

CHEMICAL DIFFERENTIATION OF HUMAN OSSEOUS, NON-
HUMAN OSSEOUS, AND NON-OSSEOUS MATERIALS USING
SCANNING ELECTRON MICROSCOPY – ENERGY DISPERSIVE X-
RAY SPECTROMETRY (SEM/EDX) AND MULTIVARIATE
STATISTICAL ANALYSIS

by

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ABSTRACT

Identification of osseous materials is generally established on gross anatomical factors; however, highly fragmented or taphonomically altered materials are often problematic and alternative methods, such as biological, histological, or chemical analysis, must be utilized. Recently, chemical methods have been proposed to sort unknown materials according to their Ca/P ratios. Ubelaker and colleagues (2002) proposed using SEM/EDX to achieve this distinction and Christensen and colleagues (2012) have validated X-ray Fluorescence Spectrometry (XRF) for this application. An alternative method of analysis involves performing principal component analysis (PCA) on element spectra to classify unknown materials based on their trace element composition. Zimmerman (2013) proposed the validity of this method with data obtained using hand held XRF. Subsequently, performing PCA on elemental data obtained using SEM/EDX demonstrates potential for material differentiation.

Elemental weight percent data were collected using SEM/EDX then processed in R, version 3.0.1, by the R Foundation for Statistical Computing using PCA and Fisher Linear Discriminant Analysis. A two-tiered analysis was undertaken to improve discrimination between sample groups. The first tier involved distinguishing between osseous and non-osseous materials. After outliers were removed overall correct classification was 98.02% with one of 1504 osseous and 39 of 520 non-osseous spectra misclassifying. Since forty spectra were collected for each sample, the single misclassifying spectra would not affect the overall classification of the sample, resulting in 100% correct classification with a 0% error rate for the osseous samples. The second tier assessed differentiation of human and non-human osseous materials but demonstrated

a poor correct classification rate of 72.41%. Finally, a blind study was conducted using 20 samples to assess the applicability for using this method to classify unknown materials as osseous or non-osseous. All of the samples were correctly classified resulting in 100% correct classification, further demonstrating the efficiency of SEM/EDX and statistical analysis for differentiation of osseous and non-osseous materials.

Due to its high specificity, small sample requirements, and relative non-destructive testing protocol, as well as its presence in most modern crime laboratories, SEM/EDX has been proposed as a laboratory method for chemical differentiation of osseous and non-osseous materials. Additionally, the proposed method does not require advanced training or knowledge of analytical chemistry as the SEM/EDX provides clear results that can be processed using publically available statistical analysis software. By assessing and improving chemical analysis methodologies used for material differentiation, forensic anthropologists might be able to identify osseous and non-osseous samples as a preemptive step in forensic investigations involving fragmentary and taphonomically modified materials, reducing time and cost investments spent on forensically insignificant samples.

This thesis is dedicated to Donnie,
for supporting (putting up with) me through this whole
process and for agreeing to do it again.

It is also dedicated to my mother, Cathy Meizel,
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LIST OF ACRONYMS

ANOVA – Analysis of Variance

Ca/P – Calcium-Phosphorus

EDS – Energy Dispersive X-Ray Spectrometer

HHXRF – Hand Held X-ray Fluorescence

ICP-MS – Inductively Coupled Plasma – Mass Spectroscopy

LDA – Linear Discriminant Analysis

LIBS – Laser Induced Breakdown Spectroscopy

NCFS – National Center for Forensic Science

PC/PCs – Principal Component/ Principal Components

PCA – Principal Component Analysis

PIXE – Proton Induced X-ray Emission

SEM – Scanning Electron Microscope

SEM/EDX – Scanning Electron Microscopy – Energy Dispersive X-Ray Spectrometry

SLICE – Spectrum Library Identification and Classification Explorer

XRD – X-ray Diffraction

XRF – X-ray Fluorescence

CHAPTER 1: INTRODUCTION

Forensic anthropologists are physical anthropologists who apply their knowledge of anthropology and osteology to aiding in legal investigations, assisting pathologists, medical examiners, or other law enforcement agents in examining skeletal remains to aid in victim identification. When presented with a set of human skeletal remains, forensic anthropologists are able to assess biologic profile (age, sex, stature, and ancestry), trauma and pathology, and taphonomy (events occurring after death). Being able to identify and interpret this information can lead to identifying the individual as well as understanding certain aspects of their life, actions surrounding their death, and events occurring between death and recovery. Determining the forensic significance of prospective skeletal materials is a multi-step process that is pivotal for the advancement of the investigation since non-human remains are not likely to be linked to criminal activities. Early determination of the forensic significance of recovered materials will allow for a more rapid exclusion of non-relevant materials or commencement of a forensic investigation.

The first step in assessing the forensic significance of unknown materials involves determining if they are osseous or non-osseous in origin (Mulhern, 2008; Schultz, 2012). Though straightforward at the macroscopic level when large, nearly complete samples exhibiting specific diagnostic criteria are involved, this can become difficult in instances concerning highly fragmented or taphonomically altered materials (Mulhern, 2008; Schultz, 2012). Highly fragmented materials can originate from mass disasters, such as plane crashes or natural disasters. These events can also result in significant taphonomic alterations such as burning, weathering, or erosion (Schultz, 2012; Sledzik, 2013). Such

events will also interfere in the second step in determining forensic significance of unknown materials: determining if bone or dental materials are human or non-human in origin.

Once a material is determined to be osseous, it is necessary to determine if the bones or teeth are human. Non-human osseous materials are not forensically significant in most instances and can therefore be excluded from further investigation (Schultz, 2012). However, in instances where materials are too fragmented or taphonomically altered to distinguish osseous from non-osseous materials it is likely impossible to determine if they are human in origin. When diagnosis cannot be accomplished through simple visual assessment, histological or biological analysis may be used. However, in instances of severe fragmentation or taphonomic alteration it becomes necessary to examine the materials in question at the chemical level.

The majority of research towards differentiating osseous from non-osseous materials has focused on calcium-phosphorus (Ca/P) ratios (Ubelaker et al., 2002; Christensen et al., 2012). This has shown to be promising for material differentiation, but is limited for discriminating between materials with similar Ca/P ratios as bone such as mineral apatites, rock phosphates, and certain types of octocoral and brachiopod shells. Other studies have assessed the chemical compositions of osseous and non-osseous materials to identify differences in trace elements and have demonstrated higher success in osseous and non-osseous material differentiation (Zimmerman, 2013). To date, Zimmerman (2013) presents the most expansive chemical differentiation study that has addressed osseous and non-osseous differentiation using handheld X-ray fluorescence (HHXRF) and includes an assessment of the method using advanced statistics. By

refining these technologies forensic anthropologists might be able to identify human osseous, non-human osseous, and non-osseous samples as a preemptive step in investigations, reducing time and cost investments spent on forensically insignificant samples. In order to accomplish this task it is necessary to evaluate the capabilities of alternative chemical analysis methods.

Due to its high specificity and small sample requirements, in addition to its presence in most crime laboratories, scanning electron microscopy-energy dispersive x-ray spectroscopy (SEM/EDX) has been suggested as a method for performing such discriminations (Ubelaker et al., 2002). Though HHXRF presents a method with a field-use potential, it is essential to examine the practicality of incorporating this method into forensic anthropological analysis. As chemical analysis is currently not widely utilized in forensic anthropological investigation, it is necessary to evaluate methods that can be easily incorporated. Since SEM/EDX is already prevalent in modern forensic laboratories and has current anthropological applications such as analysis of trace metal residues on bone (Berryman et al., 2010; Amadasi et al., 2012; Gibelli et al., 2012; Pechníková et al., 2012; Taborelli et al., 2012; Vermeij et al., 2012) it would be less complicated and more cost efficient to introduce than a method requiring new instrumentation or validation. Additionally, the proposed method using SEM/EDX does not require advanced training or knowledge of analytical chemistry as the instrument provides clear results in the form of weight percent composition that can be processed using publically available statistical analysis software.

During SEM/EDX analysis, an electron beam is scanned over the mounted sample, resulting in excitation of the electrons within the sample. Detectors within the

scanning electron microscope (SEM) perceive the excitation of these electrons and generate a high magnification image of the sample on a computer monitor. Alternatively, detectors within the energy dispersive x-ray spectrometer (EDS) measure X-rays, which are also ejected by the excited electrons, to produce a spectrograph displaying the relative intensities of all of the detectible elements within the sample (Vermeij et al., 2012). Studies have shown SEM/EDX analysis to be highly specific and minimally destructive (Gibelli et al., 2012; Pechníková et al., 2012; Vermeij et al., 2012). Assessed for its validity to differentiate osseous from non-osseous materials, Ubelaker and colleagues (2002) were the first to determine that the Ca/P ratios and trace element profiles in dental and osseous tissues could be used to differentiate osseous from non-osseous materials in their sample with the exceptions of ivory, mineral apatite, and certain types of coral – all of which are similar in composition to bone.

Considering the specific requirements associated with development and eventual implementation of a new method for differentiation of osseous and non-osseous, and potentially human and non-human osseous, materials using chemical analysis there were three main goals when preparing and executing this research:

- (1) to assess the capabilities of SEM/EDX for determining trace element concentrations within osseous and non-osseous materials
- (2) to add to previous studies by expanding sample sets to include additional osseous, non-osseous, and taphonomically altered materials
- (3) to evaluate separation of materials using a statistical analysis approach
- (4) to design and perform a blind study assessing identification of unknown materials as osseous or non-osseous

The recent study by Zimmerman (2013) used trace element analysis, rather than Ca/P ratios to discriminate osseous and non-osseous materials. This study will expand upon her analysis, concentrating on elemental weight percentages. Data were collected on a sample set expanded from Zimmerman (2013) at the National Center for Forensic Science (NCFS) at the University of Central Florida and processed in house using R, version 3.0.1, by the R Foundation for Statistical Computing. Analysis consisted of principal component analysis (PCA) and Fisher Linear Discriminant Analysis (LDA). Subsequently, a blind study was conducted that was designed to assess the validity of the method for classifying unknown fragments. A two-tiered analysis was undertaken to improve discrimination between sample groups. The primary tier involved distinguishing between osseous and non-osseous materials. The second tier assessed osseous materials to determine if human and non-human samples could be distinguished. Overall, the results of this research will serve to demonstrate the ability of SEM/EDX and statistical analysis to differentiate osseous and non-osseous materials as well as to highlight several of the complications involved with discrimination of human and non-human osseous materials when using chemical analysis.

CHAPTER 2: FOUNDATIONAL MATERIAL

Anthropology as a discipline encompasses all topics related to the study of humans. It knows no temporal bounds, no limiting themes, and integrates information and techniques from all other disciplines to help explore the human condition.

Anthropology actively incorporates methods developed in other disciplines, such as chemical analysis procedures, to assist in solving anthropological problems or questions. Research incorporating these analytical chemistry methods is needed to demonstrate how these techniques can be integrated into anthropological research and where advancement is still necessary for the field of anthropology. Additionally, in order to understand the principles used to differentiate human osseous, non-human osseous, and non-osseous materials it is first necessary to understand the structure and composition of bone and dental materials as well as potential structural and compositional variations.

Anthropology and the Use of Chemical Analysis

Anthropology is traditionally divided into four subcategories: sociocultural anthropology, biological/physical anthropology, archaeology, and linguistic anthropology (Lavenda and Schultz, 2011). Though seldom isolated from the other subareas, each sub-discipline offers a more specialized focus on their respective aspects of the human condition.

Of these four subcategories, archaeologists and biological/physical anthropologists are the most likely to be confronted with tasks that require the use of chemical analysis. Archaeological applications of chemical analysis include studies of isotopic ratios to identify dietary and migration patterns (Sandford, 1993; Mays, 2000;

Ambrose and Krigbaum, 2003; Djingova et al., 2004; Burton, 2008; Alvira et al., 2010; Katzenberg, 2012), carbon dating, chemical analysis of mummified tissues (Zimmerman, 2012), diagenesis research (Katzenberg and Harrison, 1997), paleopathological investigations (Sandford, 1993; Gernaey and Minnikin, 2000; Koztowski and Witas, 2012), analysis of building materials and soils (Liritzis et al., 2007; Uguryol and Kulakoglu, 2013) and analysis of ceramics and other artifacts (Pappalardo et al., 2003; Liritzis, 2005; Mantzourani and Liritzis, 2006; De Francesco et al., 2007; Papageorgiou and Liritzis, 2007; Centeno et al., 2012; Issi, 2012; Domench-Carbo et al., 2013; Basso et al., 2014; Robertshaw et al., 2014). Other anthropological sub-disciplines also utilize chemical analysis methods in their research.

Biological anthropologists also frequently utilize chemical analysis, investigating similar problems in relation to more recent materials. Such investigations can include pathological studies (Nagy et al., 2008) or investigations of taphonomic processes (Dirkmaat and Cabo, 2012). These applications are also seen within forensic anthropology as well as numerous others such as analysis of dental resins to assess time since death and determine forensic significance (Ksenija et al., 2013), the impact of maceration methods on DNA amplification (Lee et al., 2010), the determination of burial duration using digenetic change (McLaughlin and Lednev, 2011), or identification of metal trace elements left on bone due to trauma (Gibelli et al., 2012; Pechníková, 2012). Additional chemical analysis studies in forensic anthropology are focused on differentiating fragmentary human osseous, non-human osseous, and non-osseous materials (Brody et al., 2001; Ubelaker et al., 2002; Shimoyama et al., 2003; Bodkin et al., 2005; Vass et al., 2005; Brooks et al., 2006; Edwards et al., 2006; Bergslein et al.,

2008; Beckett et al., 2011; Dillane et al., 2011; Müller and Reiche, 2011; Christensen et al., 2012; McLaughlin and Lednev, 2012; Zimmerman, 2013). However, though these technologies are being studied, forensic anthropologists often do not use them, performing only macro-identification via visual inspection due to the ease of performing the visual identifications and the novelty of chemical methods.

Complete, undamaged bones, and often large bone fragments, can be easily identified by trained anthropologists. Additionally, specific animal species can often be identified using osteological landmarks. However, in instances where the bones are highly fragmented or taphonomically modified it may be difficult to differentiate between human and non-human bone, and frequently, even between osseous and non-osseous materials. Multiple case reports demonstrate this dilemma (Vlčke, 1978; Gantt et al., 1980; Ubelaker et al., 1991; Martinez-Navarro, 2002; Cook, 2014). In instances in which macroscopic identification is not possible, histological, biological, and chemical means of analysis are utilized. Being able to make this distinction assists in early determination of forensic significance. By doing so forensic anthropologists can identify insignificant fragments, whether they are non-human or non-osseous, and exclude them from investigations. Providing a rapid, in-house method for making this determination will assist in forensic casework and ultimately reduce the strain on our already overburdened judicial system. Furthermore, material differentiation is pertinent in other areas of anthropology, such as biological anthropology and archaeology, and viable methods could be applied in these fields as well.

Structure and Composition of Osseous Materials

Bone is a highly versatile tissue; while providing support for the musculoskeletal system and protection for multiple vital organs, bone also generates blood cells, stores fats, and maintains elemental homeostasis. It is a living organ that responds to changes in the body and exhibits extensive variation between individuals (Safadi et al., 2009; White, 2012). This is due to the highly specialized nature of this composite material. Composed primarily of hydroxyapatite, an inorganic mineral that constitutes 60-70% of the weight of dry bone, bone is a rigid structure able to withstand forces many times its weight. The secondary component of bone, the organic protein collagen, comprises 25-30% of the dry weight of bone and contributes to the elasticity of bone (Schultz, 2006). This combination, along with several other minor constituents, creates a strong but flexible structure that is continually remodeling in response to its internal and external environments.

The gross anatomical structure of bone is directly related to its ability to respond to environmental influences. There are three main bone shapes: tubular bones, such as the long bones of the arms and legs; flat bones, such as those found in the cranial vault, shoulder, pelvis, and rib cage; and irregular bones, such as the bones of the wrist, ankle, spine, and splanchnocranium (Van De Graaff, 2001; Safadi et al., 2009; White, 2012; Tersigni-Tarrant and Shirley, 2013). However, despite their variability in shape, all bones share the same two basic structural components. The first of these components is compact or cortical bone. This is dense bone that makes up the outer surfaces of all bones and provides most of bone's stability (Garner et al., 1996; Van De Graaff, 2001; Safadi et al., 2009; White, 2012; Tersigni-

Tarrant and Shirley, 2013). The second structural component of bone is spongy bone, also called trabecular or cancellous bone. Spongy bone is molecularly indistinguishable from compact bone but forms more loosely, resulting in greater porosity and lower mechanical strength. Spongy bone is found near growth centers, under protuberances for tendon and ligament attachments, in vertebral bodies, at the ends of long bones, and between the cortical layers of flat bones (Garner et al., 1996; Van De Graaff, 2001; Safadi et al., 2009; White, 2012; Tersigni-Tarrant and Shirley, 2013). These variations in the gross anatomical structures of bone are directly related to its histological qualities.

Bone can also be classified as immature or mature. Immature bone, also called woven bone, is found in areas of initial bone growth and at fracture repair sites. It is not as well organized as the mature bone that will eventually replace it and is resultantly coarse due to the disoriented arrangement of collagen fibers (Safadi et al., 2009; White, 2012; Tersigni-Tarrant and Shirley, 2013). Mature bone (compact bone) is compositionally similar to immature bone but far more structurally organized (Safadi et al., 2009; White, 2012; Tersigni-Tarrant and Shirley, 2013).

Due to compact bone's higher demand for nutrients, mature compact bone is composed of Haversian systems. Haversian systems run parallel to the long axes of bones. At the center of each Haversian system is a Haversian canal, responsible for housing blood vessels and nerve fibers. Haversian canals are lined with a membrane called the endostium (Safadi et al., 2009; White, 2012; Tersigni-Tarrant and Shirley, 2013). Perpendicular to these canals are Volkmann's canals, which allow networking of blood vessels and nerve fibers in order to support the nutritional needs of the compact bone. Also surrounding the Haversian canals are lamellae, concentric rings that provide

bone strength. Within the lamellae are tunnels called lacunae, which house osteocytes. These are connected to the main Haversian canal through canaliculi (Safadi et al., 2009; White, 2012; Tersigni-Tarrant and Shirley, 2013). These structures work together to form the cohesive living structure that is bone (Figure 1).

Also directly related to the functionality of bone structure are the three main types of bone cells: osteocytes, which are responsible for maintaining bone structure; osteoblasts, which deposit new bone; and osteoclasts, which remove damaged or unnecessary bone tissue (Schultz, 2006; Safadi et al., 2009; White, 2012; Tersigni-Tarrant and Shirley, 2013). As can be expected, the complex organization of bone is directly associated with its trace element composition.

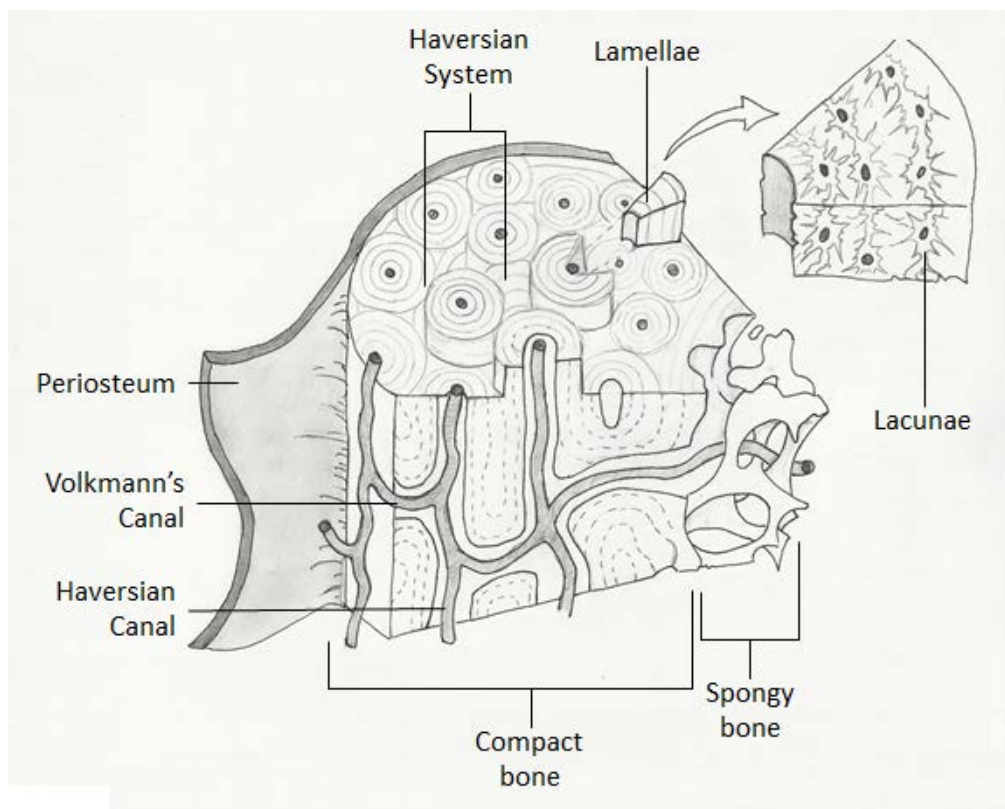


Figure 1: Bone microstructure.

Dental materials exhibit a similar composition. Teeth grow within the maxilla and mandible and only erupt once crown formation is completed. This process occurs twice at age correlated times: once for the deciduous teeth and once for the permanent teeth (White, 2012; Zinni and Crowley, 2013). Each tooth has several compositional areas (Figure 2). First, the portion visible in situ is the crown (Bawden et al., 1996). This is composed of enamel, an avascular and acellular tissue that is 99% hydroxyapatite (Burton, 2008). This higher inorganic composition results in enamel being a significantly strong material. Therefore, once a tooth has developed the main possibilities for modification are attrition (tooth wear) or fracturing –no regeneration or remodeling will occur (Hillson, 2005; White, 2012).

The tooth root anchors it into the alveoli of the maxilla or mandible. This root is coated in a layer of cementum (Bawden et al., 1996; White, 2012; Zinni and Crowley, 2013). Cementum is not as strong as enamel and is composed of approximately 70% inorganic material (Hillson, 2005). Cementum does regenerate and is laid down consistently throughout life in a layered pattern (Hillson, 2005; White, 2012).

Finally, each tooth has a layer of dentin and a central pulp chamber (Bawden et al., 1996, Zinni and Crowley, 2013). The dentin is the portion of the tooth root exposed on extracted teeth. This is composed of approximately 70-75% hydroxyapatite (Hillson, 2005; Burton, 2008; White, 2012). Turnover only occurs in dentin in the form of secondary dentin. Secondary dentin is laid down along the pulp chamber walls when tooth attrition exposes the primary dentin (Hillson, 2005).

Additional changes can occur to the components of teeth due to plaque deposits on the tooth surface. Bacteria will colonize tooth surfaces and secrete polysaccharides which aid in bacterial growth and adhesion (Hillson, 2005). As the plaque deposit increases in size the inner portion, the portion in contact with the tooth, may begin to mineralize into dental calculus. Due to the layered formation of plaque and the mineralization of the dental calculus the inner portions of these deposits can have significantly different chemical compositions than the superficial portions (Hillson, 2005).

Most dental materials, enamel and dentin, act dissimilarly to bone in regards to homeostatic exchange and remodeling in that they do not experience regeneration or remodeling. Though this does not impact the chemical analysis of enamel it is important to note that the results of such analyses will indicate environmental conditions at the time of formation rather than more recent conditions as bone does. This is a concern for analyses investigating environmental influences such as those performed in bioarchaeological or forensic studies.

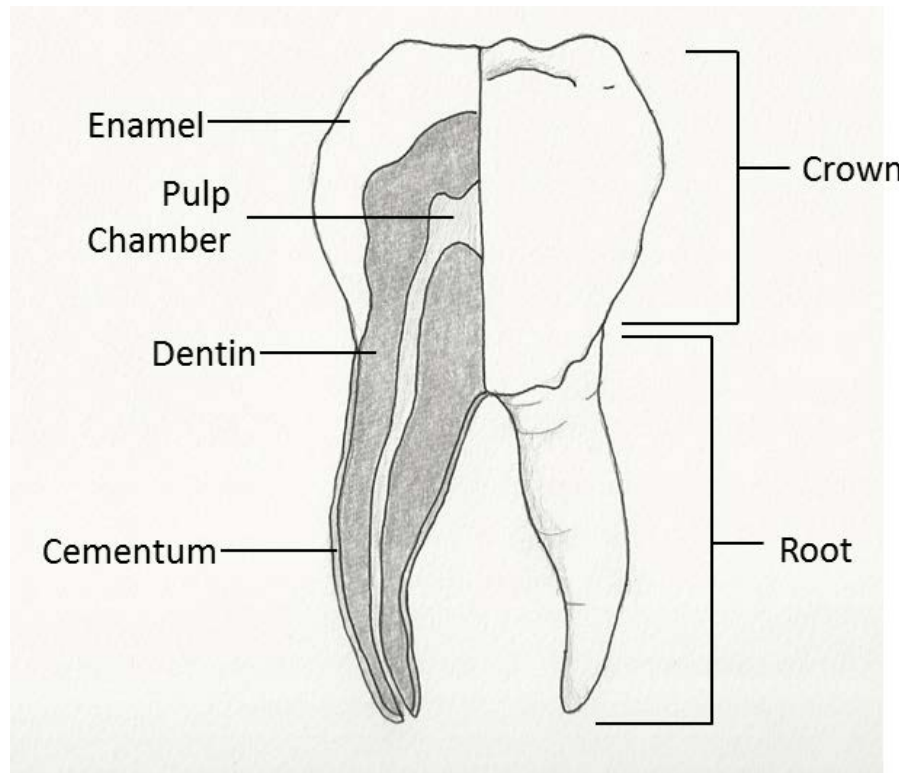


Figure 2: Tooth structure.

Variations in the Compositions of Osseous Materials

Hydroxyapatite is the primary constituent of osseous materials. It is an inorganic, crystalline calcium phosphate with a fixed composition in both human and non-human osseous materials. The chemical composition of hydroxyapatite is formally expressed as $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, though its empirical formula is $\text{Ca}_5(\text{PO}_4)_3(\text{OH})$ (Burton, 2008). Once laid down its composition can change due to ionic substitution of other elements (Blumenthal, 2000). Common substituents include carbonate, citrate, and other minor trace elements acquired during life through the dietary exchanges and after death through interaction with the burial environment (Pate, 1994; Blumenthal, 2000). This creates alternative calcium phosphate phases such as dicalcium phosphate dihydrate, $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$; octacalcium phosphate, $\text{Ca}_8\text{H}_2(\text{PO}_4)_6$; amorphous calcium phosphate,

$\text{Ca}_9(\text{PO}_4)_6$; and tricalcium phosphate $\text{Ca}_3(\text{PO}_4)_2$ (Pate, 1994). Additionally, individual ions can insert themselves into different portions of the calcium phosphate matrix. Calcium ions can be replaced by lithium, sodium, potassium, beryllium, magnesium, strontium, barium, radium, yttrium, actinium, zirconium, vanadium, niobium, chromium, manganese, iron, copper, gold, zinc, cadmium, mercury, aluminum, gallium, silicon, tin, lead, bismuth, uranium, plutonium and thorium. The phosphate group (PO_4), can be substituted by carbon tetroxide (CO_4), citrate, phosphate esters, diphosphonates, pyrophosphates, and amino acids. The hydroxyl group (OH) can be replaced by fluorine or chlorine (Pate, 1994). Finally, though less commonly encountered, bone can remove radionucleotides from the blood, depositing them at various locations within the skeleton and individual bones based on the valence levels of the radionucleotides (Priest, 2000). The majority of elements can be transferred within the skeleton or filter back out of the bone matrix depending on location of deposition, bone turn-over rates, and other environmental conditions (Bronner, 2008). However, many of these substitutions do not occur frequently and several occur in greater quantities than others. This, in addition to bone's regulatory role in overall body element homeostasis, results in a set of elements commonly found in bone both within and outside of the calcium phosphate phase.

The most common elements found in bone can be divided into essential elements and non-essential elements (Table 1). Essential elements, defined as vital to survival, include: carbon, hydrogen, oxygen, nitrogen, calcium, magnesium, phosphorus, sodium, potassium, chlorine, and sulfur. Non-essential trace elements, present mostly in low concentrations (between 10^{-6} and 10^{-9} mg/kg), are not necessary for survival but are often required to maintain optimal functioning of bodily systems. This set consists of iron,

manganese, copper, zinc, molybdenum, cobalt, selenium, iodine, fluorine, nickel, chromium, tin, silicon, vanadium, and lead (Smrčka, 2005). Similar elements have been documented in dental materials (Table 1). Oprea and colleagues (2009) analyzed human dental enamel and proposed the following elements as having significant concentrations: arsenic, barium, calcium, cerium, chlorine, cobalt, chromium, copper, iron, iodine, cadmium, potassium, lanthanum, manganese, molybdenum, niobium, neodymium, nickel, phosphorus, rubidium, tin, strontium, titanium, vanadium, and zinc (Table 1). The presence of these elements, as well as their relative concentrations, is variable since the compositions of osseous materials are influenced by numerous variables.

Table 1: Common elements found in human bone tissue and tooth enamel.

Bone Tissue	Bone Tissue and Tooth Enamel	Tooth Enamel
Carbon *	Calcium*	Arsenic
Fluorine	Chlorine*	Barium
Hydrogen *	Chromium	Cadmium
Lead	Cobalt	Cerium
Magnesium *	Copper	Lanthanum
Nitrogen *	Iodine	Neodymium
Oxygen *	Iron	Niobium
Selenium	Manganese	Rubidium
Silicon	Molybdenum	Strontium
Sodium *	Nickel	Titanium
Sulfur	Phosphorus *	
	Potassium *	
	Tin	
	Vanadium	
	Zinc	

*essential elements designated by Smrčka (2005)

(Compiled using Smrčka, 2005; Oprea et al., 2009)

Due to the high frequency of ionic substitutions, the exact Ca/P ratios within a bone will vary. A large number of species differentiation studies, discussed in Chapter 3, examines the calcium-phosphorous ratios of individual species to detect measurable changes. Through such studies average calcium-phosphorous ratios for human bone and dental materials have been established. Table 2 demonstrates several calculated Ca/P ratios established using atomic percentages, weight percentages, and peak heights.

Table 2: Calcium-phosphorous ratios of human osseous materials organized by calculation method and material type.

Atomic Percent	
Human Bone	
1.46 ± 0.12 (modern)	Ubelaker et al., 2002
1.57 ± 0.02 (archaeological 1)	Ubelaker et al., 2002
1.72 ± 0.16 (archaeological 2)	Ubelaker et al., 2002
Total Body	
1.75*	Smrvka, 2005
Weight Percent	
Human Bone	
1.88 ± 0.15 (modern)	Ubelaker et al., 2002
2.03 ± 0.03 (archaeological 1)	Ubelaker et al., 2002
2.27 ± 0.21 (archaeological 2)	Ubelaker et al., 2002
Peak Height	
Human Bone	
4.92 ± 1.19 (unaltered)	Christiansen et al., 2012
4.57 ± 1.37 (burned)	Christiansen et al., 2012
5.00 ± 1.14 (weathered)	Christiansen et al., 2012
4.58 ± 1.35 (chemically altered)	Christiansen et al., 2012
1.89 ⁺ (calcined)	Ubelaker et al., 2002
1.84 ⁺ (archaeological 1)	Ubelaker et al., 2002
1.87 ⁺ (archaeological 2)	Ubelaker et al., 2002
1.90 ⁺ (burned)	Ubelaker et al., 2002
Human Dental Materials	
4.02± 0.83 (unaltered)	Christiansen et al., 2012
3.67± 0.10 (burned)	Christiansen et al., 2012
1.82 ⁺ (enamel)	Ubelaker et al., 2002
1.72 ⁺ (dentin)	Ubelaker et al., 2002

* Smrčka (2005) provides total body calcium and phosphorus percentages, total body Ca/P ratio derived

+ Standard deviation not provided

The presence and quantities of trace elements in bone can vary significantly between individuals or even bone types (Brätter et al., 1977; Rautray et al., 2007). Variables influencing elemental disbursement include bone location and type, individual age, gender, and health, diet, growth environment, and taphonomic modifications.

Bone type is one of the strongest influential variables related to elemental distribution into the bone matrix. Trace elements are not evenly distributed within the bones of the body nor within each individual bone (Radosevich, 1993; Rautray et al., 2007). Some elements, such as zinc, vanadium, nickel, chromium, lead, manganese, cobalt, and tin, are found in higher concentrations in bone epiphyseal regions whereas elements such as calcium, strontium, sodium, and potassium are found more often in the central portion of the diaphysis (Smrčka, 2005). One of the main differences between epiphyseal bone and diaphyseal bone is their relative densities. Epiphyseal bone displays a higher spongy bone component than diaphyseal bone, resulting in a faster rate of remodeling. This causes epiphyseal bone to be more susceptible to chemical change (Allmäe et al., 2012). Directly associated are the variations in elemental composition due to individual age, gender, and health since these variables also impact bone growth and remodeling rates.

At younger ages bone remodels more rapidly, resulting in more rapid turnover of trace elements incorporated into the calcium phosphate matrix (Bronner, 2008). For example, Smrčka (2005) discusses the higher relative concentrations of zinc, tin, and lead found in individuals between the ages of birth and two years. Allmäe and colleagues (2012) provide slightly contradictory information, stating that zinc levels in bone increase with age in women but decrease with age in men. However, it is important to remember that these changes are also influenced by external variables that may appear when samples were categorically assessed, such as by gender [sex], which may impact daily activities or diet depending on the sample population (Allmäe et al., 2012). This is important for chemical analysis because it demonstrates the range of elemental

concentrations that can be found within human bone. Infant and adult human bone will have the same overall chemical compositions due to the Ca/P matrix but may exhibit significantly divergent trace element contents. Therefore, analysis must identify a range of elements and concentrations indicative of human bone as opposed to a static set of elements with fixed compositions.

Diet and growth environment also have a significant impact on the chemical composition of osseous materials (Radosevich, 1993). Abundant research effort is currently being invested in establishing historic and prehistoric dietary habits by using stable isotope analysis to detect specific trace element ratios. These ratios are then associated with particular dietary patterns. Significant concentrations of vanadium, copper, and zinc are associated with meat consumption, and high concentrations of zinc are associated with marine based meat consumption (Allmäe et al., 2012). In contrast, bone samples from herbivores will exhibit higher relative concentrations of manganese, barium, and strontium (Allmäe et al., 2012). Contaminants from the environment, such as fluorine or lead in the water supply, will also be incorporated into the bone matrix and leave markers.

One final variable influencing the trace element composition of bone is taphonomic modification. The most notable taphonomic modification is diagenesis, or the changes that result from interactions between the deposited materials and the burial environment (Radosevich, 1993; Molleson, 2000). Diagenetic changes within a bone are non-uniform both between and within individual bones and are not directly correlated with length of interment and result in bone loss and gain of biochemical components from the burial environment (Klepinger et al., 1986). There are numerous variables

impacting bone diagenesis, such as soil mineral content, environmental conditions, and peri and postmortem events or exposure.

Diagenetic changes to bone, consistent with bone degradation, are mandated by the decomposition of the organic phase (Nielsen-Marsh et al., 2000). Hydrolysis of the peptide bonds within the collagen results in unraveling of the collagen bundles and weakening of the collagen phase. In this condition, collagen fragments are lost from the bone, resulting in gross degradation and loss of bone mass (Nielsen-Marsh et al., 2000). Moisture level, pH, temperature, and atmospheric conditions all impact the rate of this process. These variables also impact the decomposition rate of the mineral phase of bone. Diagenesis of the mineral fraction is a result of dispersion of the bone apatite. Dissolution of the mineral component of bone can be diagnosed by the presence of increased porosity and crystallinity and the incorporation of exogenous ions (Nielsen-Marsh et al., 2000; King et al., 2011). Research has shown increases in iron, manganese, and copper due to leaching from the burial environment (Carvalho et al., 2004). These incorporations can lead to changes such as color change from contact with burial artifacts which will indicate a change in the chemical composition of the material (Allmäe et al., 2012).

Similar conditions impact alterations to the chemical composition of dental materials, though to a lesser degree. Due to their higher hydroxyapatite concentration dental materials are more homogenous in composition (King et al., 2011) and less susceptible to change overall (Pye, 2004). Changes to dental composition are more likely to be a result of diagenetic events due to the lack of remodeling of dental materials in vivo. As with osseous materials, trace elemental concentration in dental materials is most effected by tooth exposure. Concentrations of heavy metals increase when progressing

from the outer enamel to the inner dentin (Carvalho et al., 2000). Conversely, leached materials decrease in concentration towards the inner portion of the tooth suggesting superficial absorption (Carvalho et al., 2004). Finally, due to their formation pattern and low remodeling rate, teeth in different areas of the dental arc will reflect divergent life periods representing various environments and diets and resulting in varying trace element contents. This is most clearly demonstrated through bioarchaeological studies using trace elements in dental materials to identify migrants based on relative isotopic concentrations (Wright, 2005; Montgomery, 2010; Tung and Knudson, 2011; Wright, 2012; Beaumont et al., 2013; Kendall et al., 2013).

CHAPTER 3: DIFFERENTIATION OF HUMAN OSSEOUS, NON-HUMAN OSSEOUS, AND NON-OSSEOUS MATERIALS

Differentiation of osseous and non-osseous materials, or of human and non-human osseous materials, can be achieved using three methods: biological analysis, histological analysis, or chemical analysis. Biological analysis is the most straightforward, using proteins or DNA to identify biological materials as well as species. Histological analysis focuses on the microstructure of the bone, using organizational patterns to identify osseous materials. Finally, chemical analysis, often reserved for highly fragmented or taphonomically modified materials, discriminates materials based on their chemical compositions. Assessing these approaches illuminates the available methods that can be utilized for differentiation of human osseous, non-human osseous, and non-osseous materials while demonstrating the dire need for further research on the subject.

Biological Analysis Techniques in Forensic Anthropology

Biological analysis as a differentiation method has made significant progress over the past few decades. Early studies focused on species identification by testing for blood proteins, such as Immunoglobulin G and Albumin, using enzyme-linked immunosorbent assay (ELISA) testing (Cattaneo et al., 1992a; Cattaneo et al., 1992b; Cattaneo et al., 1994; Cattaneo et al., 1995). Enzyme-linked immunosorbent assay testing uses antibodies that seek out specific proteins and cause a color change reaction if they are present – this can easily identify species if the correct antibodies are chosen for the analysis. Albumin

has been shown to be a stronger target molecule (Cattaneo et al., 1992a) and has demonstrated utility in discriminating ancient bone (Cattaneo et al., 1992b; Cattaneo et al., 1995) and cremated bone samples (Cattaneo et al., 1994).

Solid-phase radioimmunoassay has also been proposed as a method for identifying species specific proteins (Lowenstein, 1980; Ubelaker et al., 2004). Similar to ELISA, solid-phase radioimmunoassay targets use radioactive antigens to target specific antibodies. If binding occurs then the sample matches the target species. Solid-phase radioimmunoassay has also been shown to be useful for ancient bones (Lowenstein, 1980). However, severe degradation may result in loss of organic components within the bone matrix, rendering ELISA and solid-phase radioimmunoassay testing unusable.

More recently, biological analysis has focused on the use of DNA to identify species. If non-degraded DNA is present, it is possible to discriminate between human and non-human osseous materials and identify known, non-human species using a comparative reference set. Modern DNA extraction and analysis methods for osseous tissues can be used for assessing extremely small fragments (Caputo et al., 2013) as well as ancient and weathered fragments (Benoit et al., 2013). However, DNA analysis, like other biological analysis methods, is only useful if a portion of the organic phase of the bone has maintained integrity and contamination has not occurred.

Histological Analysis Techniques in Forensic Anthropology

Using histological analysis it is easy to discriminate osseous and non-osseous materials based on their microstructural qualities. Additionally, human and non-human

bone can vary significantly on a microstructural level. Histological analysis focuses on patterns of osteon formation and deposition within the bone structure. Assessed features include bone density and osteon banding, density, circularity, and area/diameter. Using these features or combinations of these features it is generally possible to differentiate fragmentary human from non-human bone .

Bone density is perhaps the least investigated area of histological based human and non-human bone differentiation. Aerssens and colleagues (1998) compared bone mass and density between human, dog, pig, cow, and sheep femora and found that there were marked interspecies differences. On average, the mean bone mass and density for human samples was significantly lower than mean values for all other species included in the study; the maximum human bone mass and density values did not fall within the ranges of the non-human species.

Considerably more research has assessed species differentiation using osteon morphology. Qualitatively, osteon differentiation is based upon osteon banding. Osteon banding, also referred to as plexiform bone formation, occurs when primary or secondary osteons form rows within the lamellar structure. Plexiform bone is characteristically found in medium and large animals. Formerly, plexiform was used as an exclusionary variable in the differentiation of human and non-human bone, but recent studies have shown that osteon banding can also be found in humans, particularly in children experiencing rapid growth spurts (Zoetis et al., 2003). However, the placement and organization between species differs. Individual osteons within human osteon bands exhibit a rounder, less plexiform shape and exhibit a higher degree of overlapping (Martiniakova et al., 2006). Additionally, these bands tend to be shorter, averaging 5-6

osteons per band as opposed to 5-20 per band in select non-human species, and are deposited away from the endosteal edge (Mulhern and Ubelaker, 2001).

Quantitative histological differentiation between human and non-human bone focuses on the density, circularity, and area/diameter of osteons within the Haversian system. Osteon density has been assessed as the least specific method for differentiating human and non-human bone. Hillier and Bell (2006) defined upper and lower limits for osteon density in human lamellar bone. Densities falling outside of the given range can be identified as non-human, but numerous species also fall within the range, providing overall poor discrimination (Hillier and Bell, 2006).

Osteon circularity and area/diameter have been considered for species differentiation, though most studies have shown that discrimination is not reliable. Cattaneo and colleagues (2009) demonstrated low multivariate discrimination, reporting approximately 70% classification when using a formula involving Haversian canal area, maximum diameter, and minimum diameter. Using analysis of variance (ANOVA) analysis of variance, Crescimanno and Stout (2012) determined that human osteon circularity was consistently lower than in non-human bones and that when using a predictive model 76.5% classification could be achieved. Dominguez and Crowder (2012) demonstrated a lower classification rate for osteon circularity at 66.1%. However, they determined a higher classification rate for osteon area, 93.5%. Furthermore, when these osteon circularity and osteon area were combined for multivariate analysis 98.4% discrimination was achieved (Dominguez and Crowder, 2012).

Finally, research has been conducted to assess the discriminate abilities of the corticomedullary index (CMI), or the medullary cavity diameter to the diameter of the

bone. Rérolle and colleagues (2013) determined that the CMI is highly variable within species. Additionally, human bones frequently classified as being of non-human origin within their sample set, making this method highly undesirable for forensic purposes.

Though discrimination between human and non-human bone is possible using histological analysis, some methods yield classification rates only slightly higher than random probability. Influential variables for interspecies variation of osteon formation are not well enough understood to establish clear discriminatory methods (Mulhern and Ubelaker, 2012). Furthermore, taphonomic modifications can compromise lamellar structure (Hanson and Cain, 2007). Though additional studies with increased sample variation need to be conducted, existing data indicates a strong overlap between human and non-human bone microstructure and necessitates alternatives such as the exploration of chemical methods for differentiation.

Analytical Chemistry Techniques in Forensic Anthropology

Numerous modern forensic analyses utilize analytical chemistry techniques as they are relatively non-destructive and highly specific. A review of modern analytical chemistry techniques utilized in forensic investigations, including x-ray diffraction, proton induced x-ray emission, laser induced breakdown spectroscopy, Raman spectroscopy, inductively coupled plasma – mass spectroscopy, x-ray fluorescence, and scanning electron microscopy – energy dispersive x-ray spectrometry, demonstrates the methods available as well as merits and drawbacks of each. Additionally, a summary of the research investigating discrimination of human osseous, non-human osseous, and

non-osseous materials illuminates the dire need for further research and improvement of analytical chemistry techniques in forensic anthropology.

X-ray Diffraction

X-ray diffraction (XRD), also referred to as x-ray crystallography, utilizes x-ray diffraction patterns to discern the three-dimensional (3D) structures of crystalline solids. X-rays are directed towards the sample resulting in collision and refraction of the x-rays. Most of the returning x-rays have the same wavelength of the incident beam, but several are diffracted due to interference with the electrons of the crystalline solid. By measuring differences in the angles and intensities between the incident and diffracted beams, a crystallographer is able to create a three dimensional reproduction of the electron density concentration within the crystal. From this the structure of the solid, including atom position, chemical bonds, and disorder, can be determined (Waseda et al., 2011).

X-ray diffraction has been proposed for multiple applications within forensic anthropology. This method is non-destructive, requires small sample sizes, is not inhibited by elemental variations within bioapatite, and can be used to semi-quantify components of a mixed sample. The utility of XRD for identification of contaminated cremains has been demonstrated (Bergslein et al., 2008). Additionally, XRD has been suggested as a means for differentiating osseous materials on the species level based on structural differences of bone mineral upon heating (Beckett et al., 2011). However, this method does not provide information on trace element composition. There is a large amount of ionic substitution at the molecular level that XRD is unable to distinguish, resulting in the potential to misclassify structurally congruent materials with different

compositions. A more specific method of analysis is necessary to provide clear definition between species.

Proton Induced X-ray Emission

Proton induced x-ray emission (PIXE) testing is a non-destructive chemometric analysis technique. PIXE produces an x-ray spectrum of elements by directing a beam of protons at the sample and measuring the resultantly emitted ions. PIXE requires small sample amounts and is able to provide elemental concentrations within the samples (Warren et al., 2002). PIXE has been used in forensic anthropology to analyze potentially contaminated cremated remains (Fischenbeck et al., 1986; Kravchenko et al., 2001; Warren et al., 2002) as well as for the detection of gunshot residues on bone (Warren et al., 2002). More recently, PIXE has been applied as a method for discriminating ivory species (Müller and Reiche, 2011). Ivory was identifiable by its high magnesium-calcium ratio, which was on average four times higher than in bone materials (Müller and Reiche, 2011). However, though discrimination values were not provided, the authors discussed overlap of sperm whale ivory with bone samples and similarities between the magnesium-calcium ratios of multiple ivories analyzed. Additionally, difficulties were observed in identification of taphonomically modified materials resulting in the authors suggesting PIXE testing not be used for materials that have undergone diagenesis (Müller and Reiche, 2011).

Laser Induced Breakdown Spectroscopy

Laser induced breakdown spectroscopy (LIBS) is another laser excitation – emission monitoring analytical method. LIBS directs short laser pulses at the sample material to induce radiation excitation. When the laser contacts the sample surface it momentarily converts the sample material to a plasma state, which will emit radiation as the particles return to the ground state. Detectors monitor the wavelengths of the emitted radiation to determine the elemental composition of the material in question (Singh and Rai, 2011). LIBS is currently utilized in multiple forensic contexts, such as glass and paint analysis (Bridge et al., 2007; Sigman, 2010). LIBS is also employed in archaeological and forensic studies such as analysis of trace elements in calcified tissues resultant of environmental exposure (Samek et al., 2001), cremains analysis (Martin et al., 2007), composition and preservation of archaeological materials (Giakoumaki et al., 2007; Kasem et al., 2011; Rusak et al., 2011), and trace element analysis of human dental materials (Alvira et al., 2010). Additionally, LIBS has been proposed for use in differentiating human and non-human osseous materials (Vass et al., 2005). LIBS is widely utilized due to the lack of sample preparation, versatility of sample type, low destructivity, and rapid data collection (Singh and Rai, 2011). However, sample detection limit has been demonstrated as low, reducing its desirability for trace element focused discriminatory applications (Singh and Rai, 2011).

Raman Spectroscopy

Like LIBS, Raman spectroscopy uses laser excitation to determine chemical composition. A laser beam with a wavelength in the ultraviolet, visible, or near infrared region is directed at the sample. Excitation occurs and photons are expelled from the sample. The energy of these photons, which will be higher or lower than the incident photons due to vibrational coupling, is measured to produce quantifiable information on the molecular structure and composition of the sample (Larkin, 2011). Raman spectroscopy can detect wavelengths from the full vibrational spectrum, allowing for a high range of molecular identification. Additionally, Raman spectroscopy is non-destructive and Raman spectrometers are available in many forensic laboratories (Edwards, 2004). Forensically, Raman spectroscopy is utilized for analysis and identification of biomaterials such as soft tissues and bodily fluids (Edwards, 2004; Virkler and Lednev, 2009a; Virkler and Lednev, 2009b).

Raman spectroscopy has also been applied to species differentiation. Brody and colleagues (2001) demonstrated the success of Fourier Transform Raman spectroscopy, a specific type of Raman Spectroscopy, for differentiation of dentin from six mammalian ivories (African elephant, Asian elephant, hippopotamus, mammoth, sperm whale, and walrus) and three bone samples. Overlap between groups was discovered, but jack-knife classification provided 84.5-90.4% classification of samples depending on grouping classifications. Misclassification occurred most commonly between African elephant, Asian elephant, and mammoth or between hippopotamus, walrus, and sperm whale (Brody et al., 2001). These misclassifications follow both geographic and dietary divides.

Shimoyama and colleagues (2003) performed a similar analysis, attempting discrimination between mammoth, hippopotamus, sperm whale, and two types of African elephant ivories. While they do not provide specific classification data, they do assert that the five species were differentiable using three principal components. They repeated this analysis in 2004 using visible and short-wave near infrared spectroscopy (Shimoyama et al., 2004). Though classification rates were not provided, the correlation coefficient for discrimination based on specific gravity was calculated to be 0.960.

Edwards and colleagues (2006) repeated this analysis with a sample set nearly identical to Brody and colleagues (2001) using African elephant, Asian elephant, hippopotamus, walrus, sperm whale, and mammoth ivory. Overall, general mammalian species differentiation was possible and division of African and Asian elephant ivories was above 97% (Edwards et al., 2006).

McLaughlin and Lednev (2012) analyzed bone samples using Raman spectroscopy and assessed them using principal component analysis. Plotting the first two principal component scores, the authors determined that chicken, turkey, cow, and pig bone samples were completely separated with little to no overlap between 95% confidence ellipses. However, specific discrimination percentages were not provided.

Though non-destructive and readily available, the high overlap and relative inability of Raman spectroscopy to discriminate ivory demonstrates that further research, including human samples, is necessary to test the validity of this approach.

Inductively Coupled Plasma – Mass Spectroscopy

Inductively coupled plasma – mass spectroscopy (ICP-MS) also utilizes a plasma ablative source to create x-ray fluorescence. Liquid samples are loaded into the sample chambers and converted into an aerosol. The aerosol is introduced to the plasma where it undergoes decomposition into constituent atoms followed by atom ionization. These are then processed in the mass spectrometer where atoms are separated by their mass to charge ratios and identified. Isotopic ratio information is provided which can then be processed using multiple approaches (Thomas, 2013).

Inductively coupled plasma – mass spectroscopy currently has multiple anthropological and archaeological applications identifying trace elements. Preliminary studies have demonstrated that the reliability of using ICP-MS for identifying contaminated human cremains is contingent upon the ratio of cremains to contaminants (Bodkin et al., 2005; Brooks et al., 2006). Stable isotope analysis using ICP-MS has generated paleodietary and paleoenvironmental data using both bone and tooth samples (Fuller et al., 2003; Dijngova et al., 2004; Webb et al., 2005; Reynard and Hedges, 2008), as well as modern geographic origin data for unidentified skeletal remains (Rauch et al. 2007). ICP-MS also demonstrates forensic applications for mass disaster victim discrimination and species differentiation.

Castro and colleagues (2010) assess the ability of ICP-MS to group femur and humerus samples taken from 12 individuals. When analyzed together correct classification was only 42.7%; however, when the femur and humerus samples were analyzed individually classification was 75.2 and 63.1% respectively (Castro et al., 2010). Dental materials were also included in their study but classification rates were not

provided. The authors express that the low classification rates could be the result of elemental concentration variation due to bone remodeling patterns (Castro et al., 2010).

Dillane and colleagues (2011) explored species differentiation based on feeder type and domestic status. Through analysis of selective elements they were able to determine that carnivores exhibited higher concentrations of aluminum, iron, potassium, magnesium, and sodium than herbivores and omnivores. Additionally, domestic species exhibited higher concentrations of aluminum, potassium, magnesium, and sodium than wild species (Dillane et al., 2011). Classification, however, was difficult as there was a large amount of overlap between species both within and between dietary and domestication groupings. Approximately 92% of carnivores could be identified correctly, as well as 94% of wild species and 40% of domestic species (Dillane et al., 2011). Dietary or domestic status groups may be useful in differentiating human and non-human osseous materials, but this was not investigated in this study.

Though ICP-MS is highly specific and available in many crime laboratories, its ability to differentiate human from non-human osseous materials has not been demonstrated. Additionally, this method is highly destructive and therefore not as desirable for forensic applications.

X-ray Fluorescence

X-ray fluorescence (XRF) is a widely used chemical analysis method that measures x-ray fluorescence signals produced by electron excitation. Sample materials are bombarded with x-ray waves, resulting in excitation of the electrons within the sample. Ionization occurs, resulting in displacing of inner electrons. As outer electrons

fall to the inner shells to counteract the energy deficiency photons are expelled. The energy of the expelled photons is equal to the difference in energy levels between the inner and outer electron positions. This energy is quantified and compared to known excitation energies to determine the elements present and their respective concentrations (Arai, 2006; Shackley, 2011). XRF is minimally destructive, requires minimal sample preparation, and is capable of rapid detection of elements. Portable, field operational XRF devices are also available, allowing immediate chemical analysis and sample identification based on known standards (Shackley, 2011).

X-ray fluorescence has multiple applications within anthropology, including trace element distribution in modern and archaeological bone (Carvalho et al., 2004; Fleming et al., 2011; Nie et al., 2011; Swanston et al., 2012), identification of post cremation restorative dental resins (Bush et al., 2007; Bush et al., 2008), and detection of metallic transfer to bone (Williams, 2012). Additionally, the ability to differentiate osseous materials has been demonstrated (Christensen et al., 2012, Zimmerman, 2013).

Christiansen and colleagues (2012) validated the use of XRF for identification of osseous and dental materials of unknown origin. Their study expanded on the sample set used by Ubelaker and colleagues (2002) (discussed following) and included human and non-human osseous and dental materials, other biological materials such as shell or coral, and non-biological materials such as wood or stone. Materials were analyzed in both unaltered and taphonomically altered states. Samples were discriminated based on their respective Ca/P ratios calculated using peak volume. Samples lacking calcium or phosphorus were easily identifiable as non-osseous in origin. Initially, marked differences were noticed between altered and unaltered samples. However, once the top layer on

altered samples was removed to expose the subsurface for analysis Ca/P ratios consistent with unaltered materials were demonstrated. Though advanced statistics were not performed and classification rates were not provided, Christiansen and colleagues (2012) assert reliable identification of osseous materials with the exception of mineral apatite, octocoral, and brachiopod shells. The Ca/P ratios of these materials is indistinguishable from osseous ratios, but the authors state that these materials are unlikely to be confused with osseous materials due to their macroscopic and microscopic appearances.

Expanding upon Christensen and colleagues (2012), Zimmerman (2013) assessed the capabilities of HHXRF to discriminate osseous and non-osseous materials. However, rather than using Ca/P ratios, Zimmerman (2013) used trace element compositions. Discriminant analysis demonstrated an overall discrimination of 94%, with 4% of bone misclassifying as non-bone and 8% of non-bone misclassifying as bone. Misclassifying non-bone spectra included synthetic hydroxyapatite and rock apatite (Zimmerman, 2013). Additionally, it was determined that multiple taphonomic modifications did not influence proper discrimination.

Finally, XRF has also been proposed for individuation. Using XRF and principal component analysis (PCA) of elemental ratios Gonzalez-Rodriguez and Fowler (2013) were able to differentiate samples from five mediaeval skeletons (12th – 16th centuries). Such classification would be useful in response to mass burials or mass disasters. Beginning with discrimination of two individuals, classification is 100%. As more individuals were added to the sample set, the authors presented reductions in classification percentages. Classification for the full sample set of five individuals is

described as having a high percentage of accuracy, but actual percentages for discrimination were not provided.

Due to its minimally destructive nature, straightforward sample preparation, high specificity, and wide availability XRF demonstrates high potential for future applications differentiating human osseous, non-human osseous, and non-osseous materials. The low number of studies investigating the classification potentials of XRF limit implementation of the method. Supplementary research is necessary to determine discrimination rates and the applicability of XRF to forensic anthropological analyses.

Scanning Electron Microscopy – Energy Dispersive X-ray Spectrometry

Scanning electron microscopy – energy dispersive x-ray spectrometry (SEM/EDX) analysis operates by directing an electron beam at the sample. Interaction between the incident electrons and the sample result in expulsion of secondary electrons. The expelled electrons are detected by the scanning electron microscope (SEM) and an image of the sample is generated based on the energy differences between the expelled and incident electrons. X-ray photons are also generated when electron beam interacts with the substrate. As with XRF, atoms within the sample are excited resulting in expulsion of inner electrons. Photon radiation is emitted as outer electrons fall to fill the lower energy levels and this radiation is detected and quantified by the energy dispersive x-ray spectrometer (EDS). Energy differences are identified and elements are distinguished based on known excitation energies (Goldstein et al., 2003). Scanning electron microscopy – energy dispersive x-ray spectrometry is a non-destructive analysis method available in most established forensic laboratories. Sample preparation is easy

with low sample requirements and results are highly specific, making this method ideal for trace element analysis (Goldstein et al., 2003).

The primary forensic anthropological use of SEM/EDX is the identification of trace metal residues on bone as a result of gunshot, sharp force, or blunt force traumas (Berryman et al., 2010; Amadasi et al., 2012; Gibelli et al., 2012; Pechníková et al., 2012; Taborelli et al., 2012; Vermeij et al., 2012). Additional research has been conducted to assess the employment of SEM/EDX to differentiation of osseous and non-osseous materials.

Ubelaker and colleagues (2002) investigated the use of SEM/EDX to distinguish osseous tissues from non-osseous materials. Their study included human and non-human dental and bone tissues, as well as synthetic hydroxyapatite, natural hydroxyapatite (bone), octocoral, seahorse, ivory, coral, and Colgate toothpaste (Ubelaker et al., 2002). Taphonomically modified samples as well as samples from different geographic origins were assessed to determine the impact of external variables on chemical composition. Calcium-phosphorus ratios were calculated for each sample then processed using the spectrum library identification and classification explorer (SLICE) database (Ubelaker et al., 2002). The SLICE database was created by the FBI as a means of identifying unknown materials based on their chemical compositions (Ward and Colby, 2008). Results may classify the unknown to a category or to an individual sample type depending on the extent of the reference set (Ward and Colby, 2008). As with similar studies, advanced statistics were not performed and classification rates were not provided but classification patterns were identified. SEM/EDX in combination with processing through the SLICE database differentiated osseous materials from all synthetic materials

with the exception of synthetic hydroxyapatite. Non-osseous biological materials that misclassified as bone included ivory, mineral apatite, and octocoral. Finally, classification was unable to separate bone and dental tissues and species differentiation was not achieved (Ubelaker et al., 2002).

Scanning electron microscopy – energy dispersive x-ray spectrometry is well suited for osseous and non-osseous discrimination and more specific classification may be achieved using trace element analysis rather than calcium-phosphate ratio comparisons. Additional studies need to be conducted utilizing larger sample sets and alternative data processing methods.

A Summary of Analytical Chemistry in Forensic Anthropology

The incorporation of chemical analysis when examining fragmentary and taphonomically modified materials as a preemptive step in a forensic investigation would reduce the time and cost invested in forensically insignificant materials. By introducing preemptory testing, non-osseous and non-human osseous materials could be identified and removed from further analyses. This would reduce the amount of materials sent for DNA testing, effectively reducing laboratory operation costs as well as reducing processing time and removing waiting periods for negative results. Multiple methods are available to determine the chemical compositions of unknown materials, but a high degree of specificity for quantitation of trace elements is necessary for discrimination of osseous and non-osseous and of human and non-human osseous materials.

As is evident through analysis of the current literature, there exists a large gap in regards to the application of analytical chemistry for differentiation of human osseous, non-human osseous, and non-osseous materials. The majority of research has focused on Ca/P ratios or spectral analysis, though several studies have demonstrated the potential for discrimination based on trace element concentrations. Table 3 summarizes chemical analysis studies addressing material differentiation and species discrimination, methodological advantages and limitations as well as specific instrument applications and study results. Further research is necessary to determine the feasibility of incorporating a trace element based differentiation method into routine forensic investigations. Research needs to focus on expanding sample sets and establishing databases to allow for extensive comparison of unknowns. Additionally, analysis methodologies need to be expanded to include trace elements as well as Ca/P content as trace elements vary between species exhibiting similar Ca/P ratios. Future studies need to be conducted to assess the viability of each method and the advantages and disadvantages associated with each and advanced statistics need to be performed to assess the true classification potentials of the proposed methods. Finally, identification within a closed sample has been demonstrated but no blind studies have been performed to replicate real world application of these methods. Identification of an unknown through categorization, as opposed to selection from a predetermined set of known materials, may alter classification rates. It is crucial to determine whether classification rates will remain conclusive in blind tests. Each of these issues needs to be addressed with in order to evaluate the overall value of chemical differentiation in forensic anthropology and to outline implementation protocols or alternative approaches.

Table 3: Summary table of analytical chemistry methods currently utilized or previously assessed for differentiation of human osseous, non-human osseous, and non-osseous materials.

Method	Methodological Advantages	Methodological Limitations	Application	Results	References
X-Ray Diffraction	Non-destructive, small sample size	No information on elemental composition	Species differentiation (including human) of bone mineral upon heating	No classification rates provided, suggests that development of a method for distinguishing human from non-human bone is promising	Beckett et al., 2011
X-Ray Diffraction	Non-destructive, small sample size	No information on elemental composition	Identification of contaminated human cremains	Bioapatite is distinguishable from filler materials. Differentiation from geological apatite is more difficult but still possible	Bergslein et al., 2008
Proton Induced X-ray Emission Testing	Non-destructive, small sample size, high specificity	Vacuum needed for low atomic number elements	Species differentiation for ivory and bone materials based on Mg/Ca ratios	Clear identification of elephant, hippopotamus, narwhale, and walrus ivories. Sperm whale classifies as marine bone. Digenetic changes were noted but not explored	Müller and Reiche, 2011
Laser Induced Breakdown Spectroscopy	No sample preparation, versatile sample type, low destructivity, rapid data collection	Accuracy and detection limit are variable depending on the sample	Differentiation of human and non-human bone based on trace elemental analysis	No classification rates provided, state that preliminary comparisons show significant differences between human and non-human samples. Also propose identification of gender, sex, and race for human bones	Vass et al., 2005
Fourier-transform Raman Spectroscopy	Non-destructive, simple sample preparation, available in most forensic laboratories	Advanced statistical analysis may require training	Differentiation of ivory species using statistical analysis of spectral data	84.5-90.4% classification. Overlap between African and Asian elephant, mammoth and hippopotamus, and walrus and sperm whale.	Brody et al., 2001

Method	Methodological Advantages	Methodological Limitations	Application	Results	References
Fourier-transform Raman Spectroscopy	Non-destructive, simple sample preparation, available in most forensic laboratories	Advanced statistical analysis may require training	Differentiation of ivory species using statistical analysis of spectral data	Generic mammalian differentiation possible. 97% classification between African and Asian elephant samples	Edwards et al., 2006
Fourier-transform Raman Spectroscopy	Non-destructive, simple sample preparation, available in most forensic laboratories	Advanced statistical analysis may require training	Discrimination of non-human bone samples using statistical analysis of spectral data	Separation of chicken, turkey, cow, and pig samples with little to no overlap between 95% confidence ellipses	McLaughlin and Lednev, 2012
Fourier-transform Raman Spectroscopy	Non-destructive, simple sample preparation, available in most forensic laboratories	Advanced statistical analysis may require training	Differentiation of ivory species using statistical analysis of spectral data	No classification rates provided, five species differentiable using three principal components	Shimoyama et al., 2003
Visible and Short-wave Near Infrared Spectroscopy	Non-destructive, deep sample penetration	Broad spectral bands, not highly sensitive	Differentiation of ivory species using statistical analysis of spectral data	0.960 correlation coefficient	Shimoyama et al., 2004
Inductively Coupled Plasma – Optical Emission Spectroscopy	Highly specific, available in most forensic laboratories	Highly destructive	Identification of contaminated human remains using elemental profiles	No classification rates provided, human samples consistent. Expected concentration ranges for identifying human remains can be calculated.	Bodkin et al., 2005
Inductively Coupled Plasma – Optical Emission Spectroscopy	Highly specific, available in most forensic laboratories	Highly destructive	Identification of contaminated human remains using statistical analysis of elemental compositions	Classification was dependent on concentration. Samples consisting of 60-75% human remains had a classification probability of 0.14-0.51 and samples containing 90% remains consistently classified as remains	Brooks et al., 2006

Method	Methodological Advantages	Methodological Limitations	Application	Results	References
Inductively Coupled Plasma – Atomic Emission Spectroscopy	Highly Specific	Highly destructive, moderate emission interference	Feeder type based species differentiation using elemental analysis	92% classification of carnivores, 94% classification of wild species, and 40% classification of domestic species at a 95% confidence level	Dillane et al., 2011
X-ray Fluorescence	Minimally destructive, minimal sample preparation, rapid detection	Variable sensitivity to light elements or elements of low concentrations	Identification of osseous and dental materials of unknown origin based on Ca/P ratios	No classification rates provided, reliable separation of osseous materials with the exception of mineral apatite, octocoral, and brachiopod shells (all classified as osseous)	Christensen et al., 2012
Hand Held X-ray Fluorescence	Minimally destructive, minimal sample preparation, rapid detection, portable	Variable sensitivity to light elements or elements of low concentrations	Differentiation of osseous and dental tissues from non-osseous materials using statistical analysis of trace elements	94% average discrimination between bone and non-bone samples (4% of bone classified as non-bone and 8% of non-bone classified as bone).	Zimmerman, 2013
Scanning Electron Microscopy – Energy Dispersive Spectrometry	Non-destructive, minimal sample preparation, rapid detection, high specificity, low sample requirements	Select preparation methods may alter samples, time consuming	Separation of osseous and non-osseous materials based on Ca/P ratios	No classification rates provided, reliable discrimination of osseous materials with the exception of synthetic hydroxyapatite, ivory, mineral apatite, and coral (all classified as osseous). Bone and dental tissues not separated, species differentiation not achieved	Ubelaker et al., 2002

CHAPTER 4: MATERIALS AND METHODS

Samples for this study were chosen based on their physical and chemical similarity to osseous materials as well as the probability of encountering them in forensic investigations. A total of 60 samples were analyzed: 20 human osseous samples, 27 non-human osseous samples, and 13 non-osseous samples. Samples were prepared by extracting a small portion from each location using a handheld rotary tool and an engraving bit. The resulting powders were processed using SEM/EDX. After manual verification of identified elements, weight percent data provided by the EDS were processed using PCA and Fisher LDA to assess the level of discrimination at each tier. Subsequently, a blind study was conducted to assess the application of the method for identification of unknown materials as osseous or non-osseous.

Sample Selection and Preparation

The samples chosen for this project represent a mixture of osseous and non-osseous materials of similar chemical composition and appearance. Pertinent sample information is provided throughout Tables 4-6 and includes sample type, species name for non-human osseous materials, whether the sample type was included in previous material differentiation studies, the origin of the materials, and taphonomic modifications were listed if present. Samples were chosen based on the probability of encountering the material as an unknown in a forensic investigation as well as the difficulty of

differentiating fragments or taphonomically modified samples using non-chemical methods.

Samples were divided into three main categories; human osseous, non-human osseous, and non-osseous. Further categorization occurred within these groups, including the division of osseous groups into bone, dental, taphonomically modified bone, and taphonomically modified dental materials, and the division of non-osseous samples into marine, plant, stone, synthetic, and taphonomically modified materials. It is important to note that unlike previous studies, ivory and synthetic hydroxyapatite were designated as non-human osseous, as opposed to non-osseous. As ivory is composed primarily of dentin it is dental material and is osseous in origin. Synthetic hydroxyapatite, though not truly osseous in origin, is chemically identical to naturally occurring hydroxyapatite and thus not chemically distinctive. Additionally, synthetic hydroxyapatite can be used in medical procedures as medical devices (Jordan et al., 1998) or for bone grafts or adhering medical implantation devices (Cook et al., 1988). As a result, the presence of synthetic hydroxyapatite may be indicative of human remains.

The majority of samples were chosen to replicate previous chemical differentiation studies, including Ubelaker et al., (2002), Christensen et al., (2012), and Zimmerman, (2013). Samples chosen to replicate Zimmerman (2013) used the same samples and testing locations. Newly introduced samples include non-human teeth and additional taphonomically and chemically altered osseous materials.

Numerous variables were considered when choosing each sample set. When selecting human osseous samples (Table 4, Figure 3) bone type and taphonomic

modifications were taken into consideration. As discussed, bone type is shown to be one of the strongest influences on which elements are incorporated into the bone matrix. For this reason multiple bone types were chosen, as well as multiple sample location sites per bone. A similar approach was taken with dental materials, choosing teeth that develop at different ages and choosing multiple sample locations on the enamel and dentin of each tooth.

Another highly influential variable related to the chemical composition of bone is postmortem taphonomic modifications. Though there are a plethora of postmortem modifications, several commonly encountered in forensic settings were chosen. These included weathered, burnt, and archaeological bones. Weathered bone is exposed to environmental conditions and often results in staining or bleaching, depending on the specific conditions. This sample can help determine if sun exposure or organic staining impacts elemental composition, or detection of elemental composition. Burnt bone was selected because it has been shown that exposure to extreme heat or flame will alter the chemical composition of bone. Upon heating, depending on temperature, bones will lose 30-55% of their weight, presumably from water and lipid loss (Grupe and Hummel, 1991). Between 600 and 700°C carbon from the remaining organic compounds turns to CO₂. This leaves only the crystalline mineral phase of the initial bone. Above 800°C the hydroxyapatite will begin changing to β-tricalciumphosphate (Grupe and Hummel, 1991). Burnt dental materials behave similarly, though at higher temperatures, and were included in the study. Finally, though archaeological bones are not frequently mistaken as forensically significant, this can provide information useful to archaeological study.

Additional variables, such as individual biological profile, burial environment, geographic origin, or dietary practices, were not incorporated into sample selection due to difficulties associated with procurement of such samples and the reduced influence of such variables on the chemical composition of osseous materials. Knowing the burial environment, a postmortem taphonomic modification, of an osseous sample would allow better understanding of the impact of staining and elemental leaching from the soil, but it was not feasible within the parameters of this study. Finally, knowing the dietary habits or geographic location of the individuals would provide knowledge regarding individual variation and the degree of influence consumption and environment has on elemental composition. This could provide information on whether dietary components alter elemental composition or only influence concentration, providing clarity on differentiation. However, this information as well as biological profiles were unknown for the included samples and determining statistically significant results would require a considerably larger sample size.

Table 4: Human osseous materials analyzed.

Human Bone	Sample Origin or Modification
Fibula ^{1,2}	Cleaned medical specimen
Humerus ^{1,2}	Cleaned medical specimen
Metacarpal ¹	Cleaned medical specimen
Parietal ^{1,2,3}	Cleaned medical specimen
Pedal Phalanx ^{1,2}	Cleaned medical specimen
Rib ^{1,2}	Cleaned medical specimen
Zygomatic ^{1,2,3}	Cleaned medical specimen
Human Tooth	
Canine Dentin ^{1,2}	Cleaned, same as canine enamel
Canine Enamel ^{1,2}	Cleaned, same as canine dentin
Premolar Dentin ^{1,2}	Cleaned, same as premolar enamel
Premolar Enamel ^{1,2}	Cleaned, same as premolar dentin
Molar Dentin ^{1,2}	Cleaned, same as molar enamel
Molar Enamel ^{1,2}	Cleaned, same as molar dentin
Taphonomically Modified Human Bone	
Fetal Femur ¹	Archaeological
Fibula ¹	Burned (calcined)
Metacarpal ¹	Weathered
Taphonomically Modified Human Tooth	
Molar 1 Dentin ¹	Burned (charred)
Molar 1 Enamel ¹	Burned (charred)
Molar 2 Dentin ¹	Burned (charred)
Molar 2 Enamel ¹	Burned (charred)

(¹Samples used by Zimmerman, 2013; ²Analyzed by Christensen et al., 2012;

³Analyzed by Ubelaker et al., 2002)



Figure 3: Human bone and tooth samples: (a) fibula, (b) humerus, (c) rib, (d) metacarpal, (e) pedal phalanx, (f) zygomatic, (g) parietal, (h) canine, (i) premolar, (j) molar, (k) taphonomically modified molar 1, (l) taphonomically modified molar 2, (m) taphonomically modified fibular, (n) taphonomically modified fetal femur, (o) taphonomically modified metacarpal. Labels designate data collection points.

A similar approach was used when choosing non-human osseous samples (Table 5, Figure 4). Bone and dental materials were chosen from multiple species originating in various environments. Bird, reptile, and mammal samples were included to provide a broad spectrum of results. Also, specific species were chosen to reflect fauna commonly encountered in Central Florida. Dental materials were also chosen from various species and samples were collected from both the enamel and the dentin portions.

Postmortem taphonomic modifications were more extensive on non-human osseous materials due to a higher availability of non-human bones for modification. Weathered, fossilized, and boiled/chemically altered samples were included. A fossilized

sample was included due to the visual similarity between fossilized turtle shell and flat bones of the skull as well as to determine if the large number of chemical changes that occur to fossilized bones (Molleson, 2000) impact discrimination of fossilized samples. A boiled and chemically cleaned sample was included to assess the impact of postmortem soft tissue maceration.

Table 5: Non-human osseous materials analyzed.

Non-Human Bone	Sample Species or Modification
Alligator Femur ¹	<i>Alligator mississippiensis</i>
Armadillo Femur ¹	<i>Dasyus novemcinctus</i>
Bird Femur ¹	<i>Aves</i>
Deer Antler ¹	<i>Odocoileus virginianus</i>
Deer Femur ¹	<i>Odocoileus virginianus</i>
Dog Femur ^{1,2,3}	<i>Canis lupus familiaris</i>
Fish Vertebral Spine	<i>Species unknown</i>
Pig Femur ^{1,2}	<i>Sus scrofa</i>
Raccoon Femur ¹	<i>Procyon lotor</i>
Turkey Tarsometatarsus ¹	<i>Meleagris gallopavo osceola</i>
Turtle Femur ^{1,2}	<i>Testudines</i>
Turtle Shell ^{1,2}	<i>Testudines</i>
Synthetic Hydroxyapatite ^{1,3}	Ca ₅ (PO ₄) ₃ (OH)
Non-Human Tooth	
Ivory Flat ^{1,3}	<i>Species unknown</i>
Ivory Round ^{1,3}	<i>Species unknown</i>
Cow Dentin ²	<i>Bos primigenius</i>
Cow Enamel ²	<i>Bos primigenius</i>
Deer Dentin ²	<i>Odocoileus virginianus</i>
Deer Enamel ²	<i>Odocoileus virginianus</i>
Pig Dentin ²	<i>Sus scrofa</i>
Pig Enamel ²	<i>Sus scrofa</i>
Raccoon Dentin ²	<i>Procyon lotor</i>
Raccoon Enamel ²	<i>Procyon lotor</i>
Taphonomically Modified Non-Human Bone	
Chicken	Boiled/Chemically Cleaned
Raccoon	Weathered
Turtle Shell	Fossilized
Whale Rib	Weathered

(¹Samples used by Zimmerman, 2013; ²Analyzed by Christensen et al., 2012;

³Analyzed by Ubelaker et al., 2002)



Figure 4: Non-human bone and tooth samples: (a) deer femur, (b) pig femur, (c) alligator femur, (d) dog femur, (e) turkey tarsometatarsis, (f) raccoon femur, (g) bird femur, (h) armadillo femur, (i) turtle femur, (j) fish vertebral spine, (k) turtle shell, (l) synthetic hydroxyapatite, (m) taphonomically modified turtle shell, (n) taphonomically modified raccoon, (o) taphonomically modified whale, (p) taphonomically modified chicken, (q) pig tooth, (r) deer tooth, (s) cow tooth, (t) raccoon tooth, (u) ivory flat, (v) ivory round, (w) deer antler. Labels designate data collection points.

Non-osseous samples were chosen to reflect materials similar in chemical composition and appearance to osseous materials (Table 6, Figure 5). Multiple marine samples, including several species of shell and coral, were chosen due to their chemical and physical similarities to bone. Rock apatite and limestone were included due to their chemical similarity to bone. The remainder of the samples, including plant material, glass, and plastic, were chosen due to their physical resemblance with fragmentary osseous materials. Finally, several of these materials were exposed to burning to act as a control for other taphonomically modified samples.

Table 6: Non-osseous materials analyzed.

Non-Osseous, Marine	Sample Species or Modification
Atlantic Bay Scallop Shell ^{1,2}	<i>Argopecten irradians</i>
Clam Shell ¹	<i>Macrocallista nimbosa</i>
Octocoral 1 ¹	<i>Octocoralia ricordea</i>
Octocoral 2	<i>Octocoralia leptogorgia virgulata</i>
Oyster Shell ¹	<i>Ostreoidea</i>
Sand Dollar ^{1,2}	<i>Echinarchnius parma</i>
Starfish ¹	<i>Asteroidea</i>
Non-Osseous, Plant	
Twig ^{1,2}	
Non-Osseous, Stone	
Limestone ^{1,2}	
Rock Apatite ^{1,2,3}	
Non-Osseous, Synthetic	
Float Glass ^{1,2}	
Taphonomically Modified, Non-Osseous	
Plastic ¹	Burned
Wood ¹	Burned

(¹Samples used by Zimmerman, 2013; ²Analyzed by Christensen et al., 2012;

³Analyzed by Ubelaker et al., 2002)

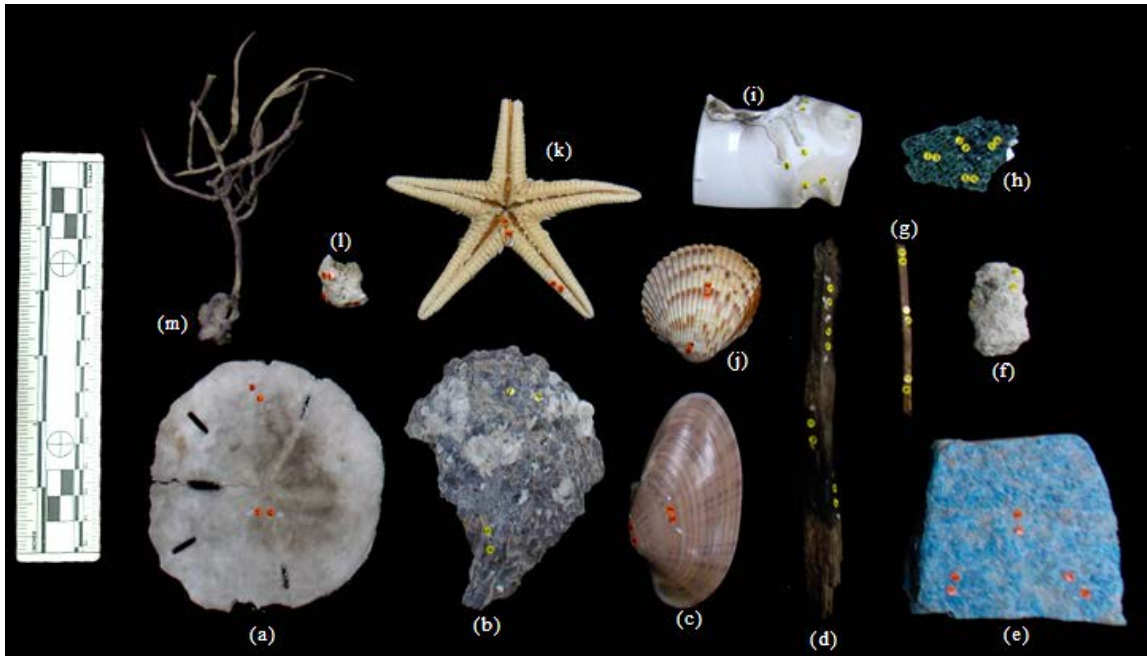


Figure 5: Non-osseous samples: (a) sand dollar, (b) oyster shell, (c) sunray clam shell, (d) taphonomically modified wood, (e) rock apatite, (f) limestone, (g) twig, (h) float glass, (i) taphonomically modified plastic, (j) scallop shell, (k) starfish, (l) octocoral 1, (m) octocoral 2. Labels designate data collection points.

A total of 60 samples were analyzed, with eight testing locations each (for the nine teeth four locations were sampled each from the enamel and dentin to total eight per tooth), resulting in 408 individual testing locations. Five spectra were collected at each location, resulting in a final total of 2040 elemental data sets. Repetitions of eight testing locations per sample and five spectra per location were selected to maintain consistency with and allow comparisons to previous data collected by Zimmerman (2013) and to allow for examination of homogeneity within and between individual bones from the same group. Additionally, this allowed a better representation of each sample and ensured that if outliers were present, potentially the result of analysis errors or contamination, that

they would not impact the overall correct classification of the samples they originated from.

Samples were prepared by removing a small amount of sample from each testing location using a Black and Decker® rotary tool with an engraving bit. Though larger samples can be analyzed in the SEM/EDX, using powders reduced the processing time and allowed for more rapid data collection. This collection method is mildly destructive, resulting in a small, circular indentation at each collection location. Collection was performed under the fume hood to prevent cross contamination. Twelve millimeter stubs with carbon dots coated in an adhesive organic resin were placed one at a time under the hood and samples were collected directly over them so that the extracted particles would fall directly onto the stub, removing the need to transfer materials and risk cross-contamination. The powers were then tamped down using a metal spatula to ensure that they were secured to the stub. The engraving bits and the metal spatula were cleaned between each sample using soap and water then viewed under a high powered light microscope to ensure there were no adhering materials to cause contamination between samples. A small piece of copper tape was placed on each stub to use for calibration. Sample letters were carved into the copper stubs to ensure proper sample designation for analysis (Figure 6).



Figure 6: Example sample set. Note final consistency of samples and copper tape placed for sample designation.

Scanning Electron Microscopy – Energy Dispersive X-ray Spectrometry

Samples were analyzed at the National Center for Forensic Science (NCFS) in Orlando, Florida using a LEO 1450VP Scanning Electron Microscope and an Oxford Energy Dispersive Spectrometer (Figure 7). Multiple chemical analysis methods have been proposed in the literature for discrimination between human osseous, non-human osseous, and non-osseous materials (Chapter 3). Scanning electron microscopy – energy dispersive x-ray spectrometry was chosen for analysis over alternative laser excitation-emission monitoring approaches due to the commonality of SEM/EDX instrumentation within established crime laboratories and several procedural advantages including non-destructive analysis, low sample requirements, ease of preparation, high specificity of results, and simplicity of data analysis. While multiple instruments promote similar advantages, preliminary testing using SEM/EDX, LIBS, and Raman Spectroscopy

identified SEM/EDX as the most discriminatory when using the proposed statistical analysis procedure.



Figure 7: LEO 1450 VP Scanning Electron Microscope and Oxford Energy Dispersive Spectrometer.

Scanning electron microscopy – energy dispersive x-ray spectrometry analysis begins with observation of the unknown materials using the scanning electron microscope (SEM). The SEM consists of two main components; the electron column and the control console. The electron column contains an electron gun, electron lenses, sample stage, and vacuum pumps. The control console is comprised of the viewing screen, computer keyboard, and additional knobs to control the stage and electron beam (Goldstein et al., 2003).

Once the chamber is vacuum pumped down and the electron gun is activated it generates a constant stream of electrons that accelerates toward the sample stage. The electron lenses direct the electrons to a focused area – the tilt of the lenses determines the spot size of the electron beam. A larger spot size will allow more electrons to interact with the sample, but if the spot size becomes too large reflected electrons may flood the system decreasing imaging resolution and element detection. As the electrons collide with the sample they interact with the positively charged particles of the material. This high velocity impact results in the expulsion of electrons from the atoms within the sample. These electrons then collide with neighboring atoms resulting in expulsion of additional electrons and the creation of a chain reaction. Eventually, multiple electrons will be directed back towards the surface of the material resulting in secondary electrons (electrons ejected from atoms outer shells) and backscattered electrons (electrons from the inciting beam) to be expelled from the sample (Figure 8) (Goldstein et al., 2003).

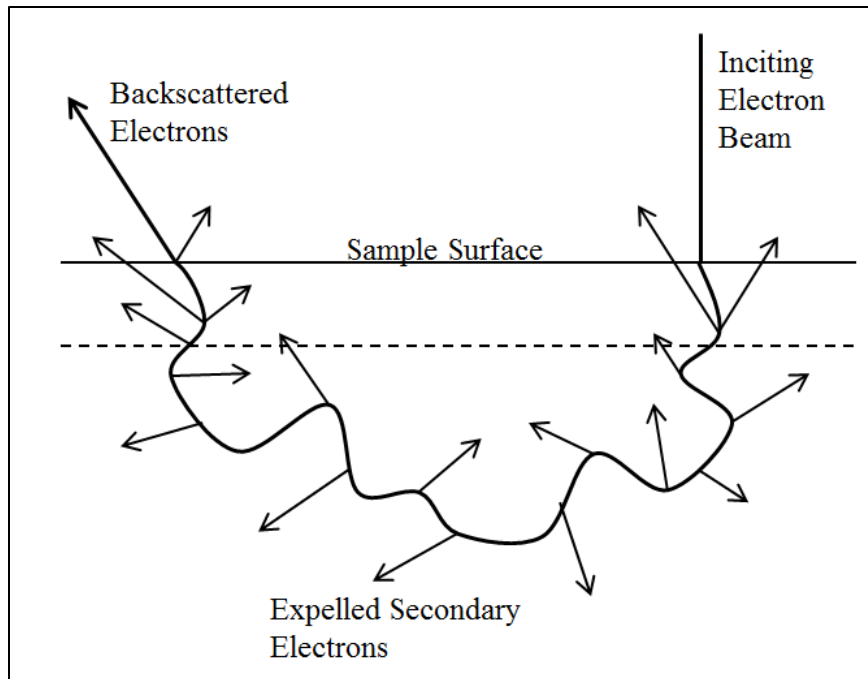


Figure 8: Electron interaction within a sample.

Electromagnetic deflection coils sweep the electron beam across the sample, creating a raster of expelled electrons that will be picked up by the electron detector. The electron detector is able to differentiate between the backscatter and secondary electrons based on their energy levels – backscattered electrons have a higher energy level since secondary electrons use large amounts of energy to discharge from their electron shell. A positive voltage is then applied to a collector screen, located in front of the detector, to capture the electron signals. By using both signals SEMs are able to produce high resolution images with strong contrast and depth of focus (Goldstein et al., 2003). The captured signal is then converted to an image, based on the energy of the electrons, and transmitted to the viewing screen of the SEM (Figure 9).

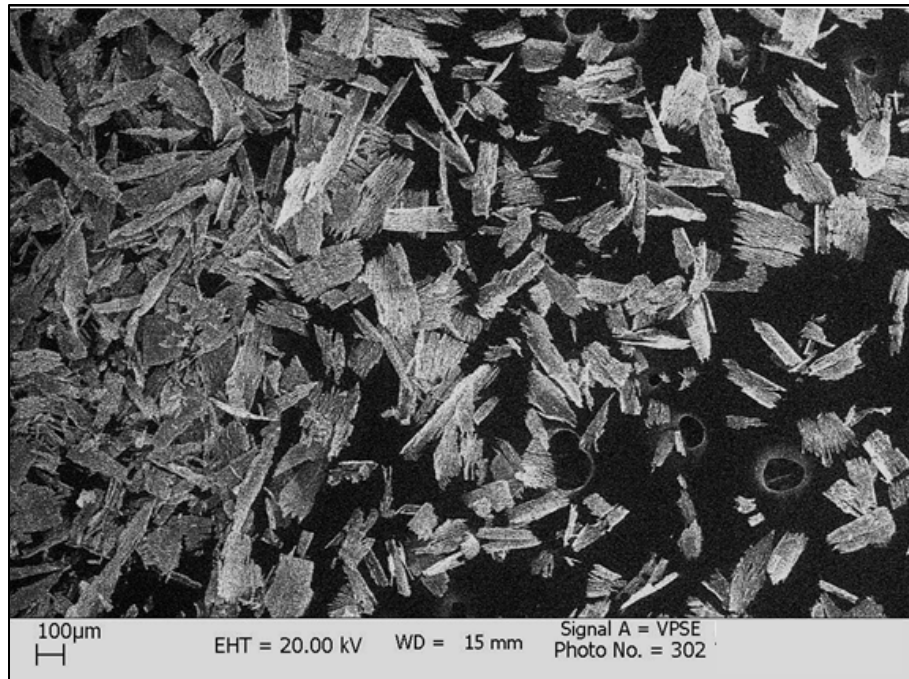


Figure 9: Example SEM image, armadillo bone.

X-ray photons are also generated when the electron beam interacts with the substrate. When the electron beam interacts with inner shell electrons it will eject an electron, leaving the atom in an excited state. The atom is required to return to ground state by moving outer shell electrons to fill the gaps, resulting in shifting of electron energy and the emission of a photon (Goldstein et al., 2003). The energy of each photon is equal to the difference in energy between the electron shells. Therefore, an emitted photon can express the $K\alpha$ x-ray energy, equal to the difference between the K and L shells; the $K\beta$ x-ray energy, equal to the difference between the K and M shells; the $L\alpha$ x-ray energy, equal to the difference between the L and M shells; and the $M\alpha$ x-ray energy, equal to the difference between the M and N shells (Figure 10). There are numerous

additional energy transitions within the sublevels of the *L*, *M*, and *N* shells but these are beyond the detection of the EDS (Goldstein et al., 2003).

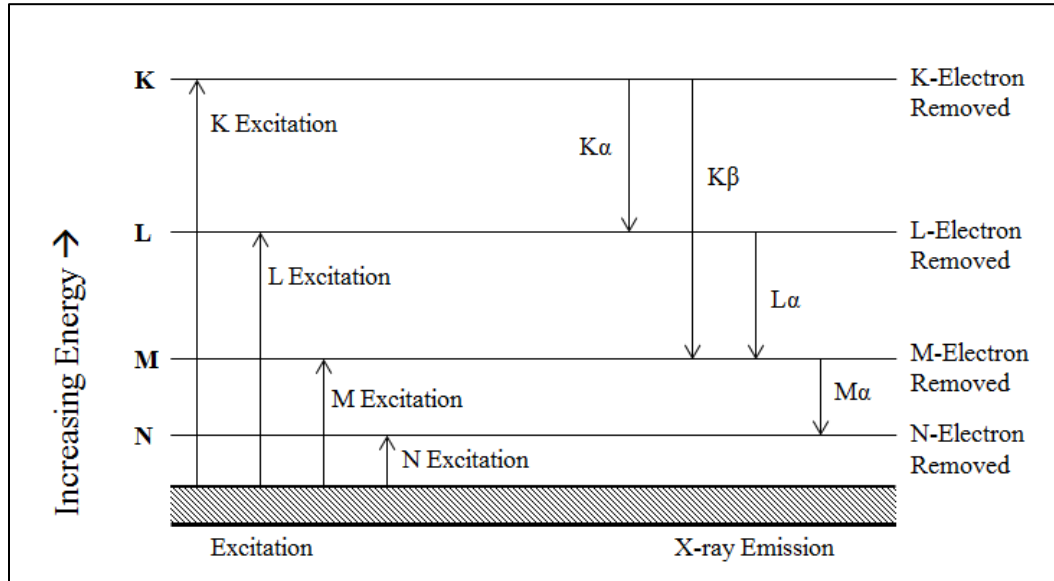


Figure 10: Atom electron shells and photon energy diagram.

The emitted photons move through a small window into a cooled reverse-bias Si(Li) crystal (Goldstein et al., 2003). This lithium coated silicon crystal functions as an intrinsic semiconductor. As such, it will not conduct a current unless an electric field is applied to cause excitement and ejection of electrons within the crystal. When the photons ejected from the sample encounter the cooled crystal they cause the ejection of a photoelectron of corresponding energy. These are pulled away from the crystal to form a charge pulse. The charge pulse is then converted into a voltage pulse by a charge-to-voltage converter. These data are finally amplified and received by a computer, which displays the voltages as a spectrum organized by signal intensity (Goldstein et al., 2003). These intensities can be further analyzed by comparing them to known intensities,

provided through a reference library and instrument calibration, to identify specific peaks and their associated elements (Figure 11). However, it is important for the instrument operator to validate detected elements due to overlap between x-ray lines of elements in close proximity to each other (Goldstein et al., 2003). This information can then be converted into quantitative data expressing the relative weight and atomic percentages of the elements contained within the sample. Accuracy for weight percent data can be within $\pm 1-2\%$, though the accuracy may decrease slightly with small particle analysis, such as was performed in this study, due to sample penetration (Goldstein et al., 2003).

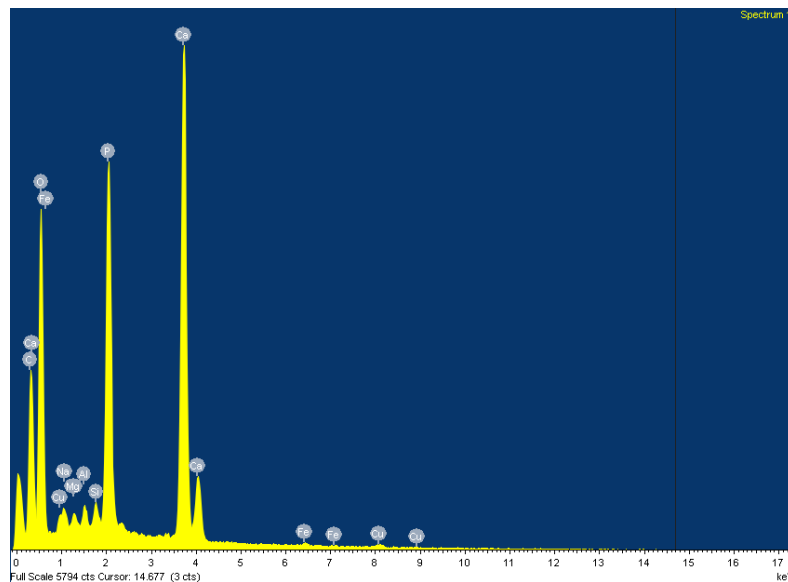


Figure 11: Example of spectral data displayed on the EDX, this spectrum is from a human phalanx.

Analysis Parameters

Instrument settings were chosen to provide the highest degree of accuracy for quantitative results while reducing sample preparation and analysis time. The organic origin of most of the samples required specialized processing due to the porous nature many of the samples (Stokes, 2008). Though dried, many retained moisture and would have delayed pumping down of the vacuum chamber. To combat this, samples were analyzed as powders, rather than fragments, and a variable pressure chamber was utilized. The increased chance of atmospheric interference within the chamber has been demonstrated as minimal (Stokes, 2008) and removing the high vacuum pumping process reduced the analysis time significantly. Pressure within the chamber was held between 40 and 70 pA for all analyses. Relatedly, using variable pressure SEM removed the necessity to coat samples in carbon. This is desirable for organic samples for imaging purposes as well as forensic samples that may need to be re-analyzed.

An accelerating voltage of 20kV was chosen because it was high enough to ensure proper excitation of heavy elements but not too strong as to completely penetrate the sample (Goldstein et al., 2003). This was essential because 99% of organic materials are formed from elements with low atomic weights, between 1 and 20, but multiple identified trace elements, such as chromium, cobalt, copper, iron, manganese, zinc, and iron, are heavy elements with atomic weights ranging from 24 to 30 (Echlin, 2009). The working distance suggested by INCA instruments, 15mm, provided adequate depth of field and high image resolution. Finally, a scan speed of 6 was chosen to ensure sufficient collection time.

Statistical Analysis

Dissimilar to other analyses, which have focused on the Ca/P ratios of materials (Ubelaker et al., 2002; Christensen et al., 2012), this analysis concentrated on elemental compositions with a focus on trace elements. Weight percentages for all contained elements were provided by and processed within the EDS software. Carbon was removed due to suspected contamination from the stub. Elements appearing in two or less spectra per sample location were also removed as contamination based outliers (most of these also fell below the visible noise threshold). The remaining elements, all of which were kept because factor extraction was performed using PCA, were normalized to 100% weight percent and final weight percent data were processed in R, version 3.0.1, by the R Foundation for Statistical Computing.

Data Processing

Data processing methods were modeled after Zimmerman (2013) and expanded to incorporate a multi-step statistical analysis approach. After the raw data were processed (removing carbon and anomalous elements) and pretreated (background removed and weight percentages normalized to 100%) within the EDS software, weight percent values were exported to an Excel® spreadsheet. Data were viewed for inconsistencies and sample “THFem1-2” was removed due to a lack of oxygen in the spectra and sample “HFib2-4” was removed due to an abnormally low phosphorus weight percentage

(0.4759%). The remaining data were analyzed using a multi-step statistical analysis procedure. First, principal component analysis (PCA) was performed to determine the number of principal components (PCs) necessary to represent 95% of the variation within the data (Appendix A). Principal component analysis is commonly used in multivariate discrimination studies as it reduces the number of variables to allow the data to be reproduced by a smaller set of variables known as latent variables or principal components (Varmuza and Filzmoser, 2009). The PCs are mathematical vectors that represent the latent variables and reproduce the desired fraction of the variance within a dataset. These are determined using a covariance matrix compiled from the data being examined and can be mathematically represented using Equation 1, with [R] representing the matrix of scores and [C] representing the matrix of loadings of each PC (Varmuza and Filzmoser, 2009). An additional term, not shown in Equation 1, is the error [E], which is removed when the desired fraction of the variance (1% in this case) is removed from the data recovered by the matrix multiplication in Equation 1.

$$[D] = [R][C] \quad (1)$$

The scores express how much of each variable is present and the loadings describe how much of each original variable is necessary to create a latent variable (Equation 2 and 3) (Varmuza and Filzmoser, 2009). When the transpose of [C] post-multiplies the original data matrix, the scores matrix is obtained.

$$R_1 = x_{i1}p_1 + x_{i2}p_2 + \dots + x_{im}p_{im} \quad (2)$$

$$C_1 = (p_1, p_2, \dots, p_m) \quad (3)$$

The first PC represents the most variance in the data, the second PC represents the second highest variance, and so on. (Anderson, 2003). More specifically, the first PC represents an average of all of the contributing variables, allowing calculation of the maximum variance for each variable, and all consecutive PCs comprise an orthonormal set (i.e., the vectors are orthogonal, or at right angles to each other, and they are normalized to a length of one). The loadings can be examined to identify the original variables that play a significant role in differentiation of the samples. Principal component analysis was conducted in R version 3.0.1 using R-code written in house (2013).

The R code calculates the scores [R] and the eigenvectors of loadings [C], in addition to generating cumulative percent variance for the PCs as well as a scree plot. Each eigenvalue is proportional to the fraction of the variance described by each PC and the sum of all the eigenvalues represents the cumulative variance in all of the PCs. A scree plot is generated as a visual representation of these values, graphing the PC eigenvalues against the PC number (Crawley, 2012). From the scree plot, the number of significant principal components (i.e., the number to be utilized in further analysis) can be determined by locating the point at which there is a break or drop in the graph and selecting the number of PCs situated before this point (Varmuza and Filzmoser, 2009). These points can be compared to the percent total variances (cumulative sums of the

principal component eigenvalues) to ensure they represent significant variance within the data.

Subsequently, Fisher Linear Discriminant Analysis (LDA) was performed to assess classification of the data into predefined classes. Discriminant analysis determines the relationships within and among groups to define the variables that contribute to group classification (Crawley, 2012). Fisher LDA was used because it does not assume that the data exhibits a normal distribution.

After defining group classification based on sample material, LDA calculates a linear boundary (line, plane or hyperplane) to separate groups using Equation 4 with \bar{x}_1 and \bar{x}_2 being the arithmetic mean vectors from the data sets and \mathbf{S}_P equal to the pooled covariance matrix (Equation 5) (Varmuza and Filzmoser, 2009).

$$\mathbf{b}_{FISHER} = \mathbf{S}_P^{-1}(\bar{x}_1 - \bar{x}_2) \quad (4)$$

$$\mathbf{S}_P = \frac{(n_1 - 1)\mathbf{S}_1 + \dots + (n_k - 1)\mathbf{S}_k}{n_1 + \dots + n_k - k} \quad (5)$$

The dimensionality of the plane is contingent on the number of principal components used for analysis. Individual data points will be classified according to their location relative to the calculated linear boundary (Figure 12).

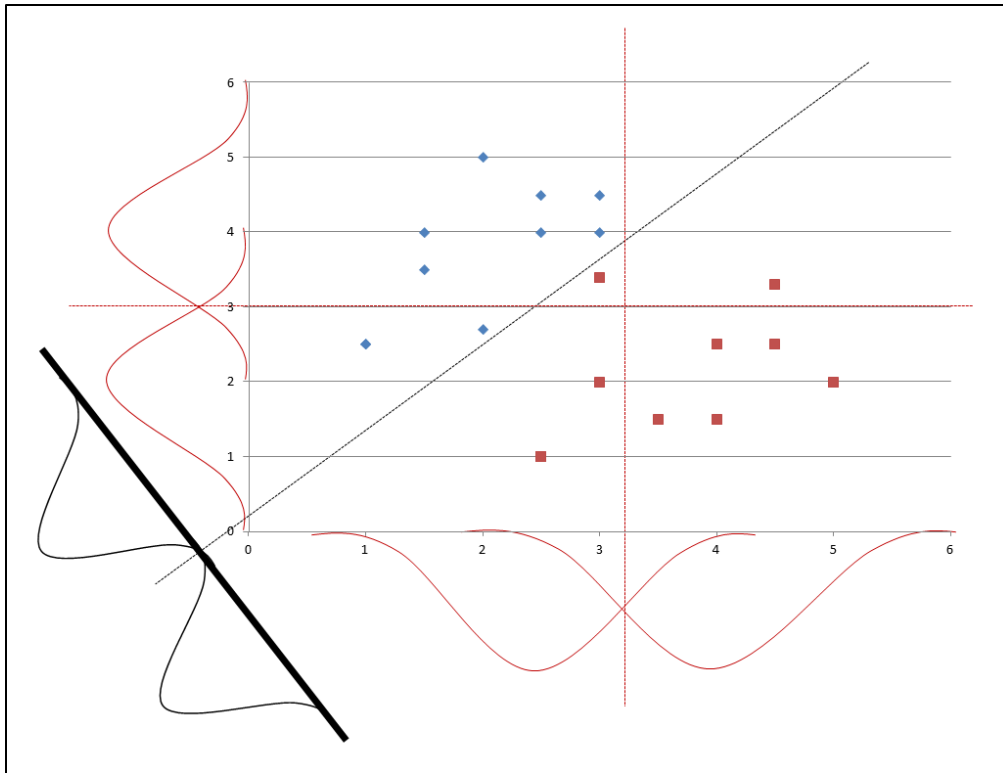


Figure 12: Hypothetic discrimination based on linear boundary calculated using LDA (not to scale). Red distribution curves and dashed linear boundaries indicate intrinsic X and Y plane based divisions of data set – these provide poor classification. The black bolded line represents the new plane created using LDA and the black distribution curves and dashed linear boundary indicate the new planar distribution and linear discriminant boundary – this line provides good classification.

Quadratic discriminate analysis was also considered, but as it consistently provided lower classification rates it was excluded from final analyses.

Blind Study

A blind study was also included as a component of this project to assess the applicability of this method for classification of unknown samples. Additionally, it

provided preliminary error rates, which are required under the Daubert standard (*Daubert v. Merrell Dow Pharmaceuticals, Inc.*, 1993).

Twenty samples were provided for the blind study. Samples were selected and prepared by Dr. John J. Schultz. The majority of the samples were provided by Mr. Frank Logiudice from the Vertebrate Zoology Laboratory in the Department of Biology at the University of Central Florida. The origin of the samples was unknown by the author prior to analysis. All samples were derived from new sources and were chosen to reflect categories within the original study (human osseous, non-human osseous, non-osseous). Additionally, samples were chosen to represent materials within the original sample set as well as new materials.

Samples were received as fragments, similar to a forensic investigation. They were mounted on stubs and processed using the same procedure as all previous samples. The engraving bits were not used as a result of their small size. However, if only one fragment was presented smaller pieces were broken off to reduce processing time and to preserve materials if future testing was required. Five spectra were collected for each unknown resulting in a total of 100 spectra. Spectra were analyzed as individual spectra and as averages of spectra within a sample to determine if this would influence discrimination results. Finally, after samples had been classified results were compared to the known sample origins to determine the accuracy of the preliminary blind study.

CHAPTER 5: RESULTS

Data analysis was performed in three steps: discrimination of osseous and non-osseous materials, discrimination of human osseous and non-human osseous materials, and analysis of the blind study.

Discrimination of Osseous and Non-Osseous Materials

After the raw data were processed and the two anomalous sample spectra (HFib2-4 and THFem1-2) were removed the data set contained 2038 spectra, 1518 osseous and 520 non-osseous. Principal component analysis was performed using code written in house and 5 principal components were identified as representing greater than 95% of the variance within the data. Figure 13 demonstrates the splom matrix generated using the first three principal components with osseous spectra shown in blue and non-osseous spectra shown in pink. The strongest visual discrimination can be seen between principal components 1 and 3 (Figure 13). Note the mild overlap between categories. Additionally, it is important to note the general congruency of the osseous materials due to their consistent calcium-phosphate base and the relative incongruence of the non-osseous samples.

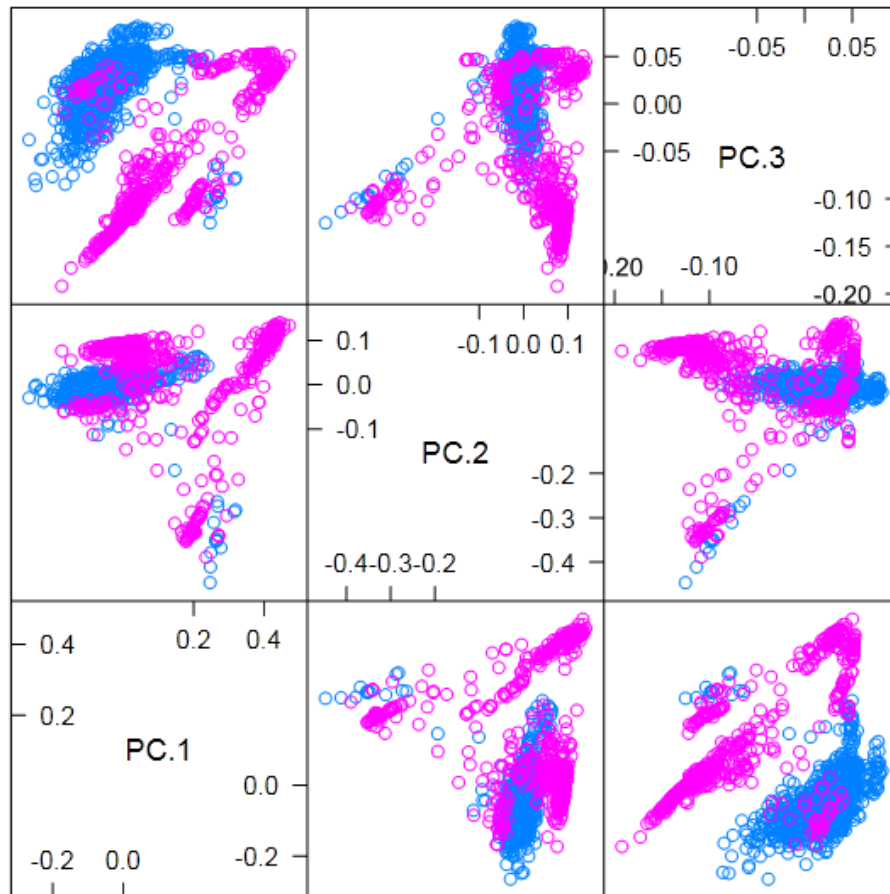


Figure 13: Splom matrix generated using the first three principal components from the initial data set. Osseous spectra are blue, and non-osseous spectra are shown in pink.

Next, Fisher LDA was performed: 1504 of the 1518 osseous (99.01%, Table 7) and 481 of the 520 non-osseous (92.50%, Table 7) spectra were correctly classified resulting in an overall correct classification of 97.35%. This represents an error rate of 0.0299, or 2.99%.

Table 7: Confusion matrix demonstrating Fisher LDA of osseous and non-osseous materials for initial data set.

	Osseous	Non-Osseous	Correct Classification
Osseous	1504	14	99.01%
Non-Osseous	39	481	92.50%

The misclassifying spectra were identified and assessed. The 14 misclassifying osseous spectra were identified as DeerD2-1, DeerD2-2, DeerD2-3, DeerD2-4, DeerE2-1, DeerE2-2, DeerE2-3, DeerE2-4, DeerE2-5, DeerE4-1, DeerE4-2, DeerE4-3, DeerE4-4, and DeerE4-5. These samples are deer dentin location 2 (spectra 1-4), deer enamel location 2 (spectra 1-5), and deer enamel location 4 (spectra 1-5). The spectra for each of these samples demonstrate abnormally elevated silicon contents in comparison to other osseous materials and other spectra collected from the same sample. This demonstrates that these spectra are not representative of their respective samples and were thus removed from analysis as outliers. The high silicon content for these deer tooth spectra (14 out of 40 spectra) could be due to foreign materials on the sample, such as dirt or sand. The 39 misclassifying non-osseous spectra were all identified as rock apatite (RA). This is similar to previous studies (Ubelaker et al., 2002; Christensen et al., 2012; Zimmerman, 2013). Since these spectra are representative of their sample they were not removed.

The fourteen osseous outliers were removed and data processing was repeated with the remaining 2024 spectra (1504 osseous and 520 non-osseous). Principal component analysis identified 6 principal components representing greater than 95% of the variation within the data and Figure 14 demonstrates the splom matrix generated

using the first three principal components with osseous spectra shown in blue, non-osseous spectra shown in pink, and rock apatite spectra shown in green. Again, the strongest visual discrimination can be seen between principal components 1 and 3 (Figure 14).

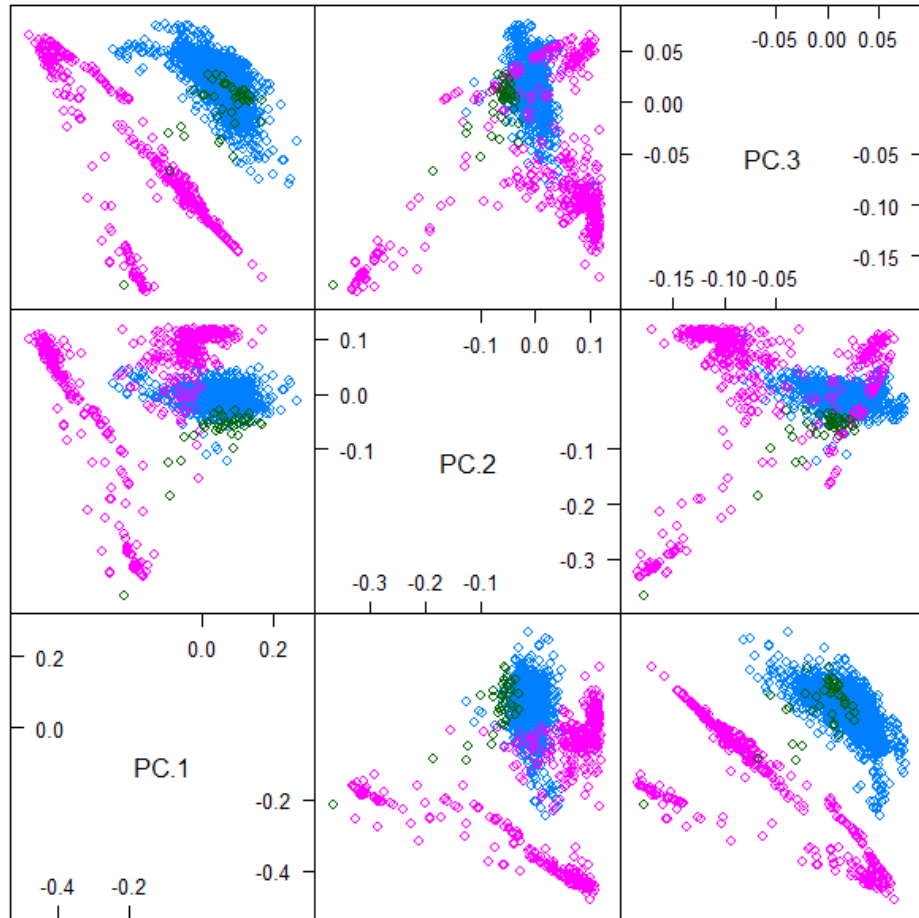


Figure 14: Splom matrix generated using the first three principal components from the initial data set. Osseous spectra are shown in blue, non-osseous spectra are shown in pink, and rock apatite spectra are shown in green.

Fisher LDA was performed again: 1503 of the remaining 1504 osseous (99.93%, Table 8) and 481 of the 520 non-osseous (92.50%, Table 8) spectra were correctly

classified resulting in an overall correct classification of 98.02%. This represents an error rate of 0.0173, or 1.73%.

Table 8: Confusion matrix demonstrating Fisher LDA of osseous and non-osseous materials for revised data set.

	Osseous	Non-Osseous	Correct Classification
Osseous	1503	1	99.93%
Non-Osseous	39	481	92.50%

Discrimination of Human Osseous and Non-Human Osseous Materials

To assess discrimination of human and non-human osseous materials a new data set was created containing only the osseous spectra. The osseous samples removed as outliers during the first analysis remained excluded, resulting in 1504 spectra, 598 human and 906 non-human. Principal component analysis identified 4 principal components representing greater than 95% of the variation within the data and Figure 15 demonstrates the splom matrix generated using the first three principal components with human shown in blue and non-human shown in pink. Unlike splom matrices generated for the osseous and non-osseous classifications there is no clear visual discrimination (Figure 15).

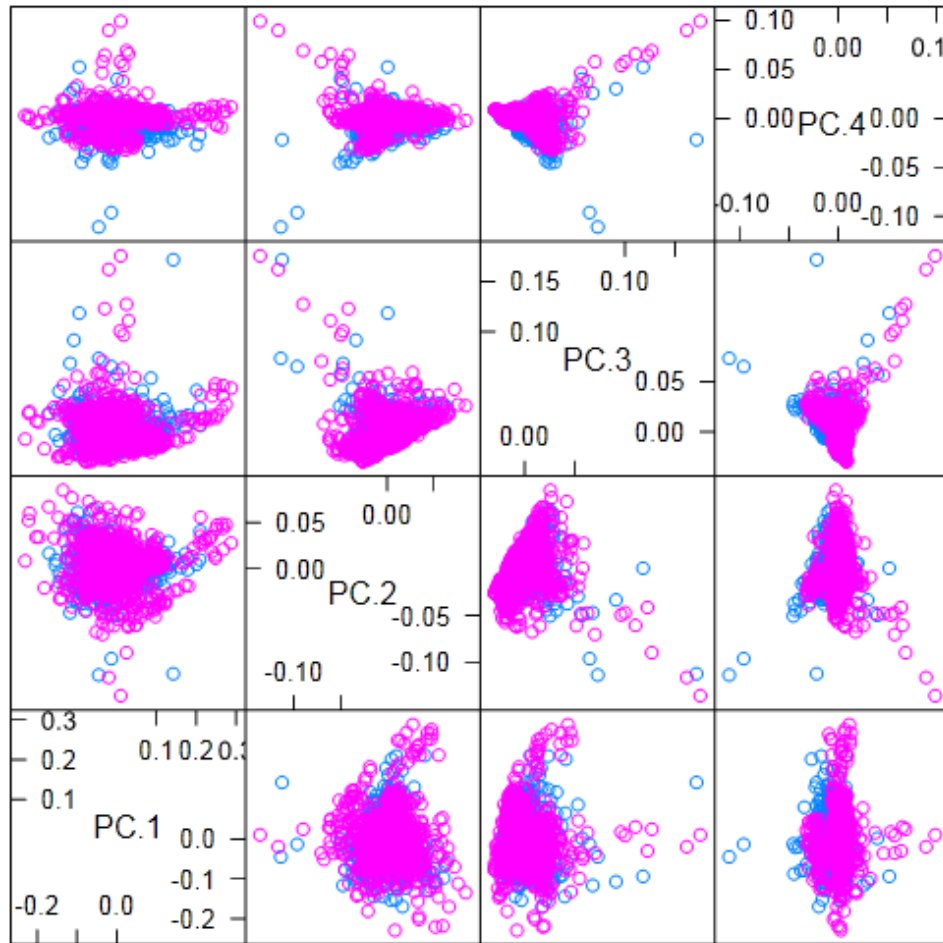


Figure 15: Splom matrix generated using the first four principal components from the osseous data set. Human osseous spectra are shown in blue, and non-human osseous spectra are shown in pink.

Fisher LDA was performed resulting in 212 of the 598 human (35.45%, Table 9) and 877 of the 906 non-human (96.80%, Table 9) spectra being correctly classified resulting in an overall correct classification of 72.41%. This represents an error rate of 0.2886, or 28.86%.

Table 9: Confusion matrix demonstrating Fisher LDA of human osseous and non-human osseous materials within the osseous data set.

	Human Osseous	Non-Human Osseous	Correct Classification
Human Osseous	212	386	35.45%
Non-Human Osseous	29	877	96.80%

Blind Study

To determine the efficacy of discrimination osseous and non-osseous materials for unknown samples, five spectra from each of the 20 blind samples (100 total spectra) were projected into the PCA space for the original data set and subjected to Fisher LDA to determine their classification, the projection shown in Figure 16 aids in visualizing the class assignments. The splom shown was generated using principal components one and three from the original and blind data sets. Osseous spectra are shown in red, non-osseous spectra are shown in blue, and the spectra from the blind study are shown in green.

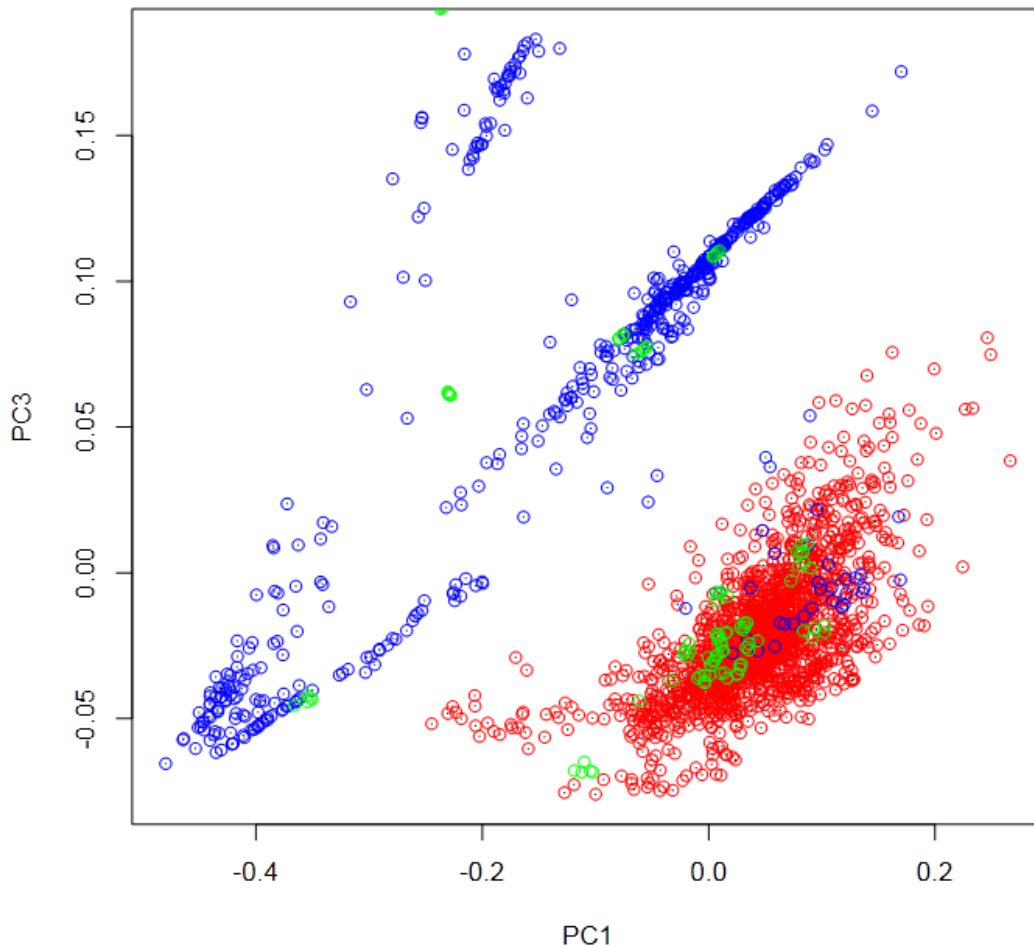


Figure 16: Splom matrix generated using principal components one and three from the original and blind data sets. Osseous spectra are shown in red, non-osseous spectra are shown in blue, and the blind data spectra are shown in green. Note the location of blind data points within both groups.

The five spectra collected for each sample classified together, demonstrating a high degree of precision. This resulted in individual and average spectra providing the same classifications. After the samples were assigned to categories, osseous or non-osseous, using predictive software in R the information concerning the material of the samples was provided and the classifications were compared to the true category of the sample. The blind study included 7 materials that were present in the original data set

(human medical specimen, deer, whale, sand dollar, scallop shell, human archaeological bone, and modern human tooth enamel) and thirteen materials that were not (green sea turtle, bottle nose dolphin, sand tiger shark cartilage, cat, alligator enamel, manatee, false killer whale, horse dentin, gar, sea rose coral, wood stork, prehistoric pottery, and lithic) to determine if classification was contingent upon pre-inclusion of the material. Table 10 indicates the sample designations and classifications as the size of each sample and whether it was included in the original study. Of the 20 unknown samples all 20 classified correctly (100 out of 100 spectra), for 100% correct classification.

The results of the blind study, as well as of the osseous and non-osseous discrimination, indicate that SEM/EDX and multivariate statistical analysis are a viable method for differentiation of osseous and non-osseous materials.

Table 10: Blind study results identifying sample origin, if the sample type was in the original data set, and the size of the analyzed fragment.

Sample Number	Assigned Category	Actual Category	Sample Origin (Common Name, Sample Location, and Species)	In Initial Set?	Approximate Size of Fragment Analyzed
1	O	O	Green Sea Turtle Clavicle (<i>Chelonia mydas</i>)	No	2x3mm
2	O	O	Bottle Nose Dolphin Scapula (<i>Tursiops truncatus</i>)	No	1.5x2mm
3	NO	NO	Sand Tiger Shark Jaw Cartilage (<i>Carcharias taurus</i>)	No	4x5mm
4	O	O	Domestic Cat Humerus (<i>Felis catus</i>)	No	1x2mm
5	O	O	Alligator Tooth Enamel (<i>Alligatoridae mississippiensis</i>)	No	2x5mm
6	O	O	Manatee Cranium (<i>Trichechus manatus</i>)	No	1.5x3mm
7	O	O	Human (Medical Specimen) Femur	Yes	0.5x1mm
8	O	O	Whale Cranium (<i>Cetacea</i>)	Yes	1.5x2mm
9	O	O	False Killer Whale Cranium (<i>Pseudorca crassidens</i>)	No	0.5x1mm
10	O	O	Horse Tooth Dentin (<i>Equus ferus caballus</i>)	No	0.5x1mm
11	O	O	Common Gar Cranium (<i>Lepisosteidae</i>)	No	5x6mm
12	O	O	Deer Rib (<i>Odocoileus virginianus</i>)	Yes	2x4mm
13	NO	NO	Sea Rose Coral (<i>Manicina areolata</i>)	No	2x3mm
14	NO	NO	Sand Dollar (<i>Echinarchnius parma</i>)	Yes	2x7mm
15	NO	NO	Scallop Shell (<i>Argopecten irradians</i>)	Yes	2x3mm
16	O	O	Wood Stork Ulna (<i>Mycteria americana</i>)	No	0.5x0.5mm
17	NO	NO	Prehistoric Pottery	No	0.5x1mm
18	NO	NO	Lithic	No	0.5x0.5mm
19	O	O	Human Archaeological Scapula	Yes	1.5x7mm
20	O	O	Modern Human Tooth Enamel	Yes	2x4mm

(O = osseous, NO = non-osseous)

CHAPTER 6: DISCUSSION

As is evident from the literature, there is a strong need for expanded research in forensic anthropology towards the discrimination of human osseous, non-human osseous, and non-osseous materials using analytical methodologies. This study serves to address this need by assessing SEM/EDX and statistical analysis as a means for differentiating between osseous and non-osseous materials as well as human osseous and non-human osseous samples.

Discrimination of Osseous and Non-Osseous Materials

In this study, discrimination between osseous and non-osseous materials was high; using Fisher Linear Discriminant Analysis correct classification was 98.02%. More importantly, correct classification of osseous materials was 99.93%, with only 1 of 1504 spectra misclassifying. Since 40 spectra were collected for each sample, 1 spectra misclassifying would not change the overall classification of the sample. As a result, all of the bone samples would be correctly classified as such, resulting in a 100% applied discrimination. As Ubelaker and colleagues (2002) and Christensen and colleagues (2012) do not provide classification rates these results can only be compared to Zimmerman (2013). Using HHXRF, trace element analysis, and statistical analysis she was able to demonstrate 94% correct classification – an applied discrimination percentage is not provided.

This study also exhibited high classification for non-osseous materials, demonstrating 92.50% correct classification with 39 out of 520 spectra misclassifying. As

all 39 spectra were from one sample, this equates to 12 out of 13 samples being correctly classified overall for an applied discrimination of 92.3%. This sample was identified as rock apatite. Previous studies have also demonstrated difficulty correctly classifying mineral apatite materials (Ubelaker et al., 2002; Christensen et al., 2012; Zimmerman, 2013).

Dissimilar to several previous studies (Ubelaker et al., 2002; Christensen et al., 2012) this method was able to discriminate multiple species of octocoral from osseous materials. However, also dissimilar to these previous studies, the octocoral included in this study did not contain phosphorus. The species of octocoral used in the earlier studies by Ubelaker and colleagues (2002) and Christensen and colleagues (2012) was *Octocoralia leptogorgia setacea*. The species used by Zimmerman (2013) and the first species of octocoral tested in this study, *Octocoralia ricordea*, did not contain phosphorus and was discriminated from bone. After the lack of phosphorus was identified efforts were made to obtain the original species used by Ubelaker and colleagues (2002) and Christensen and colleagues (2012) as their sample exhibited a Ca/P ratio similar to bone, demonstrating a significant amount of phosphorus. Unfortunately this specific species could not be located. A species of octocoral from the same family, *Octocoralia leptogorgia virgulata*, was tested but also contained no phosphorus.

Another set of samples that previous studies have demonstrated difficulty in differentiating are ivory (Ubelaker et al., 2002; Christensen et al., 2012) and synthetic hydroxyapatite (Ubelaker et al., 2002). This study was able to differentiate these samples from non-osseous materials. It is important to emphasize, however, that the category

designation for these samples is different from previous studies. Preceding studies designated ivory and synthetic hydroxyapatite as non-osseous materials due to the desire to differentiate them from bone. Though it is desirable to remove these samples from forensic investigations, they are osseous in nature and should be classified as such. Ivory is composed of enamel, which is 99% hydroxyapatite, and synthetic hydroxyapatite is chemically constructed hydroxyapatite – neither of which should display significantly divergent compositions than other osseous materials. For this study, if classified as non-osseous all of the ivory and synthetic hydroxyapatite spectra (120) misclassify. This would reduce the overall classification to 91.06% and the osseous classification to 99.20%. Non-osseous correct classification would be reduced from 92.5% to 75.16%. Though these classifications are still high, these samples will remain classified as non-human osseous materials as they are osseous in regards to their chemical compositions. Additionally, it should be noted that Zimmerman (2013) was able to successfully discriminate ivory as a non-osseous material. This may be due to the small number of dental materials represented in that study that ivory could align with. Since this study contains significantly more dental samples than previous studies, all of which were classified as osseous, it is expected for ivory to be classified similarly as they have the same base hydroxyapatite composition.

Overall, this method demonstrated high correct classification of osseous and non-osseous materials using Fisher LDA. Overall classification was 98.02% and osseous classification was 99.93% with 100% applied discrimination. This method demonstrates both a low type one error, false exclusion, and a low type two error, false inclusion, for

osseous samples. Though traditional forensic investigations strive for a low type two error to prevent false incarcerations, this method would be applied as a preemptive step and thus a low type one error is desired. The initial question a forensic anthropologist must answer when presented a set of unidentifiable fragments is 'is it bone?' The designation of the samples as osseous or non-osseous dictates the next step of the investigation, primarily whether to exclude them from further investigation or to proceed with more costly and time invested analyses. Retaining all osseous materials is desirable so that potential evidence is not discarded. Additionally, false inclusion of non-osseous materials would be corrected at later steps, such as during DNA analysis.

This high classification rate as well as the clear separation between osseous and non-osseous materials (Figure 14) suggests this method would be highly successful for differentiation of unidentified fragmentary materials in forensic investigations.

Discrimination of Human Osseous and Non-Human Osseous Materials

Discrimination between human osseous and non-human osseous materials using Fisher LDA was considered unsuccessful, demonstrating a poor overall correct classification of 72.41%. Correct classification of human osseous samples was 35.45% and correct classification of non-human osseous samples was 96.80%, indicating that the majority of the spectra were being classified as non-human. This is corroborated by the high degree of overlap seen between the human and non-human osseous samples (Figure 15).

There are numerous factors that may be influencing the high degree of overlap between these samples. Primarily, this is likely a result of their congruent base hydroxyapatite compositions. Though previous studies have not statistically assessed differentiation between human and non-human osseous materials, determined Ca/P ratios indicate that the hydroxyapatite foundations of human and non-human osseous materials are highly similar (Ubelaker et al., 2002; Christensen et al., 2012). Additionally, there is a strong overlap between the trace elements exhibited in each of the samples due to similarities in diet and environment between represented species. Finally, the degree of homogeneity within human osseous and non-human osseous materials has not been established. However, box plots demonstrating elemental variance for each sample are included in Appendix B, providing a visualization of the relative homogeneity of each element in each sample. Box plots were also generated to provide a visual assessment of the homogeneity of the principal component distribution for each sample (Appendix C). Due to external influences, the trace element constituents in bone fluctuate, producing variations between individuals of the same species. This also contributes to the overlap witnessed between different species from the same environments. Further analysis of the trace element compositions of human and non-human osseous materials is necessary to understand the high degree of overlap seen. Accounting for environment, diet, and other variables will increase the potential for identifying key constituents that could be used for species differentiation.

Blind Study

Correct classification for the blind study was 100%, demonstrating the applicability of this method for discriminating osseous and non-osseous materials of unknown origin. The inclusion of samples not represented in the original data set further demonstrates the applicability of this method for identifying unknown materials as it does not require a pre-established reference for each sample but only a pre-established data set representative of the categories.

Unlike previous studies, this study provides preliminary error rates for using SEM/EDX for discrimination of osseous and non-osseous materials. Understanding the capabilities of the method for discrimination of unknown materials will aid in advancing the technology and designing a methodology that can be applied to unidentified samples from forensic scenes. The 0% error rate is highly encouraging, though larger blind studies encompassing larger sample sets need to be assessed to ensure the proficiency of the method.

CHAPTER 7: CONCLUSIONS AND FUTURE RESEARCH

This study has demonstrated the utility of scanning electron microscopy – energy dispersive x-ray spectrometry and multivariate statistical analysis for discriminating osseous and non-osseous materials of unknown origin. Applied as a preliminary step for discriminating osseous and non-osseous materials, this would assist law enforcement in determining the potential forensic significance of unidentified materials, reducing time and monetary costs traditionally expended on analysis of non-osseous samples. Though this would not exclude all forensically insignificant materials (non-human) it would help reduce the number of forensically insignificant materials retained for further analysis. Highly fragmented or taphonomically modified materials may be difficult to identify using traditional methods (histological or biological) due to the compromised nature of the samples. However, analytical chemistry methods, such as SEM/EDX, have been shown useful in identification of taphonomically modified materials. Additionally, SEM/EDX can analyze extremely small samples sizes. Therefore, SEM/EDX may be advantageous to alternative analysis methods.

Scanning electron microscopy – energy dispersive x-ray spectrometry was first proposed for differentiation of osseous and non-osseous materials by Ubelaker and colleagues (2002). There are several methodological advantages to using SEM/EDX for chemical differentiation of osseous and non-osseous materials. The primary reasons are its high specificity, small sample requirements, and relative non-destructive testing protocol. Additionally, SEM/EDX is present in most established crime laboratories. Though other methods have been assessed and some present field-use potential, it is

essential to examine the practicality of incorporating this method into forensic anthropological analysis. Since SEM/EDX is already prevalent in modern forensic laboratories and has current anthropological and archaeological applications such as analysis of trace metal residues on bone (Berryman et al., 2010; Amadasi et al., 2012; Gibelli et al., 2012; Pechníková et al., 2012; Taborelli et al., 2012; Vermeij et al., 2012) it would be less complicated and more cost efficient to introduce than a method requiring new instrumentation or validation. Additionally, the proposed method does not require advanced training or knowledge of analytical chemistry as the instrument provides clear results in the form of weight percent composition that can be processed using publically available statistical analysis software.

This study also took a constructive approach to data collection and analysis. Previously, the majority of research towards differentiating osseous from non-osseous materials has focused on Ca/P ratios (Ubelaker et al., 2002; Christensen et al., 2012). This is useful for material differentiation, but is limited in regards to discriminating between materials with similar Ca/P ratios as bone. Other studies have assessed the trace element compositions of materials to identify divergences and have demonstrated higher success in osseous and non-osseous material differentiation (Zimmerman, 2013). This study was designed to complement and expand upon Zimmerman (2013) by increasing sample size and utilizing alternative statistical analyses to determine if higher classification could be achieved. Additionally, a blind study was incorporated.

Previous studies have set the framework for developing methods aimed at chemically differentiating osseous from non-osseous, and perhaps human from non-

human, materials, but there are areas in which the field still needs to progress. Forensic anthropologists need a rapid, non-destructive, and cost efficient method that can identify a range of elements and concentrations indicative of osseous materials. By refining chemical differentiation technologies and working towards incorporating them into criminal investigations forensic anthropologists might be able to identify osseous and non-osseous samples as a preemptive step in investigations, reducing time and cost investments spent on forensically insignificant samples. In addition, this method can be applied to other areas of anthropology or archaeology to assist in material differentiation. A rapid and non-destructive method for differentiation of recovered fragments would aid in understanding recovered artifacts and in interpreting anthropological or archaeological contexts.

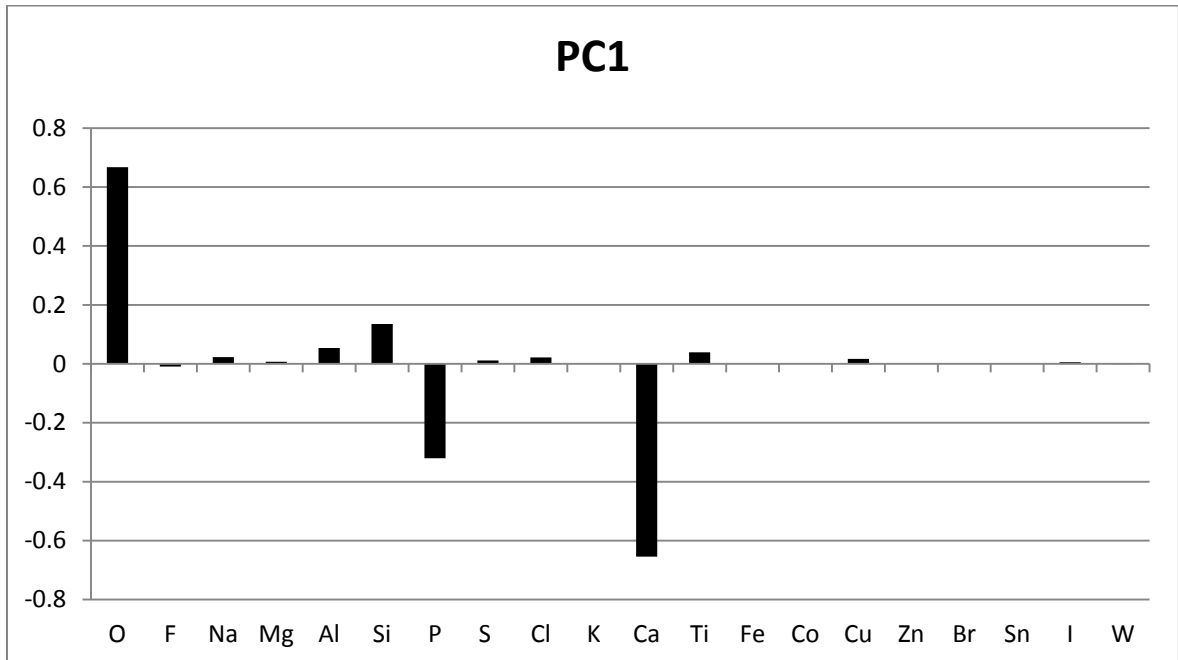
Subsequently, future research must be done to expand the data sets to include more representative samples, including additional human bones and teeth, non-human bones and teeth, non-osseous materials, and taphonomically modified materials. Homogeneity within samples and within species should be assessed to determine how this might impact classification. Additionally, the misclassifying samples should be analyzed further to determine what is causing them to misclassify and if there are alternative data analysis methods that would provide higher correct classification. Finally, additional research needs to be contributed to differentiation of human and non-human osseous materials as this is the second step in determining the forensic significance of unidentified materials.

APPENDIX A: PRINCIPAL COMPONENT LOADINGS

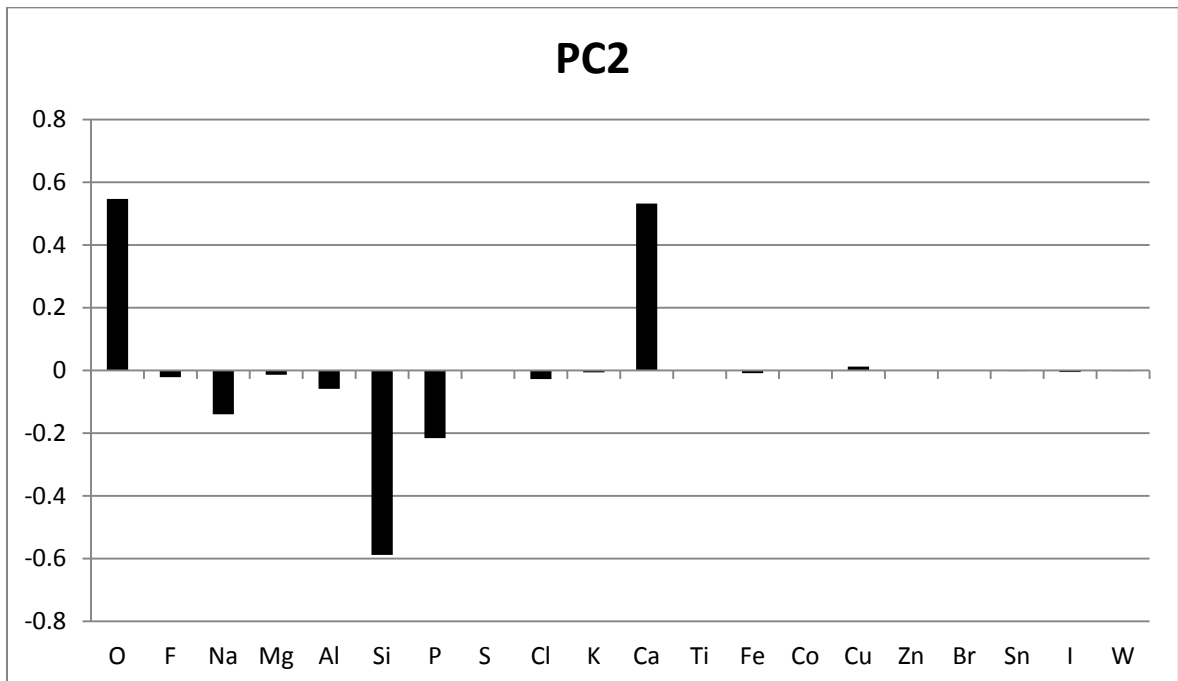
First five principal component loadings for osseous/non-osseous discrimination.

Percentages indicate the cumulative percent variation represented by each of the PCs.

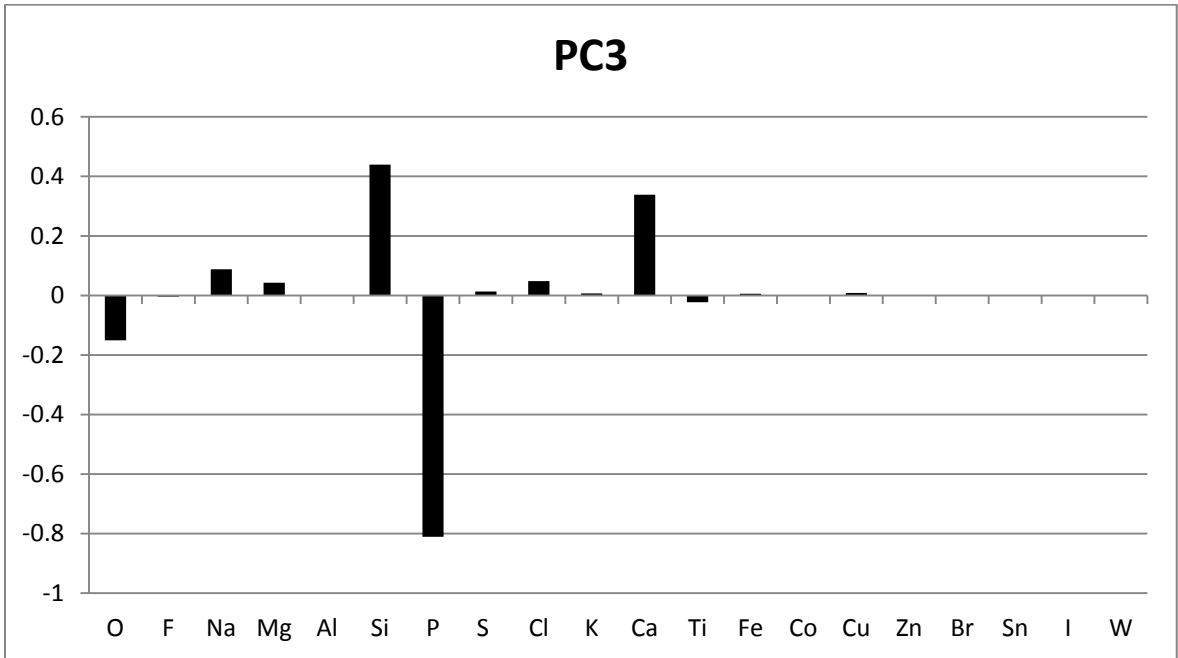
PC1 = 63.85%



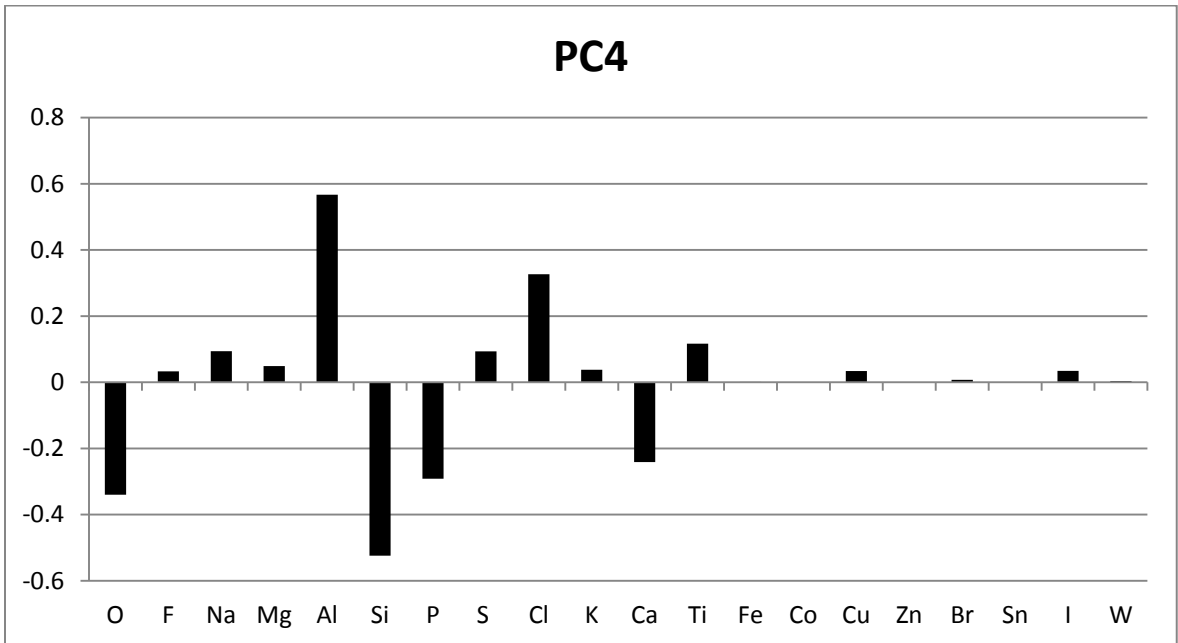
PC2 = 80.02%



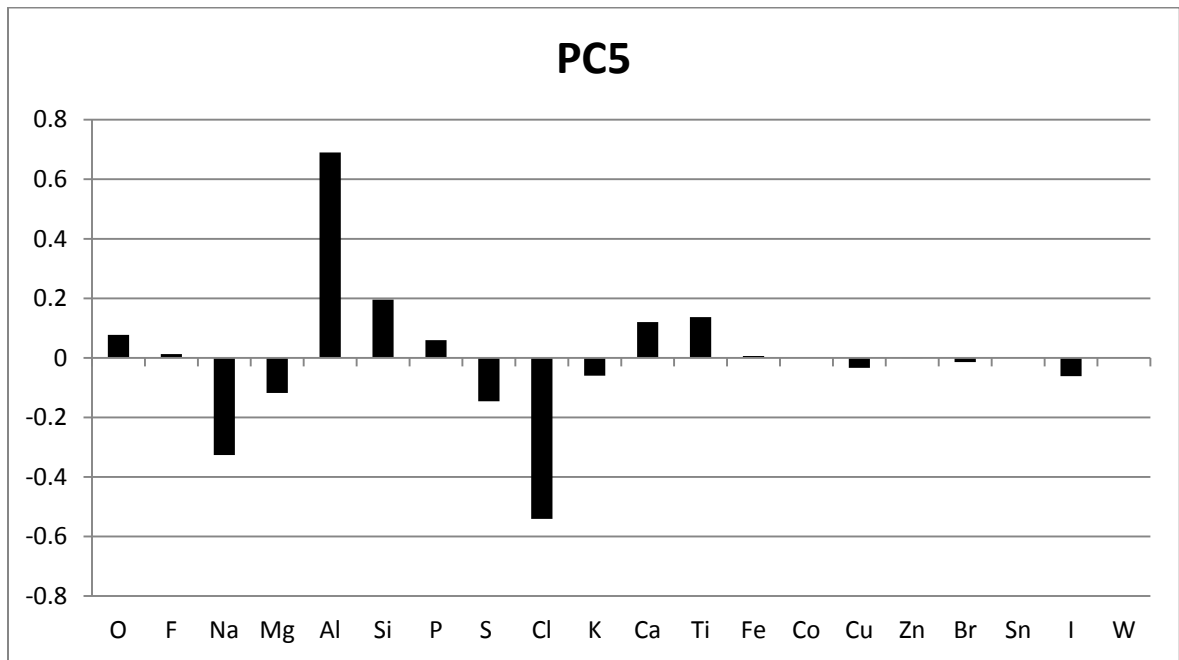
PC3 = 0.91.55%



PC 4 = 95.45%



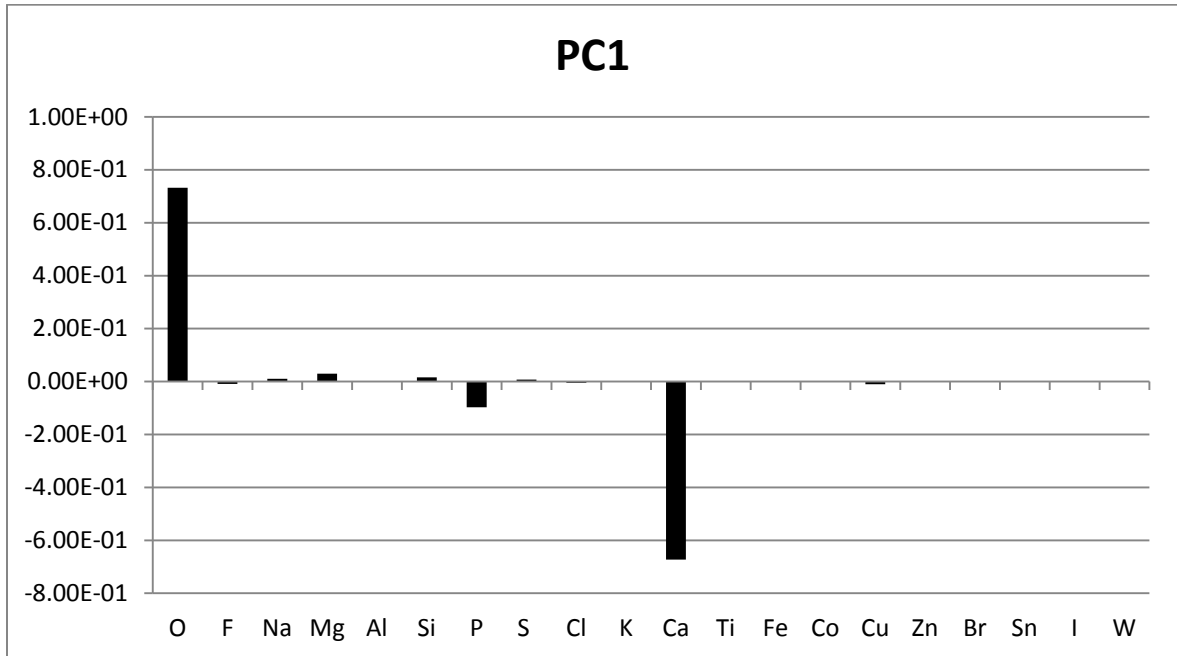
PC5 = 97.93%



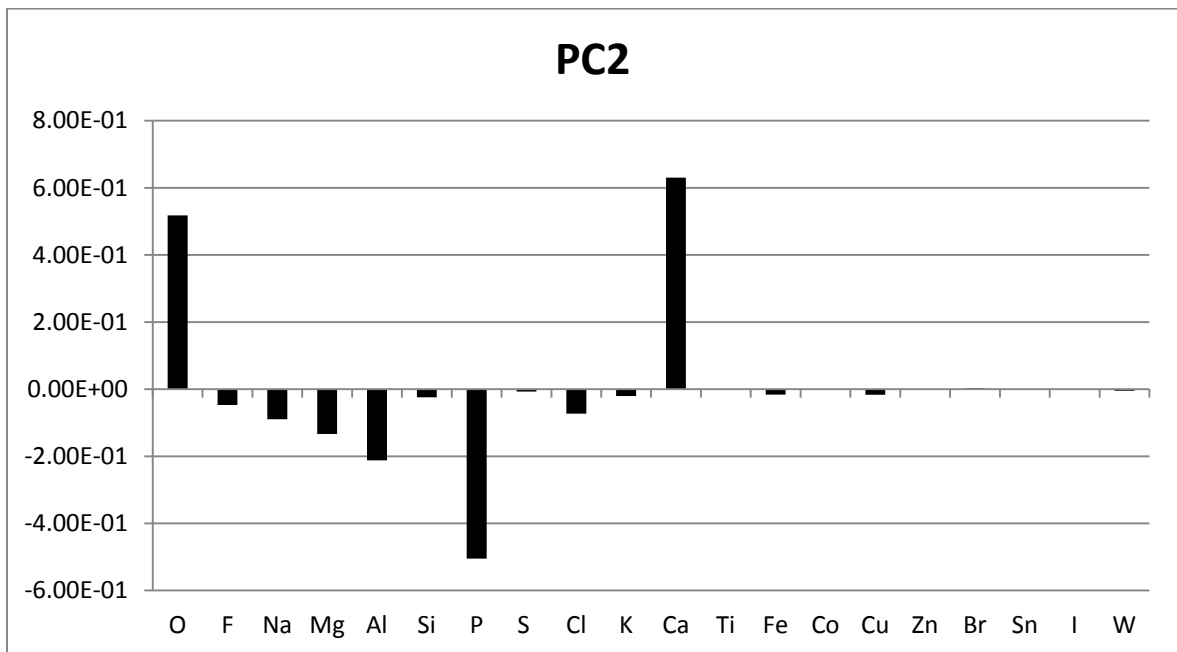
First four principal component loadings for human/non-human discrimination.

Percentages indicate the cumulative percent variation represented by each of the PCs.

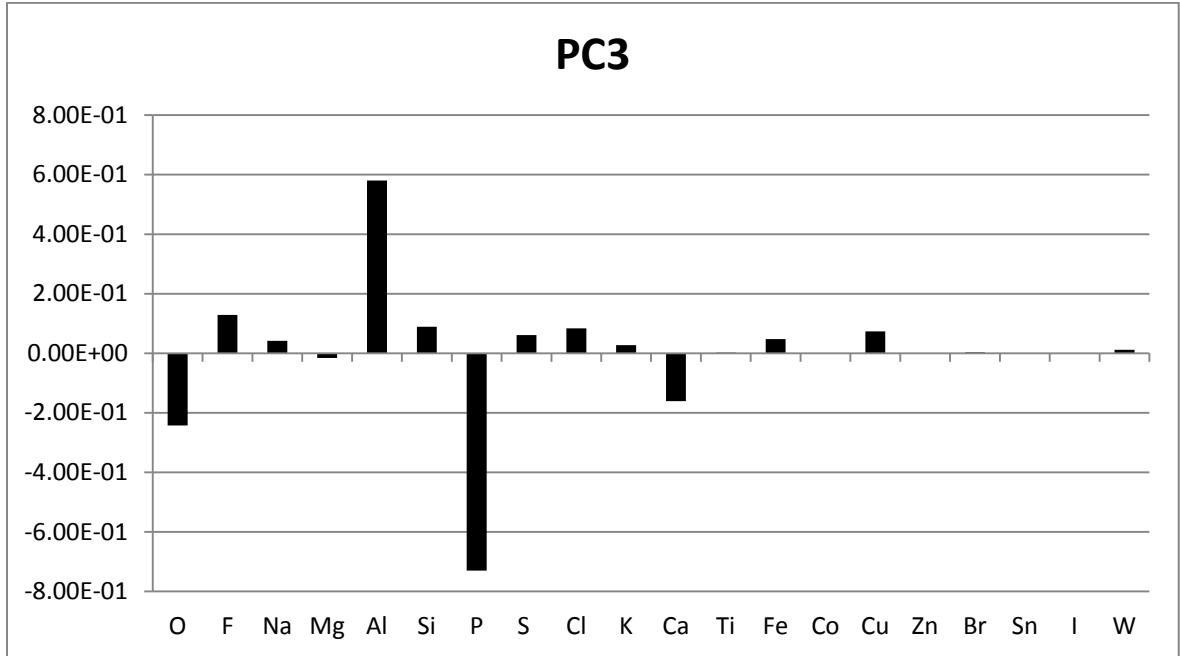
PC1 = 78.33%



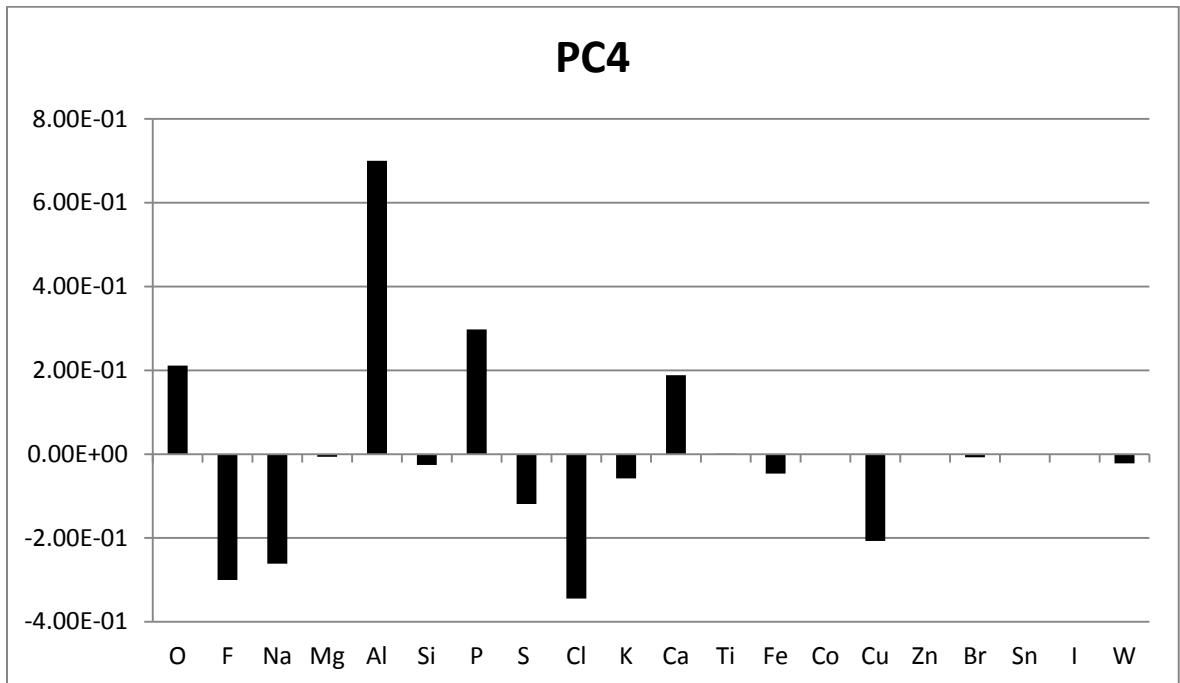
PC2 = 87.16%



PC3 = 93.54%



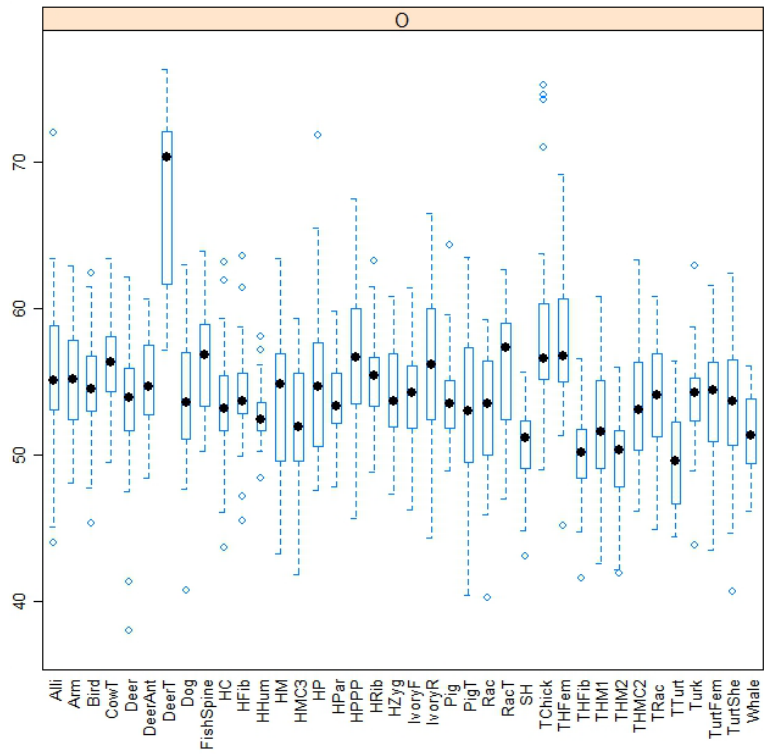
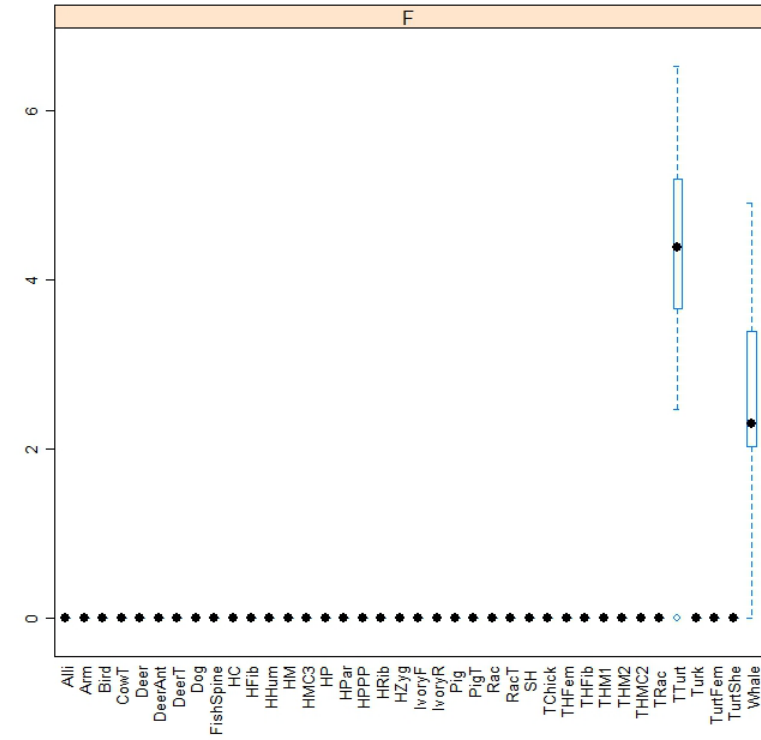
PC4 = 96.0%

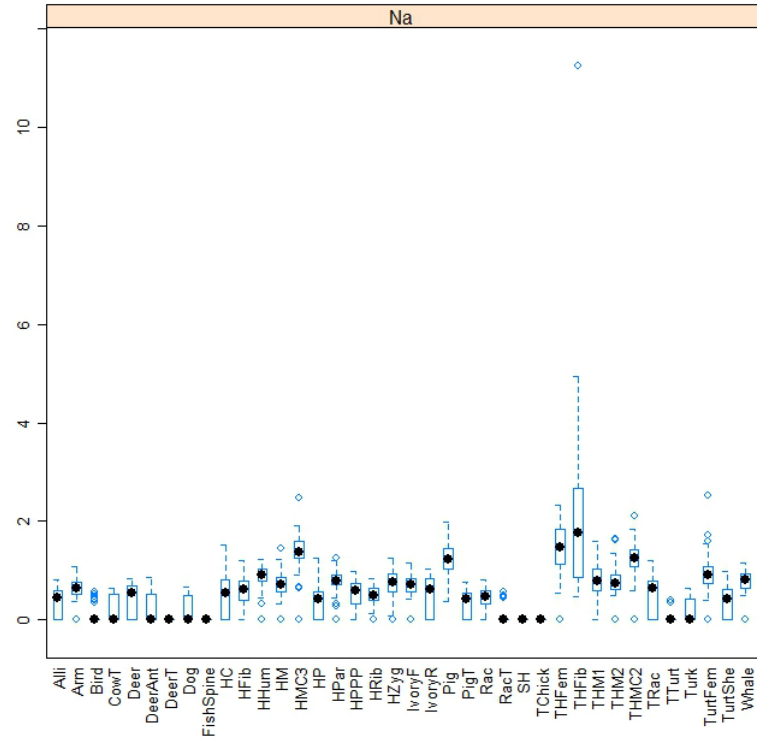
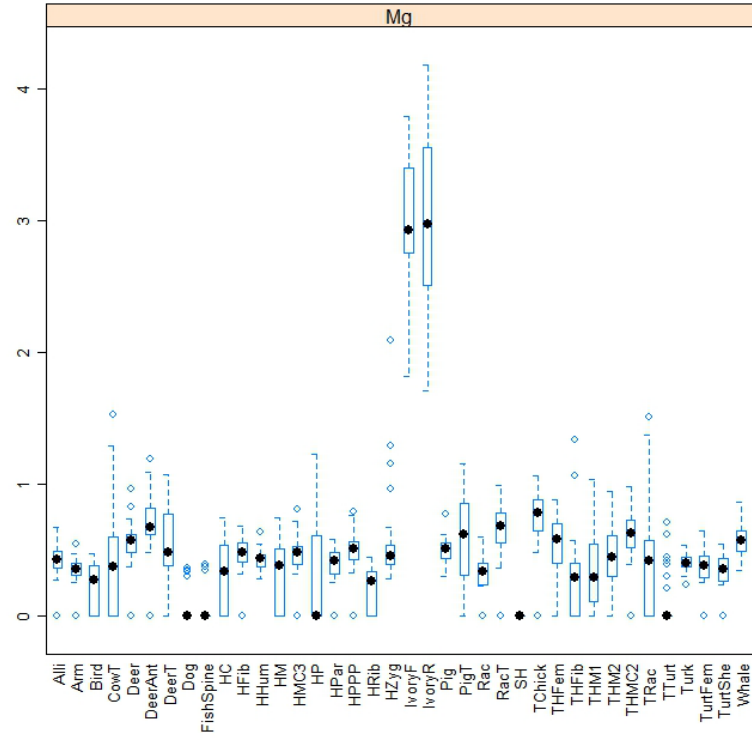


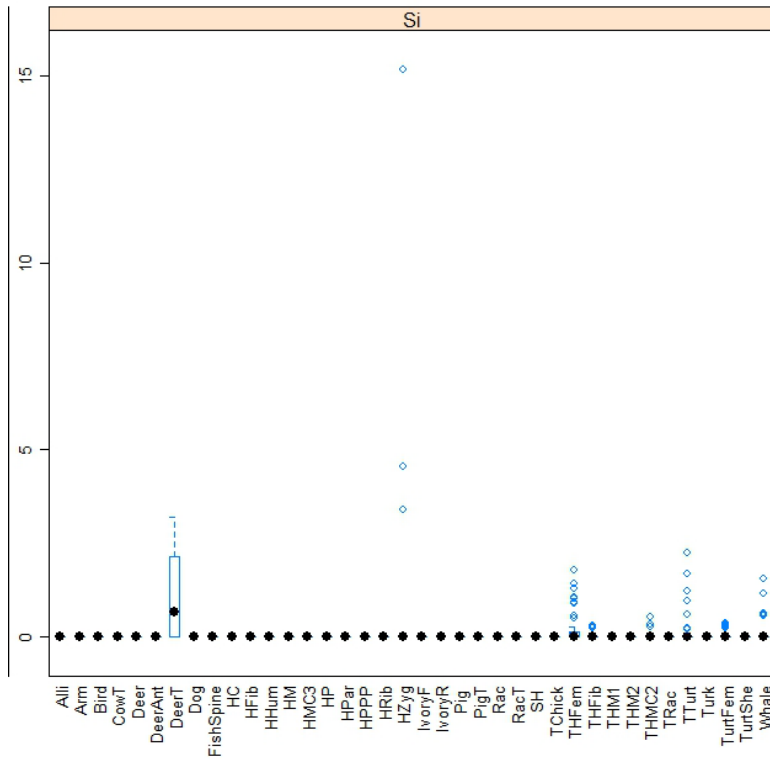
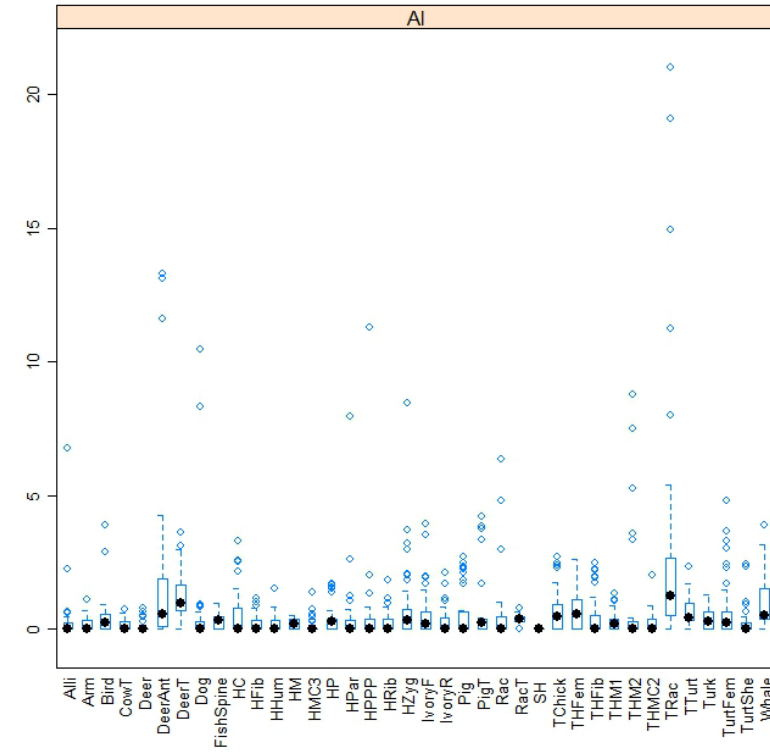
APPENDIX B: ELEMENTAL VARIANCE

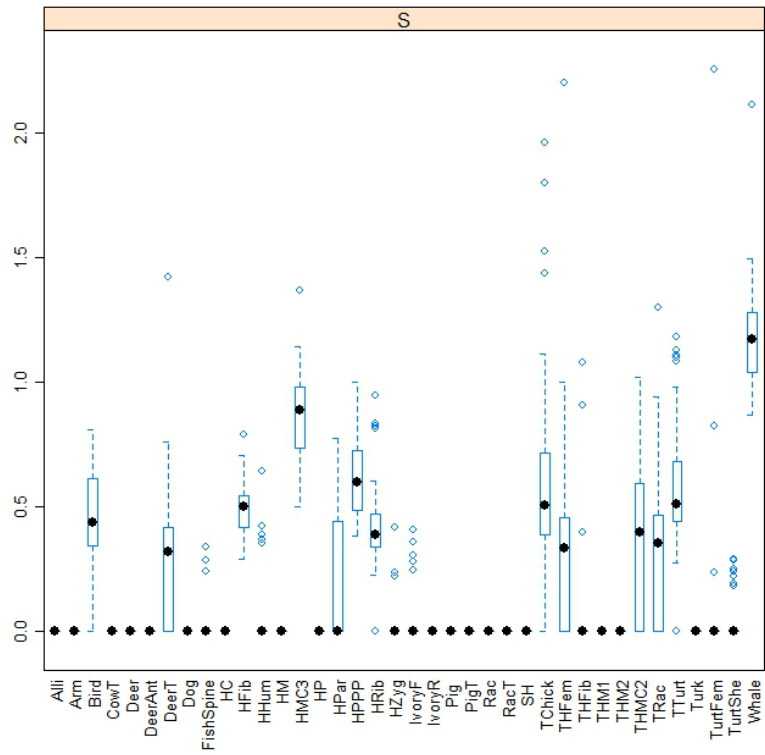
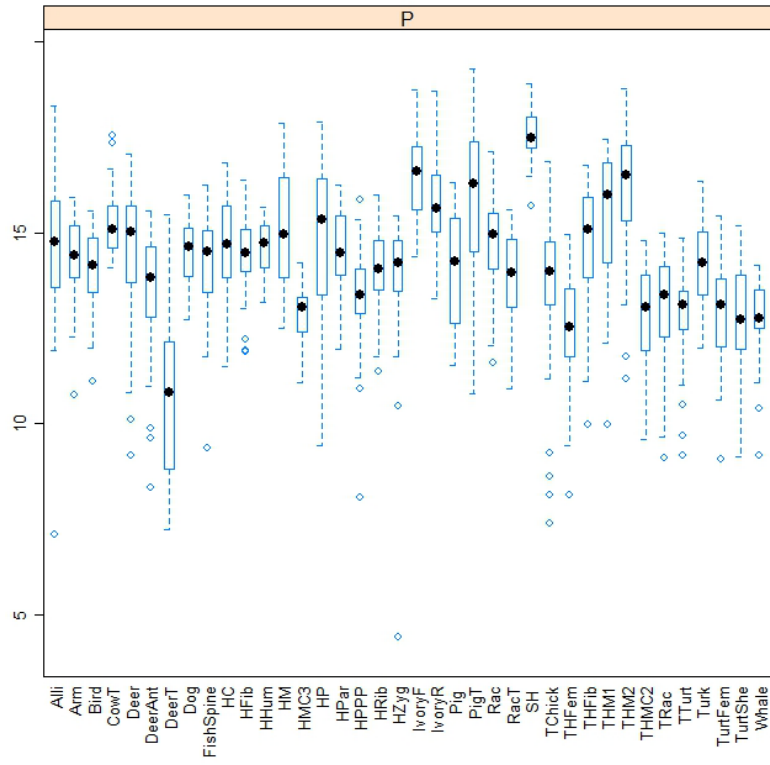
These box plots demonstrate the weight percent distributions of each element for each of the osseous samples. These demonstrate the relative homogeneity of each element. Some elements included in the final data set were not present in osseous samples (Co, Sn, I), these box plots are not presented. The black dots in the center represent the median values for each of the samples. The boxes extending from these represent the 25-75% brackets. The lines dashed extending from these boxes and the horizontal lines on the ends represent the maximum and minimum data values. Finally, the blue circles past these points represent outliers.

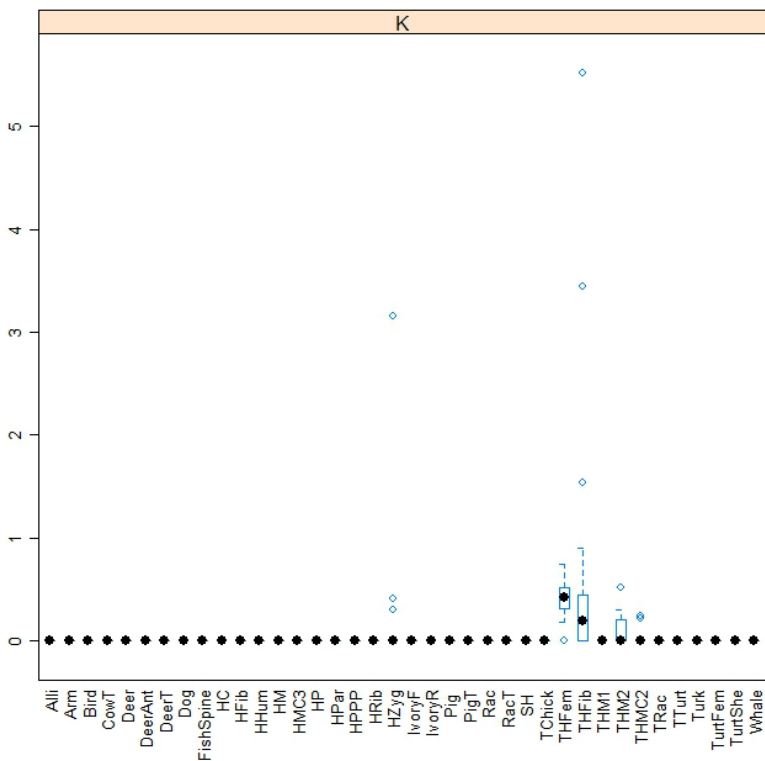
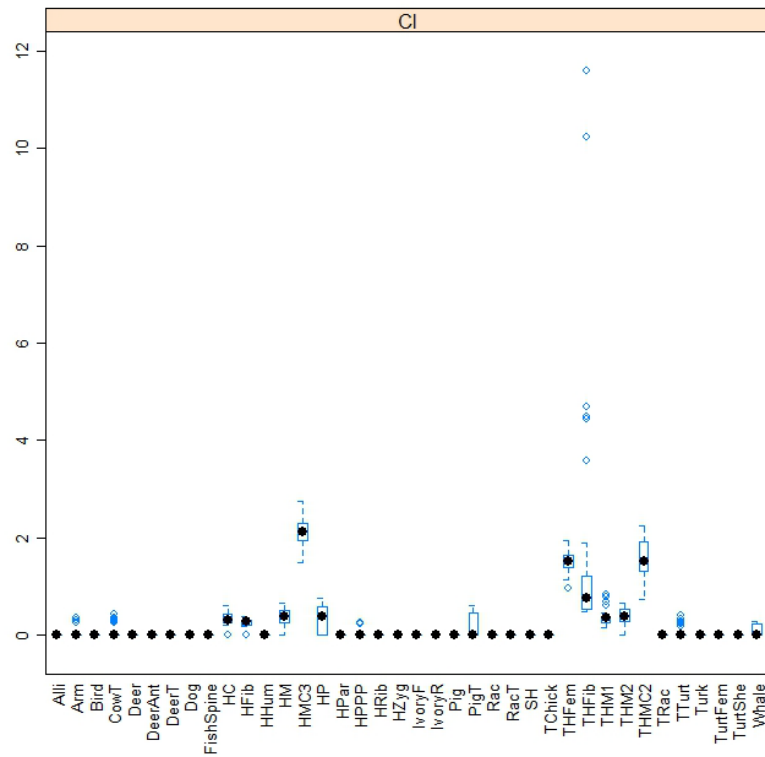
Sample designations are as follows: Alli (alligator), Arm (armadillo), Bird (bird), CowT (cow tooth), Deer (deer), DeerAnt (deer antler), DeerT (deer tooth), Dog (dog), FishSpine (fish spine), HC (human canine tooth), HFib (human fibula), HHum (human humerus), HM (human molar), HPar (human parietal), HPPP (human proximal pedal phalanx), HRib (human rib), HZyg (human zygomatic), IvoryF (ivory flat), IvoryR (ivory round), Pig (pig), PigT (pig tooth), Rac (raccoon), RacT (raccoon tooth), SH (synthetic hydroxyapatite), TChick (taphonomically modified chicken bone), THFem (taphonomically modified human femur), THFib (taphonomically modified human fibula), THM1 (taphonomically modified human molar 1), THM2 (taphonomically modified human molar 2), THMC2 (taphonomically modified human second metacarpal), TRac (taphonomically modified raccoon), TTurt (taphonomically modified turtle shell), Turk (turkey), TurtFem (turtle), TurtShe (turtle shell), Whale (whale).

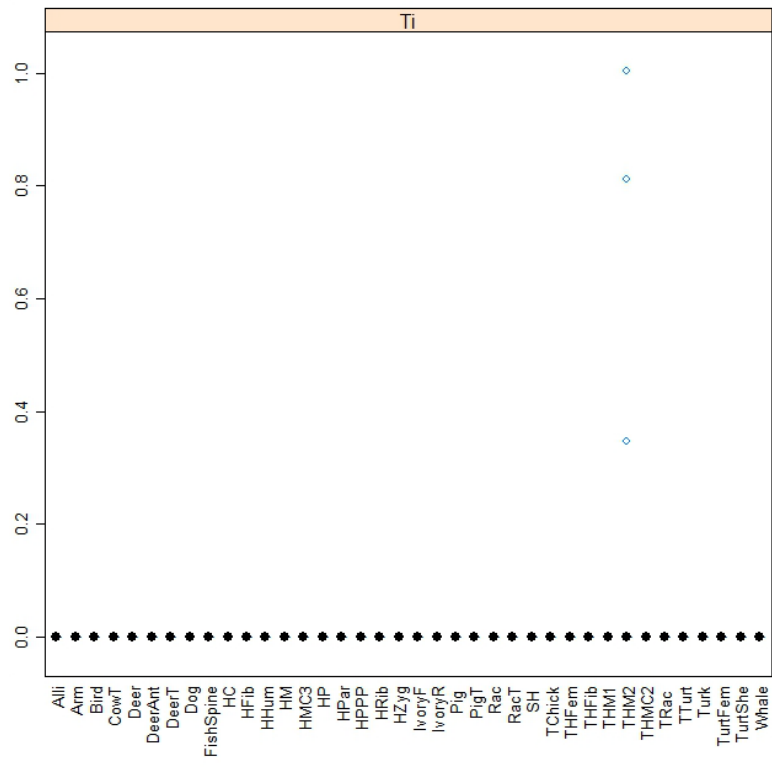
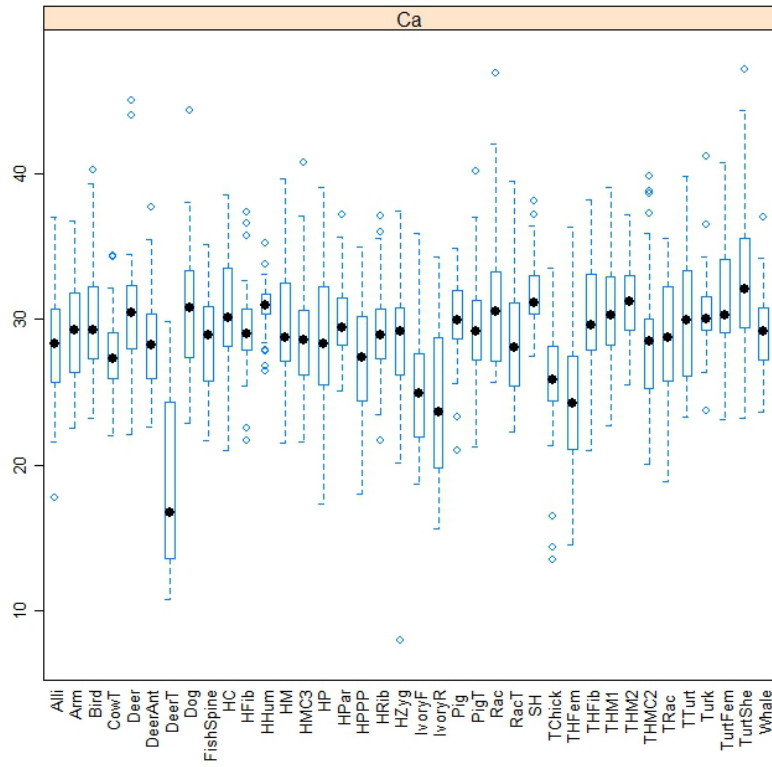


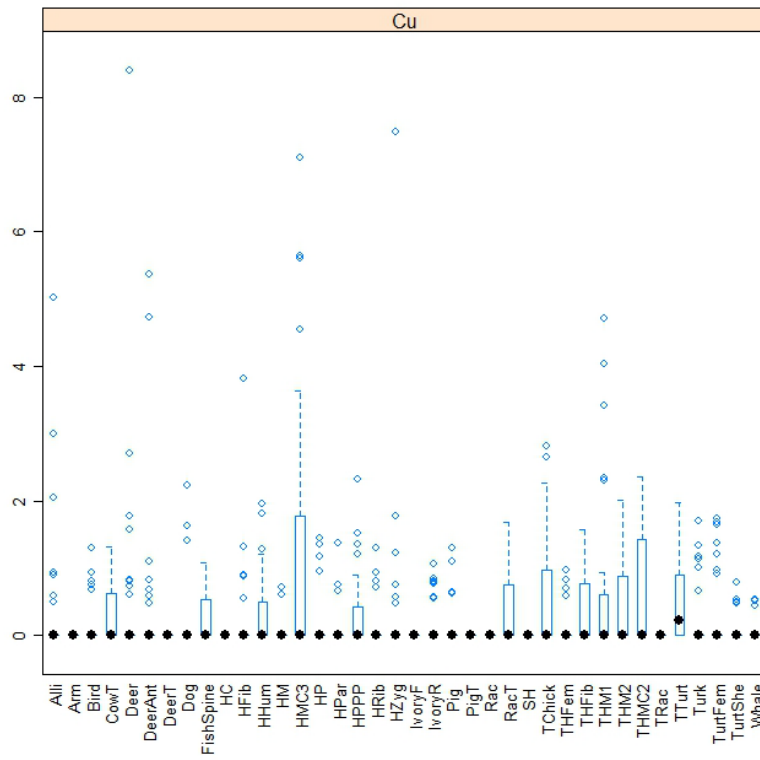
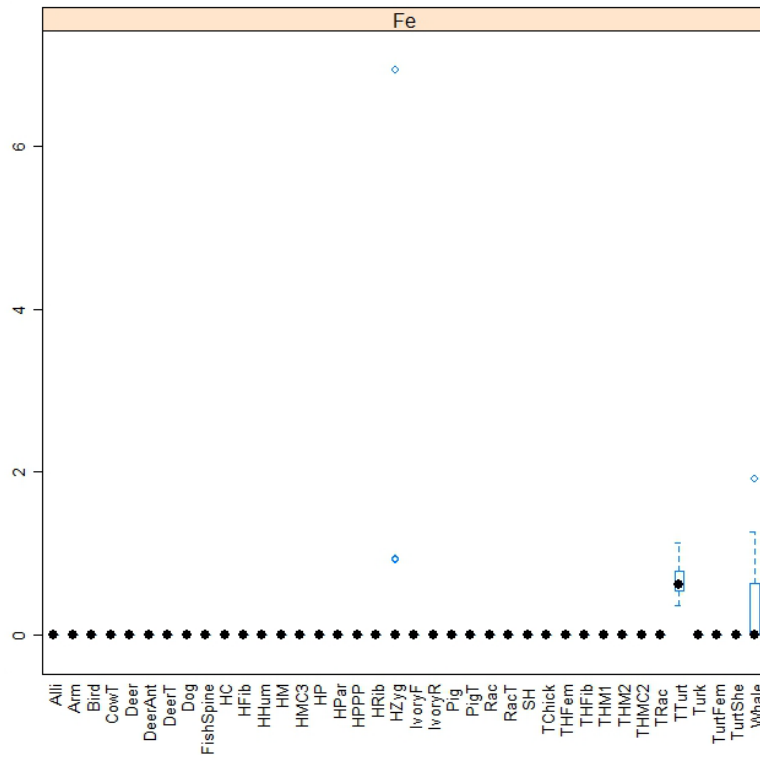


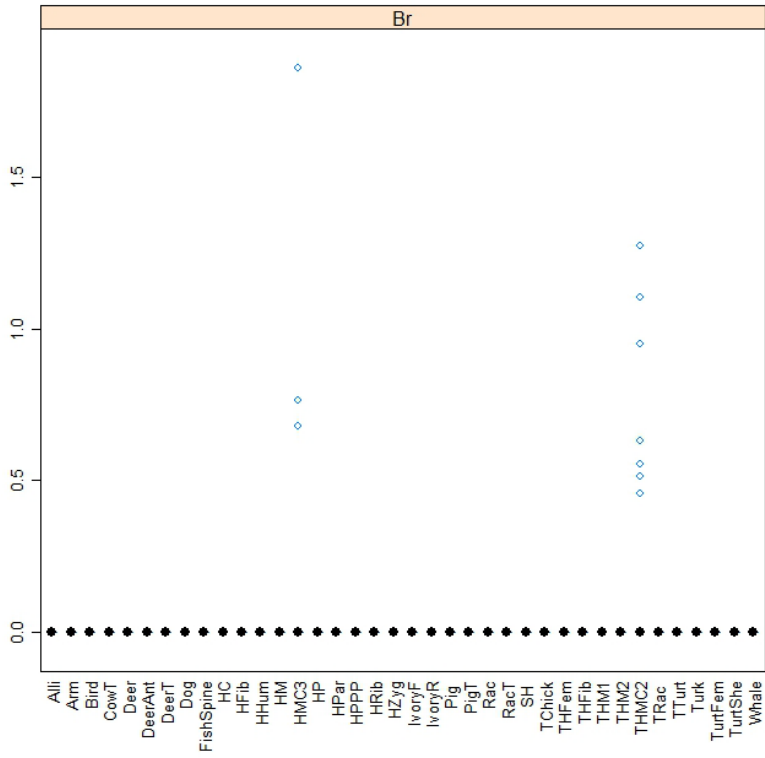
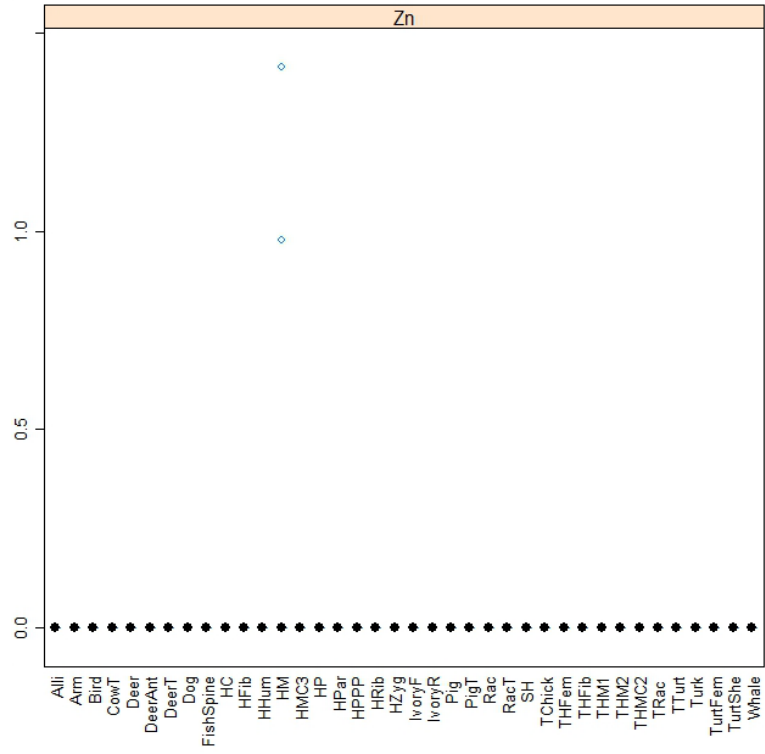


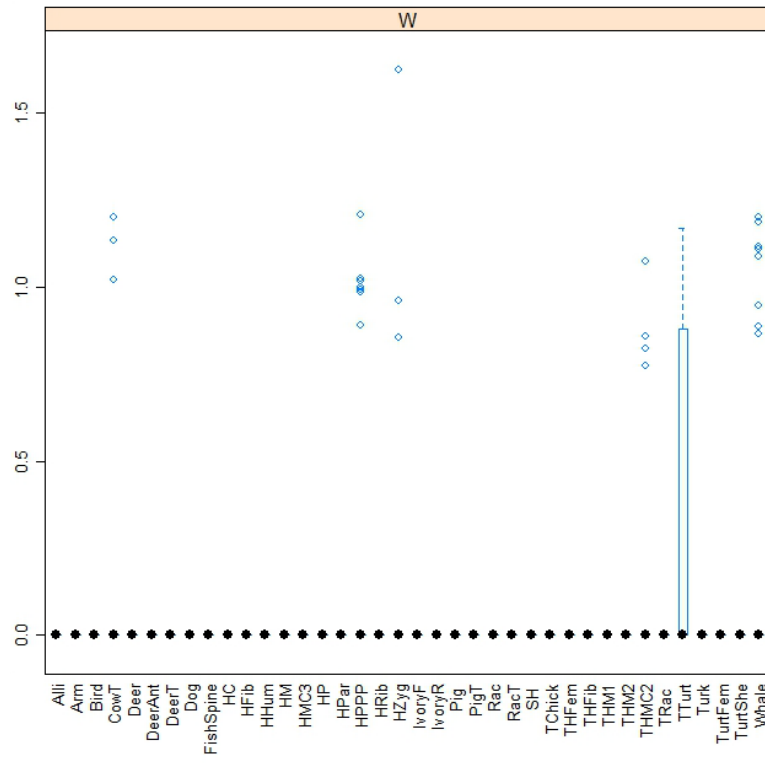






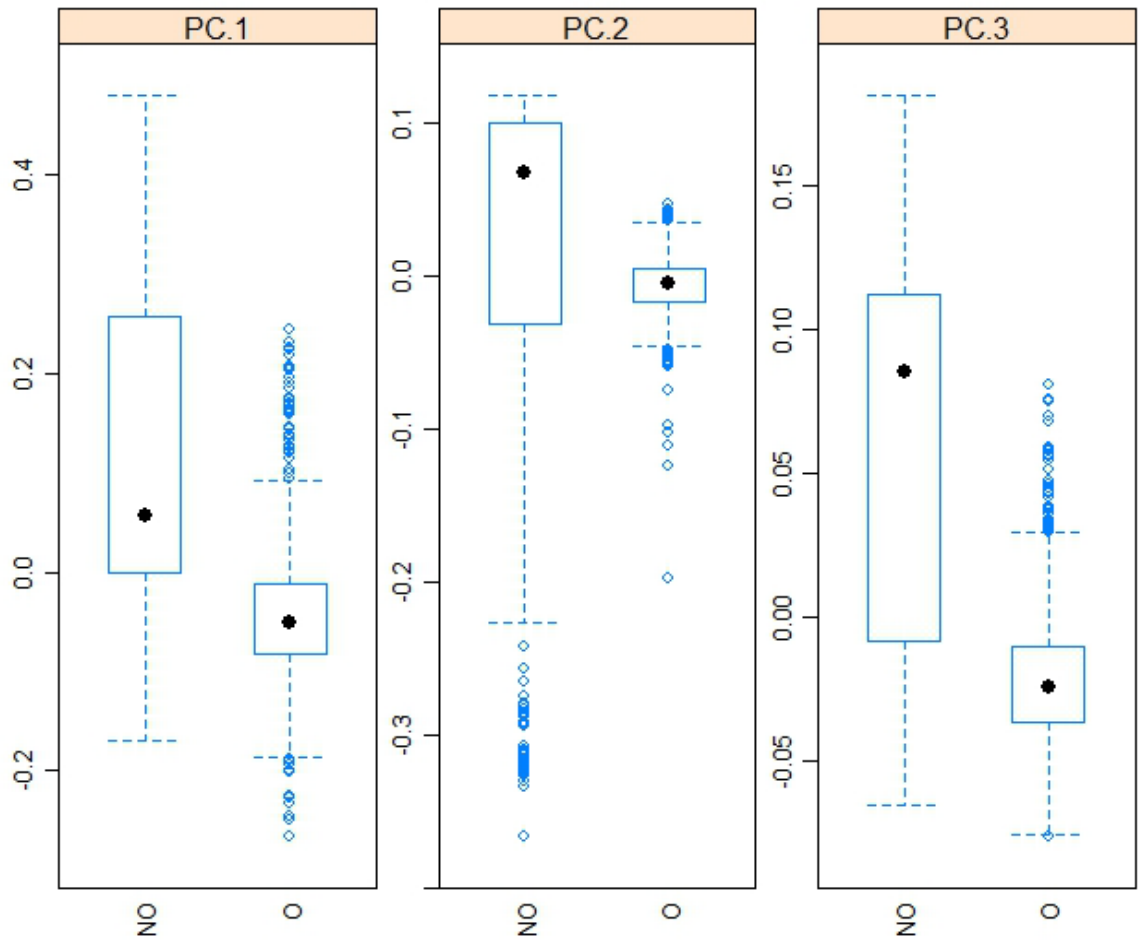




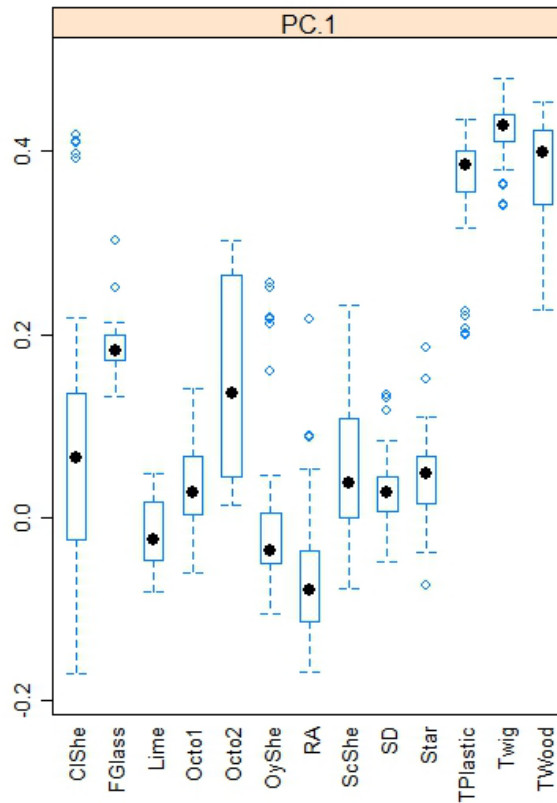


APPENDIX C: PRINCIPAL COMPONENT DISTRIBUTION

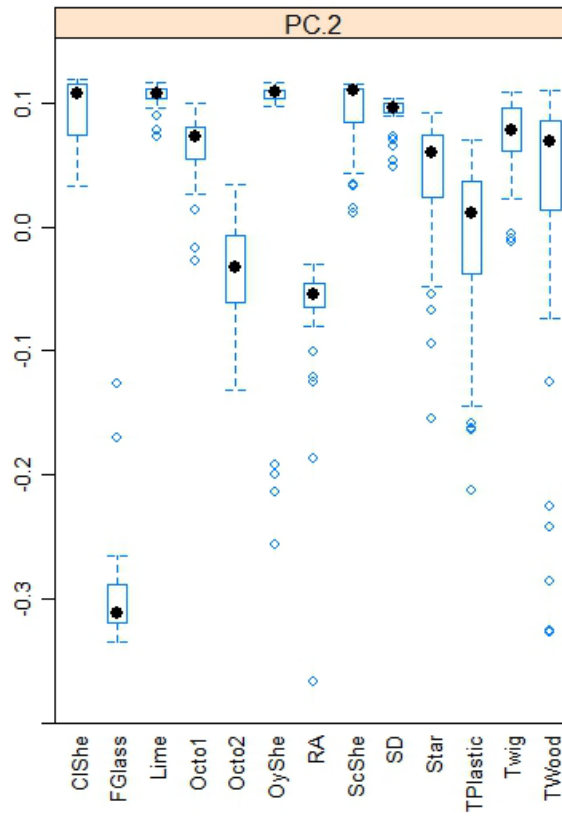
Box plots demonstrating the distribution of principal components 1-3 for osseous and non-osseous samples. NO represents all non-osseous samples and O represents all osseous samples.



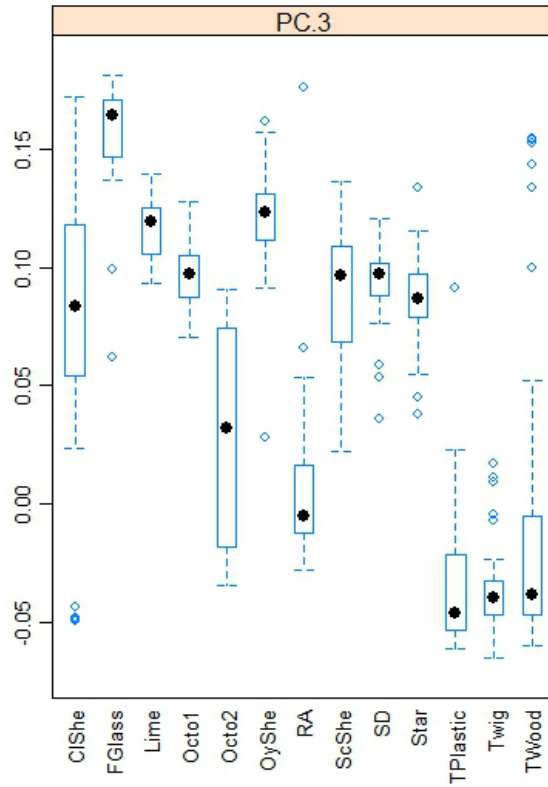
Box plot demonstrating the distribution of principal component 1 for all non-osseous materials. Sample designations are as follows: ClShe (clam shell), FGlass (float glass), Lime (limestone), Octo1 (octocoral species 1), Octo2 (octocoral species 2), OyShe (oyster shell), RA (rock apatite), ScShe (scallop shell), SD (sand dollar), Star (starfish), TPlastic (taphonomically modified plastic), Twig (twig), TWood (taphonomically modified wood).



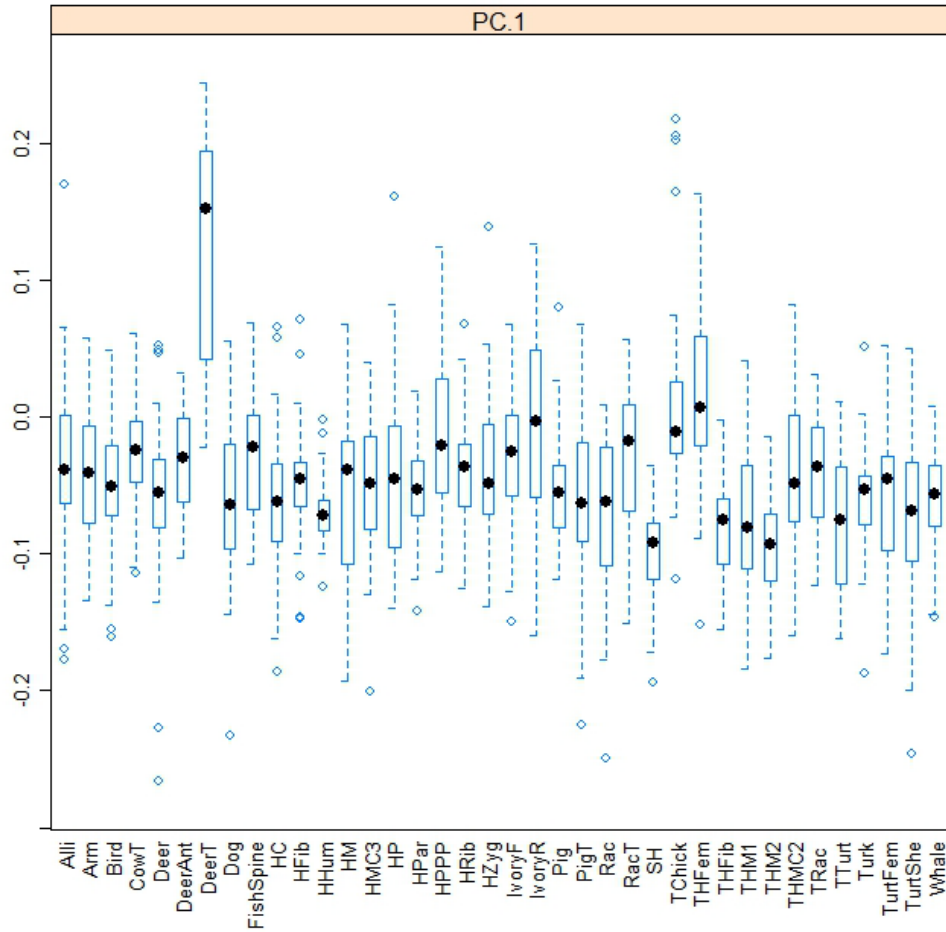
Box plot demonstrating the distribution of principal component 2 for all non-osseous materials.



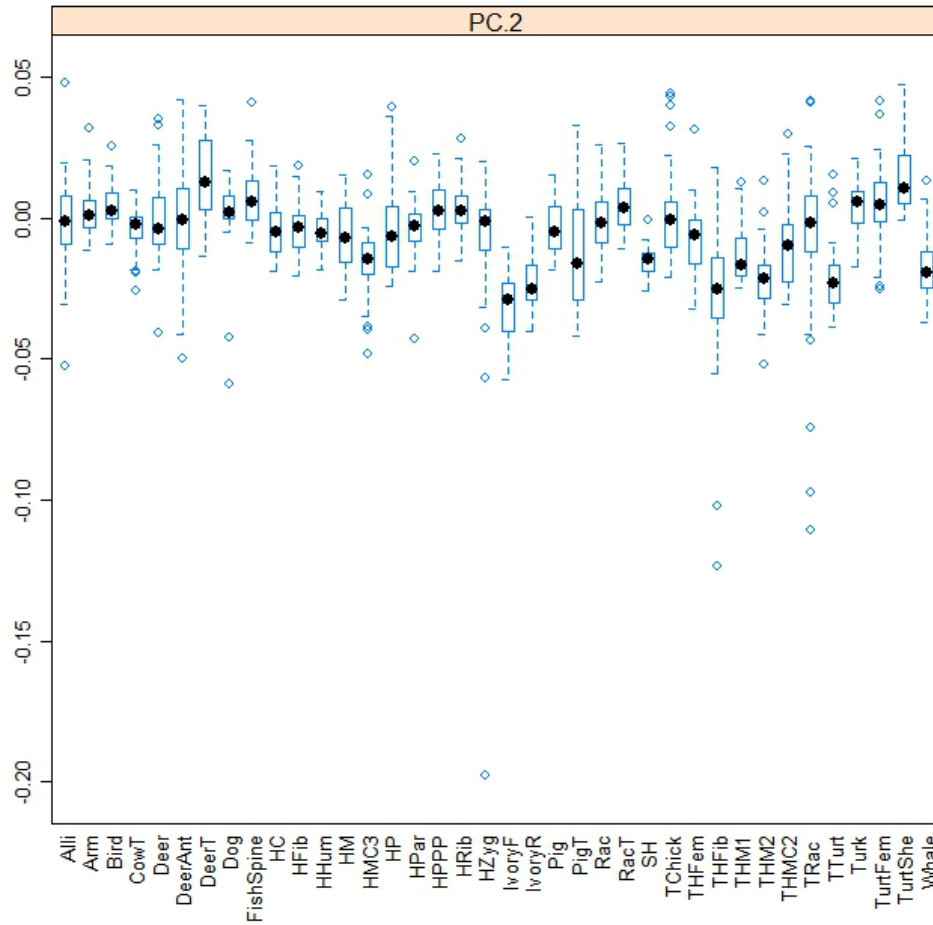
Box plot demonstrating the distribution of principal component 3 for all non-
osseous materials.



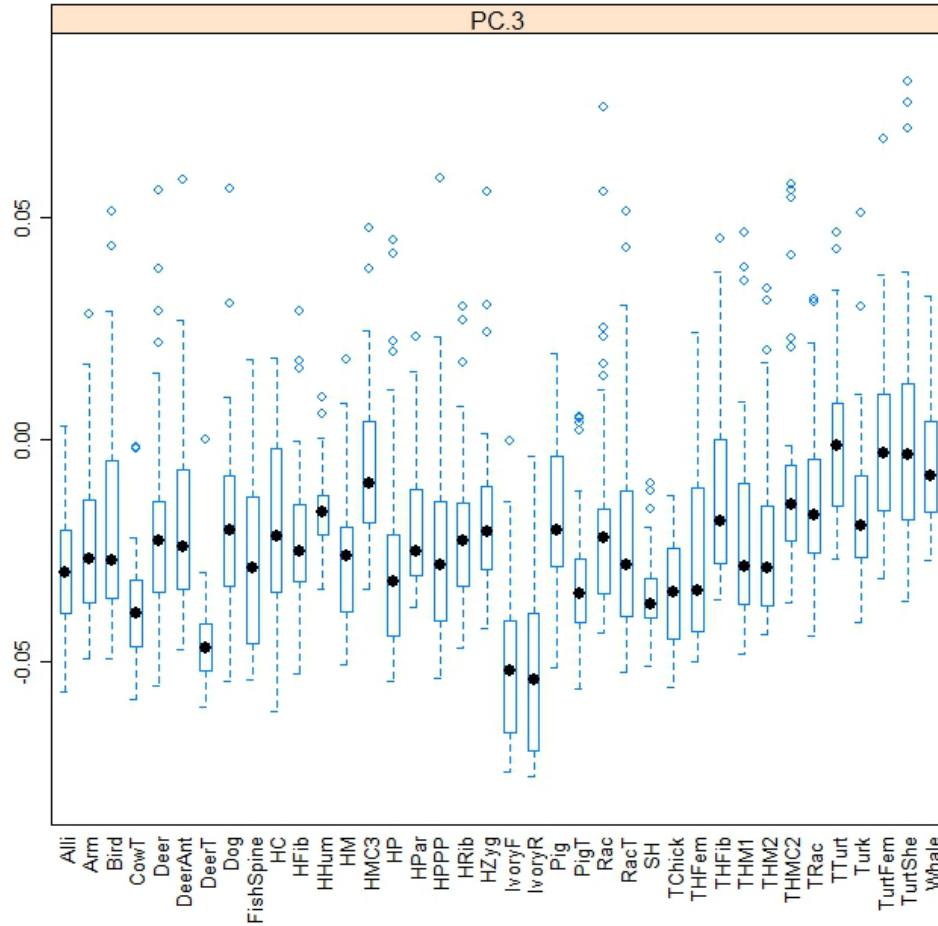
Box plot demonstrating the distribution of principal component 1 for all osseous materials. See Appendix B for sample designations.



Box plot demonstrating the distribution of principal component 2 for all osseous materials. See Appendix B for sample designations.



Box plot demonstrating the distribution of principal component 3 for all osseous materials. See Appendix B for sample designations.



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