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## CITRATE CONTENT OF BONE: A POTENTIAL MEASURE OF POSTMORTEM INTERVAL

### NIJ FINAL TECHNICAL REPORT

**Award Number: 2013-DN-BX-K031**

**Prepared by Ann W. Bunch (PI) and Michael A. Brown (Co-PI)**

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### ABSTRACT

The postmortem interval (PMI) of skeletal remains is a crucial piece of information that can help establish the time dimension in criminal cases. Unfortunately, the accurate and reliable determination of PMI from bone continues to evade forensic investigators despite concerted efforts over past decades to develop suitable qualitative and quantitative methods. Qualitative methods have come under greater scrutiny, since the publication of the National Academy of Sciences report in 2009. And the numerous quantitative methods (e.g., luminol, radionuclide,  $^{14}\text{C}$  bomb spike, DNA) that have been developed lack the accuracy and/or precision required for reliable PMI estimation.

A relatively new PMI method based on the analysis of citrate content of bone was developed by Schwarcz et al. (2010). The researchers reported that the citrate content of bone decreases with an increase in PMI and that the rate does not depend significantly on storage conditions. Kanz et al. (2014) performed an external validation study of this method using cemetery-derived bones with PMIs ranging from ~27 to 52 years. Their results suggest that the “accuracy of PMI determination was unsatisfactorily low” nevertheless this method may show promise for classifying samples as recent or historic. The main objective of our research was also to externally validate the work of Schwarcz et al.

Thirty-one bone samples were obtained from the Forensic Anthropology Center, University of Tennessee, Knoxville and the Onondaga County Medical Examiner’s Office. The samples were prepared using the procedures used by Schwarcz et al. with slight modifications to improve method performance. The citrate content (wt%) of each bone sample was determined by an ultraviolet-visible spectrophotometric enzymatic assay (UV-Vis assay) and by high-performance liquid chromatography (HPLC).

Initial studies focused on the assessment of method accuracy, precision, detection limit, and spike recovery. The accuracy for both methods was within  $\pm 5$  relative error and the precision was less than 2% relative standard deviation. The limit of quantification was estimated to be ~0.017 wt% citrate for both methods, which is similar to the value reported by Kanz et al. The method reporting limit, which is a more realistic value for detection limit, was found to be ~0.1 wt% citrate for both methods. A bone sample with a PMI of 173 years was analyzed in order to test the detection limit of the methods and resulted in a citrate value of 0.169 ( $\pm 0.006$ ) wt% for HPLC and just below the method reporting limit for the UV-Vis assay. Spike recovery values for all samples averaged in the range of 95 to 105%. Studies were also performed to establish the citrate content in remains of recently-deceased persons (PMI = 2 years or less). The average citrate content was found to be 1.21 ( $\pm 0.03$ ) wt% using HPLC and 1.19 ( $\pm 0.04$ ) wt% using the UV-Vis assay. These values were similar to the theoretical and experimental values reported by other researchers (Davies et al., 2014; Dunphy, 2014; Hu et al., 2010; Pysh, 2015; Zimmer, 2013). Results from analyzing

samples with PMI greater than 2 years suggest that the theoretical relationship between the citrate content of bone and PMI is much weaker than reported by Schwarcz et al. It was also observed that the average absolute error between the PMI value estimated using the equation proposed by Schwarcz et al. and the actual (“true”) PMI of the sample was negative indicating an underestimation in PMI. These findings are identical to those reported by Kanz et al. Despite these results this method may still serve as a technique to sort ancient from more recent skeletal cases, after further, similar validation studies have been conducted.

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## EXECUTIVE SUMMARY

The problem of determination of postmortem interval (PMI), or time since death, in questioned death cases has been a constant concern for forensic practitioners. The importance of this variable in medicolegal investigations cannot be overemphasized. The ability to establish a time frame is paramount in missing persons and unidentified remains' cases, because knowledge of the amount of time that has passed since a person has died can subsequently be utilized to identify "Jane/John Does" or to identify possible suspects that lack an alibi for that time. In a case where the PMI is determined to be greater than 50 years, typically it will be considered of non-forensic significance. The follow up protocol then will involve historic or pre-historic agencies (e.g., State Historic Preservation Office, i.e., SHPO) or local tribal authorities (under the Native American Grave Protection and Repatriation Act, i.e., NAGPRA). The reaction of investigating personnel oftentimes hinges on the PMI. If this variable is undetermined, it can delay the appropriate reaction and services. Thus this problem not only affects forensic scientists and courtroom testimony, but influences the practical considerations of the use of human resources (e.g., posting guards on 24 hour watch while PMI is being established) and fiscal responsibility (e.g., paying overtime for personnel utilized on unknown PMI cases, paying consultants and specialists to determine PMI by visual or non-standardized means).

Yet this pressing problem still has not resulted in an acceptable test or method that can firmly and precisely establish the PMI. One of the founding fathers of forensic anthropology in the United States, Wilton Marion Krogman (1973), described the problem thus:

"The problem with time-elapse since death is so complex that I'm not attempting to tackle it... There are too many unknowns, not the least of which may be careless or inexperienced exhumation. The soil may tell of primary or secondary inhumation; it may yield chemical (acid or base) evidence of the rate of decomposition; it may tell of flora or fauna (vegetation or insect action); it may tell of the mechanical factors of movement or water-seepage (a homogenous soil as in clays or glacial tills, or a heterogeneous soil as in gravels); the depth of the interment is very important; the swing of seasons and the amplitude of temperature change play their roles (in water depths temperature and factors of stasis vs. current movement are basic) (1973: 7)."

The many variables that can affect PMI determination are apparent with Krogman's summary of the problem, and his immediate focus on soil as a potential indicator of PMI is telling. When skeletal remains have been the focus, qualitative indicators such as surface texture and level of preservation have lacked precision and reliability, whereas quantitative indicators have shown to be affected by the multitude of variables at work on such cases.

More recent quantitative methods, such as those based on luminol (Ramsthaler et al., 2011), radiodecay/isotope test (Kandlbinder et al., 2009), <sup>14</sup>C bomb spike (Hodgins, 2009), and DNA (Kaiser et al., 2008) have been developed in order to improve the accuracy and precision of PMI determination. However these methods have specific issues that prevent them from being useful methods. Also centering on the skeletal remains themselves, a study by Schwarcz et al. (2010) showed that a potentially useful PMI estimation method appeared to be at hand with subsequent testing for external validation and reliability. Schwarcz et al. analyzed human and pig bone for citrate content during set time intervals to assess relationship with PMI. Schwarcz's team used ultraviolet-visible spectrophotometric enzymatic assay (UV-Vis assay) to determine the citrate content and compare that to the baseline that had been reported in the

literature for living mammals (~2.0 wt%). Schwarcz and co-workers found that citrate content of bone diminishes at a regular rate and becomes theoretically undetectable at a PMI of approximately 100 years. It was also reported that the decrease in citrate content may be unaffected by moisture and temperatures above 32 degree Fahrenheit. Dr. H. Schwarcz himself encouraged follow up studies to test the utility of this method for PMI estimation.

The PIs responded with a plan to evaluate the citrate method for human bone samples with known PMI obtained from collaborators at the Forensic Anthropology Center, University of Tennessee, Knoxville (UTK) and the Onondaga County Medical Examiner's Office (OCME) in Syracuse, New York. With additional samples with various PMIs, it was expected that a more robust study could be conducted that would support Schwarcz's findings or not. Along with the UV-Vis assay, our team utilized a high-performance liquid chromatography (HPLC) method to measure the citrate content of bone samples. This served as a confirmatory method for the UV-Vis assay throughout the project. It was shown that the HPLC method had slightly better sensitivity for citrate than the UV-Vis assay.

This study encountered challenges throughout with regard to replication of Schwarcz's method and results. Inter- and intra-sample tests resulted in varying citrate measurements in year one, so that Schwarcz's method had to be adjusted, so as to remove the pH adjustment step in order to obtain consistent data. In year two, the citrate content of 31 bone samples of known PMI was determined. None of these samples unfortunately had a low enough PMI necessary to validate the baseline citrate level (2 wt%) reported by Schwarcz et al. However analysis of fresh porcine bone samples in year one did not result in a value close to 2.0 wt%. Others report the same concerns in recent publications (Dunphy, 2014; Kanz et al., 2014; Pysh, 2015; Zimmer, 2013). In addition, the regular decline of citrate over time was not replicated in our study. Also it was seen that the relative error between the PMI value predicted by the equation proposed by Schwarcz et al. and the actual PMI of the samples was negative indicating an underestimation of PMI. Interestingly the testing of one human bone sample with a PMI of approximately 173 years did show that citrate content does decrease over time; yet, unlike Schwarcz's results, citrate is still detectable (by HPLC) post 100 years.

Our conclusions are that citrate is not a reliable and validated method for determining PMI in bone as suggested by Schwarcz et al. It certainly does not demonstrate the accuracy in PMI estimation that was implicit in Schwarcz et al.'s work. However, the pattern of decline in citrate content in bone with the passage of time was generally demonstrated with samples with PMIs and additional work should be conducted with samples with known PMIs that date between 50, 100, 150 and 200 years. Additional work must also be performed to assess the baseline (living) citrate amount of 2.0 wt% reported by Schwarcz et al. and other literature cited by these scholars. Is it possible that variables may be at work to affect living baseline citrate? Does sex of the individual have an impact? Does nutrition of the deceased, age at death, and/or bone diseases such as osteoporosis have a significant impact upon citrate content?

If follow up studies show that samples of 150 to 200 years have trace amounts of citrate or none that is determinable, this knowledge could assist in "triaging" cases in the field that require different dispositions (pre-historic, historic versus forensically significant remains). Skeletal remains of unknown PMI could thus be tested for the presence of citrate; if it is present, with further studies we could possibly determine that the bone is less than 200 years in PMI. This would necessitate follow up to rule out forensic significance. Any bone that was tested and shown to be devoid of citrate, would be ruled out for forensic significance.

## I. INTRODUCTION

### 1. Research Problem

The accurate determination of postmortem interval (PMI) of skeletonized remains continues to evade forensic investigators despite concerted efforts over past decades to use qualitative and quantitative methods. Qualitative methods have come under greater scrutiny since the publication of the National Academy of Sciences (2009) report. The work of Schwarcz et al. (2010) suggested that the citrate content of human skeletal remains could serve as a potential measure of PMI. Their results seemed to show that citrate may act like a clock, diminishing in postmortem bone at a regular rate up to a theoretical limit of approximately 100 years. Kanz et al. (2014) performed an external validation study of Schwarcz et al. citrate-based method using cemetery-derived samples. These researchers found that this method provided inaccurate results with the experimentally determined PMI being lower than the actual PMI of the skeletal remains.

The major goal of this NIJ funded project was to perform an external validation the method developed by Schwarcz et al. We employed our working relationships with collaborators at the Forensic Anthropology Center, University of Tennessee, Knoxville (UTK) and the Onondaga County Medical Examiner's Office (OCME) in Syracuse, New York, who kindly agreed to provide us with known-PMI human bone samples. Thirty-one samples were analyzed during the course of this project. This is the largest sample of known-PMI human bone analyzed by the UV-Vis assay and HPLC methods to date. We employed a larger sample of known PMI remains from both a medical examiner's office and the Forensic Anthropology Center, University of Tennessee, Knoxville. Along with Schwarcz et al.'s method, we simultaneously used a high-performance liquid chromatography (HPLC) as an alternate method for the determination of citrate content of bone. The use of two analytical methods was envisioned as a way to provide an assessment of method accuracy and reliability and to identify and correct for systematic errors if present.

### 2. Literature Review

As aforementioned, the problem of PMI has been vexing forensic scientists and death investigators for decades. Attempts have been made to develop a PMI estimation method for decomposing bodies as well as skeletal remains. These methods have been qualitative and quantitative in nature and have employed remains themselves (e.g., Berg 1963; Knight and Lauder 1969; Taylor et al. 1989; Castellano et al. 1984; Yoshino et al. 1984; Perry et al. 1989) as well as indicators from the surroundings (e.g., soil, air, plants) (e.g., Vass et al. 1992; Vass et al. 2002). To date, a method that has resulted in precise PMI estimates has been demonstrated. Schwarcz et al. presented such a method that purportedly had an estimation error of  $\pm 1$  year. A review of the literature on PMI estimation follows with a final, detailed focus on the method of Schwarcz et al.

#### 1.1 Soil Analysis and Detection of Biomarkers

The use of soil from the scene has again more recently been suggested and investigated as a possible focal point of PMI determination (Carter et al., 2008; Vass et al., 1992, 2002). Vass et al. (1992, 2002) has brought this potential method to the forefront, e.g., via analysis of soil for volatile fatty acids (VFAs) and ions, such as sodium, calcium, potassium, ammonium and magnesium ions and other biomarkers produced by a decaying body. Others have followed up on Vass' work, shifting to the analysis of volatile organic

compounds (Statheropoulous et al., 2005). Suspected grave soil may also be presumptively tested with 2, 2-dihydroxyindane-1, 3-dione (ninhydrin) (Carter et al., 2008); however the time and manner of diffusion of ninhydrin reactive nitrogen into the soil is unknown. These methods clearly are limited in scope to more recent deaths where decomposition products are actively being produced and dispersed into the surrounding environment.

### *1.2 Use of Insects (Forensic Entomology)*

Insects have also been used to determine PMI with some success (e.g., Catts & Goff, 1992; Higley & Haskell, 2001). The sequence of colonization can fine tune the timing of the deposit of remains and the amount of exposure time in cases of relatively recent deaths. It is considered one of the most accurate methods available; however the accuracy and precision of PMI estimates based on insect data breaks down rapidly with the loss of soft tissue. Once remains are completely skeletonized, the utility of insects is severely limited or negligible.

### *1.3 Qualitative and Quantitative Assessment of Decomposition Stages*

Other studies have taken a more descriptive approach, using a variety of data sources to create stages of decomposition that could be predicted in most cases (Love & Marks, 2003; Mann et al., 1990; Micozzi 1991; Rodriguez & Bass, 1985). The actualistic studies allow for investigators to trace the decomposition process in a variety of situations and contexts with nominally designated stages, e.g., “fresh,” “discoloration,” “bloat,” “skeletonization,” and “skeletal decomposition” (Love & Marks, 2003). When to expect these stages to occur is widely variable and dependent on numerous variables. Individual bodies placed within a square mile of one another (Bunch, 2009) or even a few meters (Srnska, 2005) may reach the stages at different times. Thus, the only generalizable rule that seems to come from these qualitative studies is “every case is unique” – a dishearteningly non-scientific statement.

An alternative approach to quantify PMI for skeletonized and decomposing remains cases was published by Megyesi et al. (2005). With this approach, the use of the key variable of temperature in the form of Accumulated Degree Days (ADD) is employed, matching the technique of forensic entomologists. Rather than attend to the passage of time, researchers begin by scoring the state of decomposition that the remains are in at the time of discovery. With the resulting index (based on three portions of the body) the ADD is estimated and local temperature data is used to account for each passing day’s average temperature. When the ADD total is reached, an estimate of PMI is established. This method has been tested by Michel and Moreau (2011) and others (Bunch, unpublished manuscript) with limited success.

### *1.4 Chemical Analysis of Skeletal Remains for the Determination of PMI*

The skeletal remains (bones) themselves have been focused upon by other researchers who have employed chemical analysis methods. Berg (1963) used a number of factors to assess PMI to include: carbonate levels, ultraviolet fluorescence, radiographic structure, serological protein levels, etc. Knight and Lauder (1967) and Knight and Lauder (1969) followed up on Berg’s work analyzing nitrogen content, amino acid content, anti-human serum reaction, and other variables. These biochemical tests showed promise, e.g., use of nitrogen content allowed researchers to discern bone of 50 to 100 years of age, amino acids approximately 100 years of age, and anti-human serum gel diffusion was able to distinguish remains of < five years (Sledzik, 1998).

Carbon dating is a technique that is commonly applied to dating historic or prehistoric specimens that are greater than 500 years old; however forensic scientists, such as Taylor et al. (1989) and Hodgins (2009) have utilized a related technique in the determination of PMI. Typically  $^{14}\text{C}$  is formed at a constant rate in the atmosphere when cosmic radiation bombards nitrogen resulting in a nuclear reaction. The  $^{14}\text{C}$  then is dispersed as  $^{14}\text{CO}_2$  into the atmosphere and water where it is incorporated into plants by photosynthesis and eventually into all living organisms by means of the food chain (Bulman & McLeod-Henning, 2012; Hodgins, 2009). The international nuclear testing that took place during the 1950s and 1960s resulted in a significant increase in the abundance of  $^{14}\text{C}$  in the atmosphere, thus increasing the level of  $^{14}\text{C}$  in organisms (Bulman & McLeod-Henning, 2012; Hedges et. al., 2007; Hodgins, 2009). By determination of the “bomb curve” from  $^{14}\text{C}$  in dental and osseous tissues, researchers have been able to estimate birth year and to some extent the time of death. The main advantages of this method are that it is based on a “worldwide phenomenon” that can be continually measured each year and the levels of  $^{14}\text{C}$  from this event are not “significantly altered after death by radioactive, biological, or chemical decay.” Unfortunately, the major drawback is that the best estimates of PMI come from soft tissue analysis not skeletal remains. Specimens of forensic interest (modern) can still be sorted from those that are pre-modern or non-modern. Three categories are delineated for bone age: (1) non-modern (before AD 1650), pre-modern (AD 1650 to 1950), and modern (AD 1950 to present). This method works well for triaging remains as to their forensic importance. Ubelaker et al. (2002) employed bomb curve  $^{14}\text{C}$  levels to analyze dental, cortical and trabecular bone of two (2) individuals of known birth and death years. The formation and/or remodeling of these different tissues allows for a relatively accurate estimate of age at and time of death. Yet the three different types of tissues must be available for this technique to be accurate, a luxury that many forensic cases do not provide. In addition, no studies have been done to date on taphonomic and diagenic influences that may affect the precision and accuracy of this method.

A related but different isotopic analysis method was employed by Kandlbinder et al. (2009) for determination of PMI. This method is based on the technique of determination of radionuclides (radioisotopes) in bones. Radionuclides are ingested naturally by humans over time. When an individual dies the uptake of these radionuclides ceases, and the decay process begins. The activity ratio of the decay products and the parent isotopes are determined and used to estimate the PMI. In their research, Kandlbinder et al determined the activity concentrations of the radionuclides radium-228 ( $^{228}\text{Ra}$ ), thorium-228 ( $^{228}\text{Th}$ ), thorium-232 ( $^{232}\text{Th}$ ), and thorium-230 ( $^{230}\text{Th}$ ) with spectrometry techniques sensitive for  $\alpha$ ,  $\beta$ , and  $\gamma$  particles generated from the decay process. The main advantage of this method is that nutrition, age of the deceased, and decomposition variables have minimal effect on the final results. The main disadvantages include: ~300 g of skeletal material is required, the useful range is between 0.5 to 10 years for PMI, thorium (in some soil) leads to erroneous results for PMI, the long sample preparation time (ample counting time between 6 to 15 days), high uncertainty of measurements, expensive instrumentation, high operator skill level required, and can be quite math intensive. Overall, this method does not seem well suited for PMI determination as it is currently configured. More research by Schrag et al. (2014) investigated using  $^{90}\text{Sr}$  bomb pulse dating along with  $^{210}\text{Pb}$  radionuclide content to determine PMI. Even though the accuracy was not ideal the method was able to successfully discard a third of samples as being “of archeological interest” using the method. The main drawbacks include weathering leaching effects, diagenesis factors, impact by user smoking during life, and need for normalization of calcium content.

The application of luminol for the determination of PMI has been applied by Introna et al. (1999) and Ramsthaler et al. (2011). The most common application of this method is the detection of trace blood at crime scenes, thus it was presumed to work for remains in general. It is based on the reaction between

hydrogen peroxide and luminol, which is catalyzed by hemoglobin (iron source) found in blood, bodily fluids, or tissues. The reaction results in a chemiluminescence that can be qualitatively or quantitatively measured. It has the advantages of being relatively fast, simple to use, and less expensive than most methods. However it also has the potential of having a positive reaction with other fluids and/or muscle tissue and has decreased chemiluminescence intensity as the PMI increases leading to increased negative test results, thus limiting the accuracy and precision of the method. Both Introna et al. and Ramsthaler et al. did not directly quantitate the intensity of the luminol reaction, but instead applied qualitative ratings of intensity. The authors acknowledge that a more quantitative approach is warranted. In addition to this method, Ramsthaler et al. also investigated a UV-fluorescence intensity method and commercially available screening tests designed for blood analysis. Unfortunately, the test strips did not provide positive results for any samples and the UV-fluorescence intensity measure only provided qualitative information at best. The luminol method does show promise, but will need further quantitative testing to be a viable option for the determination PMI.

Additional methods have also been developed such as those that use imaging techniques like infrared, and X-ray (Longato et al., 2015). Unfortunately, this research is somewhat preliminary and needs larger sample sets to development more robust predictor algorithms. Researchers have also investigated using DNA degradation (Kaiser, 2008) and content to estimate PMI with little success.

### *1.5 A New Method for the Determination of PMI using Citrate Content in Bone*

The exterior layer of bone is principally composed of bioapatite crystal and protein (collagen). The past work of Dickens (1941), Taylor (1960) and the work of Hu et al. (2010) has suggested that citrate (see Figure 1) is primarily bound to calcium on the surface of the apatite crystal layer, because citrate is too large to be incorporated into the crystal lattice; however the surface area required to incorporate enough citrate to equate to 2 wt% is not feasible.



**Figure 1.** Chemical structure of citrate ion.

More recent work by Davies et al. (2014) has suggested citrate bridges “between mineral platelets in bone” to give strength and stability to bone. Surprisingly, the role of citrate (citric acid) in bone has been studied for quite some time. Early research by Dickens, Dixon and Perkins (1952), Kenny et al. (1959), and Taylor (1960), investigated the level of citrate in bone as well as the role of citrate in bone metabolism and structure as well as the relationship to carbonate fractions and other factors. Despite early interest in citrate content of bone this area has been largely neglected by researchers over the past 40 years. Costello et al. (2012) recommended to the scientific community that citrate is “essential” for the formation of normal bone and “these and other relevant issues need to be addressed in order to provide a new understanding of bone biology and pathophysiology.”

Recently a study by Schwarcz et al. (2010) focused on citrate content of bone in order to establish PMI. This experimental study demonstrated that citrate decreases at a regular rate up until approximately 100 years postmortem, when it is no longer measurable (below detection limit of method or absent). The decrease in citrate interestingly does not occur if the bone is not exposed to the environment, e.g. under a hood in a laboratory. Although Schwarcz et al. did not explain the mechanism by which the citrate is being removed from the bone; they did replicate this effect in pig (*Sus scrofa domesticus*) rib samples, exposed to the environment for varying degrees of time (ranging from 30 to 180 days). The pig bone samples showed a pattern of 3 to 4 weeks of “induction period” (where nothing happens to citrate), after which the citrate began to decline. They plotted the citrate content (wt%) versus the log time (days) scale and obtained an  $R^2$  value of 0.919. The range of initial citrate was  $1.96 \pm 0.06$  wt%. When pig bone and forensic samples of known PMI were assessed together for citrate decay on a log t scale (days) the  $R^2$  value resulting was 0.980. It was noted that the temperature does affect this process. When the temperature is below  $0^\circ\text{C}$ , there is no loss of citrate. Thus a correction is necessary for frozen samples.

The results showed that the use of citrate to measure the time since death of questioned skeletal remains may be a promising approach, with an added improvement in precision. According to Schwarcz et al., it appears that it may be possible to determine PMI of skeletal remains with a precision of approximately *1% of age over a time range from 2 months to 90 years*. Furthermore, the citrate method appears to address a critical window of time for medicolegal investigators since skeletal remains with a PMI <50 years old are typically deemed forensically relevant, while older remains would be considered to be “historic”, lacking evidence to the contrary. It must be emphasized that this method is applicable to the most difficult times of remains for which to determine PMI – those that are devoid of soft tissue. As elaborated upon previously, the current methods available to investigators and forensic specialists are frequently inadequate in addressing this question, which can involve investment of manpower and concomitant pay for law enforcement officers to stand guard and protect what may or may not be a forensically significant location.

### 3. Rationale for Current Research

Clearly if the citrate method is to be applied to actual questioned death cases, it must be externally validated. Studies that replicate Schwarcz et al.’s method should be carried out on pig and human remains of varying, known PMIs. Such work was initiated during summer 2012 at The College at Brockport, SUNY, with interdisciplinary efforts of Dr. Ann W. Bunch (Criminal Justice) and Dr. Michael A. Brown (Chemistry and Biochemistry), along undergraduate researchers and continued on under the auspices of this grant funding until December 2015. The external validation of Schwarcz et al.’s promising method was carried out so that it could be (a) optimized and (b) if validated and deemed reliable, applied more broadly to medicolegal cases. This work was one of the first attempts to do just this. Funding was requested to carry on this exciting and potentially ground-breaking work that could assist medicolegal investigators, forensic experts, and law enforcement agencies in the United States and abroad. The major goals of this project are to validate the work of Schwarcz et al. Their work suggested that the citrate content of human skeletal remains could serve as a potential measure of PMI, Schwarcz et al. used an experimental approach that seemed to show that citrate may act like a clock, diminishing in postmortem bone at a regular rate over time. This project is a follow-up study with a relatively large set of human bone samples with known-PMIs. Two analytical methods were used to determine the citrate content of each sample in order to provide a better assessment of method accuracy and reliability and to identify and correct for systematic errors if present. Our working relationships with two institutions, one academic and one county forensic unit, which agreed to provide/have provided us with known-PMI human bone samples. Thirty-one samples were tested

during the course of this project. To the best of our knowledge this is the largest number of known-PMI human bones analyzed by both UV-Vis enzymatic assay and HPLC methods.

## II. METHODS AND MATERIALS

### 1. Sample Collection and Handling

The human skeletal remains used in this research were obtained from the Forensic Anthropology Center, University of Tennessee, Knoxville (UTK) and from the Office of the Medical Examiner of Onondaga County (OCME) located in Syracuse, NY. The skeletal remains from UTK are typically bequeathed by a donor and the remains from OCME are usually acquired through casework.

Collaborators at UTK collected original bulk samples from human remains (lot) by cutting small sections from rib and metatarsal bone, sealed them in individual paper envelopes, and sent to Dr. Bunch. The bulk samples from OCME were obtained directly by Dr. Bunch who used a Dremel<sup>®</sup>-type tool to cut small segments from rib and/or cranial bone. The bone sections were placed in individual paper envelopes and transported to the College at Brockport, SUNY. Once the samples were transferred to Dr. Brown he stored in a desiccator vessel maintained at room temperature. Sample information for all original bulk samples was entered into a specimen log database (MS Excel<sup>®</sup> spreadsheet) with the following information: accession number, origin (UTK, OCME), PMI, and any other relevant information regarding taphonomic history. Any identifying information (if known to UTK or OCME) was not reported to the project investigators. Samples were either consumed during the course of the project or returned to UTK or OCME. Figure 2 outlines the main sample handling scheme utilized in this project.

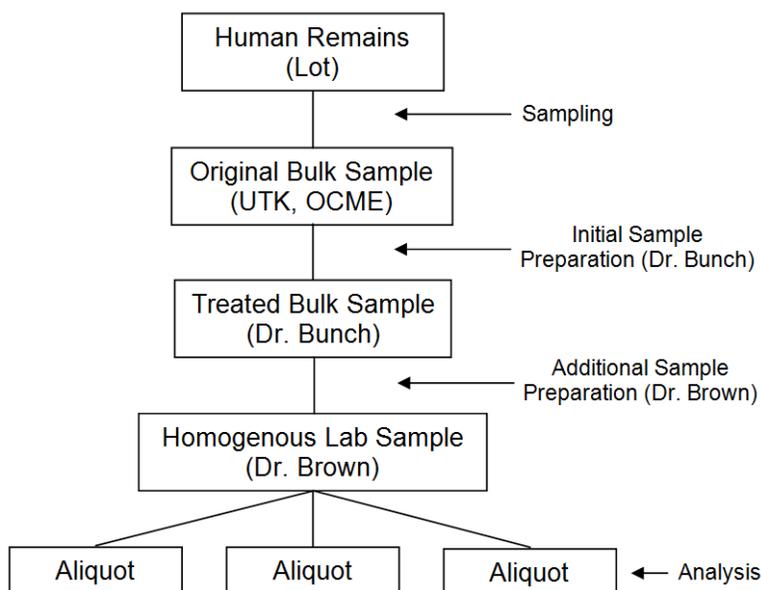


Figure 2. Sample handling scheme (adapted from Harris 2009).

In addition to human skeletal remains, domesticated pig (*Sus scrofa domesticus*) bone samples were also collected and used for method optimization studies. These samples were acquired from local vendors, were fresh, untreated with chemicals, and never frozen and were defleshed and dried at ~200 °C for two hours then stored in a desiccator vessel kept at room temperature until needed.

## 2. Sample Preparation

### 2.1 Materials and Equipment

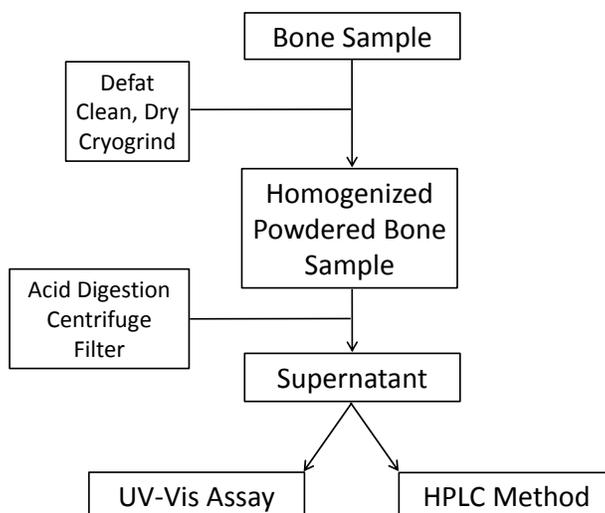
All deionized (DI) water used for sample preparation was produced by a Barnstead UV-Easypure™ system and had a resistivity of 18.2 MΩ-cm. High purity (99.9 %), ACS grade chloroform was purchased from Sigma Aldrich. High purity (99.9 %), ACS grade ethanol and concentrated hydrochloric acid (HCl) was purchased from Fisher Scientific. A stock solution (~11.7 M) of potassium hydroxide (KOH) was acquired from Ricca Chemical Company. Liquid nitrogen was obtained from Airgas. Wheaton glass scintillation vials, Amicon Ultra-4 centrifugal filters, polypropylene centrifuge tubes, a stirrer/shaker apparatus, vortex mixer, water bath, analytical balance, and various Eppendorf Research Plus adjustable micropipets were purchased from Fisher Scientific. A Thermo Scientific Orion Star A111 pH meter was purchased from Thermo Scientific. Polycarbonate grinding vials (6751) and a SPEX 6770 freezer mill were obtained from SPEX SamplePrep.

### 2.2 Preparation of Powdered Bone Samples

All original bulk bone samples from UTK and OCME and domesticated pig bone samples were cut into ~ one cm segments using surgical bone shears. The interior and exterior of each segment was manually scraped with a scalpel to remove any tissue if necessary. All samples were stored at room temperature in a dessicator vessel until needed. Approximately 1 - 2 g of bone segments, for each sample, was placed into a 20-mL glass scintillation vial containing enough 1:1 (v/v) chloroform and ethanol solution to cover the sections. The vial was then placed on an orbital type mixer/shaker for one hour to remove fat and lipophilic compounds from the bone. Afterwards each bone segment was rinsed with pure ethanol and placed on an evaporation dish and dried for 30 minutes. The dried bone sections were then place in a polycarbonate grinding tube containing a stainless steel impactor. The tube was placed in a SPEX 6770 Freezer Mill that was filled with liquid nitrogen. The grinding time was 2 min, the grinding rate was 10 cycles·s<sup>-1</sup>, and only one run was used. The powdered bone was transferred to a 50-mL polypropylene centrifuge tube and stored in a dessicator vessel at room temperature until needed. Procedures were adapted from those used by Gibbs (1991), Santos Jr. et al. (2003), Schwarcz et al. (2010), SPEX (2013), and Yu (2004). All transfers of bone powder were performed in a Labconco Xpert Biosafety cabinet.

### 2.3 Digestion of Powdered Bone Samples

Bone powder was digested by massing approximately 50 mg of powder with a 0.0001-g analytical balance into a 15-mL centrifuge tube. A 2.0 mL volume of 1.1 M hydrochloric acid was added and the tube was vortexed (setting 6) for ~15 seconds and placed in a water bath set to 60 °C for one hour. Afterwards the pH of the digestate solution was adjusted to approximately 5.0 using 0.5 M KOH delivered by a digital Eppendorf micropipette and read Orion Star A111 pH meter. Eventually, pH adjustment was omitted from sample procedure, because it led to inconsistent results. The centrifuge tube was centrifuged at 1200 g for 12 min to remove collagen and protein fragments. Approximately 1.5 mL of the aqueous supernatant was collected and sub-micron filtered using Amicon Ultra-4 centrifugal filters at 7500 g for 24 minutes then stored at 4 °C until analysis by UV-Vis and HPLC methods. A summary of these steps is shown in Figure 3. Procedures were adapted from Gibbs (1991), Nawrocki (2013), and Schwarcz et al. (2010).

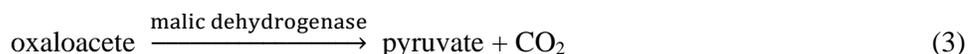
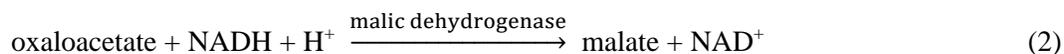


**Figure 3.** Summary of sample preparation steps.

### 3. UV-Vis Enzymatic Assay

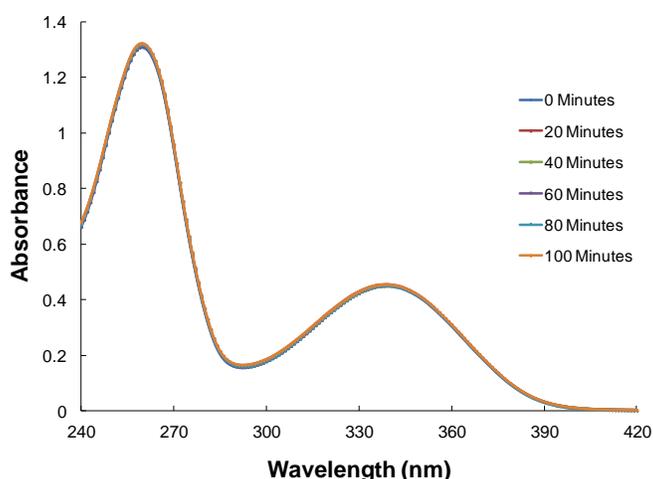
#### 3.1 Background and Theory

Dagley et al. (1955) first reported the use of the enzyme citrate lyase for the determination of citrate. Moellenberg and Gruber (1965), made improvements to this assay by using zinc as the cofactor to enhance enzyme activation and stabilization followed by the work of Blair et al. (1967). This enzymatic assay has since gone on to become a standard method used in many industries, such as food and beverage (Mori & Kadowaki, 2009), pharmaceutical, and medical technology (Petrarulo et al., 1995). The principle of this assay is based on the citric acid cycle (Kreb's cycle), which takes place in aerobic organisms (Berg et al., 2010). Within this cycle there exists multiple reactions catalyzed by various enzymes; however only the four reactions below are relevant to this assay (Moellenberg & Gruber, 1965; Sigma Aldrich, 1997).

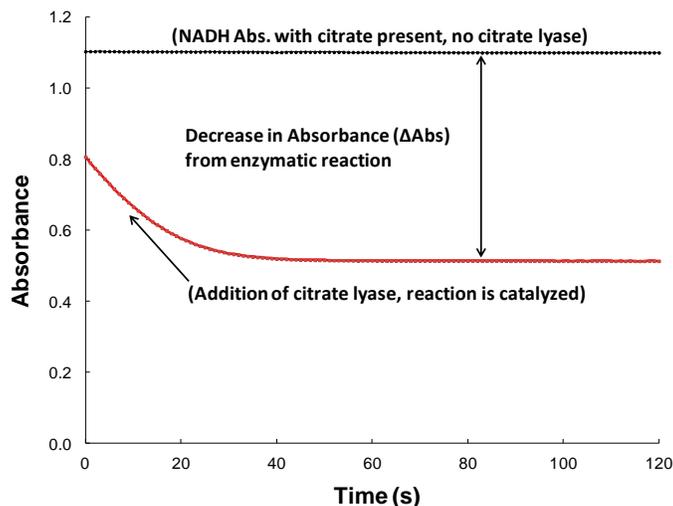


Reaction 1 involves the cleavage of citrate into oxaloacetate and acetate, which is catalyzed by the enzyme citrate lyase. The oxaloacetate is then reduced to malate upon reacting with the reduced form of beta-nicotinamide adenine dinucleotide (NADH). Consequently, NADH is oxidized to  $\text{NAD}^+$ . This reaction is catalyzed by the enzyme malic dehydrogenase (MDH). Note that  $\text{NADH}/\text{NAD}^+$  is a coenzyme pair that mediates the transfer of electrons within a catalyzed reaction. Reactions 3 and 4 are undesirable reactions that have the potential of occurring during the assay; therefore lactic dehydrogenase enzyme is added to catalyze the reaction of pyruvate with NADH. This ensures that all of citrate that is present in a standard or

sample reacts with NADH and thus maintains the 1:1 stoichiometry. Both NADH and NAD<sup>+</sup> absorb at 260 nm; however NADH exhibits a second absorption band at 340 nm (see Figure 4), which can be used for the assay. The stability of NADH at room temperature has been shown to be quite good given proper pH adjustment as reported by Rover Jr. (1998) and our research (refer to Figure 4). A stable reagent, such as NADH, is necessary for accurate enzymatic-based assays. The decrease in the absorbance of NADH at 340 nm is proportional to the citrate content in the sample or standard. The enzymatic reaction with MDH can be monitored using a scanning UV-Vis spectrophotometer in time domain mode (see Figure 5) if kinetic information is desired, though an inexpensive spectrophotometer can be used to determine the citrate content in the original sample through use of a calibration curve that relates absorbance to citrate amount. This method has also been shown by other researchers to have an accuracy of >98%, precision <1% %relative standard deviation (%RSD), and a method detection limit of <0.2  $\mu\text{g}\cdot\text{L}^{-1}$  citrate (or 5  $\mu\text{g}$ ), which is acceptable for this research project.



**Figure 4.** UV-Vis spectra of NADH taken at different time intervals (pH 7.6, ~22°C, no citrate).



**Figure 5.** Decrease in NADH absorbance at measured at 340 nm enzymatic reaction (pH 7.6, ~22°C, 50  $\mu\text{g}$  citrate).

### 3.2 *Experimental Information for the UV-Vis Assay*

#### 3.2.1. Chemicals and Reagents

All reagents and glassware utilized for this assay were of the highest quality to minimize contamination and method errors. The DI water used for preparation of assay kit and calibration solutions was produced by a Barnstead UV-Easypure™ system and had a resistivity of 18.2 MΩ·cm. Glassware and sample containers were meticulously cleaned with concentrated detergent then rinsed with tap water three times followed by DI water three to four times. All solvents were HPLC/ACS reagent grade or better and were purchased from Fisher Scientific, Pharmco-AAPER, Acros, and/or Sigma Aldrich.

Commercially available citric acid assay kits were purchased from Biocontrol (Enzyplus® EZA 785+) for determination of citrate content of pig bone samples. The kit included a glycyglycine buffer solution, zinc chloride solution, NADH, L-MDH, L-LDH, sodium azide (0.1%), citrate lyase (13 U), and citric acid standard solution (0.30 μg·μL<sup>-1</sup>). All kit solutions and reagents were stored at ~ 4°C and disposed of according to the manufacturer's expiration recommendations. Due to discontinuation of the Enzyplus® EZA 785+ citrate assay kits, new kits were purchased from R-Biopharm® and were used to determine the citrate content of all human bone samples. The kit included glycyglycine buffer, L-MDH (136 U), L-LDH (280 U), NADH (5 mg), citrate lyase (12 U), and a citric acid standard solution (0.407 μg·μL<sup>-1</sup>). Due to the limited volume of citric acid standard solution provided with the kits additional standard solution was prepared by using ACS grade citric acid trisodium salt dehydrate obtained from Acros. A 10.0 μg·μL<sup>-1</sup> citric acid solution was prepared in DI water then used to prepare a diluted standard with a concentration similar to standard included in the kit. Note both of these assay kits had nearly identical analytical performance.

#### 3.2.2. Instrumentation and Equipment

High quality, matched quartz cuvettes (1-cm) from Starna (21-Q-10) were used for absorbance measurements. Enzyme and reagent solutions were delivered using Eppendorf Research Plus adjustable micropipets and/or an Eppendorf repeater stream autopipet. A Perkin Elmer Lambda 800 UV-Vis double-beam spectrophotometer and a Perkin Elmer Lambda 3B UV-Vis spectrophotometer were used. The Perkin Elmer Lambda 800 instrument was set to time domain mode (kinetic mode) with a wavelength of 340 nm and the Perkin Elmer Lambda 3B instrument was set to absorbance mode with a wavelength of 340 nm.

#### 3.2.3. Calibration Procedure

The Lambda 800 or Lambda 3B spectrophotometer is turned on and the lamp is allowed to warm-up for 30 minutes then a background correction was performed using DI water. A blank solution containing 1000 μL of the glycyglycine buffer/enzyme kit solution and 2000 μL of DI water was placed into a standard 1-cm quartz cuvette and the absorbance is recorded after five minutes. Next a 20 μL volume of citrate lyase solution was added to the cuvette, mixed, and the absorbance value is recorded after five minutes. The change in absorbance ( $\Delta\text{Abs}_{\text{Blk}}$ ) is equal to the absorbance for the blank before citrate lyase is added subtracted from the absorbance after citrate lyase is added. Following analysis of the blank, a pre-determined volume of citric acid standard solution is added to a clean cuvette along with 1000 μL of the glycyglycine buffer containing NADH and enzymes and enough DI water to give a total volume of 3000 μL. The contents of the cuvette are thoroughly mixed and the absorbance is measured after five minutes. A 20 μL volume of citrate lyase solution is then added to the cuvette and mixed and the absorbance is recorded

after five minutes. The change in absorbance ( $\Delta\text{Abs}_{\text{Std}}$ ) is equal to the absorbance for the standard before citrate lyase is added subtracted from the absorbance after citrate lyase is added. The difference between  $\Delta\text{Abs}_{\text{Std}}$  and  $\Delta\text{Abs}_{\text{Blk}}$  is referred to as the corrected absorbance ( $\Delta\text{Abs}_{\text{corr}}$ ). Five calibration standards with citrate masses of 8, 20, 40, 60, and 80  $\mu\text{g}$  were analyzed and a calibration curve was prepared. Additional detail is included in the data treatment section.

### 3.2.4. Analysis of Samples

After performing the calibration procedure and generating a calibration curve, samples are analyzed according to the manufacturer's recommendations. An 1800  $\mu\text{L}$  DI water is added to a cuvette, followed by 200  $\mu\text{L}$  of the supernatant sample and 1000  $\mu\text{L}$  of the glycyglycine buffer solution containing NADH and enzymes. It became apparent during the course of this research that the pH of samples was inconsistent and resulted in irreproducible results; therefore it was necessary to use 350 mM glycyglycine buffer in place of DI water in all analyses. This buffer was also used for calibration and blank procedure in order to correctly match matrices. The contents of the cuvette are thoroughly mixed and the absorbance is measured after five minutes. A 20  $\mu\text{L}$  volume of citrate lyase solution is then added to the cuvette and mixed and the absorbance is recorded after 10 minutes or until the absorbance value remains constant. The change in absorbance for a sample ( $\Delta\text{Abs}_{\text{Smpl}}$ ) is calculated and corrected using the  $\Delta\text{Abs}_{\text{Blk}}$  to obtain the  $\Delta\text{Abs}_{\text{corr}}$ .

### 3.2.5. Data Treatment and Calculations

All data treatment was performed using MS Excel<sup>®</sup>. Typically one analysis per unspiked and spiked bone digestion was performed. The  $\Delta\text{Abs}_{\text{corr}}$  data for each of these samples was calculated then inputted into the calculated linear regression equation for the calibration curve to determine citrate content of the supernatant. Note that new calibration curves were generated each day and a check standard solution was analyzed every 10 analyses to ensure calibration was maintained. Corrections for dilutions are performed and the mass (mg) of citrate in the original bone powder is calculated. A Grubb's statistical test was performed to check for any outliers among  $\Delta\text{Abs}_{\text{corr}}$  values. However due to the small numbers of replicates this test was used mainly for evaluating method precision. Below is a concise summary of the calculations involved in the determination of citrate wt%.

#### *Summary of Calculations:*

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Linear regression equation:  $y = mx + b$

$y$  = blank corrected  $\Delta\text{Abs}_{\text{corr}}$ ;  $x$  = citrate mass ( $\mu\text{g}$ ) in calibration standards

Regression equation used to determine citrate mass ( $\mu\text{g}$ ) sample digestate =  $x = \frac{(y-b)}{m}$

Mass of citrate ( $\mu\text{g}$ ) in digested sample =  $\frac{\text{Total volume of digested sample (2000}\mu\text{L)}}{\text{Volume of supernatant used (200}\mu\text{L)}} \times (x)$

Mass units are converted from  $\mu\text{g}$  to mg. This value is used to calculate the wt% of citrate of bone sample.

Citrate wt% =  $\frac{\text{Total mass of citrate in bone (mg)}}{\text{Mass of bone powder analyzed (mg)}} \times 100\%$

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The sample mean ( $\bar{x}$ ), sample standard deviation ( $\sigma_{N-1}$ ), % relative standard deviation (%RSD), and the confidence interval for the 95% confidence level was calculated for each data set. Also F-tests and t-tests

were performed to statistically compare the UV-Vis assay and HPLC results. Data for each sample was saved in a unique MS Excel® spreadsheet.

#### 4. HPLC Method

##### 4.1 Background and Theory

High-performance liquid chromatography (HPLC) is a common technique applied in pharmaceutical, food, environmental, biochemical, and forensic laboratories around the world. A typical HPLC system consists of mobile phase reservoirs containing ultrapure solvents, a degasser with proportioning valve, a high pressure gradient pump, an injection valve, a guard column, an analytical column, and a detector such as UV-Vis. The most common separation mode is reversed-phase chromatography and involves using an analytical column packed with spherical silica particles with covalently bound n-alkyl chains (e.g. C-8, C-18) and a moderately polar mobile phase. This is referred to as reversed-phase HPLC. Hydrophilic compounds (polar) will elute earlier than more hydrophobic compounds (non-polar) due to interaction with the hydrophobic n-alkyl chains thus partitioning more slowly to the polar mobile phase. By changing the properties of the mobile phase or column packing, the retention of compounds can be altered. Recently, there has been a major advance in the analysis of organic acids, such as citric acid, by HPLC. Manufacturers like Restek and others now offer HPLC columns that can be operated at low pH with a 100% aqueous mobile phase (Kowaliski & Wittrig, 2013; Wittrig & Reid, 2013). Most conventional C-18 columns would undergo phase collapse after several usages with these conditions and lose function, but aqueous columns due to propriety protection of the stationary phase remain robust and stable with these conditions. For this research, citrate (predominantly citric acid at pH 2.5) was determined in bone samples using an HPLC fitted with an aqueous column.

##### 4.2 Experimental Information for HPLC Method

###### 4.2.1. Chemicals and Reagents

All DI water, chemical reagents, and glassware that were used for this method were of the highest quality to minimize contamination of the HPLC system. All solvents for mobile phase preparation were HPLC reagent grade or better and were purchased from Fisher Scientific or Pharmco-AAPER. Monobasic phosphate salt and phosphoric acid were purchased from Fisher Scientific and ACS grade citric acid trisodium salt dehydrate from Acros. Prepared stock standard and calibration solutions were stored at ~ 4°C. A 20 mM phosphate buffer (pH 2.5) mobile phase was prepared daily using DI water, monobasic phosphate salt and phosphoric acid and filtered through a 0.2 µm filter before use. All sample supernatant was filtered using Amicon Ultra-4 centrifugal filters.

###### 4.2.2. Instrumentation and Equipment

All volume deliveries for preparation of standard and sample solutions were performed using Eppendorf Research Plus adjustable micropipets and/or an Eppendorf repeater stream autopipet. All solutions were filtered through 0.2 µm Millipore Omnipore™ Membrane filter prior to analysis by HPLC. A Shimadzu Prominence LC-20 HPLC instrument with an autosampler and UV-Vis detector, and a Restek Ultra AQ C-18 analytical column (4.6 mm × 150 mm, 5 µm particle size) were used in this research. Preliminary HPLC instrument parameters were similar to those used by Kowaliski and Wittrig (2013) and Wittrig and Reid

(2013). The HPLC isocratic pump flow-rate was set at  $1.0 \text{ mL}\cdot\text{min}^{-1}$ , the UV-Vis detector was set to a wavelength of 226 nm with no response filter used (positive polarity) and had a cell temperature of  $40 \text{ }^\circ\text{C}$ . The data acquisition module was set to 3.33 Hz with a total acquisition (run) time of 6.5 minutes for calibration and 15 minutes for sample analysis. A  $20 \text{ }\mu\text{L}$  injection volume was for both calibration and sample analysis. The chromatographic retention time for citric acid was approximately 5.6 minutes.

#### 4.2.3. Calibration Procedure

The HPLC is initiated and the pump heads are primed and purged with 20 mM phosphate buffer (pH ~2.5) mobile phase for five minutes then the column is equilibrated by pumping for 20 minutes at  $1.0 \text{ mL}\cdot\text{min}^{-1}$ . The operating pressure, once the column is equilibrated, is typically between 860 to 880 psi. While the column is equilibrating the UV-Vis detector lamp is turned on and allowed to warm for at least 30 minutes before usage. Afterwards the wavelength accuracy is checked and the detector is zeroed with mobile phase pumping. The HPLC is calibrated using six citric acid calibration standard solutions with concentrations of 16.4, 32.2, 57.5, 72.1, 86.2, 99.9  $\text{mg}\cdot\text{L}^{-1}$  citric acid. The standards were prepared using a pre-filtered  $\sim 1000 \text{ mg}\cdot\text{L}^{-1}$  citric acid stock solution and 20 mM phosphate buffer. Standards were placed in 2-mL HPLC autosampler vials and analyzed three times each. The chromatographic peak area for citric acid is obtained for each standard. The average peak areas are plotted versus concentration to produce a calibration curve. A check standard with a concentration of  $21.7 \text{ mg}\cdot\text{L}^{-1}$  is analyzed seven times to test the calibration and is analyzed after every 10 analyses. The calibration curve is then plotted in MS Excel<sup>®</sup> and a linear regression equation is obtained.

#### 4.2.4. Analysis of Samples

The samples are prepared by mixing  $1200 \text{ }\mu\text{L}$  of mobile phase and  $300 \text{ }\mu\text{L}$  of filtered supernatant using Eppendorf pipets and placed in HPLC autosampler vials. After calibration and accuracy and precision checks are completed, the samples are analyzed. Following analysis the chromatographic data was integrated using the default auto integrator program included with the instrument software to obtain peak area values for the citric acid peak (retention time  $\sim 5.6$  minutes). These values were used to determine the concentration of citrate in the samples.

#### 4.2.5. Data Treatment and Calculations

All data treatment was performed using MS Excel<sup>®</sup>. Typically one analysis per unspiked and spiked bone digestion was performed. The peak area data for each of these analyses was inputted into the calculated linear regression equation for the calibration curve to determine citrate content of the supernatant. Note that new calibration curves were generated each day and a check standard solution was analyzed every 10 analyses to ensure calibration was maintained. Corrections for dilutions are performed and the mass (mg) of citrate in the original bone powder is calculated. A Grubb's statistical test was performed to check for any outliers among peak area values. However due to the small numbers of replicates this test was used more for evaluating precision. Below is a concise summary of the calculations involved in the determination of citrate wt%.

#### *Summary of Calculations:*

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Linear regression equation:  $y = mx + b$

$y$  = average chromatographic Peak Area;  $x$  = concentration ( $\text{mg}\cdot\text{L}^{-1}$ ) of citrate in calibration standards solution

Regression equation used to determine the concentration ( $\text{mg}\cdot\text{L}^{-1}$ ) of citrate in the sample digestate =  $x = \frac{(y-b)}{m}$

Mass of citrate (mg) in digested sample =  $\frac{\text{Total volume digested sample (2000 } \mu\text{L}) \times \text{Volume HPLC sample (1.5 mL)}}{\text{Volume supernatant used (300 } \mu\text{L)}} \times (x)$

This value along with the mass (mg) of bone powder analyzed is used to calculate the wt% of citrate of bone sample.

Citrate wt% =  $\frac{\text{Mass of citrate in bone (mg)}}{\text{Mass of bone powder analyzed (mg)}} \times 100\%$

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## 5. Quality Assurance, Method Validation, and Statistical Analysis Protocol

This project was governed by a quality assurance project plan (QAPP) that was prepared by the PIs. The QAPP was mainly comprised of standard operating procedures (SOPs) (see appendices) that contain specific protocol pertaining to sample acquisition/sampling, initial sample preparation, pre-analysis sample preparation, method calibration and sample analysis, data treatment and reporting of results, data archiving, and method validation and quality controls standards.

### 5.1 Method Calibration and Validation

As stated in the previous sections, it was common practice to calibrate the instrumentation using a five (UV-Vis assay) or a six point calibration curve (HPLC) using calibration standards. Each standard was replicated as many times as feasible. For HPLC this was three replicates and for the UV-Vis assay it was one replicate. The main type of calibration method utilized in this research was external calibration, which was supplemented with spike recovery analyses. Examples of calibration curves and regression data for each method are included in the Results section.

Detection limits, accuracy, and precision estimates were determined routinely for both analytical methods. Three detection limits: the lower limit of detection (LOD), the lower limit of quantitation (LOQ), and the uncertainty propagation detection limit (UDL) were determined for each method. The LOD is the lowest quantity of analyte (citrate) that is “statistically different” from a method blank. The LOQ is defined as the lowest quantity of analyte (citrate) in a sample that can be reliably reported with “reasonable” accuracy and precision. The propagated uncertainty estimated detection limit (UDL) is similar to the LOQ, but is calculated using the propagated uncertainty of the check standard data. The method reporting limit (MRL) is typically equal to the LOQ; however for this research it was selected to be the higher value of the LOQ or UDL. Any determined values below the MRL were considered to be “not reportable.” A check standard solution was prepared, containing a known citrate quantity 1 to 5 times a previously estimated LOD, and analyzed at least seven times by each method. The quantity (e.g., mass, concentration) of citrate in the standard was calculated using the calibration curves. The sample standard deviation ( $\sigma_{N-1}$ ) of this quantity data was used to calculate the LOD ( $3 \times \sigma_{N-1}$ ) and the LOQ ( $10 \times \sigma_{N-1}$ ) and the propagated uncertainty obtained from said data was used to calculate the UDL ( $10 \times \text{prop. unc.}$ ). (Green, 1996; Harris, 2009; Skoog et al., 2006; USEPA, 1996a, 1996b). Instrument accuracy, measured as % relative error from the “true” value, and precision, measured as % relative standard deviation, %RSD, was assessed by analyzing check standard solutions with a citrate amounts 2 to 10 times the MRLs. The criterion for instrument accuracy

was selected to be  $\pm 5\%$  relative error. The criterion for instrument precision was selected to be less than 5% RSD. Spiking studies were performed to determine if the sample matrix was interfering with the analysis and to also estimate the method accuracy and intra-precision for multiple digestions of the same bone powder and for the same sample. Matrix effect(s), if present, are typically of constant magnitude and are expected to have a noticeable impact upon method accuracy, commonly known as a bias, than upon method precision. Spike recovery values (calculated with the equation below) were typically within  $100 \pm 10\%$  suggesting minimal matrix effects. Typical detection limits, accuracy, and precision values are included in the Results section.

$$\text{Spike Recovery} = \frac{\text{Citrate Conc. With Spike} - \text{Citrate Conc. No Spike}}{\text{Spike Citrate Concentration}} \times 100\%$$

### 5.2 Statistical Analysis Protocol

Sample mean ( $\bar{x}$ ), sample standard deviation ( $\sigma_{N-1}$ ), % relative standard deviation (%RSD), and the confidence interval for the 95% confidence level were calculated for each data set obtained for and individual sample for each analytical method.

Comparison of the two analytical methods was periodically validated by using the statistical tests ( $F$ -test,  $t$ -test) and by computing the average bias and its standard deviation at the 95 % confidence level. Several standards and samples were analyzed to obtain these values. The student's  $t$ -test served as the primary test for making decisions on agreement or disagreement between the average results of the two methods.

Two versions of  $t$ -test were used depending on the type of comparison. For comparison of results for a single sample an unpaired  $t$ -test was utilized for comparison of results for many different samples a paired  $t$ -test was used. Note that an  $F$ -test was performed to first evaluate the variances of results obtained by both methods.

### III. RESULTS

#### 1. Method Validation Studies

Method validation studies were performed to ensure that the UV-Vis assay and HPLC method were suitable for the determination of citrate content of bone. Specific parameters that were assessed included detection limit, linearity, range, accuracy, precision, and specificity. The methods were validated for analysis of standard solution with a relatively “clean” and “simple” matrix and were also evaluated for the analysis of human bone samples, which have a “complex” matrix (see Results section #3). These studies were repeated at periodic intervals throughout the project to evaluate the need to further optimize and/or revalidate the methods. These results presented here are typical results for these methods.

##### 1.1 Calibration Results

The UV-Vis assay and the HPLC method were calibrated using an external calibration approach, which involved the analysis of five or six calibration standards solutions containing a known amount of citrate. The analytical data was then plotted versus citrate mass ( $\mu\text{g}$ ), for the UV-Vis assay, and versus citrate concentration ( $\text{mg}\cdot\text{L}^{-1}$ ) for the HPLC method. The method of least-squares was used to find the “best” fit line for each data set as was discussed in the Methods section. Figure 6 is a typical calibration curve for HPLC and Figure 6 is a typical calibration curve for UV-Vis (before optimization). Both methods yielded nearly ideal calibration curves with coefficient of determination ( $R^2$ ) values  $\geq 0.999$ , and acceptable slopes and y-intercepts. Extended linearity studies found the UV-Vis assay to have a limit of linearity of approximately 85  $\mu\text{g}$  of citrate. The HPLC method was found to be linear up to the highest concentration analyzed ( $\sim 750 \text{ mg}\cdot\text{L}^{-1}$ ). It would not have been practical to analyze more concentrated standards as the limit of linearity was sufficient for the HPLC method. The maximum citrate content expected for bone samples would be below the limit of linearity for both methods. Both methods satisfied the acceptance criteria for calibration parameters and linearity.

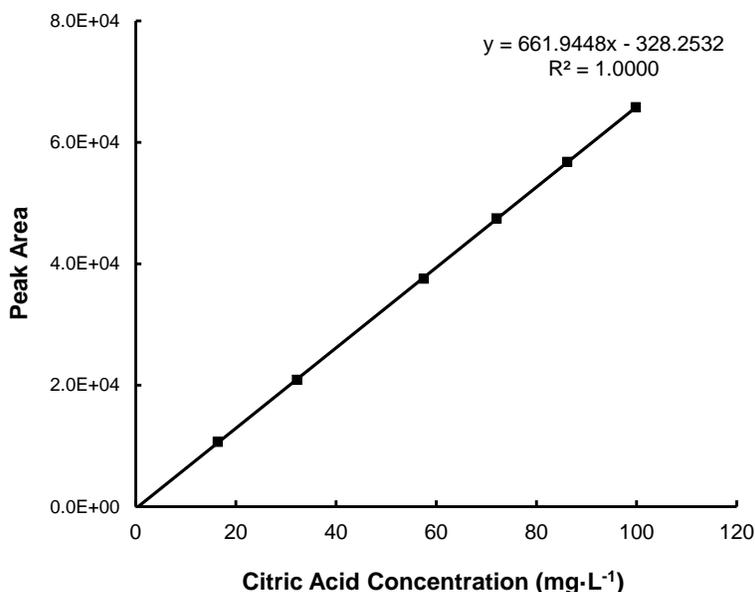
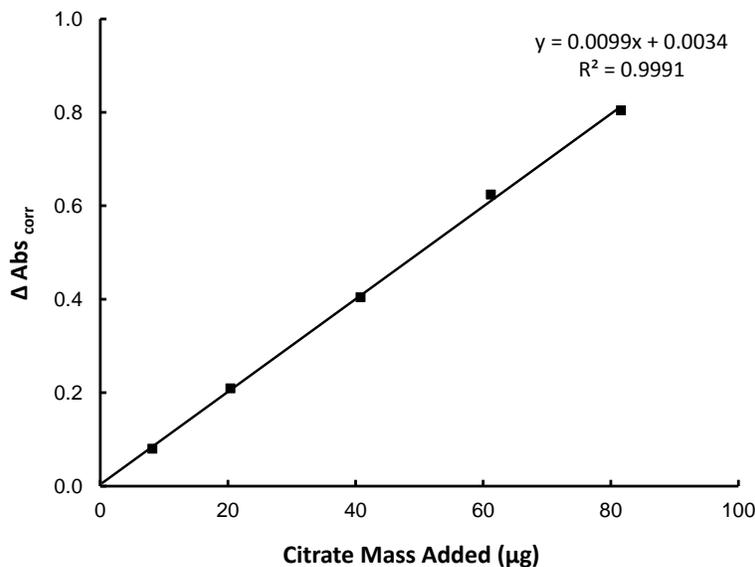


Figure 6. Typical calibration curve for HPLC method.



**Figure 7.** Typical calibration curve for the UV-Vis assay (before optimization).

## 1.2 Assessment of Detection Limit, Instrument Accuracy, and Instrument Precision

### 1.1.1. Detection Limits

Three detection limits were determined including the lower limit of detection (LOD), the lower limit of quantitation (LOQ), and the uncertainty propagation detection limit (UDL). Additional details are included in the Methods section. The UV-Vis assay had a LOD of 0.60 μg, a LOQ of 2.0 μg, and an UDL of 1.1 μg of citrate mass. The MRL for this method was selected to be 2.0 μg (or ~0.1 wt% citrate). The HPLC method had a LOD of 0.4<sub>3</sub> mg L<sup>-1</sup>, a LOQ of 1.4 mg L<sup>-1</sup>, and an UDL of 5.3 mg L<sup>-1</sup> citrate concentration. The MRL for this method was selected to be 5.3 mg L<sup>-1</sup> (or ~0.1 wt% citrate). Interestingly, both methods had similar MRLs when compared in units of wt% of citrate in fresh bone. Our estimated MRL values are several orders of magnitude higher than the estimates reported by Schwarcz et al. (2010); however the authors do mention there was an underestimation of method uncertainty due to the limited number of replicate studies performed.

### 1.1.2. Instrument Accuracy and Instrument Precision Assessment

Instrument accuracy, measured as % relative error from the “true” value, and precision, measured as % relative standard deviation, %RSD, were assessed by analyzing check standard solutions with a citrate amounts 2 to 10 times the MRLs. The criterion for instrument accuracy was selected to be ± 5% relative error. The criterion for instrument precision was selected to be less than 5% RSD. The UV-Vis assay and the HPLC method both attained % relative error results less than 3% for multiple studies. The UV-Vis assay had %RSD values less than 4% whereas the HPLC method had values less than 2% for multiple studies. Both methods had acceptable instrument accuracy and precision.

Both methods met the acceptance criteria for detection limit, instrument accuracy, and instrument precision. The linear range for the UV-Vis assay was determined to be ~2 to 85 μg and for the HPLC method 5 to 750 mg·L<sup>-1</sup>.

### *1.3 Evaluation of Method Accuracy and Intra-assay Precision by Spiking Studies*

The specific objectives of sample spiking studies were to identify matrix effects if present, to estimate the method accuracy and intra-precision for multiple digestions of the same bone powder and for the same sample. Matrix effect(s), if present, are typically of constant magnitude and are expected to have a noticeable impact upon method accuracy, commonly known as a bias, than upon method precision. The citrate content (wt%) data from spiked and non-spiked samples were used to calculate spike recoveries. Spike recovery values greater than  $100 \pm 5\%$  would suggest matrix effects are present. The %RSD values of the spike recovery data were used to assess intra-assay precision for multiple digestions of the same bone powder and for different bone powders. The analysis of bone samples is much more challenging due to the presence of biological material. Schwarcz et al. and Kanz et al. (2014) both reported acceptable accuracy and precision results using the UV-Vis assay for the determination of citrate of bone samples. Neither of the researchers utilized HPLC; but a non-published oral presentation by Zimmer, Deturi, and Muller (2013) details an attempt to incorporate this second citrate detection method. When HPLC is used to analyze biological samples one has to be cautious of chromatographic interferences, such as amino acids, short chain peptides, and proteins that remain following digestion. This bio-material has the potential to not only affect chromatography (i.e., co-elution, retention time shifts, and integration issues), but to also damage the HPLC instrument. To address this issue we have employed a centrifuge filter step and a backwash procedure. A summary of result of analysis of spike human samples is presented in section #3.

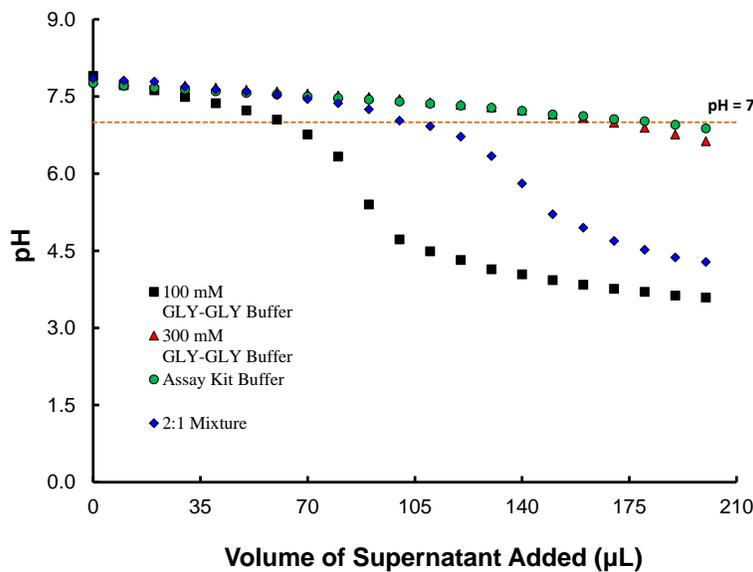
## **2. Optimization of UV-Vis Assay**

### *2.1 Adjustment of Buffer Concentration*

During the course of the project the UV-Vis assay required one major modification. The HPLC method only required very minor modification, so this is not discussed in this report. Because the UV-Vis assay is enzyme-based, it is highly sensitive to changes in pH specifically outside of the pH range of ~7 to 8. In order compensate for the acidic sample supernatant, the use of additional buffer solution was necessary. In the past, we have experimented with using phosphate and tris buffers, but have had little success. The buffer salt recommended by the manufacturer R-Biopharm is glycylglycine (GLY-GLY), because it is chemically inert and has minimal absorption at 340 nm it was the best choice. For this study we prepared a 100 mM solution of GLY-GLY and adjusted the pH to exactly 7.8 with 0.5 M KOH. We also prepared a 300 mM GLY-GLY solution (pH 7.8) and a 2:1 mixture of the 100 mM GLY-GLY buffer and the assay kit buffer. We intentionally selected a sample that would provide minimal buffering and chose to use less than 50 mg of it in order to simulate a “worse case” scenario with respect to pH control. Our goal was to have a final pH between 7 and 8 using the lowest concentration of GLY-GLY buffer possible. Higher concentrations of buffer would lead to a higher ionic strength, which consequently could inhibit the assay enzymes and result in a decrease in the rate of reaction (longer analysis times). Increasing amounts of the sample supernatant were added to separate solutions of 100 and 300 mM GLY-GLY buffer, to the assay kit buffer, and to a ~2:1 mixture.

Plots of pH versus volume of supernatant added were plotted for each test. The results are shown in Figure 8. These are fundamentally just acid-base titration plots of the basic buffer solutions being titrated with acidic sample supernatant. If the plot is more sigmoidal in shape the buffer capacity has been exceeded and a titration-type endpoint will be observed. This is not desirable. If the plot is relatively flat the buffer capacity is sufficient to compensate for added supernatant. Ideally, a supernatant volume of 200  $\mu$ L is

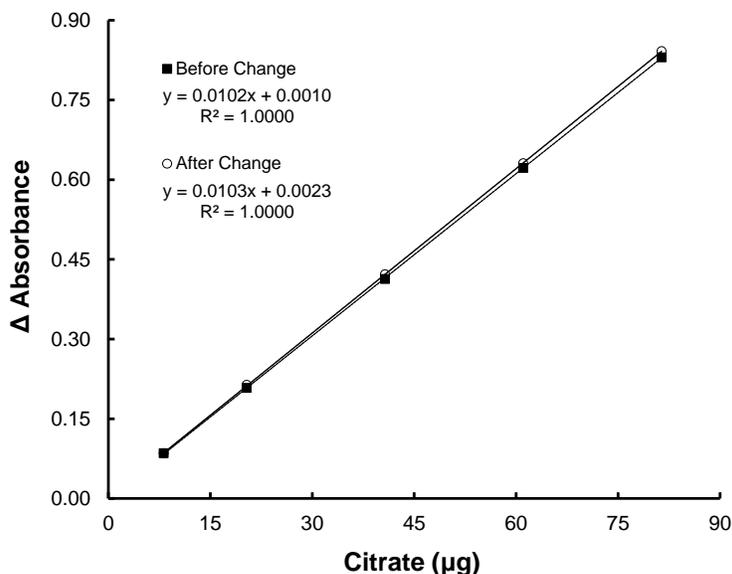
desired so that an appreciable analytical signal can be obtained during the assay. Based on the results the 100 mM GLY-GLY buffer is able to maintain a pH of 7 up to ~60  $\mu\text{L}$  of supernatant, the 300 mM GLY-GLY buffer ~170  $\mu\text{L}$ , the assay kit's buffer ~180  $\mu\text{L}$ , and the mixture of the 100 mM GLY-GLY buffer and the assay kit GLY-GLY buffer is ~100  $\mu\text{L}$ . It seems that the GLY-GLY buffer included with the assay kit is slightly more concentrated than 300 mM. After further experimentation a final optimized GLY-GLY buffer concentration of 350 mM buffer was selected.



**Figure 8.** Plots of pH versus volume of supernatant added ( $\mu\text{L}$ ). Results shown are for a 100 mM GLY-GLY and 300 mM GLY-GLY buffer solution and for the assay kit buffer and a 2:1 mixture of 100 mM GLY-GLY and assay kit buffers.

## 2.2 Reassessment of Calibration, Detection Limit, Accuracy, and Precision

Because the UV-Vis assay was altered by adding 350 mM GLY-GLY buffer it was revalidated to ensure that method performance was retained. A series of calibration and method validation studies were performed. The UV-Vis assay was calibrated using an external calibration approach, which involved the analysis of five calibration standards solutions containing a known amount of citrate. The analytical data was then plotted versus citrate mass ( $\mu\text{g}$ ). The method of least-squares was used to find the “best” fit line for each data set. It was seen that the linear regression parameters (i.e., slope, y-intercept,  $R^2$ ) were found to be statistically the same as those obtained prior to the change to the assay (see Figure 9). The kinetics of the assay also appeared to be unaffected as well.



**Figure 9.** Comparisons of calibration curves prepared before and after changes were made to the UV-Vis assay.

### 2.2.1. Detection Limit

The LOD was found to be ~0.006 wt %, the LOQ ~0.017 wt%, and the MRL was ~ 0.1 wt%. In order to be more in line with the calculations utilized by Schwarcz et al. and Kanz et al. LOQ and LOD the LOD and LOQ values were slightly adjusted; however we believe the MRL to be a more realistic estimation of the “true” detection limit of the UV-Vis assay. Overall, the detection limits were nearly the same before and after changes were made to the UV-Vis assay.

### 2.2.2. Instrument Accuracy and Precision

Instrument accuracy, measured as % relative error from the “true” value, and precision, measured as % relative standard deviation, %RSD, were assessed by analyzing citric acid standard solutions with a citrate amount 2 to 10 times the MRL. The criterion for instrument accuracy was selected to be ± 5% relative error. The criterion for instrument precision was selected to be less than 5% RSD. The % relative error results were less than 3% for n = 50 replicates collected over three weeks, which is similar before the change was made to the assay. The %RSD was less than 3% which is also similar to the results obtained prior to changes to the assay. Following these studies it was concluded that the modified UV-Vis assay performed acceptably and could be utilized for analysis of human bone samples.

### 3. Analysis of Human Skeletal Remains

A total of 31 individual bone samples with PMI values at 2 years or greater were analyzed during this project. Twenty-nine of the samples were from obtained from UTK and two were collected from OCME. Eleven of the individual samples included only metatarsal bone (long bone type), one sample included just rib bone, one sample included cranial bone, and the remaining 19 samples included both metatarsal and rib bone (flat bone type). Table 1 lists the sample ID, bone type, and PMI information for each sample. Spiked and unspiked samples were prepared, digested, and analyzed by the UV-Vis assay and the HPLC method. Note that some of the Bone Type #2 samples were analyzed months after the Bone Type #1 samples; therefore the PMI slightly different in those instances. The subsequent sections summarize the findings from the analysis of unspiked and spiked samples. A concise qualitative comparison between our results and those of Schwarcz et al. (2010) and Kanz et al. (2014) is included.

**Table 1.** Information for Human Skeletal Remains

#	Sample ID	Bone Type #1	PMI (yr) <sup>a</sup>	Bone Type #2	PMI (yr) <sup>a</sup>
1	UT53_13D	left metatarsal 3	1.8	--	--
2	UT40_13D	left metatarsal 3	2.0	--	--
3	UT35_13D	left metatarsal 3	2.0	--	--
4	UT88_12D	left metatarsal 3	2.5	--	--
5	UT69_12D	left metatarsal 3	2.8	--	--
6	UT61_11D	right metatarsal 3	4.1	--	--
7	UT92_10D	right metatarsal 3	4.7	--	--
8	UT70_10D	right metatarsal 3	4.9	--	--
9	UT115_09D	right metatarsal 3	5.6	--	--
10	UT109_09D	left rib	5.7	left metatarsal 3	5.7
11	UT62_09D	left rib	6.0	left metatarsal 3	6.1
12	UT61_08D	left rib	7.0	left metatarsal 3	7.0
13	UT34_05D	left rib	10.1	left metatarsal 3	10.1
14	UT44_04D	left rib	10.8	left metatarsal 3	10.8
15	UT26_03D	left rib	12.2	left metatarsal 3	12.2
16	UT42_02D	left metatarsal 3	12.6	--	--
17	UT24_01D	left rib	14.0	left metatarsal 3	14.0
18	UT23_00D	left rib	14.6	left metatarsal 3	14.7
19	UT13_00D	left rib	14.8	left metatarsal 3	14.8
20	UT18_98D	left rib	16.6	left metatarsal 3	16.7
21	UT04_98D	left rib	17.4	left metatarsal 3	17.5
22	UT11_97D	left rib	17.7	left metatarsal 3	17.9
23	UT08_96D	left rib	18.7	left metatarsal 3	18.9
24	UT23_94D	left rib	20.3	left metatarsal 3	20.5
25	UT07_94D	left rib	21.3	left metatarsal 3	21.5
26	UT03_93D	left rib	22.6	left metatarsal 3	22.7
27	UT02_92D	left rib	23.2	left metatarsal 3	23.4
28	UT21_90D	right rib	24.7	left metatarsal 3	24.9
29	UT22_90D	left metatarsal 3	24.7	--	--
30	OCME78-1243	rib	37.5	--	--
31	OCME15-1243	cranial	170.6	--	--

<sup>a</sup>PMI values calculated up until the day of sample preparation.

UT = Forensic Anthropology Center, University of Tennessee, Knoxville

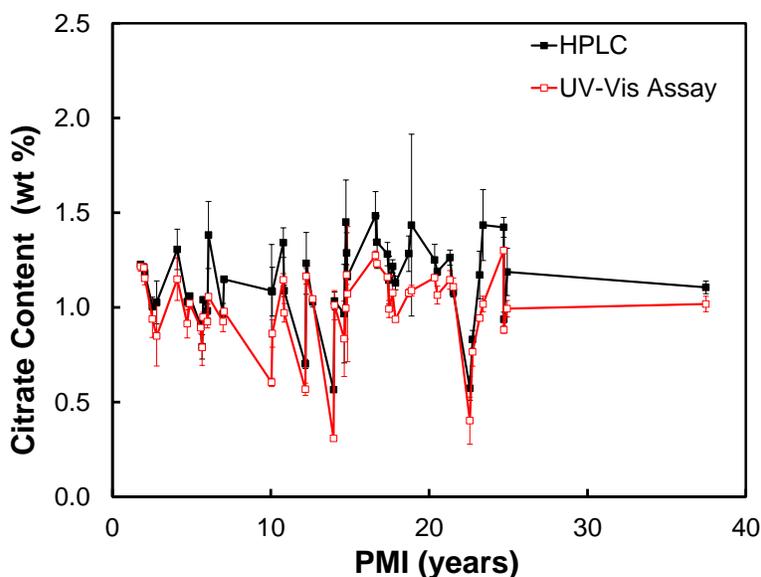
OCME = Onondaga County Medical Examiner's Office

### 3.1 Analysis of Unspiked Human Skeletal Remains

The number of analyses that could be performed for each sample was directly based on the mass of bone powder obtained from grinding. An ideal mass of ~0.6 – 0.7 g typically allows for 12 replicate digestions. Six being spiked samples and six being unspiked samples. The majority of samples; however yielded less than 0.6 grams; therefore the number of spiked samples was reduced as necessary.

#### 3.1.1. Comparison of Composite Results for Unspiked Samples for Both Bone Types

Supernatant from each digestion of an unspiked sample was analyzed by the UV-Vis assay and the HPLC method and the citrate content (wt%) was determined. The composite citrate content data was plotted versus PMI (years) as shown in Figure 10. The tracking of the two analytical methods appears to be fairly good. Overall, there was no direct correlation found between the citrate content in bone samples and PMI. Schwarcz et al. suggested there might be a possible correlation; however one must consider that their research was preliminary and only based on a limited sample size that included both human and pig bones. While these results are somewhat discouraging they are nonetheless similar to those obtained by other researchers, such as Kanz et al. Further details are included later in this report.



**Figure 10.** Plot of composite citrate content (wt%) results versus PMI (years) for 30 individual human bone samples.

The composite citrate content data for each method is summarized in Table 2. Two statistical tests were performed to compare these results. The F-test results showed that the variances for the two analytical methods were statistically the same ( $F_{\text{calculated}} = 1.05 < F_{\text{critical}} = 1.73$ ). A paired two sample t-test was performed and showed that the mean results for both methods were statistically different ( $t_{\text{stat}} = 8.329 > t_{\text{critical}} = 1.677$ ). Both tests were performed at the 95% confidence level.

The HPLC results are positively biased (larger) compared to the results for the UV-Vis assay. However the uncertainty in the data was statistically similar for both methods. The absolute error between the two methods was 16%. This difference can possibly be explained by considering that the UV-Vis assay is more susceptible to interferences from protein fragments, changes in pH, and other variables, which results in

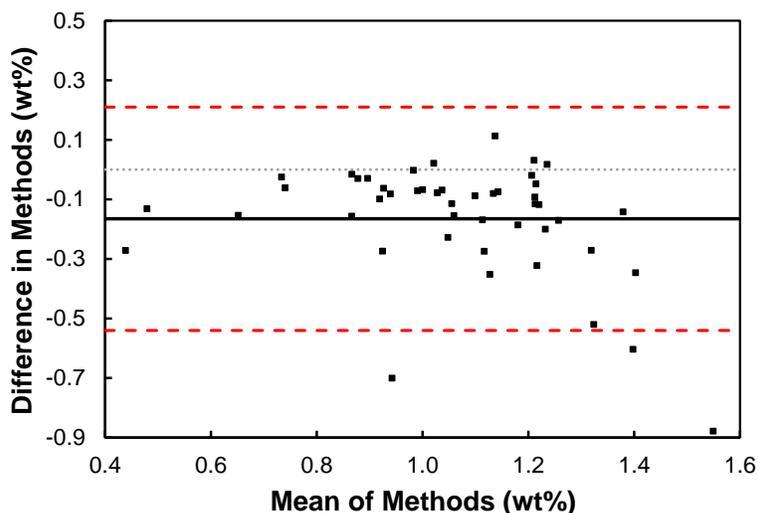
lower citrate content than expected. Another possibility is that the HPLC method could be reporting a higher citrate content than is actually present.

**Table 2.** Composite Results for Unspiked Samples

Analytical Method	HPLC Citrate wt% <sup>a</sup>	UV-Vis Citrate wt% <sup>a</sup>
Mean ( $\bar{x}$ ) Citrate wt%	1.1 <sub>3</sub>	0.9 <sub>9</sub>
Std. Dev. ( $\sigma_{n-1}$ ) Citrate wt%	±0.2 <sub>1</sub>	±0.2 <sub>0</sub>
Variance ( $\sigma^2$ )	±0.04 <sub>5</sub>	±0.04 <sub>1</sub>
%RSD	19	21

<sup>a</sup>Composite average of 30 individual samples.

A Bland-Altman analysis was performed to further compare the two methods (Bland & Altman, 1999). First a difference plot (Bland-Altman type plot) was prepared by plotting the difference in citrate content (wt%) between the UV-Vis assay and HPLC method on the y-axis versus the mean citrate content (wt%) of the two methods on the x-axis (see Figure 11). The two red dotted lines at y-values 0.21 and -0.54 are the 95% confidence levels and the solid black line is the mean of the data. This type of plot serves to detect bias (system error), and outliers, and to detect relationships between the data and magnitude of measurement. It is immediately apparent that the HPLC method has a positive bias of approximately 0.17 wt% compared to the UV-Vis assay as was mentioned in the previous section. Because the bias is higher than the method reporting limiting the difference is significant and suggests the results for the two methods are statistically different. and that there is random scattering of data about the mean line.



**Figure 11.** Bland-Altman type plot of the difference between the estimated citrate content (wt%) (UV-Vis – HPLC) versus the mean of the methods’ citrate content results. Single replicate used.

It is also apparent that there may be a slight negative correlation between the difference and the magnitude of citrate content. Also it is seen that the scatter of data becomes larger as the PMI increases; however the

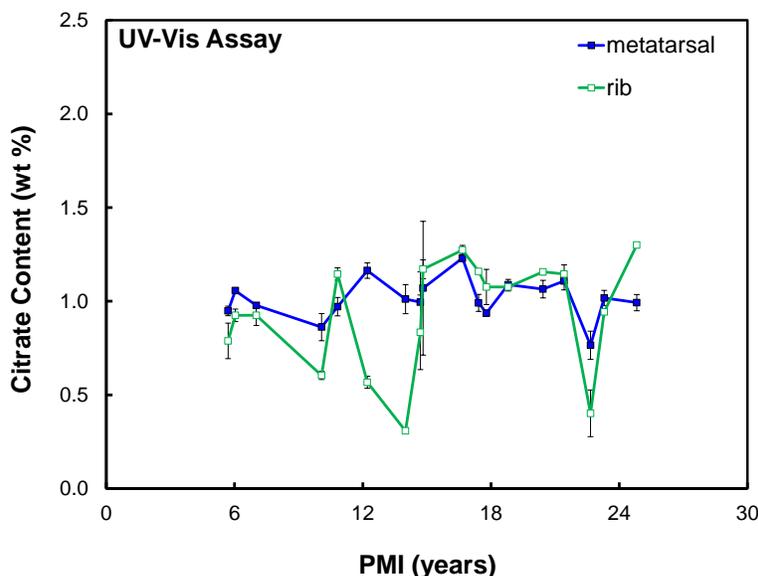
relationship is weak and is within the confidence intervals estimated from the Bland-Altman plot. The standard deviation results using two replicates of the data set suggest that the two methods do statistically disagree to a greater degree at for samples with higher PMIs thereby possibly leading to a weaker relationship between the citrate content and PMI as suggested by Schwarcz et al. Additionally, it is observed that there were three outliers, which in a data set of 49 points is probable, but will slightly skew the average data. Overall, there does not seem

### 3.1.2. Comparison of Results for Unspiked Samples for Each Bone Type

Eighteen samples included metatarsal and rib bone. The results for each bone type can therefore be statistically compared to assess for differences and overall agreement.

#### *UV-Vis Assay Results*

The citrate content data obtained from the analysis of metatarsal and rib bones was compared using two statistical tests. The results from the F-test showed that the variances in citrate content for the two types of bone were statistically different ( $F_{\text{calculated}} = 7.77 > F_{\text{critical}} = 2.27$ ). The paired two sample t-test results showed that the mean citrate content for the two methods were statistically the same for both bone types ( $t_{\text{stat}} = 1.285 < t_{\text{critical}} = 2.110$ ). Figure 12 shows a plot of citrate content versus PMI (years) for two bone types using UV-Vis assay data.

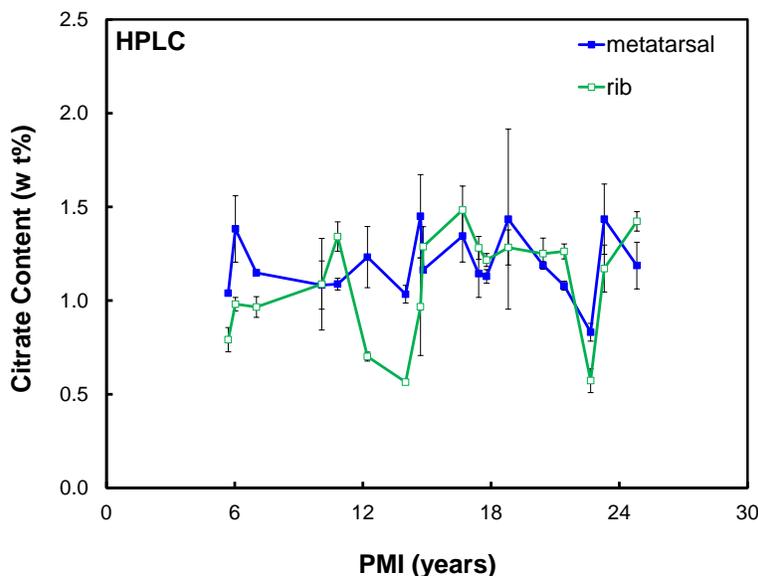


**Figure 12.** Plot of citrate content (wt%) versus PMI (years) for 18 metatarsal and 18 rib bone samples (UV-Vis assay).

Overall, the average difference between the results for metatarsal and rib bone were 0.081 ( $\pm 0.3$ ) wt%. The %RSD values for the rib samples are approximately a factor of three larger than for metatarsal. These results seem to suggest that the citrate content in long bones like metatarsal may be more homogeneously distributed within the bone matrix or perhaps preparation of metatarsal bone results in less interferences during analysis. However note that these explanations are highly speculative as there is overlap within statistical uncertainty.

#### *HPLC Method Results*

The citrate content data obtained from the analysis of metatarsal and rib bones was compared using two statistical tests. The results from the F-test showed that the variances in citrate content for the two types of bone were statistically different ( $F_{\text{calculated}} = 2.88 > F_{\text{critical}} = 2.27$ ). The paired two sample t-test was performed showed that the mean citrate content results for the two bone types were statistically the same ( $t_{\text{stat}} = 1.563 < t_{\text{critical}} = 2.110$ ). Figure 13 shows a plot of citrate content versus PMI (years) for the two bone types using HPLC method data. Overall, the average difference between the results for metatarsal bone versus rib bone was  $0.098 (\pm 0.3)$  wt%. These results were similar to those obtained utilizing the UV-Vis assay.



**Figure 13.** Plot of citrate content (wt%) versus PMI (years) for 18 metatarsal and 18 rib bone samples (HPLC).

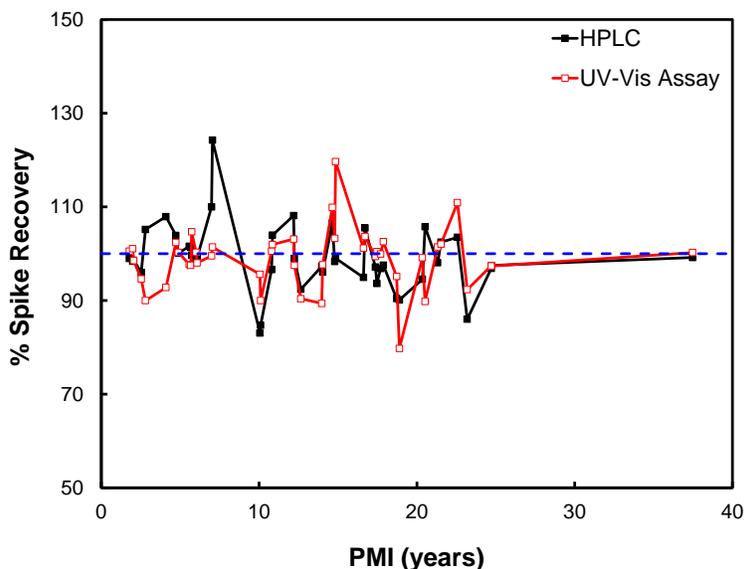
### 3.2 Analysis of Spiked Samples

The citrate content (wt%) data for the spiked samples were used to evaluate method accuracy (% spike recovery) and precision (%RSD) and to detect systematic errors if present. Three to six replicate digestions were performed for each spiked sample. To prepare a spiked sample, we added  $200 \mu\text{L}$  of  $\sim 1000 \text{ mg}\cdot\text{L}^{-1}$  citric acid standard to each centrifuge tube prior to acid digestion. This is referred to as matrix spiking and is done to increase confidence in the accuracy and validity of results and to detect systematic errors. The spike recovery was calculated by dividing the average experimental total mass of citrate determined to be the sample by the average theoretical total citrate mass and multiply by 100. A spike recovery of 100% would suggest high method accuracy and minimal systematic errors. Spike recovery values outside a range of  $\pm 5\%$  may suggest there is a matrix effect(s) or that there is a systematic error(s) with sample preparation and/or analysis procedures.

#### 3.2.1. Comparison of Composite Results for Spiked Samples for Both Bone Types

The supernatant from each digestion of spiked sample was analyzed by the UV-Vis assay and the HPLC method to determine citrate content (wt%) and % spike recovery values. A plot of % spike recovery versus PMI (years) is shown in Figure 14. The spike recovery values for HPLC ranged from 83 – 120% while the UV-Vis ranged from 80 – 120%. A few samples had recovery values that exceeded  $100 \pm 5\%$ , but the

majority of samples were within this tolerance. The analytical methods agreed (tracked) quite well and only deviated with regards to variance.



**Figure 14.** Plot of composite % spike recovery values versus PMI (years) for 30 individual human bone samples.

The composite % spike recovery results for each method are listed in Table 3. Two statistical tests were performed to compare the % recovery values. The results from the F-test showed that the variances for the two methods were statistically different ( $F_{\text{calculated}} = 1.20 < F_{\text{critical}} = 1.66$ ). A paired two sample t-test was performed, which showed that the mean % spike recovery for the two methods were statistically the same ( $t_{\text{stat}} = 0.1389 > t_{\text{critical}} = 2.0167$ ).

**Table 3.** Composite % Recovery Values from the Analysis of Spiked Samples

Analytical Method	HPLC % Spike Recovery <sup>a</sup>	UV-Vis % Spike Recovery <sup>a</sup>
Mean ( $\bar{x}$ ) % Spike Recovery	99.1	98.7
Std. Dev. ( $\sigma_{n-1}$ ) % Spike Recovery	$\pm 7.0$	$\pm 6.4$
Variance ( $\sigma^2$ )	$\pm 50$	$\pm 41$
%RSD	7	6

<sup>a</sup>Composite average of 30 individual samples.

### 3.2.2. Comparison of Results for Spiked Samples for Each Bone Type

Fourteen samples that included both metatarsal and rib bone were statistically compared to assess for differences and overall agreement.

*UV-Vis Assay Results*

Two statistical tests were performed to compare the % spike recovery values obtained for metatarsal and rib bone samples for the UV-Vis assay. The results from the F-test showed that the variances in the % recoveries for the two bone types were statistically different ( $F_{\text{calculated}} = 6.14 > F_{\text{critical}} = 2.58$ ). A paired two sample t-test was performed showed that the results for the two bone types were statistically the same ( $t_{\text{stat}} = 0.129 < t_{\text{critical}} = 2.160$ ). Both tests were performed at the 95% confidence level. The data is summarized in Table 4. Interestingly the deviation in results was larger for the metatarsal samples than the rib samples. This is opposite of what was seen for the results obtained for the unspiked samples, but still within statistical uncertainty.

**Table 4.** Average % Spike Recovery Values for the UV-Vis Assay

Bone Type	Metatarsal % Spike Recovery	Rib % Spike Recovery
Mean ( $\bar{x}$ ) % Spike Recovery	99.2	99.0
Std. Dev. ( $\sigma_{n-1}$ ) % Spike Recovery	±9.0	±3.6
Variance ( $\sigma^2$ )	±82	±13
%RSD	9	4

*HPLC Method Results*

Two statistical tests were performed to compare the % spike recovery values obtained for metatarsal and rib bone samples for HPLC. The results from the F-test showed that the variances in the % recoveries for the two bone types were statistically the same ( $F_{\text{calculated}} = 1.91 > F_{\text{critical}} = 2.58$ ). A paired two sample t-test was performed showed that the results for the two bone types were statistically the same ( $t_{\text{stat}} = 1.514 < t_{\text{critical}} = 2.160$ ). Both tests were performed at the 95% confidence level. The data is summarized in Table 5. These results suggest that no majors systematic interferences were encountered for the HPLC method.

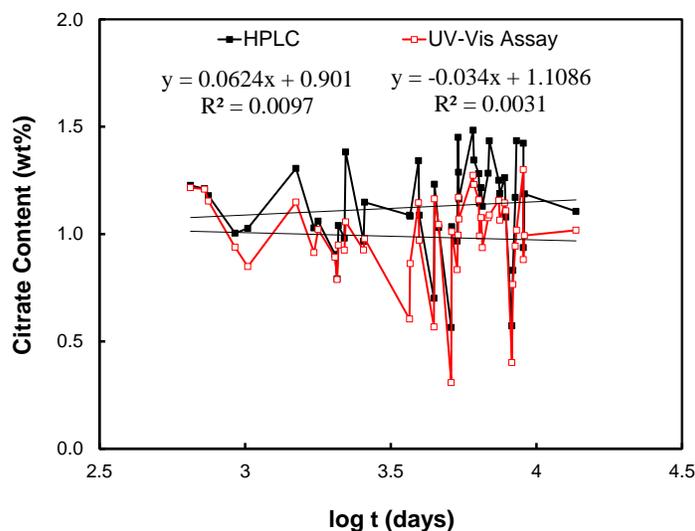
**Table 5.** Average % Spike Recovery Values for the UV-Vis Assay

Bone Type	Metatarsal % Spike Recovery	Rib % Spike Recovery
Mean ( $\bar{x}$ ) % Spike Recovery	100.1	97.5
Std. Dev. ( $\sigma_{n-1}$ ) % Spike Recovery	±9.1	±6.6
Variance ( $\sigma^2$ )	±82	±43
%RSD	9	7

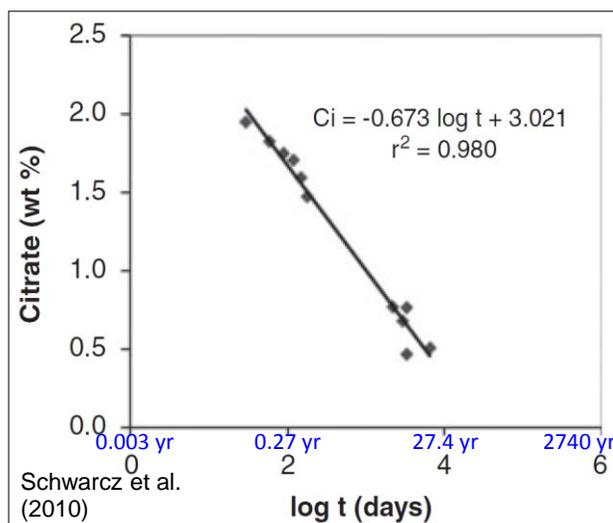
**4. Comparison to Published Results**

There was no direct correlation found between citrate content and PMI (log days) for our data (Figure 15). The  $R^2$  value was approximately 0.0097 for the HPLC data and 0.0031 for the UV-Vis assay. Schwarcz et al reported an  $R^2$  value of 0.980 when citrate content was plotted versus PMI log t (days) as seen Figure 16.

An ideal  $R^2$  value for calibration is generally above 0.980, but for this type of comparison 0.980 is quite good indeed. However the sample size used by Schwarcz et al. was much lower (~11-15) and included pig bone results as part of the data set. This does explain the discrepancy between our findings and Schwarcz, but does suggest further research by multiple labs would be required to fully validate the method. Another issue of concern is the use of a log scale for correlating citrate content with PMI. Using log scales for calibration sometimes results in issues with compression, non-linearity, and propagation of uncertainties between unequally weighted at the two extremes of the calibration fit. A polynomial weighted fit or a typical linear fit would be more desirable if possible.



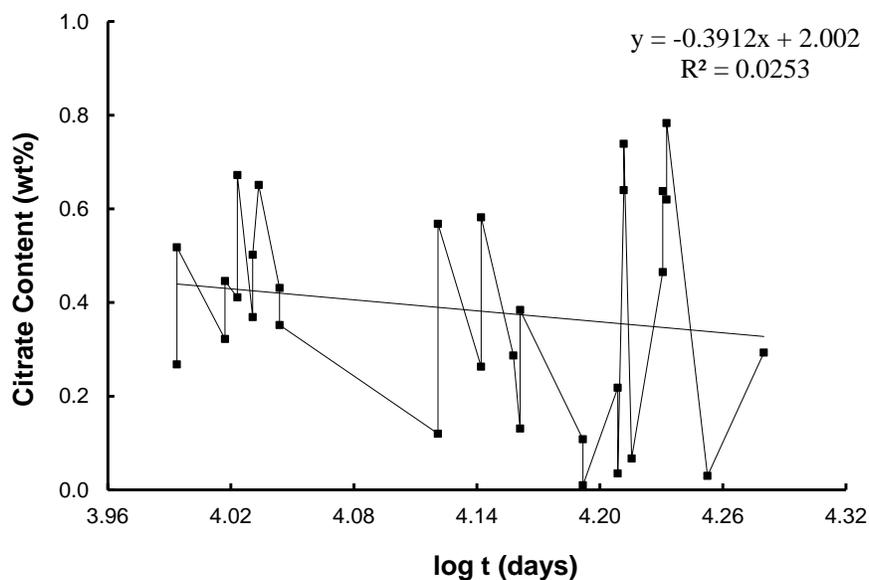
**Figure 15.** Plot of composite citrate content (wt%) versus log t (days) for 30 individual human bone samples (48 total samples).



**Figure 16.** Plot of citrate content (wt%) versus log t (days) adapted from Schwarcz et al. 2010.

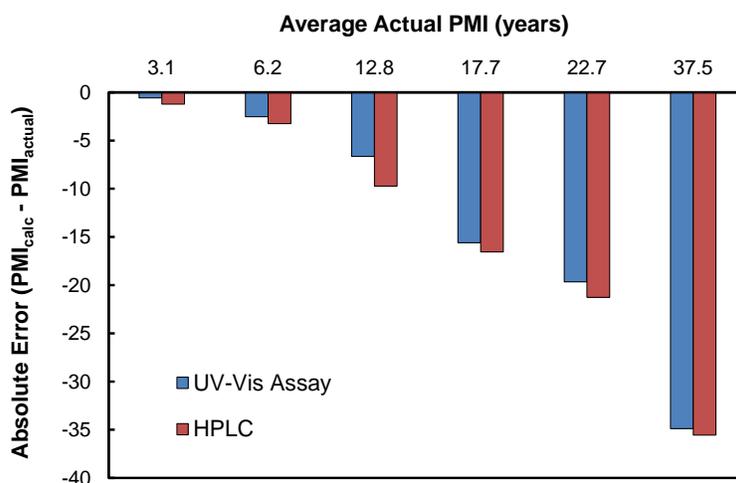
While our results do not agree with Schwarcz et al. they are similar to those reported by Kanz et al. The data reported by Kanz and coworkers was plotted in order to determine a linear regression equation (see Figure 17). This data yielded an  $R^2$  of only 0.0253, which is slightly better than our results, but not useful in the determination of PMI. Their results were also statistically different that those of Schwarcz et al. Note

Kanz et al. analyzed samples with PMI values of 27 - 52 years whereas our samples ranged from 2 to 37 years. Our data and their data appear randomly distributed with no discernable relationship between citrate content and PMI.



**Figure 17.** Plot of citrate content (wt%) versus log t (days) adapted from Kanz et al. 2014 (31 total samples).

The results for the calculated PMI using Schwarzc’s equation were well below the actual “true” PMI of the samples using either analytical method. This was also observed by Kanz et al. Figure 18 shows the average absolute error between the calculated PMI and actual PMI from 30 samples averaged into six PMI points. The average absolute error increases with an increase in PMI, which is expected as Schwarzc’s equation predicts a decreasing citrate level with an increase in PMI.



**Figure 18.** Plot of absolute error between calculated PMI and actual PMI versus average PMI intervals.

### 5. Analysis of a Human Sample with PMI > 100 years

Based on the results of Schwarcz et al. and Kanz et al. the limit of quantitation (LOQ) of PMI would be approximately 80 years. This value was calculated based on extrapolating linear (log) equation data and considering the LOQ for citrate content using the UV-Vis assay. With this information in mind, one would expect that bone samples with PMI > 100 years to have trace level citrate content below the LOQ for the UV-Vis assay and even the HPLC method. Kanz et al. analyzed a bone sample that was “several hundred years old” from an archaeological excavation and found trace level citrate at the noise level of the UV-Vis assay.

For our research, we analyzed a ~173 year old human bone sample (cranial) of known provenance and death date provided by OCME. Both spike and unspiked samples were prepared and analyzed by UV-Vis assay and the HPLC method. The summarized results are listed in Table 6 and Table 7. The citrate content determined by HPLC was above the minimum reporting level (MRL) of ~0.1 wt, so can be reported. The citrated content determined by the UV-Vis assay was below the MRL. It was also seen that the HPLC method yielded better spike recovery values than the UV-Vis assay. If a spike recovery correction factor is applied to the UV-Vis assay data the result is still below the MRL. When the HPLC result is inputted into the equation proposed by Schwarcz et al. the estimated PMI is approximately 47 years, which is an absolute error of -123 years. Despite this issue, the data does suggest that samples with PMIs above a certain threshold may be statistically discernable from samples with PMIs below said threshold. What is this this PMI threshold and would it be statistically defensible has yet to be determined by us or any researchers to the best of our knowledge. Though at this point time we can confidently report that the citrate content of the 170 year old bone sample is statistically different than any of the pig or human samples we have tested. The next oldest human sample we analyzed has a PMI of ~ 37.5 years a citrate content of ~1.10 (±0.08) wt% as determined by HPLC. Similar results and observations were reported by Kanz et al. Overall; we agree with these researchers that bone samples with PMIs > 100 years have less citrate content than “younger” samples and that further research is warranted.

**Table 6.** Results from the Analysis of Unspiked Samples of OCME\_1842\_Bone\_A

Method	HPLC Citrate wt% <sup>a</sup>	UV-Vis Citrate wt% <sup>a</sup>
Mean ( $\bar{x}$ ) Citrate wt% <sup>b</sup>	0.171 <sub>0</sub>	0.06 <sub>6</sub> *
Std. Dev. ( $\sigma_{n-1}$ ) Citrate wt%	±0.004 <sub>9</sub>	±0.01 <sub>9</sub> *
95% Conf. Interv.	±0.007 <sub>8</sub>	±0.03 <sub>1</sub> *
%RSD	3	30*

<sup>a</sup>Four separate digestions

<sup>b</sup>Minimum reporting level for HPLC and UV-Vis = ~0.1 wt%

\*Below the MRL for the analytical method

**Table 7.** Results from the Analysis of Spiked Samples of OCME\_1842\_Bone\_A

Method	HPLC % Spike Recovery <sup>a</sup>	UV-Vis* % Spike Recovery <sup>a</sup>
Mean ( $\bar{x}$ ) % Spike Recovery <sup>b</sup>	102.8	71.7
Std. Dev. ( $\sigma_{n-1}$ ) % Spike Recovery	±2.3	±3.0
95% Conf. Interv.	±3.7	±3.5
%RSD	2	3

<sup>a</sup>Four separate digestions

<sup>b</sup>Ideal %spike recovery 100 ± 5 %

\*Below the MRL for the analy

## IV. CONCLUSIONS

### 1. Discussion of Findings

Our research found that a decline in citrate did occur, yet not in the linear way that Schwarcz et al. 2010 predicted. We saw no clear, replicable relationship between our samples' citrate content and post mortem interval of 1 – 35 years. However when we tested a sample of 173 years, the citrate was nearly undetectable by HPLC and was undetected using UV-Vis method. We recommend a follow up study or studies that entail testing citrate in known PMI human remains ranging from 50 to 200 years. Thus the decline in citrate over time will be more clearly visualized if it does indeed exist.

### 2. Implications for Policy and Practice

At this time we cannot recommend utilizing citrate as a reliable and accurate predictor of PMI in skeletal cases. With the study of known PMI cases ranging from 50 to 200 years, it should be more apparent if there is a predictable relationship between these two variables. A field test could then be developed to test for citrate, with a positive result indicating that the case is less than 200 years and may need further medico legal attention, while a negative result would indicate that no medico legal agency involvement is required. The case in the latter situation would be referred to the State Historic Preservation Office or the local Tribal Authorities.

### 3. Implications for Further Research

As mentioned above, the need for a follow up study with an archaeological sample of human remains ranging from 50 to 200 years would be helpful to clarify the relationship between citrate in bone and PMI. Additional studies should also work to determine the baseline for citrate as this study and others found discrepancies between that reported by Schwarcz et al. 2010 and our own results.

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## VI. DISSEMINATION OF RESEARCH FINDINGS

### Peer reviewed Journal Articles:

In preparation. Manuscript to be submitted to *Journal of Forensic Sciences*, Fall 2016.

### Conference Presentations

Brown, M., Froome, C., Hennessy, S., **Gerling, R.**, Ellison, J. & Bunch, A. (2016). An external validation of the citrate content PMI method. Oral Communication. 68<sup>th</sup> Annual Scientific Meeting of the American Academy of Forensic Sciences, Las Vegas, NV.

**Hennessy, S.**, Brown, M., Froome, C., Gerling, R., Ellison, J. & Bunch, A. (2016). Citrate content of bone: A potential measure of postmortem interval. Oral Communication. 2<sup>nd</sup> Annual SUNY Undergraduate Research Conference (SURC), Cobleskill, NY.

**Hennessy, S.**, Brown, M., Froome, C., Gerling, R., Ellison, J. & Bunch, A. (2015). Citrate content of bone: A potential measure of postmortem interval. Poster Format. 42<sup>nd</sup> Annual Fall Scientific Paper Session for the Rochester Academy of Science, Rochester, NY.

**Froome, C.**, & Brown, M. A. (2015). Citrate content of bone: A potential measure of postmortem interval. Oral Communication. 1<sup>st</sup> Annual SUNY Undergraduate Research Conference, American Academy of Forensic Sciences Annual Meeting, The College at Brockport, SUNY, Brockport, NY.

**Bunch, A. W.**, Brown, M. A., & Froome, C. (2015). An external validation test of citrate content PMI method. NIJ R&D Grantees Meeting, "*It's a Small World: How Collaboration Leads to Innovative Research,*" American Academy of Forensic Sciences Annual Meeting, February 17, 2015, Orlando, Florida.

**Froome, C.**, & Brown, M. A. (2014). Citrate content of bone: A potential measure of postmortem interval. Oral Communication. 59<sup>th</sup> Annual 2014 Undergraduate Research Symposium, American Chemical Society (ACS), Rochester Institute of Technology, Rochester, NY.

**Ellison, J.**, & Brown, M. A. (2014) Citrate content of bone: A potential measure of postmortem interval. Oral Communication. Scholar's Day, The College at Brockport, SUNY, Brockport, NY.

**Froome, C.**, & Brown, M. A. (2014). Citrate content of bone: A potential measure of postmortem interval. Oral Communication. 28<sup>th</sup> National Conference on Undergraduate Research (NCUR), The University of Kentucky, Lexington, KY.

### Peer-reviewed Conference Proceedings

**Froome, C.**, & Brown, M. A. (2014). Citrate content of bone: A potential measure of postmortem interval. Proceedings of the National Conference on Undergraduate Research (NCUR), The University of Kentucky, Lexington, KY. pp 1100-1108.

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## **IMPACT**

**What is the impact of the project on the criminal justice system?** The impact of the project on the criminal justice system may be significant depending on results. The ability to estimate the postmortem interval with more precision could affect law enforcement activities (scene response and guarding scenes), death investigations (ascertaining whether remains are of forensic significance or not), prosecution and defense counsel (knowing time of death is crucial for the presentation of a case and defense of a case), and family members of victims (understanding what occurred with more certainty). At this time, forensic specialists have difficulty determining PMI with certainty especially if soft tissue is absent and remains are skeletonized. At the time of this writing, our team has tentatively concluded that the citrate content method has not been shown to be a reliable and valid measurement of PMI.

**How will this project contribute to crime laboratories?** The use of citrate to determine PMI may contribute to crime laboratories, depending on results. Crime laboratories may be able to use the detection methods (enzyme assay and/or HPLC) to test bone samples from unknown PMI cases in order to get a quick answer on whether or not a skeletonized case is of forensic interest or not. This may be able to be tailored to a field test kit with further work/study. Again, our team has not found that citrate decreases with predictable regularity to provide a precise/accurate/reliable method of PMI.

## **CHANGES/PROBLEMS**

### **Changes in Approach and Reasons for Changes**

The number of samples requested and obtained from the Onondaga County Medical Examiner's Office has been reduced to two samples of known age of known provenance for testing. The reason for the reduction in samples requested/obtained is that the results of the testing from the University of Tennessee Knoxville were varied and demonstrated that the Schwarcz et al. conclusions regarding the reliability of citrate as an indicator of PMI were in question. We did not want to utilize actual human remains from the medical examiner's office knowing that our prior test samples were resulting in results that appeared to show variability and lack of consistency as we had expected to see if Schwarcz et al.'s method had shown that it was externally valid.