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# Protein Residue Analysis in Archaeology: A Geological Contamination Experiment

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# PROTEIN RESIDUE ANALYSIS IN ARCHAEOLOGY: A GEOLOGICAL CONTAMINATION EXPERIMENT

by

Theresa Popp

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# ABSTRACT

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Protein residue analysis has been used by archaeologists for decades, while protein contamination of *in situ* artifacts has been hypothesized but lacked formal study. Through a contamination experiment testing different geological contexts (e.g. water movement and freeze-thaw), possible routes of *in situ* protein contamination with the ability to adversely impact protein residue analysis conducted on buried artifacts was identified. Protein was found to move through sediment columns in large enough concentrations to elicit positive crossover- immunoelectrophoresis (CIEP) results on sterile artifacts that never made direct contact with the protein source used in this study. Furthermore, protein movement was identified in the absence of simulated geological conditions. More research and greater sample sizes are needed but the result of this study is compelling enough to provide additional caution when protein residue analysis is used on artifacts from buried contexts.

(75 pages)

# PUBLIC ABSTRACT

Protein Residue Analysis in Archeology: A Geological Contamination Experiment

Theresa Popp

Protein residue analysis has been used by archaeologists for decades to explore ancient diets and hunting behaviors based on proteins deposited on tools and artifacts, however, protein contamination of buried artifacts has been assumed but lacked formal study. Through a contamination experiment testing different geological contexts (e.g. water movement and freeze-thaw), possible routes of protein contamination within soil were identified, adversely impacting protein residue analysis conducted on buried artifacts. Protein was found to move through soil in large enough concentrations to elicit positive protein residue results on sterile artifacts that never made direct contact with the protein source used in this study. Furthermore, protein movement was identified in the absence of water movement and freeze-thaw conditions. More research and greater sample sizes are needed but the result of this study is compelling enough to provide additional caution when protein residue analysis is used on artifacts from buried contexts.

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# CHAPTER I INTRODUCTION

Lithic residue analysis is an important field of study within the archaeological sciences due to the ability to link residues on artifacts with past human behaviors. Frederick Briuer (1976) published the first study on archaeologic lithic residues, concurrently introducing the use of forensic science methods and principles to archaeological research. Following this pioneering study, researchers have explored a range of methods for identifying such residues, often using techniques borrowed from the forensic sciences (Culliford 1971; Gaensslen 1983). Subsequently, archaeologists have used immunoassay protein residue analysis (IPRA) to explore prey choice based on nothing more than taxa specific proteins (Kooyman et al. 1992; Newman et al. 1996; Newman and Julig 1989; Loy and Hardy 1992; Loy and Wood 1989; Shanks et al. 1999) and more recently the use of different mass spectrometry (MS) methods, including Zooarchaeology by Mass Spectrometry (ZooMS), has opened species identification by peptide mass fingerprinting (PMF) (Ostrom et al. 2000; Solazzo et al. 2008; Heaton et al. 2009; Buckley et al. 2009; Oonk et al. 2012; Buckley et al. 2017; Hendy 2021).

IPRA is not, however, without its detractors and several researchers have questioned the accuracy and reliability of IPRA as a useful archaeological method (Eisele et al. 1995; Downs and Lowenstein 1995; Cattaneo et al. 1993; Gufinkel and Franklin 1988; Leach and Mauldin 1995; Hendy 2021), leading to an increased adoption of ZooMS analytical techniques for ancient protein species identification (Solazzo et al. 2008; Heaton et al. 2009; Buckley et al. 2009; Oonk et al. 2012; Buckley et al. 2017; Hendy 2021). These discussions do not surprise us, for within the context of our research

program, I have encountered questions about methodologies commonly associated with archaeological protein residue analysis. Our research aims to identify possible contamination issues and discern the amount of credibility that archaeologists can place on protein residue analyses.

In our case, I am currently involved with better understanding the associations between now extinct Pleistocene megafauna and projectile points recovered from Owl Cave at the Wasden site located in southern Idaho (Butler 1968; Miller and Dort 1978; Henrikson et al. 2017). Excavations at Owl Cave recovered Folsom projectile points in seeming association with mammoth remains. Moreover, IPRA identified not only mammoth protein, but also horse protein, on the projectile points (Henrikson et al. 2017). A series of AMS dates on samples of mammoth bone from Owl Cave suggests that the elephant remains predate any known Folsom occupation by at least 1,000 years and no horse remains are currently recognized in the faunal collection. These results present an interesting question. Either what we know about the chronological placement of Folsom is wrong or protein contamination issues plague the Owl Cave deposits.

Specifically, the Wasden site presents questions of potential in situ routes of protein contamination, and the foundation of this research. In situ protein contamination involves the movement of proteins from a protein source (e.g., decomposing animal carcass) and into contact with a sterile artifact by transmission through a sediment column. Protein contamination of artifacts in this manner greatly impacts the credibility of protein residue analysis, independent of the protein analysis technique being used (e.g., immunological or mass spectrometry) and the ability to effectively identify past human hunting behavior and diets in the absence of faunal material. The in situ movement of

proteins, as well as the possible contamination of artifacts through this route has been hypothesized but has yet to be studied and tested in the current literature. Additionally, Wakefield Dort Jr. identified soil mixing by cryoturbation in Owl Cave with Wood and Johnson (1987) citing aquaturbation as the mechanism of the cryoturbation phenomena; this provides a method by which proteins from an extinct, and possibly seasonally frozen mammoth carcass, could have diffused through a sediment column and into contact with a non-associated Folsom point.

The identification of in situ protein contamination would bring into question the practicality of protein residue analyses performed on artifacts from buried contexts. Additionally, if in situ movement of proteins are not identified as a possible contamination route, this study will provide further support for the reliability and use of protein residue analysis in buried contexts. However, I propose that through the decomposition process, biological material, such as proteins and DNA, are expelled from the decomposing body and are transported through the sediment column and bound to surrounding clays, silicates, and other available sediment particles. Furthermore, protein transport is aided by a fluctuating water table, while clay and other minerals protect the proteins from degradation and microbial attack. In this scenario, the transport and deposition of these proteins into surrounding sediments have created an area of contamination around the decomposing carcass affecting artifacts that have never come into direct contact with the carcass.

The Wasden Site presents a unique case study for the examination of current knowledge, and gaps in knowledge, surrounding the use of protein residue analysis (PRA) in archaeology. While the motivation for my research is founded in the

forementioned questions surrounding the Wasden Site, the intention of this thesis is twofold. First, I aim to provide a comprehensive review of the use of PRA within archaeology. By means of this literature review, I will identify and address a general gap in knowledge regarding protein survival and movement in geological contexts. Second, I conduct a geological contamination experiment based on the forementioned research. The geological contamination experiment was developed to address gaps in research surrounding the mobility and survivability of proteins within a sediment column and their ability to contaminate artifacts through a secondary context.

# CHAPTER II HISTORY OF PROTEIN RESIDUE ANALYSIS: A LITERATURE REVIEW

Research surrounding the identification of blood and bloodstains was well underway in the 1800s, with the first use of immunological antibody-antigen reaction (precipitin) tests for species identification in the medico-legal field published in 1901 (Gaenssien 1983). Since then, many variations to the precipitin test have been introduced, but the basic technique behind the precipitin reaction has remained the same (Culliford 1971). The precipitin test can exploit the sensitivity and specificity of the immune response, making it highly effective at detecting trace amounts of blood protein (Boyd 1962; Kabat and Mayer 1967).

The immunological precipitin test is a key component to immunoassay protein residue analyses conducted today, but the precipitin test has not been the sole method used in early blood residue identification. Loy (1983) and Loy and Wood (1989) explored comparative crystal morphological techniques with limited success. Additionally, many presumptive blood residue analysis techniques are used for the identification of blood proteins before further immunological tests are conducted, such as microscopy (Loy and Wood 1989; Low and Hardy 1992; Hyland et al. 1990; Briuer 1976; Newman and Julig 1989) and urinalysis strips (Loy 1983; Loy and Wood 1989; Loy and Hardy 1992; Hyland et al. 1990; Gurfinkel and Franklin 1987; Matheson and Veall 2014; Gundy et al. 2008).

After mass spectrometry (MS) became commercially available in the 1940s, its use as a scientific analysis tool grew (Griffiths 2008). Furthermore, the discovery of radiocarbon dating lead by William F. Libby propelled the use of MS as a valuable archaeological tool (Malainey 2012). The use of MS as an archaeological method has

expanded far from its uses in dating, and MS is now commonly used in stable isotope analysis providing information on diet, past climate, provenance, etc. (Malainey 2012). More recently it has been used for the identification of ancient proteins on ceramics (Solazzo et al. 2008; Heaton et al. 2009), lithic artifacts (Heaton et al. 2009), within bone fragments (Ostrom et al, 2000; Buckley et al. 2009; Buckley et al. 2017), and soil samples (Oonk et al. 2012). The technique of using peptide mass fingerprinting or peptide mass fingerprinting (PMF) for taxonomic protein identification has been coined as zooarchaeology by mass spectrometry (ZooMS). ZooMS has become increasingly popular due to the little-to-no preparation requirements of artifacts (Heaton et al. 2009), minimal to no destruction of artifacts (van Doorn et al. 2011), fine-grained taxon resolution (Buckley et al. 2009; Buckley et al. 2010), and a lack of cross-reactivity and false positives that plague immunoassay methods (Hendy 2021).

Both immunoassay and MS protein residue analysis techniques are present in the current literature, although many authors push for a shift to MS (Croft 2021b; Hendy 2021). I suggest that there is a place for both protein residue analysis techniques in archaeology and before deciding on a PRA method, the researcher should have a basic understanding of each method currently being used.

## *The Methods of PRA*

*Presumptive Blood Residue Analysis Methods*. Archaeologists have used presumptive blood tests to verify the presence of blood or blood proteins before conducting tests accurate to the taxon level. Presumptive tests only verify the presence or absence of blood resides and/or proteins and do not have the ability to provide species or taxa level results. Many of these types of tests can be done inexpensively and can be utilized in the field.

Microscopy is an easy and inexpensive way to detect areas of potential blood residue (Loy and Wood 1989; Low and Hardy 1992; Hyland et al. 1990; Briuer 1976; Newman and Julig 1989). Microscopy is also used to identify use-wear patterns on stone tools, which makes microscopic blood residue identification an easy additional step to add to lithic analysis. Although this technique can be simple, identification takes limited training and blood residues may not be visible on the surface of the artifact (Shanks et al. 2001).

Colorimetric methods are used to detect proteins using reagents that change color in the presence of proteins (Malainey 2012). Reagents that detect the presence of proteins include ninhydrin, 1-fluoro-2, dansyl chloride, and fluorescamine (Malainey 2012). Gurfinkel and Franklin (1988) found that all colorimetric tests lack specificity because they react with clay, soil, and humic acids. Colorimetric reagents change color due to weak electrostatic and/or hydrogen bonds formed between the colorimetric reagent and protein (Gurfinkel and Franklin 1988); clay, soil, and humic acids contain sites where similar bonds can form (Malainey 2012; Croft 2021a).

Urinalysis strips are a colorimetric method that is a common and inexpensive test used by archaeologist to test artifacts for the presence of blood (Loy 1983; Loy and Wood 1989; Loy and Hardy 1992; Hyland et al. 1990; Gurfinkel and Franklin 1987; Matheson and Veall 2014; Gundy et al. 2008). This is a benzidine based biochemical test that uses colorimetric test strips that change color when a chemical reaction occurs in the presence of hemoglobin. The most commonly used test strips are Chemstrip® (manufactured by Roche) or Hemastix® (Bayer).

The use of urinalysis strips has, however, been heavily criticized. Gundy et al. (2008) reference personal communications with Bayer stating these tests "are specifically designed to be utilized in a urine environment" (F-5) and they could not vouch for the efficacy of the test in any other environment. Additionally, high rates of false positives have been reported when used with 5% ammonia solution (Loy and Hardy 1992) and in the presence of contaminats such as chlorophyll, fungal and bacterial peroxidase, metals, and oxidizing substances in soils (Matheson and Veall 2014; Gundy et al. 2008). Matheson and Veall (2014) conducted a study showing positive results for the use of sodium ethylenediaminetetraacetic acid (EDTA) as a buffering agent reducing false positives caused by the most common contaminants. They did note that potential for post-excavation contamination is still an issue, and the use of urinalysis sticks needs to be used in conjunction with other forms of testing.

*Extracting Ancient Blood Proteins from Artifacts*. Once blood residue has been identified on an artifact, the proteins must be extracted before they can be tested using immunoassay methods. Protein extraction is arguably one of the most important steps in the testing process due to the minuscule levels of testable proteins present on the artifact. Dorrill and Whitehead (1979) found higher protein extraction rates from blood stains when a 5% ammonia solution was used versus water. Shanks et al. (2001) further emphasized the importance of protein extraction techniques with their study identifying blood residues deep in lithic microcracks. Through their research, Shanks et al. (2001) identified that blood residues can diffuse into microcracks on average 44 µm below the surface with uptake of blood complete within five minutes. This fairly quick and easy uptake of blood into microcracks has the potential to protect blood proteins from environmental conditions and cleaning with water or buffered saline solutions but can make the extraction of proteins for testing more difficult. Although it was identified that "treatment with 4M guanidine hydrochloride, a strong denaturant, or sonication in 5% ammonium hydroxide removed 60-18% of the DNA and protein harbored in microcracks" (Shanks et al. 2001: 971). The process of extracting blood protein residues is important for the ability to obtain useful quantities of testable material, and inadequate extraction methods can presumptively cause false negatives in blood protein residue analyses. The Shanks et al. (2001) study also identified the potential of artifacts housing valuable molecular material within museum assemblages that can still be tested and studied after routine cleaning and storage.

Craig and Collins (2000) developed a new method for analyzing proteins that have been absorbed and bound to siliceous minerals "by destroying the mineral phase

with hydrofluoric acid (HF) whilst simultaneously capturing any protein released onto the solid phase for immunological characterization" called the Digestion and Capture Immunoassay (DACIA) (Craig and Collins 2000:90). Proteins have a high affinity to bind to silicates in soil, ceramics, and stone tools preventing immunological analysis of these proteins. DACIA provides a method for breaking the bond between proteins and minerals while protecting the protein epitopes (required for immunological detection, see below) from braking down due to HF treatment using test tubes with high-binding polymer walls for DACIA protein extraction. The polymer tube walls provide a large surface area for protein sorption, which stabilizes the protein from further acid attack. Craig and Collins (2000) identified HF as a much stronger protein extraction reagent than the traditionally used reagents of urea and phosphate-buffered saline, while the DACIA method demonstrated that the harsh extraction conditions did not degrade antibody specificity of the protein.

*Methods of Protein Residue Analysis*. With the introduction of protein residue analysis, archaeologists have gained the ability to identify prey choice and diet breadth through the connection between hunting and butchering behaviors and the residual proteins left on stone tools. Much like many of the other methods archaeologists have borrowed from other disciplines, immunoassay protein residue analysis (IPRA) was first developed by forensic scientists who deployed the use of precipitin tests for medico-legal blood identification beginning in the early 1900s (Newman 1990; Newman et al. 1993; Culliford 1964; Gaensslen 1983). Biochemists have developed many new techniques since then, with the majority dominated by precipitin (antibody-antigen reaction) testing methods. To provide a basic background in the biochemistry behind these methods, here I present an overview of the different immunological and mass spectrometry protein residue analysis methods and related techniques.

*The Chemistry of Proteins and Immunoassays*. Proteins are molecules made up of linked amino acids. The functions and properties of a protein are determined by its composition and arrangement of amino acids. Amino acids are comprised of an amino group, a carboxyl group, a hydrogen atom, a carbon atom ($\alpha$-carbon), and a variable group (R group). The R group is 1 of 20 different side chains bound to the $\alpha$-carbon, resulting in 20 different amino acids (Malainey 2012). Amino acids are linked by covalent chemical bonds, or peptide bonds. A peptide is two or more joined amino acids while a polypeptide is a chain of many amino acids (Kaiser 2021). Proteins are made up of one or more polypeptides (Figure 1). Moreover, peptide mass fingerprinting (PMF) takes advantage of known taxa specific amino acid sequences and uses MS techniques for identification (Croft 2021).

Proteins can be separated into two groups, fibrous and globular proteins. Fibrous proteins are classified as water-insoluble and having “fiber-like” structure (LibreTexts 2021). Fibrous proteins play a structural role and include keratin, collagen, myosin, and elastin. Globular proteins are classified as water-soluble and “globular” or tight and compact in shape. Globular proteins have a more functional role, such as transporting oxygen and other molecules (LibreTexts 2021). The blood proteins hemoglobin, serum albumin, and immunoglobulin (antibodies) are globular proteins.

Additionally, proteins have a primary and secondary structure. The primary structure of a protein is the physical order of amino acids in the protein while the secondary structure is the spatial arrangement of the polypeptide chain(s) that produce different fiber-like or globular shapes (Malainey 2012). Many interactions occur between amino acid R groups that contribute to the shape of the protein, including ionic bonds, hydrogen bonds, covalent bonds, and hydrophobic interactions (Kaiser 2021). These interactions can be fragile when exposed to temperature and pH outside of a protein’s limited range, causing a protein to denature (secondary structure unwinds and the protein becomes biologically inactive) (Malainey 2012). Some denaturization is irreversible, such as the heating of an egg white, while in other scenarios, renaturation can occur in the blood proteins serum albumin and hemoglobin (Malainey 2012).

Blood is a complex solution of proteins that is comprised of two major parts: red blood cells (RBC) and plasma (Gurfinkel and Franklin 1988). The major protein of RBCs is hemoglobin (Hb), whereas the most abundant protein in plasma is albumin, followed by immunoglobulin (Gurfinkel and Franklin 1988). Immunoglobulins include

antibodies produced to fight pathogens; it is the diversity in these antibodies that facilitate the efficacy of precipitin tests.

Immunological precipitin tests include a set of serological tests using a precipitin reaction to detect the presence of a specific antigen (Dorril 1979). Such assays exploit the antigen-antibody reaction using a commercially produced antisera or an immunological reaction in an unimmunized rabbit, horse, or other animal (Cullford 1971). When researchers introduce foreign material into the test animal (rabbit or horse), the foreign protein stimulates the immune system and produces antibodies specific to that foreign material. Blood researchers have leveraged these reactions to produce antibodies matching different animals.

Antisera comes in two types, H-type and R-type, which is dependent on the animals used to produce it. H-type is produced by a horse and is differentiated from R-type, due to the horses' ability to produce higher quantities of antibodies in comparison to rabbits, sheep, goat, etc. The type of antisera used should match the concentration of antigens in the sample, if the concentration is unknown, R-type should be used (Culliford 1971). Consequently, if concentration of the blood extract are too high, non-specific cross reactions can occur (Culliford 1971). Therefore, it is important to dilute samples when a high concentration of blood is being tested.

In addition to H-type and R-type antiserums, there are polyclonal and monoclonal antibodies that can be used in precipitin tests and will produce different qualities of reactions. Native antisera that were produced from a reaction against a foreign invader (isolated from an animal's blood) are made up of polyclonal antibodies. Polyclonal antibodies are less specific because they will bind to a variety of different receptor

(epitopes) on different proteins (Barnard et al. 2007; Hyland et al. 1990). A purification of polyclonal antibodies can be conducted by exposing the antibodies to known cross-reactants before use. Monoclonal antibodies are developed in a laboratory and are composed of single lines of cloned B-cells (specialized immune cells), which will bind to a single epitope of a single protein. Monoclonal antibodies have a much higher species specificity than polyclonal antibodies but are much more expensive. When polyclonal antibodies are used, it is only possible to test at the family level (Gaensslen 1983). Additionally, it is important to test antisera and antibodies (especially monoclonal) for cross-reactivity before use, which will be further discussed later in this paper.

*Current Methods of Immunoassay Protein Residue Analysis*. With a basic understanding of blood chemistry and antisera origination, the concepts of precipitin tests are fairly simple. The antibodies in the antisera are attracted and attach to corresponding antigens in the blood (albumin, alpha and beta globulins) (Culliford 1964; Culliford 1971; Newman and Julig 1989). Therefore, a positive precipitant test indicates the presence of a signaled blood protein due to the precipitation, or conjoining, of the antigen and blood protein (Figure 2).

Ouchterlony double-diffusion (OCH) is the oldest and simplest of the immunoassay methods (Culliford 1964). OCH uses Ouchterlony plates (specialty plates designed for low affinity antisera with patterns of six wells that surround a single well in the center) where species-specific anti-albumin antisera is placed in one well while the test sample is placed in the other (Downs and Lowenstein 1995; Hyland et al. 1990). When left at room temperature for several hours the antibodies and antigens will disperse through a gel matrix by double-diffusion. With a positive reaction, an opaque precipitate

line will form in a zone of optimal antibody/antigen concentration. A negative reaction is represented by the absence of precipitation line. OCH has very low sensitivity when compared to other immunological precipitation methods.

Crossover-immunoelectrophoresis (CIEP) is a more sophisticated version of OCH and currently the most widely used method among archaeologists (Newman 1990; Newman et al. 1993; Kooyman et al. 1992; Newman and Julig 1989; Downs and Lowenstein 1995; Tuller and Saunders 2012; Henrikson et al. 2017). Similar to OCH, antibodies and antigens are placed in opposed wells within a gel matrix, but in a CIEP test the antigen (blood extract) is placed in a cathodic well and the antibodies (antiserum) are placed in an anodic well (Culliford 1964; Culliford 1971). The gel is then placed in electrophoretic conditions where the antigens will move toward the anode and antibodies move toward the cathode creating a precipitation between the two wells. As per Culliford (1971), electrophoresis is applied at 100–150 volts for 10–20 mins. CIEP has a higher sensitivity than OCH but has been criticized for having low discriminatory ability (identification only to family level) due to the use of polyclonal antibodies (Cattaneo at al. 1993; Gaensslen 1983; Barnard et al. 2007).

Radio-immuno-assay (RIA) is a double antibody method that is more complex than the previous two immunological techniques (Downs and Lowenstein 1995; Barnard et al. 2007). The antigen is placed in a plastic well, where some of the antigen will bind to the plastic. Species-specific antisera (monoclonal antibodies) are then placed into the well. After an incubation period the excess antibodies are washed off before a secondary set of radioactively labeled antibodies are added to the well. These secondary antibodies will adhere to the primary set of antibodies from which a radioactive measurement can be

calculated. The amount of measured radioactivity then represents the quantity of unknown proteins and its relationship to the species-specific antisera. RIA is highly sensitive and "because all reactions are numerically quantified, it is possible to discriminate objectively between their reactive strengths and thus to sharpen distinctions between false and true positive identifications" (Downs and Lowenstein 1995:14). Although RIA offers increased protein specificity and detectability, it can be expensive to run and is limited to facilities and personnel licensed for nuclear medicine (Newman 1990).

Enzyme-linked-immuno-absorbent-assay (ELISA) uses the same double antibody method as RIA but instead of using a radioactive tracer, this method employs an enzyme linked antibody to differentiate between proteins. Under positive test conditions (when the enzyme is present), the enzyme will change color in the presence of a detection reagent (Barnard et at. 2007). ELISA includes a variety of tests using monoclonal or polyclonal antibodies with results that range in specificity. The dot-blot test that uses the staphylococcal protein A (SpA) molecule has been used by Loy and Wood (1989) and Loy and Hardy (1992) when there was a lack of species-specific antisera available. SpA binds to a specific portion of an ammino acid (Fc region) on the immunoglobin G (IgG) molecule. This region on the IgG molecule is invariant among all mammalian species, making the dot-blot test useful when the presence of mammalian blood needs to be established. A wide range of ELISA tests can be conducted with a wide range of specificity and costs, although large samples are needed for testing, which can be hard to obtain from archaeological artifacts.

Gel electrophoresis is an additional method used for the analysis of protein residue that is used in the western blot test and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Barnard et al. 2007; Gurfinkel and Franklin 1988; Mahmood and Yang 2012). Similar to CIEP, gel electrophoresis separates and sorts proteins and DNA by size through a gel matrix. It differs however in that the protein molecules are first denatured through the application of heat to give them a negative charge before being added to the gel and electrophorator machine. In the western blot, proteins are transferred to a membrane after they have been separated in the electrophorator. After an incubation period in a blocking agent (to prevent non-specific bonding), the proteins are run through the double antibody method used by ELSIA with an enzyme-linked signaling second antibody. The signal is captured on film and developed, in a similar way to a photograph, to show the reaction. SDS-PAGE differs from western blot in that the proteins are not transferred onto a membrane but stained directly in the gel. They are stained with protein specific dye to identify the presence of select proteins, such as heme, but do not allow for species- or family-level identification (Figure 3). Additionally, SDS-PAGE gel electrophoresis is commonly used to sort proteins for protein specific mass spectrometry (MS) analysis (James et al. 1993; Ostrom et al. 2000).

*Methods of Mass Spectrometry Protein Residue Analysis*. It is important to note that presumptive tests focus on the identification of RBC, immunoassay and precipitin tests focus on the identification of globular proteins, specifically serum albumin, while peptide mass fingerprinting (PMF) through MS can identify peptide sequences in both globular and fibrous proteins. Recently, a lot of focus has been placed on proteomics techniques for the identification of ancient proteins within the archaeological field (van Doorn et al. 2011; Buckley et al. 2009; Buckley et al. 2010; Buckley et al. 2017).

Proteomics is a field that emerged in the 1990s, following development of soft-ionization mass spectrometry, involving the study of proteomes or the full range of proteins genetically expressed by an organism (Buckey 2019a). Paleoproteomics, or the study of ancient proteins, has become of great interest over ancient DNA analysis due to the increased survivability of proteins over DNA (Demarchi et al. 2016). PMF often used interchangeably with Zooarchaeology by Mass Spectrometry (ZooMS), arose from the field of proteomics and has gained popularity within archaeology.

PMF is a MS method for taxonomic protein identification (Croft 2021b; Hendy 2021). First, proteins are digested or broken down into smaller peptide units, commonly done using the enzyme trypsin (Buckley 2018). After the peptides have been separated, they are run through a MS to identify individual peptide masses that can be matched against reference “fingerprint masses” to identify taxonomic origin (Croft 2021b; Hendy 2021; Buckley et al. 2010; Buckley et al. 2017; Buckley 2018; Solazzo et al. 2008). Protein extraction and the pretreatment of samples varies widely based on the type of MS analysis being conducted, and since PMF and MS residue analysis is still in its infancy, there is little to no consensus on the type of MS analysis or sample pretreatment and

preparation. I will provide a brief overview of MS, then touch on the most common MS techniques and mass analyzers used in archaeology today.

Mass spectrometry (MS) is an analytical technique that identifies the mass to charge ratio (*m/z*) of molecules through the evaporation and ionization of a sample (Buckley 2019b). Soft-ionization mass spectrometry allows for the analysis of biological molecules without the extensive fragmentation that occurs with collision-based MS methods (Buckley 2019b; El-Aneed et al. 2009). MS has three main elements, the ionization source, the mass analyzer, and the detector. The ionization source is how the MS evaporates and ionizes the sample while the "mass analyzer is the part of the instrument in which ions are separated based on their *m/z* values" (El-Aneed et al. 2009) so that they hit the detector differentially. The detector is the part of the instrument that sends the signal to a computer for the analysis, a graph is generated that displays the peptide fingerprint of quantity to the mass to charge ratio (Figure 4), which are then "probabilistically matched to records of peptide sequences in large digital archives" (Barker et al. 2015: 163). Mass spectrometers can be built with a combination of ionization sources and mass analyzers based on the types of analyses being performed. I focus on the most widely used MS techniques within ancient protein studies.

Matrix assisted laser desorption ionization (MALDI) is the most widely used MS ionization source for protein and peptide identification (El-Aneed et al. 2009; Buckley et al. 2009; Buckley et al. 2010; Buckley 2019; Buckley 2019a; Buckley 2019b). With MALDI, ions are desorbed from a solid phase using a laser (El-Aneed et al. 2009). Proteins are first extracted from a sample and dissolved within a liquid solution, that is

then mixed with an appropriate matrix. The solution is placed on a MALDI plate and left to air dry, reverting to a solid state where it can be ionized through laser desorption.

Electrospray ionization (ESI) is an additional soft ionization technique that is often used with liquid chromatography (LC) to analyze proteins and peptides (El-Aneed et al. 2009). ESI pushes the sample solution through a thin needle causing it to aerosolize into ionized droplets. These droplets are then evaporated with the aid of warm natural gas before moving on into the mass analyzer (El-Aneed et al. 2009). LC is an instrument that is interfaced with MS and is used to separate compounds from mixtures before they are injected into the MS. LC increases specificity of the analysis but increases run time.

Time-of-Flight (ToF) is a commonly used mass analyzer that is paired with MALDI, (MALDI-ToF). Ionized molecules are separated based on the time it takes them to travel down a tube before reaching the detector (El-Aneed et al. 2009; Barker et al. 2015). The quadrupole (Q) mass analyzer is also commonly used, especially when paired with ESI. The quadrupole analyzer is composed of four parallel electric rods. Alternating electricity and polarity of the rods separates the ionized molecules (El-Aneed et al. 2009). A hybrid quadrupole time-of-flight (Q-ToF) analyzer has also been developed with tandem MS (MS/MS) for high resolution and sensitivity (El-Aneed et al. 2009). See El-Aneed et al. (2009) for a more in-depth discussion on the forementioned MS techniques.

Desorption electrospray ionization (DESI) mass spectrometry is a more recently developed MS technique that has shown promise among archaeological protein residue analysis (Heaton et al. 2009). DESI allows for the non-destructive analysis of proteins in situ with very little to no preparation of an artifact (Heaton et al. 2009; Yao 2012). Proteins are desorbed directly from the surface of an artifact by the application of a

solvent spray (Yao 2012). See Yao (2012) for a detailed review of DESI techniques and methods.

Much of the archaeological literature has been dedicated to MS protein analysis for the use of species level bone identification (Ostom et al. 2000; Buckley et al. 2009; van Doorn et al. 2011; Buckley et al. 2010; Buckley et al. 2017; Buckley 2016; Buckley 2018; Buckley 2019a; Buckley 2019b) with far less focused on protein residues on tools (Heaton et al. 2009) and ceramics (Solazzo et al. 2008). This has provided more questions than answers to how future protein residue analyses should be conducted, and which methods should be used.

*What is the Best Method for Archaeologists?* Archaeologists have employed many of the methods outlined above, each having their own strengths and weaknesses. Nonetheless, the feasibility of immunological protein residue analysis has received many criticisms. Many of these criticisms involve the survivability of blood and if it can be reliably identified in archaeological contexts, often involving millennia of time depth (Eisele et al. 1995; Downs and Lowenstein 1995; Cattaneo et al. 1993; Gufinkel and Franklin 1988). Others focus on contaminants and cross reactions causing false positives (Downs and Lowenstein 1995; Eisele et al. 1995; Leach and Mauldin 1995). Understanding the reasons behind these criticisms is important to archaeologists and, subsequently, I discuss them next.

An experimental study was conducted by Eisele et al. (1995) to test the survivability of blood on stone tools. A series of cross reactions occurred on experimental samples and a lack of identifiable blood residue was recorded for 152 specimens tested from archaeological sites using the GIA method. This research called into question all

previous blood residue work conducted to that point. Although, Eisele et al. (1995) did not recognize some very important and possible flaws in their research pointed out by Newman et al. (1996), such as possible bacterial contaminants from the roadkill used and the lack of testing the commercial antisera used against control samples to rule out antisera caused cross-reactions. Further criticisms involve the presence of questionable cross-reactions and the irreplaceability of different blood residue analysis methods (Leach and Mauldin 1995; Downs and Lowenstein 1995). In addition, Fiedel (1996) addressed the validity of blood protein residue methods in site interpretations. Issues with many of these studies and criticisms are reflected in inconsistent research methods and techniques that decrease the accuracy of their study.

There are many behavioral and taphonomic factors that are important to blood protein preservation including,

> intensive blood-letting, meat-cutting, or bone/skin scraping at the use-sites; artifacts with numerous flake scars to catch and preserve fragmentary residues; good preservation of residues before tool was lost or discarded… a suitable soil matrix; and reasonable protection from the elements [Cattaneo et al. 1993:41].

Cattaneo et al. (1996) also identified the differential survival between IgG and albumin in the samples they tested. This indicates that the choice of protein being tested for can greatly impact the reliability and identification of residual blood on an artifact. Methods such as ELISA, RIA and GIA may be affected by this differential preservation since these tests typically use monoclonal antibodies that test for a single protein. It may be more beneficial to use the CIEP method for archaeological material since the use of polyclonal antibodies will bind a variety of blood proteins.

Blood proteins have been shown to retain biological activity in a non-native setting over long periods of time and after extended burial periods (Sansabaugh et al. 1971; Loy and Hardy 1992). Analysis conducted at the Head-Smashed-In Buffalo Jump site in Alberta, Canada observed a 25-30% preservation rate of blood proteins on 5600-year-old lithic artifacts (Kooyman et al. 1992; Newman et al. 1996). Loy and Hardy (1992) suggested that most protein degradation occurs very shortly after its initial deposition and the structural and functional changes that occur do not affect the entire molecule. These biochemical changes include effects to protein solubility by an aggregation of proteins, this aggregation effect increases the preservation of proteins and changes Hb into a more stable form making proteins less susceptible to microbial attack (Sasanbaugh et al. 1971; Loy and Hardy 1992; Loy 1983). These studies are important because they identify the survivability of blood proteins while explaining how previously thought degradation mechanisms can instead increase survivability. However, lab tests do not account for archaeological conditionals with additional variables and mechanisms that influence the quantity and quality of blood proteins lasting on ancient artifacts.

Arguably the most important factor in immunological protein residue analysis is the quality of antisera being used, which is based on sensitivity and cross-reactivity (Shanks et al. 1999). The sensitivity of an antisera refers to the quantity of blood protein required to elicit a positive reaction. Cross-reactivity is the degree to which an antisera will react to taxa aside from what the antisera was created to identify. Antisera obtained from different manufactures can vary in quality, with antisera produced for forensic work being the highest quality (Newman et al. 1997). Not all manufacturers test for cross-reactivity, therefore routine tests against control specimens should be performed to

eliminate doubts of cross-reactivity and presence of impurities within the antisera (Newman et al. 1997; Shanks et al. 1999). In addition, the dilution of an antisera may be required to increase the specificity of the reaction (Newman and Julig 1989; Newman et al. 1997; Culliford 1971). All these control tests should be performed prior to testing unknown residue samples, which is a step that has not been controlled for by many critiques of IPRA methods.

IPRA methods have been tested and accepted by the forensic science community (Culliford 1964; Culliford 1971; Gaensslen 1983; Tuller and Sanders 2012) but have failed to be as widely accepted by the archaeological community. Many of the critiques and criticisms in archeological literature reflect inconsistencies in research methods that undermine the reliability of their studies. This issue was identified and addressed by Shanks et al. (1999) who included analysis methods of "rigorous testing of antisera quality, the use of pre-immune controls, and the practice of repeating all test runs, effectively identifying genuine positive and negative results from false reactions" (Shanks et al. 1999: 1188). Studies such as these display the reliability of immunoassay protein analyses when proper controls and methods are put in place.

MS protein residue techniques have gained acceptance due their ability to avoid the issues that plague immunological techniques (i.e., cross reactivity, false positives, targeted detection of epitope on single protein) (Barker et al. 2015; Croft 2021), though there has been little consensus about proper methods and technique. Buckley (2016) identified LC-based MS approaches to protein analysis as providing higher taxonomic resolution, although this higher resolution is not necessary for sample identification and favors the lower time and cost restraints of MALDI PMF techniques. Furthermore, DESI-

MS has been discussed as a promising new protein residue analysis technique (Croft 2021; Yao 2012) but few published studies of archaeological protein residue analysis exist in the literature (Heaton et al. 2009). More research is needed on the different MS protein residue techniques before we can better understand how they will fit into the archaeologist's toolbox.

Modern and ancient contamination mechanisms effect both immunological and MS protein analysis techniques equally. Buckley et al. (2017b) questions modern contamination issues that have influenced MS peptide identification; however, modern contamination due to the mishandling of artifacts is only one plausible route of contamination. Below, I discuss additional routes of contamination that plague both immunological and MS protein analysis techniques that have yet to be formally identified and discussed in the current literature.

*Protein Preservation in Geological Contexts*. The processes of site formation are fundamental to archaeological study (Wood and Johnson 1978; Schiffer 1995). Not only is it important to understand depositional factors but soil formation processes as well. Two processes can operate on the development of soils: horizonation or the formation of soils into horizontal profiles, and homogenization or the impediment and/or mixing of soils (Wood and Johnson 1978). Homogenization soil formation processes can affect both the location and condition that artifacts are recovered in (Hilton 2003; O'Brien 2006; Michel et al. 2019). I will focus on these homogenizing soil forming processes, specifically the effects of groundwater fluctuations and cryoturbation caused by freeze-thaw cycles, but first I will discuss the survivability of proteins and DNA molecules within soils and the decomposition processes that release proteins and DNA into the soil.

*Protein Survivability and Soil.* Soil matrices and sediments have been identified as an influencing factor in the survivability of proteins. Loy (1983) indicates that the electrostatic interactions between clay particles and proteins in soil promote clay and protein binding. In turn, this binding protects proteins from microbial attack and ground-water removal. These findings are further supported by soil chemistry studies demonstrating the absorption of proteins by silica, talc, and clay particles through both electrostatic and hydrophobic interactions based on the presence of both hydrophobic and hydrophilic regions on proteins (Docoslis et al. 1999; Docoslis et al. 2001; Chevallier et al. 2003; Cooke and Shaw 2007). Chevallier and colleagues (2003) identified how clay-bound proteins were protected from microbial attack because the bound protein was unusable by the microorganism. This protection is due to a masking of the protein through the protein's absorption into an inorganic substrate. This "masking" either prevents the recognition of the protein by a microbial attacker or by making the amino acid terminals on the protein that are needed for microbial reaction unusable (Chevallier et al. 2003).

Furthermore, studies have shown a variety of favorable soil pH levels for the binding of proteins to clay and mica particles. Bovine serum albumin has been shown to bind at relatively low pH levels (4.7; Oonk et al. 2012), while others have shown that a protein's affinity to bind to a mineral is dependent on the protein's respective isoelectric point, with myoglobin and lysosome proteins differing strongly (Docoslis et al. 1999; Docoslis et al. 2001; Chevallier et al. 2003; Cooke and Shaw 2007; Ralla et al. 2010; Moerz and Huber 2022). Clay has been recognized as having a high affinity for protein absorption (Chevallier et al. 2003; Ralla et al. 2010; Moerz and Huber 2022), although

this could be due to the higher degree of surface area present on clay particles due to their small size since greater surface area allows for higher amounts of protein absorption (Docoslis et al. 2001). Silts and sands absorb proteins based on the mineralogical composition, although these particles have a lower protein absorption rate due to their smaller surface area.

One important study on the survivability of blood proteins in soil was conducted at an ambush site where numerous individuals were killed during the war in Kosovo (Tuller and Sanders 2012). High rates of blood protein were detected in two-year-old soil samples using CIEP. Kooyman et al. (1992) were also able to detect buffalo blood proteins in the soil at a 5,600-year-old buffalo kill site. Due to the high amounts of blood protein detectable in soil, in addition to other possible soil contaminants (e.g., bacteria, lipoproteins, aluminium chromate, tannic acid) which can cause false positives, it is important to process soil samples associated with the artifacts (Newman and Julig 1989). The collection of soil samples for this type of control testing is an important procedure that is now being adopted into the artifact retrieval process when the project specifically calls for blood protein residue analysis.

*Soil Contaminants Through the Decomposition of Remains*. The decomposition of remains has been known to affect surrounding soils by increasing levels of macronutrients (e.g., nitrogen and phosphorus), the electroconductivity of soils, and pH levels (Luong et al. 2018; Barton et al. 2020). Furthermore, Baron et al. (2020) identified that these changes can be detected in a 30cm radius from human and pig remains, with these soil alterations lasting up to 700 days. Luong et al. (2018) identified a much greater movement of decomposition products through sediments over 2.5m from a human cadaver, with the spread of decomposition biomarkers moving at least 1m vertically up a 4.2-degree slope. In addition to the leaching of decompositional products, Emmons (2015) identified the presence of mtDNA in soil samples surrounding decomposing bodies. While we cannot be certain of the quality and quantity of surviving biological material that is absorbed into the soil as a body decomposes, I find it probable that some native proteins will escape a body during the decomposition process and leach into the surrounding soils.

Aside from biological material leaving the body during decomposition, contaminate transport models identify pathways and transport methods for how ‘contaminants’, such as decomposition fluids and proteins, can move through sediment columns and contaminate sterile soils. Transport models are used to identify parameters of contamination, and while multiple transport and dispersion methods can work in tandem, the sediment makeup and presence of ground water can greatly influence the spread of contaminants and increase mobility (Katyal and Morrison 2014). Through experimentation, Cooke and Shaw (2007) demonstrated that a rising water table permitted the vertical movement of proteins up a sediment column. While Cooke and

Shaw's (2007) protein soil migration experiment showed a limited vertical protein movement of 1cm, it is reasonable to expect that a larger contaminate source, such as a decomposing corpse, might result in a greater extent of vertical contamination.

*Aquaturbation and Cryoturbation.* Wood and Johnson (1987) identified soil mixing vectors for cryoturbation such as freezing and thawing, and the soil mixing vectors for aquaturbation as water. Additionally, they state that "aquaturbation occurs when water under pressure disturbs the soil" an action involved in all seasonally frozen soils and sediments (Wood and Johnson 1987: 359). In other words, the freezing of soil creates pressure that moves water through a sediment column. For this reason, the action of water (aquaturbation) and freeze-thaw (cryoturbation) mechanics will be discussed in tandem.

Many different models and theories explaining the mechanisms for cryoturbation (including aspects of aquaturbation) have been addressed and discussed in detail (Washburn 1988; Van Vliet-Lanoë 1988; Van Vliet-Lanoë 1991; Bockheim 2007; Anderson 1988). For the purposes of this paper, I will address the fundamental mechanics of cryoturbation and its relation to water movement in non-permafrost environments.

The basics of cryoturbation involve the formation of ice in soil that create pressure and push sediments vertically (Van Vliet-Lanoë 1988; Van Vliet-Lanoë 1991; Bockheim 2007;). Upon thawing, spaces are left where the ice once was, providing the movement of fine grain sediments in the spaces and creating anomalies in the sediment column (Van Vliet-Lanoë 1988; Van Vliet-Lanoë 1991; Bockheim 2007; Anderson 1988; Kaplar 1965).

Moisture, temperature, and sediment composition (Washburn 1988), and drainage (Van Vliet-Lanoë 1988; Van Vliet-Lanoë 1991) are necessary for the formation of ice in soils. Adequate water is needed for the formation of ice to occur, with high moisture producing increased susceptibility to cryoturbation. In addition, fine porous sediments allow for greater water movement and increased frequency and size of cryoturbation phenomena (Van Vliet-Lanoë 1988; Van Vliet-Lanoë 1991; Anderson 1988). Van Vliet-Lanoë (1988; 1991) with the drainage capabilities of soil and water levels through autumn as being important factors in presence and size of ice formations (i.e., wedges).

As stated above, water is important to the formation of ice during cryoturbation, and it is also essential for aquaturbation processes. As surface soil freezes, pressure is built up and placed on unfrozen ground water (Wood and Johnson 1987; Van Vliet-Lanoë 1988; Van Vliet-Lanoë 1991; Anderson 1988). As the surface soil begins to thaw, the built-up water pressure finds weak spots in the surface causing an ejection of subsurface sediments in what Wood and Johnson (1987) refers to as the "toothpaste" effect. This aquaturbation process displaces and mixes soils within a sediment column.

*Implications of Cryoturbation in Archaeological Contexts*. Proteins can survive in soils and adhere to clay particles which protect them from degradation (Loy 1983; Cattaneo et al. 1993; Chevallier et al. 2003; Tuller and Sanders 2012; Kooyman et al. 1992). The freeze-thaw cycles of cryoturbation have the capability to move sediment, objects, and water vertically through sediment columns (Hilton 2003; O'Brien 2006; Michel et al. 2019; Anderson 1988; Kaplar 1965). These sediment and water moments due to cryoturbation can alter stone tools by creating a polish that alters use-wear traces and residues (Michel et al. 2019). Studies have looked at the removal of residues from

artifacts through water movement (Michel et al. 2019; Shanks et al. 2001), although there is a lack of knowledge surrounding the contamination of artifacts through sediment columns and if water and/or sediments can carry viable blood proteins with them. Further research and testing is needed to address these unknowns.

*Other Contamination Mechanisms*. While environmental contaminants are an important aspect of our research program, I have identified other forms of contamination that archaeologists need to be aware of. Field handling and care of artifacts often include the placing of artifacts into one's mouth or the use of saliva as a cleaning agent. These field methods have high contamination rates for later PRA and can produce questionable analysis pointing toward human cannibalism based on human proteins deposited by the field archaeologist. Additionally, Lowenstein et al. (2006) identified low levels of immunological reactions on stone tools contaminated by human sweat. It is important to keep artifacts away from both humans sweat a saliva, when possible, to prevent false positives of future PRA.

Animal excrement can also be a vector of secondary protein contamination because it is a rich source of biological material that can cause secondary contamination of sediments and artifacts under favorable conditions. Henrikson et al. (2017) identified positive CIEP results for sheep on surface artifacts that were noted to be likely the result of historic sheep pasturing in the area. Murchie et al. (2021) identified 30,000-year-old sedimentary ancient DNA showing the collapse of extinct species, such as the woolly mammoth, in the steppe-tundra ecosystem of Yukon, Canada.

In summary, I have identified the preservation of proteins within sediments, soil contamination though the leaching of decompositional products, and geological

conditions that can further move proteins through sediment. I propose that through the decomposition process, biological material, such as proteins and DNA, are expelled from decomposing bodies and are transported through the sediment column and bound to surrounding clays, silicates, and other available sediment particles. Furthermore, protein transport is aided by a fluctuating water table, while clay and other minerals protect the proteins from degradation and microbial attack. The transport and deposition of these proteins into surrounding soils have created an area of contamination around the decomposing carcass affecting artifacts that have never come into direct contact with that carcass. Based on this hypothesis, I created an experiment to test secondary artifact contamination across a sediment profile. I attempted to mimic the natural processes of a fluctuating water table and freeze-thaw conditions to transport proteins across a sediment column to sterile artifacts from a known protein source.

# CHAPTER III CONTAMINATION EXPERIMENT

Previously, I hypothesized that protein transport in sediment can contaminate artifacts with protein from a contextually unassociated source. I discussed the preservation potential of proteins within sediments and protein transport through the leaching of decompositional products and geological processes of aquaturbation (water) and cryoturbation (freeze-thaw). From these discussions, I develop the following hypotheses for test within the context of a contamination experiment:

Ho: Protein transports poorly through sediment columns.

Prediction: Only artifacts in direct association with meat will display contamination.

H1: If contamination is found, it results from free protein contained in contaminated soil, not the experimental protein samples.

Prediction: Artifacts in a sediment column will display contamination that distributes randomly relative to a protein source.

H2: If contamination is found, it results from protein transported through the sediment column.

Prediction: If so, then contamination should decrease with distance from protein source.

H3: Protein preservation and movement is greater within sediments of higher clay content.

Prediction: Higher rates of movement and contamination will be present in sediments of higher clay content.

H4: Water can carry higher quantities of proteins over large distances.

Prediction: If so, then the water movement experiments should have the highest contamination rates across experiments and maintain high rates of contamination further away from the protein source.

H5: Geological conditions, such as aquaturbation and cryoturbation, move proteins through sediment columns.

Prediction: Less contamination will occur in control experiments that are not put through simulated geological conditions.

Based on those hypotheses, I created a contamination experiment to identify the contamination potential of water movement and freeze-thaw processes on sterile artifacts placed within a sediment column together with a known protein source. I test my hypotheses within six experimental contexts, three using a sandy clay loam for an experimental matrix, and the other three a slightly sandy loam, and do so to identify any differentiation in protein preservation, movement, and contamination between different sediment types. Additionally, each sediment type is exposed to three different experiments testing 1) water movement, 2) freeze-thaw, and 3) a control for decomposition.

*Methods*

Experiments were divided into a series of specific geological contexts designed to mimic fluctuating water tables and freeze-thaw conditions that are predicted to move proteins from a known protein source to sterile artifacts within the same sediment column. Six individual experiments were conducted, two for each of the following circumstances: 1) water movement, 2) freeze-thaw, and 3) control for decompositional leaching, were all conducted within two different soil matrixes. First, a gravely, slightly sandy (medium) loam derived from the Middle and Lower Ordovician Garden City Formation (Utah Geological Survey 2022) was collected from a road cut northeast of Logan, Utah. Second, a gravely sandy (coarse) clay loam derived from the Pleistocene Alpine and Bonneville Formations (Utah Geological Survey 2022) was collected from toe slope deposits within Right Hand Fork Canyon, east of Logan, Utah.

Experiments were conducted within six individual experimental devices (boxes) constructed of 5mm-thick plywood and 2.54cm (1 inch) by 2.54cm (1 inch) furring strips, measuring 65 centimeters (cm) high x 45cm$^2$ and lined with 6-mil plastic sheeting. Drain plugs were placed in bottom of the wet (water movement and freeze-thaw) experimental boxes to drain the water added during experimentation. An average of 12cm of pea gravel was added to the base of each box to aid in draining and placed in the control boxes for uniformity across experiments.

Eight “artifacts” were placed in each experimental box at varying intervals and distances from a known protein source (Figure 5). The experimenter knapped all artifacts using copper tipped tools, denim clothes, and rubber gloves to prevent possible outside protein contamination. Artifacts were made from Glass Buttes obsidian and averaged

47mm long, by 31.8mm wide, and 9.9mm thick with an average of 83 percent of the edges containing some degree of modification through pressure flaking. Edges were modified to create microcracks and aid in the absorption of proteins (Shanks et al. 2002). Bovine rump roast was used for the protein source and sliced at an average of 2.7cm thick with an average weight of 164.3 grams. Three incisions were made on the top and bottom of each protein source at a depth of approximately one cm to increase surface area and protein emission. The six experimental boxes were setup in accordance to Figure 5, with artifact #4 in each box acting as a control with placement directly on the protein source. Nitril gloves were used to handle all artifacts and protein sources with gloves replaced before the handling of each artifact and protein source. Furthermore, each artifact and protein source was photographed with their locations measured and recorded upon placement and excavation.

After experimental boxes were setup, the water movement and freeze-thaw boxes were exposed to different protocols to mimic the geological processes under test. Water movement experimental boxes were exposed to ten cycles of being completely saturated with room temperature tap water, followed by draining. These boxes were saturated for a full 12-hour period, then drained for an additional 12-hour period before the next saturation cycle was started. Freeze-thaw experimental boxes included a temperature probe placed below the protein source to measure the internal temperature of the boxes and were placed on moving dollies for transport in and out of a walk-in freezer. These boxes were fully saturated with water before undergoing five complete freeze-thaw cycles of the center probe reaching at least 25 degrees Fahrenheit (F) before removal from the freezer and thawing to the ambient room temperature, between 60 and 73

degrees Fahrenheit, before the next freeze cycle was started. Control boxes that were setup to control for decompositional leaching were stored at an ambient room temperature between 60 and 73 degrees F for 35 days before being excavated.

The location of each artifact and protein source was measured at placement and excavation with easting (x-axis) and northing (y-axis) measurements relative to a box datum, along with a depth from surface measurement, to identify potential soil compaction and artifact movement. Additionally, all artifacts were handled with fresh nitril gloves to limit any possible handling contamination. Artifacts were placed in individually labeled bags after excavation and stored in the freezer until shipped to Archaeological Investigations Northwest, Inc. (AINW) for CEIP analysis. Artifacts were stored in a freezer to prevent possible protein degradation before materials were analyzed.

*Results*

As predicted, contamination did occur within the different experiments, with almost half of the specimens (43%) signaling some level of protein contamination (Table 1). First, to depict the pattern of protein movement across experiments, artifacts were placed into four zones, with Zone 1 comprised of all artifacts within 3cm of the protein source, Zone 2 comprised of all artifacts between 3–6cm from the protein source, Zone 3 comprised of all artifacts between 6–9cm from the protein source, and Zone 4 includes all artifacts >9cm from the protein source. Viewing all experiments in aggregate illustrates a negative relationship between distance and percent contamination (Figure 6a).

Experimental results from the two different sediments, as well as between the different experiment types, did not follow expectations. First, control tests were conducted on the two sediments with Sediment 2 (Alpine and Bonneville gravely sandy (coarse) clay loam testing positive for bovine protein and Sediment 1 (Garden City gravely, slightly sandy (medium) loam) testing negative. Additionally, experiments conducted using Sediment 2 resulted in approximately the same rate of contamination as those using Sediment 1 (11 and 10 respectively). When experimental results are separated out by sediment type, both depict a distance decay pattern with Sediment 2 experiments exhibiting a stronger distance decay trend than experiments conducted using Sediment 1 (Figure 6b-c). Rates of contamination differed across the three experimental contexts: water, freeze-thaw, and control. In this case, 62 percent of the decomposition control specimens, 43 percent of the freeze-thaw specimens, and 25 percent of the water movement specimens tested positive for protein (Table 1).

The overall experimental results depict a strong distance decay pattern (Figure 6a); however, this pattern is not as strong across all experimental contexts. A Spearman's rank correlation of contamination and distance failed to find a significant relationship ($r_s$ = -0.1712, $p$ = 0.2447) when all experimental contexts are combined. This statistical result, however, likely reflects the small sample sizes available for analysis.

The decompositional control experiments depict the strongest distance decay pattern, even when separated between the two sediment types (Figure 7a-c). A Spearman's rank correlation of contamination and distance failed to find a significant relationship for the combined decompositional control experiments ($r_s$ = -0.2924, $p$ = 0.2719). It is also interesting to note that the decompositional control experiments not

only depict a distance decay pattern of protein movement but also an upward movement through the sediment column with all artifacts above the protein source testing positive for bovine protein and only one artifact below the protein source (position 2) testing positive for contamination (Table 1; Figure 5).

The water movement experiments also depict a strong distance decay pattern (Figure 8a); however, this pattern is not as strong in the Sediment 1 water movement experiment (Figure 8b). A Spearman's rank correlation of contamination and distance found a significant and negative relationship for the combined water movement experiments ($r_s$ = -0.5229, $p$ = 0.03765). Curiously, one of the artifacts in position #4 (in direct contact with protein source) tested negative for contamination, adversely impacting the negative relationship between contamination and distance.

The freeze-thaw experiments depict the weakest distance decay patterning when considered together (Figure 9a) and when separated out by sediment used (Figure 9b-c). A Spearman's rank correlation of contamination and distance failed to find a significant relationship for the combined freeze-thaw experiments ($r_s$ = 0.2282, $p$ = 0.3952). Table 2 provides a detailed breakdown of the experimental results.

*Discussion*

Artifact contamination occurred at an overall rate of 43 percent across experiments, rejecting the null hypothesis that proteins transport poorly through sediment columns. My results, however, did not unanimously support the remining hypotheses and predictions. For example, hypothesis 1 is rejected despite the contamination of Sediment 2, protein contamination is the result of protein transport, higher clay content did not

result in higher contamination rates, water movement did not result in higher contamination rates, and contamination occurred in the absence of simulated geological conditions.

Hypothesis 1, stating contamination would result from free proteins in contaminated soil and not from the experimental protein samples, was rejected despite Sediment 2 testing positive for bovine protein. If protein contamination was the result of free proteins contained in contaminated soil, it was predicted that artifacts would display random distributions relative to the protein source. Despite Sediment 2 testing positive for free protein contamination before the introduction of the experimental protein, the combined experiments using Sediment 2 display a strong trend of decreased contamination with distance from the protein source (Figure 6c). This pattern supports hypothesis 2 that contamination is a result of protein transport through the sediment columns. Therefore, I find it unlikely that sediment contamination had a significant impact on the experimental results. Additionally, the combined experiments using the 'contaminated' Sediment 2 display a stronger distance decay trend then the combined experiments using the uncontaminated Sediment 1 (Figure 6b-c), making a strong case that the free protein contamination of Sediment 2 did not have a significant influence on the experimental results.

Hypothesis 2, stating contamination is the result of protein transport, is accepted. It was predicted that contamination should decrease with distance from protein source, which is evident in the strong distance decay patterning of the experimental results (Figure 6a). Not all experimental contexts exhibited the same strong distance decay pattern, discussed further below.

Hypothesis 3 predicted that sediment with the higher clay content (Sediment 2) would have a higher contamination rate. Experiments conducted using Sediment 2 did result in a higher quantity of contaminated artifacts (11 artifacts compared to 10 contaminated artifacts in Sediment 1); however, this slight increase is not likely significant. The similar number of contaminated artifacts between Sediment 1 and Sediment 2 experiments (10 and 11 respectively) further supports the claim that the protein contamination in Sediment 2 did not have a significant impact on the experimental results. Furthermore, it is possible that the higher clay content of Sediment 2 influenced protein preservation from historic sources that lead to the sediment's contamination prior to experimentation.

Hypothesis 4 predicted the water movement experiments to have the highest contamination rates across experiments and maintain high rates of contamination further away from the protein source which was not supported with the experimental results. The water movement experiments received the lowest level of contamination of the three experimental contexts at 25 percent (Table 1). Additionally, contamination did not occur outside of zones 1 and 2 the water movement experiments (Figure 7a-c) or further than 6cm from the protein source. However, the water movement experimental results exhibited a statistically significant negative relationship between contamination and distance ($r_s$ = -0.5229, $p$ = 0.03765), the only statistically significant findings within the experiments. Interestingly, these results are statistically significant even though one of the artifacts in position #4 (in direct contact with protein source and in Zone 1) tested negative for contamination (Table 1, Table 2), adversely impacting the negative

relationship between contamination and distance. This anomaly may be the result of how the lab tested the artifacts.

I find it is possible that testing one side of an artifact could yield a negative test result when the other side of the artifact is saturated with the protein based on the methods the lab used to test the artifacts. Personal communication with lab personnel that conducted the CIEP tests indicated that artifacts were tested in a single location based on the lab personnel's discretion (e.g., close to edges that would have likely been used and have the highest likelihood to yield a positive test result) (personal communication 2023). This arbitrary method of testing artifacts may have skewed test results based on the side of the artifact undergoing testing. I find it possible that protein movement through sediments may reflect a similar pattern as to air movement around an object in a wind tunnel, the air encounters the side facing the fan, but the air does not fully encompass and flow over the backside of the object. If proteins move through sediments in a similar fashion, it is possible that testing the backside of an artifact could yield a negative test result when the frontside of the artifact is saturated with the protein. If this is true, it would explain how an artifact placed directly on top of the protein source tested negative for bovine proteins.

Hypothesis 5 predicted less contamination to occur in control experiments that are not exposed to simulated geological conditions, however, the control boxes exhibited the highest levels of contamination at 62 percent (Table 1). Experimentation did not show that the mechanisms of aquaturbation and cryoturbation do not move protein, just that proteins move regardless of the circumstances; therefore, Hypothesis 5, stating geological conditions, such as aquaturbation and cryoturbation, move proteins through sediment

columns cannot be rejected despite my failing prediction. Results of the control boxes were unexpected, especially the pattern of contaminated artifacts showing an upward movement of proteins (Table 1). Bacteria and microorganisms are known to move through soil which may have acted as "carriers" of proteins causing an upwards displacement, however, a vertical movement of these organisms in a mixed substrate without the added influence of a root systems is unknown and lacking research (Yang and van Elsas 2018; Yokota et al. 2022). Possible outside contamination issues many plague the control boxes, however, a similar patter across the two experiments of differing sediment types decreases that likelihood.

The freeze-thaw experiments exhibited the least distance decay patterning of the three experimental contexts. Additionally, the lack of contamination within Zone 2 (three to six cm from protein source) with contamination of artifacts in Zone 3 and 4 (6–9+cm from protein source) in the freeze-thaw experiments is peculiar (Figure 9a-c). This peculiar patterning may be the result of arbitrary testing practices by the lab, discussed above. Additionally, the freeze-thaw and water boxes could have undergone a washing effect that kept proteins from adhering onto the artifact surfaces, but it is unclear how this effect could have caused such opposing patterning between the water movement and freeze-thaw boxes. Additionally, due to the statistically significant distance decay contamination patterning in the water movement boxes compared to the non-significant patterning of the freeze-thaw boxes, the water in these experimental boxes causing a washing effect on artifacts is unlikely.

Due to the low level of artifact movement during experimentation, it is unlikely the compaction of sediment and slight movement of artifacts impacted the contamination

results. Artifacts were measured at placement and removal to identify any movement during experimentation. Vertical movement due to sediment compaction was the plane that artifacts were most likely to move during experimentation with an average vertical movement of 1.14 cm. The average horizontal movement of artifacts was 0.33 cm. Vertical movement was greatest among artifacts in the water movement boxes at 1.47 cm, followed by the freeze-thaw boxes at 1.26 cm, and the control boxes at 0.72 cm. The greatest movement of a single artifact was vertically at 3.2 cm in a freeze-thaw experimental box.

*Conclusion*

My experiments have identified routes of blood protein residue contamination of in situ artifacts, however, the extent of this issue and the reasons behind it are not fully understood. This research has exposed some additional questions and concerns regarding PRA when used in archaeological contexts, such as if PRA remains a valid analytical tool for the analysis of in situ artifacts and if there are methods to identify secondary in situ contamination of blood proteins verses the archaeological deposition of blood protein on artifacts. Additional future testing is needed to address the validity issues of PRA on in situ artifacts and identify the genuine mechanisms of protein movement within sediment columns and the extent of protein movement.

Experimentation has identified the movement of protein through sediment columns in a distance decay pattern, regardless of geological conditions and contexts (Figure 6-9). In situ artifacts were contaminated with protein from a secondary source within water movement, freeze-thaw, and decompositional control boxes; however, the

greater rates of contamination within the decompositional control experiments were unexpected and not fully understood. Water movement experiments exhibited the least amount of contamination across all experimental context yet was the only context with statistically significant results for contamination and distance ($r_s$ = -0.5229, $p$ = 0.03765). Furthermore, the freeze-thaw experiments resulted in the weakest distance decay patterning with artifacts three to six cm from the protein source testing negative for protein contamination while protein contamination occurred in high rates at further distances from the protein source. Sediment 2 was found to have been contaminated with protein prior to experimentation; however, this was not found to have influenced the experimental results. Finally, the lab methods for how artifacts are tested for blood protein residues has been scrutinized based on the absence of protein on artifacts placed in direct contact with the protein source being tested for.

My experimentation was not exhaustive and further research and experimentation is needed to address questions and concerns that arose during this research. First, more experimentation needs to be conducted to increase the dataset for stronger and more reliable results and lower margins of error. Based on the results of the control experiments, future research is needed to identify and address the cause of increased contamination in contexts solely exposed to decompositional leaching and the upwards protein contamination within this context. Additionally, control experiments should be conducted in the absence of a protein source to identify if any outside contamination may have been present within my experiments, or if pre-contaminated sediments, such as sediment 2, can contaminate sterile artifacts. Further testing should also be conducted at the collection site of sediment 2 to identify the scale of contamination within the

sediment used. Moreover, further experimentation and testing are needed to identify if the arbitrary testing of artifacts at a single location is to blame for the contamination patterning across experiments.

Based on this research alone I am unable to discredit the full use of PRA in archaeological contexts but provide additional caution to researches planning to use PRA as an analytical tool. This research further exemplifies the importance of testing sediments that surround an artifact, as well as the artifact, to identify any *in situ* contamination; however, it is unclear if this testing method can fully identify *in situ* protein contamination verses the archaeological deposition of blood protein on artifacts. Researches should use a full range of analytical techniques when studying archaeological sites and artifacts and should never solely rely on the results of a single analytical method when making inquiries and drawing conclusions.

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# APPENDICES

*Appendix A. Tables*

Table 1. Contamination experiment results based on geological context and artifact position.

| Position* | #Contaminated | Position | #Contaminated |
|---|---|---|---|
| Overall | 43% Contamination | | |
| 8 | **3** | | |
| 6 | **3** | 7 | **3** |
| 4 | **5** | 5 | **2** |
| 2 | **2** | 3 | **1** |
| 1 | **2** | | |
| Water | 25% Contamination | | |
| 8 | **0** | | |
| 6 | **1** | 7 | **0** |
| 4 | **1** | 5 | **1** |
| 2 | **1** | 3 | **0** |
| 1 | **0** | | |
| Freeze/Thaw | 43% Contaminated | | |
| 8 | **1** | | |
| 6 | **0** | 7 | **1** |
| 4 | **2** | 5 | **0** |
| 2 | **0** | 3 | **1** |
| 1 | **2** | | |
| Control | 62% Contaminated | | |
| 8 | **2** | | |
| 6 | **2** | 7 | **2** |
| 4 | **2** | 5 | **1** |
| 2 | **1** | 3 | **0** |
| 1 | **0** | | |

*"Position" defined as shown in Figure 5.

Table 2. Contamination experiment results.

| Sediment | Experiment | Artifact Position* | Bovine CIEP Results |
|---|---|---|---|
| S1 | Freeze/Thaw | 1 | + |
| S1 | Freeze/Thaw | 2 | - |
| S1 | Freeze/Thaw | 3 | - |
| S1 | Freeze/Thaw | 4 | + |
| S1 | Freeze/Thaw | 5 | - |
| S1 | Freeze/Thaw | 6 | - |
| S1 | Freeze/Thaw | 7 | - |
| S1 | Freeze/Thaw | 8 | + |
| S2 | Freeze/Thaw | 1 | + |
| S2 | Freeze/Thaw | 2 | - |
| S2 | Freeze/Thaw | 3 | + |
| S2 | Freeze/Thaw | 4 | + |
| S2 | Freeze/Thaw | 5 | - |
| S2 | Freeze/Thaw | 6 | - |
| S2 | Freeze/Thaw | 7 | + |
| S2 | Freeze/Thaw | 8 | - |
| S1 | Water | 1 | - |
| S1 | Water | 2 | - |
| S1 | Water | 3 | - |
| S1 | Water | 4 | - |
| S1 | Water | 5 | + |
| S1 | Water | 6 | + |
| S1 | Water | 7 | - |
| S1 | Water | 8 | - |
| S2 | Water | 1 | - |
| S2 | Water | 2 | + |
| S2 | Water | 3 | - |
| S2 | Water | 4 | + |
| S2 | Water | 5 | - |
| S2 | Water | 6 | - |
| S2 | Water | 7 | - |
| S2 | Water | 8 | - |
| S1 | Control | 1 | - |
| S1 | Control | 2 | + |
| S1 | Control | 3 | - |
| S1 | Control | 4 | + |
| S1 | Control | 5 | - |
| S1 | Control | 6 | + |

| | | | |
|---|---|---|---|
| S1 | Control | 7 | + |
| S1 | Control | 8 | + |
| S2 | Control | 1 | - |
| S2 | Control | 2 | - |
| S2 | Control | 3 | - |
| S2 | Control | 4 | + |
| S2 | Control | 5 | + |
| S2 | Control | 6 | + |
| S2 | Control | 7 | + |
| S2 | Control | 8 | + |

*"Position" defined as shown in Figure 5.

*Appendix B. Figures*

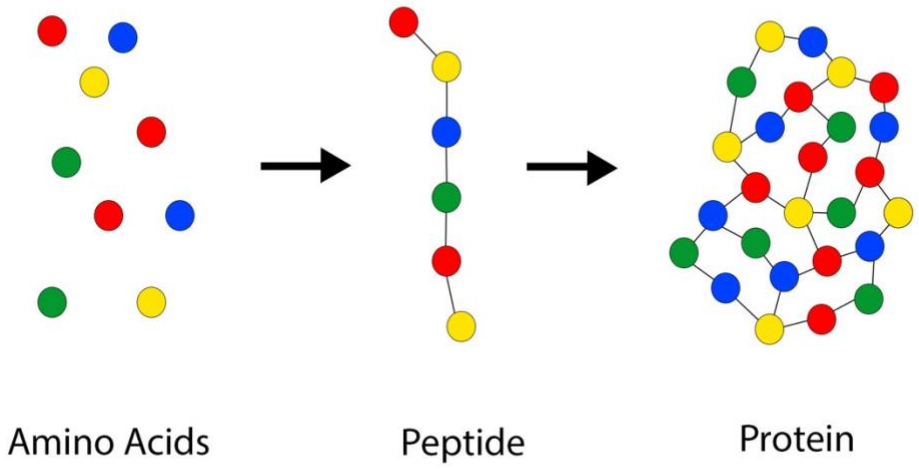


Figure 1. Protein structure.

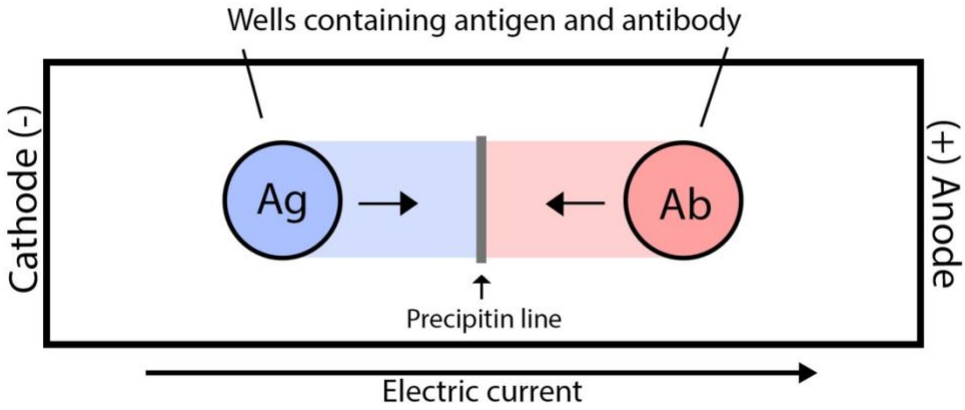


Figure 2. Crossover-immunoelectrophoresis (CIEP) depicting precipitin reaction.

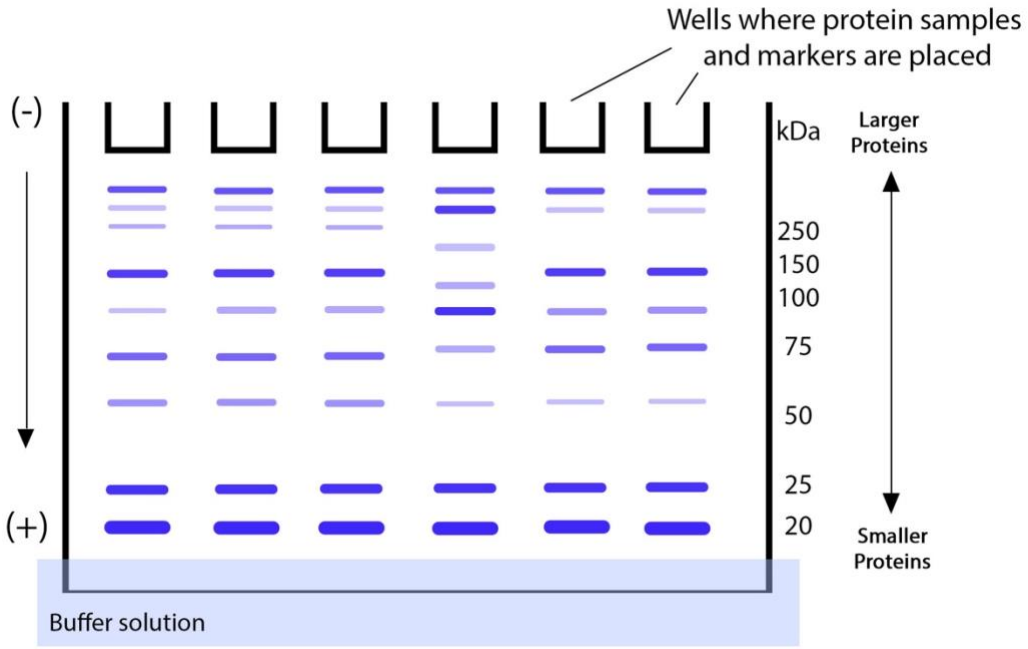


Figure 3. SDS-PAGE gel electrophoresis.

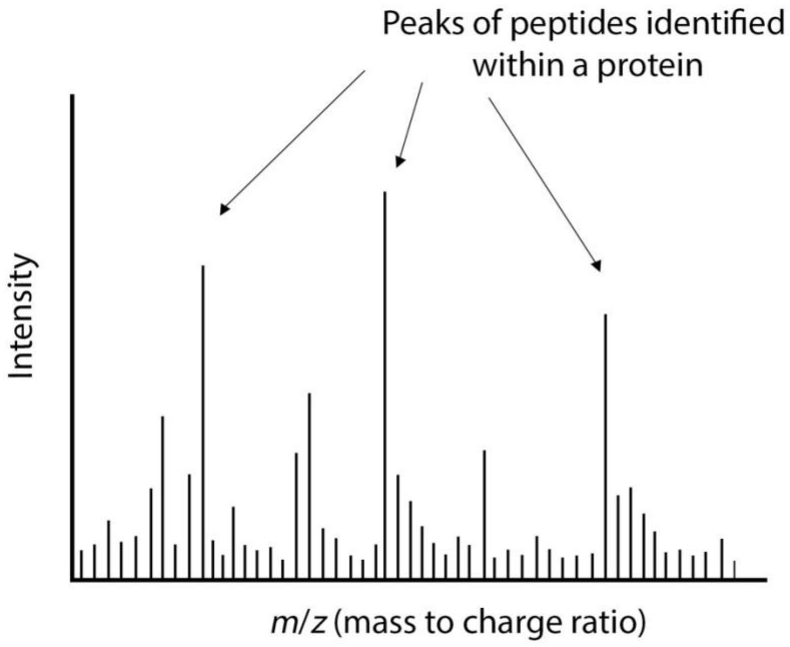


Figure 4. Peptide Mass Fingerprinting sequence.

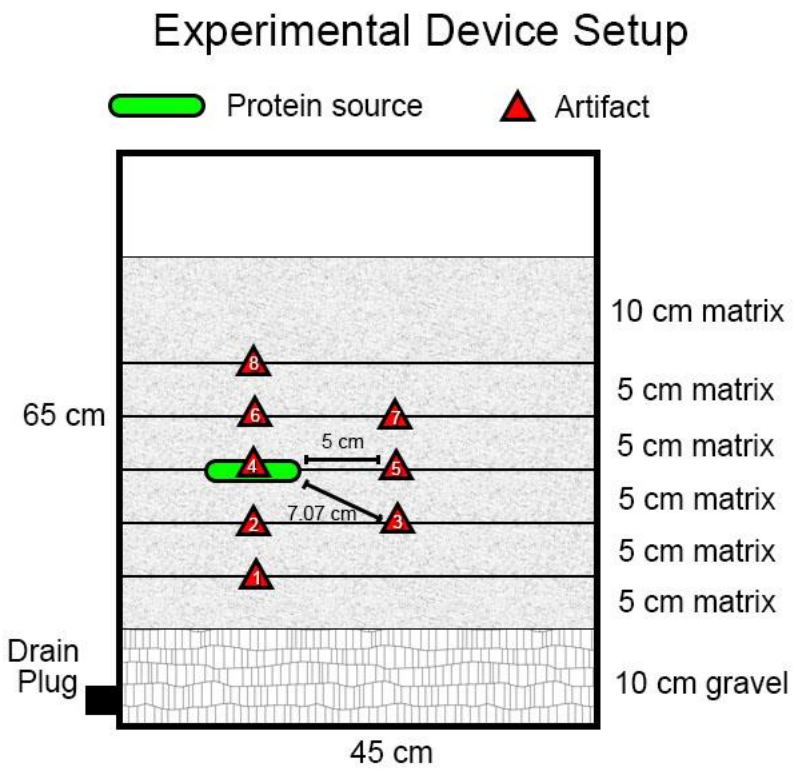


Figure 5. Experimental design.

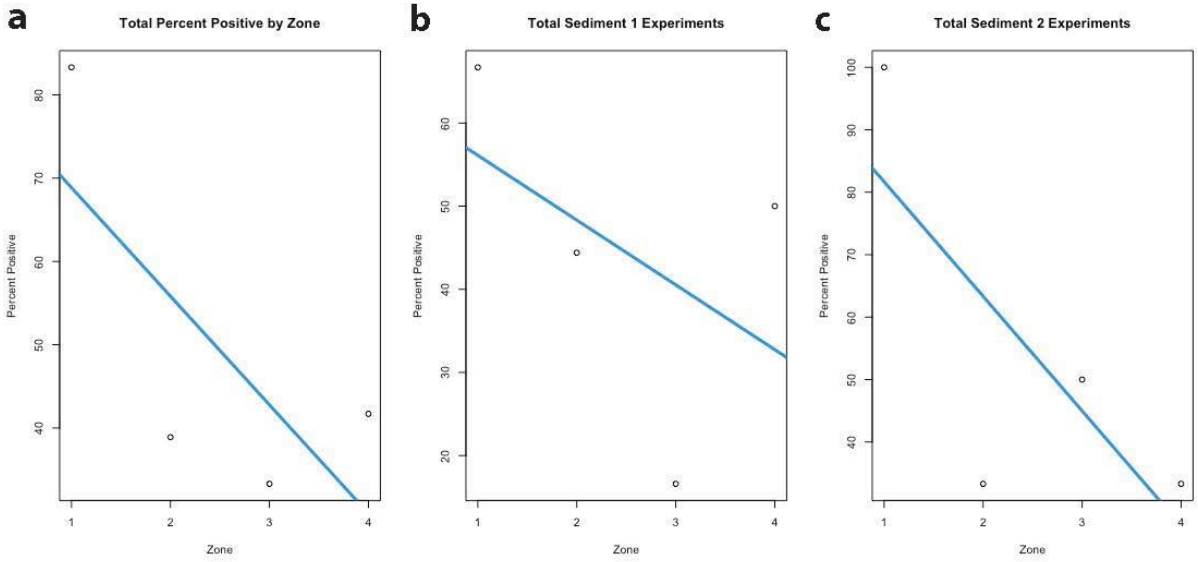


Figure 6. Percent of CIEP positive artifacts by zone: (a) Overall experimental results, (b) Results of all sediment 1 experiments, (c) Results of all sediment 2 experiments.

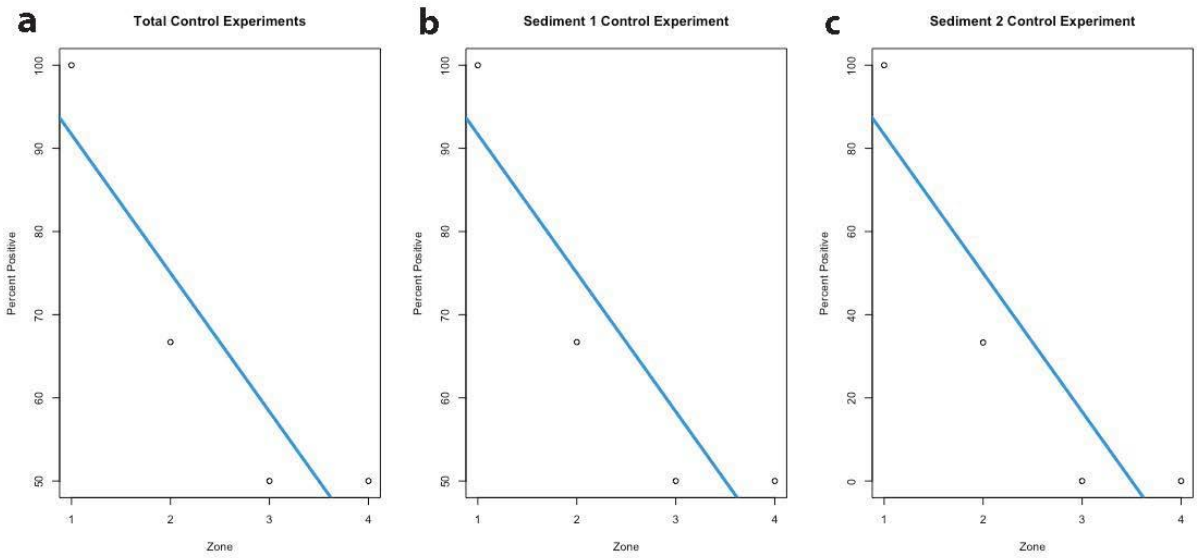


Figure 7. Percent of CIEP positive artifacts by zone for control experiments: (a) Overall control experimental results, (b) Sediment 1 control experiment results, (c) Sediment 2 control experiment results.

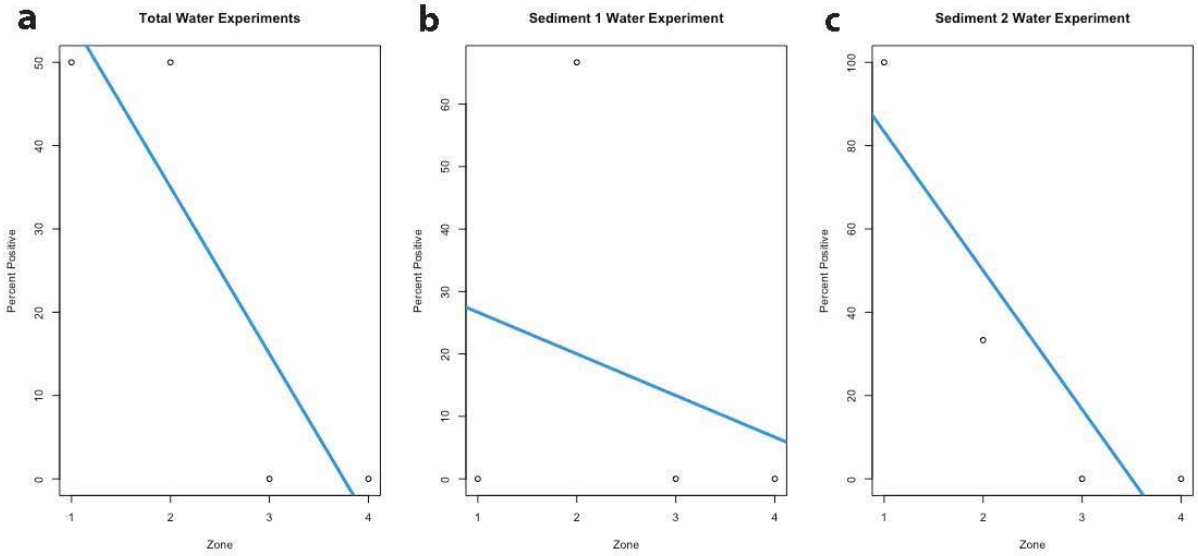


Figure 8. Percent of CIEP positive artifacts by zone for water movement experiments: (a) Overall water movement experimental results, (b) Sediment 1 water movement experiment results, (c) Sediment 2 water movement experiment results.

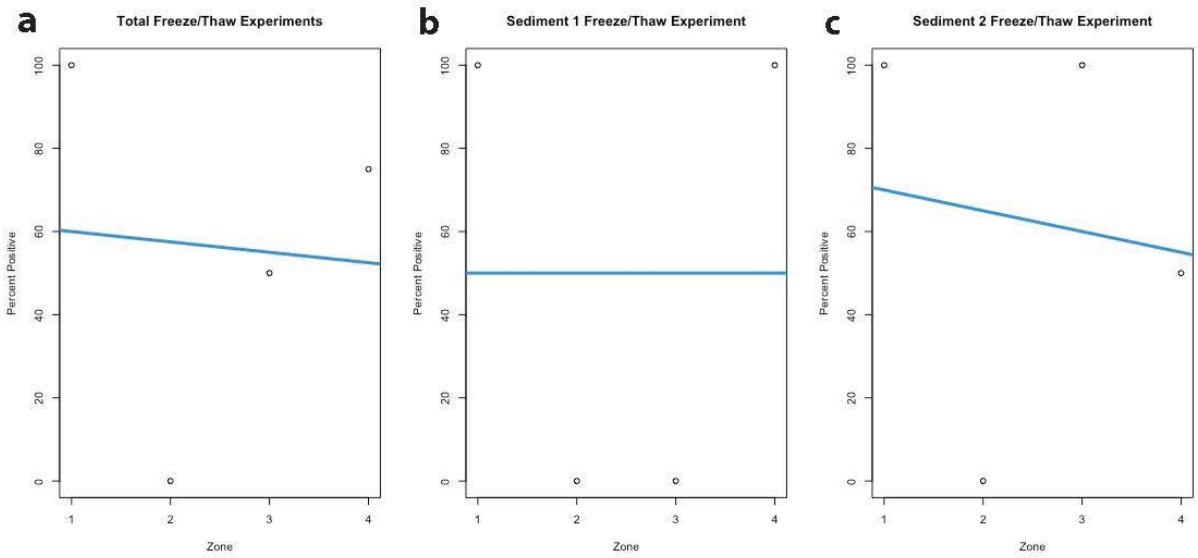


Figure 9. Percent of CIEP positive artifacts by zone for freeze-thaw experiments: (a) Overall freeze-thaw experimental results, (b) Sediment 1 freeze-thaw experiment results, (c) Sediment 2 freeze-thaw experiment results.