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Citrate Content of Bone: A Potential Measure of Postmortem Interval

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Abstract

The reliable and accurate determination of the postmortem interval (PMI) of human skeletal remains continues to be a major problem in forensic science. Recent research has suggested that the citrate content of bone may provide a promising approach to PMI determination of skeletal remains; therefore, it warrants further investigation. The main goals of this research are to validate, further optimize, and develop the method and eventually apply it to the analysis of human skeletal remains. The research project consists of three phases; however, only results from phase one are presented here. Results from initial optimization and calibration studies, and preliminary results from the analysis of fresh pig bone (PMI < 1 month) are discussed. The detection limit of the UV-Vis assay was approximately 4 µg of citrate (~0.008 wt%) and approximately 2 $mg \cdot L^{-1}$ of citrate for the HPLC method (~0.05 wt%). Acid digestions were performed with fresh pig bone and analyzed by each method; the wt% of citrate of fresh pig bone was 1.02 ± 0.08 wt% for the UV-Vis assay and 1.12 ± 0.06 wt% for the HPLC method.

Keywords: Forensic Science, Postmortem Interval, Citrate

1. Introduction

The postmortem interval (PMI), the time elapsed since death, is a crucial piece of information for forensic scientists and medicolegal investigators, because the knowledge of the PMI can lead to identification of victim(s) and/or suspects by establishing the time dimension in investigations. An interdisciplinary research effort between entomologists, anthropologists, biochemists, geochemists, botanists, pathologists, and others have investigated the remains of the decedent to assess PMI. From these research efforts various qualitative and quantitative methods have been developed. Qualitative methods looked at correlated variables, such as insect colonization and temperature to estimate PMI. Various qualitative methods have been utilized; however the majority of these methods are not able to determine the PMI beyond several weeks. The majority of quantitative methods are chemical based assessments of PMI, for example radio decay testing. The quantitative methods that have been developed have issues with accuracy and/or precision and have not been extensively validated.

1.1 Qualitative Methods

Qualitative methods utilized for estimating PMI include the evaluation of body appearance (decomposition stages) and the use of insects (forensic entomology). Unfortunately, these approaches are unreliable and are dependent on the presence of soft tissue, which makes them unsuitable for estimating PMI of skeletal remains. They generally have a limited PMI range of approximately 0 to 60 days.

When a body decomposes it exhibits distinct changes in appearance (e.g., “fresh,” “discoloration,” “bloat,” “skeletonization,” and “skeletal decomposition”), referred to as decomposition stages.[1] Prediction of stages is variable and dependent on environmental conditions, making accuracy an issue. Bodies placed within a mile[2] or

even a few meters[3] of each other may reach the stages at different times. Use of insects have also been used to asses PMI with some success.[4, 5] By examining the sequence of colonization, the remains' deposition and exposure times can be elucidated. Although accurate in many cases, the quality of PMI estimates based on entomology begins to decrease rapidly with loss of soft tissue. Once remains become fully skeletonized, the use of entomology assessment is severely limited or impossible. An alternative approach[6] utilizes temperature in the form of Accumulated Degree Days (ADD) to estimate PMI for skeletonized and decomposing remains. 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, and local temperature data to account for the average temperature of each day, the ADD is estimated.[6] When the ADD total is reached, an estimate of PMI is established. This method has been extensively evaluated with limited success.[7] Although various qualitative methods have been utilized; the majority are not able to determine the PMI accurately beyond several weeks.

1.2 Chemical-Based Quantitative Methods

The most common chemical-based quantitative methods for the determination of PMI include luminol, radiodecay, and ^{14}C bomb spike testing. Luminol-based methods use of the reaction between hydrogen peroxide and luminol, catalyzed by the iron in hemoglobin found in bodily fluids and tissues. The reaction results in chemiluminesence light that can be measured quantitatively. Luminol testing has the advantages of being inexpensive and easy to perform; however, there are potential interferences with human fluids and body tissues. Also, there is a weak correlation between luminescent intensity and PMI.[8] Radiodecay testing involves the determination of radionuclides in bones (e.g., strontium, radium, thorium) that remain after death. Because these elements decay at a known rate and the activity ratio of the decay between the decay products and parent isotopes is used to estimate the PMI.[9] Nutrition, age of the deceased, and decomposition variables have minimal effects upon results. However, large amounts (i.e., ~300 g) of skeletal material, long preparation times, and expensive instrumentation are required. The useful PMI range is 6 months to 10 years, with large uncertainties. The ^{14}C-bomb spike method is a radioisotopic analysis. Normally ^{14}C is formed at a constant rate in the atmosphere and is dispersed into the atmosphere and water where it is incorporated into plants by photosynthesis and living organisms through the food chain.[10, 11] The international nuclear testing conducted during the 1950s and 1960s resulted in significant increases in the abundance of ^{14}C in the atmosphere, resulting in an increase in this isotope in most organisms.[10, 11] Determination of the "bomb curve" from ^{14}C in dental and osseous tissues has allowed researchers to estimate the PMI with promising results. However the best results are still obtained from using soft tissue.

1.3 Citrate Content Of Bone As A Measure Of PMI

A recent chemical-based method uses citrate content of bone as a potential measure of PMI.[12] The exterior layer of bone is principally composed of bioapatite crystal and protein (i.e., collagen). Earlier and more recent work suggests that, because citrate is too large to be incorporated into the crystal lattice, citrate is primarily bound to calcium on the surface of the apatite crystal layer. [13, 14, 15] This fact has been used to analyze domesticated pig (*Sus scrofa*) and a few human bone samples for citrate content.[12] Results suggest citrate content of the bone decreases with an increase in PMI, and that PMI of skeletal remains can be determined with a precision of ~1% for a time range of 2 months to 90 years.[12] The citrate method also appears to address a critical window of time for medicolegal investigators, because skeletal remains with a PMI of <50 years old are typically deemed forensically relevant, while older remains are considered to be "historic".[12] Recent work suggests that this method has potential, but requires improvement in accuracy.[16] Further work is necessary to validate this method.[12, 16]

1.4 Research Objectives

The overall goals of this research are to validate, further optimize, and develop the method and eventually apply it to the analysis of human skeletal remains. Previous work only utilized an UV-Vis biochemical assay this work aims to utilize both a UV-Vis assay and an HPLC method to determine the citrate content of bone samples. There are three distinct phases to the research project. Phase one involves the optimization of bone sample preparation and an UV-Vis based biochemical assay, and development of a high performance liquid chromatography (HPLC) method for analysis of citrate content of pig bone samples. Phase two will involve the analysis of fresh and aged pig bone and phase three will focus on the analysis of human skeletal remains with various PMIs. This paper will detail the

research performed thus far involving the optimization and calibration of the UV-Vis based biochemical assay and HPLC method along with initial results from the analysis of fresh pig bone (PMI < 1 month).

2. UV-VIS Citrate Assay Method Background

The first reported use of the enzyme citrate lyase for citrate determination was in 1955.[17] Improvements have been made to the assay over the years by other researchers[18, 19] and the enzymatic assay has since gone on to become a standard method used in many industries, such as food and beverage,[20] pharmaceutical, and medical technology.[21] The principle of this assay is based on the citric acid cycle (Kreb's cycle), which takes place in aerobic organisms.[22] Within this cycle there exists multiple reactions catalyzed by various enzymes; though only the four main reactions are discussed.[15, 23]

$$\text{citrate} \xrightarrow{\text{citrate lyase}} \text{oxaloacetate} + \text{acetate} \quad (1)$$

$$\text{oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{malic dehydrogenase}} \text{malate} + \text{NAD}^+ \quad (2)$$

$$\text{oxaloacetate} \xrightarrow{\text{oxaloacetate decarboxylase}} \text{pyruvate} + \text{CO}_2 \quad (3)$$

$$\text{pyruvate} + \text{NADH} \xrightarrow{\text{lactic dehydrogenase}} \text{lactate} + \text{NAD}^+ \quad (4)$$

Reaction 1 involves the cleavage of citrate into oxaloacetate and acetate, which is catalyzed by the enzyme citrate lyase. The oxaloacetate that is produced is then reduced to malate upon reacting with the reduced form of beta-nicotinamide adenine dinucleotide (NADH) (see Reaction 2). Consequently, NADH is oxidized to NAD^+. This reaction is catalyzed by the enzyme malic dehydrogenase (MDH). Note that $NADH/NAD^+$ 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. The lambda max (λ_{max}) for NADH and NAD^+ are significantly different using UV-Vis spectrophotometry, so this is the most widely used technique for this assay. The decrease in the absorbance of NADH at 340 nm is proportional to the citrate content in the sample or standard.

3. Methodology

3.1 Chemicals And Reagents

All deionized (DI) water used for solution preparation and rinsing of glassware was produced by a Barnstead UV-EasyPure system and had an 18.2 MΩ·cm resistivity. High purity ACS grade chloroform and ethanol were purchased from Sigma Aldrich and Fisher Scientific, respectively. A 0.5 M potassium hydroxide (KOH) solution was prepared from a ~11.7 M stock solution purchased from Ricca Chemical Company. Calibration buffers (i.e., 4, 7, 10) for pH were purchased from Fisher Scientific. ACS reagent grade trisodium citrate was purchased from Acros Organics and monobasic sodium phosphate from Certified Fischer Scientific. These were used to prepare citrate standards and phosphate buffer (HPLC mobile phase). Commercially available citric acid assay kits were purchased from R-Biopharm®. These kits consisted of three components: The first component was a buffer solution containing 1.4 g of lyophilized glycylglycine buffer (pH approx.7.8) mixed with L-malate dehydrogenase (136 U), L-lactate dehydrogenase (280 U), and NADH (5 mg). The second component was a citrate lyase enzyme solution containing 50 mg of lyophilized citrate lyase (12 U) and the third component was a citric acid calibration standard solution (0.407 µg μL^{-1}).

3.2 Bone Sample Supernatant Preparation

Rib bones from domesticated pig (*Sus scrofa*) were acquired from local vendors. All samples were fresh, untreated with chemicals, and never frozen. These samples were defleshed and dried at ~200 ºC for 2 hr. The samples were cut into 1-cm segments and stored in a desiccator vessel. All bone samples were first soaked in a 1:1 mixture of chloroform and ethanol for one hour to remove fat. The samples were then rinsed with ethanol and left to dry for 30 min. The samples were powderized using a SPEX 6770 Freezer Mill (2 min, 10 cycles s^{-1}). Approximately 50-mg of bone powder was placed in a 15-mL centrifuge tube along with 2.0 mL of 1.0 M hydrochloric acid and digested for 1 hr in a 60 ºC water bath. Afterwards the pH of the solution was adjusted to approximately 5 using 0.5 M KOH delivered by a digital Eppendorf micropipette. The samples were then centrifuged at 1200 g for 12 min to remove collagen and protein fragments. Approximately half of the aqueous supernatant was collected and stored at 4 ºC until analysis by the UV-Vis assay. Additional aliquots of the supernatant were sub-micron filtered using Amicon Ultra-4 centrifugal filters at 7500 g for 24 minutes then stored at 4 ºC until analysis by the HPLC method.

3.3 UV-Vis Assay

A Perkin Elmer Lambda 800 UV-Vis double-beam spectrophometer set at 340 nm was operated in kinetics mode. A five-point external calibration curve was used to calibrate the UV-Vis assay. A reagent blank containing just buffer and enzymes was analyzed prior to the analysis of five citric acid standards containing ~8, 20, 40, 60, and 80 µg of citrate. A volume of citric acid standard was added to a quartz cuvette along with glyclygylcine buffer mixture and DI water using Eppendorf micropipettes. The contents of the cuvette were thoroughly mixed and the initial absorbance was measured after 5 min. Then lyophilized citrate lyase was added to the cuvette, mixed thoroughly and the absorbance was measured after 5 min. The difference in the two absorbance values denoted as ΔAbs (see figure 1) was calculated for each standard and corrected using the ΔAbs of the reagent blank. The corrected ΔAbs values were then plotted versus the citrate mass (µg) to produce a calibration curve.

Following calibration, supernatant samples were analyzed using a similar procedure. The glyclygylcine buffer mixture, DI water, and supernatant sample were all added to the cuvette and mixed. The initial absorbance was recorded after 5 mins. The lyophilized citrate lyase was added to the cuvette mixed thoroughly and the absorbance recorded after 5 mins. The ΔAbs was calculated and corrected. The corrected ΔAbs value was substituted into the linear regression equation to determine citrate mass in the diluted sample. Appropriate calculations were performed to correct for dilution and to calculate the wt% of citrate in the original bone powder.

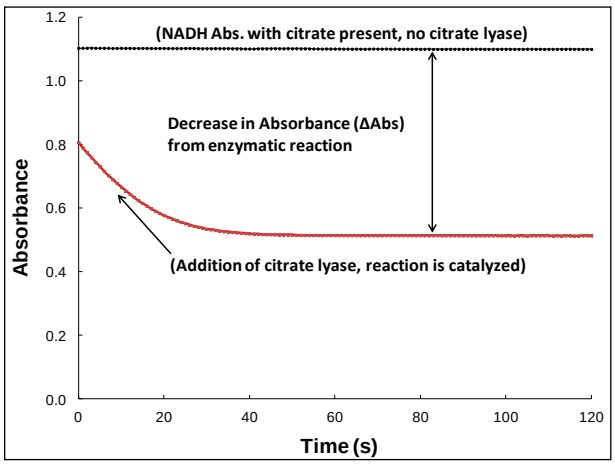

Figure 1. decrease in NADH absorbance ΔAbs data, enzymatic reaction (~pH 7.6, ~22°C, 50 µg citrate)

3.4 HPLC method

A Shimadzu Prominence LC-20A HPLC system with a Restek Ultra AQ (4.6 mm × 150 mm × 5µm) chromatography column, a Sil-20A autosampler, and a SPD-20A UV-Vis detector was used. The mobile phase was 25 mM phosphate buffer (~2.5 pH) at 1.0 mL min^{-1}. The UV-VIS detector was set to 226 nm with a cell temperature of 40 °C.

The method was calibrated using an external calibration approach for initial studies and using standard addition for sample analyses due to matrix effects. Calibration standards were analyzed five times each. Linear regression was used to determine a "best-fit" line. Following calibration supernatant aliquots from fresh pig bone samples were analyzed for citrate. Appropriate calculations were performed to correct for dilution and to calculate the wt% of citrate in the original bone powder. These results were compared to those from the UV-Vis assay.

4. Results

4.1 Calibration Studies

Figure 2 shows a typical calibration curve for the UV-Vis assay. Five citric acid calibration standards were analyzed and the corrected ΔAbs data was plotted versus citrate mass (µg). From previous studies the upper limit of linearity was determined to be approximately 80 µg of citrate, which was consistent with reported values. The linearity for this method is quite good as suggested by a R^2 value of 0.9999. Figure 3 in comparison shows a typical calibration curve using the HPLC method. Like the UV-Vis assay citric acid standards were analyzed the peak area data was obtained and plotted. In addition each citric acid standard was replicated five times for the HPLC method. The upper limit of linearity for the HPLC method was higher than the UV-Vis assay. The linearity for the HPLC method was also quite good suggested by a R^2 value of 0.9998.

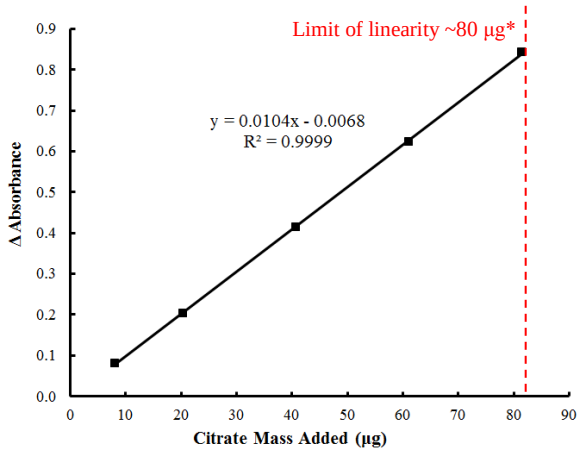

Figure 2. external calibration curve for the UV-VIS assay

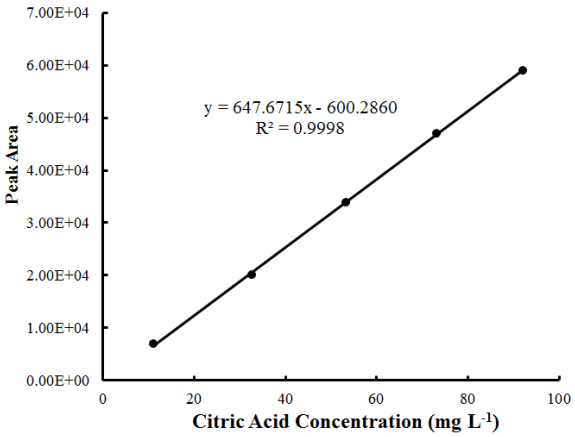

Figure 3. external calibration curve for the HPLC method

4.2 Detection Limit Studies

A preliminary estimate of minimum detectable mass (MDM) or minimum detectable concentration (MDC) of citrate (see equation 5) and lower limit of quantitation (LOQ) was determined using the uncertainties from calibration curve linear regression data for both methods (see equation 6). The theoretical estimate of the MDM was found to be 1.05 µg of citrate and the LOQ was found to be 3.51 µg of citrate for the UV-Vis assay. For the HPLC method the theoretical estimate of the MDC was found to be 1.68 $mg \cdot L^{-1}$ of citrate and the LOQ was found to be 5.59 $mg \cdot L^{-1}$ of citrate.

$$\mathrm{MDM/MDC} = \frac{3\times\text{regression error}}{\text{regression slope}} \quad (5)$$

$$\mathrm{LOQ} = \frac{10\times\text{regression error}}{\text{regression slope}} \quad (6)$$

Based on these preliminary estimates an experimental study was performed to determine more accurate values for the MDM, MDC and LOQ by analyzing replicates of a low level standard. For UV-Vis 7 replicates of a 4.07 µg citrate standard were analyzed and for the HPLC method 10 replicates of a 6.71 $mg{\cdot}L^{-1}$. The results are given in Tables 1 and 2. Note that the second replicate in Table 1was found to be an outlier using the Grubb's test, therefore was omitted in all calculations. Equations 5 and 6 were used to calculate the MDM/MDC and the LOQ though the standard deviation of the replicate data was used instead of the regression error and slope was not required. The MDM and LOQ were found to be 0.4 and 1.4 µg of citrate respectively, for the UV-Vis assay. A realistic LOQ for the method was deemed to be ~4 µg based on these calculations and prior work. For the HPLC method the MDC and LOQ were found to be 0.58 and 1.94 $mg{\cdot}L^{-1}$ of citrate respectively.

Table 1. detection limit results for UV-Vis assay 7 replicates of a 4.07 µg[a]

Replicate #	Corr. Δ Abs	Citrate Mass (µg)
1	0.0402	4.53
2	0.0502	5.49
3	0.0370	4.22
4	0.0375	4.27
5	0.0370	4.22
6	0.0370	4.22
7	0.0357	4.09
Calculated statistics		
mean (µg)		4.26
standard deviation (µg)		0.14
%RSD		3.39
MDM (µg)		0.433
LOQ (µg)		1.443
Realistic Detection limit (µg)		≈ 4

[a]The row shaded in yellow is a statistical outlier

Table 2. detection limit results for HPLC method, 10 replicates (6.71 $mg{\cdot}L^{-1}$)

Replicate #	Peak Area	Citrate Conc. (mgL^{-1})
1	4441	7.70
2	4623	7.98
3	4433	7.69
4	4199	7.33
5	4248	7.40
6	4269	7.44
7	4303	7.49
8	4273	7.44
9	4282	7.46
10	4318	7.51
Calculated statistics		
mean (mgL^{-1})		7.54
standard deviation (mgL^{-1})		0.20
%RSD		2.58
MDM (mgL^{-1})		0.58
LOQ (mgL^{-1})		1.94

4.3 Analysis Of Bone Samples

Analysis of fresh pig bone is presented for the UV-Vis assay in Table 3 and in Table 4 for the HPLC method. Bone from the same individual was analyzed by each method, but separate replicate digestions of the bone were performed to assess method precision. Bone powder masses of 59.9, 50.5, 47.9, and 45.9 mg were digested and the resultant supernatant was analyzed by the UV-Vis assay (Table 3). Replicate analysis of each digestion was also performed.

The citrate wt% across the four different digestions was consistent for the UV-Vis assay, with an average of 1.02 ± 0.07 wt%. Compared to the theoretical weight percent value 1.20 wt% reported[15] this value falls 0.18% lower. Bone powder masses of 44.7, 45.5, and 50.4 mg were digested and the resultant supernatant was analyzed by the HPLC method (Table 4). Replicate analysis of each digestion was also performed. The citrate wt% values for the three

digestions were also consistent for the HPLC method, with an average of 1.12 ± 0.06 wt%. Compared to the theoretical weight percent value this value falls 0.08% lower.

There was greater variation among digestions compared to replicate analyses of supernatant of each digestion (Table 3 and 4). The F-test was utilized to compare variances and the t-test was used to compare the means of the methods. The results these tests are summarized in Table 5. There was not a statistical difference between the variances of the two methods, but there was a statistical difference between the means.

Table 3. results of pooled analysis of fresh pig bone by UV-Vis assay

Corr. Δ Abs	Citrate Mass (µg)	Total Citrate Mass Digested Sample (µg)	Mass of Bone Analyzed (mg)	wt%
0.241	22.0	549.1	59.9	0.93
0.247	22.5	562.1	59.9	0.95
0.241	22.3	558.1	59.9	0.93
0.234	21.6	540.5	50.5	1.07
0.235	21.7	543.5	50.5	1.08
0.211	19.5	488.4	47.9	1.02
0.216	20.0	500.0	47.9	1.04
0.222	20.6	514.8	45.9	1.12
0.214	19.8	495.2	45.9	1.08
Calculated Statistics				
Mean				1.02
Stdev				0.07
Precision (%RSD)				7.08
Comparsion to theortical value (1.20 wt%)[15]				-0.18

Table 4. results of pooled analysis of fresh pig bone by HPLC method

Citrate Conc. (mgL^{-1})	Total mass of citrate in Digested Sample (mg)	Mass of Bone Analyzed (mg)	wt %
243.47	0.49	44.7	1.09
246.07	0.49	44.7	1.10
241.63	0.48	45.5	1.06
245.83	0.49	45.5	1.08
299.96	0.60	50.4	1.19
298.55	0.60	50.4	1.18
Calculated Statistics			
Mean			1.12
Stdev			0.06
%RSD			4.95
Comparsion to theortical value (1.20 wt%)[15]			-0.08

Table 5. F-Test and t-test results for the UV-Vis and HPLC method

UV-Vis vs. HPLC			
F-Test Two-Sample for Variances		**t-Test: Two-Sample Assuming Equal Variances**	
Fcalc =	1.72	tcalc=	2.666
Fcrit =	4.82	tcrit value =	2.160
Statistical Difference	no	Statistical Difference	Yes

5. Discussion

From the calibration studies' results, the linearity of each method and linear range for each method was quite good and more than adequate for bone analysis of citrate. The citrate content of all of the fresh pig bone samples analyzed fell well within the linear range of the calibration studies for each method. Considering all fresh pig bone samples analyzed fell within the linear range of the methods aged bone, although not analyzed in this study, should also fall within the linear range of the methods, assuming that citrate content does indeed decrease with increasing PMI.

In analysis of aged bone the detection limits of the methods need to be taken into consideration. If ultimately, a relationship between increasing PMI and decreasing citrate content is established from analysis of aged bone the rate at which the citrate decreases and the detection limits of the methods will affect the useful range of PMI determination. The detection limit of the UV-Vis assay was approximately 4 µg of citrate and for HPLC 2 $mg \cdot L^{-1}$ of citrate. This would be a citrate weight percent of ~0.008 wt% and ~0.05 wt% respectively (assuming 50 mg of bone powder was used to prepare the sample). The theoretical reported value for citrate content of fresh bone is approximately 1.20 wt%[15]. The faster the rate at which bone reaches a value of ~0.008 wt% or ~0.05 wt% of citrate will result in a shorter range for PMI assessment, whereas, a slower rate would result in a greater range for PMI assessment.

Before investigating the relationship between PMI and citrate content of aged bone establishing a baseline value of citrate for fresh bone is necessary. If the value of citrate is not relatively constant among fresh bone samples this will affect PMI determination. From the results of the analysis of fresh pig bone the there was a statistical difference between the mean weight percent of the two methods suggesting there is a systematic error that has yet to be determined. Considering that the digestions were all prepared from bone taken from the same individual it is not expected that there would be a statistical difference in the means. The average citrate weight percent for each method was also slightly lower than the theoretical reported value 1.20 wt%[15]. This warrants further investigation into sample preparation, specifically digestion and pH adjustment of samples, in attempt to improve precision and accuracy among replicate digestions.

6. Conclusions and Future Research

The detection limit of the UV-Vis assay is approximately 4 µg of citrate (~0.008 wt%) and is approximately 2 $mg \cdot L^{-1}$ of citrate for the HPLC method (~0.05 wt%). From analysis of fresh pig bone the average citrate wt% was 1.02 ± 0.08 wt% for the UV-Vis assay and 1.12 ± 0.06 wt% for the HPLC method. Additional sample analysis with fresh pig bone is needed to fine tune sample preparation and establish a baseline value of citrate. Once this has been accomplished, aged pig bones will be analyzed to further validate the methods prior analysis of human skeletal remains for assessment of PMI. Future research will also investigate using the surface area of samples to normalize citrate content (wt%) data.

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8. References

1. Love, Jennifer C. and Murray K. Marks. 2003. "Taphonomy and Time: Estimating the Postmortem Interval" In Hard Evidence: Case Studies in Forensic Anthropology, Edited by Steadman, Dawnie W., 112-120. Upper Saddle River, NJ: Pearson Education, Inc.
2. Bunch, Ann. 2009. "The Effect of Cold Climate on the Decomposition Process." *Journal of Forensic Identification* 59 (1): 26-44.
3. Srnka, C. 2005. "The Effect of Sun and Shade on the Early Stages of Human Decomposition." Master's Thesis. University of Tennessee, Knoxville. Catts, E.P. and M. Lee Goff. 1992. "Forensic Entomology in Criminal Investigations." *Annual Review of Entomology* 37 (1): 253-272.
4. Catts, E.P. and M. Lee Goff. 1992. "Forensic Entomology in Criminal Investigations." Annual Review of Entomology 37 (1): 253-272.
5. Higley, Leon G. and Neal H. Haskell. 2002. "Insect development and forensic entomology" In *Forensic Entomology: The Utility of Arthropods in Legal Investigations,* Edited by Byrd, Jason H. and James L. Castner, 287-302, Boca Raton, FL: CRC Press.
6. Megyesi, M.; Nawrocki, S.; and N. Haskell. 2005. "Using Accumulated Degree Days To Estimate Postmortem Interval from Decomposed Human Remains." *Journal Of Forensic Sciences* 50 (3): 1-9.
7. Michaud, J. and G. Moreau. 2011. "A Statistical Approach Based on Accumulated Degree Days to Predict Decomposition-related Processes in Forensic Studies." *Journal of Forensic Sciences* 56 (1): 229-232.
8. Ramsthaler, F.; Ebach, S.C.; Birngruber, C.G.; and M. A. Verhoff. 2011. "Postmortem Interval of Skeletal Remains through the Detection of Intraosseal Hemin Traces. A Comparison of UV-fluorescence, Luminol, Hexagon-OBTI®, and Combur® Tests." *Forensic Science International* 209 (1-3): 59-63.
9. Kandlbinder, R., V.; Geissler, R.; Schupfner, O. Wolfbeis, and B. Zinka. 2009."Analysing of 228Th, 232Th, 228Ra in Human Bone Tissues for the Purpose of Determining the Post Mortal Interval." *Journal of Radioanalytical and Nuclear Chemistry* 280, no. 1, 113-119.

10. Bulman, Philip and Danielle McLeod-Henning. March 2012. "Applying Carbon-14 Dating to Recent Human Remains." *National Institute of Justice Website.* Accessed March 2013. *https://www.ncjrs.gov/pdffiles1/nij/237722.pdf*.

11. Hodgins, Gregory W.L. 2009 *Measuring Atomic Bomb-derived 14C Levels in Human Remains to Determine Year of Birth and/or Year of Death.* Final Report Grant 2005-IJ-CX-KO13, Washington D. C.: U.S. Department of Justice.

12. Schwarcz, Henry, P.; Agur, Kristina; and Lee Meadows Jantz. 2010. "A New Method For Determination of Postrmortem Interval: Citrate Content in Bone." *Journal of Forensic Sciences* 55 (6): 1516-1522.

13. Dickens, Frank. 1941. "The Citric Acid Content of Animal Tissues, with References to its Occurrence in Bone and Tumour." *The Biochemical Journal*. 35 (8-9): 1011- 1023.

14. Taylor, R.; Suchery, J.; Payen, C.; and P. Slota, Jr. 1989. "The Use of Radiocarbon (^{14}C) to Identify Skeletal Materials of Forensic Science Interest." *Journal of Forensic Sciences* 34: 1196-1205.

15. Hu, Y-Y.; Rawal, A. and K. Schmidt-Rohr. 2013. "Strongly Bound Citrate Stabilizes The Apatite Nanocrystals in Bone." *Proceedings of the National Academy of Sciences of the United States of America, Early Edition*. 1-5.

16. Kanz, F., Reiter, C. and Risser, D. U. 2014, "Citrate Content of Bone for Time Since Death Estimation: Results from Burials with Different Physical Characteristics and Known PMI." *Journal of Forensic Sciences*, 59: 613–620.

17. Dagley, S. and E. A. Dawes. 1955. "Citridesmolase, its Properties and Mode of Action." *Biochemica Physica Acta* 17: 177-184.

18. Moellering, Hans and Wolfgang Gruber. 1966. "Determination of Citrate with Citrate Lyase." *Analytical Biochemistry* 17: 369-376.

19. Blair, J. M.; Datta, Satya P.; and S. S. Tate. 1967. "Reversible Inactivation of Citrate Lyase; Effect of Metal Ions and Adenine Nucleotides." *European Journal of Biochemistry* 1 (1): 26-28.

20. Mori, Hisakazu, and Takashi Kadowaki. "Determination of Citrate Using Immobolized Citrate Lyase and Malate Dehydrogenase in a Flow System and its Application to Analyze Citrate Content in Beverages." *Journal of Health Science*, 2009: 128-131.

21. Petrarulo, M.; Facchini, P.; Cerelli, E.; Marangella, M.; and F. Linari. 1995 "Citrate in Urine Determined with a New Citrate Lyase Method." *Clinical Chemistry*, 1518 - 1521.

22. Berg, S. 1963. "The determination of bone age." *Methods in Forensic Science* 2: 231- 252.

23. SigmaAldrich. 1997. "Enzymatic Assay of Citrate Lyase" Accessed June 12, 2012. *http://www.sigmaldrich.com/etc/medialib/docs/Sigma/.../citrate_lyase.pdf*.

24. Rover, Laercio, Jr; Fernandes, J.; Oliviera Neto, Graciliano de; Kubota, Lauro T.; Katekawa, Edson; and Silva H. P. Serrano. 1998. "Study of NADH Stability Using Ultraviolet-visible Spectrophotometric Analysis and Factorial Design." *Analytical Biochemistry* 260 (1): 50-55.