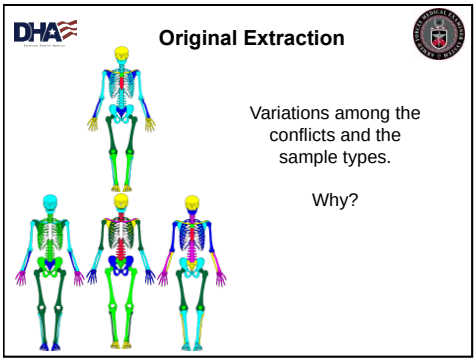
- 90 – 100%
- 80 – 89.9%
- 70 – 79.9%
- 60 – 69.9%
- 50 – 59.9%
- 40 – 49.9%
- 30 – 39.9%
- 0 – 20%



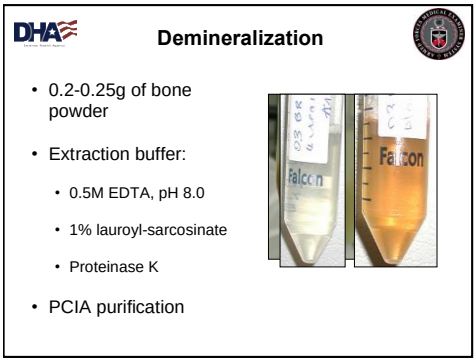
Korean War

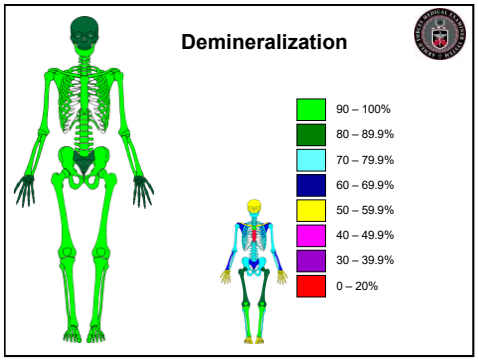
- 90 – 100%
- 80 – 89.9%
- 70 – 79.9%
- 60 – 69.9%
- 50 – 59.9%
- 40 – 49.9%
- 30 – 39.9%
- 0 – 20%





In 2006, we changed our extraction procedure, which changed this train of thought.



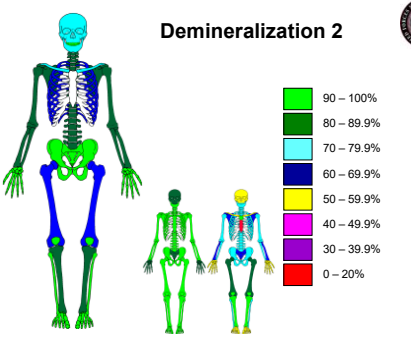


In 2013, we changed our extraction procedure again in an attempt to be safer for the staff.

Demineralization 2

- 0.2-0.25g of bone powder
- Extraction buffer:
  - 0.5M EDTA, pH 8.0
  - 1% lauroyl-sarcosinate
  - Proteinase K
- Inorganic column purification

Demineralization 2



- The change to Demineralization 2 caused a drop in success for mitochondrial DNA analysis from ~92% to ~83%.
- What is it about the extraction method that is compromising success?

Enter mass spectrometry

The Plan

- Collect the bone powder from over 400 casework samples.
- Collect 10ul of DNA extract from the same 400 samples.
- Run this all through a mass spec and see what is in the bone that is co-extracting with the DNA.

The Plan

Correlate the information from the mass spec

To the relative success and failure of

- mtDNA
- YSTR
- Autosomal STR

Caveats

- I'm not a chemist.
- Why wouldn't this work?
- If I can see tetracycline in the DNA extract, why can't I see it in the bone too?

Enter the Collaborators

- Drs. Mecki Prinz and Marcel Roberts of John Jay College of Criminal Justice, City University of New York.

Trial and Error

- Agilent 7890B
- How to volatilize whatever is in the samples?

Trial and Error

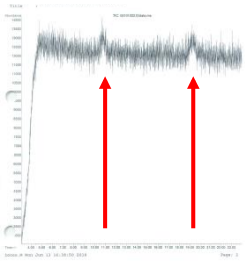
- About 0.25g of this powder was soaked in methanol for a 2 hours.
- Vortexed and removed 500ul of the liquid.

WWII (10-5) Vertebra	Mito	YSTR	Autosomal STR

Trial and Error

- Modified the run parameters.

WWII (10-5) Vertebra	Mito	YSTR	Autosomal STR



Trial and Error

WWII (10-5) Vertebra	Mito	YSTR	Autosomal STR

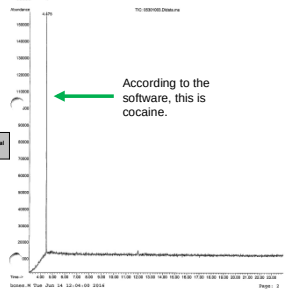
- Concentrated over night.
- Resuspended in methanol.

Korea (3-13) Ulna	Mito	YSTR	Autosomal STR

- ~1.0g of powder.
- Incubated over night in methanol

Trial and Error

WWII (10-5) Vertebra	Mito	YSTR	Autosomal STR



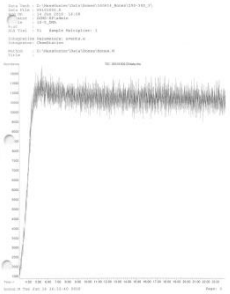
According to the software, this is cocaine.

What about the DNA?

WWII (10-5) Vertebra	Mito	YSTR	Autosomal STR
Korea (3-13) Ulna	Mito	YSTR	Autosomal STR
WWII-PNG (2-12) Vertebra	Mito	YSTR	Autosomal STR

- Extracted DNA resuspended in 500ul of methanol and loaded under the same parameters as the previous bone powder runs.

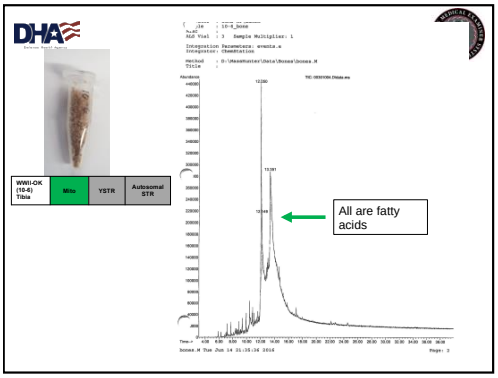
What about the DNA?

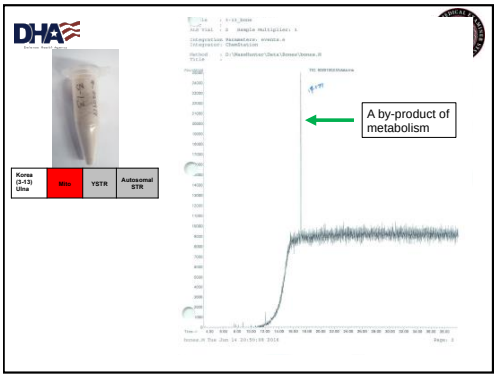


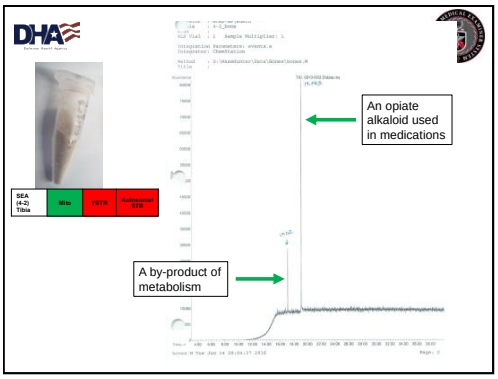
What to do?

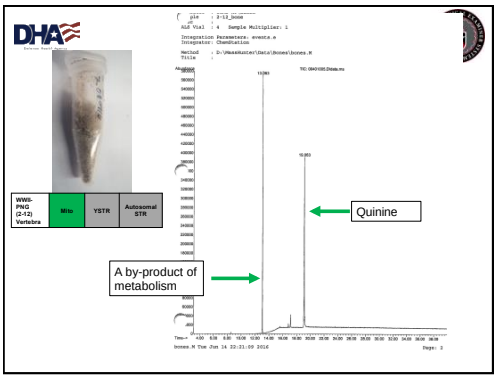
Next Trial

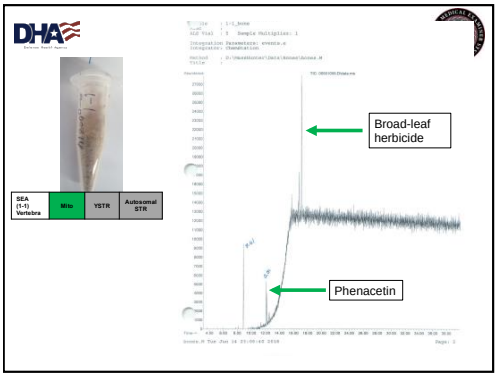
- Multiple samples chosen.
  - Different conflicts
- 3 tubes for each sample:
  - ~0.25g each tube
  - 1.5mL methanol
- Incubated on a nutator for an hour
- Dried on a watch glass
- Load parameters changed to a lower temperature.











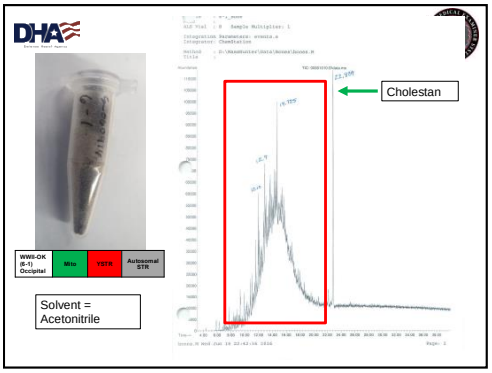
What about the DNA?

- 5 DNA samples run under the same conditions as the bone.

- Nope.

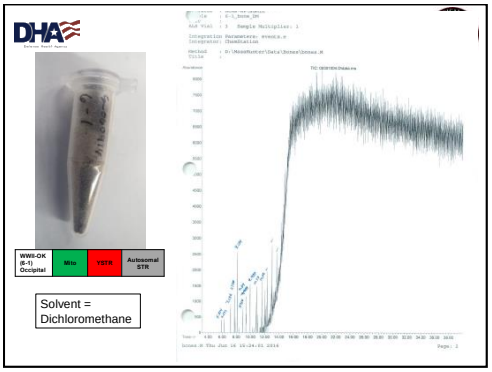
What now?

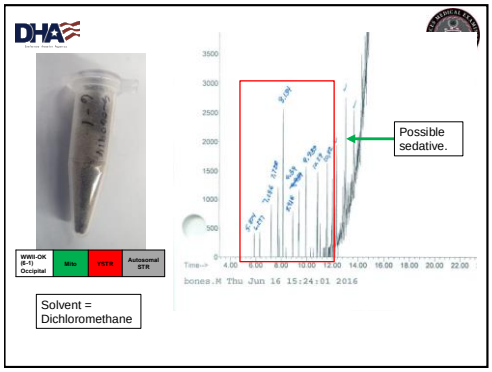
- Continued modifications of:
  - Solvents
    - Methanol
    - Acetonitrile
  - Volumes of bone powder
  - Injection times and temperatures
- Selection of DNA and bone samples from different conflicts

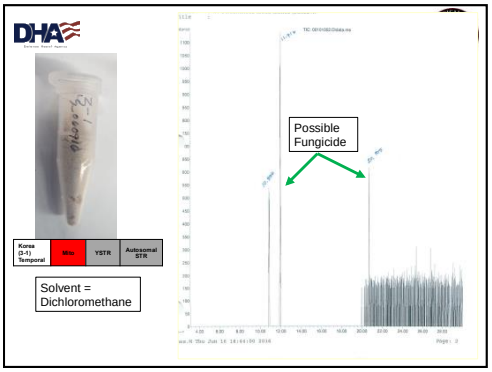


What now?

- After such a promising start, this last set of results was a little frustrating.
  - Acetonitrile should be a better solvent than methanol, but it provided fewer results.
- Tried a final solvent: dichloromethane.
  - ~0.5g bone powder and 1.5mL of solvent.
  - Incubated for 1hr and dried to concentrate.







Why keep showing slides of what may be in the bone samples?

Wasn't this supposed to be about DNA?

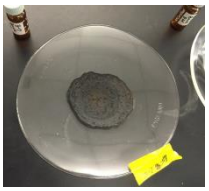
Components in the bone sample could provide information as to why the DNA testing is not working.

Even if we can't see it in the DNA sample itself.

Part of the problem with detecting co-extracted materials in the DNA extract is simply that there is not enough sample.

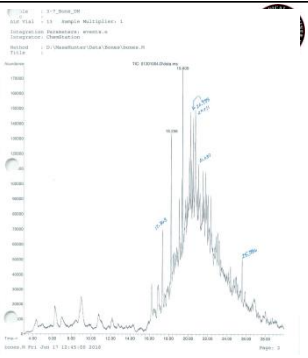
We tried one final push to get the samples to work. We hadn't done it before, because there was the risk that the "machine would break."

Highly 'Dirty' Sample

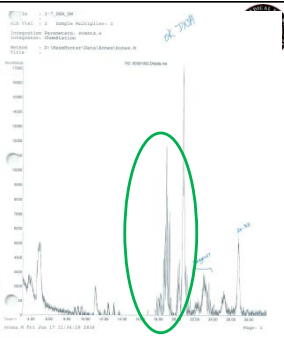


WWII-OK (3-1) Vertebra	Mito	YSTR	Autosomal STR

- This is a diagnostic series of peaks for a fuel/ accelerant.



- Present in the DNA extract at a reduced signal strength.



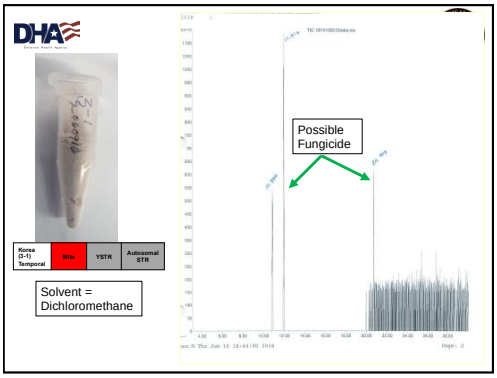
Proof of Concept

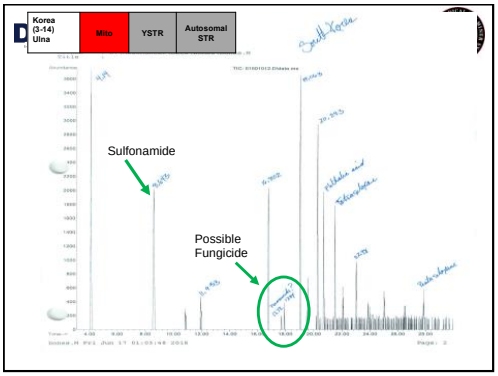
This works. It just needs to be fine tuned.

Implications

Materials Detected

- Products of decomposition.
- Fuels/accelerants
- Herbicides and Pesticides
- Fat soluble medications
- Environmental components.





- These two samples are purported to be from the same location.
  - Both contain a fungicide, but not the medication.
- Even if there is no DNA, could the possible medications be enough to say it's two different people?
- And what does the fungicide tell us about the storage/post-mortem conditions of the remains?

Implications

- Testing of bone samples in this manner can potentially provide a great deal of information about how the individual was treated prior to death and the conditions under which the remains were stored/treated.

Implications

- Components in the bone sample could provide information as to why the DNA testing is not working.
- Fine-tuning of this technique and further testing of DNA samples will allow for detection of materials that may be inhibiting DNA testing.

Future Work

A test sample has been run on a more sensitive mass spec.

Over 1500 possible compounds were detected in a single sample.

Testing will continue….

Acknowledgements

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  - Dr. Tim McMahon
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  - Mr. Jim Canik
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  - Darlene Murphy
- John Jay
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Any Questions?

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Chapter 7

GC/MS Analysis of Skeletal Remains: What's There?

7.1 INTRODUCTION

Compounds endogenous to skeletonized human remains have thought to inhibit downstream PCR analysis of the DNA extracted from those elements (Barta, et al., 2014b; Kemp, et al., 2014). This chapter expands upon the testing presented in Chapter 6 (Edson and Roberts, *in preparation*) by testing 426 samples recovered from skeletonized remains. The skeletonized materials used for testing were recovered by the Defense POW/MIA Accounting Agency (DPAA) from locations world-wide, and involve the presumptive war-time losses of United States service members. The skeletal elements themselves were not used for testing, but rather the powdered osseous materials removed from the exterior of the elements prior to DNA extraction. Post-mortem intervals range from 45 to 75 years.

By performing such testing, the possible inhibitory materials present may be evaluated and compared to similar analysis of the associated DNA extractions. The primary goal of this series of tests to determine how efficient DNA extraction protocols are at removing compounds that are present in the osseous materials.

7.1.1 INTRODUCTION REFERENCES

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Kemp BM, C Monroe, KG Judd, E Reams, C Grier. 2014. Evaluation of methods that subdue the effects of polymerase chain reaction inhibitors in the study of ancient and degraded DNA. Journal of Archaeological Science 42:373-380.

Edson SM, M Roberts. *Submitted*. Determination of Materials Present in Skeletonized Human Remains and the Associated DNA: Development of a GC/MS Protocol. Submitted to Forensic Science International: Synergy.

7.2 PUBLICATION

The results of this study were published in Forensic Science International: Genetics Supplemental Series.

SM Edson. (2017). DNA Typing from Skeletal Remains: A Study of Inhibitors using Mass Spectrometry. Forensic Science International: Genetics Supplemental Series 6: e337-e339.

Journal Impact Factor: 0.8

7.3 TITLE PAGE

DNA Typing from Skeletal Remains: A Study of Inhibitors using Mass Spectrometry

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This work was presented at the 2017 International Society of Forensic Genetics meeting in Seoul, South Korea.

7.3.1 DISCLAIMER:

The opinions or assertions presented are the private views of the author and should not be construed as official or as reflecting the views of the Department of Defense; the Defense Health Agency; the Armed Forces Medical Examiner System; ARP Sciences, LLC; or the Defense POW/MIA Accounting Agency.

7.3.2 ACKNOWLEDGEMENTS:

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7.4 ABSTRACT

This project examines the materials co-extracting with DNA from skeletonized remains that have been in the environment for greater than 50 years. A total of 435 samples with known loss locations were collected for this study in the course of ongoing HID processes. During preparation for DNA extraction, a fine powder containing osseous materials and associated environmental detritus was collected from these skeletal elements. Initial results indicate that accelerants and other fuels are not completely removed from DNA extracts using an organic extraction protocol. Portions of this skeletal residue were extracted with multiple solvents and evaporated to concentrate available materials. Samples were rehydrated in methanol and analyzed using a GC/MS. Additionally, the purified DNA from the associated remains was suspended in methanol for comparison. The skeletal materials were a mix of materials present in the environment, by-products of decay (e.g., lipids), and fat-soluble compounds inherent to the remains. Fat-soluble medications (e.g., quinine) were detectable, as were fuels and accelerants. Site-specific biological materials, such as oils from local plants, were also detected. Comparison of skeletal elements from the same site, but not the same individual, showed similar patterns of compounds present with personal variations. Not only is it possible to qualitatively study the presence of DNA inhibitors in real-world situations using GC/MS, but there is the potential to provide an additional metric for individuation or identification of unknown human remains.

7.5 KEY WORDS

Skeletonized remains; GC/MS; DNA; Human identification

7.6 ARTICLE INTRODUCTION

While the extraction of DNA from skeletonized human remains has been modified and significantly improved since the establishment of the Armed Forces DNA Identification Laboratory (AFDIL) in 1992 [1,2,3] there yet remains some challenges to be met. Success rates for mitochondrial DNA (mtDNA) and STR testing remains static at approximately 92% and 45%, respectively (internal data). Improving the efficiency of DNA extraction by removing inhibitors should serve to likewise improve DNA testing results.

Moreno and McCord [4] successfully used mass spectrometry to analyze the presence of inhibitors in DNA extracted from blood spiked with a variety of inhibitors. As the inhibitors present in the samples can be assumed from the environment [5], limited studies have been performed on real-world samples [e.g. 6, 7]. Using GC/MS, this study seeks to ascertain the specific materials present in skeletal remains and whether they are efficiently removed by the DNA extraction protocols currently available.

7.7 METHODS AND MATERIALS

Skeletal samples are received at AFDIL from the Defense POW/MIA Accounting Agency – Laboratory (DPAA-Lab) for DNA extraction and processing for DNA analysis (mtDNA, autosomal STR, and Y-STR). During the course of regular casework, the exterior of skeletal sample was removed using a Dremel® sanding tool (Dremel, Racine, WA). Rather than discarding the detritus generated from this process, it was collected and retained at -20°C. The skeletal sample itself continued through one of two DNA extraction protocols as described in Edson and McMahon [3].

Of the 426 'powder' samples collected, 100 were prepared for GC/MS analysis. A fraction of the powder (~0.1 g if possible) was treated with 1.0 mL of acetonitrile and allowed to incubate for an hour. Samples were spun down and the liquid fraction removed to a cleaned glass beaker for volatilization until dry. The dried materials were resuspended in 500 μL of methanol (≥99.9%, HPLC grade, Sigma-Aldrich, St. Louis, MO, USA). This fraction of powder was subsequently treated in the same fashion with dicholoromethane. A 10 μL aliquot of the DNA extracts associated with the 100 samples was resuspended in 150 μL of methanol.

All samples were loaded onto an Agilent 7890A/5875C GC/MS System with a 20 m column (Agilent, Santa Clara, CA). The splitless injection proceeded under the following conditions: 2.5 minute solvent delay, initial oven temperature of 150°C with a ramp to 250°C over 10 minutes at 20°C/minute (Edson and Roberts, *in preparation*). Non-targeted data analyses were performed via ChemStation (Agilent) using the NIST2005/2011 spectral library supplemented with the Cayman Spectral Library (Cayman Chemicals, Ann Arbor, MI).

7.8 RESULTS

A wide variety of materials were detected from the bones themselves. These include, but are not limited to: products of decomposition (e.g., hexadecanoic acid, cholestan); fuel elements (e.g., phenanthracene, anthracene, nonane); plant materials (e.g., longicamphenylone, terpines); site-specific elements (e.g., naphthalene); and individual specific elements (e.g., quinolone). The DNA samples produced limited information; however, carry-over from parent bone as well as elements of the chosen extraction protocol were detected (i.e., phenol, butanol, and guanidinium). Analytes from the two solvents (acetonitrile and dichloromethane) generated different traces and provided a great depth of information about each bone sample (Figure 7.1).

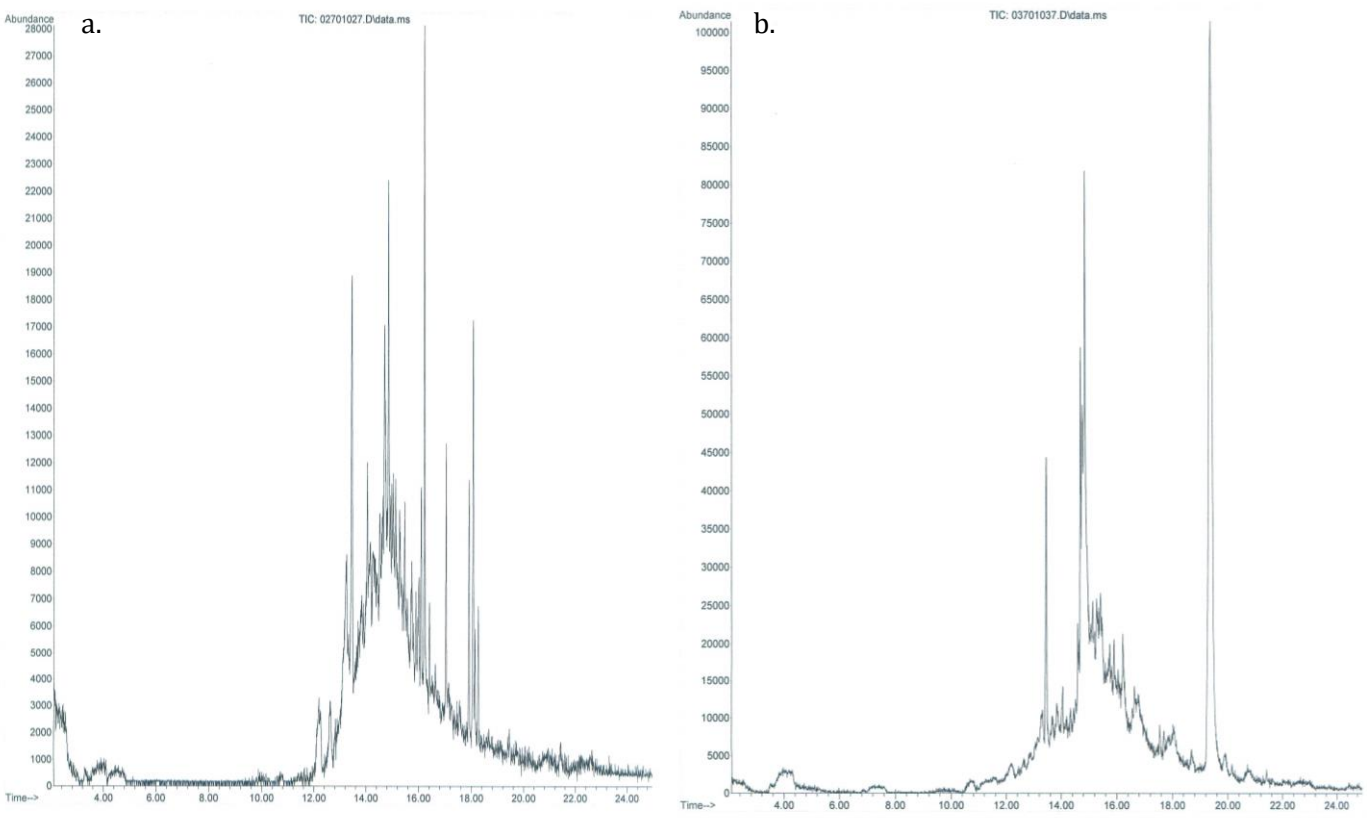

Figure 7.1. Examples of GC/MS traces generated. a.) A trace generated from the acetonitrile fraction of a sample recovered from a ship sunk in 1942 (the USS *Oklahoma*). The larger series of peaks in the middle consists of fats, esters, and materials from the fuel oil found on the ship. b.) A trace generated from the acetonitrile fraction of a sample recovered from Vietnam. The large peak at approximately 20 minutes is a by-product of decomposition.

7.9 DISCUSSION

It is widely accepted that inhibitors present in the environment will continue from the skeletal sample tested into the DNA and inhibit downstream processing of the sample if they are not removed [8,9]. This work indicates the probability that extraction procedures are very efficient, and there is little to no carry-over of environmental inhibitors from skeletal elements to the DNA. While there were some instances of high carry-over, it appears this has more to do with the skill of the analyst performing the extraction rather than being symptomatic of the extraction protocol itself. Realistically, it is possible that the GC/MS program parameters are

not yet well enough defined to detect the materials. Modifications of the program parameters will be undertaken to verify the results thus far.

A wealth of information was derived from the 100 skeletal samples examined. Given it has been shown to be possible with plants [e.g., tea: 10], it was believed that materials associated with the location of burial would be able to be detected; however, the depth of detectable elements was unexpected. Not only location-specific materials were detected, but materials that may be attributable to the decedent. Fat-soluble medications were detected, and further work will be done to determine if multiple elements from the same individual show the same medications. It is of note that elements from different individuals within the same incident show a different composition. While many elements are the same, different metals and plant materials were detected, which could lead to specifying a more exact location of mortality within a given incident. Coupled with the detection of medications, these results give promise that GC/MS analysis of skeletal elements may be low-cost method by which materials from a mass fatality may be sorted.

7.10 CONCLUSIONS

Current extraction protocols appear to be generally efficient with regards to the removal of environmental materials. While issues with downstream DNA processing may be due to inhibitors inherent to the skeletal element, it may simply be due to low-quality/low-quantity DNA being recovered from the remains. Detection of location or individual specific materials in skeletal elements is an unexpected, yet exciting result. Mass spectrometry is low-cost compared to DNA analysis and may be of great use for screening elements once initial DNA profiles have been generated. Analysis of the remaining collected samples is being undertaken to verify these conclusions and perhaps provide an extremely valuable addition to the tool-kit of human identification

7.11 REFERENCES

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[4] L.I. Moreno, B.R. McCord, The use of direct analysis in real time (DART) to assess the levels of inhibitors co-extracted with DNA and the associated impact in quantification and amplification, Electrophoresis 37 (2016) 2807-2816.

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7.12 SUPPLEMENTAL MATERIALS

What follows is additional information on the tests done and the results achieved.

7.13 SUPPLEMENTAL METHODS

All processing of the skeletal materials was performed in the manner of regular casework. This included standard precautions for preventing contamination of samples with exogenous DNA, including the use of 8.5% (v/v) bleach for the cleaning of surfaces and the UV irradiation of consumable materials. Scientists performing tests typically wear personal protective gear, such as gloves, lab coat, protective sleeves, and mask. All sanding of samples took place within bench-top hoods outfitted with a vacuum system that prevented the flow of bone powder out into the laboratory space.

The handling of remains in the field cannot be controlled. The field teams do not wear gloves and may wash or otherwise examine the remains. In addition, samples returned by locals (known colloquially as "unilateral turn-overs") may have been carried about as good-luck charms or used in the kitchen (L. Freas, pers. comm.).

7.13.1 SAMPLES

During the course of regular casework, osseous materials and teeth from unidentified remains were recovered from burial locations world-wide by the Defense POW/MIA Accounting Agency (DPAA). Following examination by a forensic anthropologist, a window of bone was removed from the parent element for DNA testing (Figure 7.2). The teeth recovered were examined by a forensic odontologist, and a tooth lacking damage or caries was selected for DNA testing. Samples of both types were submitted to the Armed Forces Medical Examiner System – Armed Forces DNA Identification Laboratory (AFMES-AFDIL, aka AFDIL) for DNA testing.

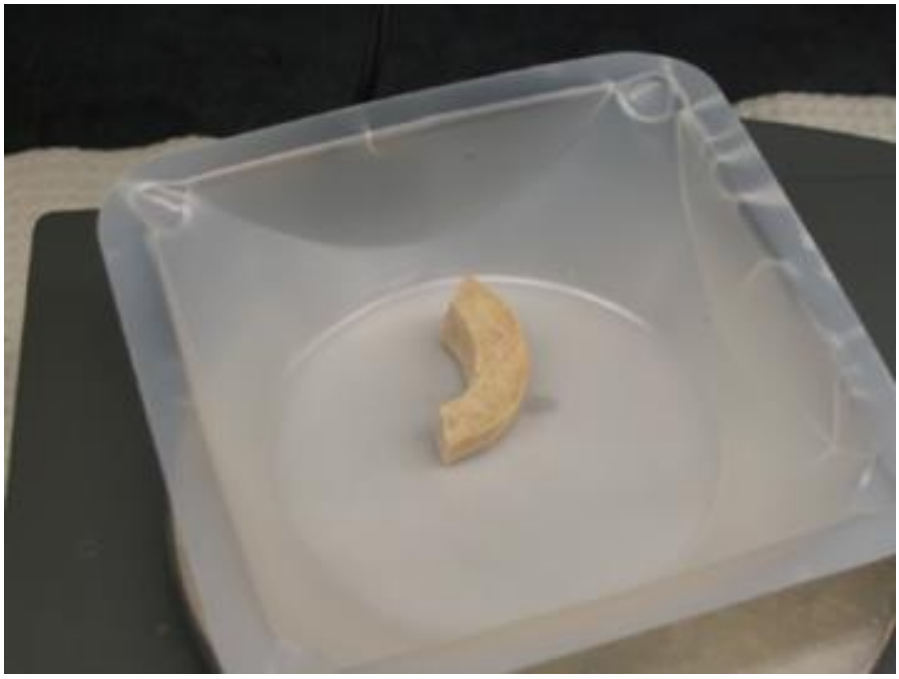

Figure 7.2. An example of a sample submitted for DNA testing to AFDIL from DPAA. The sample above is a typical window of bone recovered from a long bone and has already been cleaned.

7.13.1.1 Osseous Materials

Osseous samples submitted to AFDIL from DPAA between 6 June 2015 and 3 December 2015 were randomly selected for inclusion in this study. Not all of the samples processed for DNA extraction during this time frame were collected in order to provide a wider variety of recovery locations. In July of 2015, the first of the modern recoveries for the USS *Oklahoma* was initiated. During the same six months of collection, nearly 5,000 osseous fragments from the USS *Oklahoma* were sent to AFDIL for DNA testing. Although 208 samples from this incident are included in the study, the remainder were from a variety of recovery locations (Table 7.1). Samples ranged in size from 0.3 g to 16.2 g.

In preparation for DNA testing, the exterior of the samples was removed using a foot-pedal operated Dremel® tool (Bosch, Stuttgart, Germany) fitted with aluminum oxide sanding bits. The sanding bits were used one time only and then discarded. The Dremel tool itself was

cleaned with 8.5% (v/v) bleach and a Kimwipe to remove any adhering powder or other materials.

Table 7.1. A summary of the samples included in this study. The "Location" indicated is either a specific incident or a specific region where a conflict occurred. A Joint Recovery Operation in one in which the recovery of the remains was undertaken by a field team from DPAA operating in concert with a team from that particular country. All samples tested from the Korean War were returned by the North Koreans between 1990 and 1992, except for those from South Korea or Joint Recovery Operations.

Conflict	Location	Number Tested
Korean War	Joint Recovery Operation (JRO)	5
Korean War	Kaljon-Ri	1
Korean War	Namjong-gu	6
Korean War	Ryongpho-ri	1
Korean War	Sinhung-ri	1
Korean War	South Korea	6
Korean War	Unspecified	2
Other	Unspecified	3
Southeast Asia	Cambodia	7
Southeast Asia	Laos	16
Southeast Asia	Unspecified	8
Southeast Asia	Vietnam	13
World War II	Battle of Tarawa	21
World War II	Italy	3
World War II	Kiribati	7
World War II	Papua New Guinea	4
World War II	Philippines	2
World War II	Solomon Islands	72
World War II	USS Oklahoma	208
World War II	Yugoslavia	1

Table 7.2. A summary of the tooth samples tested in this study. As with Table 7.1, "Location" refers to a specific country or event within that conflict.

Conflict	Location	Number Tested
Korean War	Joint Recovery Operation (JRO)	3
Korean War	Kaljon-Ri	3
Korean War	Namjong-gu	5
Other	Alaska	3
World War II	Belgium	5
World War II	Cabanatuan	8
World War II	Japan	2
World War II	Kiribati	2
World War II	Northern Mariana Islands	2
World War II	Papua New Guinea	4

For the purposes of this study, the bone powder generated from the sanding of the remains (Figure 7.3) was collected by the analyst and placed in a 15 mL polypropylene Falcon™ conical polypropylene tube (Corning, Corning, NY). The amount of powder generated ranged from 0.01 g to 3.04 g. Samples were stored at -20°C until such time as they were needed. Samples were portioned into 1.7 mL polypropylene tubes (Costar, Corning) in preparation for solvent analysis and returned to storage (Figure 7.4)

Figure 7.3. An example of the powder generated by the cleaning of skeletal materials prior to DNA extraction. The sample in the above image is a parietal sample. Cranial samples are split in half and the diploë removed.

Figure 7.4. A selection of samples recovered from the exterior of the skeletal samples. Samples ranged from a light talc-like powder to a sticky, black substance. Most of those recovered from the USS *Oklahoma* fall into the latter category and carry with them the odor of fuel. However, all samples in this figure, with the exception of the circled sample, were recovered from that incident.

7.13.1.2 Teeth

Tooth samples were not selected during regular casework, but were from cases that had been designated as closed by the laboratory. Thirty-seven teeth were included for analysis (Table 7.2), none of which were from the Southeast Asia conflict.

Prior to DNA testing, the teeth are cleaned by placing in a sonicating water bath while submerged in 8.5% (v/v) bleach in a 50 mL Falcon™ conical polypropylene tube. After a 5 minute sonication, the samples were removed from the bleach solution and wiped clean with a 4 x 4 cm gauze pad moistened with 8.5% (v/v) bleach. A second wiping with a 4 x 4 cm gauze pad moistened with 100% (v/v) ethanol (Pharmco, Greenfield Global, Brookfield, CT) follows and then the tooth was allowed to dry under a UV light for 15 minutes. If a tooth presented with caries, other damage, or restorations, the sonication step was eliminated.

Once the tooth was dry, the crown was separated from the root of the tooth using a round bur attached to a high-performance brushless motor (Brasseler USA, Savannah, GA). During crown separation, a fine powder was generated. This external cut powder was collected by the analysts and not used for DNA extraction; however, it was stored on the chance that the internal drillings of the tooth were contaminated or produced questionable results. In those cases the external cut powder could be tested. It is this powder that was used for the GC/MS testing. The amount of tooth powder generated ranged from 0.01 g to 0.21 g.

Powder was placed in 15 mL conical polypropylene tubes and stored at -20°C until needed.

7.13.2 NAMING CONVENTION

Samples were anonymized with a code number to reduce the chance of sample bias in the analysis. Samples were numbered by the bag in which they were collected and then the random draw from that bag. For example, Bag 1 was the first set of collections done. Collections continued until the bag was full, and then that bag was sealed and the next bag initiated. Sample 1-1 was the first sample drawn from Bag 1. Tooth samples were collected from completed cases and are named with a "T" in front of the name (e.g., T1-1).

7.13.3 PREPARATION OF SAMPLES

Two different solvents, acetonitrile (HPLC grade, Sigma-Aldrich) and dichloromethane (HPLC grade; Pharmco AAPER, Brookfield, CT, USA), were chosen based on a previous proof of concept study (Edson and Roberts, submitted). Approximately 0.1 g of osseous powder was removed from the storage tube and placed in a 1.7 mL polypropylene tube (Costar, Corning, Corning, NY). The solvents were added sequentially, with acetonitrile being added first, and proceeding through the entire process of solvent extraction before the addition of dichloromethane.

Solvent addition proceeded in the same fashion for both of the solvents used. For each solvent, 1.0 mL of solvent was added to 0.1 g of osseous powder. The sample was then vortexed for approximately 30 seconds and allowed to incubate at room temperature for at least one hour. At completion of incubation, the sample was spun down for 2 minutes at 13,000 x rpm in order to pellet the osseous material.

After pelleting, the liquid was poured into a clean 10 mL glass beaker and allowed to volatilize in a chemical fume hood (Figure 7.4). Once the solvent has completely evaporated, the remaining material was suspended in 500 µL of methanol (≥99.9%, HPLC grade, Sigma-Aldrich, St. Louis, MO, USA), and placed in 1.7 mL polypropylene tubes for storage at -20°C until loading on the GC/MS instrument. The process was then repeated with dichloromethane.

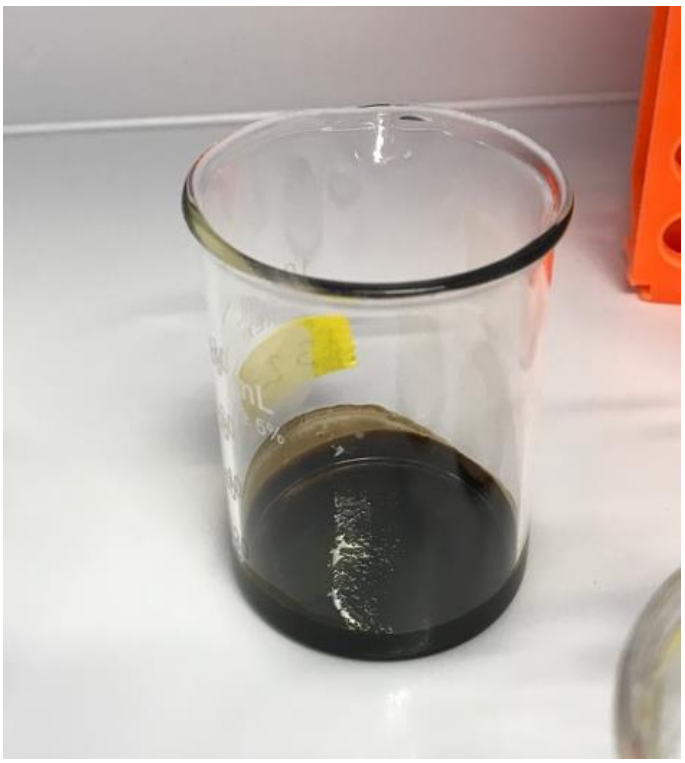

Figure 7.5. A sample from the USS *Oklahoma* during volatilization. Regardless of the solvent, samples from this incident tended to form a sticky, black substance upon being poured into the beaker for drying.

Prior to loading the samples were again spun at 13,000 x rpm. Pelleting of any flocculants or floating materials is critical at this stage. Introduction of solid materials into the injection port of the GC/MS instrument may cause damage to the instrument or destruction of the column. After pelleting, the liquid fraction was removed to a 9 mm glass vials (Thermo Fisher, Walther, MA, USA) with crimp caps.

7.13.4 GC/MS LOADING

Samples were loaded onto an Agilent 7890A/5875C GC/MS System with a 20 m column (Agilent, Santa Clara, CA). The starting oven temperature was 150°C, and remained at that temperature for a 20 minute hold before ramping at 20°C / min to 250°C for an additional 30 minute hold. The injection was splitless, meaning the entire fraction was injected into the run, which provided the best possible detection of low-level compounds. A full scan of the injection was performed with no subtraction of known elements.

7.13.5 ANALYSIS OF DATA

Automatic analysis was performed by the instrument software using ChemStation and MassHunter (Agilent), and a comparison of the NIST2005/2011 spectral libraries. Should the software not designate or correlate a compound with a peak, the analyst visually compared the spectra of the peak to that of the reference libraries. For each peak, the software assigned a score as to the probability that the material detected is the compound chosen. For example, during analysis of a peak, the analyst is presented with a choice of 20 – 30 different compounds that are most likely to be the identity of the compound detected. Samples are rated from best to worst, with scores between 100 and one, with 100 being the best. Some low scoring compounds were chosen during analysis of the samples, but were eliminated after further review.

7.14 SUPPLEMENTAL RESULTS

The full spectrum read of the samples generated some rather complex traces for the samples tested. During typical GC/MS testing, a filter can be used to focus on certain types or peaks or to eliminate others. Given that the components of the osseous materials were largely unknown, a specific choice was made to ascertain all of the materials possibly present in the remains. Therefore, a large number of compounds were detected during GC/MS testing.

The two solvents used, acetonitrile and dichloromethane, provided different chemical profiles for the samples tested. Samples recovered from the USS *Oklahoma* typically contained more compounds than the other incidents tested (Table 7.3). Samples from other locations contained fewer overall, with samples from the Korean War containing the least (Table 7.4). Samples from this conflict were either recovered *in situ* or had been curated in unknown conditions by the DPRK (Democratic People's Republic of Korea, aka North Korea). Samples recovered from the Southeast Asia region (Table 7.5) and other World War II era incidents (Table 7.6) were largely equivalent in the number of compounds detected.

The origin of each of the materials could not be accurately determined. Some materials, such as siloxanes, could have originated from the instrument column, the sanding bits used in sample preparation, or the environment from which the remains were recovered.

Due to the overall size of the tables they are listed after the References for this section.

7.15 SUPPLEMENTAL DISCUSSION

7.15.1 DETECTION OF MATERIALS

At the initiation of this study, the researchers anticipated that it would be a fairly simple study. While there would be some compounds detected, there would be just a few and the GC/MS traces would be fairly simple to analyze. Fats, esters, and some environmental materials such as humic or tannic acid or those from adjacent structures were expected. These expectations were founded on observations made during testing of several hundred osseous samples during casework and color changes that occurred during the addition of extraction buffer or phenol. Powdered osseous remains containing copper often present a turquoise color upon addition of demineralization buffer (0.5M EDTA, pH 8.0; 1% *N*-

Lauroylsarcosine) and those containing tetracycline (a water-soluble, broad-spectrum antibiotic) would turn a bright yellow.

A wide-range of compounds were detected in the remains tested. A number of materials, such as fats, esters, oils and alcohols, could be derived from the decomposition of the body itself (Dent, et al., 2004; Notter, et al., 2009). However, materials from the environment immediately surrounding the body were also detected.

This is most apparently obvious in the remains from the USS *Oklahoma*. Primary decomposition occurred within the hull of the overturned ship, which was filled with fuel oil and salt water. The GC/MS trace from the majority of the samples tested from this incident shows an aggregate of fats and oils. Elements recovered from other WWII incidents, Southeast Asia region, and Korea contained fewer compounds, on average than those from the USS *Oklahoma*. Yet the trend of recovering location specific materials continued. For example, sample 1-1, recovered from Cambodia in a loss dating to the 1970's, contained traces of 18-Norabietane and a napalm component and sample 6-23 lost during WWII in Yugoslavia contained DDT.

Determination of the point of origin of these materials is challenging. Medications could originate from the remains or from the surrounding environment, or even from the recovery team. For the purposes of this particular study, it was sufficient to be aware that the materials were present, so as to determine if they co-extracted with the DNA.

7.15.2 CLEANING PRECAUTIONS AND THE DETECTION OF MODERN MATERIALS

GC/MS is a highly sensitive platform for the detection of compounds. During preparation of the preparation of the samples for loading, precautions were taken to keep both the samples

and the preparation materials free of contamination. The beakers and watch glasses used for volatilization were cleaned using Alconox solid detergent (Alconox, White Plains, NY) and successive washes of deionized water and 95% ethanol. The glassware dried under UV irradiation in a chemical fume hood. The glassware was virgin at the time of initial use and was not allowed to be used by other laboratory members during the study.

The compounds recovered from skeletal materials that were largely fats or fuel oil tended to stick to the bottom of the glassware. Removal was difficult even with the use of Alconox. This is of note mainly as a recommendation to laboratories who are performing this type of testing. The glassware needs to be very clean, or there is potential for carry-over to the next sample prepared using that particular piece of glass.

All samples prepared for this study were prepared using the cleanliness guidelines for ancient DNA testing laboratories, in order to reduce the possibility of modern contamination. However, the conditions during recovery or anthropological examination could not be controlled. This was evidenced by the detection of a broad spectrum sunscreen in sample 2-2 during the proof of concept study (Edson and Roberts, *in preparation*).

Caffeine was detected in sample 1-2 recovered from a site in Vietnam. This was originally discounted as having come from ground-water contamination (Seiler, et al., 1999; Knee, et al., 2010). However, local residents had been observed to use skeletal fragments along with small stones to aid in the heating of water or tea (L. Freas, pers comm). Even though the compound may be modern in origin, it is still relevant to the identification of the material. MtDNA testing of this particular sample indicated that the fragment was not from a missing soldier, but rather that of a local resident.

7.15.3 ANIMAL REMAINS

Animal remains are frequently recovered along with human remains during excavations. Oftentimes, the skeletal materials are so fragmented that they cannot visually be determined to be human or non-human in origin; therefore, the samples are sent for DNA testing along with other skeletal materials. Since 2010, 256 samples received for testing have been tested using a 12S rRNA strategy and shown to be non-human in origin (Edson, et al., *in press*).

One of those samples, sample 3-22, recovered from a site in Cambodia, was included in the GC/MS testing. The sample appeared to be no different than other remains recovered from the same region. Although additional studies can be done to confirm this, it is unlikely that GC/MS could be used as a screening tool to eliminate non-human samples.

7.15.4 THE MECHANICS OF TESTING

In order to perform this type of testing, the laboratory must be willing to destroy at least a small portion of the recovered remains. The testing strategy in this case tested a small portion (~0.1 g) of the osseous materials removed from the remains during cleaning. This detritus would typically have been discarded, thus none of the parent sample was damaged for the test. Larger elements, or those containing a high degree of cancellous bone, would have more waste material to be tested, and might provide more reliable results, although that has yet to be verified.

Different solvents may also be evaluated. For the purposes of this study, two different solvents were chosen as they appeared to provide the greatest amount of data. This is not intrinsically necessary. If the purpose is simply to evaluate the presence of possible inhibitory materials in an osseous sample, testing by a single solvent would be sufficient. During the proof of concept study (Edson and Roberts, *in preparation*), methanol alone was found to

generate very limited data. A stronger solvent, such as acetonitrile or dichloromethane, would provide a better data profile.

There are other forms of sample ionization that can be used rather than dissolving compounds into solvents and injecting those onto the instrument for GC/MS analysis. DART (Direct Analysis in Real Time) was used by Moreno and McCord (2016) to study inhibitors co-extracting with DNA from whole blood. DART allows for no preparation of the sample and a simple direct introduction of the materials into the ionization stream of the instrument. However, a DART based protocol was deemed to not be useful for this study, as the skeletal matrix is more dense and complex than blood, and might not correctly ionize. At the beginning of this project, it was hoped to be able to use LAESI (Laser Ablation with Electrospray Ionization). LAESI uses a laser to directly ionize a substrate with no preparation. However, a skeletal matrix may be unable to volatilized under such a laser and would be ineffective.

7.16 SUPPLEMENTAL CONCLUSIONS

Gas chromatography / mass spectrometry has been shown to be an effective tool for the evaluation of materials present in skeletonized human remains. Testing of remains prior to DNA extraction would provide a laboratory with a profile of the chemicals needing to be removed from the remains; thereby allowing the lab to make a decision on which DNA extraction pathway to take. For example, samples containing a high amount of fats would be better served by being purified with an organic purification method and samples with an elevated mineral content would be best extracted with an inorganic purification.

Regardless of the practical applications of GC/MS and skeletal materials, the number and diversity of compounds present within the elements was unexpected. Determination that

there may be compounds specific to a locality or individual was additional unforeseen result. Further work will be necessary to ensure that the results are repeatable and accurate.

7.17 SUPPLEMENTAL REFERENCES

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7.18 SUPPLEMENTAL TABLES

The tables associated with the Supplemental Results can be found on the following pages.

Table 7.3. Compounds recovered from skeletal elements sampled from the USS *Oklahoma.* Materials developed from acetonitrile eluates are marked in blue. This is a summary of compounds recovered; therefore, there is some duplication of compounds between the solvents, although this is minimal. In total, 512 compounds were detected. Compounds are listed alphabetically.

COMPOUND	FORMULA
.alpha.-Cedrene oxide	C15H24O
(-)-cis,3,4-Dimethyl-2-phenyltetrahydro-1,4-thiazine	C12H17NS
(+)-Longicamphenylone	C14H22O
(+)-Thujylidene-.beta.-alanine, methyl ester	C14H23NO2
(1R,2S,8As)-8-oxo-1-carboxymethyl-1,2,5,5-tetramethyl-trans-decalin	C16H26O3
(2,3-diphenylcyclopropyl)methyl phenyl sulfoxide, trans-	C22H20OS
(4-Methyl-6-phenylpyrimidin-2-yl)(4,6,8-trimethylquinazolin-2-yl)amine	C22H21N5
(t-Butyl-dimethylsilyl)[2-methyl-2-(4-methyl-pent-3-enyl)-cyclopropyl]-methanol	C17H34OSi
[1,2,4] triazolo[1,5-a]pyrimidin-5-ol, 7-methyl-6-nitro	C6H5N5O3
[1]Benzopyrano[4,3-b]indole, 6,11-dihyrdo	C15H11NO
[14]Annulene, 1,6:8,13-bis(methano)-, syn	C16H14
1-(2-hydroxyethyl-1,2,5,5-tetramethyl-cis-decalin(1R,2S,4as,8as)	C16H30O
1-(2-Hydroxyethyl)-1,2,5,5-tetramethyl-cis-decalin(1R,2S,4as,8as)	C16H30O
1-Benzazirene-1-carboyxlic acid, 2,2,5a-trimethyl-1a-[3-oxo-1-butenyl] perhydro-, methyl ester	C15H23NO3
1-Bromo-11-iodoundecane	C11H22BrI
1-Bromo-11-iodoundecane	C11H22BrI
1-Bromodocosane	C22H45Br
1-Decanol, 2-hexyl-	C16H34O
1-Decanol, 2-hexyl-	C18H38O
1-Docosanethiol	C22H46S
1-Dodecano, 3,7,11-trimethyl-	C15H32O
1-Dodecanol, 2-hexyl-	C18H38O
1-Dodecanol, 2-octyl-	C20H42O

1-Dodecanol, 3,7,11-trimethyl-	C12H24Br2
1-Formyl-2,2,6-trimethyl-3-(3-methyl-but-2-enyl)-6-cyclohexane	C15H24O
1-Heneicosanol	
1-Hentetracontanol	C41H84O
1-Heptanol, 2,4-diethyl	C11H24O
1-Hydroxy-4-(1',1',2'-trichloroallyl)benzene	C9H7Cl3O
1-Monolinoleoylglycerol trimethylsilyl ether	C27H54O4Si2
1-Nonene, 4,6,8-trimethyl-	C12H24
1-Octadecene	C18H36
1-Penanthrenecarboxylic acid, ,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-,methyl ester, [1R-1.alpha,4a.beta	C21H30O2
1-Phenanthrenecarboxylic acid, 1,2,3,4,4a,9,10,10a-octahydro-1, 4a-dimethyl-7-(1-methylethyl)-, methyl ester, [1R-(1.alpha.,4a.beta)	C21H30O2
1-propanol, 2,3[(3,7,11,15-tetramethylhexadecyl)oxy]-	C43H88O3
1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	C13H40O5Si6
1,1,3,3-Tetraallyl-1,1,3-disilacyclobutane	C14H24Si2
1,1'-Bicyclohexyl, 4-methoxy-4'-pentyl	C18H34O
1,1'-Biphenyl, 3,4-diethyl-	C16H18
1,2-Dithiolane-3-pentanoic acid	C8H14O2S2
1,3-Dioxolane, 4-ehthyl-5-octyl-2,2-bis(trifluoromethyl)-, cis-	C15H24F6O2
1,3-Dioxolane, 4-ehthyl-5-octyl-2,2-bis(trifluoromethyl)-, trans-	C15H24F6O2
1,3-Pentadiene, 1,1-diphenyl-, (Z)-	C17H16
1,4-Methanoazulen-3-ol, decahydro-1,5,5,8a-tetramethyl-,[1S-(1.alpha.,3.beta.,3a.beta.,4.alpha.,8a.beta.)]-	C15H26O
1,6,10,14,18,22-Tetracosahexaen-3-ol, 2,6,10,15,19,23-hexamethyl-, (all-E)-	C30H50O
1,7-Dimethyl-4-(1-methylethyl)cyclodecane	C15H30
1,7-Dimethyl-4-(1-methylethyl)cyclodecane	C15H30
1(2H)-Naphthalenone, 6-acetyloctahydro-8a-methyl-, (4a.alpha.,7.beta.,8a.beta.)-	C13H20O2
1(2H)-Pentalenone, hexahydro-4-(phenylmethylene)-	C15H16O
10-Methylanthracene-9-carboxaldehyde	C16H12O
10-Methylanthracene-9-carboxaldehyde	C16H12O

11-Eicosenoic acid, methyl ester	C21H40O2
11-Octadecenoic acid, methyl ester	C19H36O2
11,13-dimethyl-12-tetradecen-1-ol acetate	C18H34O2
12-Tricosanone	C23H46O
13-Tetradecen-1-ol acetate	C16H30O2
17-Pentatriacontene	C35H70
1H-2,6-Methano-2,3-benzodiazocine, 3,4,5,6-tetrahydro-8-methoxy-3,6,11-trimethyl-	C15H22N2O
1H-Inden-1-one,2-(2,3-dihydro-1H-inden-1-ylidene)-2,3-dihydro-	C18H14O
1H-Indene, 2-butyl-5-hexyloctahydro-	C19H36
1H-Indene, 2-butyl-5-hexyloctahydro-	C19H36
1H-Indene, 5-butyl-6-hexyloctahydro-	C19H36
1H-Indene,2,3-dihydro-2-methoxy-1-phenyl-, cis-	C16H16O
1H-Indole, 1-methyl-2-phenyl-	C15H13N
1H-Indole, 2-methyl-3-phenyl	C15H13N
1H-Purine-8-propanoica acid, .alpha.-amino-2,3,6,7-tetrahydro-1,3,7-trimethyl-2,6-dioxo-	C11H15N5O4
1H-Pyrazole-4-carbaldehyde, 3-(4-methoxyphenyl)-	C11H10N2O2
2-(3,5-Dichloro-4-methoxymethylphenyl)propan-2-ol	C11H14Cl2O2
2-(4a,8-Dimethyl-6-oxo-1,2,3,4,4a,5,6,8a-octahydro-naphthalen-2-yl)-propionaldehyde	C15H22O2
2-(Acetoxymethyl)-3-(methoxycarbonyl)biphenylene	C17H14O4
2-Chloro-5,10-dihydro-5,10-ethanophenazine	C24H22O2
2-Chloropropionic acid, octadecyl ester	C21H41ClO2
2-Dedecen-1-yl(-)succinic anhydride	C16H26O3
2-Dodecen-1-yl (-) succinic anhydride	C16H26O3
2-Ethylacridine	C15H13N
2-Heptadecanone	
2-Heptadecanone	C17H34O
2-Isopropenyl-4,4,7a-trimethyl-2,4,5,6,7,7a-hexahydro-benzofuran-6-ol	C14H22O2
2-Isopropyl-5-methyl-1-heptanol	C11H24O

2-Methoxy-2'-methyl-stilbene	C16H16O
2-Methyl-3-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-oxetane	C15H26O
2-Methyl-cis-7,8-epoxynonadecane	C20H40O
2-Methyl-E-7-hexadecene	C17H34
2-Methyl-Z-4-tetradecene	C15H30
2-Methyl-Z-4-tetradecene	C15H30
2-methylhexacosane	C27H56
2-methyloctacosane	C29H60
2-methyltetracosane	C25H52
2-Methylthio-4-oxo-4H-quinolizine-3-carboxamide	C11H10N2O2S
2-Myristynoyl-glycinamide	C16H28N2O2
2-Myristynoyl-glycinaminde	C16H28N2O2
2-Oxazoline, 4,4-dimethyl-2-(1-hydroxy-heptadec-8-enyl)-	C22H41NO2
2-Phenyl-2,3-dihydrobenzo[b]thiophene	C14H12S
2-Piperidnone, N-(4-bromo-n-butyl)-	C9H16BrNO
2-Undecanone, 6,10-dimethyl-	C13H26O
2-Undecene, 4,5-dimethyl-, [R*,S*-(Z)]-	C13H26
2,1,3-Benzoselenadiazole-5-carboxylic acid	C7H4N2O2Se
2,2,6-Trimethyl-1-(2-methyl-cyclobut-2-enyl)-hepta-4,6-dien-3-one	C15H22O
2,3-Pentadienoic acid, 2-ethyl-4-phenyl-, ethyl ester	C15H18O2
2,4-cyclohexadien-1-one, 3,5-bis(1,1-dimethyl)-4-hydroxy-	C14H22O2
2,4-Dimethyl-5,8-dimethoxy-6-aminoquinoline	C13H16N2O2
2,4'-dihydroxy-stilbene	C14H12O2
2,5-di-tert-Butylnitrobenzene	C13H21NO2
2,5-di-tert-Butylnitrobenzene	C14H21NO2
2,5-Furandione, 3-dodecyl-	C16H26O3
2,6,10,11,14-Tetramethyl-7-(3-methylpent-4-enylidene) pentadecane	C25H48
2,6,10,14,18-Pentamethyl-2,6,10,14,18-eicosapentaene	C25H42
2,6,10,14,18-Pentamethyl-2,6,10,14,18-eicosapentaene	C25H42
2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)-	C30H50

2,8-Dimetyldibenzo(b,d)thiophene	C14H12S
2'-Acetonaphthone, 1'2'.alpha.,3',4',4'a,5',6',7',8',8'a.alpha.-decahydro-5'.beta.-hydroxy-4'a.beta.8'.beta.-dimethyl-,(.+-.)-	C14H24O2
2(1H)-Naphthalenone, 4a,5,6,7,8,8a-hexahydro-6-[1-(hydroxymethyl)ethenyl]-4,8a-dimethyl-, [4ar-(4a.alpha.,6.alpha.,8a.beta.)]-	C15H22O2
2(1H)-Naphthaleone, 4a,5,6,7,8,8a-hexahydro-6-[1-(hydroxymethyl)ethenyl]4,8a-dimethyl-,[4ar-(4a.alpha.,6.alpha.,8a.beta.)]-	c15H22O2
2(1H)-Napththalenone, octahydro-4a-methyl-7-(1-methylethyl)-, (4a.alpha, 7.beta, 8a.beta)	C14H24O
26-Nor-5-cholesten-3.beta.-ol-25-one	C26H42O2
2H-3,9a-Methano-1-benzoxepin, octahydro-2,2,5a,9-tetramethyl-,[3R-(3.alpha.,5a.alpha.,9.alpha.,9a.alpha.)]-	C15H26O
2H-Pyran-2-one, tetrahydro-6-tridecyl-	C18H34O2
3-[p-Methoxyphenyl]-5-methylrhodanine	C11H11NO2S2
3-Bromo-5-ethoxy-4-hydroxybenzaldehyde	C9H9BrO3
3-buten-2-one, 4-(5,5-dimethyl-1-oxaspiro[2,5]oct-4-yl	C13H20O2
3-Buten-2-one, 4-mesityl-, semicarbazone	C14H19N3O
3-Eicosene, (E)-	C20H40
3-Heptadecene, (Z)-	C17H34
3-Heptyl-1,1,1-triphenyl-decan-2-one	C35H46O
3-Hexadecanol	C16H34O
3-Methyl-4-(phenylthio)-2-prop-2-enyl-2,5-dihydrothiophene 1,1-dioxide	C14H16O2S2
3-Octadecene, (E)-	C18H36
3-Trifluoroacetoxy-6-ethyldecane	C8H13F3O2
3,5-Octadiene, 4,5-diethyl-3,6-dimethyl-	C14H26
3(4H)-Phenanthrenone, 4a,4b,5,6,7,8,8a,9,10,10a-decahydro-4b,8,8-trimethyl-, [4aS-(4a.alpha.,4b.beta.,8a.alpha.,10a.beta.)]-	C17H26O
4-(3-Chlorophenylamino)cytosine	C10H8ClN3O
4-Acetylphenanthrene	C16H12O
4-Amino-7-diethylamino-chromen-2-one	C13H16NO2
4-Chloro-2-(.alpha.-methylbenzyl)phenol	C14H13ClO
4-Chloro-2,6,8-trimethyl-quinolin-5-ylamine	C12H13ClN2
4-Dehydroxy-N-(4,5-methylenedioxy-2-nitrobenzylidene)tyramine	C12H17NO2

4-Fluoro-3-tribluoromethylbenzoic acid, 6-tridecyl ester	C21H30F4O2
4-Hexen-2-one, 3,3-diethyl-4,5-dimethyl-	C12H22O
4-n-Hexylthiane, S,S-dioxide	C11H22O2S
4-Nitro-1H-pyrazole-3-carboxylic acid (2,4-difluoro-phenyl)-amide	C10H6F2H4O3
4-Trifluoromethylcinnamic acid	C10H7F3O2
4,5-Dimethyl-3,6-dihydro-N-(7-chloro-4-quinolinyl)-1,2-oxazine	C15H15ClN2O
4,8-Dimethylbicyclo[3.3.1]nonane-2,6-dione	C11H16O2
4'-Bromobenzo[1',2'-b]-1,4-diazabicyclo[2.2.2]octene	C10H11BrN2
4'-Methoxy-2-hydroxystilbene	C15H14O2
4a,7-Metahno-4aH-naphth[1,8a-b]oxirene, octahydro-4,4,8,8-tetramethyl	C15H24O
4H-Pyran-3-carboxylic acid, 2-amino-5-cyano-6-ethyl-4-(3-pyridinyl)-, methyl ester	C15H15N3O3
4H-Thiazolo[5,4-b]indole, 2,5,7-trimethyl-	C12H12N2S
5-(2-Propenylidene)-10,11-dihydro-5H-debenzo[a,d]cycloheptene	C18H16
5-(3,3-Dimethyl-5-methylthio-3,4-dihydropyrrol-2-ylidenemethyl)-1,4,4,5-tetramethylpyrrolidine-2-thione	C16H26N2S2
5-(Prop-2-enylidene)-10-oxa-10,11-dihydro-5H-dibenzo[a,d]cycloheptane	C17H14O
5,6-Dihydro-2-(p-toluenesulfonamido)-4H-benzo(3,4)cyclohepta(2,1-d)thiazole	C19H18N2O2S2
5.beta.,7.beta.H,10.alpha.-Eudesm-11-en-1.alpha.-ol	C15H26O
5H-Dibenzo[a,d]cyclohepten-5-ol, 10,11-dihydro	C15H14O
6-bromohexanoic acid, tetradecyl ester	C20H39BrO2
6-Chloromethyl-2-dimethylamino-6,7-dihydro-4H-oxazolo[3,2-a]-1,3,5-triazin-4-one	C8H11ClN4O2
6-Hydroxy-7-meethyl-oct-3-enedithioic acid, isopropyl ester	C12H22OS2
6-Hydroxy-7,7-dimethyl-oct-3-enedithioic acid, isopropyl ester	C13H24OS2
6-Isopropenyl-4,8a-dimethyl-4a,5,6,7,8,8a-hexahydro-1H-naphthalen-2-one	C15H22O
6-Isopropenyl-4,8a-dimethyl-4a,5,6,7,8,8a-hexahydro-1H-naphthalen-2-one	C15H22O
6-Ocetenal, 3,7-dimethyl-	C10H18O
6-Octadecenoic acid	C18H34O2
6-Octadecenoic acid, (Z)-	C18H34O2
6-Octadecenoic acid, (Z)-	C18H34O2

6-Octadecenoic acid, methyl ester, (Z)-	C19H36O2
6-Octenal, 3,7-dimethyl-	C10H18O
6,6-Diphenylfulvene	C18H14
7-Hexadecenal, (Z)-	C19H30O
7-Oxabicyclo[4.1.0]heptane, 1,5-dimethyl	C8H14O
7-Pentadecyne	C15H28
7,8-Diphenylbicyclo[4.2.1]nona-2,4,7-triene	C21H18
8-Dodecen-1-ol, acetate, (Z)-	C14H26O2
8-Octadecenoic acid, methyl ester	C19H36O2
8H-Pyrano[2,3-e]benzothiophen-8-one, 4-formamido-6-methyl-	C13H9NO4
9-Cedranone	C15H24O
9-Cedranone	C15H24O
9-Hexadecenoic acid, methy ester, (Z)-	C17H32O2
9-Octadecanoic acid (Z)-, methyl ester	C19H36O2
9-Octadecanoic acid, methyl ester	C19H36O2
9-Octadecanoic acid, methyl ester, (E)-	C19H36O2
9-Octadecemide, (Z)-	C18H35NO
9-Octadecenamide, (Z)-	C18H35NO
9-Octadecenoic acid (Z)-, methyl ester	C19H36O2
9-Octadecenoic acid, (E)-	C19H34O2
9-Octadecenoic acid, methyl ester, (E)-	C19H36O2
9-Octadecenoic acid, methyl ester, (E)-	C19H36O2
9-Undecen-2-one, 6,10-dimethyl-	C13H24O
9-Undecen-2-one, 6,10-dimethyl-	C13H24O
9-Undecenal, 2,6,10-trimethyl	C14H26O
9-Undecenoic aci, 2,6,10-trimethyl-	C14H26O2
9-Undecenoic acid, 2,6,10-trimethyl-	C14H26O2
9,10-Dimethylanthracene	C16H14
9,12-Octadecadienoic acid (Z,Z)-, methyl ester	C19H34O2
9,9-Dimethoxybicyclo[3.3.1]nona-2,4-dione	C11H16O4

9H-Fluorene, 2-ethyl-	C15H14
Acetic acid, 3,7,11,15-tetramethyl-hexadecyl ester	C22H44O2
Aciphyllyl alchohol	C15H24O
Androst-5,16-diene-3.beta.-ol	C19H28O
Androst-7-ene, (5.alpha.)-	C19H30
Androstan-3-one, 17-hydroxy-, (5.alpha.,17.beta.)-	C19H30O2
Androstane, (5.alpha.)-	C19H32
Anthracene, 1-methyl-	C15H12
Anthracene, 9-dodecyltetradecahydro-	C26H48
Anthracene, 9-methyl-	C15H12
Anthracene, 9-nitro-	C15H9NO2
Antrhacene, tetradecahydro-	C13H24
Benzenamine, 3-chloro-N-(2-pyridinylmethylene)-	C12H9ClN2
Benzenamine, 4-bromo-N-(phenylmethylene)-	C13H10BrN
Benzene, 1-[(4-ethoxyphenyl)ethnyl]-4-propyl-	C19H20O
Benzene, 1,1'-(2-cyclopropen-1-yldene)bis-	C15H12
Benzene, 1,2,3,5-tetrachloro-4-methoxy	C7H4Cl4O
Benzene, 1,2,3,5-tetrachloro-4-methoxy-	C7H4Cl4O
Benzene, ethenylpentaethyl-	C18H28
Benzene, hexaethyl-	C18H30
Benzenmaine, 2-chloro-N-(3-pyridinylmethylene)-	C12H9ClN2
Benzo[a]pyrene	C20H12
Benzo[b]selenophene-3-carboxylic acid, 2-formyl-	C10H6O3Se
Bicyclo(3.1.1)heptan-3-one, 6,6-dimethyl-2-(2-methylpropyl)-	C13H22O
Bicyclo[3.2.0]heptan-3-one, 2-hydroxy-1,4,4-trimethyl-, O-acetyloxime	C12H19NO3
Bismuthine, trimethyl-	C3H9Bi
Borane, 2,3-dimethyl-2-butyl- (dimer)	C12H30B2
Bromoacetic acid, octadecyl ester	C20H39BrO2
Butanediamide, 2-methylene-	C5H8N2O2
Butylphosphonic acid, hexyl 2-phenylethyl ester	C18H31O3P

Carbamic acid, N-phenyl-, 1,5-dimethyl-1-vinyl-4-hexenyl ester	C17H23NO2
Carbonic acid, hexadecyl 2,2,2-trichloroethyl ester	C19H35Cl3O3
Cholest-22-ene, (5.alpha.)-	C27H46
Cholest-5-en-3-ol (3.beta.)-	C27H46O
Cholest-5-en-3-ol (3.beta.)-	C27H46O
Cholesta-3,5-dien-7-one	C27H42O
Cholestan-3-ol, (3.alpha.,5.beta.)-	C27H48O
Cholestan-3.alpha.-ol acetate	C29H50O2
cis-11-Eicosenoic acid, methyl ester	C21H40O2
cis-13-Octadecenoic acid, methyl ester	C19H36O2
cis-9-Hexadecenal	C16H30O
cis-Vacceric Acid	C18H34O2
Citronellol epoxide (R or S)	C10H20O2
Corymbolone	C15H24O2
Cyclodeca[b]furan-2(3H)-one, 3a,4,5,6,7,8,9,11a-octahydro-3,6,10-trimethyl-	C15H24O2
Cycloheanone, 2,6-bis(2-methylpropylidene)-	C14H22O
Cyclohesasiloxane, dodecamethyl-	C12H36O6Si6
Cyclohexadecane	C16H32
cyclohexadecane	C16H32
Cyclohexane, (1,2-dimethylbutyl)-	C12H24
Cyclohexane, (1,2-dimethylpropyl)-	C11H22
Cyclohexane, 1-(1,5-dimethylhexyl)-4-(4-methylpentyl)-	C20H40
Cyclohexane, 1-(cyclohexylmethyl)-2-ethyl-, trans-	C15H28
Cyclohexane, 1-(cyclohexylmethyl)-2-ethyl-, trans-	C15H28
Cyclohexane, 1-ehtyl-2-propyl-	C11H22
Cyclohexane, 1,1',1''-(1-propanyl-2-ylidene)tris-	C21H38
Cyclohexane, 1,2,4-trimethyl	C9H18
Cyclohexane, 1,2,4,5-tetraethyl-	C14H28
Cyclohexane, 1,2,4,5-tetraethyl-, (1.alpha, 2.alpha.,4.alpha.,5.alpha.)-	C14H28
Cyclohexanebutanoic acid, 2,2-dimethyl-6-methylene-, methyl ester	C14H24O2

Cyclohexanecarboxylic acid, decyl ester	C17H32O2
Cyclohexasiloxane, dodecamethyl-	C6H18O3Si3
Cyclohexasiloxane, dodecamethyl-	C12H36O6Si6
Cyclohexene, 4-(4-ethylcyclohexyl)-1-pentyl-	C19H34
Cyclononasiloxane, octadecamethyl-	C18H54O9Si9
Cyclopentadecanone, 3-methyl-	C16H30O
Cyclopentadecanone, 3-methyl-	C16H30O
Cyclopentane, (4-octyldodecyl)-	C25H50
Cyclopentane, (4-octyldodecyl)-	C25H50
Cyclopentane, 1-pentyl-2-propyl-	C13H26
Cyclopentane, 1,1'-[3-(2-cyclopentylethythyl)-1,5-pentanediyl]bis-	C22H40
Cyclopentane, 1,2-dipropyl	C11H22
Cyclopentane, propyl-	C8H16
Cyclopentanecarboxamide, N-(4-fluorophenyl)-	C12H14FNO
Cyclopentanecarboxylic acid, 2-phenylethyl ester	C14H18O2
Cyclopentaneethanol, beta.,2,3-trimethyl	C10H20O
Cyclopentanetridecanoic acid	C19H36O2
Cyclopentanetridecanoic acid, methyl ester	C19H36O2
Cyclopropanecarboxylic acid, 2-[4-(1,1-dimethylethyl)phenyl]-, ethyl ester	C16H22O2
Cyclotetradecan, 1,7,11-trimehtyl-4-(1-methylethyl)-	C20H40
Cyclotetradecane, 1,7,11-trimethyl-4-(1-methylethyl)-	C20H40
Cyclotetradecane, 1,7,11-trimethyl-4-(1-methylethyl)-	C20H40
D-Homoandrostane, (5.alpha.,13.alpha.)-	C20H34
d-Norandrostane (5.alpha.,14.alpha.)	C18H30
D,D-Dihomoandrostane, (5.alpha.)-	C21H36
Decahydro-8a-ehtyl-1,1,4a,6-tetramethylnaphthalene	C16H30
Decane, 1,1'-oxybis-	C20H42O
Decane, 1,10-dibromo-	C10H20Br2
Decane, 2,3,8-trimethyl-	C13H28
Di-n-decylsulfone	C20H42O2S

Dibenzo[c,e]thiepin, 5,7-dihydro-	C14H12S
Disulfide, di-tert-dodecyl	C24H50OS2
Ditetradecyl ether	C28H58O
DL-Xylitol, cyclic 1,4:2,3-bis(ethylboronate)	C9H18B2O5
Docosanoic acid, methyl ester	C23H46O2
Dodecahydropyrido(1,2-b)isoquinolin-6-one	C13H21NO
Dodecahydropyrido[1,2-b]isoquinolin-6-one	C13H21NO
Dodecane	C12H26
Dodecane, 1,12-dibromo-	C12H24Br2
Dodecane, 2,6,10-trimethyl	C15H32
Dodecanoic acid	C12H24O2
Dotraicontyl pentafluoropropionate	C35H65F5O2
Dotriacontane	C32H66
Dotriacontyl heptafluorobutyrate	C36H65F7O2
Dotriacontyl heptafluorobutyrate	C36H65F7O2
Dotriacontyl trifluoroacetate	C34H65F3O2
E-11-Methyl-12-tetradecen-1-ol acetate	C17H32O2
E-11-Methyl-12-tetradecen-1-ol acetate	C17H32O2
E-6-Tetradecen-1-ol acetate	C16H30O2
E-8-Methyl-7-dedecen-1-ol acetate	C17H32O2
E-8-Methyl-9-tetradecen-1-ol acetate	C17H32O2
E-8-Methyl-9-tetradecen-1-ol acetate	C17H32O2
E,Z-2,15-Octadecadien-1-ol acetate	C20H36O2
Eicosane	C20H42
Eicosane	C20H42
Eicosanoic acid, methyl ester	C21H42O2
Estra-1,3,5(10)-trien-17.beta.-ol	C18H24O
Ethanol, 2-(dodecyloxy)-	C14C30O2
Ethanol, 2-(dodecyloxy)-	C14H30O2
Ethanol, 2-(hexadecyloxy)-	C18H38O2

Ethanone, 1-(1,2,3,4,7,7a-hexahydro-1,4,4,5-tetramethyl-1,3a-ethano-3aH-inden-6-yl)-	C17H26O
Ethanone, 1-(2-phenyl-1H-indol-3-yl)-	C16H13NO
Ethanone, 1-[4-(4-nitrophenyl)methylene]-	C15H12N2O3
Ethyl Acetate	C4H8O2
Fumaric acid, hexadecyl octyl ester	C28H52O4
Heneicosane	C21H44
Heneicosane	C21H44
Heneicosane, 11-cyclopentyl-	C26H52
Heneicosanoic acid, methyl ester	C22H44O2
Hentriacontane	C31H64
Heptacosane	C27H56
Heptacosane, 1-chloro-	C27H55Cl
heptacosyl pentafluoropropionate	C30H55F5O2
heptadecane	C17H36
Heptadecane, 1-bromo-	C17H35Br
Heptadecanoic acid	C17H34O2
Heptadecanoic acid, 10-methyl-, methyl ester	C19H38O2
Heptadecanoic acid, 10-methyl-, methyl ester	C19H38O2
Heptadecanoic acid, 14-methyl-, methyl ester	C19H38O2
Heptadecanoic acid, 15-methyl-, methyl ester	C19H38O2
Heptadecanoic acid, 16-methyl-, methyl ester	C19H38O2
Heptadecanoic acid, 16-methyl-, methyl ester	C19H38O2
Heptadecanoic acid, methyl ester	C18H36O2
Heptadecyl heptafluorobutyrate	C21H35F7O2
Heptafluorobutanoic acid, heptadecyl ester	C21H35F7O2
Heptafluorobutyric acid, pentadecyl ester	C19H31F7O2
Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	C14H44O6Si7
Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	C14H44O6Si7
Hexadecane	C16H34
Hexadecane	C16H34

Hexadecanoic acid, 10-hydroxy-, methyl ester	C17H34O3
Hexadecanoic acid, 10-hydroxy-, methyl ester	C17H34O3
Hexadecanoic acid, 14-methyl-, methyl ester	C18H36O2
Hexadecanoic acid, methyl ester	C17H34O2
Hexadecanoic acid, methyl ester	
Hexadecanoic acid, methyl ester	C17H34O2
Hexane, 3,3-dimethyl-	C8H18
Hexatriacontane	C36H74
Indeno[2,1-a]indene, 4b,5,9b,10-tetrahydro	C16H14
l-(+)-Ascorbic acid 2,6-dihexadecanoate	C38H68O8
Mannosamine	C6H13NO5
Mercury, dimethyl-	C2H6Hg
Methane, methoyxbis(2-vinylphenyl)-	C18H18O
Methanone, (2-amino-5-chlorophenyl)phenyl-, oxime	C13H11ClN2O
Methyl 10-methyl-hexadecanoate	C18H36O2
Methyl 10-trans,12-cis-octadecadienoate	C19H34O2
Methyl 2-hydroxy-eicosanoate	C21H42O3
Methyl hexadec-9-enoate	C17H32O2
methyl hexadec-9-enoate	C17H32O2
Methyl stearate	C19H38O2
Methyl Sterate	C19H38O2
Methyl tetradecanoate	C15H30O2
Methyl Z-11-tetradecenoate	C15H28O2
Molybdenum, (acetato-O,O')tris(.eta.3-2-propehyl)-	C11H18MoO2
Muscone	C16H30O
n_Nonenylsuccinic anhydride	C13H20O3
N-(3-Chloro-4-fluorophenyl)-5-methyl-4,5-dihydro-1,3-thiazol-2-amine	C10H10ClFN2S
N-Decanoylmorpholine	C14H27NO2
n-Hexadecanoic acid	C16H32O2
N-Methyladrenaline, tri-TMS	C19H39NO3Si3

n-Nonenylsuccinic anhydride	C13H20O3
Naphthalene, 1,2-dihydro-4-phenyl	C16H14
Naphthalene, 1,2,3,4-tetramethyl-	C14H16
Naphthalene, 1,4-bis(methylthio)-	C12H12S2
Naphthalene, 1,6,7-trimethyl-	C13H14
Naphthalene, 4-chloro-1,5-dinitro-	C10H5ClN2O4
Naphthalene, decahydro-1,4a-dimethyl-7-(1-methylethyl)-, [1S-(1.alpha.,4a.alpha.,7.alpha.,8a.beta.)]-	C15H28
Naphthalene, decahydro-1,4a-dimethyl-7-(1-methylethyl)-, [1S-(1.alpha.,4a.alpha.,7.alpha.,8a.beta.)]-	C15H28
Naphtho[1,2-b]norbornadiene	C15H12
Nonadecane	C19H40
Nonadecane, 2,3-dimethyl-	C21H44
Nonahexacontanoic acid	C69H138O2
Nonahexacontanoic acid	C69H138O2
Octacosane	C25H58
Octacosyl trifluoroacetate	C30H57F3O2
Octadec-9-enoic acid	C18H34O2
Octadecane, 1-(ethenyloxy)-	C20H40O
Octadecane, 1-bromo-	C18H37Br
Octadecane, 1-chloro	C18H37Cl
Octadecane, 1,1'-[1,3-propanediylbis(oxy)]bis-	C39H80O2
Octadecanenitrile	C18H35N
Octadecanoic acid	C18H36O2
Octadecanoic acid, 10-methyl-, methyl ester	C20H40O2
Octadecanoic acid, 10-oxo-, methyl ester	C19H36O3
Octadecanoic acid, 10-oxo-, methyl ester	C19H36O3
Octadecanoic acid, 11-methyl-, methyl ester	C20H40O2
Octadecanoic acid, methyl ester	C19H38O2
Octadecanoic acid, methyl ester	C19H38O2
Octanoic acid, morpholide	C12H23NO2

Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	C16H50O7Si8
Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	C16H50O7Si8
Octatriacontyl pentafluoropropionate	C41H77F5O2
Oleic acid	C18H34O2
Oleic acid	C19H34O2
Oleic diethanolamide	C22H43NO3
Oxalic Acid, 6-ethyloct-3-yl ehtyl ester	C14H26O4
Oxalic acid, 6-ethyloct-3-yl ethyl ester	C14H26O4
oxalic acid, 6-ethyloct-3-yl heptyl ester	C19H36O4
Oxalic acid, allyl hexadecyl ester	C20H36O4
Oxalic Acid, allyl octadecyl ester	C23H42O4
Oxalic Acid, allyl tricecyl ester	C18H32O4
Oxalic acid, allyl tridecyl ester	C18H32O4
Oxalic acid, cyclobuyl heptadecyl ester	C23H42O4
Oxirane, [(dodecyloxy)methyl]-	C15H30O2
Oxirane, [(hexadecyloxy)methyl]-	C19H38O2
Oxirane, [(hexadecyloxy)methyl]-	C19H38O2
Oxirane, 2-decyl-3-(5-methylhexyl)-, cis-(.+/-.)	C19H38O
Oxirane, tetradecyl-	C16H32O
Oxirane,[(hexadecyloxy)methyl]-	C19H38O2
p-Menth-1-en-3-one, semicarbazone	C11H19N3O
Palmitic acid vinyl ester	C18H34O2
Penanthrene, 2,3,5-trimethyl-	C17H16
Penanthrene, 2,5-dimethyl-	C16H14
Penanthrene, 3,6-dimethyl-	C16H14
Pentadecane	C15H32
Pentadecane	C15H32
Pentadecane, 2,6,10-trimethyl-	C18H38
Pentadecanoic acid	C15H30O2
Pentadecanoic acid, 14-methyl-, methyl ester	C17H34O2

Pentadecanoic acid, 14-methyl-, methyl ester	C17H34O2
Pentadecanoic acid, methyl ester	C16H32O2
Pentafluoropropionic acid, octadecyl ester	C21H37F5O2
Perhydro-hlx-2-one, 2-depentyl-, acetate ester	C16H27NO3
Phenanthracene, 3.6-dimethyl-	C16H14
Phenanthrene	C14H10
Phenanthrene	C14H10
Phenanthrene, 1-methyl-	C15H12
Phenanthrene, 2-methyl	C15H12
Phenanthrene, 2,3,5-trimethyl-	C17H16
Phenanthrene, 2,5-dimehtyl-	C16H14
Phenanthrene, 2,5-dimethyl-	C16H14
Phenanthrene, 3,6-dimethyl-	C16H14
Phenanthrene, 3.6-dimethyl-	C16H14
Phenanthrene, 4-methyl-	C15H12
Phenanthrene, 4-methyl-	C15H12
Phenanthridine 6-chloro-2-methyl-7,8,9,10-tetrahydro-	C14H14ClN
Phenmethylcynid, .alpha.,.alpha.-dimethyl-2-methoxy-6-nitro	C11H12N2O3
Phenol, 2,4-bis(1,1-dimethylethyl)-	C14H22O
Phenol, 2,5-bis(1,1-dimethylethyl)-	C14H22O
Phenol, 2,6-bis(1,1-dimethylethyl)-	C14H22O
Phenol, 3,5-bis(1,1-dimethylethyl)-	C14H22O
Phenol, 4-4'-(1-methylethylidene)bis-	C15H16O2
Phosphole, 1-chloro-2,3,4,5-tetraethyl-	C12H20ClP
Phytol	C20H40O
Propanamide, 3-bromo-N-(4-bromo-2-chlorophenyl)-	C9H8Br2ClNO
Purine-2,6-dione, 8-(3-ethoxypropylamino)-1,3-dimethyl-3,9-dihydro-	C12H19N5O3
Pyrene, 1-methyl-	C17H12
Pyrene, 1-methyl-	C17H12
Pyrene, 1,3-dimethyl-	C18H14

Pyrene, 1,3-dimethyl-	C18H14
Pyrene, 2-methyl-	C17H12
Pyrene, 2-methyl-	C17H12
Pyridine-3-carboxamide, oxime,N-(2-trifluoromethylphenyl)-	C13H10F3N
Quinoline, 3-methyl-2-phenyl-, 1-oxide	C16H13NO
Ridecanoic acid, 12-methyl-, methyl ester	C15H30O2
Silane, dimethyl(2,2,2-trochloroethoxy)nonyloxy-	C13H27Cl3
Sulfurous acid, 2-propyl tetradecyl ester	C17H36O3S
Sulfurous acid, 2-propyl tetradecyl ester	C17H36O3S
Sulfurous Acid, butyl dodecyl ester	C16H34O3S
Sulfurous acid, butyl pentadecyl ester	C19H40O3S
Sulfurous acid, octadecyl 2-propyl ester	C21H44O3S
Sulfurous acid, pentadecyl 2-propyl ester	C18H38O3S
Sulfurous Acid, pentadecyl 2-propyl estr	C18H38O3S
Surlfurous acid, butyl heptadecyl ester	C21H44O3S
tert-hexadecanethiol	C16H34S
Tetracosyl pentafluoropropionate	C15H28O2
Tetradecanamide	C14H29NO
Tetradecanamide	C14H29NO
Tetradecane	C14H30
Tetradecane	C14H30
Tetradecanoic acid	C14H28O2
tetrahydroionyl acetate	C15H28O2
Tetrapentaconane, 1,54-dibromo-	C54H108Br2
Tetrapentacontane	C44H90
Tetrapentacontane, 1,54-dibromo	C54H108Br2
Tetratetracontane	C44H90
Tetratetracontane	C44H90
Thiocarbamic acid, N,N-dimethyl, S-1,3-diphenyl-2-butenyl ester	C19H21NOS
Thiophene, 2,2'-(1,2-ethenediyl)bis- (E)-	C10H8S2

trans-13-Docosenamide	C22H43NO
Triacontyl pentafluoropropionate	C33H61F5O2
Triarachine	C36H122O6
Trichloromethane	CHCl3
Tricosane	C23H48
Tricyclo[5.3.1.1(2,6)]dodecane-11,12-dione,(1.alpha.,2.beta.,6.beta.,7.alpha.)-	C12H16O2
tricyclo[9.2.2.2(4,7)]heptadeca-1(14),2,4(17),5,7(16),11(15),12-heptane	C17H16
Triethyl 4-phosphonobutanoate	C10H21O5P
Trifluoroacetic acid, n-heptadecyl ester	C17H31F3O2
Trifluoroacetic acid, pentadecyl ester	C17H31F3O2
Tritetracontane	C43H88
Undecane, 5-methyl-	C12H26
Undecanoic acid, 10-methyl-, methyl ester	C13H26O2
Vitamin E	C29H50O2
Z-11-Pentadecenol	C15H30O
Z-5-Methyl-6-heneicosen-11-one	C22H42O
Z-5-Nonadecene	C19H38
Z-5-Nonadecene	C19H38
Z-8-Methyl-9-tetradecen-1-ol acetate	C17H32O2

Table 7.4. Compounds recovered from skeletal elements sampled from the Korean War. Samples recovered from all locations within this conflict are included. This includes samples that were recovered *in situ* as well as elements known to have been curated by the DPRK. Materials developed from acetonitrile eluates are marked in blue. This is a summary of compounds recovered; therefore, there is some duplication of compounds between the solvents, although this is minimal. In total, 78 compounds were detected. Compounds are listed alphabetically.

COMPOUND	**FORMULA**
(1R,2S,8R,8Ar)-8-acetoxy-1-(2-hydroxyethyl)-1,2,5,5-tetramethyl-trans-decalin	C18H32O3
1-Decanol, 2-hexyl-	C16H34O
1-Hentetracontanol	C41H84O
1-Hentetracontanol	C41H84O
1-Heptanol, 2,4-diethyl-	C11H24O
1-Hexyl-1-nitrocyclohexane	C12H23NO2
1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	C13H40O5Si6
1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester	C20H30O4
1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl)ester	C16H22O4
1,3-Dioxolane, 4-ethyl-5-octyl-2,2-bis(trifluoromethyl)-, cis-	C15H24F6O2
1,3,5,7,9-Pentaethylbicyclo[5.3.1]pentasiloxane	C10H28O6Si5
1,6;3,4-Dianhydro-2-deoxy-beta-d-ribo-hexopyranose	C6H9O3
10,13-Octadecadienoic acid, methyl ester	C19H34O2
11-Tricosene	C23H46
17-Pentatriacontene	C35H70
17-Petnatriacontene	C35H70
2-Dodecen-1-yl(-)succinic anhydride	C16H26O3
2-methyl-4-ethoxycarbonyl-3H-imidazo[1,5)	C10H11N3O4
2,2-Dimethyl-3-heptene trans	C9H18
3-Chloropropionic acid, hetadecyl ester	C20H39ClO2
3-Eicosene, (E)-	C20H40
3-Ethyl-3methylheptane	C10H22
4-Hydroxyphenylacetic acid, ethyl ester, tert-butyldimethylsilyl	C16H26O3Si
7-Hydroxy-3-(1,1-dimethylprop-2-enyl)coumarin	C14H14O3
8-Heptadecene	C17H34

9-Ocetadecenoic acid (Z)-, methyl ester	C19H36O2
9-Octadecenoic acid (Z)-, methyl ester	C19H36O2
Bromoacetic acid, tridecyl ester	C15H29BrO2
Cholest-5-en-3-ol (3.beta.)-	C27H46O
Cholestan-3-one, (5.besta.)-	C27H46O
Cyclodecasiloxane, eicosamethyl-	C20H60O10Si1
Cycloheptasiloxane, tetradecamethyl-	C14H42O7Si7
Cyclohexane, 1-ethyl-2-propyl-	C11H22
Cyclohexane, 1,2,4-trimethyl-	C9H18
Cyclohexasiloxane, dodecamethyl-	C12H36O6Si6
Cyclononasiloxane, octadecamethyl-	C18H54O9Si9
Cyclopentane, (2-methylbutyl)-	n/r
Cyclopentasiloxane, decamethyl-	C10H30O5Si5
Decanoic Acid, 5-ethyl-3,5,9-trimethyl-,	C16H32O2
Disulfide, di-tert-dodecyl	C24H50S2
Dodecane, 2,6,10-trimethyl-	C15H32
Heneicosane	C21H44
Hentriacontane	C31H64
Heptadecanoic acid, 10-methyl-, methyl ester	C19H38O2
Hexadecane, ,6,11,15-tetramethyl-	C20H42
Hexadecane, 2,6,10,14-tetramethyl-	C20H42
Hexadecanoic acid, methyl ester	C17H34O2
Hexadecanoic acid, methyl ester	C17H34O2
Hexasiloxane, tetradecamethyl-	C14H42O5Si6
Isotridecanol-	C13H29O
Ketone, 2,2-dimethylcyclohexyl methyl	C10H18O
Nonahexacontanoic acid	C69H138O2
Octadecanoic acid, methyl ester	C19H38O2
Octadecanoic acid, methyl ester	C19H38O2
Oxalic acid, 6-ethyloct-3-yl ethyl ester	C14H26O4
Oxalic acid, allyl octadecyl ester	C23H42O4

Oxalic Acid, cyclobutyl dodecyl ester	C18H32O4
Oxalic acid, cyclobutyl pentadecyl ester	C21H38O4
Oxirane, 2-decyl-3-(5-methylhexyl)-, cis-	C19H38O
Oxirane, 2-decyl-3-(5-methylhexyl)-, cis-(.+/-.)-	C19H38O
Pentacosane	C25H52
Pentadecanoic acid, 14-methyl-, methyl ester	C17H34O2
Phenol, 2,5-bis(1,1-dimethylethyl)-	C14H22O
Phenol, 2,6-bis(1,1-dimethylethyl)-	C14C22O
Phthalic acid, isobutyl undecyl ester	C23H36O4
Propenoic acid, 3-(5-ethoxycarbonyl-2,4-dimethyl-3-pyrrolyl)-	C12H15NO4
Pyrimidin-4-one, hexahydro-3-hydroxy-2-(4-nitrophenyl)-	C10H11N3O4
Silane, [[4-[1,2-bis[(trimethylsilyl)oxy]ethyl]-1,2-phenylene]bis(oxy)]bis[trimethyl-	C20H42O4Si4
Sulfurous Acid, 2-propyl tridecyl ester	C16H34O3S
Sulfurous acid, butyl dodecyl ester	C16H34O3S
Sulfurous acid, butyl heptadecyl ester	C21H44O3S
Tetracosane	C24H50
Tetradecane, 1-bromo-	C14H29Br
Tetrapentacontane, 1,54-dibromo-	C54H108Br2
Tetrapentacontane, 1,54-dibromo--	C54H108Br2
Tetratetracontane	C44H90
Tetratriacontane	C34H70
Thiocarbamic acid, N,N-dimethyl, S-1,3-diphenyl-2-butenyl ester	C19H21NOS

Table 7.5. Compounds recovered from skeletal elements sampled from the Southeast Asia conflict. Samples recovered from all locations within this conflict are included. This includes samples that were recovered *in situ* as well as returned to the DPAA via local individuals. Materials developed from acetonitrile eluates are marked in blue. This is a summary of compounds recovered; therefore, there is some duplication of compounds between the solvents, although this is minimal. In total, 288 compounds were detected. Compounds are listed alphabetically.

COMPOUND	FORMULA
(1,2,3,3a,4,6a-Hexahydropentalen-2-yl)-dimethyl-amine	C10H17N
1-Chloroeicosane	C20H41Cl
1-Decanol, 2-hexyl-	C16H34O
1-Decanol, 2-hexyl-	C16H34O
1-Dodecanol, 2-hexyl-	C18H38O
1-Dodecanol, 3,7,11-trimethyl-	C15H32O
1-Ethyl-2,2,6-trimethylcyclohexane	C11H22
1-Heptafluorobutyryloxy-2-methylpentane	C10H13F7O2
1-Heptanol, 2,4-diethyl	C11H24O
1-Heptanol, 2,4-diethyl-	C11H24O
1-Monolinoleoylglycerol trimethylsilyl ether	C27H54O4Si2
1-Nonene, 4,6,8-trimethyl-	C12H24
1-Octanol, 2-butyl-	C12H26O
1-Octanol, 2-butyl-	C12H26O
1-Pentanol, 2,3-dimethyl	C7H16O
1-Phenanthrenecarboxylic acid, 1,2,3,4,4a,9,10,10a-octahydro-,1,4a-dimethyl-7-(1-methylethyl)-, methyl ester, [1R-(1.alpha.,4a.beta.,10a.alpha.)]-	C21H32O2
1-Phenanthrenecarboxylic acid, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-,methyl ester, [1R-(1.alpha.,4a.beta.)]	C21H30O2
1,1-Dodecanediol, diacetate	C16H30O4
1,1,1,5,7,7,7-heptamethyl-3,3-bis(trimethlsiloxy)tetrasiloxane	C13H40O5Si6
1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	C13H40O5Si6
1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	C13H40O5Si6
1,2-Benzenedicarboxylic acid, bis(8-methylnonyl) ester	C28H46O4
1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	C16H22O4

1,2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester	C22H34O4
1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	C16H22O4
1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl)ester	C16H22O4
1,3-Dioxolan-4-one, 2-t-butyl-5-methyl-5-(4,4-dimethoxypentyl)-	C15H28O5
1,3-Dioxolane, 2-tert-butyl-2-phenyl-	C15H20O4
1,7-Dimethyl-4-(1-methylethyl)cyclodecane	C15H30
10-Methylnonadecane	C20H42
10-Octadecenoic acid, methyl ester	C19H36O2
11-Octadecenoic acid, methyl ester	C19H36O2
17-Pentatriacontene	C35H70
17-Pentatriacontene	C35H70
18-Norabietane	C19H34
1H-Pyrrole-2,5-doine, 1-(hydroxymethyl)-	C5H5NO3
2-Acetyl-1,3,3,4,4-pentamethylcyclopentene semicarbazone	C13H23N3O
2-Chloropropionic acid, octadecyl ester	C21H41ClO2
2-Dodecen-1-yl(-)succinic anhydride	C16H26O3
2-Ethoxy-1-methyl-6-oxo-1,-azaphosphinane 2-oxide	C7H14NO3P
2-Isopropyl-5-methyl-1-heptanol	C11H24O
2-Methyl-4-ethyoxycarbony-3H-imidazo[1,5-b]pyridazine-5,7-(6H)-dione	C10H11N3O4
2-methyltetracosane	C25H52
2-octanol, 2-methyl-6-methylene-	C10H20O
2-Piperidinone, N-[4-bromo-n-butyl]-	C19H16BrNO
2-Propenamide	C3H5NO
2-Pyrazoline-1-carboxaldehyde, 5-tert-butyl-5-hydroxy-3-methyl-	C9H16N2O2
2,3-Anhydro-d-mannosan	C6H8O4
2,5-Furandione, 3-dodecyl-	C16H26O3
3-(2-Methoxyphenoxy)lactic acid, O,O'-bis(trimethylsilyl)-	C16H28O5Si2
3-Eicosene, (E)-	C20H40
3-Ethyl-3-methylheptane	C10H22
3-Ethyl-3-methylheptane	C10H22
3-Methyl-4-(phenylthio)-2-prop-2enyl-2,5-dihydrothiophene 1,1-dioxide	C14H16O2S2

4-Hyrdoxyphenylacetic acid, ethyl ester, tert-butyldimethylsilyl	C16H26O3Si
5-Fluoro-2-trifluoromethylbenzoic acid, 2-pentadecyl ester	C23H34F4O2
5-Methoxy-.alpha.,.alpha.,.alpha.-trifluoro-m-toluidine	C8H8F3NO
5,5-Dimethyl-cyclohex-3-en-1-ol	C11H24O3S
5,5-Dimethyl-cyclohex-3-en-1-ol	C11H24O3S
5,7,9(11)-Androstatriene, 3-hydroxy-17-oxo	C19H24O2
5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)-	C21H34O2
6,6-diethylhoctadecane	C22H46
7-Hexadecanal, (Z)-	C16H30O
7-Octadecenoic acid, methyl ester	C19H36O2
8-Octadecenoic acid, methy ester, (E)-	C19H36O2
8-Octadecenoic acid, methyl ester	C19H36O2
8-Octadecenoic acid, methyl ester	C19H36O2
9-Hexadecanoic acid, methyl ester, (Z)-	C17H32O2
9-Hexadecenoic acid, eicosyl ester, (Z)-	C36H70O2
9-Octadecanoic acid (Z)-, methyl ester	C19H36O2
9-Octadecenoic acid (Z)-, methyl ester	C19H36O2
Acetaldehyde	C2H4O
Acetic acid, trichloro-, methyl ester	C3H3Cl3O2
Acetic acid, trifluoro-, 3,7-dimethyloctyl ester	C12H21F3O2
Adenosine, 2-methyl-	C11H15N5O4
Androst-1-en-3-one, 4,4-dimethyl-, (5.alpha.)-	C21H32O
Androst-5-en-3-ol, 4,4-dimethyl-,(3.beta.)-	C21H34O
Anilazine	C9H5Cl3N4
Azetidine, 1,2-dimethyl-	C5H11N
Azetidine, 1,2-dimethyl-	C5H11N
Benzene, 1-isothiocyanato-2-methyl-	C8H7NS
Benzeneethanamine, .alpha.,3,4-trimethyl-	C11H17N
Benzo[b]selenophene-2-carbonitrile, 3-(hydroxymethyl)-	C10H7NOSe
Benzoic acid, 2-propenyl ester	C10H10O2
Benzoic acid, 2,4-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	C16H30O4Si3

Benzoic acid, 2,4-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	C16H30O4Si3
Benzothiazole, 2-methyl-	C8H7NS
Bicyclo[4.1.0]heptan-2-ol, 3,7,7-trimethyl-, (1.alpha.,2.alpha.,3.beta.,6.alpha.)-	C10H18O
Bicyclo[4.3.0]none, 2,2,6,7-tetramethyl-7-hydroxy-	C13H24O
Bis(tridecyl) phthalate	C34H58O4
Bromoacetic acid, tridecyl ester	C15H29BrO2
Caffeine	C8H10N4O2
Carbamic acid, N-(2,3-dimethylphenyl)-, oxiranylmethyl ester	C13H23N2O6P
Carbonic Acid, dodecyl 2,2,2-trichloroethyl ester	C15H27Cl3O3
Cholest-7-ene, (5.alpha.)-	C27H46
Cholestan-3-one, (5.beta.)-	C27H46O
Cholestan-3-one, (5.beta.)-	C27H46O
Cholestan-3.alpha.-ol acetate	C29H50O2
Cyanamide, dibutyl-	C9H18N2
Cyclobutanone, oxime	C4H7NO
Cyclodecane	C10H20
Cyclodecasiloxane, eicosamethyl-	C20H60O10Si!
Cyclododecane	C12H24
Cyclododecanol, 1-ethenyl-	C14H26O
Cycloheptasiloxane, tetradecamethyl-	C14H42O7Si7
Cyclohexane, 1-ethyl-2-propyl-	C11H22
Cyclohexane, 1,1,2-trimethyl	C9H18
Cyclohexane, 1,2,4-trimethyl-	C9H18
Cyclohexane, 1,2,4,5-tetraehtyl-, (1.alpha.,2.alpha.,4.alpha.,5.alpha.)-	C14H28
Cyclohexane, undecyl-	C17H34
Cyclohexasiloxane, dodecamethyl-	C12H36O6Si6
Cyclohexasiloxane, dodecamethyl-	C12H36O6Si6
Cyclohexene, 4-(4-ethylcyclohexyl)-1-pentyl-	C19H34
Cyclononasiloxane, octadecamethyl-	C18H54O9Si9
Cyclononasiloxane, octadecamethyl-	C18H54O9Si9
Cyclooctane, ethyl-	C10H20

Cyclopentadecanone, 2-hydroxy-	C15H28O2
Cyclopentane, 1-pentyl-2-propyl-	C13H26
Cyclopentane, 1,1,3-trimethyl-	C8H16
Cyclopentane, 1,2-butyl-	C13H26
Cyclopentane, propyl	C8H16
Cyclopentasiloxane, decamethyl-	C10H30O5Si5
Decane, 1,1'-oxybis-	C20H42O
Decane, 2,3,5-trimethyl-	C13H28
Dibutyl phthalate	C16H22O4
Dibutyl phthalate	C16H22O4
Dichloroacetic acid, nonyl ester	C11H20Cl2O2
Dichloroacetic acid, tridecyl ester	C15H28C12O2
Dichloroacetic acid, undecyl ester	C13H24Cl2O2
didodecyl phthalate	C32H54O4
Diethyl phthalate	C12H14O4
Diethyl phthalate	C12H14O4
Diethylene glycol monododecyl ether	C16H34O3
Disulfide, di-tert-dodecyl	C24H50S2
Docosanoic acid, 1,2,3-propanetriyl ester	C19H134O6
Docosanoic acid, docosyl ester	C44H88O2
Dodecane	C12H26
Dodecane, 1-fluoro-	C12H25F
Dodecane, 2,6,10-trimethyl-	C15H32
Dodecanoic acid, 11-hydroxy-, methyl ester	C13H26O3
Dodecanoic acid, methyl ester	C13H26O2
E,E,Z-1,3,12-Nonadecatriene-5,14-diol	C19H34O2
Eicosane	C20H42
Eicosane, 7-hexyl-	C26H54
Eicosane, 9-cyclohexyl-	C26H52
Eicosene, (E)-	C20H40
Ethanamine, N-methyl-	C3H9N

Ethanol, 2-(dodecyloxy)-	C14H30O2
Ethanol, 2-(hexadecyloxy)-	C18H38O2
Ethanol, 2-(octadecyloxy)-	C20H42O2
Ethanol, 2-(tetradecyloxy)-	C16H34O2
Heneicosane	C21H44
Heneicosane, 3-methyl-	C22H46
Heneicosanoic acid, methyl ester	C22H44O2
Hentriacontane	C31H64
Heptacosane	C27H56
Heptadecanoic aci, 16-methyl-, methyl ester	C19H38O2
Heptadecanoic acid, 10-methyl-, methyl ester	C19H38O2
Heptadecanoic acid, 16-methyl, methyl ester	C19H38O2
Heptafluorobutanoic acid, hetadecyl ester	C21H35F7O2
Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	C14H44O6Si7
Heptasiloxane, hexadecamethyl-	C16H48O6Si7
Hexacontane	C60H122
Hexacosane	C26H54
Hexadecane-1,2-diol	C16H34O2
Hexadecane, 1-chloro	C16H33Cl
Hexadecane, 1-chloro- (extra peak at 191)	C16H33Cl
Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	C35H68O5
Hexadecanoic acid, 14-methyl- methyl ester	C18H36O2
Hexadecanoic acid, methyl ester	C17H34O2
Hexadecanoic acid, methyl ester	C17H34O2
Hexane, 2,3,4-trimethyl-	C9H20
Hexane, 3,3-dimethyl-	C8H18
Hexasiloxane, tetradecamethyl-	C14H42O5Si6
Hexasiloxane, tetradecamethyl-	C14H42O5Si6
Hexatriacontane	C36H74
Indolo[2,3-b]quinoxaline, 1-fluoro-	C14H8FN3
Isotridecanol-	C13H28O

l-(+)-Ascorbic acid 2,6-dihexadecanoate	C38H68O8
Methanone, (3,4-dimethylphenyl)(2,4,6-trimethylphenyl)	C18H20O
Methoxyacetic acid, tridecyl ester	C16H32O3
Methyl 7-methylhexadecanoate	C18H36O2
Methyl 8-oxooctanoate	C9H16O3
Methyl 9-methyltetradecanoate	C16H32O2
Methyl stearate	C19H38O2
Methyl tetradecanoate	C15H30O2
Morphinan, 7,8-didehydro-4,5-epoxy-17-methyl-3,6-bis[(trimethylsilyl)oxy]-, (5.alpha.,6.alpha.)-	C23H35NO3Si2
Morpholine, 4-phenyl-	C10H13NO
Morpholine, 4-phenyl-	C10H13NO
N-Benzoylglycine ethyl ester	C11H13NO3
n-Hexadecanoic acid	C16H32O2
n-Hexadecanoic acid (extra peak at 149)	C16H32O2
N-Methyladrenaline, tri-TMS	C19H39NO3Si3
Naphthalene, 6-ethyl-1,2,3,4-tetrahydro-	C19H28
Nonacosane	C29H60
Nonahexacontanoic acid	C69H138O2
Octacosane	C28H58
Octacosanoic acid, methyl ester	C29H58O2
Octadecane, 1-(ethenyloxy)-	C20H40O
Octadecane, 1,1'-[(1-methyl-1,2-ethanediyl]bis(oxy)]bis-	C39H80O2
Octadecane, 2,2,4,15,,17,17-hexamethyl-7,12-bis(3,5,5-trimethylhexyl)-	C42H86
Octadecane, 5-methyl-	C19H40
Octadecanoic acid	C18H36O2
Octadecanoic acid, 2-(octadecyloxy)ethyl ester	C38H76O3
Octadecanoic acid, 2,3-bis[(1-oxotetradecyl)oxy]propyl ester	C49H94O5
Octadecanoic acid, 3-hydoxy-2-tetradecyl-, methyl ester	C33H66O3
Octadecanoic acid, methyl ester	C19H38O2
Octadecanoic acid, methyl ester	C19H38O2
Octane, 2,4,6-trimethyl-	C11H24

Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	C16H50O7Si8
Oleic acid	C18H34O2
Oxalic acid, 6-ehtyloct-3-yl hexyl ester	C18H34O4
Oxalic acid, 6-ethyloct-3-yl ethyl ester	C14H26O4
Oxalic acid, 6-ethyloct-3-yl heptyl ester	C19H36O4
Oxalic acid, 6-ethyloct-3-yl isobutyl ester	C16H30O4
Oxalic acid, 6-ethyloct-3-yl isohexyl ester	C14H30O3S
Oxalic acid, 6-ethyloct-3-yl propyl ester	C15H28O4
Oxalic acid, 6-thyloct-3-yl hexyl ester	C18H34O4
Oxalic Acid, allyl octadecyl ester	C23H42O4
Oxalic acid, allyl tetradecyl ester	C19H34O4
Oxalic acid, allyl tridecyl ester	C18H32O4
Oxalic acid, allyl tridecyl ester	C18H32O4
Oxalic acid, bis(6-ethyloct-3-yl) ester	C22H42O4
Oxalic acid, cyclobutyl hexadecyl ester	C22H40O4
Oxalic acid, cyclobutyl tridecyl ester	C19H34O4
Oxalic Acid, cyclobutyl tridecyl ester	C19H34O4
Oxalic acid, isobutyl tetradecyl ester	C20H38O4
Oxirane, [(dodecyloxy)methyl]-	C15H30O2
Oxirane, 2-decyl-3-(5-methylhexyl)-, cis(.+/-.)-	C19H38O
Oxiraneundecanoic acid, 3-pentyl-, methyl ester, trans-	C19H36O2
Palmitic acid	C16H32O2
Pentacosane	C25H52
Pentacosanoic acid, methyl ester	C26H52O2
Pentadecane, 2,6,10-trimethyl-	C18H38
Pentadecanoic acid	C15H30O2
Pentadecanoic acid, 14-methyl-, methyl ester	C17H34O2
Pentadecanoic acid, 14-methyl-, methyl ester	C17H34O2
Pentafluoropropionic acid, hexadecyl ester	C19H33F5O2
Pentatriacontane	C35H72
Phenol, 2,4-bis(1,1-dimethylethyl)-	C17H30OSi

Phenol, 2,4-bis(1,1-dimethylethyl)-	C17H30OSi
Phenol, 2,5-bis(1,1-dimethylethyl)-	C14H22O
Phenol, 2,6-bis(1,1-dimethylethyl)-	C13H22O
Phenol, 3,5-bis(1,1-dimethylethyl)-	C14H22O
Phosphonofluoridic acid, methyl-, nonyl ester	C10H22FO2P
Phthalic acid, butyl tricedyl ester	C25H40O4
Phthalic acid, isobutyl tridec-2-yn-1-yl	C25H36O4
Phthalic acid, monoamide, N-ethyl-N-(3-methylphenyl)-, ethyl ester	C19H21NO3
Phthalic acid, nonyl tridec-2-yn-1-yl ester	C30H46O4
Propanamide	C3H7NO
Propenoic acid, 3-(5-ethoxycarbonyl-2,4-dimethyl-3-pyrrolyl)-	C12H15NO4
Pyrazolo[5,1-c][1,2,4]triazine-3-carboxy	C8H9N5O2
Pyrimidin-4-one, hexahydro-3-hydroxy-2-(4-nitrophenyl)-	C10H11N3O4
silanamine, N-[2,6-dimehtyl-4-[(trimethylsilyl)oxy]phenyl]-1,1,1-trimethyl-	C14H27NOSi2
Silane, [[4-[1,2-bis[(trimethylsilyl)oxy…	C20H42O4Si4
Spiro[4,5]decan-7-one, 1,8-dimethyl-8,9-epoxy-4-isopropyl	C15H24O2
Sulfurous acid, 2-propyl tridecyl ester	C16H34O3S
Sulfurous acid, 2-propyl tridecyl ester	C16H34O3S
Sulfurous acid, 2-propyl undecyl ester	C14H30O3S
Sulfurous acid, 2-propyl undecyl ester	C14H30O3S
Sulfurous acid, butyl tridecyl ester	C17H36O3S
Sulfurous acid, dodecyl 2-propyl ester	C15H32O3S
Sulfurous acid, hexyl petnadecyl ester	C21H44O3S
Sulfurous acid, octadecyl 2-propyl ester	C21H44O3S
Sulfurous acid, octadecyl 2-propyl ester	C21H44O3S
Sulfurous acid, pentadecyl 2-propyl ester	C18H38O3S
Tetracosane	C24H50
Tetradecanoic acid	C14H28O2
Tetrapentacontane	C54H110
Tetrapentacontane, 1,54-dibromo-	C54H108Br2
Tetrapentacontane, 1,54-dibromo-	C54H108Br2

Tetratetracontane	C44H90
trans-2-Hexadecenoic acid	C16H30O2
trans-2,3-Epoxydecane	C10H20O
Triacontane, 1-bromo-	C30H61Br
Triacontanoic acid, methyl ester	C31H62O2
Trichlormethane; aka Chloroform	CHCl3
Trichloroacetic acid, hexadecyl ester	C18H33Cl3O
Trichloroacetic acid, pentadecyl ester	C17H31Cl3O2
Trichloroacetic acid, tridecyl ester	C15H27Cl3O2
Trichloromethane	CHCl3
tricholoacetic acid, undecyl ester	C13H23Cl3O2
Tridecanoic acid, 12-oxo-	C13H24O3
Tridecanoic acid, 4,8,12-trimethyl-, methyl ester	C17H34O2
Triethylene glycol monododecyl ether	C18H38O4
trifluoroacetic acid, n-heptadecyl ester	C17H31F3O2
Tritetracontane	C43H88
Tritriacontane	C33H68

Table 7.6. Compounds recovered from skeletal elements sampled from all World War II incidents with the exception of the USS *Oklahoma*. This includes samples that were recovered the Solomon Islands, the Battle of Tarawa, battle locations in the Philippines, Italy, Yugoslavia, and Papua New Guinea. Materials developed from acetonitrile eluates are marked in blue. This is a summary of compounds recovered; therefore, there is some duplication of compounds between the solvents, although this is minimal. In total, 267 compounds were detected. Compounds are listed alphabetically.

COMPOUND	FORMULA
(2,3-Diphylcyclopropyl)methyl phenyl sulfoxid, trans-	C22H20OS
1-(Cyclopropyl-nitro-methyl)-cyclopentanol	C9H15NO3
1-Bromo-11-iodoundecane	C11H22BrI
1-Chloroeicosane	C20H41Cl
1-Decanol, 2-hexyl-	C16H34O
1-Docosanethiol	C22H46S
1-Dodecanol, 2-octyl-	C20H42O
1-Heptadec-1-ynyl-cyclohexanol	C23H42O
1-Heptanol, 2,4-diethyl-	C11H24O
1-Hexacosanol	C26H54O
1-Octanol, 2-butyl-	C12H26O
1-Pentanol, 2,3-dimethyl-	C7H16O
1-propanol, 2,3-bis[(3,7,11,15-tetramethylhexadecyl)oxy]-	C43H88O3
1-Sec-butyldiaziridine	C5H12N2
1,1-Dichloro-2,2-bis(p-chlorophenyl)ethane	C14H10Cl4
1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy) tetrasiloxane	C13H40O5Si6
1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	C13H40O5Si6
1,2-Benzenedicarboxylic acid, butyl octyl ester	C20H30O4
1,2-Benzenedicarboxylic acid, diisooctyl ester	C24H38O4
1,3-Dioxolan-4-one, 2-t-butyl-5-methyl-5-(4,4-dimethoxypentyl)-	C15H28O5
1,3-Doxolane, 4-ethyl-5-octyl-2,2-bis(trifluoromethyl)-, cis-	C15H24F6O2
1,3,5,7,9,11-Hexaethylbicyclo[5.5.1]hexasiloxane	C12H34O7Si6
1,6;3,4-Dianhydro-2-deoxy-.beta.-d-lyxo-hexopyranose	C6H8O3
10-Methylnonadecane	C20H42
10-Octadecenoic acid, methyl ester	C19H36O2

17-Pentatriacontene	C35H70
1H-Indole, 1-methyl-2-phenyl-	C15H13N
2-Acetyl-1,3,3,4,4-pentamethylcyclopentene semicarbazone	C13H23N3O
2-Bromopropionic acid, 6-ehtyl-3-octyl ester	C13H25BrO2
2-Bromopropionic acid, pentadecyl ester	C18H35BrO2
2-Hexanal, (E)-	C6H10O
2-Hexyl-1-octanol	C14H30O
2-Isopropyl-5methyl-1-heptanol	C11H24O
2-Methyl-7-phenylindole	C15H13N
2-Methyldocosane	C23H48
2-methylhexacosane	C27H56
2-methyltetracosane	C25H52
2-p-Nitrophenyl-oxadiazol-1,3,4-one-5	C8H5N3O4
2-Piperidinone, N-[4-bromo-n-butyl]-	C9H16BrNO
2-Propen-1-one, 1-cyclopropyl-	C6H80
2-Propenamide	C3H5NO
2-Undecene, 4,5-dimehtyl-, [R*,S*-(Z)]-	C13H26
2,4(1H,5H)-Imidazoledione, dihydro-5-(2-fluoro-4,5-dimethoxybenzyl)-5-methyl	C13H15FN2
2,5-Isoxazolidinedicarboxylic acid, 2-ethyl 5-methyl ester	C8H13NO5
2H-Indeno[1,2-b]furan-2-one, 3,3a,4,5,6,7,8,8b-octahydro-8,8-dimethyl	C13H18O2
3-Ethyl-3-methylheptane	C10H22
3-Heptyl-1,1,1-triphenyl-decan-2-one	C35H46O
3-n-Heptyl-7-methyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraneal	C26H40O
3,4-Dihydroxymandelic acid, ethyl ester, tri-TMS	C19H36O5Si3
4-Fluoro-3-(trifluoromethyl)acetophenone	C9H6F4O
4-Hydroxyphenylacetic acid, ethyl ester, tert-butyldimethylsilyl	C16H26O3Si
5-Acetomido-4,7-dioxo-4,7-dihydrobenzofurazan	C8H5N3O4
5-Methyl-2-phenylindolizine	C15H13N
5-Methyl-2-phenylindolizine	C15H13N
6-Octadecenoic acid, methyl ester, (Z)-	C19H36O2
6-octenal, 3,7-dimethyl	C10H18O

7-Hexadecenal, (Z)-	C16H30O
7-Octadecenoic acid, methyl ester	C19H36O2
7-Oxabicyclo[4.1.0]heptane, 1,5-dimethyl-	C8H14O
8-Octadecenoic acid, methyl ester	C19H36O2
9-Hexadecenoic acid, eicosyl ester, (Z)	C36H70O2
9-Hexadecenoic acid, methyl ester, (Z)-	C17H32O2
9-Octadecenoic acid (Z)-, methyl ester	C19H36O2
9-Octadecenoic acid, methyl ester, (E)-	C19H36O2
9-Octadecenoic acid, methyl ester, (E)-	C19H36O2
9,12-Octadecadienoic acid (Z,Z)- methyl ester	C19H34O2
9,12-Octadecadienoic acid (Z,Z)-, methyl ester	C19H34O2
9,12-Octadecadienoic acid, methyl ester, (E,E)-	C19H34O2
9,15-Octadecadienoic acid, methyl acid, (Z,Z)-	C19H34O2
9H-purine, 6-methyl-9-(trimethylsilyl)-	C9H14N4Si
Acetaldehyde, methyl(2-propenyl)hydrazone	C6H12N2
Acetic acid, trichloro-, methyl ester	C3H3Cl3O2
Acetic acid, trichloro-, nonyl ester	C11H19Cl3O2
Acetic acid, trifluoro- tetradecyl ester	C16H29F3O2
Acetic acid, trifluoro-,3,7-dimethyloctyl ester	C12H21F3O2
Benz(b)-1,4-oxazepine-4(5H)-thione, 2,3-dihydro-2,8-dimethyl-	C11H13NOS
Benzoic acid, 2,4-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	C16H30O4Si3
Borane, 2,3-dimethyl-2-butyl- (dimer)	C12H30B2
Butane, 2,2-dimethyl-	C6H14
Carbonic acid, dodecyl 2,2,2-trichloroethyl ester	C15H27Cl3O3
Carbonic acid, tetradecyl 2,2,2-trichloroethyl ester	C17H31Cl3O3
Carbonic acid, tridecyl 2,2,2-trichloroethyl ester	C16H29Cl3O3
Cholest-2-ene	C27H46
Citronellol epoxide (R or S)	C10H20O2
Cyclodecasiloxane, eicosamethyl-	C20H60O10Si1
cycloheptasiloxane,tetradecamethyl	C14H42O7Si7
Cyclohexanamine, N-cyclooctylidene-	C14H25N

Cyclohexane, 1-ethyl-2-propyl	C11H22
Cyclohexane, 1,1'-(1,2-dimethyl-1,2-ethanediyl)bis-	C16H30
Cyclohexane, 1,2,4-trimethyl	C9H18
Cyclohexane, 1,2,4,5-tetraethyl-, (1.alpha.,2.alpha.,4.alpha.,5.alpha.)-	C14H28
Cyclohexane, 1,2,4,5-tetraethyl-,(1.alpha.,2.alpha.,4.alpha.,5.alpha.)-	C14H28
Cyclohexanecarboxamide	C7H13NO
Cyclohexasiloxane, dodecamethyl-	C12H36O6Si6
Cyclononasiloxane, octadecamethyl-	C18H54O9Si9
Cyclononasiloxane, octadecamethyl-	C18H54O9Si9
Cyclooctane, butyl-	C12H24
Cyclooctasiloxane, hexadecamethyl-	C16H48O8Si
Cyclopentane, (2-methylbutyl)	C10H20
Cyclopentane, 1-pentyl-2-propyl	C13H26
Cyclopentane, 1,1'-[3-(2-cyclopentylethyl)-1,5-pentanediyl]bis-	C22H40
Cyclopentasiloxane, decamethyl-	C10H30O5Si5
Cyclopropene-3-carboxamid, 1,2-diphenyl-N-(3-methoxyphenyl)-	C23H19NO2
d-Mannitol, 1-O-(22-hydroxydocosyl)-	C28H58O7
DDMU	C14H9Cl3
Decane, 1-iodo-	C10H21I
Decane, 1,1'-oxybis-	C20H42O
Decane, 1,1'oxybis-	C20H42O
Dichloroacetic acid, undecyl ester	C13H24Cl2O2
Disulfide, di-tert-dodecyl	C24H50S2
Docosane	C22H46
Docosanoic acid, docoyl ester	C44H88O2
Dodecahydropyrido[1,2-b]isoquinolin-6-one	C13H21NO
dodecane, 1-chloro-	C12H25Cl
Dodecane, 1,1'-oxybis-	C24H50O
Dodecane, 2-methyl-	C13H28
Dodecane, 2,6,11-trimethyl-	C15H32
Dodecanoic acid, 3-hyrdoxy-	C12H24O3

Eicosane	C20H42
Eicosane, 9-octyl-	C28H58
Estra-1,3,5(10)-trien-17.beta.-ol	C18H24O
Ethanol	C4H8O2
Ethanol, 2-(dodecyloxy)-	C14H30O2
ethyl acetate	C4H8O2
Fumaric acid, 2-decyl tridecyl ester	C15H24F6O2
Geranyl ethyl ester 2	C12H22O
Heneicosane	C21H44
Heneicosane	C21H44
Heneicosane, 3-methyl-	C22H46
Heneicosanoic acid, methyl ester	C22H44O2
Hentriacontane	C31H64
Heptacosane	C27H56
Heptacosane, 1-chloro	C27H55Cl
Heptacosanoic acid, methyl ester	C28H56O2
Heptadecane	C17H36
Heptadecanoic acid, 16-methyl-, methyl ester	C19H38O2
Heptadecanoic acid, 16-methyl-, methyl ester	C19H38O2
Heptadecyl heptafluorobutyrate	C21H35F7O2
Heptane, 2,4-dimethyl-	C9H20
Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	C14H44O6Si7
Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	C14H44O6Si7
Heptasiloxane, hexadecamethyl-	C16H48O6Si7
Heptasiloxane, hexadecamethyl-	C16H48O6Si7
Hexacontane	C60H122
Hexacosane	C26H54
Hexadecane	C16H34
Hexadecane, 1-chloro	C16H33Cl
Hexadecane, 1,1-bis(dodecyloxy)-	C40H82O2
hexadecane, 2,6,10,14-tetramethyl	C20H42

Hexadecane, 2,6,11,15-tetramethyl-	C20H42
Hexadecanoic acid, 1-(hydroxymehtyl)-1,2-ethanediyl ester	C35H68O5
Hexadecanoic acid, 14-methyl-, methyl ester	C18H36O2
Hexadecanoic acid, 2-(octadecyloxy) ethyl ester	C36H72O3
Hexadecanoic acid, methyl ester	C17H34O2
Hexadecanoic acid, methyl ester	C17H34O2
Hexadecen-1-ol, trans-9-	C16H32O
Hexane, 1-(hexyloxy)-5-methyl-	C13H28O
Hexane, 3,3-dimethyl-	C8H18
Hexasiloxane, tetradecamethyl-	C14H42O5Si6
hexasiloxane, tetradecamethyl-	C14H42O5Si6
Hexatriacontane	C36H74
Isophthalic acid, allyl dodecyl ester	C23H34O4
l-(+)-Ascorbic acid 2,6-dihexadecanoate	C38H68O8
l-(+)-Ascorbic acid, 2,6-dihexadecanoate (peaks at 355, 429, & 147 that don't fit)	C38H68O8
Methadone N-oxide	C21H27NO2
Methanone, (3,4-dimethylphenyl)(2,4,6-trimethylphenyl)-	C18H20O
Methoxyacetic acid, 2-tetradecyl ester	C17H34O3
Methyl 5,9-dimethyldecanoate	C13H26O2
Methyl 9-cis,11-trans-octadecadienoate	C19H34O2
Methyl stearate	C19H38O2
Methyl stearate	C19H38O2
N-Methyladrenaline, tri-TMS	C19H39NO3Si3
n-Nonadecanoic acid, pentamethyldisilyl ester	C24H52O2Si2
n-Nonadecanoic acid, pentamethyldisilyl ester	C24H52O2Si2
n-Nonadecanol-1	C19H40O
Nonacosane	C29H60
Nonahexacontanoic acid	C69H138O2
o,p'-DDT	C14H9Cl5
Octacosane	C28H58
Octadecane	C18H38

Octadecane, 1-(ethenyloxy)-	C20H40O
Octadecane, 1-chloro-	C18H37Cl
Octadecane, 1,1'-[1,3-propanediylbis(oxy)]bis-	C39H80O2
Octadecane, 2,2,4,15,17,17-hexamethyl-7,	C42H86
Octadecane, 3-ethyl-5-(2-ethylbutyl)-	C26H54
Octadecane, 5-methyl-	C19H40
Octadecanoic acid, 10-oxo-, methyl ester	C19H36O3
Octadecanoic acid, 10-oxo-, methyl ester	C19H36O3
Octadecanoic acid, methyl ester	C19H38O2
Octadecanoic acid, methyl ester	C19H38O2
Octadecanoic acid, trimethylsilyl ester	C21H44O2Si
Octane, 1,1'-oxybis-	C16H34O
Octane, 2,4,6-trimethyl-	C11H24
Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	C16H50O7Si8
Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	C16H50O7Si8
Oxalic acid, 6-ethylocat-3-yl isobutyl ester	C16H30O4
Oxalic acid, 6-ethyloct-3-yl ethyl ester	C13H26O4
Oxalic acid, 6-ethyloct-3-yl ethyl ester	C14H26O4
Oxalic acid, 6-ethyloct-3-yl heptyl ester	C19H36O4
Oxalic acid, 6-ethyloct-3-yl hexyl ester	C18H34O4
Oxalic acid, 6-ethyloct-3-yl isohexyl ester	C18H34O4
Oxalic acid, 6-ethyloct-3-yl propyl ester	C15H28O4
Oxalic acid, allyl dodecyl ester	C17H30O4
Oxalic acid, allyl octadecyl ester	C23H42O4
Oxalic acid, allyl pentadecyl ester	C20H36O4
Oxalic acid, allyl tridecyl ester	C18H32O4
Oxalic acid, allyl tridecyl ester	C18H32O4
Oxalic acid, bis(6-ethyloct-3-yl) ester	C22H42O4
Oxalic acid, bis(6-ethyloct-3-yl) ester	C22H42O4
Oxalic acid, butyl 6-ethyloct-3-yl ester	C16H30O4
Oxalic acid, butyl 6-ethyloct-3-yl ester	C16H30O4

Oxalic acid, isohexyl neopentyl ester	C13H24O4
Oxetane, 2-methyl-4-propyl-	C7H14O
p,p'-DDE	C14H8Cl4
p,p'-DDT	C14H9Cl5
Pentacosane	C25H52
Pentacosanoic acid, methyl ester	C26H52O2
Pentadecane, 2-methyl-	C16H34
Pentadecane, 2,6,10-trimethyl	C18H38
Pentadecanoic acid, 14-methyl-, methyl ester	C17H34O2
Pentadecanoic acid, 14-methyl-, methyl ester	C17H34O2
Pentaecane, 2,6,10-trimethyl-	C18H38
Pentafluoropropionic acid, actadecyl ester	C21H37F5O2
Pentafluoropropionic acid, hexadecyl ester	C19H33F5O2
Pentafluoropropionic acid, octadecyl ester	C21H37F5O2
Pentafluoropropionic acid, undecyl ester	C14H23F5O2
Pentane, 2,2-dimethyl-	C7H16
Pentanoic acid, 1,1-dimethylpropyl ester	C9H18O2
Phenethylamine, N-methyl-.beta.,3,4-tris	C18H37NO3Si3
Phenol, 2,4-bis(1,1-dimethylethyl)-	C14H22O
Phenol, 2,5-bis(1,1-dimethylethyl)-	C14H22O
Phenol, 2,6-bis(1,1dimethylethyl)-	C14H22O
Phenol, 3,5-bis(1,1-dimethylethyl)-	C14H22O
Pyrazolo[5,1-c][1,2,4]triazine-3-carboxylic acid, 4-amino, ethyl ester	C8H9N5O2
Pyrimidin-4-one, hexahydro-3-hydroxy-2-(4-nitrophenyl)-	C10H11N3O4
Pyrimidin-4-one, hexahydro-3-hydroxy-2-(4-nitrophenyl)-	C10H11N3O4
Silanamine, N-[2,6-dimethyl-4-[(trimethylsilyl)oxy]phenyl]-1,1,1-trimethyl-	C14H27NOSi2
Silane, trichlorooctadecyl-	C18H37Cl3Si
Squalane	C30H62
Sulfurous acid, butyl dodecyl ester	C16H34O3S
Sulfurous acid, butyl heptadecyl ester	C21H44O3S
Sulfurous acid, butyl tetradecyl ester	C18H38O3S

Sulfurous acid, dodecyl 2-propyl ester	C15H32O3S
Sulfurous acid, hexyl octyl ester	C14H30O3S
Sulfurous acid, hexyl octyl ester	C14H30O3S
Sulfurous acid, hexyl pentadecyl ester	C21H44O3S
Sulfurous acid, isohexyl 2-pentyl ester	C11H24O3S
Sulfurous acid, octadecyl 2-propyl ester	C21H44O3S
Sulfurous acid, pentadecyl 2-propyl ester	C15H32O3S
Tetracosane	C24H50
Tetrapentacontane, 1,54-dibromo-	C54H108Br2
Tetratetracontance	C44H90
Tetratriacontane	C34H70
Tetratriacontane	C34H70
trans-2,3-Epoxydecane	C10H20O
Triacontane, 1-bromo-	C30H61Br
Triacontanoic acid, methyl ester	C31H62O2
Triarachine	C63H122O6
Trichloroacetic acid, hexadecyl ester	C18H33Cl3O2
Trichloromethane	CHCl3
tricholoacetic acid, hexadecyl ester	C18H33Cl3O2
Tricosanoic acid, 10,14,18,22-tetramethyl-, methyl ester	C28H56O2
Tricosanoic acid, methyl ester	C24H48O2
Tridecanol, 2-ethyl-2-methyl-	C16H34O
Trifluoroacetic acid, 4-methylpentyl ester	C8H13F3O2
Trifluoroacetic acid, N-heptadecyl ester	C17H31F3O2
Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]-	C12H36O4Si5
Undecane, 5-cyclohexyl-	C17H34

7.19 PRESENTATION

A presentation entitled “DNA Typing from Skeletal Remains: A Study of Inhibitors using Mass Spectrometry” was presented at the International Society of Forensic Genetics meeting in Seoul, South Korea on 2 September 2017. The presentation can be found on the following pages.

DNA Typing from Skeletal Remains: A Study of Inhibitors using Mass Spectrometry

Suni M. Edson[1,2]

ISFG
Seoul, South Korea
2 September 2017

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Commercial Products

Commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible, and does not imply that any of the commercial products identified are necessarily the best available for the purpose.

Outline

- Introduction to the mission
- Why this project?
- The protocol
- The results
  - Bone
  - DNA
- What does it mean?
- What can we do with it?
- The road to come.

Armed Forces DNA Identification Laboratory (AFDIL)

- Established in 1991 as the DoD DNA Registry
  - With the primary purpose of identifying the remains of missing US service members.
- A subdivision of the Armed Forces Medical Examiner System (AFMES)
- Mission Partner with Defense POW/ MIA Accounting Agency (DPAA)

Why THIS project?

Why Mass Spec Analysis of Bone Samples and DNA?

The goal is to more fully optimize extraction of DNA from skeletal materials

- Increased yield of DNA
- Decrease loss of DNA
- Remove inhibitors
  - But what is really there?

Extraction of DNA from Skeletal Materials

- Since 1992, AFDIL has reported 17,280 analyses of skeletonized remains.
- Encompasses:
  - Three different extraction protocols
  - Sanger sequencing of mitochondrial DNA
  - STR analysis
    - Y-STR (Modified and Neat)
    - Minifiler
    - Identifiler, Identifiler Plus, PP16, Fusion

Extractions

	Original Extraction	Demineralization 1 (Organic)	Demineralization 2 (Inorganic)
Weight of Bone	2.0-2.5g	0.2-0.25g	0.2-0.25g
Extraction Buffer Composition	10mM Tris, pH8.0 50mM EDTA, pH8.0 0.5% SDS Proteinase K	0.5M EDTA, pH8.0 1% lauroyl-sarcosinate Proteinase K	0.5M EDTA, pH8.0 1% lauroyl-sarcosinate Proteinase K
Incubation Time	Overnight at 56°C	Overnight at 56°C	Overnight at 56°C
Purification	PCIA Wash with Butanol	PCIA Wash with Butanol	QiaQuick Column/Wash
Concentration	Centrifugal filters	Centrifugal filters	Centrifugal Filters

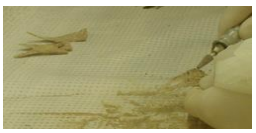
Success Rates

	Original Extraction		Demineralization 1 (Organic)		Demineralization 2 (Inorganic)	
	Number tested	%Success	Number tested	%Success	Number tested	%Success
Mitochondrial DNA	5809	75%	6256	89%	1805	80%
Minifiler	103	32%	839	29%	411	48%
Modified Y-Filer	173	40%	988	31%	634	57%
Identifiler Plus	1	0%	25	60%	30	60%
PowerPlex Fusion			81	77%	50	96%

Protocol

Protocol - Bone

- In the course of regular casework, the powder sanded from the exterior of more than 400 samples was collected.
- Volumes ranged from 0.01g to 1.71g.

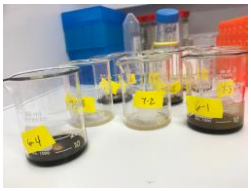


Protocol - Bone

- Powder collected ranged from fine powdered white to black and oily.
- Samples were from Korea, Southeast Asia, and world-wide sites involved in World War II.

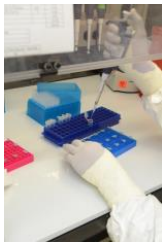
Protocol - Bone

- ~0.1g of bone powder was soaked in 1mL of solvent for 1 hour.
  - Samples were treated sequentially with:
    - Acetonitrile
    - Dichloromethane
- Volatilized until dry at room temperature.
- Resuspended in 500ul of Methanol



Protocol - DNA

- 10ul of extracted DNA was suspended in Methanol
  - 500ul initially
  - 150ul recently.

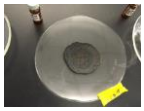


Protocol

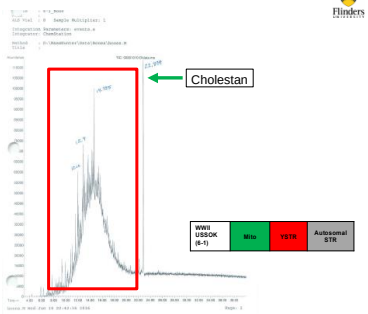
- Loaded on the Agilent 7890A – 5975C GC/MSD (Single Quadrupole)
  - -- 30cm column / 20cm column
  - -- 2.5min solvent delay
  - -- 150ºC 10min
  - -- Ramp to 250ºC over 10min at 20ºC/min
  - -- Splitless
- Analyzed using Chemstation
  - -- Mass Hunter
  - -- NIST 05 Library

Detected Materials -- Bone

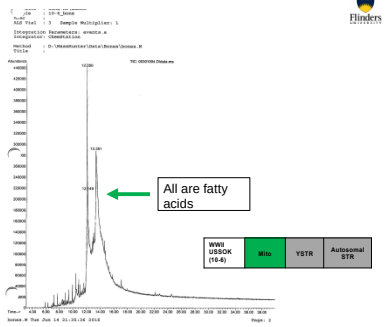
- By-products of decay
  - Cholestan
  - Various esters
  - Multiple fats
- Site/individual specific materials
  - Quinoline
  - Cedranone
  - Naphthalene
- Fuel
  - Decane
  - Phenanthracene
- Metals
  - Molybdenum
  - Mercury

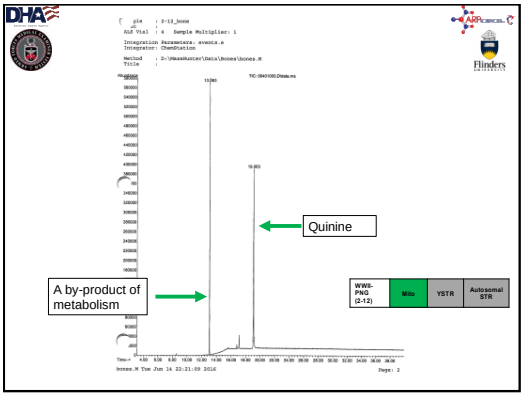


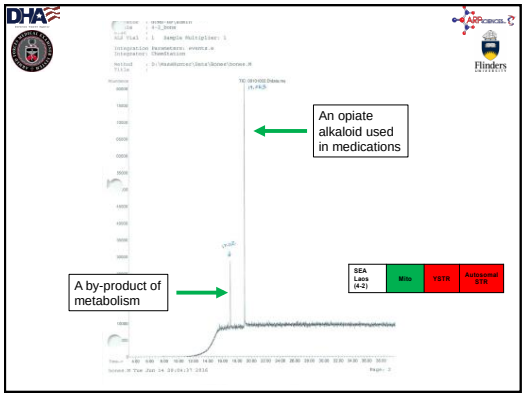
Detected Materials -- Bone



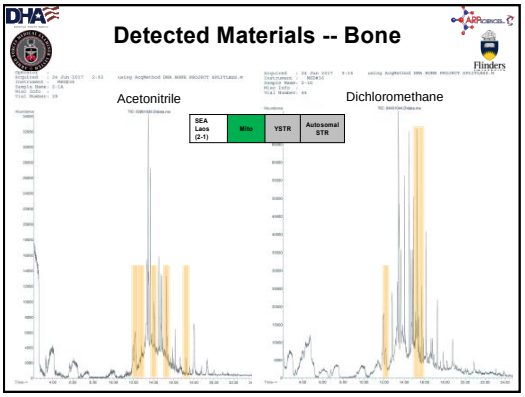
Detected Materials -- Bone



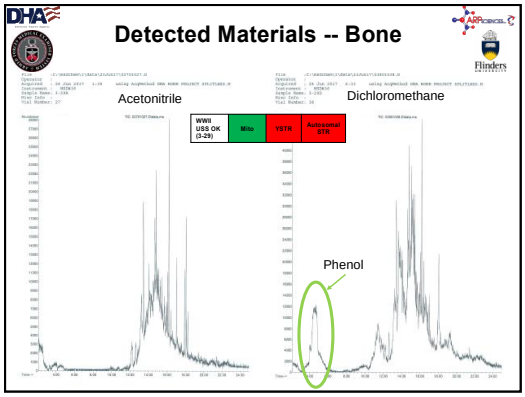




Detected Materials -- Bone



Detected Materials -- Bone



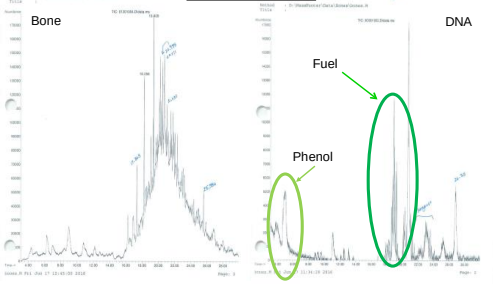
Detected Materials -- DNA

- Mostly products from extraction
  - Butanol
  - Phenol
- There is some carry-over from the samples themselves.

Detected Materials -- DNA

WWII USS OK (3-7) | Mito | YSTR | Autosomal STR

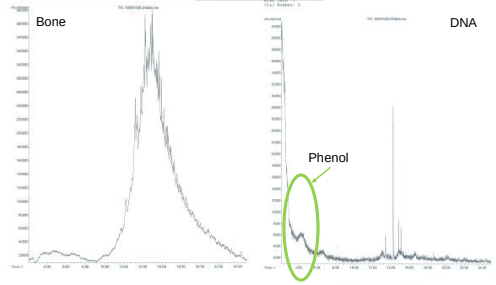
Organic Extraction



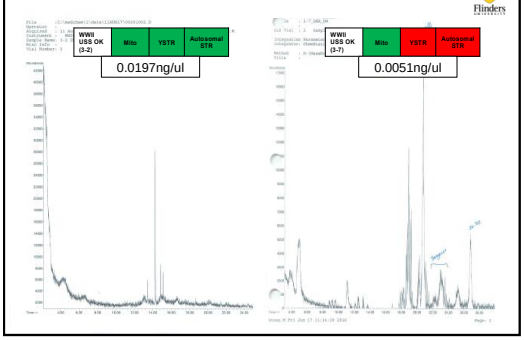
Detected Materials -- DNA

WWII USS OK (3-2) | Mito | YSTR | Autosomal STR

Organic Extraction

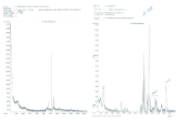


Detected Materials -- DNA



Detected Materials -- DNA

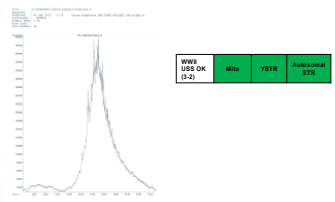
- What is the trend?
  - The bone samples look similar, but the DNA samples worked differently.
  - Is it the quality of the extraction, or the quality of the bone?
- Need additional samples from different locations to build a more robust model.



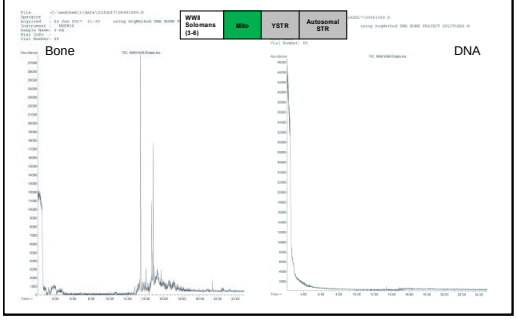
What can we learn from this data?

Are the components of the bone a predictor of success?

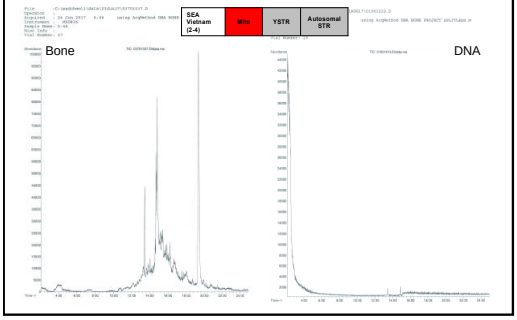
- A large number of fats in the bone may indicate DNA testing will go well.



Are the components of the bone a predictor of success?

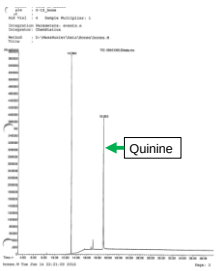


Are the components of the bone a predictor of success?

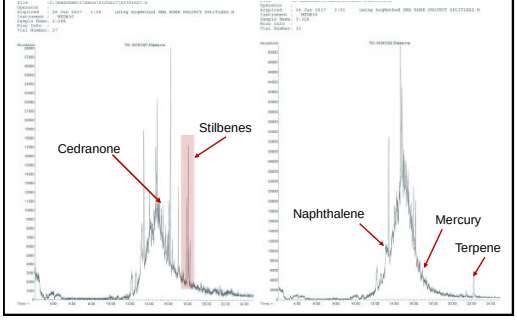


Are the components of the bone a predictor of an individual?

- Fat-soluble medications can be detected in the bones
- Components from the environment can be detected.
- GC/MS has been used to predict location of origin in plants



Are the components of the bone a predictor of an individual?



What does this all mean?

- There is an enormous wealth of data to be found within the dried skeletal remains themselves.
  - Provides information on DNA testing.
  - Provides information on the location of recovery and possible individually identifiable information.

Continuing Work

- Complete loading and analysis of the initial 435 bone and DNA pairs.
- Comparative analysis of the same fractions loaded on different platforms
  - Shimadzu GC/MS
  - Agilent GC/MS
  - Thermo Fisher Q-Exactive Plus
- Evaluation of extraction protocols for efficiency.

Modeling of Data Continues

- More data = A better model
- Determination of materials that are present in every sample
  - Although it is important to note when they are not there.
- What trends can be elucidated?
  - Is there a predictor of DNA success?
  - Can data aid in identification?

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- FTI – Jon Norris
- DPAA – Stephanie Ah Sam, Dr. Greg Berg, Dr. Alec Christensen, Dr. Laurel Freas
- NIST – Dr. Mike Coble
- ARP Sciences, LLC – Cynthia Thomas
- And everyone who said it would work….

….and those that said it wouldn't.

Any Questions?

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AFMES Additional Information: http://www.health.mil/afmes

DPAA Additional Information: http://www.dpaa.mil/

Chapter 8

The Presence of Inhibitory Materials in DNA from Skeletal Remains: Is it the Sample or the Extractor?

8.1 INTRODUCTION

From the results that were presented in Chapter 7, there is clear evidence of materials absorbing into skeletal material during decomposition of the fleshy remains. The compounds present consist of presumptive biological compounds from the remains themselves (i.e., fats and esters), materials from the environment (i.e., plant materials, DDT), and compounds from the event in which the individual perished (i.e., fuel oil, explosive residue). Even samples recovered more than 70 years post-mortem showed evidence of many of these compounds.

What remains to be seen is how the compounds that carry-over effect the recovery of DNA from the osseous materials or the downstream processing of DNA. Many studies have shown that inhibitors are not efficiently removed from liquefied skeletal or other biological materials. Kemp, et al. (2014) studied aged salmonid vertebrae recovered from archaeological sites in Canada. Their tests indicated that while there were inhibitors present in the bone itself, they could be efficiently removed during the extraction. However, the 'cleanliness' of the extract came at a cost. With increase in washes through a silica column, came an increase in the loss of DNA. Up to 60% of the DNA present had been lost. Moreno and McCord (2016) used DART (Direct Analysis in Real Time) coupled with AccuTOF to detect the carry-over of known inhibitors (e.g., phenol, EDTA, bile salts) spiked into blood samples. Not only did they detect carry-over of phenol, but the other inhibitors as well.

It is accepted within the forensics community that there is this carry-over from the parent material and the extraction protocol itself. But does it really occur? The following study seeks to evaluate DNA recovered from the real-world, forensic case samples presented in the previous chapter. A GC/MS protocol was used to examine DNA extracted during the course of regular casework by scientists who perform this task on almost a daily basis. The

compounds detected in both inorganic and organically purified DNA from skeletal remains and teeth are shown and the potential impact of such materials are evaluated.

8.1.1 INTRODUCTION REFERENCES

Kemp BM, C Monroe, KG Judd, E Reams, C Grier. 2014. Evaluation of methods that subdue the effects of polymerase chain reaction inhibitors in the study of ancient and degraded DNA. Journal of Archaeological Science 42:373-380.

Moreno LI, BR McCord. 2016. The use of direct analysis in real time (DART) to assess the levels of inhibitors co-extracted with DNA and the associated impact in quantification and amplification. Electrophoresis 37(21):2807-2816.

8.2 TITLE PAGE

The Presence of Inhibitory Materials in DNA from Skeletal Remains: Is it the Sample or the Extractor?

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8.2.1 ACKNOWLEDGEMENTS

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8.2.2 DISCLAIMER

The opinions or assertions presented are the private views of the author and should not be construed as official or as reflecting the views of the Department of Defense; the Defense Health Agency; the Armed Forces Medical Examiner System; or the Defense POW/MIA Accounting Agency.

8.2.3 FINANCIAL DISCLOSURE

The author received no monies in the completion of this work other than a salary as an employee.

8.3 ABSTRACT

Carry-over of exogenous and endogenous materials from a parent osseous sample into a DNA extract has been cause for concern in forensic DNA analysis. DNA extraction protocols have been designed to remove as many inhibitors as possible, while maintaining an acceptable level of recovered DNA. A number of studies have been done to examine samples spiked with inhibitors and specific collections of osseous samples. However, few studies have been done to evaluate what materials and compounds carry-over to extracted DNA in real-world situations. This study presents the results of the gas chromatography / mass spectrometry (GC/MS) analysis of 412 DNA samples extracted during the course of regular casework. The laboratory processing the remains performs, on average, four to six thousand DNA extractions from skeletal materials in the course of a single year and 200 – 650 in a single month. The goal of this study was to provide information on the co-extraction of materials endogenous to the osseous materials, as well as elements of the extraction protocol itself, to provide guidance on optimizing DNA extraction techniques. However, the results would seem to show that there is little to no co-extraction of chemical compounds, thereby indicating that the extraction protocols are highly efficient.

8.4 KEY WORDS

DNA; skeletonized human remains; DNA extraction; inhibition; GC/MS

8.5 INTRODUCTION

Skeletonized human remains continue to be a challenge for forensic analysts world-wide. In the United States alone, there are over 100,000 sets of unidentified remains currently in storage at medical examiners offices or buried unnamed (NCIC 2018). This number may not include the 500-1000 migrants lost along the US/Mexico border each year, where the remains are often fully skeletonized prior to discovery (Baker 2019). In truth, this is not a problem isolated to the US. Mass graves or large assemblages of unidentified persons created during times of conflict/genocide (Varas and Leiva, 2012; Ríos, et al., 2012; Tyner, 2014; Baeta, et al., 2015), disaster (Morgan, et al., 2006; Hartman, et al., 2011), human trafficking (Noor, et al., 2017), migration (Cattaneo, et al., 2015; Kovras and Robins 2016) or famine/disease (Gerber and Larsen 2015) may involve immeasurable numbers of commingled and skeletonized remains. In many of these events, DNA from the remaining materials may the only manner by which identifications can be made (Primorac, 2004; Marjanović, et al., 2007; Goodwin, 2017; Ossowski, et al., 2017).

Skeletonized human remains are often exposed to a wide variety of insults during decomposition that may impact downstream DNA testing. The circumstances surrounding the death may cause a decreased recovery of DNA. Exposure of the remains to high heat through burning (Tsuchimochi, et al., 2002; Zgonjanin, et al., 2015) or even simple long-term burial at elevated ambient temperatures (Smith, et al., 2003; Nieves-Colón, et al., 2018) may reduce or destroy the endogenous DNA. Materials within the burial environment or on the person at time of death may inhibit PCR or reactions involving fluorescent dyes. Known PCR inhibitors include heme (Akane, et al., 2004), humic acid (Braid, et al., 2003), and melanin (Eckhart, et al., 2000). Indigo dye, while frequently cited as an inhibitor to PCR, is more likely to prevent efficient fluorescent detection of labeled materials due to the dye itself interacting with laser detection systems (Opel, et al., 2010).

Studies using gas chromatography/mass spectrometry (GC/MS) have shown that osseous materials may accrete compounds from both the body and the surrounding environment (Edson 2017; Edson and McMahon 2019), which may carry-forward into the DNA itself, as there is thought to be a marked co-extraction of both inhibitors and the chemicals used in extraction (i.e., phenol). Kemp, et al. (2014) examined ancient salmonid vertebrae and found a transfer of inhibitors from the bone to the DNA extract. Studies done by Barta, et al. (2014) on seal ribs likewise found a transfer of inhibitors under certain circumstances. More recently, Moreno and McCord (2016) spiked blood samples with indigo, phenol, EDTA, bile salts, melanin, and tannic acid and found that there was a distinct carry-over of both phenol and other inhibitors into the extracted DNA.

The belief that inhibitory materials co-extract with DNA has led to modification of downstream testing strategies. Laboratories have implemented a variety of more sensitive testing modalities including reducing amplicon size (Gabriel, et al., 2001; Sprecher, et al., 2009; Welch, et al., 2011), increasing the amount of polymerase added to a reaction (Sturk, et al., 2009), or increasing the number of PCR cycles (Petricevic, et al., 2010). In addition, new methodologies have been developed to avoid traditional Sanger sequencing of mitochondrial DNA (mtDNA) or STR analysis (i.e., mtDNA SNPs: Palencia-Madrid, et al., 2019; XSTR: Prieto-Fernandez, et al. 2015, Diegoli, et al., 2016; NGS: Marshall, et al., 2017).

However, few studies have examined how to improve DNA extractions. As a field, forensics has a decided lack of consistency among protocols for the extraction of DNA from osseous materials. Even when the protocols have been designed from the same parent protocol, there are differences in volume of bone used or even the components of the extraction buffer itself (Edson 2019b). While there is an understood goal in the field to extract the greatest volume

of high-quality DNA possible with the fewest co-extracting inhibitors, there is a lack of understanding as to which inhibitors actually co-extract in real-world situations. The purpose of the following study was to use GC/MS to examine DNA extracted from real-world osseous materials and determine which compounds, if any, transfer from the remains to the extracted DNA.

8.6 METHODS AND MATERIALS

Samples included in this testing were submitted by the Defense POW/MIA Accounting Agency (DPAA) to the Armed Forces Medical Examiner – Armed Forces DNA Identification Laboratory (AFMES-AFDIL, aka AFDIL) in the course of typical casework. The DNA tested was extracted from these osseous or tooth materials in the manner described below. While genetic data were recovered from these remains, none of that information is included here, as it is only relevant to this study inasmuch as confirming whether the samples came from different individuals.

The DNA samples chosen for GC/MS analysis are associated with 426 bone and tooth samples randomly chosen for GC/MS testing. Some of the data generated from these skeletal elements are presented in previous publications (Edson, 2017; Edson and McMahon, 2019; Edson and Roberts, *in preparation*). All testing was completed on the DNA extracted from these remains prior to being used for GC/MS analysis so as not to interfere with casework. In some instances, the DNA extract was exhausted at the completion of the case and therefore those samples are not included in this study.

8.6.1 Sample Preparation

DNA was extracted from osseous and tooth material collected from a variety of locations world-wide. The skeletal elements range in age from 45 to 75 years post-mortem. The

extraction protocols used are described in detail in Edson and McMahon (2016) and Edson (2019a,b) and are briefly summarized here. Details with regards to the cleaning of the osseous materials are mentioned as it was expected to be relevant to what might carry-over into the DNA extract.

8.6.1.1 Skeletal Materials

A portion of an intact skeletal element was submitted to the AFMES-AFDIL laboratory from DPAA. This portion was cleaned thoroughly using a foot-pedal operated Dremel® tool (Bosch, Stuttgart, Germany) and the appropriate aluminum oxide sanding bit. Approximately 0.2 g of this parent sample was removed for further cleaning by agitation in a 50 mL Falcon™ conical polypropylene tube (Corning, Corning, NY) containing deionized water (diH_2O). Water washes were repeated until the liquid appeared largely clear. A final wash was undertaken with 100% (v/v) ethanol (Pharmco, Greenfield Global, Brookfield, CT) and the sample allowed to air dry at room temperature. Following cleaning, the bone sample was pulverized using a MC2 Waring® blender cup (Waring, Torrington, CT).

8.6.1.2 Teeth

Intact teeth were cleaned by agitation in a 50 mL Falcon™ conical polypropylene tube containing 8.5% (v/v) bleach. Following agitation, the teeth were wiped clean with a 4 x 4 cm gauze pad moistened with 8.5% (v/v) bleach and a second gauze pad moistened with 100% (v/v) ethanol (Pharmco). If the tooth was cracked, had extensive caries, or was restored, the agitation step was eliminated and the tooth was simply wiped clean with the gauze pads. Once cleaned, the tooth was allowed to dry under a UV light for approximately 15 minutes.

The crown was removed using a dental hand-tool (Brasseler USA, Savannah, GA) and the appropriately sized bur. The powder generated from the removal of the crown was retained for possible future extractions, but was not included in the powder used for standard casework testing. The interior dentine of the tooth was removed using a straight bur attached to the dental hand-tool.

8.6.2 DNA Extraction

The bone or tooth powder was placed in a 15 mL polypropylene conical tube containing 3.0 mL demineralization buffer (0.5 M EDTA, pH 8.0; 1% *N*-Lauroylsarcosine) and 100 µL Proteinase K (200 mg/mL). Samples were incubated overnight at 56°C with agitation.

Purification took place using either an inorganic or organic method. Using the organic pathway, the liquefied material was subjected to 2 - 3 washes of phenol:chloroform:isoamyl alcohol (PCIA) (25:24:1) followed by a single wash using *n*-Butanol. In the inorganic pathway, samples were purified using the QIAquick PCR purification Kit (QIAGEN, Hilden, Germany). Both methods involved a concentration of the liquid fraction using Amicon Ultra-4/30K centrifugal filter units (Millipore, Billerica, MA). In the organic pathway, the liquid fraction was concentrated after the PCIA and *n*-Butanol washes. In the inorganic pathway, the liquid fraction was concentrated prior to the QIAquick purification. In both pathways, the purified DNA extract was brought to a final volume of 50 - 200 µL with TE^{-4} (10 mM Tris, 0.1 mM EDTA; pH 7.5).

8.6.3 Selection of DNA Extracts for Testing

DNA samples were assigned a code corresponding to that of the osseous samples tested. This was designed to eliminate possible bias in analysis. The osseous materials were assigned a

number representing when the sample was collected over a four-month period. For example, the samples collected in the first two weeks of collection were numbered as "1-XX". The number after the dash would be simply the order in which the samples were randomly drawn from that group of samples (e.g., 1-1 was the first sample chosen). Tooth samples are labeled with a "T" in front of the first number (e.g., T1-1).

Supplemental Table 8.1 lists all of the samples tested, the location from which the originating bone sample was recovered, and the DNA extraction protocol used. As mentioned, DNA extracts were used only after the required casework analysis was completed. Therefore, not all of the samples designated for testing were available. In total, 412 samples were examined in GC/MS.

8.6.4 GC/MS Parameters

In preparation for GC/MS analysis, a portion of the DNA extract was suspended in methanol (≥99.9%, HPLC grade, Sigma-Aldrich, St. Louis, MO, USA). The amount of both varied, depending on the test being done, with an increase in the amount of DNA being used, and a corresponding decrease in the amount of methanol. These modifications were undertaken in an attempt to increase the probability of detecting inhibitors present in the DNA extract. Modifications to the strategies are described in Table 8.1.

Table 8.1. The volumes of DNA and methanol used for GC/MS analysis of DNA extracts. Glass autosampler inserts were used for all of the runs described in this paper; however, the initial proof of concept paper did not use these.

Volume of DNA (µL)	**Volume of Methanol (µL)**	**Ratio**	**Number of Samples**
10	250	1:25	115
10	150	1:15	17
20	150	1:7.5	103
10	100	1:10	5
20	100	1:5	172

All samples were prepared in 9 mm glass vials (Thermo Fisher, Walther, MA, USA) with crimp caps. The original work presented in Edson and Roberts (*in preparation*), used 10 µL of DNA extract in 500 µL of methanol. In order to increase the probability of recovering a chemical profile from the DNA samples, glass autosampler inserts were added to the vial, to allow for less than 500 µL of total volume to be tested.

Analysis was performed using an Agilent 7890A/5875C GC/MS System with a 20 m column (Agilent, Santa Clara, CA) with a full scan and no subtraction of possible known elements. The starting oven temperature was 150°C, and remained at that temperature for a 20 minute hold before ramping at 20°C / min to 250°C for an additional 30 minute hold. The injection was splitless with no subtraction for "known" elements. A splitless injection involves injecting the entire fraction of the injected portion rather than splitting the injection. A splitless injection has the potential to damage the column if the materials being tested include particulates; however, it also provides the greatest chance of detecting low-levels of materials.

After the initial run, DNA samples were loaded on the instrument independent of the associated osseous samples to reduce the chance of carry-over during the run. Samples were not loaded in order of their code number, rather they were randomly placed on the instrument. A total of 412 runs were performed with DNA extractions suspended in methanol, 236 of which were DNA extractions generated using an organic purification.

8.6.5 GC/MS Analysis

Analysis was performed using ChemStation and MassHunter (Agilent). Comparison was made to NIST2011 Spectral Library. From previous work (Edson 2017; Edson and McMahon 2019; Edson and Roberts, *in preparation*), it was expected that the instrument

would not automatically call detectable peaks given the overall quality of the trace. The analyst manually selected the peaks for analysis, and determined which compounds were present based on comparison of the trace generated to the spectral library.

8.7 RESULTS

There was very little carry-over observed in any of the injections of extracted DNA (Figure 8.1). The most commonly seen material was tromethamine, also known as Tris ($C_4H_{11}NO_3$), which is to be expected given the addition of TE to the final volume of extracted DNA. Of the 412 GC/MS injections performed, 296 (71.8%) contained detectable levels of Tris. Tris was more commonly seen in organically extracted DNA samples (205 of 236 vs. 91 of 176).

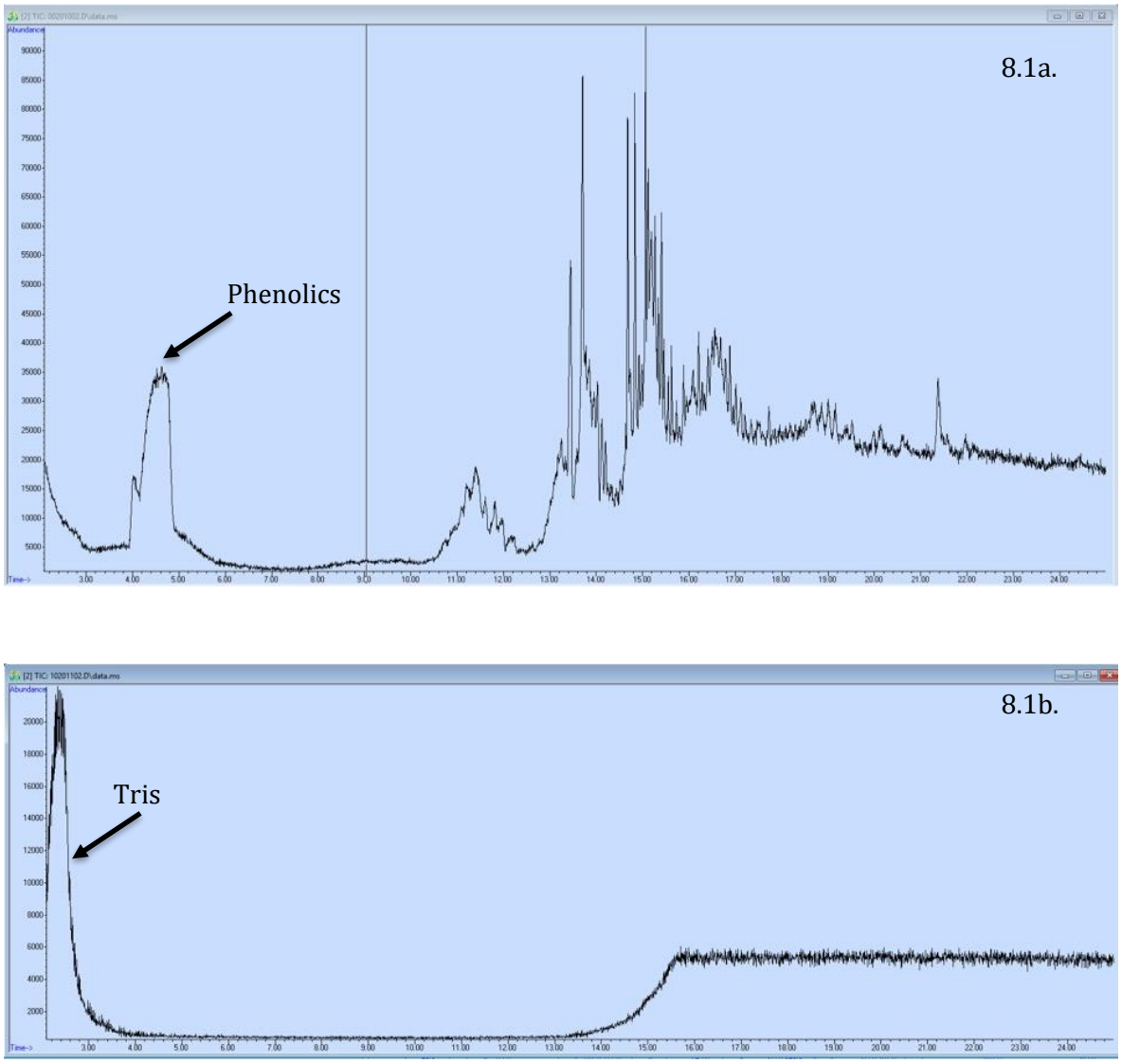

Figure 8.1. An example of a lack of carry-over from the associated bone sample. Figure 1a is the trace generated from a dichloromethane extract of a bone sample recovered from the USS *Oklahoma* (Sample 6-32). The peaks in the center are primarily fats and components of fuel. The poorly defined peak at the beginning of the trace are phenolics derived from either the fuel or putrefaction. Figure 1b is the DNA extracted from that bone sample.

The compounds observed in both organic and inorganic extractions are somewhat similar (Tables 8.2 and 8.3). There was almost no carry-over from the extraction process itself, with only four organically extracted samples showing evidence of the presence of phenol (Figure

8.2) and no inorganically extracted samples containing guanidinium or other compounds from the QIAquick kit. Some minimal carry-over of fats or environmental material was observed in both extraction protocols (Figures 8.3 and 8.4). There is a great deal of silica-based materials found in the extracts, although this is most likely due to either the GC/MS column or the sanding wheel, as they are found in both organic and inorganic DNA extracts.

Samples injected at lower ratios of DNA to methanol exhibited mainly background or an elevated baseline (Figure 8.5). While there appear to be peaks present, these may be due to the excessive noise within the background. During analysis, the software used (ChemStation) assigns a value to a potential call of 1-100. The larger the value, the more likely the identity of the designated compound. Those visible peaks in Figure 8.5 scored less than 12, and are therefore unlikely to be authentic.

Table 8.2. Compounds detected in organically extracted DNA samples. Compounds with a ChemStation score of less than 20 are not included. Most scores were in the 80's and 90's. The number of injections where the compound was seen is included in the final column. 236 individual DNA extracts generated from osseous materials using an organic purification method were tested. Compounds marked with a "*" were seen in both types of DNA extracts.

Compound	**Formula**	**Descriptor**	**# Occurrences**
*Tromethamine	C4H11NO3	Tris – from the final suspension of DNA	205
*Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	C16H50O7Si8	Possible column component or from environment	31
*Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	C14H44O6Si7	Possible column component or from environment	11
*Octadecanoic Acid, methyl ester	C19H38O2	Fatty acid	9
Cyclotrisiloxane, hexamethyl-	C6H18O3Si3	Possible column component or from environment	7
*Hexadecanoic acid, methyl ester	C17H34O2	Fatty acid	7
*Silicic acid, diethyl bis (trimethylsilyl) ester	C10H28O4Si3	Fatty acid	7
*Tetrasiloxane, decamethyl-	C10H30O3Si4	Possible column component or from environment	7
4,4'-(Hexafluoroisopropylidene)diphenol	C15H10F6O2	Phenol	4
*Oxalic acid, 6-ethyloct-3-yl propyl ester	C15H28O4	Fatty acid	4
*Tetrasiloxane, 1,7-diallyoctadecyl-	C14H34O3Si4	Possible column component or from environment	4
*Heptadecanoic acid, 16-methyl-, methyl ester	C19H38O2	Fatty acid	3
*(5-Isopropyl-2-methylphenoxy)trimethylsilane	C13H22OSi	May be related to the instrument	2
*Hexahydropyridine, 1-methyl-4(4,5-dihydroxoyphenyl)-	C12H17NO2	Plant compound	2
Phenol, 4,4'-(1-methylethylidene)bis	C15H16O2	Phenol	2
1,3-Oxathiolane	C3H6OS	nucleoside	1
2-Ethylacridine	C15H13N	hydrocarbon	1
2-Propanol, 1-[2-(2-methoxy-1-methylethoxy)-1-methylethoxy]-	C10H22O4	Solvent	1
3-Ethyl-3-methylheptane	C10H22	Alcohol	1
7,7,9,9,11,11-Hexamethyl-3,6,8,10,12,15-hexaoxa-7,9,11-trisilaheptadecane	C14H36O6Si3	Possible human decomposition by-product	1

Butanoic acid, 4-ethoxy-, methyl ester	C7H14O3	Fatty acid	1
Compound	**Formula**	**Descriptor**	**# Occurrences**
*Decane, 1,1'-oxybis-	C20H42O	Possible plant material	1
Dodecane, 1-iodo-	C12H25I	Possible plant material	1
Dodecanedioic acid, dimethyl ester	C14H26O4	Fatty acids	1
Ethanamine, N-ethyl-N-nitroso	C4H10N2O	Plastics stabilizer or gasoline additive	1
Hexadecanamide	C16H33NO	Fatty acid	1
*Indole-2-one, 2,3-dihydro-N-hydroxy-4-methoxy-3,3-dimehtyl-	C11H13NO3	Aromatic compound; alcohol	1
N-Aminomorpholine	C4H10N2O	Possible fuel additive	1
Nonane, 4,5-dimethyl-	C11H24	Hydrocarbon	1
Pentadecanoic acid, 14-methyl-, methyl ester	C17H34O2	Fatty acid	1
Trimethylsilyl 2-[2-[2-[2-[2-methoxyethoxy)ethoxy)ethoxy)ethoxy)aceate	C14H30O7Si	Possible column component or from environment	1

Table 8.3. Compounds detected in inorganically extracted DNA samples. Compounds with a ChemStation score of less than 20 are not included. Most scores were in the 80's and 90's. The number of injections where the compound was seen is included in the final column. Compounds marked with a "*" were seen in both types of DNA extracts.

Compound	**Formula**	**Descriptor**	**# Occurrences**
Tromethamine	C4H11NO3	Tris – from the final suspension of DNA	91
*Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	C16H50O7Si8	Possible column component or from environment	14
*Hexadecanoic acid, methyl ester	C17H34O2	Fatty acid	9
*Octadecanoic acid, methyl ester	C19H38O2	Fatty acid	9
*Silicic acid, diethyl bis(trimethysilyl) ester	C10H28O4Si3	Fatty acid	4
*Heptadecanoic Acid, 16-methyl- methyl ester	C19H38O2	Fatty acid	3
*Tetrasiloxane, decamethyl-	C10H30O3Si4	Possible column component or from environment	3
*Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	C14H44O6Si7	Possible column component or from environment	2
Methanamine, N-methyl-N-nitro	C2H6N2O2	Primary amine. An ammonia derivative	2
(5-Isopropyl-2-methylphenoxy)trimethylsilane	C13H22OSi	May be related to the instrument	1
1-Nitro-9,10-dioxo-9,10-dihyrdo-anthracene-2-carboxylic acid diethylamide	C19H16N2O5	Aromatic amine	1
1,1,1,3,5,5,5-Heptamethyltrisiloxane	C7H22O2Si3	Catalyst for hydrosilylation	1
3-Ethyl-3-methylheptane	C10H22	Hydrocarbon	1
Butanamide, N-ethyl	C6H13NO	A metabolite of a fatty acid	1
*Decane, 1,1'-oxybis-	C20H42O	Possible plant material	1
*Hexahydropyridine, 1-methyl-4-[4,5-dihydroxyphenyl]-	C12H17NO2	Plant compound	1
*Indole-2-one, 2,3-dihydro-N-hydroxy-4-methoxy-3,3-dimethyl-	C11H13NO3	Aromatic compound; alcohol	1
*Oxalic Acid, 6-ethyloct-3-yl ethyl ester	C15H28O4	Fatty acid	1
*Tetrasiloxane, 1,7-diallyoctadecyl-	C14H34O3Si4	Possible column component or from environment	1
Thieno[2,3-b]pyridine-2-carboxamide, 3-amino-6-methyl-	C9H9N3OS	A kinase inhibitor	1
Thiourea, methyl-	C2H6N2S	Used in the production of flame retardant resins	1
Trans-2,3-Epoxydecane	C10H20O	Possible plant material	1

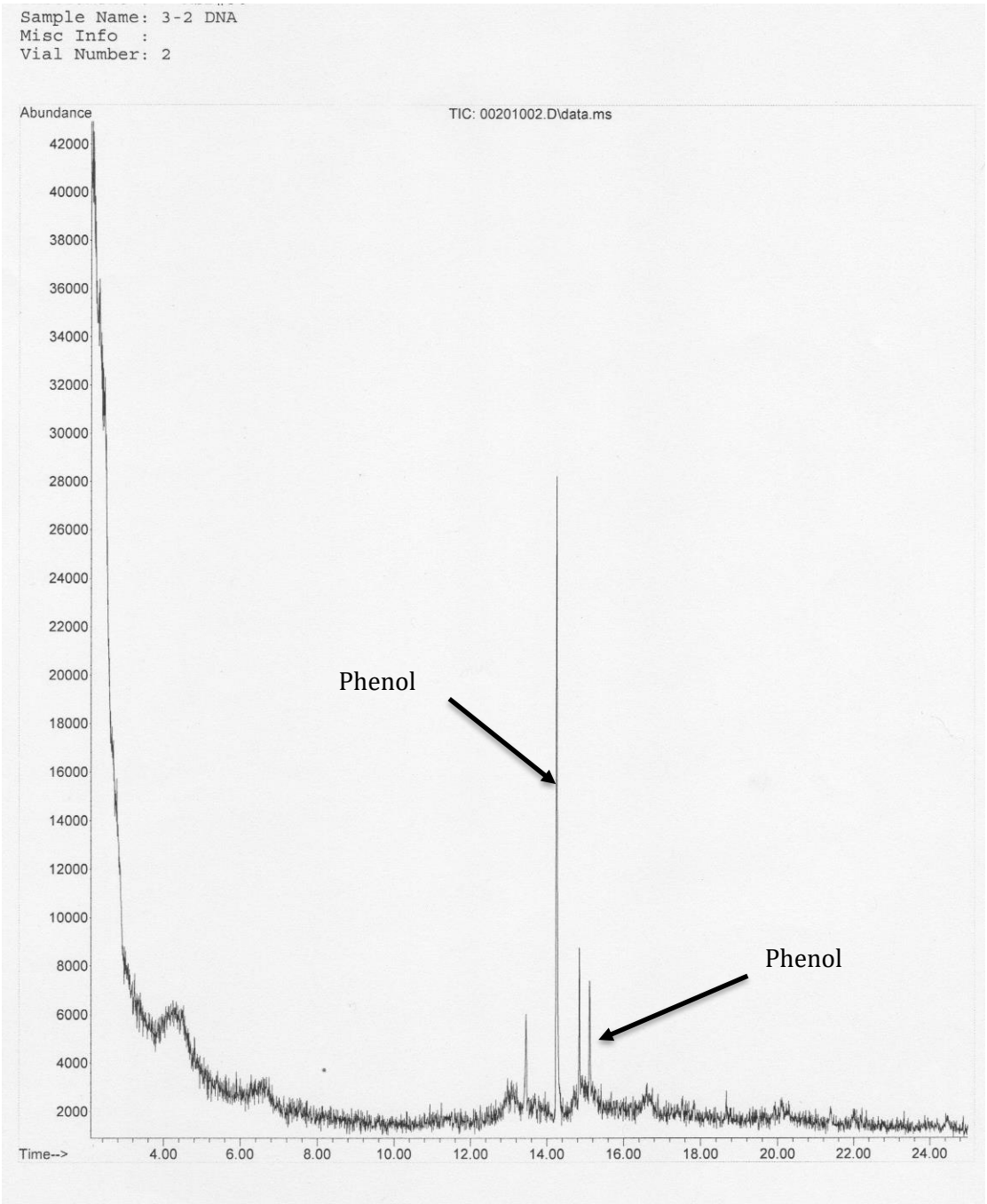

Figure 8.2. An example of phenol carry-over. This the GC/MS trace of a DNA sample extracted from a temporal bone recovered from the USS *Oklahoma* (Sample 3-2 – See Supplemental Table 1). The two marked peaks are two different types of phenol. The peak at the beginning of the trace that is undefined, was not able to be scored by the instrument and was not called; however, in the corresponding osseous samples, it has been shown to be phenol.

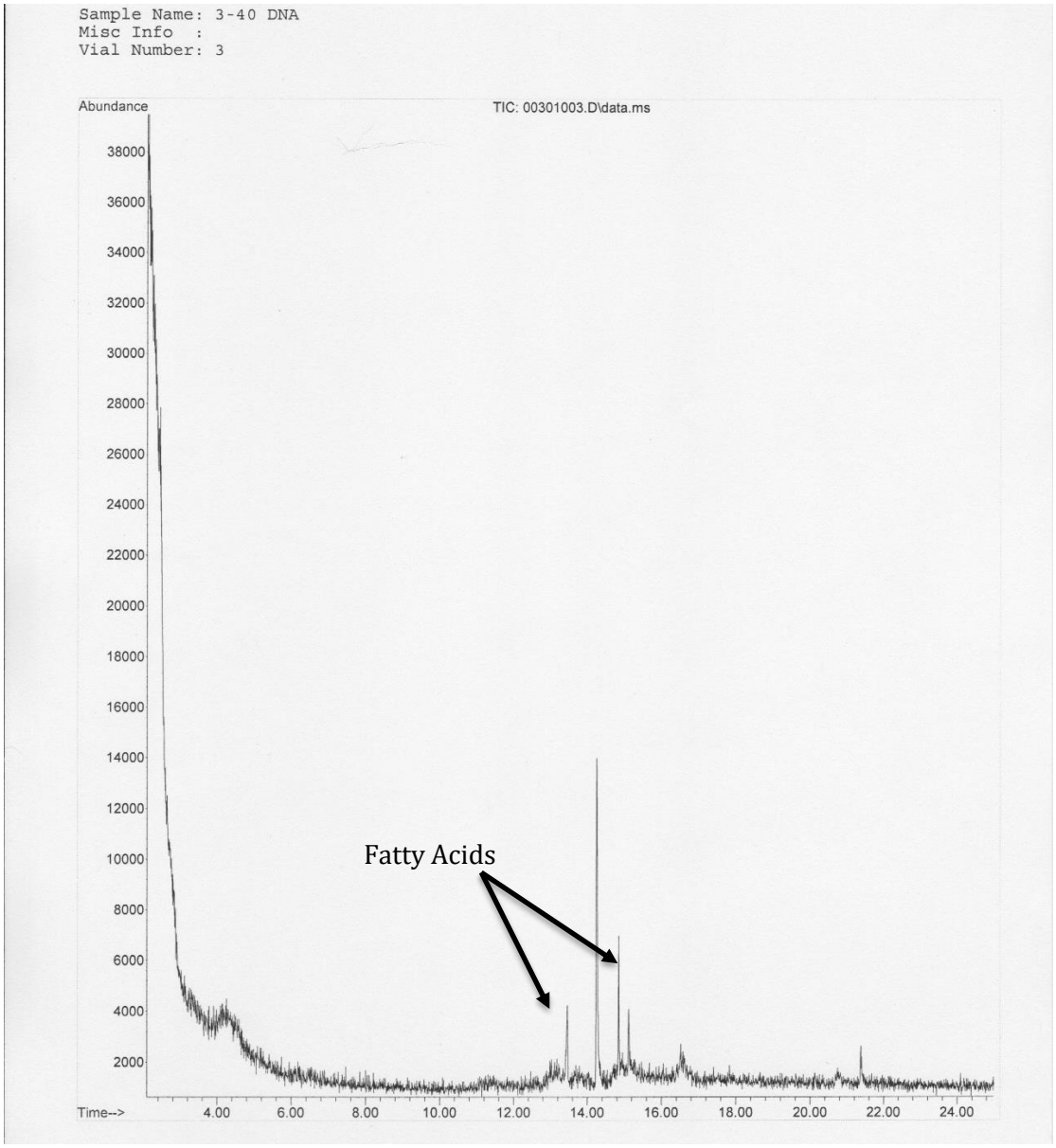

Figure 8.3. Low-level carry-over of fatty acids in a DNA trace extracted using an organic purification. For reference, this is sample 3-40, extracted from a sample recovered from the USS *Oklahoma*. It is very similar to Figure 2, and indeed the peaks are mostly of the same origin.

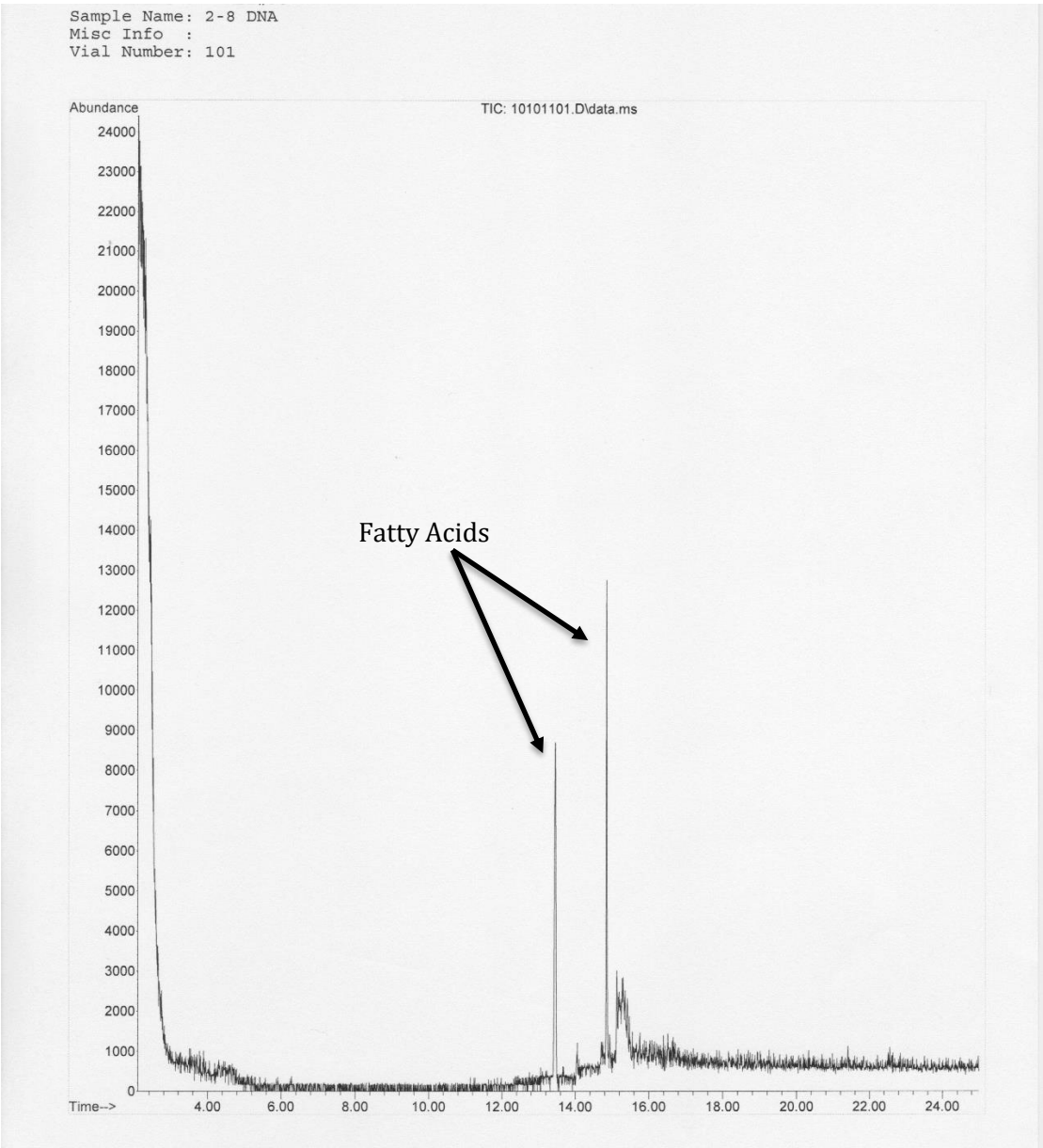

Figure 8.4. An example of carry-over from the osseous sample into the DNA extract. This DNA was extracted using an inorganic purification from a cranial fragment recovered from Southeast Asia (sample 2-8).

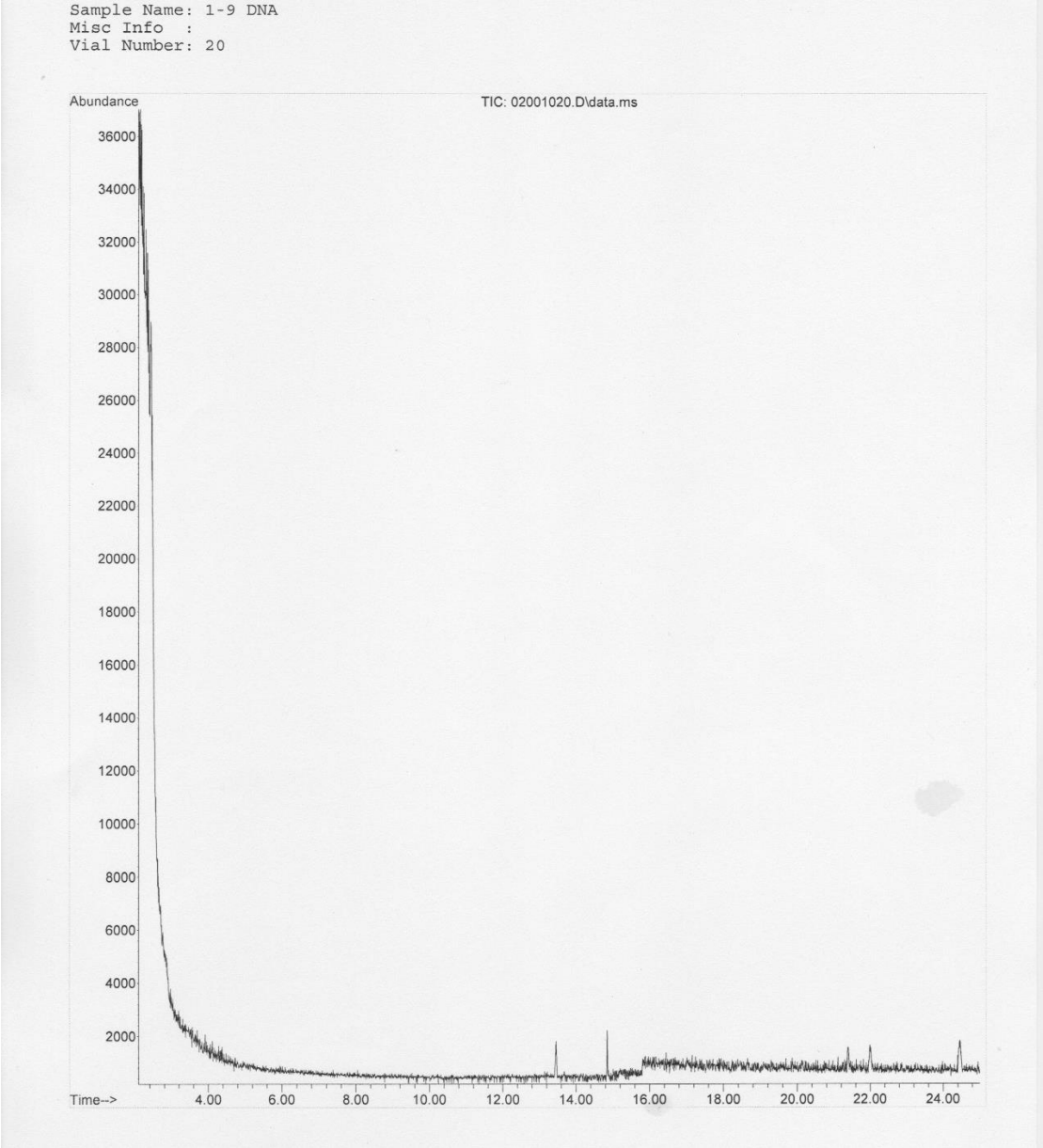

Figure 8.5. An example of a trace with peaks that are too low in signal strength to be called accurately. This sample is a DNA extract recovered with an inorganic purification from a World War II era case in the Solomon Islands (Sample 1-9). The peaks present do not have a ChemStation station score higher than 12.

8.8 DISCUSSION

8.8.1 Carry-Over and Experience

Unlike other papers that have shown carry-over of materials from the osseous elements into the DNA (Akane, et al., 1994; Barta, et al., 2014; Moreno and McCord, 2016;), there appears to be little to no carry-over in the 412 samples examined. For almost all samples examined, tromethamine, or Tris, is the only detectable compound. Given the initial testing presented in Chapter 6 (Edson and Roberts, *in preparation*), there was some expectation that there would be carry-over, particularly from the USS *Oklahoma* samples exhibited elevated levels of fats and oils; however, this was seen to not be the case. Of the 236 DNA organic extracts tested, 202 were from the USS *Oklahoma*. In those samples, fatty acids were observed only 34 times.

There was some degree of carry-over from the kits themselves, with an occasional occurrence of phenol, but this was seen in only four of 236 samples. It appears that the carry-over has more to do with the experience of the analysts performing the extraction. Analysts performing extractions for this set of samples had casework experience ranging from the first day out of training to over seventeen years. Prior to being released into casework, analysts undergo an intensive training involving six to nine months; however, this provides a relatively limited amount of practice at the extraction protocols. Once released to perform casework, an analyst may extract sets of one to four bones or teeth multiple times a week. When studies on inhibition or carry-over are preformed at other laboratories, the extractions may not be performed by individuals who constantly extract DNA from osseous materials. While they are most certainly skilled scientists, they most likely do not have constant exposure to DNA extractions.

The first set of samples extracted by a new analyst is included in this set. Samples at the lab are extracted as either solo samples or up to sets of four. The four samples prepared by this

novice extractor appear markedly different than other samples. All four have almost no detectable Tris and trace rapidly degrades into a simple solvent front (Figure 8.6a). The traces from these samples are different when compared to those of an analyst with 17 years of experience (Figure 8.6b), indicating that there is at least some difference in technique.

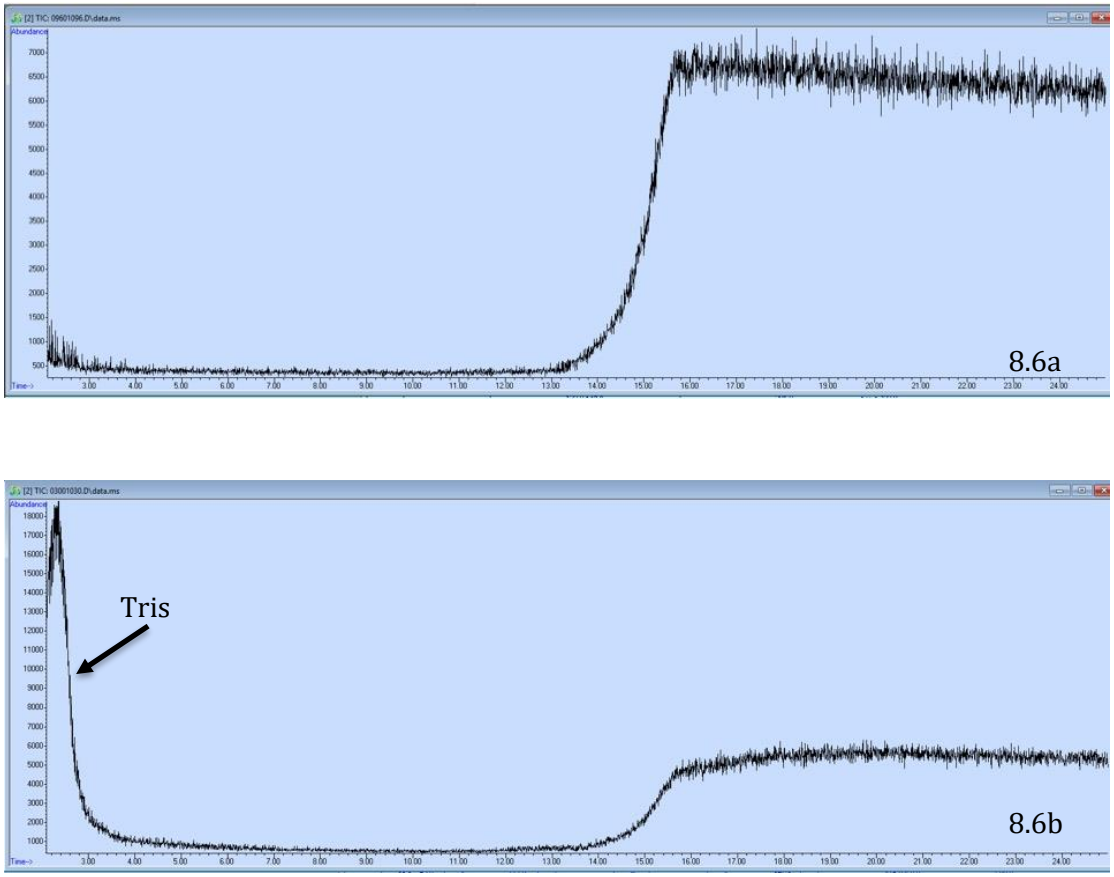

Figure 8.6. A comparison between DNA samples extracted by a novice extractor on their first day of extraction and an extractor with over 17 years of experience. Figure 8.6a is a trace of one of four samples extracted by a novice extractor (Sample 7-29). This sample was from the USS *Oklahoma* and was purified using an organic purification. There is almost no evidence of Tris, and the solvent front is very high. Figure 8.6b is from the same incident but extracted by a more experienced analyst (Sample 4-33). This trace exhibits a high peak of Tris at the beginning of the run and a lower solvent front. Both extracts were prepared with the same ratio of DNA to methanol and were loaded on the instrument on the same day.

8.8.2 Compounds

Samples extracted using an organic purification had 31 detectable compounds across the 236 samples tested. Inorganically purified DNA extracts had 21 detectable compounds across the 176 samples tested. Fourteen of these compounds were seen in both sets of extracts, most of which were fatty acids. It is, of course, possible that the methodology is simply not sensitive enough to detect all of the materials present; however, given the volume of samples tested and that there are samples with detectable fats and phenol, it is unlikely.

It is difficult to determine the source of the compounds present. While they are clearly present in the DNA extract, and therefore came from the osseous sample itself, the extraction procedure, or the instrument, the compounds may have originated from any of those three or the recovery location. For instance, phenol is present in the organic extraction protocol; although it should be noted that this was also present in the fuel in which the USS *Oklahoma* samples were soaked. Further, forms of phenol are also by-products of putrefaction and decomposition. The silicates present in the DNA extract may have come from the column of the GC/MS instrument or the sanding tool. While it is possible that they were derived from the QIAquick column, they are also present in samples extracted without silica columns.

Another example of a material that is difficult to characterize are the decanes, which can be derived from plant materials or different types of fuel. In studies of the osseous materials for identification purposes, it may be relevant to know the specific origin of a material (Edson and McMahon 2019); however, for DNA analysis it is merely necessary to know that the compound is present and whether it might impede downstream DNA testing. It was therefore considered unnecessary for this study to determine the origin of a compound.

8.9 CONCLUSIONS

As a community, forensic scientists have operated under the assumption that the extraction protocols we use are designed to remove all possible inhibitors from the DNA extracted from skeletal remains. Yet we do not have an agreed upon extraction protocol. Researchers and industry groups continue to modify downstream testing protocols based on the assumption that no matter how good the extraction protocol is, there will be materials that co-extract. But what if we're wrong? This current study shows that there is very little or no carry-over from either an organic or inorganic protocol. Phenol was seen infrequently, as were fats and esters from the decomposing body, and quanidinium not at all. The most commonly seen compound was Tris, which was to be expected.

The present study does not examine the carry-over of microbial DNA, which may be the cause of some of the inhibition being seen. However, additional work needs to be done to examine the stringency of the protocols currently in use on skeletonized human remains. It is possible that the current protocols are extremely efficient at the removal of inhibitors, so much so that DNA is also being lost. Perhaps a retooling of the available extraction protocols would permit recovery of greater amount of high quality DNA and improve our overall rates of successful DNA testing.

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Supplemental Table 8.1. List of DNA Samples Tested. Samples recovered from the same conflict and location are not necessarily from the same incident. The 'location' designator is mostly the region or country from which the remains were recovered, unless the incident was large enough to warrant a specific designator. "Korea" refers to the Korean War and "Southeast Asia" refers to the conflict in Southeast Asia, known colloquially as the "Vietnam War". If there are two listings for a sample, the sample was loaded twice to verify results from a previous injection. For the duplicated entries, if the volumes of DNA and methanol do not change, the same fraction was re-injected.

Sample	Conflict	Location Recovered	Skeletal Element	Extraction Protocol	Volume of DNA Extract (µL)	Volume of MeOH (µL)
1-1	Southeast Asia	Cambodia	Thoracic Vertebra	Inorganic	10	250
1-2	Southeast Asia	Cambodia	Temporal	Inorganic	10	250
1-3	World War II	Solomon Islands	Humerus	Inorganic	10	250
1-3	World War II	Solomon Islands	Humerus	Inorganic	10	250
1-4	World War II	Solomon Islands	Metatarsal	Inorganic	10	250
1-4	World War II	Solomon Islands	Metatarsal	Inorganic	10	250
1-5	World War II	Solomon Islands	Fibula	Inorganic	10	250
1-5	World War II	Solomon Islands	Fibula	Inorganic	10	250
1-6	World War II	Solomon Islands	Long Bone	Inorganic	10	250
1-7	World War II	Solomon Islands	Long Bone	Inorganic	10	250
1-8	World War II	Solomon Islands	Long Bone	Inorganic	10	250
1-9	World War II	Solomon Islands	Long Bone	Inorganic	10	250
1-10	World War II	Solomon Islands	Long Bone	Inorganic	10	250
1-11	World War II	Solomon Islands	Fibula	Inorganic	10	250
1-12	World War II	Solomon Islands	Long Bone	Inorganic	10	250
1-13	World War II	Solomon Islands	Parietal	Inorganic	10	250
1-14	World War II	Solomon Islands	Humerus	Inorganic	10	250
1-15	World War II	Solomon Islands	Femur	Inorganic	10	250
1-16	World War II	Solomon Islands	Humerus	Inorganic	10	250
1-17	World War II	Solomon Islands	Tibia	Inorganic	10	250
1-18	World War II	Kiribati	Metatarsal	Inorganic	10	250
1-19	World War II	Kiribati	Fibula	Inorganic	10	250
1-20	World War II	Kiribati	Fibula	Inorganic	10	250
1-21	Korea	Namjong-gu	Clavicle	Inorganic	10	250
1-23	Southeast Asia	Cambodia	Clavicle	Inorganic	10	250
1-24	Southeast Asia	Cambodia	Clavicle	Inorganic	10	250
1-25	Southeast Asia	Vietnam	Cranium	Inorganic	10	250
1-26	Southeast Asia	Vietnam	Cranium	Inorganic	10	250
1-27	Southeast Asia	Vietnam	Cranium	Inorganic	10	250
1-28	Southeast Asia	Vietnam	Cranium	Inorganic	10	250
1-29	World War II	Solomon Islands	Clavicle	Inorganic	10	250
1-30	World War II	Solomon Islands	Clavicle	Inorganic	10	250
1-31	World War II	Solomon Islands	Clavicle	Inorganic	10	250
1-32	World War II	Solomon Islands	Clavicle	Inorganic	10	250
2-1	Southeast Asia	Laos	Femur	Inorganic	10	250
2-2	World War II	Philippines	Occipital	Inorganic	10	250
2-3	Southeast Asia	Vietnam	Cranium	Inorganic	10	250
2-4	Southeast Asia	Vietnam	Parietal	Inorganic	10	250

Sample	Conflict	Location Recovered	Skeletal Element	Extraction Protocol	Volume of DNA Extract (µL)	Volume of MeOH (µL)
2-5	Southeast Asia	Laos	Frontal	Inorganic	10	250
2-6	World War II	Kiribati	Thoracic Vertebra	Inorganic	10	250
2-7	World War II	Solomon Islands	Occipital	Inorganic	10	250
2-7?	World War II	Solomon Islands	Occipital	Inorganic	10	250
2-8	Southeast Asia	Laos	Cranium	Inorganic	10	250
2-8	Southeast Asia	Laos	Cranium	Inorganic	10	250
2-9	Southeast Asia	Laos	Long Bone	Inorganic	10	250
2-10	Southeast Asia	Vietnam	Cranium	Inorganic	10	250
2-11	World War II	Papua New Guinea	Humerus	Inorganic	10	250
2-12	World War II	Papua New Guinea	Lumbar Vertebra	Inorganic	10	250
2-13	Southeast Asia	Laos	Rib	Inorganic	10	250
2-13	Southeast Asia	Laos	Rib	Inorganic	10	250
2-14	Korea	South Korea	Femur	Inorganic	10	250
2-15	Korea	South Korea	Tibia	Inorganic	10	250
2-16	Southeast Asia	Laos	Radius	Inorganic	10	250
2-17	Southeast Asia	Laos	Long Bone	Inorganic	10	250
2-19	Southeast Asia	Laos	Long Bone	Inorganic	10	250
2-20	Southeast Asia	Laos	Long Bone	Inorganic	10	250
2-21	Southeast Asia	Laos	Ulna	Inorganic	10	250
2-22	Southeast Asia	Laos	Long Bone	Inorganic	10	250
2-23	Southeast Asia	Laos	Long Bone	Inorganic	10	250
2-24	World War II	Kiribati	Tibia	Inorganic	10	250
2-25	World War II	Kiribati	Fibula	Inorganic	10	250
2-26	World War II	Kiribati	Humerus	Inorganic	10	250
2-27	World War II	Philippines	Femur	Inorganic	10	250
2-28	World War II	Papua New Guinea	Fibula	Inorganic	10	250
2-29	World War II	Solomon Islands	Cervical Vertebra	Inorganic	10	250
2-30	World War II	Solomon Islands	Fragment	Inorganic	10	250
3-1	Korea	South Korea	Temporal	Inorganic	10	250
3-2	World War II	USS Oklahoma	Temporal	Organic	10	250
3-3	Southeast Asia	Vietnam	Cranium	Inorganic	10	250
3-4	World War II	Solomon Islands	Os Coxa	Inorganic	10	250
3-5	Korea	North Korea	Occipital	Inorganic	10	250
3-6	World War II	Solomon Islands	Sacrum	Inorganic	10	250
3-7	World War II	USS Oklahoma	Lumbar Vertebra	Organic	10	250
3-8	Southeast Asia	Vietnam	Cranium	Inorganic	10	250
3-9	Southeast Asia	Vietnam	Cranium	Inorganic	10	250
3-10	World War II	USS Oklahoma	Temporal	Organic	10	250
3-11	World War II	Solomon Islands	Os Coxa	Inorganic	10	250
3-12	World War II	Solomon Islands	Os Coxa	Inorganic	10	250
3-13	Korea	South Korea	Ulna	Inorganic	10	250
3-13	Korea	South Korea	Ulna	Inorganic	10	250
3-14	Korea	South Korea	Humerus	Inorganic	10	250
3-15	World War II	USS Oklahoma	Radius	Organic	10	250
3-16	Southeast Asia	Cambodia	Cranium	Inorganic	10	250
3-17	Southeast Asia	Cambodia	Cranium	Inorganic	10	250
3-18	World War II	Italy	Mandible	Inorganic	10	250

Sample	Conflict	Location Recovered	Skeletal Element	Extraction Protocol	Volume of DNA Extract (µL)	Volume of MeOH (µL)
3-19	World War II	Italy	Mandible	Inorganic	10	250
3-20	World War II	Italy	Cranium	Inorganic	10	250
3-21	Southeast Asia	Vietnam	Cranium	Inorganic	10	250
3-22	Southeast Asia	Cambodia	Rib	Inorganic	10	250
3-23	World War II	USS Oklahoma	Femur	Organic	10	250
3-24	World War II	USS Oklahoma	Humerus	Organic	10	250
3-25	Korea	Unknown	Humerus	Inorganic	10	250
3-26	World War II	Solomon Islands	Cranium	Inorganic	10	250
3-27	World War II	Solomon Islands	Cranium	Inorganic	10	250
3-28	World War II	USS Oklahoma	Humerus	Organic	10	250
3-29	World War II	USS Oklahoma	Rib	Organic	10	250
3-30	World War II	USS Oklahoma	Occipital	Organic	10	250
3-31	World War II	USS Oklahoma	Humerus	Organic	10	250
3-32	World War II	USS Oklahoma	Radius	Organic	10	250
3-33	World War II	USS Oklahoma	Lumbar Vertebra	Organic	10	250
3-34	World War II	USS Oklahoma	Rib	Organic	10	250
3-35	World War II	USS Oklahoma	Humerus	Organic	10	250
3-36	World War II	USS Oklahoma	Occipital	Organic	10	250
3-37	World War II	USS Oklahoma	Humerus	Organic	10	250
3-38	World War II	USS Oklahoma	Radius	Organic	10	250
3-39	World War II	USS Oklahoma	Ulna	Organic	10	250
3-40	World War II	USS Oklahoma	Rib	Organic	10	250
4-1	World War II	USS Oklahoma	Occipital	Organic	10	250
4-2	Southeast Asia	Laos	Tibia	Inorganic	20	100
4-3	Korea	Namjong-gu	Temporal	Inorganic	10	150
4-4	World War II	USS Oklahoma	Occipital	Organic	20	100
4-6	World War II	USS Oklahoma	Lumbar Vertebra	Organic	10	100
4-7	World War II	USS Oklahoma	Tibia	Organic	20	100
4-8	World War II	USS Oklahoma	Occipital	Organic	20	100
4-9	World War II	USS Oklahoma	Tibia	Organic	20	100
4-10	World War II	USS Oklahoma	Tibia	Organic	20	100
4-11	World War II	USS Oklahoma	Femur	Organic	20	100
4-14	Southeast Asia	Laos	Cervical Vertebra	Inorganic	20	100
4-15	World War II	USS Oklahoma	Femur	Organic	20	100
4-16	World War II	USS Oklahoma	Tibia	Organic	20	100
4-17	World War II	USS Oklahoma	Fibula	Organic	20	100
4-18	World War II	USS Oklahoma	Humerus	Organic	20	100
4-19	World War II	USS Oklahoma	Humerus	Organic	20	100
4-20	World War II	USS Oklahoma	Radisu	Organic	20	100
4-22	World War II	USS Oklahoma	Fibula	Organic	20	100
4-23	World War II	USS Oklahoma	Humerus	Organic	20	100
4-24	World War II	USS Oklahoma	Rib	Organic	20	150
4-25	World War II	USS Oklahoma	Humerus	Organic	20	150
4-26	World War II	USS Oklahoma	Radius	Organic	20	150
4-27	World War II	USS Oklahoma	Ulna	Organic	20	150
4-28	World War II	USS Oklahoma	Femur	Organic	20	100
4-29	Korea	Namjong-gu	Clavicle	Inorganic	20	150

Sample	Conflict	Location Recovered	Skeletal Element	Extraction Protocol	Volume of DNA Extract (µL)	Volume of MeOH (µL)
4-30	Korea	Namjong-gu	Radius	Inorganic	20	150
4-31	World War II	USS Oklahoma	Ulna	Organic	20	150
4-32	World War II	USS Oklahoma	Tibia	Organic	20	150
4-33	World War II	USS Oklahoma	Fibula	Organic	20	150
4-34	World War II	USS Oklahoma	Fibula	Organic	20	100
4-36	World War II	USS Oklahoma	Tibia	Organic	20	100
4-37	World War II	USS Oklahoma	Rib	Organic	20	100
4-38	World War II	USS Oklahoma	Ulna	Organic	20	100
4-39	World War II	USS Oklahoma	Fibula	Organic	20	100
4-40	World War II	USS Oklahoma	Radius	Organic	20	100
4-41	World War II	USS Oklahoma	Ulna	Organic	20	100
4-42	World War II	USS Oklahoma	Femur	Organic	20	100
5-1	World War II	Tarawa	Lumbar Vertebra	Inorganic	20	150
5-3	World War II	USS Oklahoma	Humerus	Organic	20	150
5-4	World War II	USS Oklahoma	Humerus	Organic	20	150
5-5	World War II	Solomon Islands	Humerus	Inorganic	20	150
5-6	World War II	USS Oklahoma	Tibia	Organic	20	150
5-7	World War II	Tarawa	Occipital	Inorganic	20	150
5-8	World War II	USS Oklahoma	Occipital	Organic	20	100
5-9	World War II	USS Oklahoma	Humerus	Organic	20	100
5-10	World War II	USS Oklahoma	Radius	Organic	20	100
5-11	World War II	USS Oklahoma	Ulna	Organic	20	100
5-12	World War II	Solomon Islands	Radius	Inorganic	20	150
5-14	World War II	Solomon Islands	Humerus	Inorganic	20	150
5-15	World War II	Solomon Islands	Humerus	Inorganic	20	150
5-16	World War II	Tarawa	Tibia	Inorganic	20	150
5-17	World War II	Tarawa	Humerus	Inorganic	20	150
5-18	World War II	USS Oklahoma	Radius	Organic	20	150
5-19	World War II	USS Oklahoma	Ulna	Organic	20	150
5-20	World War II	USS Oklahoma	Femur	Organic	20	150
5-21	World War II	USS Oklahoma	Tibia	Organic	20	150
6-1	World War II	USS Oklahoma	Occipital	Organic	20	150
6-2	World War II	USS Oklahoma	Lumbar Vertebra	Organic	20	100
6-3	World War II	USS Oklahoma	Femur	Organic	20	100
6-4	World War II	USS Oklahoma	Occipital	Organic	20	150
6-5	World War II	USS Oklahoma	Tibia	Organic	10	150
6-6	World War II	USS Oklahoma	Tibia	Organic	10	150
6-7	World War II	USS Oklahoma	Humerus	Organic	20	100
6-7	World War II	USS Oklahoma	Humerus	Organic	10	250
6-8	World War II	USS Oklahoma	Occipital	Organic	20	100
6-9	World War II	Solomon Islands	Ulna	Inorganic	20	150
6-10	World War II	USS Oklahoma	Tibia	Organic	20	150
6-11	World War II	USS Oklahoma	Occipital	Organic	20	100
6-12	World War II	USS Oklahoma	Femur	Organic	20	150
6-13	World War II	USS Oklahoma	Tibia	Organic	20	150
6-13	World War II	USS Oklahoma	Tibia	Organic	10	250
6-14	World War II	USS Oklahoma	Fibula	Organic	10	150

Sample	Conflict	Location Recovered	Skeletal Element	Extraction Protocol	Volume of DNA Extract (µL)	Volume of MeOH (µL)
6-14	World War II	USS Oklahoma	Fibula	Organic	10	250
6-15	Southeast Asia	Vietnam	Bone Fragment	Inorganic	20	150
6-16	Southeast Asia	Vietnam	Bone Fragment	Inorganic	20	150
6-17	World War II	Solomon Islands	Humerus	Inorganic	20	150
6-18	World War II	Solomon Islands	Ulna	Inorganic	20	150
6-19	World War II	Solomon Islands	Ulna	Inorganic	20	100
6-20	World War II	USS Oklahoma	Occipital	Organic	20	100
6-21	World War II	USS Oklahoma	Occipital	Organic	20	100
6-22	World War II	USS Oklahoma	Occipital	Organic	20	150
6-23	World War II	Yugoslavia	Metatarsal	Inorganic	10	100
6-24	World War II	USS Oklahoma	Fibula	Organic	20	150
6-25	World War II	USS Oklahoma	Humerus	Organic	20	150
6-26	World War II	USS Oklahoma	Rib	Organic	20	100
6-27	World War II	USS Oklahoma	Occipital	Organic	20	100
6-28	World War II	USS Oklahoma	Humerus	Organic	20	100
6-29	World War II	USS Oklahoma	Radius	Organic	20	100
6-30	World War II	USS Oklahoma	Ulna	Organic	20	100
6-31	World War II	USS Oklahoma	Lumbar Vertebra	Organic	20	150
6-32	World War II	USS Oklahoma	Rib	Organic	20	150
7-1	World War II	USS Oklahoma	Vertebra	Organic	20	100
7-2	World War II	Solomon Islands	Cranium	Inorganic	20	100
7-3	World War II	Solomon Islands	Cranium	Inorganic	20	100
7-4	World War II	Solomon Islands	Patella	Inorganic	20	100
7-5	World War II	USS Oklahoma	Occipital	Organic	20	100
7-6	World War II	Solomon Islands	Patella	Inorganic	20	100
7-7	World War II	USS Oklahoma	Tibia	Organic	20	100
7-8	World War II	Solomon Islands	Cranium	Inorganic	20	100
7-9	World War II	USS Oklahoma	Humerus	Organic	20	100
7-10	World War II	Solomon Islands	Femur	Inorganic	20	100
7-11	World War II	USS Oklahoma	Fibula	Organic	20	100
7-12	World War II	USS Oklahoma	Humerus	Organic	20	100
7-13	World War II	Solomon Islands	Patella	Inorganic	20	100
7-14	World War II	Solomon Islands	Cranium	Inorganic	20	100
7-15	World War II	USS Oklahoma	Occipital	Organic	20	100
7-16	World War II	USS Oklahoma	Occipital	Organic	20	100
7-17	World War II	USS Oklahoma	Occipital	Organic	20	100
7-18	World War II	USS Oklahoma	Occipital	Organic	20	100
7-19	World War II	USS Oklahoma	Femur	Organic	20	100
7-20	World War II	USS Oklahoma	Tibia	Organic	20	100
7-21	World War II	USS Oklahoma	Fibula	Organic	20	100
7-22	World War II	USS Oklahoma	Humerus	Organic	20	100
7-23	World War II	USS Oklahoma	Tibia	Organic	20	100
7-24	World War II	USS Oklahoma	Occipital	Organic	20	100
7-25	World War II	USS Oklahoma	Occipital	Organic	20	100
7-26	World War II	USS Oklahoma	Occipital	Organic	20	100
7-27	World War II	USS Oklahoma	Occipital	Organic	10	150
7-28	World War II	USS Oklahoma	Occipital	Organic	10	150

Sample	Conflict	Location Recovered	Skeletal Element	Extraction Protocol	Volume of DNA Extract (µL)	Volume of MeOH (µL)
7-29	World War II	USS Oklahoma	Occipital	Organic	10	150
7-30	World War II	USS Oklahoma	Occipital	Organic	10	150
7-31	World War II	USS Oklahoma	Vertebra	Organic	20	100
7-32	World War II	USS Oklahoma	Tibia	Organic	20	100
7-33	World War II	USS Oklahoma	Humerus	Organic	20	100
7-34	World War II	USS Oklahoma	Fibula	Organic	20	100
7-35	World War II	USS Oklahoma	Tibia	Organic	20	100
7-36	World War II	USS Oklahoma	Humerus	Organic	20	100
7-37	World War II	USS Oklahoma	Rib	Organic	20	100
7-38	World War II	USS Oklahoma	Ulna	Organic	20	100
7-39	World War II	USS Oklahoma	Radius	Organic	20	100
7-40	World War II	USS Oklahoma	Rib	Organic	20	100
7-41	World War II	Solomon Islands	Cranium	Inorganic	20	100
7-42	World War II	Solomon Islands	Cranium	Inorganic	20	100
7-43	World War II	Solomon Islands	Bone Fragment	Inorganic	20	100
7-44	World War II	Solomon Islands	Unknown	Inorganic	20	100
7-45	World War II	Solomon Islands	Long Bone	Inorganic	20	150
7-46	World War II	Solomon Islands	Bone Fragment	Inorganic	20	100
7-47	World War II	USS Oklahoma	Femur	Organic	20	100
7-48	World War II	USS Oklahoma	Ulna	Organic	20	100
7-49	World War II	USS Oklahoma	Radius	Organic	20	100
7-50	World War II	Solomon Islands	Patella	Inorganic	20	100
7-51	World War II	Solomon Islands	Femur	Inorganic	20	100
7-52	World War II	Solomon Islands	Femur	Inorganic	20	100
7-53	World War II	Solomon Islands	Femur	Inorganic	20	100
7-54	World War II	Solomon Islands	Mandible	Inorganic	20	100
7-55	World War II	Solomon Islands	Mandible	Inorganic	20	100
7-56	World War II	Solomon Islands	Mandible	Inorganic	20	100
7-57	World War II	Solomon Islands	Zygomatic	Inorganic	20	100
7-58	World War II	Solomon Islands	Cranium	Inorganic	20	100
7-59	World War II	Solomon Islands	Cranium	Inorganic	20	100
8-1	World War II	Tarawa	Temporal	Inorganic	20	100
8-3	World War II	Solomon Islands	Cranium	Inorganic	20	100
8-6	World War II	USS Oklahoma	Fibula	Organic	20	150
8-7	World War II	Tarawa	Occipital	Inorganic	20	100
8-8	World War II	USS Oklahoma	Tibia	Organic	20	150
8-9	Korea	Namjong-gu	Mandible	Inorganic	10	150
8-10	World War II	USS Oklahoma	Humerus	Organic	20	150
8-11	World War II	USS Oklahoma	Femur	Organic	20	150
8-12	World War II	USS Oklahoma	Mandible	Organic	20	100
8-13	World War II	USS Oklahoma	Mandible	Organic	20	100
8-14	World War II	Solomon Islands	Unknown	Inorganic	20	150
8-15	World War II	Solomon Islands	Unknown	Inorganic	20	100
8-16	World War II	Tarawa	Humerus	Inorganic	20	100
8-17	World War II	Tarawa	Tibia	Inorganic	20	100
8-18	World War II	Tarawa	Humerus	Inorganic	20	100
8-19	World War II	Tarawa	Femur	Inorganic	20	100

Sample	Conflict	Location Recovered	Skeletal Element	Extraction Protocol	Volume of DNA Extract (µL)	Volume of MeOH (µL)
8-20	World War II	USS Oklahoma	Radius	Organic	20	150
8-21	World War II	USS Oklahoma	Ulna	Organic	20	150
8-22	World War II	USS Oklahoma	Femur	Organic	20	150
8-23	World War II	Solomon Islands	Radius	Inorganic	20	100
8-24	World War II	Solomon Islands	Radius	Inorganic	20	100
8-25	World War II	Solomon Islands	Radius	Inorganic	20	100
8-26	World War II	Solomon Islands	Zygomatic	Inorganic	20	100
8-27	World War II	Solomon Islands	Zygomatic	Inorganic	20	100
8-28	World War II	Solomon Islands	Zygomatic	Inorganic	20	100
8-29	World War II	USS Oklahoma	Femur	Organic	20	100
8-30	World War II	USS Oklahoma	Tibia	Organic	20	100
8-31	World War II	USS Oklahoma	Fibula	Organic	20	100
8-32	World War II	USS Oklahoma	Humerus	Organic	20	100
8-34	World War II	USS Oklahoma	Rib	Organic	20	150
9-1	World War II	Tarawa	Temporal	Inorganic	20	100
9-2	World War II	USS Oklahoma	Vertebra	Organic	20	150
9-3	World War II	USS Oklahoma	Occipital	Organic	20	100
9-4	Korea	Joint Recovery Operation	Tibia	Inorganic	20	100
9-5	World War II	USS Oklahoma	Mandible	Organic	20	100
9-6	World War II	USS Oklahoma	Occipital	Organic	20	100
9-7	World War II	USS Oklahoma	Occipital	Organic	20	100
9-8	World War II	USS Oklahoma	Occipital	Organic	20	100
9-9	World War II	USS Oklahoma	Occipital	Organic	20	100
9-10	World War II	USS Oklahoma	Occipital	Organic	20	150
9-11	World War II	USS Oklahoma	Occipital	Organic	20	150
9-12	World War II	USS Oklahoma	Occipital	Organic	20	150
9-13	World War II	USS Oklahoma	Occipital	Organic	20	100
9-14	World War II	USS Oklahoma	Occipital	Organic	20	100
9-15	World War II	USS Oklahoma	Occipital	Organic	20	100
9-16	World War II	USS Oklahoma	Occipital	Organic	20	100
9-17	World War II	Tarawa	Tibia	Inorganic	20	100
9-18	World War II	Tarawa	Tibia	Inorganic	20	100
9-19	World War II	Tarawa	Tibia	Inorganic	20	100
9-20	World War II	Tarawa	Tibia	Inorganic	20	100
9-21	World War II	Tarawa	Tibia	Inorganic	20	100
9-22	World War II	Tarawa	Tibia	Inorganic	20	100
9-23	World War II	Tarawa	Tibia	Inorganic	20	100
9-24	World War II	Tarawa	Tibia	Inorganic	20	100
9-25	World War II	Tarawa	Tibia	Inorganic	20	100
9-26	World War II	Tarawa	Tibia	Inorganic	20	100
9-27	World War II	USS Oklahoma	Humerus	Organic	20	150
9-28	World War II	USS Oklahoma	Radius	Organic	20	150
9-29	World War II	USS Oklahoma	Femur	Organic	20	150
9-30	World War II	USS Oklahoma	Ulna	Organic	20	150
9-31	World War II	USS Oklahoma	Tibia	Organic	20	150
9-32	World War II	USS Oklahoma	Fibula	Organic	20	150
9-33	World War II	USS Oklahoma	Tibia	Organic	20	150

Sample	Conflict	Location Recovered	Skeletal Element	Extraction Protocol	Volume of DNA Extract (µL)	Volume of MeOH (µL)
9-34	World War II	USS Oklahoma	Radius	Organic	20	100
9-35	World War II	USS Oklahoma	Ulna	Organic	20	100
9-36	World War II	USS Oklahoma	Femur	Organic	10	100
9-37	World War II	USS Oklahoma	Tibia	Organic	20	100
9-38	World War II	USS Oklahoma	Fibula	Organic	20	100
9-39	World War II	USS Oklahoma	Humerus	Organic	20	100
9-40	World War II	USS Oklahoma	Femur	Organic	20	100
9-41	World War II	USS Oklahoma	Vertebra	Organic	20	100
9-42	World War II	USS Oklahoma	Femur	Organic	20	100
9-43	World War II	USS Oklahoma	Tibia	Organic	10	100
9-44	World War II	USS Oklahoma	Fibula	Organic	10	150
9-45	World War II	USS Oklahoma	Humerus	Organic	10	150
10-1	Southeast Asia	Not Specified	Os Coxa	Inorganic	20	100
10-2	Southeast Asia	Not Specified	Scapula	Inorganic	20	100
10-3	Other	Not Specified	Lumbar Vertebra	Inorganic	20	100
10-4	World War II	USS Oklahoma	Rib	Organic	20	100
10-5	World War II	USS Oklahoma	Vertebra	Organic	20	150
10-6	World War II	USS Oklahoma	Tibia	Organic	20	150
10-7	World War II	USS Oklahoma	Femu	Organic	20	100
10-8	Korea	Not Specified	Tibia	Inorganic	20	150
10-9	World War II	USS Oklahoma	Ulna	Organic	20	100
10-10	Southeast Asia	Not Specified	Cranium	Inorganic	20	100
10-11	Southeast Asia	Not Specified	Thoracic Vertebra	Inorganic	20	100
10-12	Southeast Asia	Not Specified	Rib	Inorganic	20	100
10-13	Southeast Asia	Not Specified	Long Bone	Inorganic	20	100
10-14	Southeast Asia	Not Specified	Os Coxa	Inorganic	20	100
10-17	World War II	Solomon Islands	Long Bone	Inorganic	20	100
10-18	Korea	Joint Recovery Operation	Cervical Vertebra	Inorganic	20	150
10-19	World War II	USS Oklahoma	Mandible	Organic	20	100
10-20	World War II	USS Oklahoma	Mandible	Organic	20	100
10-21	World War II	USS Oklahoma	Mandible	Organic	20	100
10-22	Southeast Asia	Not Specified	Scapula	Inorganic	20	100
10-23	World War II	USS Oklahoma	Occipital	Organic	20	100
10-24	World War II	USS Oklahoma	Occipital	Organic	20	100
10-25	World War II	USS Oklahoma	Occipital	Organic	20	100
10-26	Other	Not Specified	Humerus	Inorganic	20	100
10-27	Other	Not Specified	Os Coxa	Inorganic	20	100
10-28	Other	Not Specified	Tibia	Inorganic	20	100
10-29	World War II	USS Oklahoma	Occipital	Organic	20	150
10-30	World War II	USS Oklahoma	Occipital	Organic	20	150
10-31	World War II	USS Oklahoma	Occipital	Organic	20	150
10-32	World War II	USS Oklahoma	Occipital	Organic	20	150
10-33	Korea	Ryongpho-ri	Humerus	Inorganic	10	150
10-34	World War II	USS Oklahoma	Rib	Organic	20	150
10-35	World War II	USS Oklahoma	Humerus	Organic	20	150
10-36	World War II	USS Oklahoma	Radius	Organic	20	150
10-37	World War II	USS Oklahoma	Femur	Organic	20	100

Sample	Conflict	Location Recovered	Skeletal Element	Extraction Protocol	Volume of DNA Extract (µL)	Volume of MeOH (µL)
10-38	World War II	USS Oklahoma	Vertebra	Organic	20	100
10-39	World War II	USS Oklahoma	Humerus	Organic	20	100
10-40	World War II	USS Oklahoma	Radius	Organic	20	150
10-41	World War II	USS Oklahoma	Ulna	Organic	20	150
10-42	World War II	USS Oklahoma	Femur	Organic	20	150
10-43	World War II	USS Oklahoma	Tibia	Organic	20	150
10-44	World War II	USS Oklahoma	Rib	Organic	20	100
10-45	World War II	USS Oklahoma	Humerus	Organic	10	100
10-46	World War II	USS Oklahoma	Radius	Organic	20	150
10-47	World War II	USS Oklahoma	Ulna	Organic	20	150
10-48	World War II	USS Oklahoma	Femur	Organic	20	150
T1-1	World War II	Philippines	Tooth #11	Organic	10	150
T1-2	World War II	Philippines	Tooth #15	Organic	10	150
T1-3	World War II	Papua New Guinea	Tooth #4	Organic	20	150
T1-3	World War II	Papua New Guinea	Tooth #4	Organic	10	250
T1-4	World War II	Papua New Guinea	Tooth #31	Organic	20	150
T1-4	World War II	Papua New Guinea	Tooth #31	Organic	10	250
T1-5	World War II	Papua New Guinea	Tooth #6	Organic	20	150
T1-5	World War II	Papua New Guinea	Tooth #6	Organic	10	250
T1-6	World War II	Papua New Guinea	Tooth #20	Organic	20	150
T1-6	World War II	Papua New Guinea	Tooth #20	Organic	10	250
T1-10	World War II	Northern Mariana Island	Tooth #21	Organic	20	150
T1-11	World War II	Northern Mariana Island	Tooth #7	Organic	20	150
T1-12	World War II	Philippines	Tooth #22	Organic	20	150
T1-13	World War II	Philippines	Tooth #11	Organic	20	150
T1-14	World War II	Kiribati	Tooth #9	Organic	20	150
T1-15	World War II	Kiribati	Tooth #32	Organic	20	150
T1-16	World War II	Philippines	Tooth #11	Organic	10	150
T1-17	World War II	Philippines	Tooth #15	Organic	10	150
T1-18	World War II	Philippines	Tooth #22	Organic	10	150
T2-1?	Korea	Namjong-gu	Maxillary Canine	Organic	20	150
T2-1?	World War II	Belgium	Tooth #6	Organic	20	150
T2-3	Korea	Namjong-gu	Tooth #22	Organic	20	150
T2-4	Korea	Joint Recovery Operation	Tooth #2	Organic	20	150
T2-5	Korea	Joint Recovery Operation	Tooth #14	Organic	20	150
T2-6	Korea	Joint Recovery Operation	Maxillary Premolar	Organic	20	150
T2-7	Korea	Joint Recovery Operation	Tooth #6	Organic	20	150
T2-8	Korea	Namjong-gu	Tooth #17	Organic	20	150
T2-9	Korea	Kaljon-ri	Maxillary Premolar	Organic	20	150
T2-10	World War II	Japan	Tooth #8	Organic	20	150
T2-11	World War II	Japan	Tooth #28	Organic	20	150
T2-12	Korea	Namjong-gu	Tooth #4	Organic	20	150
T2-13	Korea	Namjong-gu	Tooth #27	Organic	20	150

Sample	Conflict	Location Recovered	Skeletal Element	Extraction Protocol	Volume of DNA Extract (µL)	Volume of MeOH (µL)
T2-17	World War II	Belgium	Tooth #31	Organic	20	150
T2-18	World War II	Belgium	Tooth #18	Organic	20	150

Chapter 9

GC/MS Analysis of Skeletal Remains II: Creation of a Personal Chemical Profile

9.1 INTRODUCTION

During the gas chromatography / mass spectrometry (GC/MS) analysis of osseous materials as presented in Edson (2017), an interesting trend was noted: a different series of compounds was found in different samples recovered from the same incident. For example, plant materials, particularly pine tar, were found in some osseous materials from the USS *Oklahoma* and others contained none. Similar trends were seen in samples recovered from the same incidents from the Korean peninsula and areas of Southeast Asia. When coupled with the DNA testing results of the skeletal elements, there was an indication that the different chemical profiles from a single incident were from different individuals.

Mass spectrometry has been used in horticulture to differentiate locations of origin in plants or varietals (tea: Zhang, et al., 2011; apples: Giannetti, et al., 2017; sapple juice: Gan, et al., 2014; dates: Khalil, et al., 2017). While these are differentiations between varietals and locations, it would seem to be a logical leap to think that individual persons could be differentiated by GC/MS. Given the relatively large mass of a human being, it would stand to reason that the compounds ingested by an individual and absorbed into the fats and tissues of the body would absorb into the skeletal material during decomposition. Compounds from the environment should also absorb into the skeletal remains, although this may depend on the environment itself.

The following chapter presents the framework of this idea and supporting examples. However, additional work needs to be done in order to create a well-defined model for the use of GC/MS analysis of volatilized skeletal materials as a tool to aid in human identification. As the sampling strategy for the collection of the osseous materials tested was random, the bulk of the samples tested came from the USS *Oklahoma*. As the ship was sunk early on a Sunday morning, not all individuals were at their designated stations. Even though a DNA profile has been assigned to the remains, an identity may not yet be determined. With the

extensive commingling within the ship, and a lack of awareness of the location of decomposition, a model could not be developed from the remains tested.

Ideally, all samples from a single case, involving well-mapped sets of remains would be sampled. This would allow for a statistical analysis of the results and a determination of whether GC/MS could be used in this manner. Regardless, the technique shows great promise, and additional work will be done.

9.1.1 INTRODUCTION REFERENCES

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Zhang J, W Zhang, Z Zhou, Y Bai, H Liu. 2011. In situ and rapid identification of tea by direct analysis in real time mass spectrometry. Chinese Journal of Chromatography 29(7): 681-686.

9.2 PUBLICATION

The results of this study were published in the Australian Journal of Forensic Sciences.

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9.2.1 ATTRIBUTION OF TASKS

Ms. Edson developed the concept for this project, provided the samples, performed the testing in the laboratory and analyzed the data. In addition, she wrote the publication.

Dr. McMahon provided the laboratory space and aided in editing of the publication.

9.3 TITLE PAGE

Testing of Skeletonized Human Remains Using GC/MS – Development of a Personal Environmental Profile

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9.3.1 DISCLAIMER

The opinions or assertions presented are the private views of the author and should not be construed as official or as reflecting the views of the Department of Defense; the Defense Health Agency; the Armed Forces Medical Examiner System; or the Defense POW/MIA Accounting Agency.

9.3.2 ROLE OF FUNDING

Authors are employees of their respective agencies and otherwise received no monetary support.

9.3.3 CONFLICT OF INTEREST

None

9.4 ABSTRACT

Gas chromatography/mass spectrometry (GC/MS) is well used in forensic science for the identification of materials present in toxicology, pharmacology, and arson investigation. However, GC/MS has been underused in human identification. In this study, highly commingled skeletonized remains from a mass fatality incident involving over 400 individuals were examined. Environmental compounds and those present in the remains themselves were extracted from osseous materials removed from the surface of the skeletal elements using two different solvents, dichloromethane and acetonitrile. Solvent extracts were concentrated and resuspended in methanol prior to injection on the GC/MS instrument. Compounds present from the environment surrounding the individual post-mortem, as well as biological materials present in the individual antemortem, are detectable. Traces generated from the GC/MS instrument provide distinctive images and analysis of those materials shows patterns that are specific to the individual and may be used for individuation. Results indicate that GC/MS analysis of skeletonized remains may be a new tool for human identification.

9.5 KEY WORDS

Gas chromatography / mass spectrometry; skeletonized remains; human identification; mass fatalities

9.6 INTRODUCTION

In mass fatality events involving a high degree of commingling, sorting and reassociation of elements is a time-consuming and expensive process, involving multiple disciplines within forensic science. The difficulty of this task is compounded when the remains are skeletonized. Gas chromatography/mass spectrometry (GC/MS) is used routinely to detect trace amounts of materials in toxicology, pharmacology, and fire investigations; however, it has rarely been used in the field of human identification. The only comparable study was of tea leaves, where mass spectrometry was found to be able to determine the geographical location from which the tea was gathered [1]. If the compounds within osseous materials could be concentrated enough to be detectable, it might be possible to use GC/MS to provide ante-, peri-, and post-mortem information about any given set of skeletonized remains. Preliminary work performed by Edson [2] indicated that GC/MS trace analysis of skeletonized remains from individuals within the same incident have different patterns chemical components found within them.

On 7 December 1941, the USS *Oklahoma* capsized after being struck by multiple torpedoes during the Japanese Imperial Army attack on the US Naval Base in Pearl Harbor, Hawaiian Territory. Rather than sinking, the ship rolled, trapping 429 sailors and Marines within the hull. The human remains were removed from the ship in 1943 and multiple attempts at identification were made until they were finally laid to rest at the National Memorial Cemetery of the Pacific (NMCP) in 1951. The modern project to identify the remains began in 2003 and is ongoing.

To evaluate the use of GC/MS, 208 skeletal samples from the loss of the USS *Oklahoma* were examined. Mitochondrial DNA analysis has determined that these 208 samples represent a minimum of 121 individuals. Individuals were unknown at the time of collection and

elements were randomly selected during the course of regular casework to eliminate possible bias.

9.7 MATERIAL & METHODS

Remains were disinterred and examined by anthropologists. A small window of bone was removed from a desired element and sent for DNA testing. As described in [3], each sample was sanded using a Dremel® tool (Dremel, Racine, WI) to remove any exogenous contamination. This detritus, comprised of osseous, biological, and environmental materials, was collected and placed in 15mL Falcon™ polypropylene tubes (Corning, Corning, NY). The volume of material collected varied (0.01 - 1.68 g) and was dependent on the size and type of element. A fraction of the osseous detritus (0.1 g or less) was treated with 1.0mL of acetonitrile. Samples were vortexed vigorously after the addition of the solvent and allowed to incubate for one hour. Samples were spun down to pellet the solids and the liquid fraction removed to a clean glass beaker for volatilization. The dried fraction was removed from the beaker using 500 µL methanol (≥99.9%, HPLC grade, Sigma-Aldrich, St. Louis, MO, USA). This process was repeated using dichloromethane (HPLC grade; Pharmco AAPER, Brookfield, CT, USA).

All samples were loaded onto an Agilent 7890A/5875C GC/MS System with a 20 m column (Agilent, Santa Clara, CA) using a splitless injection and the following conditions: 2.5 minute solvent delay, initial oven temperature of 150°C with a ramp to 250°C over 10 minutes at 20°C/minute. Traces were analyzed by non-targeted data analysis via ChemStation (Agilent) using NIST 2005/2011 spectral libraries supplemented with Cayman Spectral Library (Cayman Chemicals, Ann Arbor, MI).

9.8 RESULTS

A wide variety of materials were found to be detectable in the eluate from the osseous materials. These included, but were not limited to: fuel residue (e.g., anthracene); by-products of decomposition (e.g., oleic acid); possible plant materials (e.g., phytol, citronellol); and possible medications (e.g., quinolone). Compounds found within each samples were similar; however, they were not the same.

Figure 1 shows examples of four different traces generated from a dichloromethane elution of osseous detritus. Mitochondrial DNA testing determined that each of these traces was from a different individual.

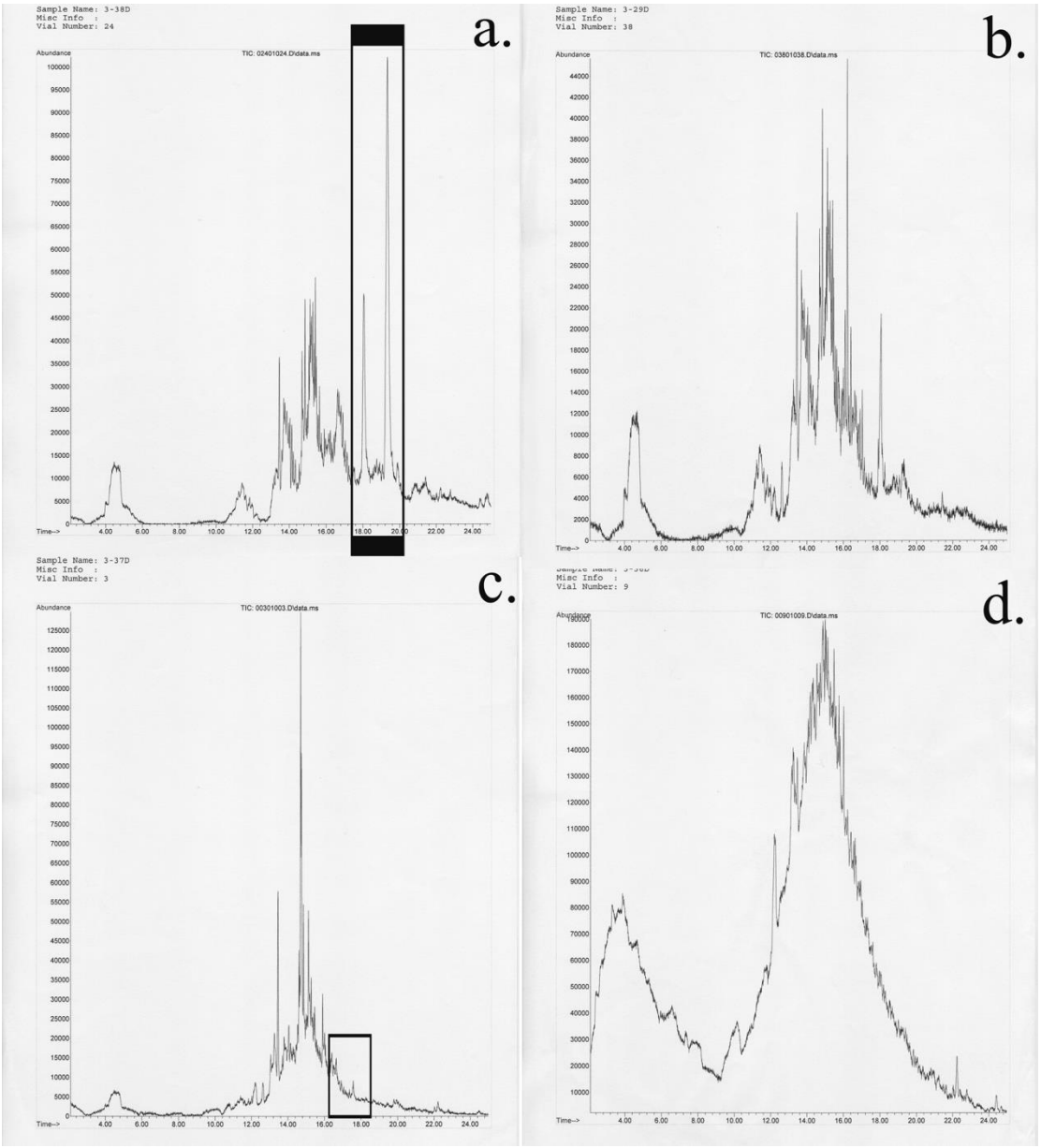

Figure 9.1. Four GC/MC traces generated from different osseous elements from the USS *Oklahoma*. Each trace is from a different individual as determined by mitochondrial DNA testing. The samples were recovered from the same burial yet yield different trace profiles. The collection of peaks in the center of each trace is fats, esters, and fuel components. The highlighted group of peaks in 1a and 1c indicate the presence of plant materials.

9.9 CONCLUSIONS

GC/MS analysis of osseous detritus via extraction of compounds using solvents can potentially be used to separate individuals in mass fatality events. While the testing of the 208 osseous detritus samples from the USS *Oklahoma* has been completed, the analysis of the specific components present has not yet been fully compiled. The finished data shows a marked difference in compounds present in the remains of different individuals. There is also a consistency in pattern between elements of the same individual. Although there are subtle differences, this could be attributed to positioning of the body during initial decomposition and proximity to exogenous materials.

GC/MS analysis could be a valuable tool to be included in the identification of the remains of missing persons. Once an initial trace of materials can be linked to a DNA profile, samples could be reassociated by GC/MS analysis. Coupled with DNA analysis and other forensic modalities, GC/MS may prove to be an efficient and inexpensive manner by which osseous remains can be quickly sorted for identification.

9.10 REFERENCES

[1] Zhang, J, W Zhang, Z Zhou, et al. 2011. In situ and rapid identification of tea by direct analysis in real time mass spectrometry. Chinese Journal of Chromatography 29(7): 681-686.

[2] Edson, S. 2017. DNA typing from skeletal remains: A study of inhibitors using mass spectrometry. Forensic Science International: Genetics Supplemental Series 6:e337-e339.

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9.11 SUPPLEMENTAL DATA

The supplemental data for this section contain expanded images from Figure 9.1. Data are presented in a larger format, so as to more clearly present the data. The compounds detected in each sample are listed in Table 9.1. Some of the same compounds are present in each, but there are also diagnostic elements as well. Sample 3-29 is the only of the four to contain pine resin, indicating that decomposition occurred in proximity to either the carpentry shop or another location that contained an elevated amount of pine resin.

The source of the materials can be difficult to ascertain. For example, forms of phenol may derive from both fuel break-down or decomposition of human remains. The purpose of this study was merely to detect the compounds, rather than to determine to source. Additional historical studies will need to be done to aid in the understanding of the materials present and the point of origination.

DNA testing results indicate that each of these skeletal elements originated from a different individual. Due to limitations with the family reference materials collected for comparison, the persons have not yet been identified.

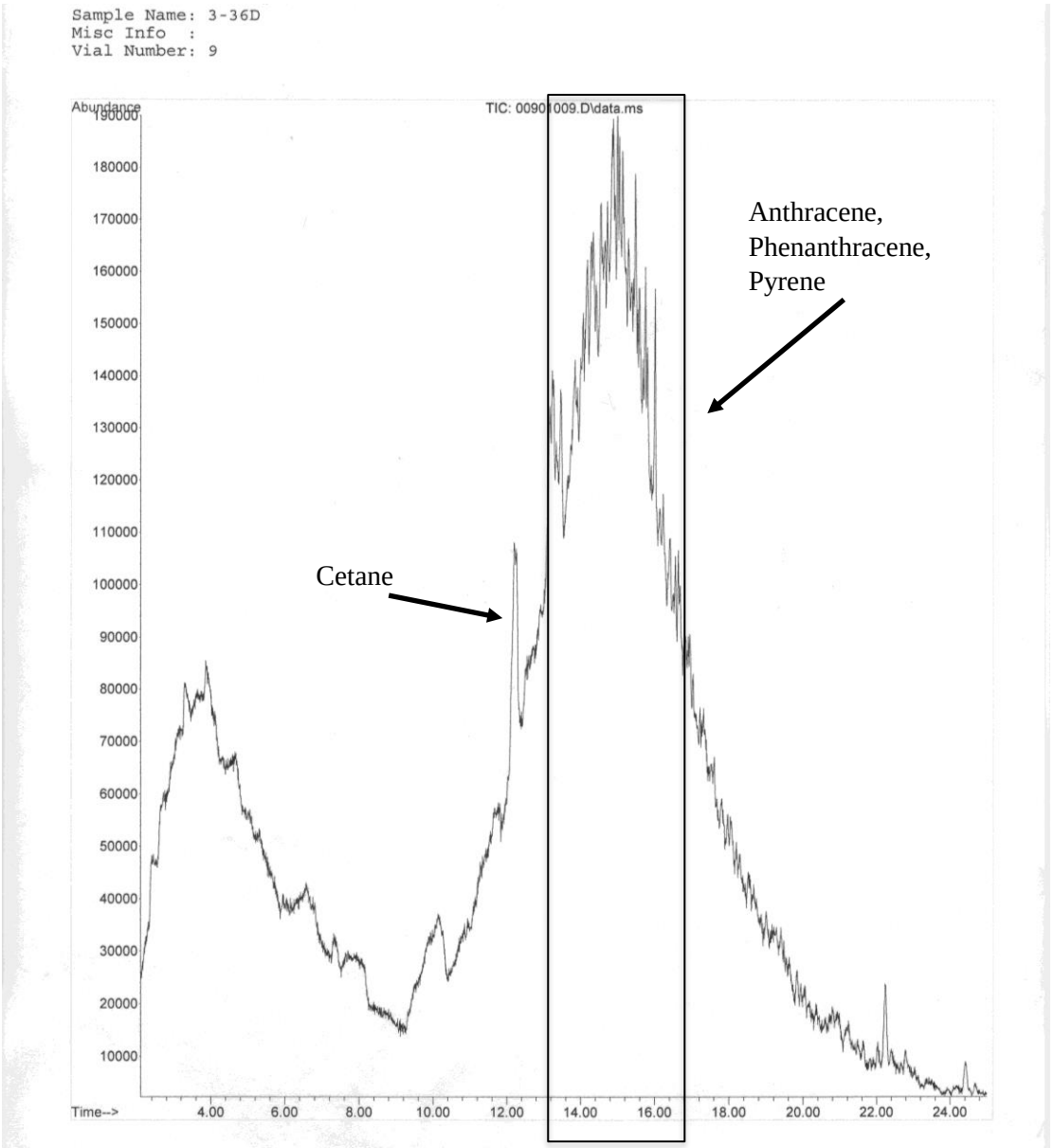


Figure 9.2. Sample 3-36 treated with dichloromethane. The centre range of peaks is anthracene, phenanthracene, and pyrene. Cetane, a hydrocarbon, is also present. This is the same sample as in Figure 9.1d.

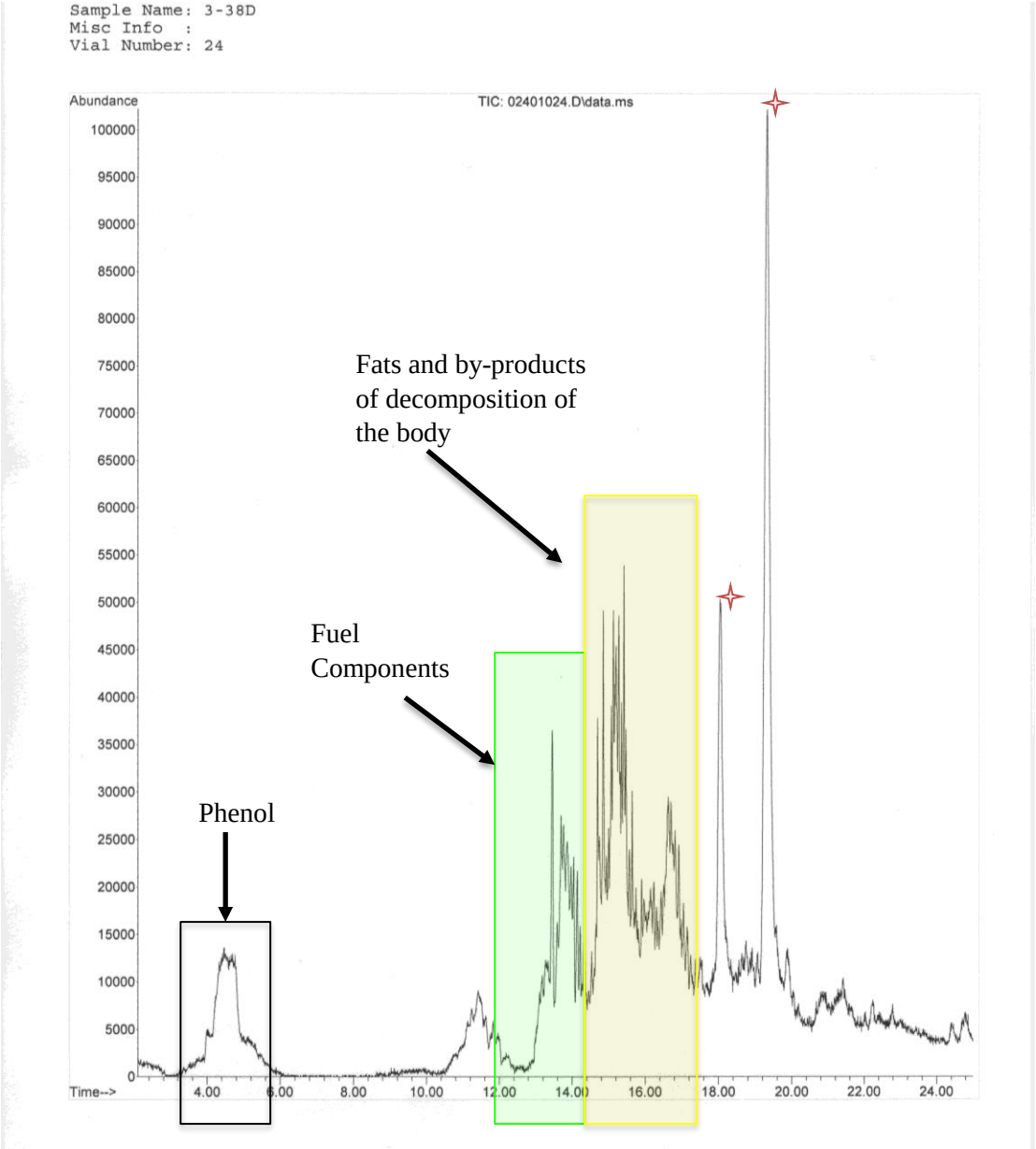


Figure 9.3. Sample 3-38 treated with dichloromethane. The two large peaks marked with stars are forms of cholesterol derivatives. The presence of phenol derives from either human decomposition or the breakdown of fuel products. This is the same sample as in Figure 9.1a.

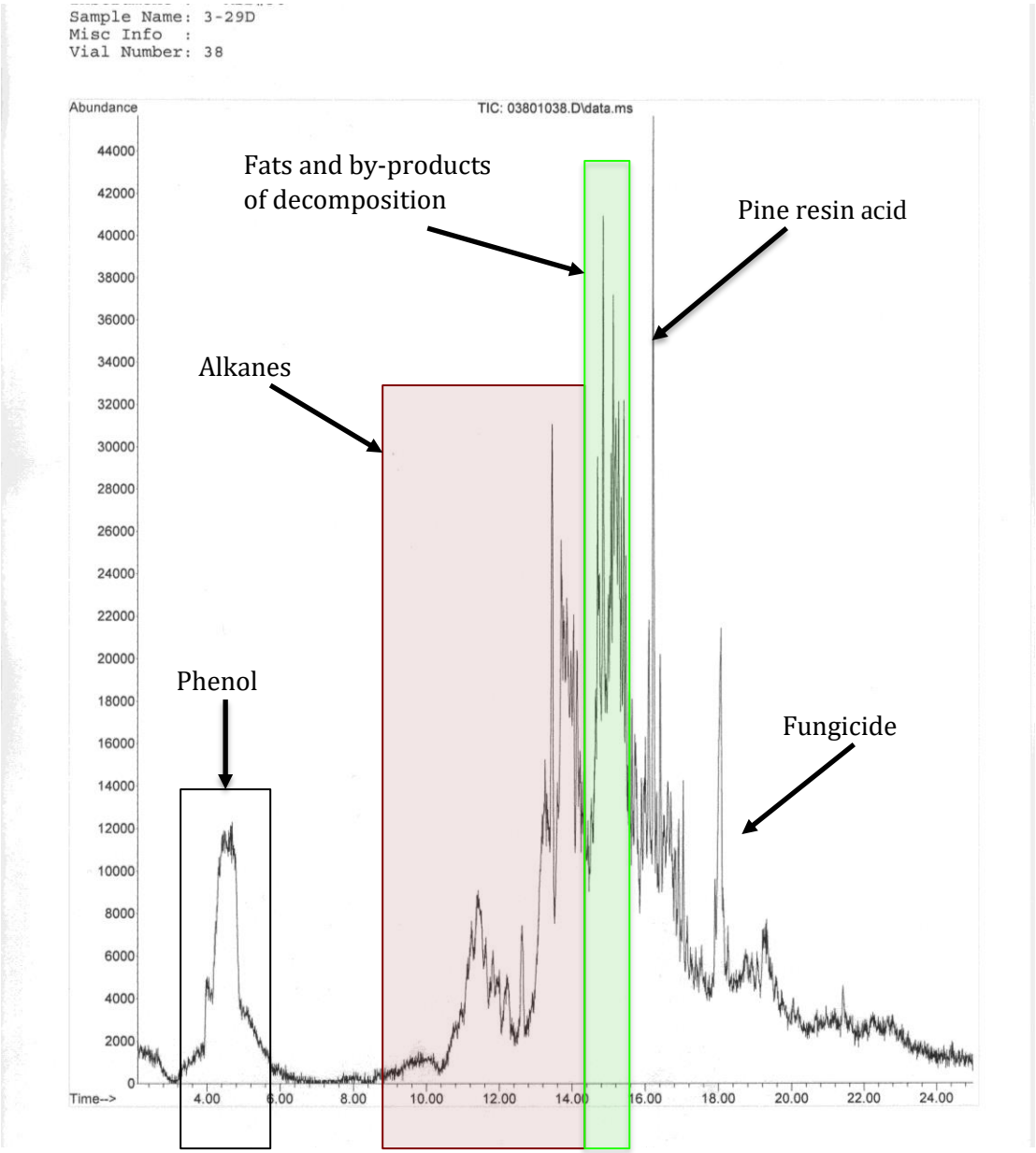


Figure 9.4. Sample 3-29 treated with dichloromethane. A group of phenol peaks is present in this sample as with the other samples. The alkane group can be both from fuel and from fatty acids. The pine resin acid is likely from the break-down of the pine decking of the ship or the pine resin used to seal the decking and ropes of the ship. The fungicide is thiocarbamic acid. This is the same sample as in Figure 9.1b.

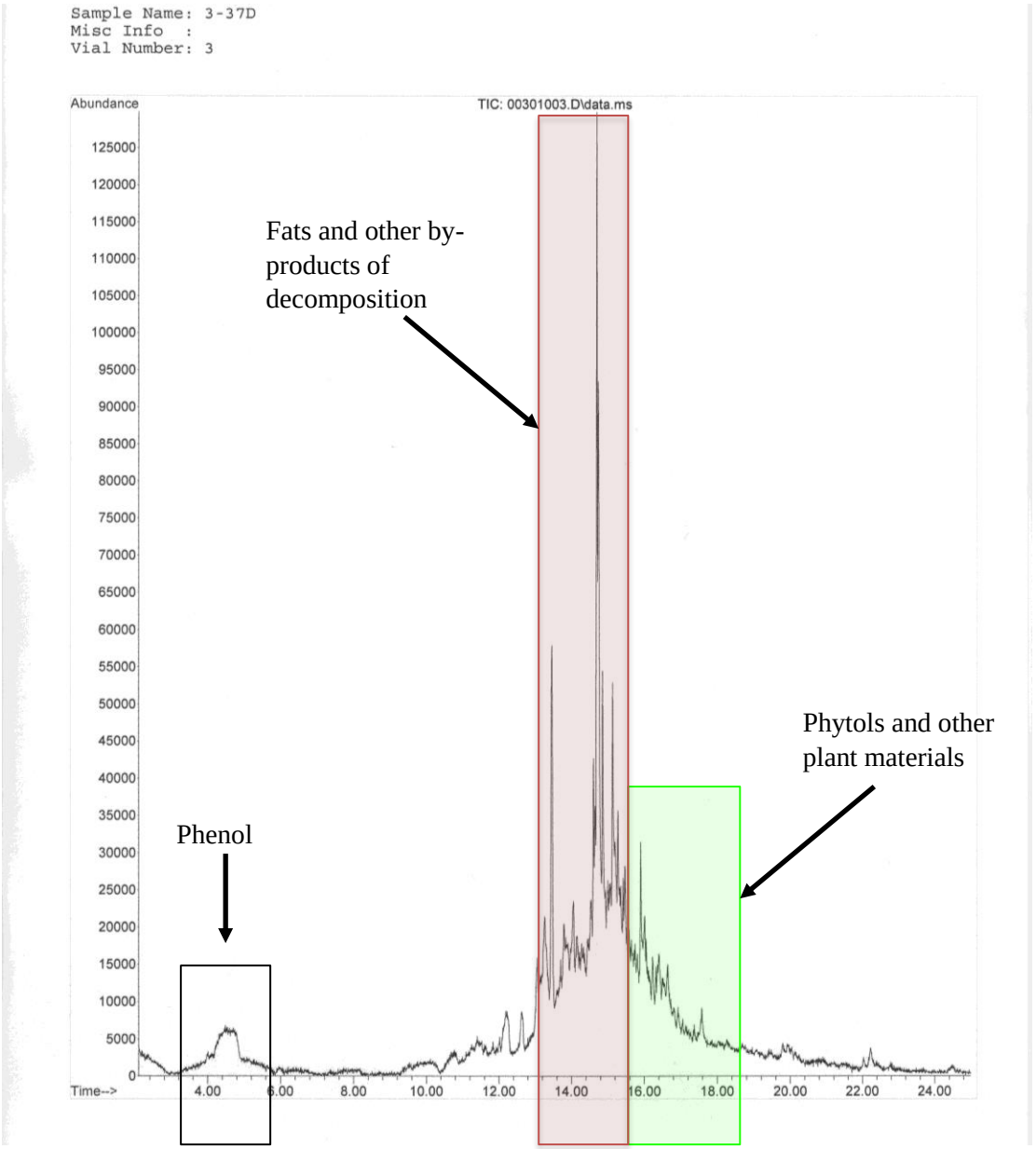


Figure 9.5. Sample 3-37 treated with dichloromethane. The peaks are mainly fats with no apparent materials related to fuels or oils. There is a cluster of phytols and other plant materials such as citronellol. This is the same sample as in Figure 9.1c.

Table 9.1. The chemical compounds detected four osseous samples recovered from the USS *Oklahoma*. The samples are the same as those represented in Figures 9.2-9.5. The compounds are arranged in alphabetical order. Specific compounds shared between samples are marked in blue.

Sample 3-36 Chemical Components	Sample 3-38 Chemical Components	Sample 3-29 Chemical Components	Sample 3-37 Chemical Components
1-(2-Hydroxyethyl)-1,2,5,5-tetramethyl-cis-decalin(1R,2S,4as,8as)	1-Decanol, 2-hexyl-	1-Decanol, 2-hexyl-	1-Decanol, 2-hexyl-
1,2-Dithiolane-3-pentanoic acid	1-Dodecanol, 3,7,11-trimethyl-	1-Dodecano, 3,7,11-trimethyl-	1-Dodecano, 3,7,11-trimethyl-
1,4-Methanoazulen-3-ol, decahydro-1,5,5,8a-tetramethyl-,[1S-(1.alpha.,3.beta.,3a.beta.,4.alpha.,8a.beta.)]-	1-Heptanol, 2,4-diethyl-	1-Heptanol, 2,4-diethyl	1,7-Dimethyl-4-(1-methylethyl)cyclodecane
10-Methylanthracene-9-carboxaldehyde	3-Heptyl-1,1,1-triphenyl-decan-2-one	1-Octadecene	17-Pentatriacontene
1H-Indene, 2-butyl-5-hexyloctahydro-	4-n-Hexylthiane, S,S-dioxide	1-Phenanthrenecarboxylic acid, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-,methyl ester, [1R-(1.alpha.,4a.beta)]	3-Hexadecanol
1H-Pyrazole-4-carbaldehyde, 3-(4-methoxyphenyl)-	6-Ocetenal, 3,7-dimethyl-	2-Isopropyl-5-methyl-1-heptanol	7-Hexadecenal, (Z)-
2-Methylthio-4-oxo-4H-quinolizine-3-carboxamide	7-Hexadecenal, (Z)-	2-Undecene, 4,5-dimethyl-, [R*,S*-(Z)]-	9-Octadecenoic acid (Z)-, methyl ester
2,5-di-tert-Butylnitrobenzene	7-Oxabicyclo[4.1.0]heptane, 1,5-dimethyl	3-Eicosene, (E)-	9-Octadecenoic acid, methyl ester, (E)-
2,6,10,14,18-Pentamethyl-2,6,10,14,18-eicosapentaene	9-Octadecenoic acid (Z)-, methyl ester	3-Octadecene, (E)-	Citronellol epoxide (R or S)
4'-Bromobenzo[1',2'-b]-1,4-diazabicyclo[2.2.2]octene	Acetic acid, 3,7,11,15-tetramethyl-hexadecyl ester	6-Hydroxy-7,7-dimethyl-oct-3-enedithioic acid, isopropyl ester	Cyclohexane, 1,2,4-trimethyl
9-Cedranone	Cholest-5-en-3-ol (3.beta.)-	8H-Pyrano[2,3-e]benzothiophen-8-one, 4-formamido-6-methyl-	Cyclopentane, (4-octyldodecyl)-
Anthracene, 9-methyl-	Cholestan-3-ol, (3.alpha.,5.beta.)-	9-Undecenoic aci, 2,6,10-trimethyl-	E-8-Methyl-9-tetradecen-1-ol acetate
Benzene, 1,2,3,5-tetrachloro-4-methoxy-	Cyclohexane, 1-ethyl-2-propyl-	9,9-Dimethoxybicyclo[3.3.1]nona-2,4-dione	Heptacosane, 1-chloro-
Cyclodeca[b]furan-2(3H)-one, 3a,4,5,6,7,8,9,11a-octahydro-3,6,10-trimethyl-	Decane, 1,1'-oxybis-	Benzenmaine, 2-chloro-N-(3-pyridinylmethylene)-	Octadecane, 1-bromo-
Cyclopentadecanone, 3-methyl-	Decane, 1,10-dibromo-	Borane, 2,3-dimethyl-2-butyl-(dimer)	Octadecanoic acid, methyl ester
Cyclotetradecane, 1,7,11-trimethyl-4-(1-methylethyl)-	Disulfide, di-tert-dodecyl	Cyclohexane, 1-ehtyl-2-propyl-	Pentadecanoic acid, 14-methyl-, methyl ester
d-Norandrostane (5.alpha.,14.alpha.)	Dodecane, 1,12-dibromo-	Cyclohexane, 1,2,4-trimethyl-	Phenol, 2,5-bis(1,1-dimethylethyl)-
Dodecane, 2,6,10-trimethyl	Ethanol, 2-(dodecyloxy)-	Cyclopentadecanone, 3-methyl-	Phytol
Hexadecane	Heptadecanoic acid, 16-methyl-, methyl ester	Cyclopentane, 1-pentyl-2-propyl-	Tetrapentacontane, 1,54-dibromo-

Sample 3-36 Chemical Components	Sample 3-38 Chemical Components	Sample 3-29 Chemical Components	Sample 3-37 Chemical Components
Naphthalene, 1,2,3,4-tetramethyl-	Hexadecanoic acid, methyl ester	Cyclopentane, 1,2-dipropyl	Trifluoroacetic acid, n-heptadecyl ester
Nonahexacontanoic acid	Nonahexacontanoic acid	Decahydro-8a-ehtyl-1,1,4a,6-tetramethylnaphthalene	
Phenanthrene, 2,5-dimethyl-	Octacosane	Decane, 1,1'-oxybis-	
Phenanthrene, 3,6-dimethyl-	Oleic acid	Dodecahydropyrido[1,2-b]isoquinolin-6-one	
Pyrene, 1-methyl-	Phenol, 2,4-bis(1,1-dimethylethyl)-	Dodecane, 1,12-dibromo-	
Pyrene, 1,3-dimethyl-	Phenol, 3,5-bis(1,1-dimethylethyl)-	Dotriacontane	
Tetradecane	Sulfurous acid, 2-propyl tetradecyl ester	Ethanol, 2-(dodecyloxy)-	
Tetrapentacontane, 1,54-dibromo	Sulfurous acid, butyl pentadecyl ester	Ethanol, 2-(hexadecyloxy)-	
Thiophene, 2,2'-(1,2-ethenediyl)bis- (E)-	Sulfurous acid, octadecyl 2-propyl ester	Heptadecane, 1-bromo-	
	Tetrapentacontane	Heptadecanoic acid, 16-methyl-, methyl ester	
	Tetrapentacontane, 1,54-dibromo-	Heptafluorobutanoic acid, hetadecyl ester	
	Tetratetracontane	Hexadecane	
	Tritetracontane	Hexadecanoic acid, methyl ester	
		Octadecane, 1-(ethenyloxy)-	
		Octadecane, 1-chloro	
		Oxirane, [(hexadecyloxy)methyl]-	
		Pentadecane	
		Pentadecane, 2,6,10-trimethyl-	
		Pentafluoropropionic acid, octadecyl ester	
		Phenol, 2,5-bis(1,1-dimethylethyl)-	
		Phenol, 2,6-bis(1,1-dimethylethyl)-	
		Sulfurous acid, 2-propyl tridecyl ester	
		Tetrapentacontane, 1,54-dibromo	
		Tetratetracontane	
		Thiocarbamic acid, N,N-dimethyl, S-1,3-diphenyl-2-butenyl ester	

9.12 POSTER PRESENTATION

The following poster presentation was given at the Gordon Research Conference in Sunday River, ME in June 2018.

Testing of Skeletonized Human Remains Using GC/MS: Development of a Personal Environmental Profile

Suni M. Edson[1,2], Timothy P. McMahon[3], and Adrian Linacre[2]

[1]Armed Forces DNA Identification Laboratory, American Registry of Pathology, LLC, Armed Forces Medical Examiner System, 115 Purple Heart Drive, Dover AFB, DE 19902
[2]Flinders University, School of Biological Sciences, Adelaide, South Australia
[3]Armed Forces DNA Identification Laboratory, Armed Forces Medical Examiner System, 115 Purple Heart Drive, Dover AFB, DE 19902

Presented at the Gordon Research Conference, Sunday River, Maine / June 2018

INTRODUCTION

Recovering DNA from skeletonized human remains is a constant challenge in human identification. DNA extraction and purification techniques are regularly developed and modified to both increase the amount of DNA recovered from osseous materials and to remove any potential environmental inhibitors present in the remains.

Initial study results indicate that DNA extraction methods are extremely efficient at removing any environmental materials present in the bone. An unexpected outcome was the ability to construct a personal environmental profile from the skeletal residue. The collective GC/MS data provided a chemical profile that is specific to the individual, as well as the incident. Results showed skeletal elements retain fat soluble medications present in the individual at the time of death. In addition, environmental materials, such as plant residues and fuel components, are absorbed into the skeletal elements during decomposition. Individuals lost and subsequently commingled in the same event can be separated based on the GC/MS profile.

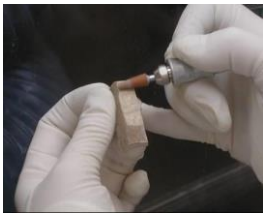
This poster presents a selection of the GC/MS data produced, to provide a demonstration of the wealth of data that may be recovered from skeletal samples. In the event of a mass fatality, this personal chemical profile may be coupled with DNA data and allow for a rapid segregation of remains by GC/MS testing.

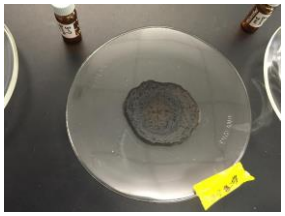
METHODS AND MATERIALS

To evaluate the presence of possibly inhibitory compounds in skeletal remains, 435 osseous samples, ranging from 40 to 70 years in age, were selected for testing. Skeletal residues (e.g., bone powder, soil), removed from the exterior of the bone during regular cleaning, were treated sequentially with acetonitrile and dichloromethane. A small subset was also treated with water. Solvent incubation was 1-2 hours in length. The liquid fraction was removed from the skeletal powder and volatilized until dry. Methanol (1mL) was used to recover the concentrated materials.

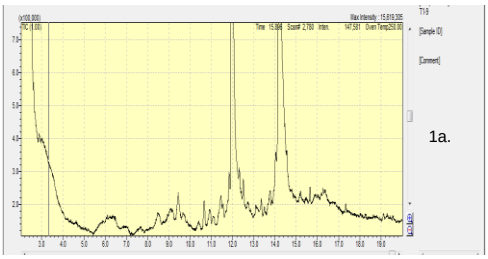
The recovered eluate were loaded onto an Agilent 7890A – 5975C GC/MSD Single Quadrupole or Shimadzu GC/MSD Single Quadrupole with the following parameters: 20/30cm column; 2.5 min solvent delay; 150°C 10min; Ramp to 250°C over 10min at 20°C/min.

Analysis was performed using ChemStation and comparison to the NIST 2005 Spectral Library.





RESULTS



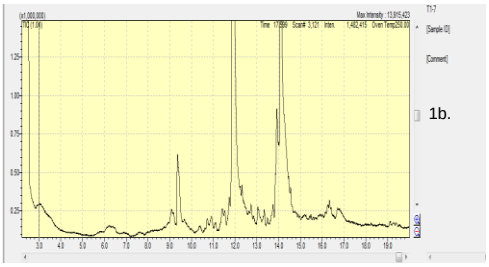


Figure 1a and b: Samples taken from external cuttings of two molars recovered from a 1952 plane crash on the Colony Glacier (Alaska). Powder was treated with dichloromethane and loaded onto the Shimadzu in the same run. Traces vary slightly in intensity, however, the materials recovered are the same pattern and classes of materials: a mix of esters, fats, and by-products of decay. After DNA testing was completed, samples were determined to be from the same individual.

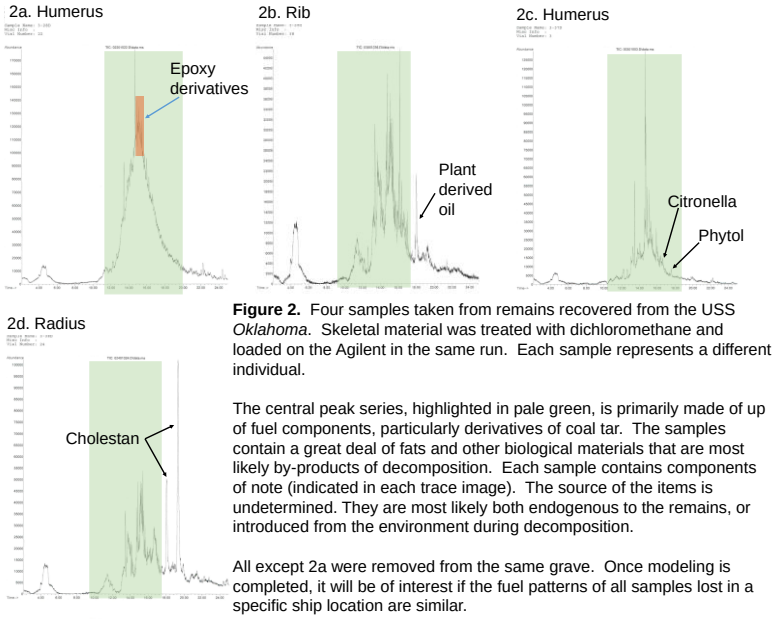


Figure 2. Four samples taken from remains recovered from the USS *Oklahoma*. Skeletal material was treated with dichloromethane and loaded on the Agilent in the same run. Each sample represents a different individual.

The central peak series, highlighted in pale green, is primarily made of up of fuel components, particularly derivatives of coal tar. The samples contain a great deal of fats and other biological materials that are most likely by-products of decomposition. Each sample contains components of note (indicated in each trace image). The source of the items is undetermined. They are most likely both endogenous to the remains, or introduced from the environment during decomposition.

All except 2a were removed from the same grave. Once modeling is completed, it will be of interest if the fuel patterns of all samples lost in a specific ship location are similar.

DISCUSSION

GC/MS analysis allows for the determination of compounds present in skeletal remains. It is not simply the materials themselves, but the pattern in which they are presented, that can provide a personal chemical profile. Items recovered from the same incident may not exhibit the exact same traces of the primary materials present. The samples from the USS *Oklahoma* (Figure 2) shows slightly different traces of the fuel oil. As they were all recovered from the same incident, the same fuel pattern should be seen in each. However, the differences could be due to the degree of exposure to the fuel (i.e., location on the ship) or a combination of the fuel with other compounds.

Analysis of samples tested is ongoing. The final stages of the project will include compiling data from each solvent tested into a single profile for a sample. Samples may be retested to verify reproducibility of profiles. Modeling will be done with SPSS to verify the patterns observed.

ACKNOWLEDGEMENTS

The authors are grateful to all of those involved in the mission to bring fallen service members home. Particular thanks to Marcel Roberts and Mecki Prinz of John Jay College of Criminal Justice.

DISCLAIMER

The opinions or assertions presented here are the private views of the speaker and should not be construed as official or as reflecting the views of the Department of Defense; the Defense Health Agency; the Armed Forces Medical Examiner System; ARP Sciences, LLC; the Defense POW/MIA Accounting Agency; or Flinders University.

9.13 PRESENTATION

The following presentation was given at the American Academy of Forensic Sciences meeting in Baltimore, MD on 21 February 2019. An earlier, less detailed version of this presentation was given at the Australia New Zealand Forensic Sciences Symposium in Perth, Australia on 11 September 2019. The ANZFSS version was presented with Timothy McMahon and Adrian Linacre as co-authors.

Data from this presentation were also shown at the Promega Technology Tour, Bode Annual Meeting, Phoenix, AZ, 23 April 2019.

Coupling DNA and GC/MS Analysis of Skeletal Remains:
A Case Study of the USS Oklahoma

Suni M. Edson[1,2]

AAFS Annual Meeting
Baltimore, MD
21 February 2019

[1]Armed Forces DNA Identification Laboratory, Armed Forces Medical Examiner System, Dover AFB, DE, USA
[2]Flinders University, College of Science and Engineering, Adelaide, South Australia

Disclaimer

The opinions or assertions presented hereafter are the private views of the speaker and should not be construed as official or as reflecting the views of the Department of Defense; the Defense Health Agency; the Armed Forces Medical Examiner System; or the Defense POW/MIA Accounting Agency.

Commercial Products

Commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible, and does not imply that any of the commercial products identified are necessarily the best available for the purpose.

Outline

- Introduction to the Mission
- Project description
  - Details regarding GC/MS
    - Protocol used for testing bone samples and DNA
  - GC/MS results
- Application of protocol to the USS *Oklahoma*

Armed Forces Medical Examiner System – Armed Forces DNA Identification Laboratory (AFMES-AFDIL)

Our express purpose is to aid in the identification of the remains of US service members

Source: www.15wing.af.mil Photo Credit: Petty Officer 2nd Class Seth Coulter

Armed Forces Medical Examiner System – Armed Forces DNA Identification Laboratory (AFMES-AFDIL)

- A subdivision of the Armed Forces Medical Examiner System (AFMES)
- Established in 1990 as the DoD DNA Registry
- Mission Partner with Defense POW/MIA Accounting Agency (DPAA)

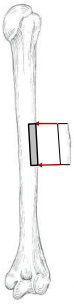
Sample Collection

Sample Collection

- Samples are recovered by scientists from DPAA or partner agencies.
- Samples are typically 40-70 years post-mortem
- Elements may be found under a variety of conditions:
  - Buried in soil
  - Surface
  - Unilateral turnovers
  - Submerged in water

DPAA website. Photo credit: SSgt Erik Cardenas

Sample Collection

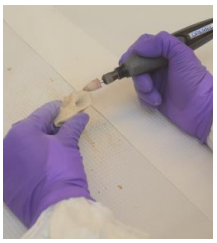


- Samples are returned to the lab.
- Elements are sorted and nominated by an anthropologist
- A small fragment is removed for DNA testing
  - Samples are typically 2.0 - 5.0 g
  - But can range from 0.06 – 33.0 g

GC/MS Analysis

GC/MS Analysis

- 438 samples of bone powder removed from the exterior of the element during regular casework were collected

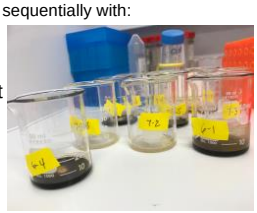


GC/MS Analysis

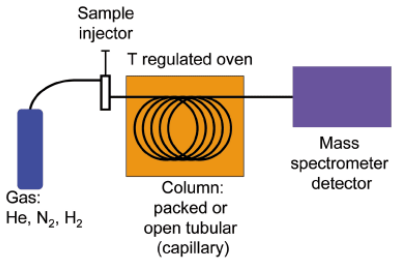
- Powder was collected at random ranged from fine powdered white to black and oily.
- Samples were from Korea, Southeast Asia, and world-wide sites involved in World War II.

GC/MS Analysis

- ~0.1g of bone powder was soaked in 1mL of solvent for 1 hour.
  - Samples were treated sequentially with:
    - Acetonitrile
    - Dichloromethane
- Volatilized until dry at room temperature.
- Resuspended in 500ul of Methanol



GC/MS Analysis



GC/MS Analysis

- Loaded on the Agilent 7890A – 5975C GC/MS (Single Quadrupole)
  - -- 30m column / 20m column
  - -- 2.5min solvent delay
  - -- 150ºC 10min
  - -- Ramp to 250ºC over 10min at 20ºC/min
  - -- Splitless
  - -- Full scan
- Analyzed using Chemstation
  - -- Mass Hunter
  - -- NIST 05/11 Library

GC/MS Analysis

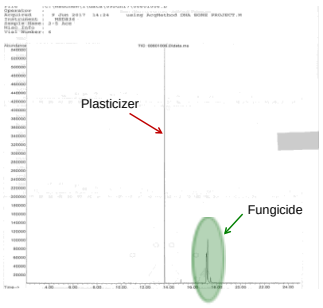
A full scan analysis meant everything in the sample was detected.

And it turns out there is a LOT of information there.

GC/MS

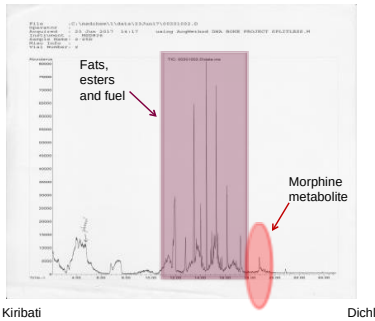
- There is a wealth of data in the bones.
  - Samples may have fats, oils, alcohols, esters, and sugars from the body itself,
    - Including fat soluble medications.
  - Fuel, pesticides, and plant oils from the environment are also present.

GC/MS



SEA-Laos Acetonitrile

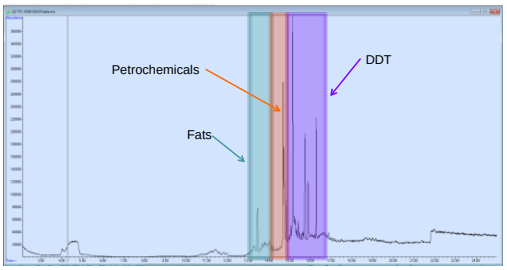
GC/MS



WWII – Kiribati

Dichloromethane

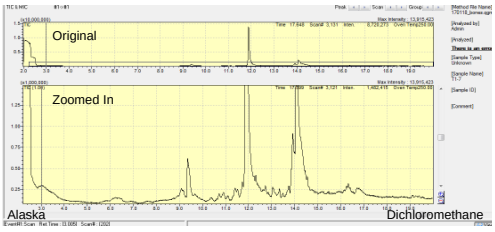
GC/MS



WWII – Yugoslavia

Dichloromethane

GC/MS



Alaska

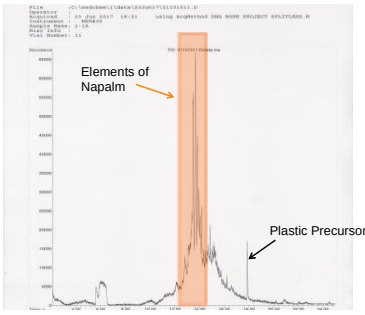
Dichloromethane

Some samples have lower signal strength, but can still be analyzed.

Solvents

- Different solvents remove different compounds from the skeletal samples
  - Samples need to be soaked in solvents from least stringent to most stringent.
  - Allows a fraction of the bone powder to be tested more than once.

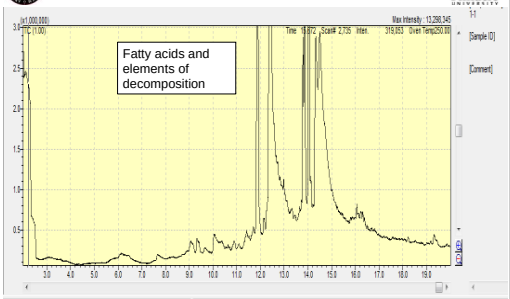
Acetonitrile



Southeast Asia – Laos (Unilateral Turnover)

Acetonitrile

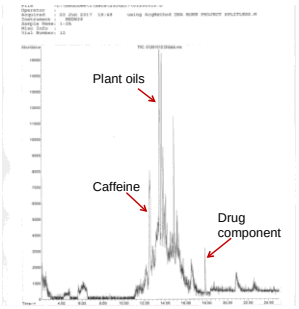
Dichloromethane



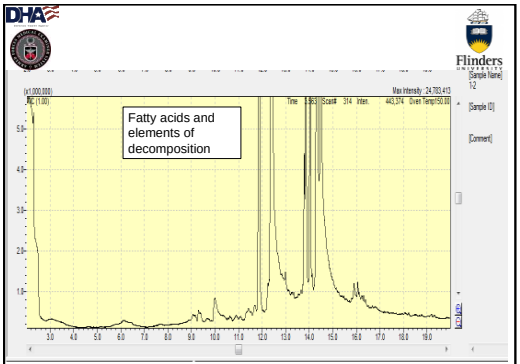
Southeast Asia – Laos (Unilateral Turnover)

Dichloromethane

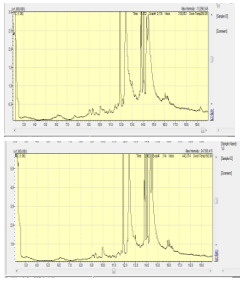
Acetonitrile



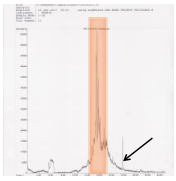
Southeast Asia – Laos (Unilateral Turnover) Acetonitrile

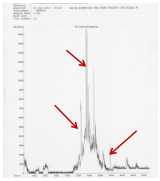


Southeast Asia – Laos (Unilateral Turnover) Dichloromethane



- One solvent alone gives a fairly similar profile.
- The compounds present are not the same, but the traces are similar
- These are two different samples from the same 'site'

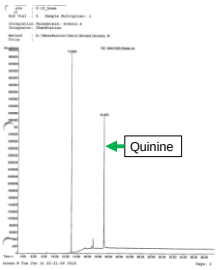




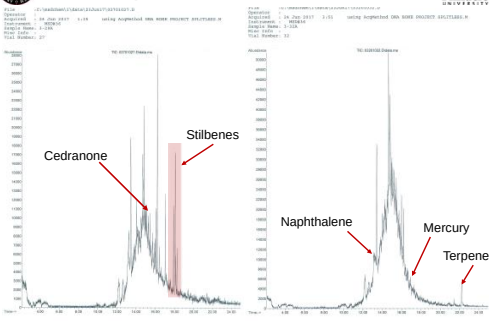
- The 2nd solvent shows distinct differences between the samples.
- DNA testing supported that they are 2 different people
- The compounds indicate they may not be from the same site.

Are the components of the bone a predictor of an individual?

- Fat-soluble medications can be detected in the bones
- Components from the environment can be detected.
- GC/MS has been used to predict location of origin in plants

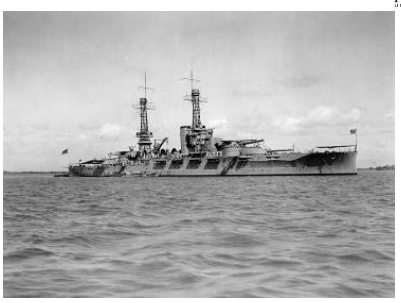


Are the components of the bone a predictor of an individual?



If the GC/MS trace from a skeletal element is distinct to an individual, can it be used as a tool for disaster victim identification in mass fatalities?

USS *Oklahoma*: A Case Study



USS *Oklahoma*

On 7 December 1941, the USS *Oklahoma* was torpedoed during an attack on Pearl Harbor, HI.

USS *Oklahoma*

- The ship rolled, trapping 429 sailors and marines inside.
- The ship was righted in 1943.
  - Remains were recovered and interred in two cemeteries in Hawaii.

USS *Oklahoma*

- Remains were disinterred in 1947, in an attempt at identification.
- Commingling was extensive.
- Remains reburied in Hawaii in 65 coffins.

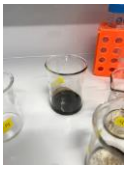
USS *Oklahoma*

- The first casket was disinterred in 2003, with the remaining 64 caskets being disinterred starting in 2015.
  - The first casket was believed to be 5 individuals, yet in testing 107 samples, 96 distinct mtDNA sequences were reported
- To date, 3319 samples have been processed for Sanger mtDNA.
  - Samples work remarkably well, with a 99% success rate for any element tested.

USS *Oklahoma* & GC/MS

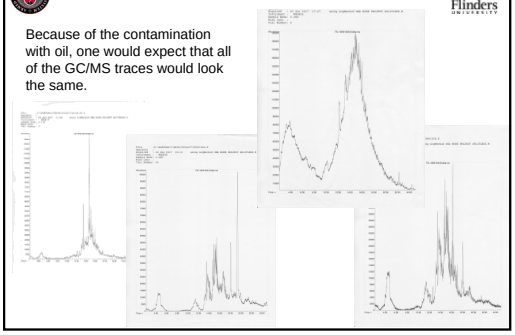
- 208 samples collected for GC/MS were from the USS OK
- "Powder" was oily and black
  - Elements themselves retained an odor of oil



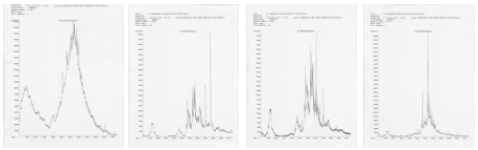


USS *Oklahoma* & GC/MS

Because of the contamination with oil, one would expect that all of the GC/MS traces would look the same.



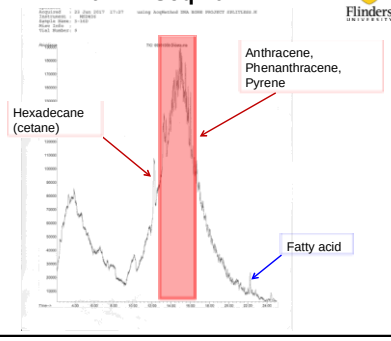
USS *Oklahoma*, GC/MS, & DNA



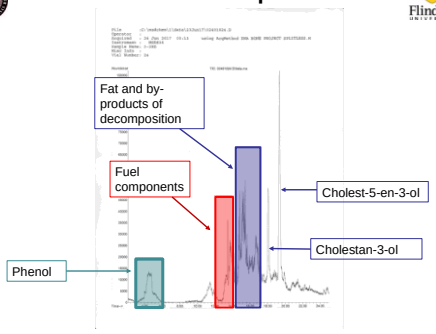
MtDNA Seq 102 MtDNA Seq 99 MtDNA Seq 87-C MtDNA Seq 29-2

All of these were disinterred from the same tertiary grave, so it is expected that the profiles should be a predictor of where on the ship the individuals lost their lives.

MtDNA Seq 102



MtDNA Seq 99



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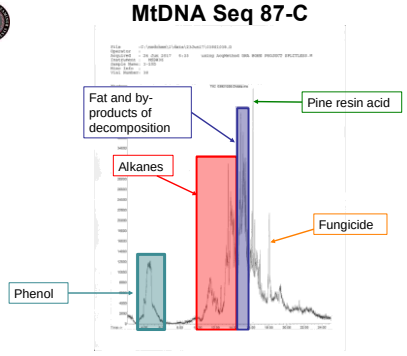

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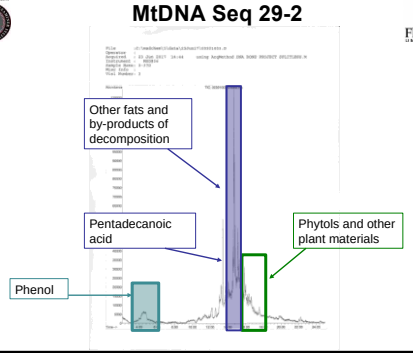
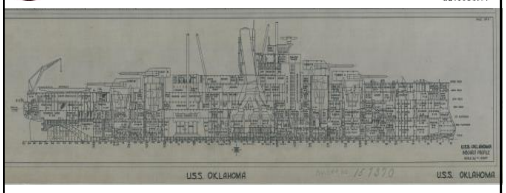
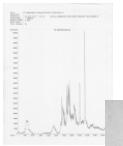


USS *Oklahoma*, GC/MS, & DNA



- Not all of the skeletal remains have been associated with an individual yet, so it has been difficult to make a prediction with the remains GC/MS tested.
- Project is ongoing.

USS *Oklahoma*, GC/MS, & DNA



MtDNA Seq 99

- If the GC/MS profile can be tied to the DNA profile, the rate with which identifications could be made in mass fatality incidents could be increased.

Potential of the Technique

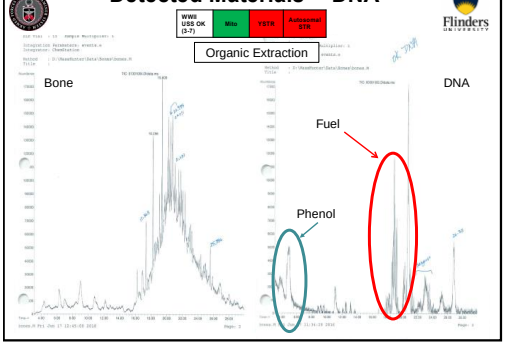
- The chemical profiles provide information about the person and the site of burial.
- Inexpensive.
- Can be coupled with DNA analysis to more rapidly separate skeletonized remains.
  - Once a chemical profile is paired with DNA, one only need chemically test the remains. Every bone recovered in a mass fatality incident could be tested if needed.

(It's more time consuming to do the analysis than to do the actual test.)

Associated DNA Testing

How does the presence of these chemicals impact the success of the DNA testing?

Detected Materials -- DNA



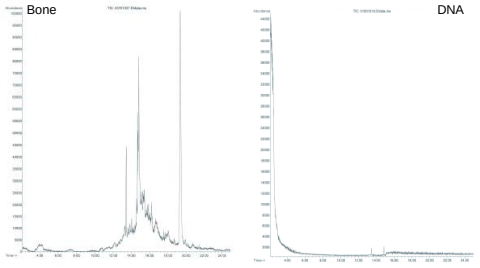
Detected Materials -- DNA

SEA Vietnam (2-4) | Mito | YSTR | Autosomal STR

Inorganic Extraction

Bone

DNA



Continuing Work

- Verification that the profiles can be replicated.
- Continue the study with the USS *Oklahoma* to confirm correlation between loss location and GC/MS profile
- Efficiently model the profiles themselves to make the data more accessible.
  - Complex GC/MS can obscure some information.

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- ForTox – Jeff Chmiel
- FTI – Jon Norris
- DPAA – Stephanie Ah Sam, Dr. Greg Berg, Dr. Alec Christensen
- Administrative Support – Carole Anderson, James Canik, Dr. Mike Coble

Any Questions?

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AFMES Additional Information:
http://www.health.mil/afmes

DPAA Additional Information:
http://www.dpaa.mil/

Chapter 10

Concluding Statements & Future Work

10.1 INTRODUCTION

The statements described in this final chapter are an overall summary of the work preformed, the ideas presented, and the future direction that the work can take. The thesis has taken a broad survey of the DNA analysis of skeletonized remains and attempted to derive recommendations for the forensic community, and specifically for those who are working towards human identification in mass fatality circumstances. Sampling strategy choices can be made from the data gathered herein, but there is a need to also reevaluate the manner by which DNA from skeletal remains is recovered. Gas chromatography / mass spectrometry (GC/MS) provided an insight into what is not carried over into the DNA extracted from skeletonized remains, and perhaps recasts how inhibitors in extracted DNA should be considered.

10.2 SAMPLING OF THE SKELETON FOR DNA

The foundational technologies of the DNA process need to be evaluated for efficacy and efficiency. DNA extraction protocols are typically not considered as part of a process improvement for DNA testing of skeletonized human remains. Industry, universities, and practitioner laboratories have spent a great deal of time improving the downstream processes, but have rarely looked back to the start. The focus has been on sampling strategies of skeletonized remains (Barta, et al., 2014a; Hines, et al., 2014; Mundorff and Davoren, 2014); however, as the results in this study have indicated, the element chosen is irrelevant should the extraction protocol be optimal for the downstream DNA process (Figure 10.1).

A single DNA extraction protocol is not always optimal for all downstream tests. In Table 10.1, it is can be seen that almost all skeletal elements produce over a 90% success in Sanger sequencing of mitochondrial DNA when paired with a complete demineralization and organic purification (EP#2). However, this success does not carry-over to STR analysis. STR analysis is better served by an inorganic purification of completely demineralized bone (EP#3) (Table 10.2).

Teeth exhibited a different pattern than that of the osseous material. Considered an optimal source of DNA in a plethora of situations (Miloš , et al., 2007; Prinz, et al., 2007), teeth may be sampled without obvious damage by removing the crown and removing the dentin or pulp from the interior of the tooth. The high mineral content of teeth provided the best results when purified using a silica column purification rather than an organic purification (Figure 10.3). Silica columns are designed to retain minerals and remove them from the DNA extraction, whereas phenol:chloroform purification more effectively removes fats and proteins.

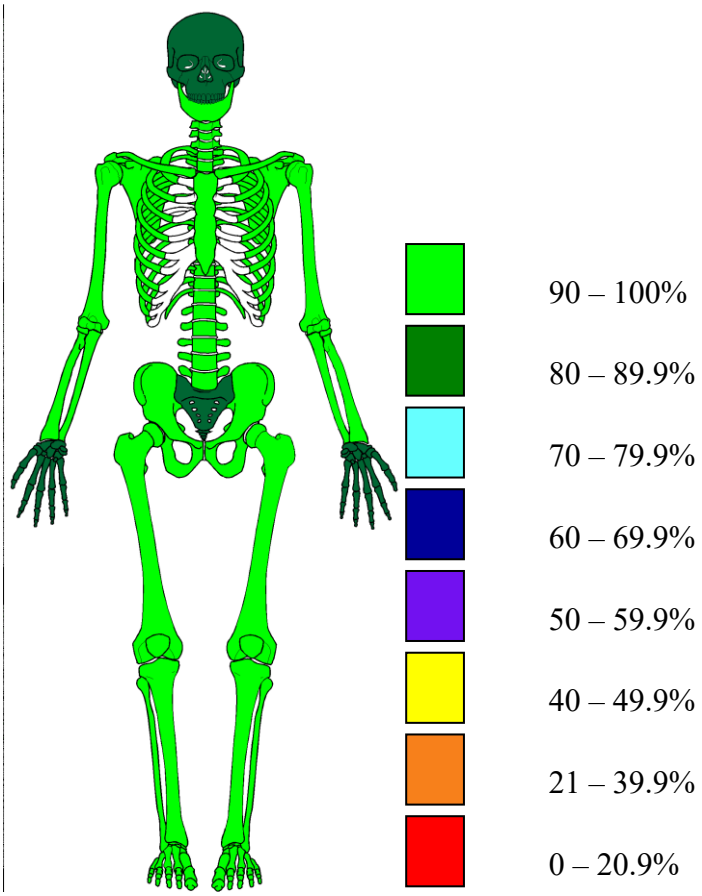

Figure 10.1. A graphical representation of the success of the Sanger sequencing of mitochondrial DNA from individual skeletal elements in which the DNA was extracted using a complete demineralization coupled with an organic purification.

Table 10.1. A summary of the Sanger sequencing of mtDNA success for individual skeletal elements tested at AFMES-AFDIL between 1990 and 2016. Only the success percentage of those samples extracted using demineralization coupled with an organic purification is contained in the table. Samples are listed alphabetically.

	Sanger Sequencing Extraction Protocol #2		
	# Tested	# Success	% Success
All	7110	6528	92
Acetabulum			
Calcaneus	26	24	92
Capitate			
Clavicle	344	328	95
Cuboid	1	0	0
Cuneiform			
Femur	805	749	93
Fibula	536	524	98
Fragments	492	269	55
Hallux			
Humerus	876	824	94
Jaw	154	140	91
Manubrium			
Metacarpal	64	58	91
Metatarsal	161	154	96
Navicular	1	1	100
Patella	14	14	100
Pelvis	566	549	97
Phalanx	13	12	92
Radius	464	443	95
Rib	433	397	92
Scapula	454	437	96
Sphenoid	2	2	100
Sternum	1	1	100
Talus	15	12	80
Tibia	746	704	94
Trapezium	2	1	50
Ulna	519	493	95
Vertebra	390	375	96
Zygomatic	10	9	90

Table 10.2. The success of individual skeletal elements tested in AmpFlSTR® MiniFiler™. Samples were submitted to AFMES-AFDIL between 1990 and 2018 and are included in the table alphabetically. Extraction Protocol #2 is demineralization coupled with an organic purification. Extraction Protocol #3 is a demineralization coupled with an inorganic purification.

	Extraction Protocol #2			**Extraction Protocol #3**		
	# Tested	# Success	% Success	# Tested	# Success	% Success
All	637	216	34	628	324	52
Clavicle				40	1	3
Femur	85	52	61	91	78	86
Fibula	51	28	55	38	28	74
Fragments	18	7	39	22	10	45
Humerus	87	30	34	88	51	58
Jaw	26	10	38	20	18	90
Metacarpal	2	0	0	10	1	10
Metatarsal	13	0	0	12	4	33
Pelvis	28	0	0	53	20	38
Phalanx				2	1	50
Radius	57	6	11	56	18	32
Rib	44	4	9	11	4	36
Scapula	21	2	10	35	7	20
Talus				1	1	100
Tibia	94	55	59	75	63	84
Ulna	58	5	9	52	20	38
Vertebra	38	17	45	19	9	47

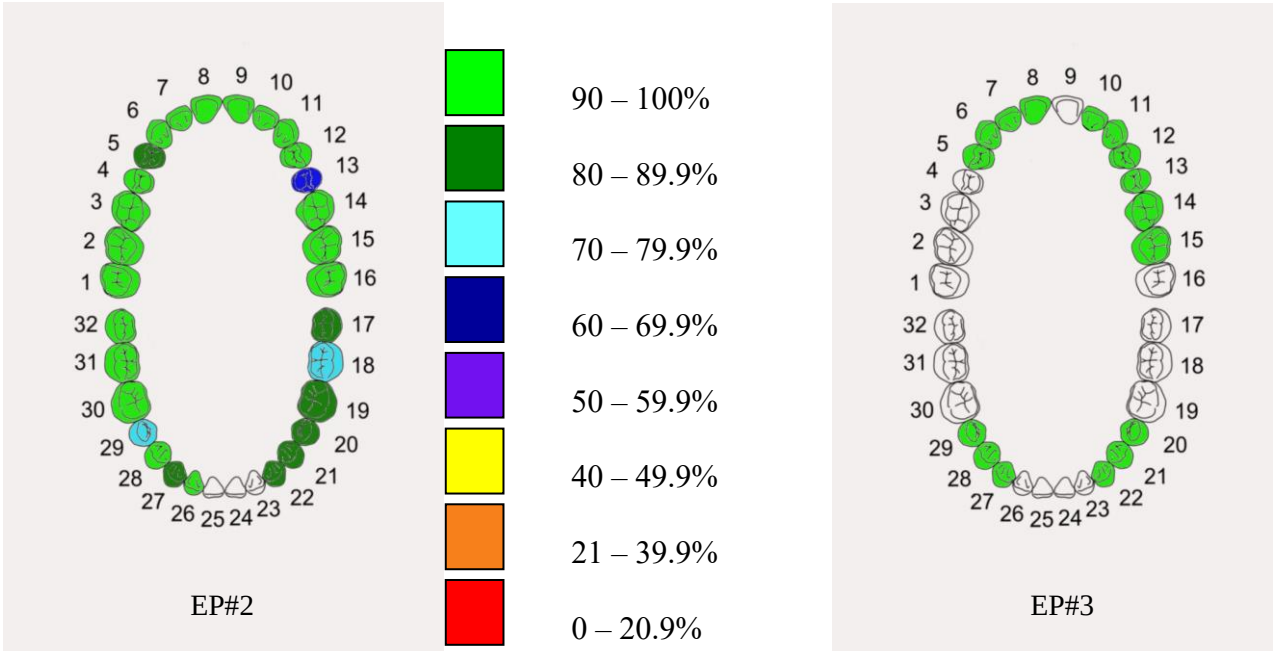

Figure 10.2. A graphical representation of the success of teeth using Sanger sequencing of mitochondrial DNA. Teeth were extracted using Extraction Protocol #2 (EP#2) and Extraction Protocol #3 (EP#3). Teeth are numbered starting from the upper left. Only teeth numbered by a forensic odontologists are included in these diagrams.

Should laboratories be able to have more than one extraction protocol validated at any given time, they could employ a check-list on which extraction strategy should be employed, based on the type of samples they are working with and the desired downstream DNA testing. For example, a femur could be extracted with a complete demineralization coupled with an organic purification for any downstream DNA processing. A femur might not always be the best post-cranial skeletal element from which to sample, but it the most reliable. This is most likely due to the protection the density of its bone structure provides to the DNA present. In cases where the skeletal remains are in discrete burials and there is minimal commingling, metatarsals and metacarpals work well with either an organic or inorganic purification, but in this study, these elements worked most reliably with mtDNA Sanger sequencing.

10.2.1 Sampling Bias or the Curiosity of the USS Oklahoma

The sampling guidelines presented in Chapters 2 and 3 derived from an aggregate of all of the casework performed at AFMES-AFDIL since the inception of the laboratory in 1990. The initial goal of this survey was to provide a sampling strategy that could be used across a wide-variety of circumstances and recoveries of remains. There are a number of papers that have suggested sampling strategies of skeletonized human remains, but they tend to be recommendations based on specific types of incidents (Mundorff, et al., 2009; Mundorff and Davoren, 2014; Hines, et al., 2014). By examining a broad base of sample types and circumstances, a more generalized strategy should be obtained. While this is true, it was noted during the data analysis that there is a marked difference in success rates for some of the incidents contained within the casework. For example, in Chapter 2, there appeared to be a very high level of success among the ribs. At closer examination, 19 of those ribs (36% of the ribs sampled) were recovered from the same location in Vietnam and all provided successful testing results.

The effects of sampling bias were further bolstered during the analysis of the cranial elements in Chapter 3, where it was noted that there was a preferential sampling of the occipital during the testing of a single incident, that of the sinking of the USS *Oklahoma* (Figure 10.3). Given the number of samples processed overall in the past 25 years, the success rates of the cranium should not have been markedly skewed. However, of the 436 occipitals tested at AFMES-AFDIL since 1992, 347 (79.5%) were from the USS *Oklahoma*. Of these, 329 generated a result in mtDNA analysis, thereby dramatically skewing the overall success of that particular element. Removal of the samples from the USS *Oklahoma* from the accounting of the cranial elements tested, reduced the Sanger sequencing of mtDNA success of the occipitals to 78% (Table 10.3).

Table 10.3. A summary of mitochondrial DNA Sanger sequencing from a selection of samples recovered from the USS *Oklahoma*. The data here demonstrate the sampling bias that is inherent in the testing of the samples.

		mtDNA Sanger sequencing		
Sample type and Recovery Location		# tested	# successful	% successful
Mandible	All samples	153	139	91%
	USS Oklahoma only	32	32	100%
	All samples minus USS Oklahoma	121	107	88%
Temporal	All samples	189	165	87%
	USS Oklahoma only	21	19	90%
	All samples minus USS Oklahoma	168	146	87%
Occipital	All samples	436	398	91%
	USS Oklahoma only	347	329	95%
	All samples minus USS Oklahoma	89	69	78%
Teeth	All samples	686	618	90%
	USS Oklahoma only	36	35	97%
	All samples minus USS Oklahoma	650	583	90%

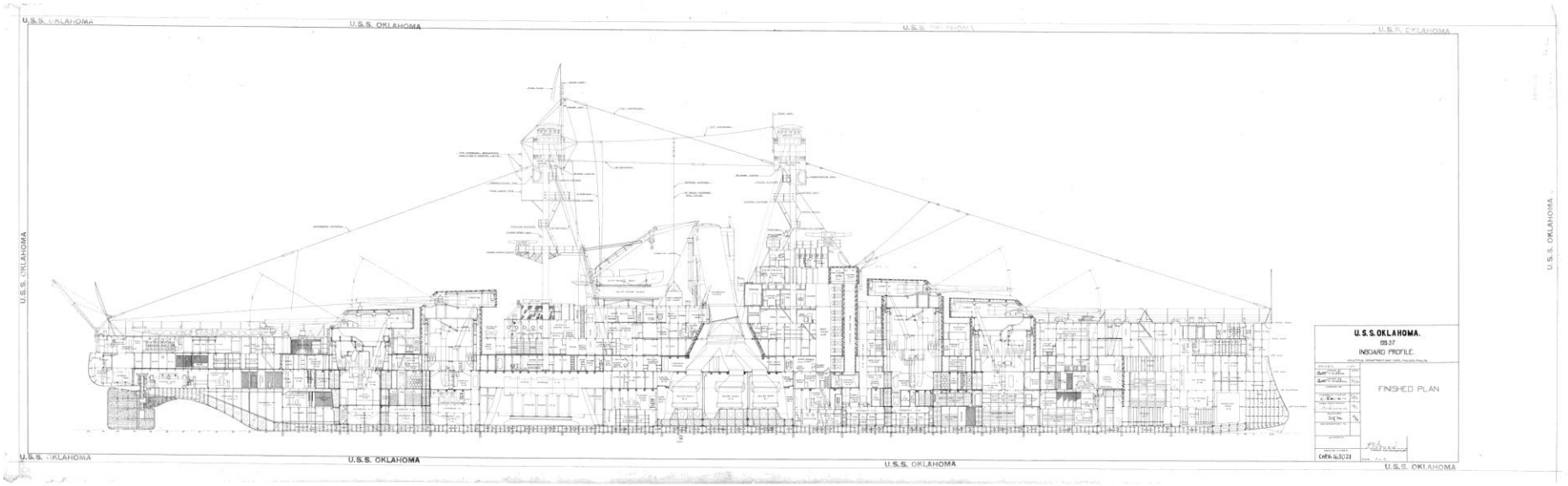

Figure 10.3. An inboard profile of the USS *Oklahoma* (National Archives). The ship was a *Nevada* class battleship that was one the first in the United States Navy to run exclusively on fuel oil.

The success of the USS *Oklahoma* samples across all modalities of DNA testing is somewhat curious under the prevailing wisdom that elevated temperatures and chemical contamination should cause rapid degradation of DNA in skeletonized remains (Smith, et al., 2003; Hofreiter, et al., 2014; Nieves-Colón, et al., 2018). As a brief summary, torpedoes struck the USS *Oklahoma* on 7 December 1941 during a raid on the US Naval Base in Pearl Harbor, Hawaii (then the Hawaiian Territories). Rather than sinking to the bottom, the ship rolled, trapping 439 sailors and Marines in the belly of the ship, which rapidly filled with seawater and liquid fuel. The bodies remained within the ship until 1943, when the ship was righted for salvage. Despite efforts at identification, the remains were eventually buried collectively as unknowns in the National Memorial Cemetery of the Pacific (NMCP) in Hawaii. Modern identification efforts started in 2015.

Chapter 5 provided a comparison of the USS *Oklahoma* samples to two other sets of World War II era losses, one of which had no chemical contamination (Battle of Tarawa) and one extensive chemical contamination (Cabanatuan Prison Camps). The samples from the USS *Oklahoma* show a marked success across all modalities (with the exception of Next Generation Sequencing), even when compared to contemporary samples. Next Generation Sequencing (NGS) success was only evaluated between the Battle of Tarawa samples and the Cabanatuan Prison Camps, as this strategy is employed primarily for samples showing marked inhibition or those sharing a common mitotype.

10.3 GC/MS ANALYSIS OF SKELETAL REMAINS

The comparison of these three sets of World War II remains led to a question, how does chemical contamination impact downstream processing? In the field of forensic DNA analysis, there are constant reminders of the inhibitory effects of certain materials (e.g., haem: Akane, et al., 2004; humic acid: Braid, et al., 2003; indigo: Opel, et al., 2010) and the need to

remove them during the DNA extraction protocol. Co-extraction of chemical compounds, inhibitory or not, is a prevailing problem, especially among those working on skeletonized human remains. The remains from the USS *Oklahoma* would seem to run counter to the common wisdom: they were subjected to extensive chemical exposure, yet they still are more successful than samples from contemporaneous events, or even samples recovered more recently. The fuel oil contamination was so extensive that its presence can still be detected through visual and olfactory means to this day. How effective are the current DNA extraction protocols at removing this exogenous material? Clearly the DNA testing is successful, yet is the oil being removed during extraction, or does it simply not affect PCR?

A novel technique for examining skeletal materials and the co-extracted DNA was developed to answer not only this question, but also the larger question of the co-extraction of inhibitors. There is the general assumption that materials from the environment seep into the osseous materials and co-extract with the DNA, but few studies have examined real-world situations involving human remains. Several studies have been done on animal bones (e.g., salmonid vertebrae: Kemp, et al., 2014; seal ribs: Barta, et al., 2014) or samples spiked with inhibitory materials (blood: Moreno and McCord 2016). The intent in the study presented in Chapter 6 was to develop a method by which any laboratory could evaluate the efficacy of their extraction protocols, by testing both the parent bone and the associated DNA extract.

Gas chromatography / mass spectrometry (GC/MS) is used in a number of different forensic disciplines, such as toxicology (e.g., Skender, et al., 2002), questioned documents (e.g., Yao, et al., 2009), and accelerants / arson (e.g., Dhabbah, et al., 2014), to describe the component parts or contaminating compounds of a target material. The osseous material is a complex matrix and might not retain many exogenous materials; therefore, a solvent extraction of compounds and subsequent concentration was anticipated to be the most effective technique

for detection of materials. A similarly type of testing is used in arson investigations (Pert, et al., 2006), where the materials are reduced in concentration and may be extremely volatile.

The protocol developed is a simple technique. During preparation for extraction of DNA from a skeletal element, the outer surface is removed using a sanding tool. The osseous detritus was collected and exposed to a sequence of washes with two different solvents, acetonitrile and dichloromethane. The treatments were performed independently, with the solvent fraction removed for concentration before the next solvent was added. The solvent eluate was allowed to volatilize in a fume hood until dry and the remaining materials recovered with methanol. The methanol fraction was injected onto the Agilent 7890A/5875C GC/MS System with a 20 m column (Agilent, Santa Clara, CA). The treatment of the DNA was simpler, and involved suspending DNA extracted from over 400 individual skeletal elements into methanol for injection on the GC/MS instrument. Data were analyzed using ChemStation and MassHunter and comparison to the NIST2005/2011 spectral libraries.

The profiles generated form the osseous materials are complex. Compounds from the surrounding environment and the remains of the individual were detected. Fats, esters, and by-products of the decomposition of the body were detected, in addition to plant oils from the remains and traces of petrochemicals. The remains tested from the USS *Oklahoma* primarily showed an intricate mixture of petrochemical and fats. The results of saponification of the bodies during the initial entombing within the ship were unable to be removed by washing or even sanding. During cleaning, outer surface of the bone, although appearing solid, would often liquefy under the heat of the sanding bit. The fats and oils were readily apparent in the majority of these remains, forming an arc of results rather than a series of discrete peaks that could be individuated (Figure 10.4).

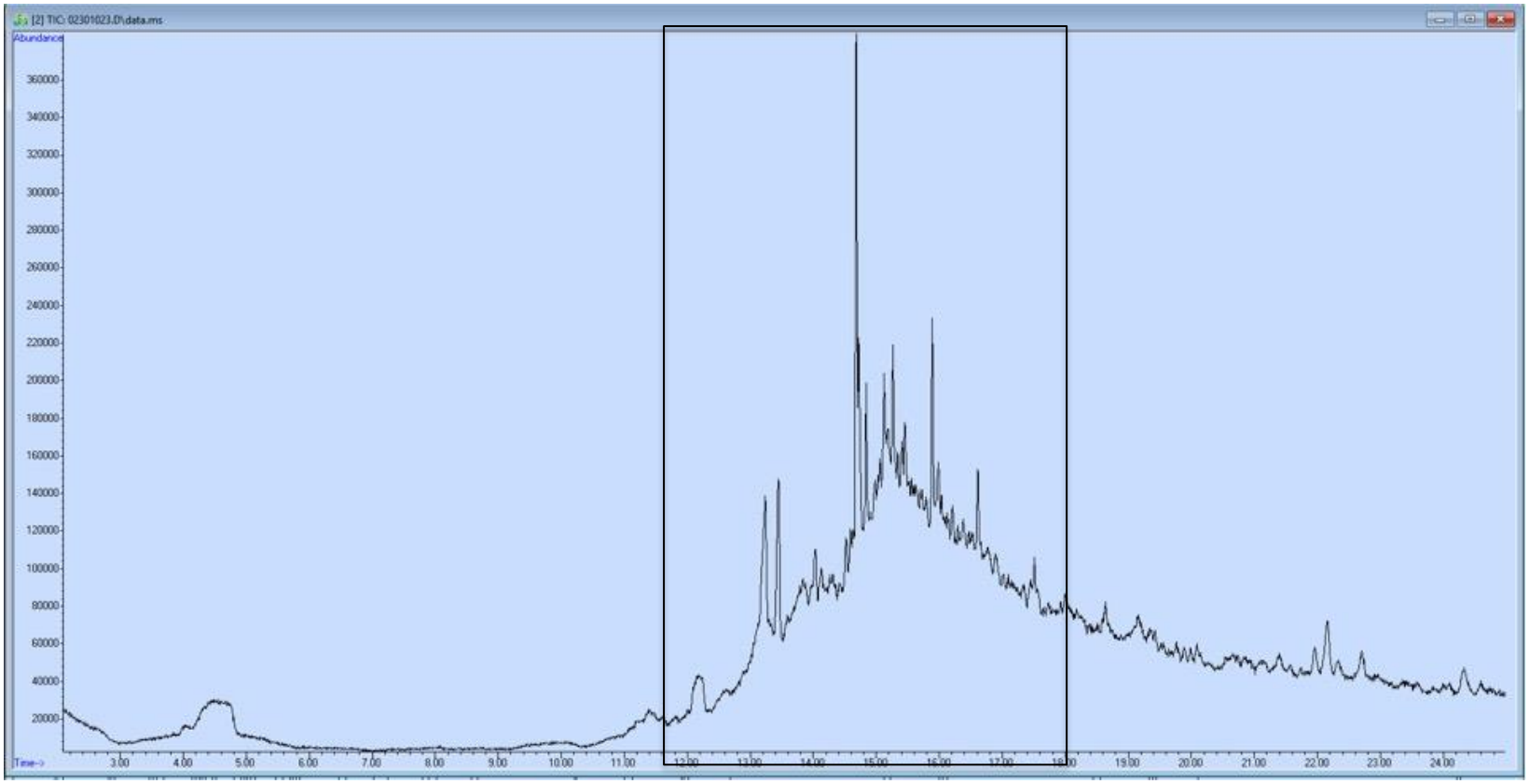

Figure 10.4. A GC/MS trace generated from a sample recovered from the USS *Oklahoma*. This particular image was generated form osseous materials treated with dichloromethane. The centre peaks, marked with a rectangle, are a combination of fats and petrochemicals.

An unexpected result from the remains was the ability to detect materials that may have been specific to the individual or the primary location of decomposition. For the USS *Oklahoma* remains it was the detection of pine tar in several samples. Historical research was needed to determine the source of this was from either the decks or the ropes of the ship, which were constructed of pine and needed regular maintenance (L. Freas, pers. comm.). Another example of location or individual specificity was the detection of DDT in a WWII era sample recovered from Yugoslavia (Figure 10.5). During World War II, soldiers were regularly treated with DDT to combat infestations with lice or other parasites and prevent the spread of typhus (Friedman, 1992). The treatment was apparently so pervasive as to have incorporated into the fats of the individual during life, thus soaking into the bones during decomposition.

There is the distinct possibility that the chemical profiles generated from the skeletal remains can be assigned to a single individual. Examination of sets of remains from the USS *Oklahoma* indicates that there are clear differences in the chemical profiles of individuals from the same event. In order to more fully develop a robust model, a collection of samples from known locations of decomposition need to be gathered and tested. There was an initial thought in this study that the remains from the USS *Oklahoma* could be used to design such a model. While there are distinct differences in the chemical profiles from known individuals (as determined by their DNA profiles), there is limited knowledge of where the persons were at time of death. In addition, as the samples were collected at random during the collection period, there may be only one bone from and individual in the collection of samples tested. Further work needs to be done on the development of a chemical profile, but it holds much promise.

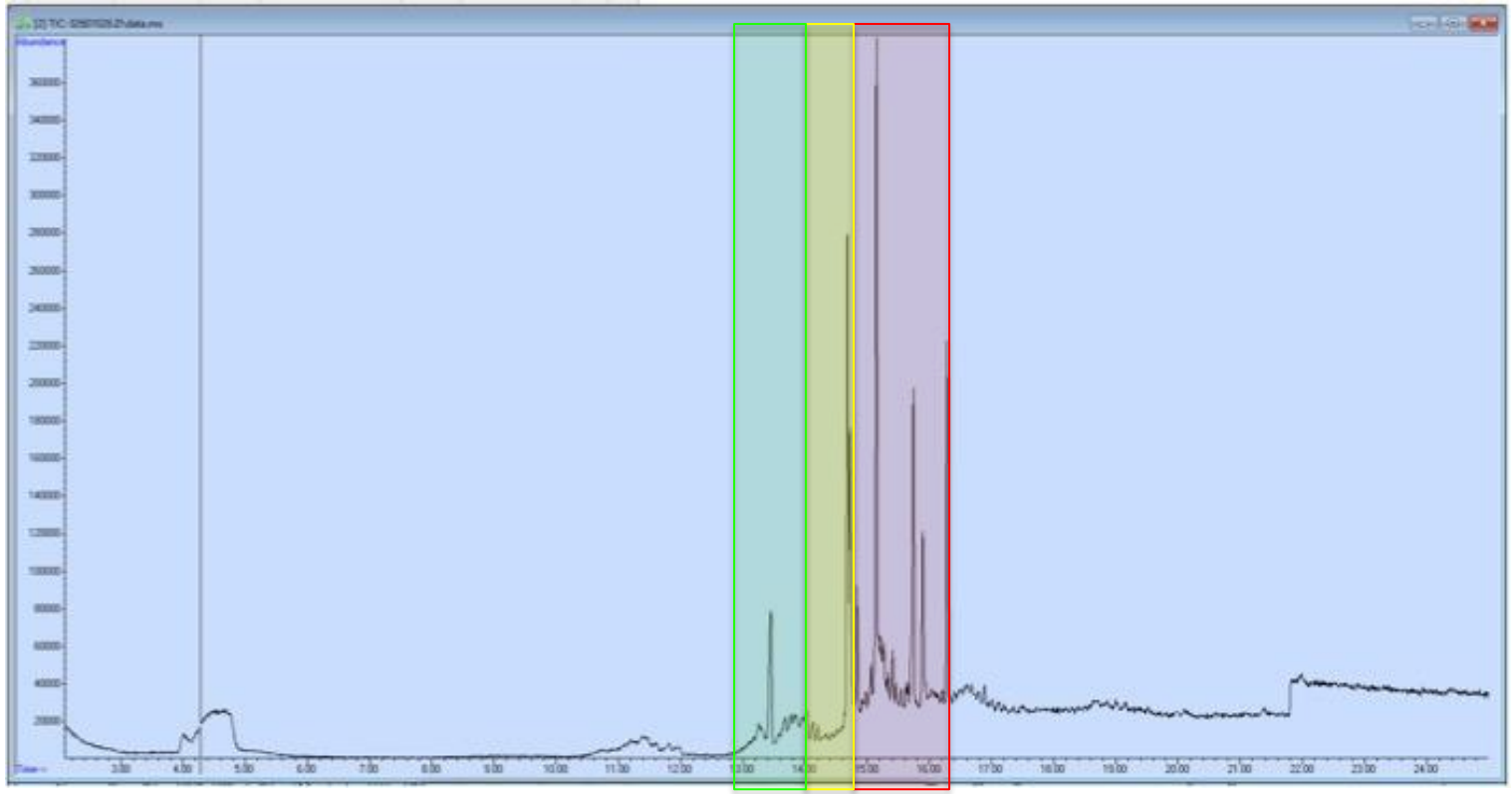

Figure 10.5. The GC/MS trace generated from the dichloromethane extraction of osseous material recovered from a World War II era case found in Yugoslavia. The peaks in the red square originate from DDT and associated metabolites. The peaks in the yellow square are petrochemicals and the peaks in the green are fats.

10.4 IS THERE CARRY-OVER FROM SKELETAL MATERIALS TO DNA?

With the amount of materials to be seen in the osseous materials, there was an expectation that there would be a high degree of carry-over to the associated DNA extracts. Even if none of the environmental materials transferred from the bone to the DNA, other studies lead to the expectation of carry-over from the extraction technique (Kemp, et al., 2014; Moreno and McCord, 2016). However, this was found to not be so. With the 412 DNA extracts tested via GC/MS, only four samples showed evidence of phenol carry-over from an organic purification. No guanidinium or other materials associated with the inorganic extraction were detected. There is very limited carry-over from either the bone or the extraction protocol. It appears that the DNA extraction protocols commonly used in the forensic DNA community are quite effective at removing potential inhibitors.

However, the efficacy of the removal of inhibitors comes at a cost. There is a loss of DNA with each additional wash step within a DNA extraction protocol (Kemp and Smith 2006; Qiagen, 2008; Barta, et al., 2014b). With this realization that there is minimal carry-over from the skeletal remains to associated DNA extract, we have come full circle back to the original purpose of this study. How do we improve the extraction of DNA from skeletal remains? The targeting of specific inhibitors or even the simple re-evaluation of extraction strategies could maintain the purity of the DNA extract without the associated DNA loss.

10.5 FURTHER WORK

Further work should be done to examine the stringency or efficiency of DNA extraction protocols currently in use. From the data presented here, it would appear that at the least the protocols in use at AFMES-AFDIL are very efficient. Removal of some of the wash steps could increase the amount of DNA recovered, while still efficiently removing inhibitors and other co-extracting materials.

In addition, the data recovered from the GC/MS analysis of the skeletal materials needs to be further analyzed to determine if there are corollaries between the amount of detected materials and the recovery of DNA. Also, sets of remains from known individuals and losses should be analyzed with the GC/MS techniques to determine the amount of peri-mortem information that can be recovered from osseous materials. Other locations within the AFMES-AFDIL data set can be analyzed to determine if there are other trends that could be gleaned from the loss/recovery location and the DNA success.

10.6 CONCLUDING STATEMENTS AND IMPACT ON THE FIELD

There are a few specific points that can be taken away from the work contained within this thesis:

- Firstly, DNA extraction protocols are highly efficient at the removal of DNA. Laboratories should seek to redefine extractions and retool them to maintain the purity of the extracted DNA without the concordant DNA loss. GC/MS analysis of extracted DNA indicates there is almost no carry-over from either the bone or the extraction protocol.
- DNA extraction protocols should be chosen based on the downstream DNA processing. In general, organic extractions work better for Sanger sequencing of mitochondrial DNA.
- In cases where remains are both skeletonized and highly commingled, sampling of a femur or the temporal bone for DNA are the most reliable choices for the generation of a DNA profile regardless of the extraction protocol or downstream testing platform.
- In cases where the remains are skeletonized but in discrete burials, sampling of smaller elements such as the metatarsals and metacarpals can be sampled for DNA. Caution should be taken as these elements will not aid in anthropological reassociation of other skeletal elements.

- GC/MS analysis of skeletal remains and the associated DNA is a useful tool that has potential to aid in identifications.
- Additional work should be done on the GC/MS analysis of osseous remains and the generation of a personal chemical profile. This innovative technique has the potential provide an additional tool in the identification of skeletonized human remains.

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Appendix A

Flexibility in Testing Skeletonized Remains for DNA Analysis can Lead to Increased Success: Suggestions and Case Studies

A.1 INTRODUCTION

In a forensic laboratory, analysts are often restricted to a certain set of parameters for processing of cases. Due to the requirements of accreditation, standard operating procedures (SOP) need to be rigorously followed, and approval received from supervisors before deviations occur. The intent of this publication is to provide DNA analysts and anthropologists with a framework in which they can think laterally from the SOPs.

In mass fatality events, the scientists involved in the human identification process are often faced with the challenge of processing large groups of remains quickly. Mass fatality events involving skeletonized remains may also be faced with the confounding factor of commingling, necessitating DNA testing of most, if not all, of the remains tested. While laboratory guidelines must be followed for accurate reporting of results, the scientists involved should consider carefully the conditions surrounding the remains being tested. By doing so, DNA recovery may proceed in a more efficient manner. This chapter was designed to present anthropologists and DNA analysts with alternatives to DNA processing while remaining within the framework of casework requirements.

A.2 PUBLICATION

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Flexibility in Testing Skeletonized Remains for DNA Analysis can Lead to Increased Success: Suggestions and Case Studies.

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A.2.1 ATTRIBUTION OF TASKS

Ms. Edson wrote the Introduction, Conclusions and the Extraction of DNA from Skeletal Remains sections. She also edited the chapter overall and bolstered some sections with additional text. Pieces of the Literature Review presented in Chapter 1 of this thesis were published as the introduction of this Appendix.

Ms. Root wrote the section on Small Samples and the Possibility of Non-Human Remains.

Ms. Kahline wrote the Sampling of Teeth section.

Ms. Dunn wrote the Skeletal Samples Retrieved from Water section.

Ms. O'Rourke and Ms. Trotter wrote the Commingling of Remains: Intentional and Coincidental Section.

A.3 TITLE PAGE

Flexibility in Testing Skeletonized Remains for DNA Analysis can Lead to Increased Success: Suggestions and Case Studies

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A.3.1 DISCLAIMER: The opinions or assertions presented in this document are the private views of the authors and should not be construed as official or as reflecting the views of the Department of Defense, its branches, the Defense POW/MIA Accounting Agency, the US Army Medical Research and Material Command, the Armed Forces Medical Examiner System, the Armed Forces DNA Identification Laboratory, or the American Registry of Pathology. Commercial equipment, instruments, and materials are identified in order to specify experimental procedures as completely as possible, and does not imply that any of the commercial products identified are necessarily the best available for the purpose.

A.4 ABSTRACT

DNA analysis of skeletonized human remains is often seen to be a challenging undertaking. As practitioners, we are constantly looking to expand the toolbox of what is available to us for testing. While following SOPs is required in most forensic laboratories, it is important to remember and retain the processes that have preceded the newest practices. This chapter attempts to present how lateral thinking in protocols and assemblage analysis may provide solutions to current day challenges to producing quality DNA results from skeletonized remains.

A.5 KEY WORDS

DNA, mitochondrial DNA, STR analysis, skeletonized human remains, DNA extraction

A.6 ARTICLE INTRODUCTION

Constant adaptation to new methods and new techniques is a hallmark of science. In the last few decades, there have been enormous advancements in the area of DNA science and human identification. It was almost 100 years from the theories of inheritance developed by Gregor Mendel to the description of the physical structure of DNA by Watson and Crick in the 1950's (Watson and Crick, 1953). It was another 32 years before Sir Alec Jeffreys, Peter Gill, and associates released two seminal papers that revolutionized human identification (Jeffreys et al., 1985; Gill et al., 1985). From there, techniques and technologies available have changed rapidly: Kary Mullis and polymerase chain reaction (Mullis and Faloona 1987); the descriptions of various minisatellites in nuclear DNA for human identification (Hammond et al., 1994; Urquhart et al., 1994); and the standardization of nomenclature of short tandem repeat (STR) analysis (Bar et al., 1994). Not to be ignored is Sanger sequencing of mitochondrial DNA (mtDNA) (Anderson et al., 1981; Holland et al., 1993; Holland and Parsons, 1999; Sanger et al., 1977) and STR analysis of the Y-chromosome (Butler, 2003).

As a field, forensic DNA analysis for human identification has continued to grow and expand and is largely unrecognizable from the early years of manual manipulation of samples during PCR analysis. No longer are tubes manually transferred from water baths or hot blocks of specific temperatures in order to amplify DNA. Thermal cyclers do it with little human input other than to push a button. Southern blot analysis of hypervariable regions is mostly a thing of the past, and indeed, most college students in forensic DNA analysis or biochemistry courses today would be puzzled to realize that such analysis took days rather than 8 hours or less. With the advent of rapid DNA analysis equipment (among many: Bienvenue et al., 2010; Hopwood et al., 2010; Tan et al., 2013), the "instant science" of CSI has become a reality.

While the new technologies are exciting and will continue to change the 'face' of human identification, it is important for the practitioner to remember that many of these new technologies are rooted in the original fundamentals of the science. STRs would probably not have been discovered so rapidly without the initial work of Jeffreys. New techniques exist because of the history of the field. It is beneficial to keep an eye on the past in order to continue to move forward. This chapter will serve as an examination of some of the testing protocols used for DNA analysis of skeletonized remains and a reminder that flexibility in protocols can lead to more successful results.

A.7 EXTRACTION OF DNA FROM SKELETONIZED REMAINS

Dried skeletal specimens and teeth are the typical sample types that the Past Accounting Section at the Armed Forces DNA Identification Laboratory (AFDIL) receives from the Defense POW/MIA Accounting Agency (DPAA) Laboratory, formerly Joint POW/MIA Accounting Command – Central Identification Laboratory (JPAC-CIL). AFDIL assists the DPAA Scientific Analysis Division (more commonly called DPAA-Lab) in identifying service members from past military conflicts such as World War II, Korean War, Southeast Asia conflict, the Cold War, and other incidents by processing the DNA analysis from the remains. From its inception in 1992, AFDIL used an organic extraction method in the extraction of total genomic DNA from skeletonized remains. This protocol, described in Edson et al., (2004), typically used 2.5 g of pulverized osseous material dissolved overnight at 56°C in an extraction buffer (10 mM Tris, pH 8.0; 100 mM NaCl; 50 mM EDTA, pH 8.0; 0.5% SDS) and proteinase K, followed by purification with 25:24:1 phenol:chloroform:isoamyl alcohol and, the now obsolete, Centricon-100® centrifugal filters (Millipore). At the time, the only DNA platform testing used was Sanger sequencing of mitochondrial DNA (mtDNA). In a survey of skeletal samples tested from 1992 to 2003, success was found to be somewhat predictable: Femora were the most successful element for mtDNA testing and should be sampled preferentially.

In 2006, AFDIL validated a new demineralization technique ("Demin1": Loreille et al., 2007; Edson and McMahon, 2016) that reduced the input of skeletal material from 2.5 g to 0.25 g. The extraction buffer itself was modified to be primarily EDTA (0.5 M EDTA, pH 8.0; 1% *N*-Lauroylsarcosine), but otherwise the protocol did not change significantly. Purification of the extract still occurred using PCIA and a purification filter, now Amicon® Ultra-4 Centrifugal Filter Units (EMD Millipore, Germany). The fundamentals of the procedure itself remained largely unchanged, even with the reduction in input of the sample. However, the success rates for mitochondrial DNA testing increased markedly. Gone was the preferential selection of the femur or other compact bones. Any skeletal sample selected would tend to give a reportable mtDNA sequence (Edson et al., 2011).

At the same time, AFDIL was expanding testing to include STR analysis. Modified PowerPlex® 16 (Promega Corporation, Madison, WI) or AmpFLSTR® Yfiler® (Thermo Fisher Scientific, Waltham, MA) protocols were successfully used to identify the remains of soldiers from the Vietnam War, Korean War, and World War II (Irwin, et al., 2007a,b). While useful, these modified protocols were not broadly incorporated into casework use at the time. It took another change in the extraction protocol for STR analysis to be fully implemented for use on a daily basis.

In 2011, the Past Accounting Section of AFDIL adopted a modification of the inorganic purification protocol (Edson and McMahon 2016) that was already in use by the Current Accounting Section for use on fresh skeletal remains and other agencies and laboratories, such as the ICMP, on aged remains (Amory et al., 2012; Davoren et al., 2007; Kim et al., 2010; Rohland and Hofreiter 2007). The AFDIL protocol remains the same as the demineralization technique adopted in 2006, with the introduction of silica column purification step using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA) and the elimination of any PCIA purification step. In theory, this protocol would be faster, more

efficient, more successful, and less harmful to the staff. While the last is certainly true, the remaining did not necessarily turn out as expected.

Examination of the mtDNA success rates for all samples showed that the inorganic purification technique, known as “Demin2” in-house, gave an 80% success rate across all skeletal samples tested. This is understandably disappointing after the almost 90% success for Demin1. In addition, the overall quality of the data being reported was decreased. The target for reporting in the Control Region of mtDNA is 611-705 bases. Demin2 generated an average of 543 bases reported. While not as low as the average for the original extraction method (459 bases), it is still rather disappointing. What was markedly more successful was STR testing. Demin2 proved to provide a marked improvement in almost all STR platforms tested over either Demin1 or the original extraction protocol (Table A.1).

As with many labs, AFDIL is increasing the output of degraded skeletal remains tested with STR kits. Demin2 would seem to be a relatively decent fit to the workflow of the laboratory: success with STR analysis is needed; and mtDNA analysis is becoming less dominant, despite the make-up of the family reference database. However, some samples have been exposed to environmental conditions immediately antemortem, perimortem, or postmortem that may inhibit PCR processing should the materials co-extract with the DNA.

Table A.1. Summary of testing done at AFDIL from 1992 until the spring of 2016. MtDNA Sanger Sequencing testing is of the hypervariable regions I and II (HVI and HVII) of the Control Region. The target to be considered successful is 100bp or more of DNA amplified in duplicate and confirmed to be consistent by two independent analyses. Identifiiler (AmpFLSTR® Identifiler™: Thermo Fisher Scientific), MiniFiler (AmpFLSTR® MiniFiler™ PCR Amplification Kit: Thermo Fisher Scientific), and PowerPlex® Fusion (Promega Corporation, Madison, WI) reactions are unmodified from the manufacturers' recommendations. YFiler (AmpFLSTR® Yfiler®: Thermo Fisher Scientific) is a combination of low copy number (LCN) testing and unmodified. All STR testing platforms are considered 'successful' with the reporting of 4 or more loci that are confirmed through duplicate amplifications. Not all kits and protocols used at AFDIL are included in this table.

	Original Extraction	Demin1	Demin2
MtDNA Sanger Sequencing			
Number of samples tested	5809	6256	1805
% successfully reported	75%	89%	80%
Avg. # bases reported	459	611	543
MiniFiler Testing			
Number of samples tested	103	839	411
% successfully reported	32%	29%	48%
Avg. # loci reported	3	2.4	3.9
YFiler Testing			
Number of samples tested	173	988	634
% successfully reported	40%	31%	57%
Avg. # loci reported	4	2.8	6.7
Identifiler Testing			
Number of samples tested	7	24	37
% successfully reported	86%	50%	73%
Avg. # loci reported	10	5.9	7.3
Fusion Testing			
Number of samples tested	0	81	50
% successfully reported	n/a	77%	96%
Avg. # loci reported	n/a	8.9	15.8

In 2015, the DPAA disinterred 45 graves from the National Memorial Cemetery of the Pacific (NMCP) containing the highly commingled remains of sailors and Marines who died on the USS *Oklahoma* 7 December 1941. Since 1941, the remains had undergone a series of burials and disinterments, including an extended period within the hull of the breached ship. During this time, the fuel from the ship had leaked into the water and the hull and extensively contaminated the remains. Even with time and cleaning, skeletal samples sent to AFDIL for DNA testing retain the scent of fuel.

The first set of skeletal samples sent to AFDIL was extracted twice according to our standard SOP; however, Demin1 was used for the first extraction and Demin2 for the second. The goal was to determine which of the extraction protocols would work consistently better for mtDNA and STR testing on this particular set of samples. Given the presence of fuel, it should not be surprising that the Demin2 extraction protocol did not work as well as could be expected for this specific set of samples. The fuel could bind to the silica column and prevent the DNA from binding during the wash steps, thus increasing the amount of DNA lost. Previous work on other cases that have been exposed to inhibitory materials, as will be discussed later in this chapter, has shown that Demin1 tends to work better overall when the remains are chemically compromised. As with those cases, the samples from the USS *Oklahoma* tended to work better overall with an organic purification.

GC/MS analysis of bone powder removed from the remains during the cleaning process indicates that there is fuel oil still present on the remains along with some by-products of decay (Figure A.1). The fuel itself cannot be characterized, as the US Navy does not have on file mass spectrometry data on the fuels used in the 1940s; however, components of the fuel, including anthracene and its derivatives, can be identified. GC/MS analysis of the DNA extracted from the same sample indicates that the fuel is not completely removed during the extraction procedure and a small amount is co-extracted with the DNA. It does not appear to have a deleterious effect on the process of PCR.

Demin1 is now commonly used at AFDIL when working on chemically compromised skeletal samples such as those from the USS *Oklahoma*. Demin2 is used on all other sets of skeletal samples. This particular incident is a good example of how it is useful to laboratories to keep ‘older’ methods as active Standard Operating Procedures (SOP). Flexibility in thought and activity served to save time and increase the chance of identifying the sailors and marines

involved in this incident and others. As this chapter continues, additional possible alternatives to the 'common wisdom' will be discussed.

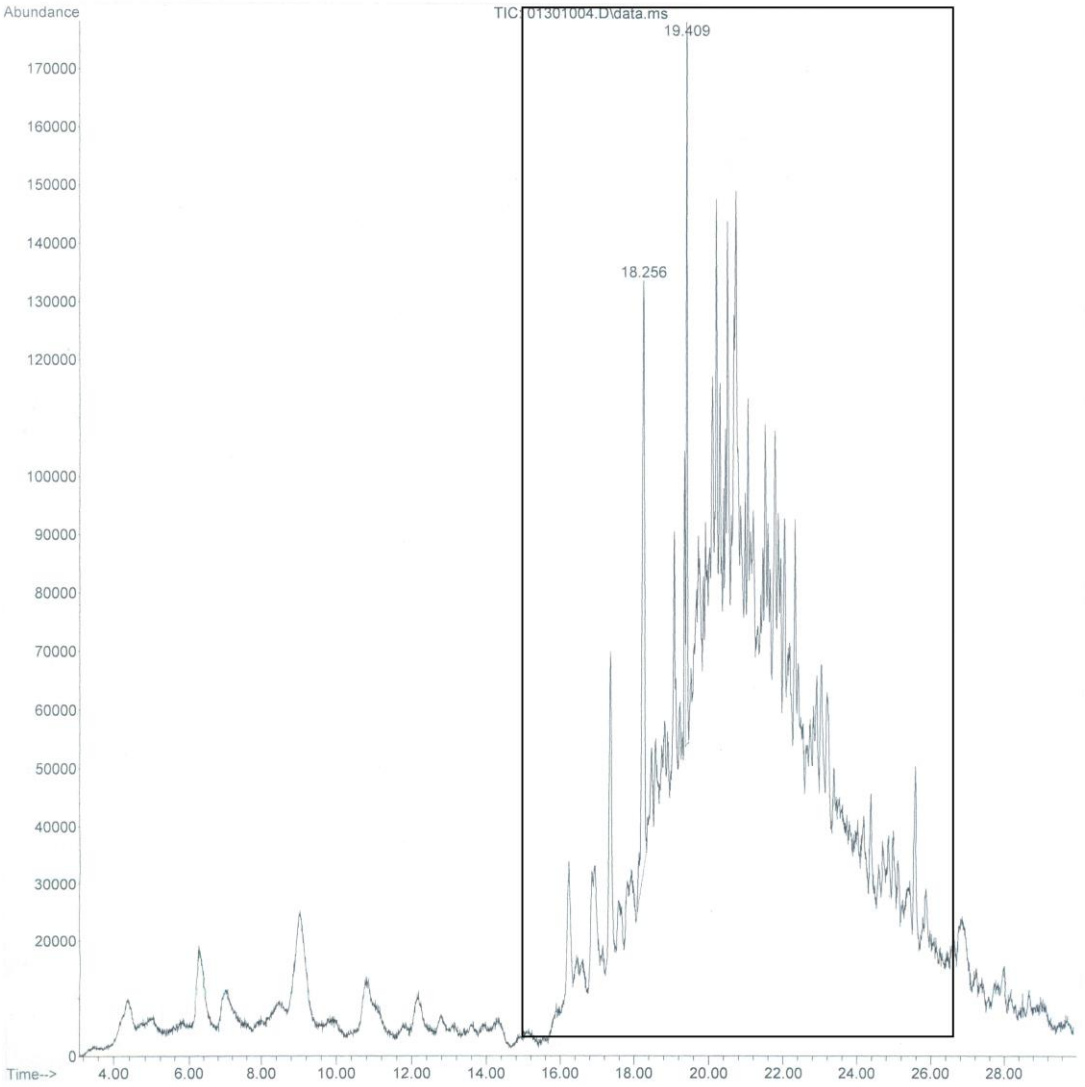

Figure A.1. A GC/MS trace of bone powder removed from the exterior of a skeletal sample from the USS *Oklahoma*. The area marked with the box is an accelerant trace that would be able to be associated with the specific fuel used on the USS *Oklahoma*. However, GC/MS analysis of that specific fuel was not done at the time. Components of the fuel, such as anthracene, can be identified in the trace itself.

A.8 SMALL SAMPLES AND THE POSSIBILITY OF NON-HUMAN REMAINS

Extracting DNA from extremely small osseous samples can be extremely challenging. The focus here is on samples that are submitted for DNA extraction that are under 0.30 g in gross weight. Due to the size, the standard cleaning methods used have to be altered to accommodate the samples. Typically, samples received from DPAA are sanded using a small

sanding bit attached to a Dremel® tool (Bosch, Mt. Prospect, IL). Because of the small size, one way in which these samples are cleaned is by just lightly sanding them, instead of a vigorous sanding, to remove any modern contaminates on the outer layer. However, this method may not be enough in some circumstances. Sonication of the sample, usually in a 50 mL conical tube containing either sterile water or a 10% bleach solution, for a period of time between 30 seconds and 5 minutes has been used at AFDIL. Sonication can be for a single or multiple sessions, depending on the amount of debris perceived to be present (Figure A.2). This method is well suited for small samples with a large amount of trabecular bone that may contain pockets of dirt or other exogenous materials.

Figure A.2. Scapula, 0.23 g. Sample was cleaned by lightly sanding and sonicating. A full control region mtDNA sequence (565 bp) was obtained for this sample.

Another challenge of small samples comes with the lower quality that is generally associated with these types of samples. In most instances, these samples are small due to severe fragmentation of the bones, which often goes hand in hand with lower quality DNA. This severe fragmentation may be due to disintegration at time of death (e.g., loss of personnel in high speed plane crashes), long periods of time, or environmental factors, such as the acidity of soil or high temperatures, which may cause the bone to be brittle and break resulting in the small fragments (Figure A.3).

Figure A.3. Fragment, 0.18 g. Sample was cleaned by light sanding and washing. Osseous material was very brittle. Inconclusive mtDNA testing results were obtained.

Samples of this size are typically consumed within a single extraction. After cleaning, the fragments are usually less than 0.15 g. At AFDIL, it is preferred to have to duplicate extractions of an osseous sample to confirm the mtDNA sequence or STR profile obtained; however, with a standard target powder weight of 0.20 - 0.25 g per extraction it is not possible to obtain a second extraction. In general, most samples submitted have additional intact bone associated: either there is remaining bone to sample from or material could be taken from a bone that can be articulated to the initial sample. The first sample sent for testing is often the best available and any subsequent submissions are not expected to give any better quality data. In some instances, bone that is submitted is all that is available. These samples are deemed “critical” (Figure A.4). The critical designation is a simple manner for the submitting agency to inform the testing laboratory that no additional materials remain for analysis.

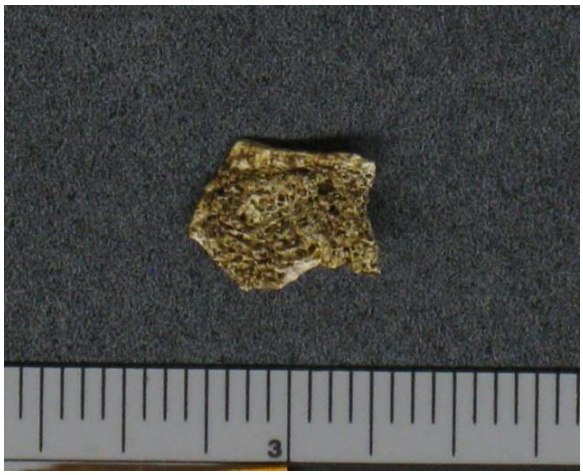

Figure A.4. **Bone fragment, 0.14 g**. Submitted to AFDIL from DPAA as a critical sample. Testing using 12S primers indicated that the sample originated from *Sus scrofa*.

If it cannot be determined to which skeletal element the fragment belongs, samples will be submitted as “bone fragment” or “long bone”. With these bones, the possibility of a non-human origin is exists. While there are other methods for species identification of biological materials (melt curve analysis: Kitpipit et al., 2016; cytochrome b: Ciavaglia et al., 2015; Linacre and Lee 2016; Tobe and Linacre 2010), AFDIL uses a rapid screen of samples with in-house designed primers to amplify a section of the 12S region of mtDNA. The region is approximately 100 bp in size, which is small enough to amplify most highly degraded human samples, and is not human specific. After Sanger sequencing, the data are compared to a human reference sequence. If the sequence is 100% consistent with the reference, the sample is believed to be of human origin. If the sequence is not 100% consistent with the reference, the sequence is searched against the nucleotide BLAST (Basic Local Alignment Search Tool) on the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The BLAST is not peer reviewed and individuals can upload the sequences they have into the tool; therefore, results may be skewed or incorrect, although this is rare.

Melton and Holland (2007) determined that the BLAST can supply a high degree of confidence when determining an animal species based on the sequence obtained from a 12S analysis. They also found that primate species can be up to 98% homologous (as with *Pan troglodytes*, the chimpanzee) however, that 2% difference is enough to be able to differentiate between the chimpanzees and humans.

Cryptids may be found when searching the BLAST tool. The kting voar (*Pseudonovibos spiralis*), also known as the snake eating cow or spiral-horned ox, is one such cryptid. Some believe that the kting voar is a cow-like animal with twisting horns and spotted fur that has a primary distribution range in Cambodia. This designation is the source of some controversy (summarized in Olson and Hassanin, 2003). Most supposed specimens have been determined to be cow horns, artificially shaped by locals for ritualistic or medicinal purposes (Brandt et al., 2001). DNA studies that have been done on historical samples concur that the remains are closely related to or are domestic cows (Hassanin et al., 2001) or water buffalo (Kuznetsov et al., 2001). What is unfortunate is that some 12S sequence data for the "kting voar" has made its way into GenBank (e.g., GenBank Accession No. AF231029) and may occasionally match results from DPAA samples.

AFDIL uses the 12S testing for primarily two situations: the first being when a sample is submitted specifically for the 12S testing to be performed because the anthropologist is unsure of the species origin. For these samples, 12S is the first amplification that is attempted on the sample. If the sample is shown to be non-human, the sample is re-amplified for verification and the case is completed. If the sample is consistent with the human reference, then routine mtDNA testing is continued. It is sufficient for the purposes of the lab to simply identify a sample as human or non-human, although it is interesting to determine the specific species sampled. Since implementation of this protocol, 384 samples have been analyzed using 12S. Only 68 were determined to be human.

The second use of 12S testing is when a sample fails to amplify using the normal processing methods. In these cases, 12S amplification is used an investigative tool to determine if the sample is failing due to being non-human or to severe degradation or inhibition. Most of the samples that are determined to be non-human are bone fragments or long bones. Nevertheless, there have been occasions in which a bone is submitted as a human bone but is later determined to be non-human in origin (see Figures A.5 and A.6). During normal processing, if a sample does not amplify, the analyst has the option to attempt a 12S amplification. There are three different outcomes an analyst would expect to see. The first is that the sample data are consistent with *Homo sapiens* (human). In this scenario, the sample is believed to be human; however, caution is exercised due to the possibility of exogenous contamination by scientists or locals. The second is that the sample data are determined to be non-human in origin (after a BLAST search is performed). The third is that the 12S amplification does not produce a positive amplification and the analyst is unable to determine species. However, this outcome does provide some insight, as the quality of the sample is also likely very poor if the 12S region does not amplify.

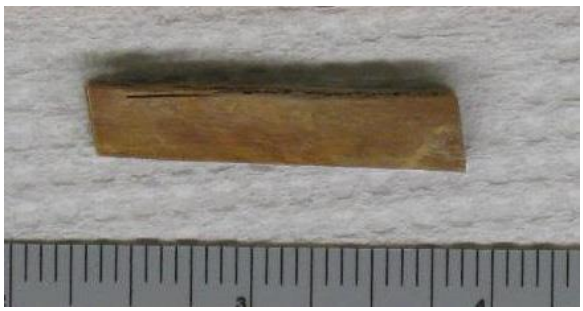

Figure A.5. Sample submitted as a rib. 12S rRNA testing determined the sample to have originated from a cow (*Bos taurus*).

a.  b. 

Figure A.6. Samples submitted as a rib (a) and a humerus (b) from two different cases. 12S rRNA testing determined the samples to have originated from a pig (*Sus scrofa*).

A.8.1 Case Study #1

A sample was submitted to the AFDIL listed as a "fragment" with a gross weight of 0.30 g (Figure A.7). The sample was noted as being very spongy and brittle, dirty and partially blue-green in color. After sanding, the sample was sonicated in water three times. The resulting 12S sequence data appeared to be a mixture of *Homo sapien*s and a species within the family Bovidae. A resampling of the fragment was obtained with a gross weight of 0.05 g (Figure A.8). The sample was not sanded due to the size, but rather cleaned by a 2 minute sonication in each of the following solutions: twice in a bleach dilution, twice with sterile water, and twice with ethanol. The 12S data obtained from the resampling was a clean sequence consistent with a species of the family Bovidae. The human data observed was believed to be from handling of the bone sample at some point prior to examination and the sample was reported as non-human.

Figure A.7. Sample submitted a fragment weighing 0.3 g. 12S testing produced a mixture of sequences: *Homo sapiens* and a species in the family Bovidae.

Figure A.8. A resampling of the bone fragment in Figure 7. 12S testing determined the fragment to have originated from a species in the family Bovidae.

A.8.2 Case study #2

A set of 13 samples from the same site were submitted to the AFDIL. The letter from the client stated that the samples were sent specifically for 12S testing and subsequent mtDNA testing if the 12S results were positive for human. Of the 13 samples, eight were submitted as "Long Bone" and five submitted with specific bone designations. Four of the long bones were inconclusive, in both mtDNA and 12S testing, three were human and reported with mtDNA sequences, and one was determined to be non-human. Two of the samples with human bone designations were inconclusive and two were human and reported. The fifth sample was sent in as a metacarpal (Figure A.9). After 12S testing, the sample was determined to have originated from *Canis lupus*, or part of the same genus as the domestic dog and wolf.

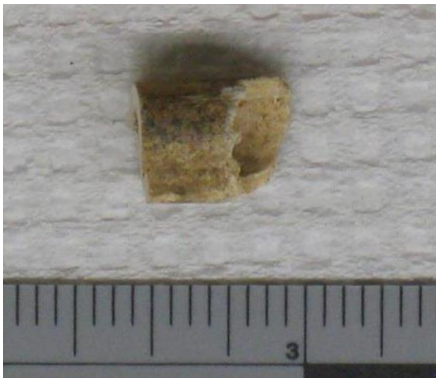

Figure A.9. Sample submitted as a 0.53 g metacarpal. 12S testing determined the sample to have originated from *Canis lupus* (dog).

A.9 Sampling of Teeth

Since AFDIL started processing samples from DPAA-Lab, 12% of the samples submitted have been teeth. When working with skeletonized human remains, teeth are often ideal due to the success of the sample type in recovering DNA. The success of teeth is due in part to its structure. A whole tooth is surrounded by enamel and cementum which helps protect the DNA of the tooth from environmental and modern contaminants (Adler et al., 2011). Prior to 2008, the DPAA Laboratory would prepare the tooth sample by drilling the tooth and sending the tooth powder to the AFDIL. Contamination issues were consistently observed in the powdered tooth and the AFDIL decided to validate and implement their own protocol of preparing teeth for extraction of DNA.

The DPAA-Lab has numerous factors they consider when choosing a tooth to send to the AFDIL for any type of DNA testing. Teeth will not typically be sampled if identification can be made based on antemortem dental X-rays. Intact, whole teeth are preferred, whereas teeth with cavities, fractures, or are otherwise structurally compromised are generally avoided due to possible contamination of the interior of the tooth. If several teeth are attached to a jaw fragment, only one tooth needs to be sampled. The remaining teeth are available for testing in

case inconclusive DNA results are obtained or if the sample becomes contaminated, as it is presumed that all teeth articulated in the same jaw belong to the same individual.

Loose teeth that cannot be associated with a mandible or maxillary bone may be submitted for DNA testing. Previously, a tooth would be chosen for sampling and the DPAA-Lab odontologist would drill the interior of the tooth structure to obtain as much dentin as possible. The powder was collected in a 15 mL conical tube that was packaged and sent to AFDIL for DNA testing. All of the tooth powder removed by the odontologists would be needed for the original extraction protocol, leaving AFDIL only one opportunity to extract the DNA successfully. Over time, AFDIL began to note that a certain percentage of samples received showed either gross or low-level contamination. AFDIL believed that the contamination was potentially being introduced when the odontologists were drilling the teeth. In some cases, the profile that was produced from the sample was consistent with the odontologist that drilled the tooth. Due to the manner in which the powdered tooth was sent to AFDIL, there was the potential for additional contamination. Tooth powder frequently was found in the threads of the 15 mL tube used for shipping, allowing for the possibility of other materials entering the tube. There was also some tooth powder loss and the potential for contamination by the DNA analyst. To eliminate these issues, AFDIL decided to validate and implement an SOP for the preparation of teeth prior to DNA extraction.

The teeth received by AFDIL from DPAA-Lab have been exposed to varying environmental conditions. They can be covered in dirt, debris, and as with many of the samples from the USS *Oklahoma,* in fuel (Figures A.10a and A.10b). The exterior condition of the samples is not always optimal, but due to the enamel and cementum that surrounds the interior of the tooth, the DNA rich dentin (Figure A.11) is preserved and ideal for yielding DNA results. The exterior of the tooth simply needs to be thoroughly cleaned in order to prevent transfer any of the exterior contaminants to the interior portion of the tooth. By 2008, AFDIL developed a

method for the preparation of teeth samples in-house and had applied the procedure to casework for the extraction of DNA.

The tooth preparation and extraction process became more fluid by incorporating tooth preparation as the Day 1 of the extraction procedure followed by Day 2 the organic extraction of DNA. Since the implementation of this protocol, the success rate for the reporting of samples has increased from 74% to approximately 90%. The AFDIL SOP mirrors the tooth preparation done by DPAA-Lab (Shiroma et al., 2004) with some slight modifications to accommodate the AFDIL workflow. The portion of the tooth targeted for DNA extraction is the dentin (Figure A.11). The dentin is protected by the hard enamel and cementum, which makes it an excellent source of DNA. The pulp is typically completely desiccated in the samples that the AFDIL receives due to age and extended environmental exposure. In fresh tooth samples the pulp is the recommended source for DNA because that is the source of blood flow to the tooth.

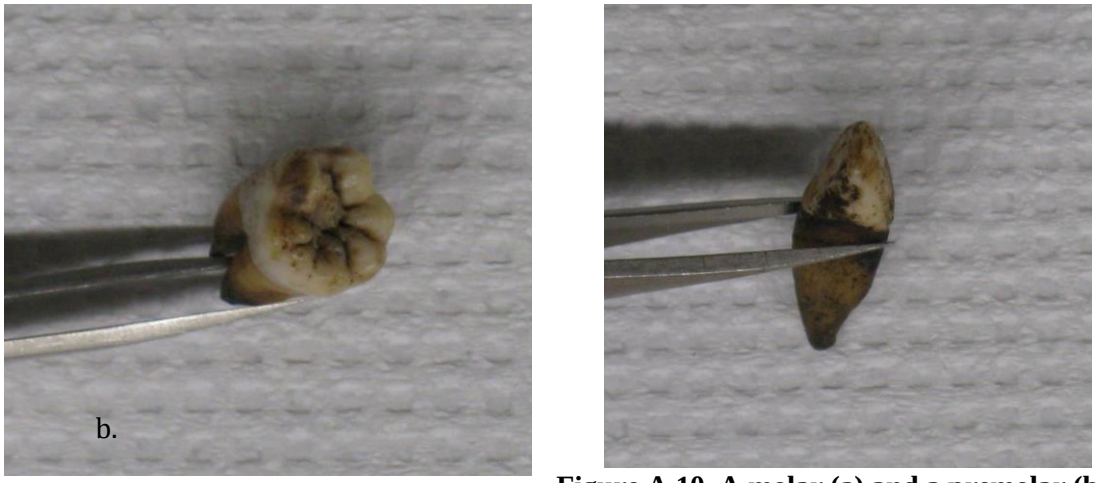

Figure A.10. A molar (a) and a premolar (b) submitted from remains recovered from the USS *Oklahoma*.

The preparation of teeth begins by cleaning the tooth with an 8.5% (v/v) bleach solution. If the tooth has a detached root or fractures in the structure, it is cleaned with a gauze pad moistened with the bleach solution and wiped down with a gauze pad moistened with absolute ethanol. If the tooth is intact, it is sonicated in the bleach solution and rinsed with absolute ethanol. In both cases, the tooth is then allowed to air dry for 15 minutes under a UV light. A horizontal cut around the base of the crown at the cemento-enamel junction is made with a dental bur allowing for the eventual separation of the crown from the root (Figure A.11). Separating the tooth structure in this manner allows for better re-orientation of the crown to the root after the dentin has been drilled out of the root and crown. Following the drilling of the tooth, the tooth powder that is collected can proceed to the extraction of DNA and the remaining tooth structure is returned to DPAA-Lab for reassembly and eventual return to the family members upon identification.

The choice to drill the dentin from the interior of the tooth can be seen as somewhat counter to the common wisdom, as it is simpler and less time-consuming to pulverize the roots of the tooth, or the entire tooth. Some publications (Adler et al., 2011; Baker et al., 2001) espouse the crushing of the entire tooth out of ease and the reduction of the possibility of contamination of the sample. However, the crushing of the crown will introduce higher levels of enamel into the DNA extract, leading to inhibition of the downstream PCR testing. Higgins et al. (2015) recommended selecting tooth tissue depending on the type of DNA testing to be done. Cementum is preferred for STR testing; whereas, testing found that mitochondrial DNA was better preserved in the dentin and the roots. Pulverization of the root may provide sufficient DNA for both types of testing; however, retention of the entire tooth is often seen to be preferable in human identification casework so as to have some biological material to return to a family member.

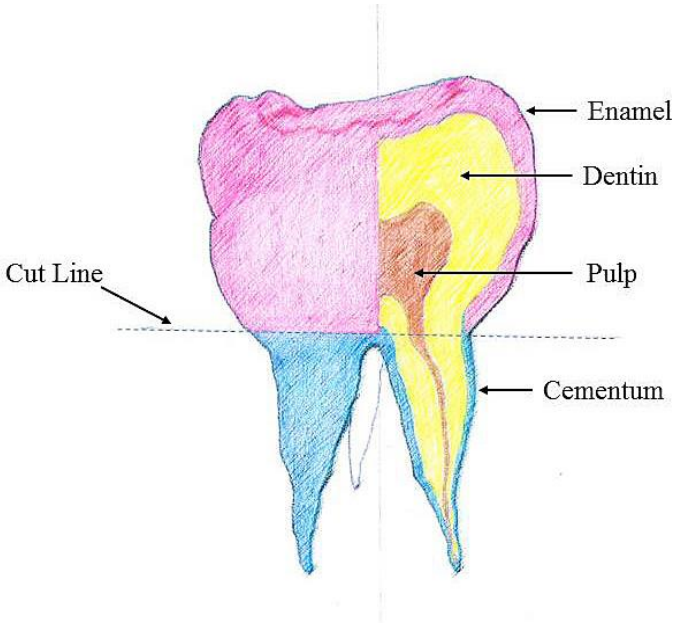

Figure A.11. Diagram of the structure of a human tooth.

A.10 SKELETAL SAMPLES RETRIEVED FROM WATER

The advances in technologies for DNA extraction and analysis have allowed for testing on a variety of specimens previously considered unviable. This has increased the opportunity to successfully identify missing individuals, victims of mass disasters, and victims of crime. Post-mortem interval (PMI), or time passed since death, is one of the factors used to assist in the identification. Pursuant to this, numerous studies have been conducted on tissue decomposition of remains that have been buried, exposed to the open air, scattered by scavengers, and defleshed by insects and microorganisms. Fewer studies have made aquatic environments their focus, thereby creating a gap in the information necessary to efficiently collect, examine, and process specimens found in wet locations. Of the few, most are dedicated to determining PMI with very little data on DNA recoverability. Given that other bodily tissues would be compromised or missing, bone is likely the best candidate for DNA testing of samples found in water.

Bone is composed of collagen (protein), hydroxyapatite (mineral), and organic compounds. The collagen and hydroxyapatite are strongly bound together, which is why bone persists long after the soft tissue is gone. Degradation of bone, or diagenesis, will begin when collagenases (enzymes) attack collagen, reduce it to amino acids, and weaken the protein-mineral bond. The minerals are vulnerable to leaching into the environment, a process exacerbated by water and microorganisms. This continues until the bone eventually disintegrates.

In order for diagenesis to occur, the enzymes and microorganisms that break down bone must obtain access to it, thus the decomposition of the rest of the body must also be understood. Some factors affecting aquatic decomposition include bacterial content of the water, temperature, salinity, presence or absence of scavengers, and water movement. The literature for decomposition in water does little to distinguish between the possible environments - some freshwater data are from bodies found in pools and bath tubs, which could have a drastically different bacterial content than a lentic water source (such as a pond), which could be drastically different from seawater. Due to the lack of diverse experimentation and conclusions, the probity of bone samples recovered from these sites is not well understood.

A.10.1 Soft Tissue Decomposition in Water

In an attempt to test generalizations made regarding bodies found in water, a project by Ayers (2010) at Texas State University – San Marcos used pig carcasses to observe decomposition in different environments, including freshwater and saltwater. The major differences observed between them were that freshwater bodies had abdominal protrusions, which attracted insects/scavengers and released bacteria. This was attributed to an osmotic effect that would differ between the fresh and salt waters. The saltwater bodies had no insect activity and suffered mainly from skin slippage, which allowed the bones to sink without observation or documentation. In neither case was adipocere present.

Adipocere, also known as grave wax, is formed by saponification, or the hydrolysis of fatty acids. Hydrolysis is the chemical breakdown of a compound due to a reaction with water. Certain conditions must be met for its creation: presence of adipose, anaerobic/warm/wet environment, and putrefactive bacteria. The hydrolysis is aided by anaerobic bacteria, which generate ammonia-rich waste, thereby creating an alkaline environment. The alkalinity prevents further bacterial activity and stops putrefaction (O'Brien and Kuehner 2007). Soft tissue becomes waxy and pale; the body is preserved and stable due to the increased melting temperature of the adipocere. When it dries, the adipocere does not further decay but rather becomes brittle, making PMI approximation exceptionally difficult. The presence of adipocere preserves the bone as well as internal organs, which will desiccate over time. Due to the ideal circumstances required for its formation, O'Brien and Kuehner (2007) use the term "Goldilocks Phenomenon": If too dry or too cold the tissue will desiccate, if too wet or too hot it will soften and liquefy, but when it's "just right," adipocere forms and soft tissue does not decompose. This can occur within a few weeks or up to a few years. The first individual to investigate adipocere was a French chemist named Antoine Fourcroy. In 1789 he exhumed bodies from the *Cimitiere des Innocents* in Paris and noticed a fatty and waxy tissue formation most noticeable in the cheeks and breasts. Fourcroy named it "*adipocire*" using the Latin terms for fat and wax, "*adeps*" and "*cera*", respectively.

A.10.2 Case Study #3

A body was found in a dam on the Seine River outside of Paris, France, with a document in the clothing suggesting it was a man who had been missing for three years. Since the head and limbs were missing, DNA testing was necessary for identification. Saponified muscle and a clavicle were tested for nuclear DNA. The saponified tissue did not yield results but the clavicle produced a partial auSTR profile that was sufficient for identification (Crainic et al., 2002). The presence of saponified tissue gives some clues to the water environment the body

was left in and the successful DNA testing indicates that those aquatic conditions may be favorable to bone preservation.

A.10.3 Wet Tissue's an Issue, What About Bone?

An important question for researchers has been: what is the relationship of DNA in bone with other components of the bone tissue and how might that affect its survivability? DNA has a high affinity to water; however, DNA has been recovered from bone samples in aquatic environments, in higher quantity and quality than other tissues in the same conditions. This indicates that there is something affecting DNA in bone that increases its survivability, even after years of bones being submerged in water.

There have been several studies focusing on the binding of DNA to hydroxyapatite. Götherström et al. (2002) used modern bovid samples, artificially degraded in a laboratory, and ancient horse samples. The ancient samples showed that collagen preservation was linked to hydroxyapatite and that with an increase in crystallinity of hydroxyapatite, there was a decrease in amplifiable DNA. Bone dissolution, or the dissolving of bone minerals, renders DNA susceptible to degradation, supporting their theory that the complex to preserve DNA includes collagen, and DNA is adsorbed and stabilized by apatite.

Despite Götherström et al. (2002) linking increased crystallinity to decreased DNA yield, Salamon et al. (2005) found intergrown crystal aggregates that are resistant to separation may create a barrier and thus protect DNA from environmental degradation - basically DNA becomes trapped within an impenetrable ring of crystals. Environmental and biotic degraders would be thwarted, but the DNA extraction techniques used in laboratories include demineralization steps that release the trapped DNA. Salamon et al. (2005) found that while the quality of the DNA extracted from such samples was better, there were fewer DNA

molecules recovered. The benefit is greatest for fossil bones or other remains for which modern contamination is a concern, but could limit forensic testing, which requires reproducibility.

Another experiment testing the relationship of DNA and hydroxyapatite was conducted by Brundin et al. (2013) in which extracted bacterial DNA was added to ceramic hydroxyapatite, then incubated for three months in water, sera, and DNase I - an enzyme that nonspecifically cleaves DNA. At intervals during the three months, they tested for the presence of DNA. It was detectable in all samples after three months. Extracted DNA without hydroxyapatite was used as a control and also added to water, sera, and DNase I. With the exception of sera, which had a faint positive result, none had detectable DNA after three weeks. These data demonstrate that DNA does have a binding affinity for hydroxyapatite which appears to stabilize and prevent the DNA from binding to the water, otherwise a significant loss of DNA in water would have been true for both the experimental and control samples. Sera gave a weak result for detectable DNA but it demonstrates protein may also play a role in DNA preservation. The collagen protein and hydroxyapatite mineral complex in bone likely serves as a strong stabilizer for DNA, aiding in its preservation in water.

Given the strength of the protein mineral bond, what leads to its breakdown and is there a way to screen for viability prior to testing? While the type of bone sampled has been shown to correlate to the quality and quantity of DNA (Edson et al., 2004; Misner et al., 2009; Johnston et al., 2016), visible skeletal weathering has not. The appearance of the bone has not been shown to be predictive of DNA viability, since damage to the bone would not necessarily affect DNA, and damage to DNA does not necessarily result in visible deterioration of the bone (Misner et al., 2009). Whether this translates to bones damaged in water remains to be seen. A study conducted at the University of New Haven examined the changes of morphology of sharp force trauma when abraded by sand and diatomaceous earth in a moving

water environment (Appleton 2014). Diatomaceous earth was more abrasive, resulting in more bone tissue loss, but the effects on DNA recoverability were not examined.

As remains decay, a danger to bone tissue is bioerosion. Bioerosion of the microstructure of bone has been observed in ancient bone samples, and there is some debate as to whether the bacteria responsible are endogenous or exogenous. An experiment conducted by White and Booth (2014) used stillborn and juvenile pig carcasses (buried and above ground) to investigate which of the two is the culprit. Their findings support endogenous bacteria, and also suggest that since the bacteria are aggressive, with prolonged putrefaction there would be little to no well-preserved bone. The extensive bioerosion of the microstructure would expose DNA to enzymes, bacterial attack, and total degradation. Given that there is a high incidence of well-preserved bone, rapid skeletonization likely occurred to prevent putrefaction, which aids in DNA yield. The freshwater bodies of Ayers (2010) did release bacteria due to the abdominal protrusions while the saltwater bodies did not. DNA testing was not performed for that study, so the question remains of whether the presence of endogenous bacteria from a putrefying corpse may have an effect on the DNA yield of freshwater bones once the body has skeletonized.

Thus far, the research regarding DNA in bones submerged in water mimics the advice for compromised bone samples on land:

- Cortical bone is denser and a better option for DNA testing than cancellous since the microstructure in cancellous bones makes them more susceptible to degradation (Misner et al., 2009).
- Femur, teeth, tibia, fibula, and the petrous portion of temporal bones are the best to sample given they are dense cortical bone or well protected from the surrounding

environment (Edson et al., 2004; Edson et al., 2009; Johnston et al., 2016), although this can be highly dependent on extraction procedures.

- High heat during bone sampling (i.e., prolonged high speed drilling) and high stress during DNA extraction should be avoided since they may further degrade fragile DNA (Courts and Madea 2011).
- Increased bone powder in extraction and an added concentration step can increase DNA yield (Mameli et al., 2014).

Further research is necessary for specific data and suggestions to clarify the reasons for possible loss in yield, to distinguish challenges of freshwater versus saltwater specimens, and to truly optimize protocols for DNA extraction of samples in various aquatic environments.

A.10.4 Case Study #4:

December 15, 1942: A group of United States Army Air Force (USAAF) B-26 Marauders, a twin-engine medium bomber used in World War II, were sent to bomb an Axis-controlled airport in Tunis. After their attack, one plane was witnessed crashing into the water near Tunis. Since they were lost over water, the crewmen were not recovered.

The crash site was located in Lac Sud in 1948 by the U.S. Army Graves Registration Service; however, only large pieces of the aircraft were able to be retrieved since methods for underwater recovery were decades away from being developed. The crew of the crashed aircraft was deemed “non-recoverable.”

November 2000: The US Embassy in Tunis contacted the US Army Central Identification Laboratory-Hawaii (CILHI: currently known as the Defense POW/MIA Accounting Agency Laboratory), informing them that an American aircraft and some human remains had been found in Lac Sud during a dredging project. CILHI sent a team to evaluate the site, and

subsequent collaborations amongst CILHI, US Navy divers, and Tunisian Navy divers through 7 February 2001 lead to the recovery of material evidence and human remains.

Lac Sud is a portion of Lake Tunis that is separated from the rest of the lake by a causeway. The water at the excavation site was described as brackish, muddy, and shallow. The state of the recovered remains was documented as being excellent with attribution to the anaerobic state of the soil on the lake floor and the presence of fuel. Both of these factors slowed decomposition, thus the remains were well-preserved. Twenty-eight bone (cranial, humerus, and os coxa) and tooth samples were sent to AFDIL for mitochondrial DNA (mtDNA) testing. The testing was successful on all but one tooth and compared to the mtDNA references obtained from the missing aircrew's families. The mtDNA results, along with anthropological and odontological evidence, resulted in the identification of the entire crew in 2002.

Almost 60 years had passed since the crewmen were lost and viable mtDNA was still recoverable and reportable. In the 14 years since those identifications, DNA methods have improved significantly, further advancing the argument that bone is a strong candidate for DNA testing of samples in water and worthy of a more thorough look in the forensic community.

A.11 COMMINGLING OF REMAINS: INTENTIONAL AND COINCIDENTAL

As is known, human identification efforts cannot be done based upon one method alone. It may be necessary to partner with different agencies, personnel and even countries in order to obtain the correct information. Such partnership has developed between the DPAA and AFDIL over many years to incorporate anthropological, archaeological, DNA, and other

identification methods. Though many cases can provide quick answers, others are not as straight forward.

The tedious task of skeletal re-association is further hindered when sites unexpectedly contain the remains of more than one individual. Commingling of remains can either be accidental or intentional. Elements belonging to one individual may be spread across multiple sites, which may not be collected at the same time and may introduce the risk of failing to identify all elements belonging to that individual. In these cases, this issue moves beyond the field, and into the laboratory. Sample selection, the number of specimens to process, and the possibility of sample to sample contamination are all confounding factors that can cause delays in generating DNA results. The importance of communication between the different agencies, a sharing of the history of the site and recovery, and the availability of different scientific methods is crucial in such scenarios. While initial DNA testing can be done in the blind, sharing of site conditions and incident circumstances will aid the DNA analysts in making choices about the methods to be used. As demonstrated at the beginning of the chapter, extraction protocols may vary depending on what the skeletal remains may have been exposed to. Examples of such situations and strength of partnership are seen in the identification process of service members from World War II and Korean War.

A.11.1 Unintentional Commingling during World War II – Cabanatuan

Following the attack on Pearl Harbor, the Japanese military attacked United States and Filipino forces in the Philippines, overrunning their positions by 9 April 1942. Survivors were taken prisoner and marched northward from the Bataan Peninsula to prison camps in central Luzon in the 65 mile "Bataan Death March", followed by a 25 mile train car journey to Capas, and a nine mile march to POW Camp O'Donnell. As result of overcrowding and an

excessively high death rate at Camp O'Donnell, the Japanese Army began transferring POWs to Camp Cabanatuan, 60 miles north of Manila, a few months later.

Upon entrance into the camp, the personal property of the prisoners, including dog tags, was confiscated. Those individuals who were able to keep their identification tags often gave them to friends in the hopes of getting the tags home to their families. As a result many individuals died without identification while others were found with multiple tags on their person. Attempts were made by other soldiers to identify decedents by placing slips of paper bearing the name of the person in his hand or mouth before burial. Unfortunately, most of these slips of papers had disintegrated by the time American Graves Registration Service (AGRS) began disinterring graves at the camp following the war. As a result, most individuals passed away with no individually identifiable information on their body.

At Camp Cabanatuan burials were conducted daily. All individuals who died in a given 24-hour period were buried together in a mass grave. However, due to a great deal of disorder at the camp and the high death rate, many graves went undocumented and record keeping was incomplete at best. Following the war, AGRS created *The Cabanatuan Death Report*. This was a list of dates, times, causes of death, and some basic biographical information for those who died at the camp. Information on the grave number in which individuals were buried was not indicated and the report was limited in other information.

Between December 1945 and March 1946, AGRS exhumed interments at Camp Cabanatuan, a process hampered by overgrowth of vegetation as well as a high water table. Remains were reinterred 12 miles north of Manila at a temporary collection facility before being moved to the Manila Mausoleum for analysis and storage.

One particular grave from Camp Cabanatuan had records indicating that fourteen bodies had been interred within it. Of these, one was identified with dog tags and three others were identified in the 1940s, leaving ten unknown individuals. The 10 unidentified remains were reinterred briefly at the temporary collection facility before being moved to the Manila Mausoleum. Skeletal and dental charts of the remains were compiled, but the re-association of the remains with the ten individuals believed to be in the grave was unsuccessful. The remains were individually buried as unknowns in the Manila American Cemetery and Memorial in February 1950 and 1952.

In August 2014, the caskets of these ten individuals were disinterred for identification purposes. Identification by anthropological means was hampered due to circumstances of the original burial and the previous disinterments and analyses, increasing the likelihood that the remains had been extensively commingled. In addition, dental comparisons were complicated by the fact that postmortem dental records could appear significantly different from the last available official chart as malnutrition and violence at the camp could have contributed to tooth loss and work could have been performed during captivity. Thus, DNA analysis was considered highly useful for the sorting of the remains and possible identification.

A.11.2 Cabanatuan Sample Processing

Small cuttings of skeletal elements were submitted for testing. Initially extractions of the bone samples were performed using the Demin2 inorganic extraction protocol. However, after the first round of extractions and subsequent mtDNA amplifications the success rate of obtaining data was very low. Further investigation uncovered that the samples buried in the Manila American Cemetery had been treated with a preservative powder believed to contain magnesium sulfate ($MgSO_4 \cdot 7H_2O$), ammonia alum [$(NH_4Al(SO_4)_2 \cdot 12H_2O$], plaster of Paris, and other compounds that was inhibiting the release of DNA during the extraction process.

Rather than continue to work with Demin2, AFDIL changed to using Demin1 to improve the quantity and quality of the DNA obtained from the samples.

Resulting data from the DNA analysis using mtDNA and modified Y-STR testing indicate the presence of at least 16 different individuals present among the bone samples submitted. Given the historical documents, the remains of only ten individuals were thought to be present after the identification of burial of four of the original 14 persons buried in the grave. This discrepancy from the historical record further illustrates the fact that unintentional commingling of the remains occurred both at the time the grave was initiated and later during the numerous handlings of the remains in the 1940s and 1950s.

The treatment of the samples also underpins the importance of maintaining a variety of processing tools available for use. By maintaining numerous validated testing methods, the laboratory was able to quickly and smoothly transition from one testing method to another with minimal loss of time, reagents, and sample extract. This case also demonstrates how important it is for laboratories doing testing of unknown remains to have as much information as possible about any previous processing or storage of submitted samples so the best method of extraction can be utilized to maximize the success rate for the requested samples.

A.11.3 Suspected Intentional Commingling during the Korean War

According to the DPAA, over 7800 US service members remain missing from the Korean War. Though there is still much to do to identify the missing, this number is relatively small due in large part to the efforts of soldiers and anthropologists of the Army Central Identification Unit (CIU), AGRS, Quartermaster and 108th Graves Registration Platoon during the war. Throughout the Korean War, causalities were being recovered, transported, or buried in temporary cemeteries. It was one of the first attempts by the US to return and

possibly identify war dead while battle was on-going. In the beginning, lack of resources hindered appropriate recordkeeping; however, as the recovery effort continued the necessity to maintain detailed information on each individual became critical for proper processing of the casualties. A mortuary was established at Camp Kokura in Japan, which allowed for a uniform method of processing remains prior to shipment back to the US. The availability of personal effects, eye witness accounts, available service, medical and dental records, along with accessioning with a new Information Business Management system (IBM), made identifications faster than before.

A historic battle of the Korean War occurred at the Chosin Reservoir in what is now North Korea, or the Democratic Peoples' Republic of Korea (DPRK). This area was known for its tundra-like conditions during late fall and early winter. At times, it was referred to as the "Frozen Chosin" or "Frozen Hell" and those who survived the conditions were known as the "Chosin Few". In late 1950, soldiers from the US Army were positioned along the eastern side of the Chosin, providing assistance and relief to the 1st Marines. In an effort to move south out of the region, the units came under attack by Chinese forces and an intense battle occurred for several days. The commander of the 1st Battalion, 32nd Infantry distinguished himself in combat, but was mortally wounded during the battle. While some soldiers and Marines were able to retreat to safety, thousands of others were captured or left for dead.

In particular to this battle, casualties that were unable to be recovered at the time were removed or buried on site by the opposing forces. After the war ended in 1953, the UN and Communist coalitions developed three repatriation pacts; the first two, Operation Little Switch and Operation Big Switch, were for the return of POWs immediately after war. The final pact, Operation Glory, was to return war dead of the opposing side. Commencing September 1954, the exchanges occurred in demilitarized zones and continued through the

end of October 1954. A total of 4219 UN remains were returned, of which, 2944 were considered American.

A.11.4 Korean War Sample Analysis

After the completion of Operation Glory, questions lingered about whether more unrecovered remains were still in the combat area. Beginning in 1990 and continuing through 1994, 208 boxes were repatriated to the US by North Korea, each believed to contain the remains of a single US service member. It was reported that the remains were excavated from 21 different locations, including former POW camps and battlegrounds. As anthropologists began the task of identifying the remains, the supposed single individuals were found to be highly commingled. Initial observations estimated there were 200-400 individuals distributed between all containers; however, the current MNI is around 600 (Jin et al., 2014).

The advancement of scientific technology provided an opportunity to utilize, what was then a new and novel, mitochondrial DNA technique in the identification process. Working together, DPAA and AFDIL partnered to process this large set of remains. DPAA would undertake the task of separating and associating remains, based upon anthropological techniques. A cutting would be sent to AFDIL, where scientists would conduct the DNA analysis and provide testing on the evidence and any available family reference samples. The first set of bones and teeth were received by AFDIL in the mid-1990s. Current work continues on this set of remains, with nearly 50 identifications being made from the K208 this year (FY2016).

Almost 50 years after the Battle of the Chosin Reservoir, DPAA was able to perform recoveries from that region. Between 1994 and 2005, the US conducted joint field activities with the DPRK to visit suspected areas of unrecovered US service members. In 2004, the recovery team traveled to a location close to the initial retreat point during the 1950 battle of

the Chosin Reservoir. A primary site of burial was reportedly disturbed during a construction project and remains were moved by a construction worker to a different location. The secondary burial site was excavated and remains were recovered, with an estimated MNI of five. Along with the skeletal elements, personal effects were recovered and accessioned for further evaluation. The purported primary burial was examined by the recovery team, and no additional objects were recovered. Due to the conditions of recovery, there was some suspicion by the team that the recovered remains might be highly commingled.

Upon receipt of the samples at DPAA, the specimens were sampled and sent to AFDIL for DNA analysis. A total of 103 specimens in six submissions were submitted from 2005-2012. Results showed 32 different mitochondrial DNA sequences from the initial set of remains. The advancement of DNA technology has grown tremendously throughout the years, giving even greater testing sensitivity for environmentally challenged and degraded specimens. However, DNA testing alone is not enough. If family references are not available for comparison, samples will remain unidentified. By using available dental records, anthropological estimations, and available family references, only ten identifications have been made from the potential 32 individuals. As protocols continue to improve and advance, and as more family references are received, it is hoped that the others will be identified in the future.

A.12 CONCLUSIONS

The challenges presented in identifying individuals from war or from other large- or long-scale disasters are unique. What may appear to be obvious at first glance may not be true after analysis is undertaken. Records may be poor. Methods once thought to be sound, such as the treatment of samples with a preservation compound, can, unfortunately, hamper future attempts at identification.

What is hopefully apparent through the course of this chapter is that DNA testing of skeletonized remains does not always take a straight course. As scientists we need to fully consider the circumstances of death and post-mortem conditions before making a decision on how to proceed and not accept that the standard technique is the best. Techniques that we use now are rooted firmly in the past techniques and the future may be lateral moves in thought or a return to an older technique. Demin1 proving to be of better use in some cases for DNA extraction from skeletonized remains is an excellent example of the latter. By keeping this as an active SOP in the laboratory, AFDIL was able to return to the procedure and improve the testing results of compromised samples.

The former, lateral moves in protocols, are fast upon the forensic identification community. Next Generation Sequencing (NGS), or Massively Parallel Sequencing (MPS), has the possibility of completely changing the approach of HID using DNA. AFDIL recently incorporated NGS into the normal workflow for compromised samples. In six months of regular usage, 78 DPAA samples have been reported. While the success rate (28%) may seem to be low, consider that many of the samples tested had been unsuccessfully processed multiple times with standard protocols. Other techniques, such as mass spec analysis of the materials contained within the bone samples themselves, may lead to increased efficiencies in DNA extraction and provide additional information on the post-mortem conditions of the remains.

It is the task of the scientist to communicate with their partners in the identification process and to provide the best science possible in their efforts. Identification of a missing person, be they a soldier or a civilian, is crucial to family members. Not only do we do a disservice to

ourselves in not fully thinking about the scientific choices we can make, but to the families as well.

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Appendix B

National Institutes of Justice (NIJ) Grant Application – 2016

B.1 INTRODUCTION

The following grant was written for submission to the NIJ Research Opportunity: Research and Development in Forensic Science for Criminal Justice Purposes. The goal of the application was to seek funding for the GC/MS testing of the skeletal detritus and DNA generated from skeletal remains in support of this thesis.

The majority of the grant application is included in this Appendix. The budget worksheet is not included, but is explained in the budget narrative. The forms associated with grant submission are not included as they are simply signatory in nature and not relevant to the information being presented.

The application was ultimately unsuccessful and the research was supported through other means.

B.2 TITLE PAGE

Analysis and Mitigation of Materials Co-Extracting With DNA from Degraded Skeletal Remains

Submission Date:
30 January 2016

Funding Opportunity:
NIJ-2016-4305 – Research and Development in Forensic Science for Criminal Justice Purposes
OMB No. 1121-0329

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B.3 PROJECT ABSTRACT

Recovery of DNA from ancient human skeletal remains has been one of the more challenging concerns in forensic science in recent years. The Armed Forces DNA Identification Laboratory (AFDIL) has been tasked with the DNA analysis of the skeletonized remains of missing US service members from past conflicts. It is paramount that quality DNA be recovered from these samples with the aim of entering the profiles into searchable databases. However, in many sets of remains, subsequent analyses indicate that the DNA present is not only damaged or degraded, but are also inhibited by materials present in the environment or on the remains themselves.

Modification to the extraction protocol at AFDIL has resulted in a success rate for mtDNA analysis of approximately 92%, irrespective of conflict or skeletal element processed. We hypothesize that the failure rate of 8% is due to either low quality DNA or inhibitors co-extracted with the DNA. While there have been studies that target a broad-based removal of inhibitors from staged burials, none have examined the presence of specific inhibitors by evaluating real-world samples. This study attempts to qualify the chemical elements and inhibitors that co-extract with the DNA from the skeletal remains.

In Phase 1, skeletal residue, removed from the exterior of the sample during standard cleaning, from over 400 samples and the purified DNA extract will be tested using mass spectrophotometry. Comprehensive DNA typing of the same samples using mtDNA, autosomal STR, and Y-STR analysis allows a correlation between DNA success and the types and quantity of inhibitors. Two different types of extraction purification, organic and inorganic, will also be compared to determine if one is more effective for the removal of certain inhibitors.

In Phase 2, different methods of DNA extraction from skeletonized human remains will be tested in an attempt to find a more focused means of removing potential inhibitors. Most

extraction protocols are designed to remove all inhibitors. The goal of Phase 2 is to remove only the inhibitors causing issues in downstream processing, thereby potentially improving both the quality and quantity of DNA recovered.

The outcome of this work is to report on inhibitors co-extracting with DNA recovered from skeletal remains, the impact on downstream DNA testing, and possible protocols for removal of detected inhibitors.

B.4 STATEMENT OF THE PROBLEM

Recovery of DNA from human skeletal remains has been one of the more challenging concerns in forensic science in recent years. With the continued build-up of unidentified skeletonized remains in medical examiner offices across the United States and world-wide, it is becoming paramount that quality DNA be recovered from these samples with the aim of entering the profiles into searchable databases. However, in many sets of remains, the DNA present is not only damaged or degraded, subsequent analyses are also inhibited by materials present in the environment or on the remains themselves (Alaeddini, 2014; Kreader, 1996). While there have been studies that target a broad-based removal of inhibitors (Eilert and Foran, 2009; Kemp *et al*., 2006; Kemp, *et al*., 2014;), none have examined a targeted removal of specific inhibitors by evaluating real-world samples for the presence of inhibitory materials.

This particular research meets all three of the NIJ Research and Development in Forensic Science for Criminal Justice Purposes Program goals. Phase One is primarily a Fundamental/Basic Research goal that is applicable across multiple disciplines: DNA, Anthropology and Medico-Legal Death Investigations. The impact of the co-extraction of metals and other elements with DNA from degraded skeletal remains will be evaluated. In addition, the predictive value of the elemental content of the remains will be considered as it pertains to location or circumstances of the post-mortem interval; thereby meeting the

Development Goal of the solicitation. Isotopic analysis will not be undertaken, but rather the analysis will be conducted into the distinct composition of the surrounding environment as it impacts the decay of the remains. As the location and circumstances since death are largely known for the remains to be used in this study, it is reasonable to anticipate that a predictive model can be generated.

Phase Two meets the Applied Research Goal in that the focus here is to mitigate the downstream impact of the co-extracted materials. Various modifications to the extraction protocol will be examined in an attempt to remove elements that were shown to have the most deleterious effect on amplification of DNA.

The Armed Forces DNA Identification Laboratory (AFDIL) works in conjunction with the Defense POW/MIA Accounting Agency Laboratory (DPAA-Lab) with the primary purpose of identifying the remains of missing U.S. service members from past military conflicts. These samples typically range in time since death of 40 to 70 years. Remains may have lain *in situ* until recovery by teams from DPAA or have been curated in unknown locations. Thus the quality of the skeletal elements varies markedly depending on the environmental conditions to which they have been exposed. It should be stressed that in no way are we suggesting that we experiment on the remains of missing U.S. service members. Rather we are taking elements as they are received into the laboratory in the course of regular casework and analyzing the exterior surface of the remains that would be removed during the cleaning process prior to the extraction of DNA.

Modifications to the extraction strategy has resulted in a success rate for mitochondrial DNA (mtDNA) analysis of approximately 92%, irrespective of conflict or skeletal element processed (Edson, *et al*., 2011; Loreille, *et al*. 2007). As commercially available autosomal analysis kits become more sensitive to degraded DNA, it is becoming more feasible to

process DNA recovered from skeletonized human remains for autosomal STR and Y-STR. Until recently, the success rates for these kits had remained at approximately 20% (when used at AFDIL). With the implementation of a non-organic extraction method in 2013 (Edson and McMahon, in press; Huel, *et al*., 2012), success rates have steadily improved for STR analysis; however, mtDNA success slowly declined (Edson and Ah Sam 2015).

Previous studies on inhibition and bone density have used animal bone (Salmonid vertebrae: Kemp, *et al*. 2014 & Monroe, *et al*. 2013; seal ribs: Barta, *et al*., 2014; bovine: Antinick and Foran, 2015) and synthetic bone (Barta, *et al*., 2014) as substitutes for human remains. Even if studies have used *in situ* human remains, they are typically of small sample size (Yang, *et al*. 1998); recovered from a single location (Keyser-Tracqui, *et al*., 2003; Misner, *et al*., 2009) or a single event (Mundorff *et al*., 2008; Mundorff *et al*., 2009); or staged (Mundorff and Davoren, 2014). There is limited variability among the samples, and while this may allow for a development of a model for that specific time and place, extrapolation to other events is limited. By involving real-world samples, these predictive models can be evaluated within the framework of this project.

B.5 PROJECT DESIGN AND IMPLEMENTATION

B.5.1 Phase One

Phase One of the proposed work is basic research to quantify what materials co-extract with DNA from skeletonized human remains and the impact of these materials on downstream processing. Three different modalities of DNA analysis will be evaluated: mitochondrial DNA (mtDNA), autosomal STR (auSTR), and Y-chromosome STR (Y-STR). All of these modalities will be examined in course of regular casework with no modifications to analytical protocols. It should be stressed that there will be no interruption to normal casework. No new samples are to be taken for this work. The majority of materials to be collected for analysis would typically be discarded as waste in the course of regular casework processing.

B.5.1.1 Phase One, Part One

AFDIL reports 1300 independent analyses each year. These analyses account for all three modalities indicated above and does not represent discrete samples. Rather than set a goal to collect an entire year's worth of samples, Phase One of this work will focus on unique samples prepared in the course of three months, or 400 unique samples, whichever is greater. Samples will not be submitted specifically for this work. All samples taken will be taken in the context of regular casework processing at AFDIL.

Preparation of skeletal elements will proceed as per AFDIL standard operating protocols (SOPs) (Edson et al., 2004; Edson and McMahon, in press; Loreille et al., 2007). The exterior of the remains will be sanded off using a Dremel® tool with sanding attachments. This usually accounts for approximately the top 1 mm of material from the skeletal element. In some instances where the majority of the element is trabecular (spongy) bone, additional material may be removed. This material is removed to eliminate the possibility of contamination from both modern DNA and any environmental inhibitors the sample may have been exposed to in time since death. The bone dust, combined with exogenous dirt and biological material, is considered to be a waste product and is typically discarded. For the purposes of this study, this will be collected and retained for analysis. The remainder of the extraction will proceed as normal. Given the types of samples selected two different extraction protocols will be used: an inorganic protocol and a standard organic (phenol:chloroform) protocol. At the completion of the extraction, 1-5 µL of the extract will be removed for the purposes of this study.

The bone dust will be collected using a spatula, weighed, and stored within a 15 mL tube. Samples can be stored at room temperature (RT) or a -20°C freezer until submission to the

contract lab for analysis. At this time, all samples to be analyzed in Phase One have already been collected and stored.

Depending on the laboratory to which the samples are submitted, the 'dust' needs to be dissolved prior to submission for analysis in order to aid in the volatilization of the materials in the mass spectrometry protocol. Prior to submission to the contract laboratory, the following steps will take place:

1) A portion of the bone dust will be transferred to an anonymized 15 mL tube.
2) The solution required for dissolution of the sample will be added to this portion of the bone dust and allowed to dissolve prior to shipment. This solution will change depending on the requirements of the contract laboratory.
3) The remainder of the bone dust will be stored until needed or until the study is completed and it can be discarded.

The key for the anonymized samples will be kept with the PI and not distributed outside the laboratory. It is the protocol of AFDIL to not release case numbers to outside entities. The associated extract will likewise be placed in an anonymized and coded tube prior to submission to the contract lab.

Tubes of dissolved bone dust and purified extract will be submitted to an as yet to be determined contract lab for mass spectrophotometer analysis.

B.5.1.1.a Possible pitfalls:

Submission of bone dust for mass spectrophotometer analysis is an as yet unknown process. It may be necessary to modify the preparation protocol after the first batch of samples is

submitted for analysis. Samples will be submitted in small groups of 50 or less to allow for modification of how samples are prepared for submission.

By selecting samples processed in the course of regular casework, this testing is dependent on what samples are submitted by DPAA-Lab for analysis. It is possible that only a single conflict or incident may be submitted during the sampling period. If that is so, the sampling period may need to be shifted to encourage a wider variety of incidents.

B.5.1.1.b Scholarly Products

Preliminary Phase One data will be presented at the Australia New Zealand Forensic Science Society meeting in September 2016. While this is outside the funding period of this grant, it is relevant to the proposal.

B.5.1.2 Phase One, Part Two

Part Two of Phase One involves data analyses. There are two forensically relevant outcomes to this part of the research: 1) determination of elemental co-extraction during DNA purification and the impact on downstream processing and 2) developing a predictive model based on the location of recovery and the elemental content of the skeletal dust.

In addition to the information generated from the mass spectrophotometer analysis, the following data will be collected at AFDIL for correlation:

1) Location of recovery. Previous work (Edson, 2007) has indicated that there is some variability in success rate based on the location of the recovery that is not

correlated with age. A survey of all samples submitted is currently underway and should be completed prior to initiation of Part Two.

2) Conflict and approximate time since death.
3) Modalities tested. As testing will be recorded based on standard casework, not all samples will be tested in each of the given modalities (mtDNA, auSTR, YSTR).
4) Success metrics for each modality tested. "Success" will be measured based upon whether or not the sample is considered reportable according to the measures set forth in the AFDIL SOPs.
    a. Mitochondrial DNA – Samples are tested through direct Sanger sequencing. To be considered reportable, the strand sequenced must be duplicated in two independent amplifications. In previous studies (Edson, et al., 2004; Edson, et al., 2009; Edson and Ah Sam 2015), 'success' was considered to be a minimum of 100 reportable base pairs. This metric will be continued in this study. Samples that are later determined to be animal bones will be removed from the 'success' metric, but will still be tracked for co-extraction of materials.
    b. Autosomal STR – Samples are tested through the use of un-modified, commercially available kits. At the time of writing, the most commonly used kits by AFDIL are AmpFlSTR MiniFiler™ and AmpFlSTR Identifiler® (Life Technologies); however, AFDIL has recently converted to using PowerPlex® Fusion (Promega Corporation, Madison, WI). Given that the samples tested are low quality samples, a system of replication similar to that with sequencing is used before a sample is considered reportable. Samples are amplified a minimum of three times and an allele must be seen at least twice before it can be reported. To be considered successful for statistical analysis, a minimum of four loci (not including amelogenin) must be reported.

c. Y-Chromosome STR – Samples are tested using a commercial kit (AmpFlSTR Yfiler®: Life Technologies) and a modified protocol of increased cycle numbers and increased Taq DNA polymerase. Reporting is similar to that of autosomal STR analysis. To be considered successful for statistical analysis, a minimum of four loci must be reported.

5) The minimum and maximum amplicon sizes. This is applicable for mitochondrial DNA testing only and should be indicative of relative fragment size.
6) Protocol used to generate STR data. As indicated above, different protocols are currently used to generate STR data. They vary in sensitivity and necessary input.

Storage conditions prior to submission of the sample to AFDIL will not be considered.

Data will be combined together to determine trends in between processing, location of recovery and elemental composition.

B.5.1.2.a Possible Pitfalls

Pitfalls for Phase One Part Two are extremely limited. Part Two is largely data analysis and modeling, which are only dependent upon the contract laboratory completing analysis in a timely fashion and the PI.

B.5.1.2.b Scholarly Products

It is believed that four papers can be generated for Phase One. The first two papers will detail the on-going analysis of sample selection relative to success of individual skeletal elements and the existence of possible trends in DNA extraction methods and age of samples. A third paper will detail the materials found within the skeletal dust, which of these materials co-

extract with the DNA, and which have an impact on the downstream processing. The fourth paper will describe any location based trends for the presence of elements and other materials.

Abstracts will be submitted for the International Society of Forensic Genetics Meeting in September 2017. Funding for attending this meeting is requested.

B.5.2 Phase Two

The activities to take place in Phase 2 are largely dependent on the results of Phase One and fall within the category of Applied Research. By using mass spectrophotometer, the specific inhibitory materials can be identified in the original sample and the resulting DNA extraction. With this knowledge, it will be possible to identify specific protocols for reducing the presence of inhibitors. Rather than taking a single method in an attempt to reduce all possible inhibitors, individual protocols for specific inhibitors will be examined.

Protocols include, but are not limited to:

1) Chelex® resin purification of dissolved bone prior to inorganic clean-up
2) Use of EGTA in place of EDTA
3) Additional purification by means of a centrifugal filter
4) Addition of a phenol:chloroform:isoamyl alcohol was step prior to an inorganic purification.
5) Eliminating the inorganic purification.
6) Inorganic purification using a different commercially available kit.

The danger in additional purification steps is the reduction in the quantity and quality of DNA remaining.

Samples that have been retained at the AFDIL for training will be used for Phase 2. These include a variety of skeletal elements from differing locations. The original success of the samples will be used as a baseline and correlated to predicted trends generated in Phase 1. Skeletal dust will need to be collected from these samples and analyzed via mass spectrophotometer prior to extraction.

The specific number of extracts to be tested will depend largely upon the results of Phase One. Ideally, ten samples exhibiting contamination by a specific elemental inhibitor or combination of inhibitors will be tested for elimination of said inhibitor. For example, out of the 400 samples initially tested, 30 exhibit high levels of iron. Only ten samples would be tested for the effectiveness of the chosen purification method. By selecting samples as they are received in the course of regular casework, there should be a wide variety of elemental inhibitors. It is of course possible that there will be only one or two primary elements seen. It is anticipated that at least 50 samples will need to be tested in Phase 2.

After the samples are purified and extracted, 5 µL will be submitted for mass spectrophotometer analysis and the remainder will be processed in the same modalities as had been previously, and the relative improvement assessed. Sample processing at this phase will fall outside normal casework analysis, and thus the costs associated will not be accounted for in the regular AFDIL budget.

B.5.2.1 Possible Pitfalls

Phase Two contains the most significant pitfall. Mitigation of inhibition is a difficult process at best. By incorporating additional steps in the standard DNA extraction protocol, the possibility of reducing the amount of DNA recovered increases (Doran and Foran, 2014; Kemp, *et al*., 2006). Even the extraction protocol itself may lead to a fairly significant loss of

quality DNA (Barta, *et al*., 2014; Kemp, *et al*., 2015). It is possible that in attempting to reduce the inhibitors, a large proportion of DNA will be lost. While this would be a less than optimal outcome to the study, the knowledge that over-cleaning of a sample can lead to complete failure is still a result worth knowing.

B.5.2.2 Scholarly Products

Data will be disseminated through presentations at national and international conferences. The target will be to present Phase 2 data at the American Association of Forensic Sciences Meeting in 2018.

At a minimum, one journal article will be generated from Phase 2.

B.6 OUTCOMES

Inhibition of DNA analysis in 'ancient' skeletal remains is a widely recognized problem in the forensics community. While some work has been done to ameliorate the issues, very little work has sought to qualify what materials are specifically co-extracted with DNA. As practitioners, we have sought to eliminate or reduce the impact of the problem with a one-size fits all approach. From a budgeting and casework stand-point, this is certainly more cost effective and can serve to streamline laboratory processing. However, treatments specific to elemental contaminants can be developed. Many may be very simple to implement, will improve results, and save time and money.

Data from both phases of the study can be disseminated to a wide audience. Any crime laboratory in the country or world-wide that extracts DNA from degraded human skeletal remains can use the data generated from this study to improve their own analysis methods.

Further, materials on the surface of the skeletal elements may be used as a predictor of burial location, which would greatly assist in medico-legal death investigations.

B.7 DATA MANAGEMENT

All data will be maintained on the secure servers at AFDIL. Any case related information will remain at AFDIL and DPAA-Lab. Samples to be sent out will be anonymized by having casework associated identifiers removed, with the key being retained by the PI. Upon completion of the project, relevant data will be transferred to NIJ in a manner of their choosing; however, case related data, such as case numbers will not be released.

No personally identifiable information is being collected during the course of this study.

B.8 CAPABILITIES AND COMPETENCIES

The staff of the Armed Forces DNA Identification Laboratory is a highly trained group of individuals. We are relying on the regular processing of cases to generate DNA results in Phase One. They are also responsible for the collection of the 'bone dust' that will be submitted for mass spec analysis. All individuals are proficiency tested.

The PI of this project has worked at AFDIL for 17 years and has 11 publications relating to forensic DNA analysis of skeletonized human remains. She will be responsible for data collection and analysis as well as the preparation of the 'dust' for submission to the as yet to be determined contract lab and the lab work in Phase 2. The PI has not previously applied for an NIJ grant; however, AFDIL has previously managed NIJ grants as have other members of the advisory group.

The advisory group consists of highly trained and respected members of the forensic community.

Please see the personnel attachment the list of individuals and their *Curriculum Vitae*.

B.9 IRB

The project is not subject to IRB approval for the following reasons:

1) The samples being tested come from deceased humans.
2) The samples being tested are obtained from unknown/unidentified individuals lost during past military conflicts. Samples tested are generally fragmented and/or from highly commingled sets of remains.
3) The skeletal 'dust' being tested is generally considered a waste product and would otherwise be discarded.

B.10 DISCLAIMER

The opinions or assertions presented are the private views of the authors and should not be construed as official or as reflecting the views of the Department of Defense, its branches, the U.S. Army Medical Research and Materiel Command, the Armed Forces Medical Examiner System, the Armed Forces DNA Identification Laboratory, the American Registry of Pathology, Defense Health Agency, or Defense POW/MIA Accounting Command.

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B.12 PROJECT PERSONNEL

Principal Investigator: Suni M. Edson, Assistant Technical Leader, Past Accounting Section, Armed Forces DNA Identification Laboratory (AFDIL), Dover, DE

Budget Officer: LtCol Laura L. Garner, USAF, Armed Forces Medical Examiner System (AFMES), Dover, DE

Project Advisory Group:

Dr. Timothy McMahon, Scientific Director, Armed Forces DNA Identification Laboratory (AFDIL), Dover, DE

Dr. Adrian Linacre, Chair in Forensic DNA Technologies, School of Biological Sciences, Flinders University, Adelaide, South Australia

Dr. Michael Coble, Forensic Biologist, Biochemical Science Division, Applied Genetics, National Institutes of Science and Technology (NIST), Gaithersburg, MD

Dr. Greg Berg, Laboratory Manager, Defense POW/MIA Accounting Agency – Laboratory (DPAA-LAB), Joint Base Pearl Harbor – Hickam, HI

Dr. Duncan Taylor, Principal Scientist for Forensic Statistics, School of Biological Sciences, Flinders University, Adelaide, South Australia

**CV for the above listed personnel were included in the NIJ proposal, but are not included here.

B.13 PROJECT TIMELINE

Summer and Fall of 2015:

1) Sample collection has been completed. 421 skeletal dust samples have been collected. Samples will be evaluated for testing and not all will be submitted for analysis.
2) Contract core facilities for processing of mass spec will be sought. Collaborative partners will be sought in an attempt to lessen costs.

Winter/Spring 2016:

1) Success metrics of samples previously tested through standard case work will be generated. This will allow for a comparison of relative success on multiple DNA platforms to the outcomes from chemical analysis.
2) Contract core facilities identified.
3) A small number of samples to be submitted for analysis. Initial cost to be borne by AFDIL/ARP.
4) Abstract submitted for ANZFSS meeting.

Summer 2016:

1) Award granted
2) Initial data analysis and generation of models.

Fall/Winter 2016:

1) Attendance at ANZFSS. Presentation of model strategy for skeletal sampling based on location of recovery and correlation to initial results from mass spec
2) Adjustment of volume/treatment of samples for submission.
3) The remainder of the samples will be submitted to the core facility.
4) Preparation of manuscripts from Phase One
5) Design of project analysis for Phase Two

6) Abstraction submission for ISFG meeting

Spring/Summer 2017:

1) Sample processing.
2) Modification of purification strategies based upon results
3) Presentation of final results of Phase One at ISFG
4) Abstract submitted for AAFS meeting

Fall 2017/Winter 2018:

1) Analysis of data
2) Preparation of manuscripts relating to Phase 2
3) Presentation of Phase 2 results at AAFS

B.14 BUDGET NARRATIVE

**A budget worksheet was also included in the NIJ submission but is not included here.

B.14.1 Phase One

B.14.1.1 Phase One, Part One

Funding is requested for the processing of samples for mass spectrophotometer analysis. The core facility where this will be done has yet to be determined. We are seeking laboratories that may be interested in entering into a collaborative partnership. The cost of analysis listed below is based upon the price for a similar analysis at NMS Labs. This is anticipated to be much greater than the actual cost of analysis.

To be submitted for analysis:

400 samples of prepared skeletal dust

400 corresponding DNA extract

800 x $200 = $160,000

Shipping of samples to contract laboratory will be incurred. Materials will be sent via FedEx or other traceable, priority shipping. Cost will potentially be less than anticipated.

8 packages x $20 = $160

Other costs incurred in Phase One Part One will be covered by AFDIL. This includes the costs for Eppendorf tubes, pipet tips, and the solution for dissolving the bone powder.

B.14.1.2 Phase One, Part Two

There are no associated costs for the analysis of the data. Salary and associated overhead is not being requested for the PI.

Travel costs for the PI to present preliminary Phase One data at the Australia New Zealand Forensic Science Society International Symposium is not being requested. The meeting is being held in Auckland, New Zealand 18-23 September 2016 and will provide an opportunity to meet with advisory board members, Professor Adrian Linacre and Dr. Duncan Taylor. Dr. Linacre will request funding through Flinders University with in-kind support of approximately $3,000 AUD.

Travel costs for the PI to present Phase One results at the International Society of Forensic Genetics is being requested. The meeting is being held in Seoul, South Korea, 28 August to 1 September, 2017. This will provide an opportunity for the yearly required meeting with Professor Adrian Linacre and Dr. Duncan Taylor. Prices listed were calculated from similar dates in 2016.

Coach Class Flight – Philadelphia, PA to Seoul, South Korea = $1400.00

Meeting registration: $500.00 -- meeting cost is estimated based upon the registration fees for 2015 and early registration.

The following cost+500s are based upon the US Department of Defense per diem costs as of 28 December 2015. Additional nights are included based on the possibility of attending workshops prior to the start of the meeting. Anticipated meeting costs may be less:

8 nights in hotel: 8 x $230.00 = 1840.00

7.5 days of local meals: 7.5 x $108.00 = $810.00

7.5 days of incidentals: 7.5 x $27 = $202.50

Total cost for meeting travel: $4752.50

Total cost for Phase One: $164,912.50

B.14.2 Phase 2

Funding is requested for the processing of samples for mass spectrophotometer analysis. The core facility where this will be done has yet to be determined. We are seeking laboratories that may be interested in entering into a collaborative partnership. The cost of analysis listed below is based upon the price for a similar analysis at NMS Labs. This is anticipated to be much greater than the actual cost of analysis.

To be submitted for analysis:

40 samples of prepared skeletal dust

40 samples of purified DNA extract

80 x $200 = $16,000

Shipping of samples to contract laboratory will be incurred. Materials will be sent via FedEx or other traceable, priority shipping. Cost will potentially be less than anticipated.

1 packages x $20 = $20

The specific cost of laboratory supplies to be used in the purification of samples, extraction of DNA, and the downstream processing is difficult to determine as these are dependent upon the outcomes of Phase 1. The cost per sample is generated from the AFDIL's billable costs to process a single skeletal element for mitochondrial DNA, minus personnel costs.

40 samples x $1000 = $40,000

Travel to the American Academy of Forensic Sciences Meeting in February 2018 and publication costs will be borne by the PI.

Total cost for Phase 2: $56,020

B.15 LETTER OF SUPPORT

OFFICE OF THE ASSISTANT SECRETARY OF DEFENSE
HEALTH AFFAIRS
ARMED FORCES MEDICAL EXAMINER SYSTEM
115 PURPLE HEART DRIVE
DOVER AFB, DE 19902-5051

MEMORANDUM FOR RECORD 20 January 2016

FROM: DEPUTY DIRECTOR DoD DNA REGISTRY

SUBJECT: Letter of Intent to Support Ms. Suni Edson application for NIJ Grant (NIJ-2016-4305)

1. The Armed Forces DNA Identification Laboratory (AFDIL) is pleased to offer its support to Ms. Suni Edson in her response to a US Department of Justice, Office of Justice Programs, National Institute of Justice (NIJ) grant proposal (NIJ-2016-4305- Research and Development in Forensic Science for Criminal Justice Purposes).

2. Ms. Edson is applying as an individual for funds, which will be managed by the Armed Forces Medical Examiner System as the supervisory agency for scientific contractors performing human remains identification for the Armed Forces Medical Examiner System (AFMES).

3. This study is of particular importance because Ms. Edson's technical approach addresses the evaluation of practical importance to the targeted forensic community.

4. The Deputy Director of DoD DNA Registry as the contracting officer representative (COR) manages all funds supplied to scientific contractors supporting the identification of human remains mission for the Armed Forces Medical Examiner System. This includes an annual budget of xx million dollars per year.

5. Questions related to the above should be addressed to Lt Col Laura Garner at (302) 346-8911.

B.16 DATA MANAGEMENT FOR ARCHIVAL PURPOSES

Data Management for Archival Purposes

Data will be transmitted for archival purposes in multiple formats.

Data correlating the quality of the original DNA extraction and the success rates of multiple DNA testing modalities (mtDNA, YSTR, auSTR) will be compiled into an Excel spreadsheet. Internal AFDIL case numbers will be removed. Instead, the anonymized numbers used when submitting the samples to the contract lab will be used to label each sample. Actual DNA profiles will not be made available for archiving as they are not relevant to the grant.

Data generated from the mass spectrophotometry analysis will be provided in the raw data file format. A summarized table of results will also be provided, most likely in an Excel format. Future researchers will be able to generate their own conclusions from the mas spec results as well as view the conclusions of the researchers.

Appendix C

International Association of Forensic Sciences Poster Presentation

C.1 POSTER PRESENATION

The following poster is not related to the work contained within the thesis. However, the work was presented while actively working towards completion of the thesis.

The poster was presented at the International Association of Forensic Sciences Meeting in Toronto, Canada, August 2018.

Mutation Rates of Y-STR Loci and Incidences of Non-Paternity Among Purported Paternal Relatives

Suni M. Edson, MS[1,2]

[1]Armed Forces DNA Identification Laboratory, American Registry of Pathology, LLC, Armed Forces Medical Examiner System, 115 Purple Heart Drive, Dover AFB, DE 19902
[2]Flinders University, School of Biological Sciences, Adelaide, South Australia

Presented at IAFS, Toronto, Canada / August 2017

INTRODUCTION

Collection of family reference samples (FRS) for comparison to unidentified human remains relies heavily upon the family members providing a valid family history. Oftentimes, relatives may be unaware of certain familial relationships or they chose to not share this information with the reference collector. Since 1992, the Armed Forces Medical Examiners System's Armed Forces DNA Identification Laboratory (AFMES-AFDIL) has collected family reference samples, primarily whole blood and buccal swabs, to aid in the identification of U.S. service members missing from past military conflicts.

The authenticity of the reference samples submitted is paramount when determining the identity of missing persons or those involved in a mass fatality event. Mutation rates for YSTR analysis have been largely calculated from father-son pairings. While valuable, long-scale mass fatalities, such as the identification of service members from past conflicts often rely on references from distant relatives. This research examines the paternal references submitted as Y-STR reference materials for individuals missing from the Vietnam War, Korean War, and World War II.

METHODS AND MATERIALS

Family references are collected by Service Casualty Officers based on service records, genealogical research, and anecdotal evidence from family members.

Samples are sent to the AFDIL for processing in appropriate DNA platforms:

- Mitochondrial DNA
  - Control region
  - Whole genome
- **YSTR**
- Autosomal STR

Generated profiles are compared to other purported familial references for authenticity. Individuals of non-paternity were removed from the mutation calculations, resulting in 1129 meioses events per locus.

MUTATION RATES AND INCIDENTS

all 10x-3	This Study Admixed (United States)	Gusmao, et al. (2005) (8) Argentina, Brazil, Colombia, Portugal, Spain, & Venezuela	Kayser and Sajantila (2001) (1) Admixed (Europe)	Weng, et al. (2013) (9) South China Han	Willems, et al. (2016) (10) Admixed (Global)	Dominges, et al. (2007) (11) Sub-Saharan Africans in Brazil	Hohoff, et al. (2007) (12) Germany	Goedbloed, et al. (2009) (13) Germany and Poland	Lee, et al. (2007) (14) Korea
DYS456	5.31	-	-	2.2	3.77	-	-	4.5	5.4
DYS389I	3.54	1.115	2.35	0	-	1.9935	0.97	5.1	2.7
DYS390	4.42	1.065	8.58	3.3	-	2.2072	1.95	1.1	2.7
DYS389II	8.85	1.123	4.71	5.4	-	2.1798	4.87	3.4	5.4
DYS458	10.6	-	-	8.7	9.2	-	-	8	8.1
DYS19	1.77	1.425	2.01	0	-	1.7774	5.84	4	5.4
DYS385a/b	9.74	1.819	2.1	4.9	-	2.3513	1.95	-	2.7
DYS393	0	1.275	0	0	-	0.7275	0.97	1.7	2.7
DYS391	2.65	3.197	4.82	0	-	3.4104	1.95	2.8	0
DYS439	4.42	6.873	-	7.6	5.08	4.896	6.82	3.5	5.4
DYS635	3.54	3.436	-	2.2	-	-	-	3.5	8.1
DYS392	0	1.07	0	0	-	0.5961	0	0.6	0
Y GATA H4	3.54	2.286	-	0	-		-	2.8	2,7
DYS437	2.65	1.739	-	0	-	2.0517	0	1.1	2.7
DYS438	1.77	0.824	-	0	-	0.4039	0.98	0.6	0
DYS448	1.77	-	-	0	-	-	-	0	0

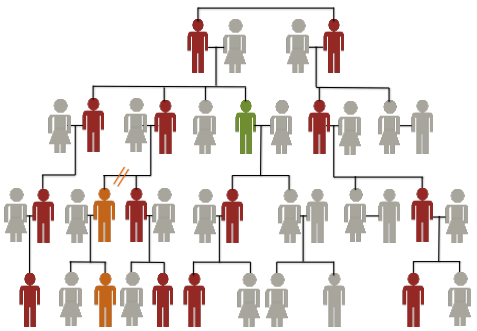
Figure 1. Comparison of mutation rates from different worldwide populations. Mutation rates are similar at some loci (i.e., DYS458 and DYS439) and highly variable at others (i.e., DYS389II). Elevated mutation rates are expected in this group examined due to the wide variety of population groups sampled from and the relative familial distance of the references to each other.

Allele	locus 1	locus 2	References	Allele	locus 1	locus 2	References	Allele	locus 1	locus 2	References
DYS 456	16	17	brother - brother	DYS 458	17	18	son - son	DYS 391	11	12	brother - brother
	15	15,16	brother - brother		16	17	brother - brother		9	10	nephew(p) - brother
	15	16	brother - brother		16	17	brother - brother		10	11	brother - brother
	15	16	cousin(p) - cousin(p)		17	18	cousin(p) - grand nephew(p)	DYS 439	12	13	brother - brother
	16	17	brother - brother		16	17	nephew(p) - nephew (p)		12	13	brother - brother
	15,16	15	brother - brother		18	19	cousin(p) - 2nd cousin(p)		12	13	brother - brother
DYS 389I	14	15	grand nephew(p) - nephew (p)		15	16	brother - brother		11	12	nephew(p) - brother
	14	15	half brother - half brother		16	17	brother - brother		12	13	brother - brother
	14	14,15	brother - brother		18	19	son - brother	DYS 635	21	22	brother - brother
	12	13	cousin(p) - cousin(p)	DYS 19	14	15	brother - brother		23	24	brother - brother
DYS 390	23,24	24	nephew(p) - brother		15	16	brother - brother	Y GATA H4	12	13	nephew(p) - nephew (p)
DYS 389II	29	30	brother - brother	DYS 385a/b	14	15	brother - brother		12,13	12	brother - brother
	29	30	nephew(p) - nephew (p)		15	16	brother - brother		12	13	brother - brother
	29	30	nephew(p) - brother		14	15	brother - brother	DYS 438	11	12	nephew(p) - nephew (p)
	30	31	nephew(p) - nephew (p)		16	17	brother - brother	DYS 448	20	21	brother - brother
	31	32	grand nephew(p) - nephew (p)		10,11,14	10,11	brother - brother				
	31	32	half brother - half brother		13	14	brother - brother				
	31	31,32	brother - brother		14	15	son - son				
	28	29	cousin(p) - cousin(p)		11	13	grand nephew(p) - nephew(p)				

Figure 2. Variations seen among references. Relationships are noted in reference to the missing person.

RELATEDNESS AND NON-PATERNITY

Of the 636 purported paternal pairs collected and compared, 44 show indication of being paternally unrelated by having more than 3 differences in their YSTR profiles [as recommended in (1)]. This indicates a non-paternity percentage within this random population of 6.92%. This is higher than many studies [1.4% in England (2); 0.8% in Switzerland (3); and 2.3% in Hawaii (4)], but lower than other studies [9% among Yanomamo (5)]. It is thought that the incidence of non-paternity in Western populations should be approximately 1% (6,7). However, the population in this database is admixed and spread across several generations. Reporting of familial relationships relies heavily on the donor's understanding of their heredity across possibly multiple generations.



Families may be contacted to verify a relationship, and may state that it was collected with an incorrect designation of relatedness (i.e., paternal vs. maternal). This can be due to a lack of understanding of what the designation of "paternal" means if the reference is not a brother, son, or other relationship that does not carry a gender specific designation.

In other cases, the non-paternity is cryptic, and cannot be resolved, even with further DNA testing. Given that individuals may be falsely excluded from identification due to a cryptic non-paternity, it is important to collect from multiple paternal relatives and not just a father/son or brother/brother pair.

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ACKNOWLEDGEMENTS

The author is grateful to all of those involved in the mission to bring our fallen service members home.

DISCLAIMER

The opinions or assertions presented here are the private views of the speaker and should not be construed as official or as reflecting the views of the Department of Defense; the Defense Health Agency; the Armed Forces Medical Examiner System; ARP Sciences, LLC; or the Defense POW/MIA Accounting Agency.

Appendix D

Bibilography

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