ORGANIC RESIDUE ANALYSIS AND ARCHAEOLOGY: A CRITICAL EVALUATION OF ORA METHODS

by

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ABSTRACT

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Under the Supervision of Professor Bettina Arnold

In recent years the field of archaeology has been increasingly interested in applying hard science techniques to different aspects of ancient life in new ways. One area of research that has been pushing the boundaries of hard science applications to understanding past societies is the archaeology of ancient alcohol. The rise of Organic Residue Analysis (ORA) has resulted in increased interdisciplinary collaborations between anthropologists, archaeologists, biochemists and other ancillary disciplines interested in revealing the mysteries of ancient alcohol. However, the application of these new technologies and methodological advances has also increased miscommunication between the chemists and biochemists, who understand the hard science, methodologies, and data presented, and the anthropologists and archaeologists, who understand the social implications and impacts of these data. Furthermore, as these new technologies continue to emerge, their interpretive limitations are still poorly understood. This thesis provides an overview and establishes protocols and guidelines for scientists in these collaborating disciplines who are navigating the appropriate use of ORA in the study of ancient alcohol.

Keywords: Scientific Revolutions, Organic Residue Analysis, Archaeology of Fermentation, Ancient Alcohol

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Chapter 1: Introduction

In recent years there has been increased interest in the application of hard science methods and technologies within the field of archaeology, including in the area of organic residue analysis. Through the use of these new technologies, archaeologists have been able to make great advances and have generated new insights into past societies and cultures, learning more about their diets, both food and drink, and the complex ways that commensality contributes to what makes us human. In addition to learning more about diet and nutrition, these advances have also allowed archaeologists to explore social organization, including access to commodities and how these may have been unequally distributed and associated with various pre- and proscriptions, such as age, gender and status. These advances have also increased interdisciplinary cooperation between anthropologists, specifically archaeologists, and members of the natural science community. However, to some extent this interdisciplinarity has resulted in a disconnect between the scientists, who understand the methodology, data, and experimental design applied in the natural science paradigm, and the anthropologists and archaeologists who understand the social implications that arise from the results of these interdisciplinary studies (Driscoll and Damm 2023; Kristiansen 2022).

Therefore, it is imperative that archaeologists and anthropologists gain a better understanding of how these technological approaches operate, as well as how to properly design experiments, select appropriate methods of analysis, and appropriately interpret the data that arise from these studies. Additionally, it is important for members of the hard science community to understand the social and cultural implications of the results of these interdisciplinary studies. Organic residue analysis and other archaeometric advances have led to a great deal of new valuable information including the various uses for ceramics created by ancient civilizations

(Evershed 1996); however, the disconnect between the scientists conducting the research and the archaeologists interpreting the data remains a challenge. This thesis was designed to help bridge the gap between scientists and archaeologists by exploring the concepts of scientific revolutions and paradigm shifts in relation to the archaeology of ancient alcohol, especially how to appropriately conduct experiments and produce research designs based on hard science methodologies. This is accomplished through a comparative analysis of case studies and literature illustrating the appropriate use of organic residue analysis and archaeometric techniques, as well as highlighting the potential pitfalls of improper planning and predetermined research designs.

Scientific Revolutions, Paradigm Shifts and Good Explanations

An appropriate starting point is an examination of scientific revolutions, paradigm shifts, and good explanations, as outlined by Thomas Kuhn (2015) and David Deutsch (2011). Kuhn discussed the concepts of scientific revolutions and what he referred to as paradigm shifts in his work, *The Structure of Scientific Revolutions*, first published in 1962. The focus is on how changes in the scientific community are driven by paradigm shifts, meaning that for a period of time normal science prevails until there is an alteration in perspective that leads to a scientific revolution. Kuhn defines normal science as "research firmly based upon one or more past scientific achievements, achievements that some particular scientific community acknowledges for a time as supplying the foundation for its further practice" (Kuhn 2015: 10). Over time as individuals continue to pursue "normal science" the probability of a paradigm shift occurring increases. Through their pursuits these individuals develop a new scientific concept or theory that is fundamentally incompatible with the current scientific viewpoint. This incompatibility then induces the paradigm shift in which the current scientific viewpoint is rejected in favor of

the newly proposed concept or theory. This in turn leads to a conceptual revolution within that field of study, allowing scientists to gain new insights as well as generate new and crucial information, which can eventually lead to more paradigm shifts and additional scientific revolutions. These paradigm shifts change the fundamental concepts of science and impact research and experiment design. Shifts and revolutions that follow this pattern can be seen in the theories and concepts proposed by scientific giants such as Copernicus (heliocentrism), Newton (laws of gravity), Lavoisier (stoichiometry, and the chemical revolution), Darwin (evolution by means of natural selection), Einstein (theory of relativity), and many more. These well-known examples help to illustrate the role of the period of normal science, leading up to the incompatible theory, causing the paradigm shift that questions the established foundation of that scientific field, and then leading to the revolution within that scientific field where the new theory is adopted as standard and establishes a new foundation for the scientific field. Archaeometry, or the application of physical sciences to archaeological materials, has experienced several paradigm shifts since 1945, including the radiocarbon revolution, paleogenetics, isotope analysis and mobility; organic residue analysis is a growing area of research that has been contributing to this phenomenon.

To understand the importance of paradigms and revolutions it is important to define the role of what Kuhn refers to as “normal science.” As mentioned previously Kuhn describes normal science as research based on previous scientific achievements that supply the foundational knowledge for that field of science (Kuhn 2015). In addition, Kuhn discusses how “normal science” doesn’t tend to produce radical changes within scientific fields, focusing instead on adding scope and precision to how the current paradigm can be implemented. Normal science is focused mainly on problem and puzzle-solving. As scientists and researchers conduct

normal science and work on solving these problems and puzzles, adding precision and scope to the existing foundational paradigm, the discovery of other problems forces additional paradigm shifts that leading to scientific revolutions, further maturing that particular field of study. Normal science plays a critical role in creating and strengthening the network of theoretical, conceptual, methodological and instrumental commitments that are derived from the particular foundational paradigm, ultimately leading to the next paradigm shift that revolutionizes the field yet again.

Furthermore, crises within scientific fields are the driving forces behind these revolutions and paradigm shifts. What this means is that as new observations are made by individuals, a variety of discrepancies occur that do not quite line up with the current theories and beliefs. This eventually builds towards a crisis that the current paradigm cannot answer appropriately, allowing for emergence of new ideas. In the aftermath of the crisis, the paradigm shift allows the new theory to establish itself, causing the rejection of the current paradigm. The acceptance of the new paradigm allows the revolution to play out and come to a conclusion (Kuhn 2015).

A good example of how crises are the driving forces behind paradigm shifts is Copernican astronomy. Prior to the advent of Copernican astronomy, its predecessor, the Ptolemaic system, was fairly accurate at predicting the movement of planets and stars. However, these predictions, particularly regarding planetary position and the procession of the equinoxes, never quite aligned with the available observations. These discrepancies eventually led Ptolemy's successors to identify problems within the theory. This recognition of the problem allowed the paradigm shift and the subsequent scientific revolution to occur. As the Ptolemaic system continued to break down and fail to provide solid explanations, the model that Copernicus suggested gained traction and favor amongst his contemporaries, eventually being adopted as the best explanation for astronomy and replacing the Ptolemaic system. The revolution completed its

cycle and established Copernican astronomy as the new “normal” science for the field of astronomy.

Now one might look at this as evidence that hard science fields can be revolutionized by such concepts but question what sort of benefit a field like anthropology can receive from employing such an approach. The answer to this question is a great deal. Anthropology, more specifically archaeology, has undergone various revolutions during its history as an academic discipline, and both fields have experienced paradigm shifts that have led to new understandings of the ancient world. One recent paradigm shift and revolution revolves around the use of ancient DNA (aDNA).

Archaeology has undergone at least three major revolutions or paradigm shifts (Kristiansen 2022). The first of these was the Darwinian Revolution, which introduced the principles of stratification, deep time, and evolution (Kristiansen 2022: 1). This revolution brought about several changes. First, it allowed archaeology to transition from a subfield to an independent discipline through collaboration with other fields such as biology and geology. Secondly, it gained new methodological principles, including excavation and classification techniques, from biology, zoology, and geology. These concepts were then further developed by Oscar Montelius, leading to classification of material culture and sites via a new methodological approach known as typology, which would become the dominant tools of the field. Finally, the concept of evolution helped to develop and push to the forefront new perceptions regarding the social evolution of human culture (Kristiansen 2022).

The next revolutions to impact archaeology were the environmental and radiocarbon revolutions, which gave archaeologists access to absolute dating of artifacts (Kristiansen 2022). This revolution occurred during the 1940s and 1950s and was comprised of two major

breakthroughs, modern pollen analysis and the rise of environmental archaeology, and the development of C^{14} absolute dating. These revolutions changed the dating of prehistory before written sources, which were previously necessary to date artifacts and events even though problems with such educated guesses were clear. Now, however, archaeologists could explicitly date artifacts and ancient materials in absolute rather than relative terms due to the principles of C^{14} decay rates. This allowed archaeology to leap forward and become what could be considered a modern science-based discipline (Kristiansen 2022: 7).

The third revolution occurred with the rise of strontium and aDNA analyses, which allowed archaeologists to gain a better understanding of population genomics and migrations of ancient peoples (Kristiansen 2022: 7) With the publication of the first full human genome as well as the development of short read sequencing technologies, this revolution began to ramp up in the 2010s and allowed prehistoric sequencing to occur, with the first prehistoric genome being published in 2010 by two teams of scholars, the Copenhagen team of Rasmussen et al. (2010) and the Max Planck team of Green et al. (2010). In addition, advances in strontium isotope analysis and dating plus the digitalization of archaeological data that drove this revolution led to the birth of archaeogenetics and Big Data, giving archaeologists and anthropologists a much greater understanding of prehistoric genetics, population dynamics, and migrations.

Finally, ORA is beginning to show signs of laying the foundations for the next paradigm shift and scientific revolution within the field of archaeology. With the introduction of ORA technologies and methodologies, archaeologists are beginning to gain a better understanding of the role of food and drink in different cultures, including the make-up of ancient diets, and the impact of various nutritional ailments. Whether or not ORA will usher in a paradigm shift will largely rely on its application and refinement by archaeologists, and more importantly, the

continued interdisciplinary collaboration between archaeologists and the laboratory sciences, with a specific focus on tackling new challenges and problems. By working together archaeologists and chemists can design experiments that target new molecules and biomarkers to further enhance ORA. This is why it is important to establish the appropriate protocols regarding experiment design and research planning, as well as how to appropriately interpret scientific data, to create a bridge of communication between archaeologists and anthropologists, and the chemists, biochemists, botanists, and biologists who may be involved in the interdisciplinary projects that have become more common due to these emerging technologies.

As one revolution leads to the establishment of a new dominant theory, there is always a counterrevolution that occurs that pushes back against it. This is also common in the field of archaeology. These counterrevolutions can be best described as "a discursive reaction from practitioners of humanities and cultural history against science-based interpretations, or rather about the role of science, which in their view should be supporting archaeology, rather than playing an equal, collaborative role—if it has a role at all" (Kristiansen 2022:7). The role of science has always been a contentious debate within the field of archaeology. The early post-processualists, for example, wanted to eliminate science and quantification because they believed that they dehumanized history (Kristiansen 2022; Shanks and Tilley 1987).

It is this author's opinion that science should take a more collaborative and equal role within the field of archaeology and anthropology for many reasons, but the main one is that it provides robust and powerful explanations, as outlined in David Deutsch's (2011) book, *The Beginning of Infinity*. In this book Deutsch argues that one of the roles of science is to provide the best explanation possible given all factors, variables, and our current understanding of that

particular scientific field. One note of particular importance regarding these explanations is that they must be testable.

> When a good explanation has been falsified by new observations, it is no longer a good explanation because the problem has expanded to include those observations. Thus, the standard scientific methodology of dropping theories when refuted by experiment is implied by the requirements for a good explanation. The best explanations are the ones that are most constrained by existing knowledge – including other good explanations as well as other knowledge of the phenomena to be explained. That is why testable explanations that have passed stringent tests become extremely good explanations, which is in turn why the maxim of testability promotes the growth of knowledge in science (Deutsch 2011: 25).

This is a good summary of how science works and it exemplifies why the application of scientific methodology can have beneficial effects for the field of archaeology. By seeking the best explanation based on currently known observations and continuously testing those explanations to ensure they are the most robust available, science helps to eliminate weak explanations, and ensure that the current explanation is supported by solid evidence. Therefore, as earlier explanations are discarded, scientific revolutions and paradigm shifts continue to push forward scientific fields. These sample principles can easily be applied to the field of anthropology, and more specifically archaeology, to ensure that the theories proposed produce the best explanations based on the available evidence. However, for these principles to be applied effectively, scientific methodologies must play a larger, more equal and collaborative role in archaeology and anthropology, as opposed to simply being used to support existing conclusions.

Furthermore, through the appropriate use of scientific instrumentation and methodology, science can help correct potential errors in observation in archaeological and anthropological data. Within the philosophy of science, and science in general, there is a great deal of debate regarding the perception of reality. Deutsch illustrates this in his description of an event that

occurred during his time in graduate school looking at negatives of the night sky to observe galaxies and stars:

> As I reached one particularly ambiguous image I asked my hosts, 'Is that a galaxy or a star?' 'Neither,' was the reply. 'That's just a defect in the photographic emulsion.' The drastic mental gear change made me laugh. My grandiose speculations about the deep meaning of what I was seeing had turned out to be, in regard to this particular object, about nothing at all… (Deutsch 2011: 35).

After having this revelation, Deutch continues by saying,

> "But wait. Was I ever looking at a galaxy? All the other blobs were in fact microscopic smudges of silver too. If I misclassified the *cause* of one of them, because it looked too like the others, why was that such a big error? Because an error in experimental science *is* a mistake about the cause of something. Like an accurate observation, it is a matter of theory" (Deutsch 2011: 36-37).

Many phenomena within the natural world cannot be observed by the human eye without some sort of aid due to variation in speed, the size of the event, or the location of the event. Take, for example, a video of a plant growing. If an individual were to sit and stare at the plant to make observations as to what happens during its lifecycle, one would be hard pressed to see all the minute details as the plant grows, as well as the various movements the plant makes as it orients itself towards a light source. However, with the use of time-lapse photography and video editing software one can see how the seed germinates and grows roots as well as how the main body of the plant emerges from the soil, develops leaves and completes its life cycle. Scientific instrumentation and scientific methodology allow humans to make accurate observations of reality and it is this concept that can be applied to the field of archaeology as it relates to the topic of this thesis, the study of the production of alcohol in ancient societies. Due to the nature of these observations, and the difficulty of seeing them unaided, scientific instrumentation and methodologies allow more accurate observations to be made minimizing the possibility of errors in our observations.

Scientific instrumentation, methodologies and technologies all bring us closer to observing reality, while simultaneously separating us further in a physical sense. To best explain this, let us again imagine we are looking at galaxies and stars in the night sky. If one were to go out on a dark night to observe these galaxies and individual stars, one might see several twinkling lights within the sky, but one could not determine which lights were stars as opposed to planets, galaxies, or long-dead stars whose light has yet to reach earth. By using a telescope, one might identify at least some of the celestial bodies within the night sky. The appropriate camera equipment along with the appropriate lenses and telescopes to create the plates seen under a microscope would reveal a subset of the various celestial bodies in the heavens.

To best understand how these concepts can be applied beneficially to ORA and the archaeology of ancient alcohol, it is helpful to consider an example such as the site of El Olivillo in Cadiz, Spain. El Olivillo was a Roman settlement located next to the Roman port of Gades from which Cadiz gets its name. One feature of the site is an artificial trash mound dating back to the Roman period and consisting mostly of amphorae. While some of the amphorae were long distance imports, most of the transport vessels found within this trash pile were local varieties. In addition to the amphorae, the remains of fish and murex shells were found at the site, leading archaeologists to believe that the amphorae here were used once and then discarded. ORA via gas chromatography mass spectrometry (GC-MS) was conducted on 24 pottery sherds from local ovoid amphora assumed to be made within the region to test this idea.

The results of the ORA revealed several key factors, but one of the more important ones was that three of the sherds sampled contained biomarkers for both wine and its derivative products and plant oil products. Since these materials would not have been stored together in the amphorae simultaneously, this indicates that the amphorae in the trash pile were reused for

different products prior to disposal as opposed to being single-use items. This example illustrates how observations can be deceptive. The archaeologists were deceived by their initial observations and operated on the assumption that the amphorae that were discarded were only used once prior to being disposed of. However, using ORA and the appropriate instrumentation to analyze the preserved residues, the archaeologists were able to revise their initial observations and determine that at least some of the amphorae were used more than once prior to being discarded. Scientific instrumentation and methodologies can help to bring archaeologists closer to determining how alcohol was produced, distributed, and consumed by ancient peoples, as well as how it impacted the development of civilizations and state-level societies.

I have demonstrated the importance of understanding how scientific revolutions occur, and the role of science in providing robust explanations for cultural phenomena as well as the collaborative role it can play within the fields of archaeology and anthropology. In the following chapters we will delve a little further into the scientific methodologies that have historically been used for organic residue analysis, how the technology functions, and what it can be used for. Chapter Two will focus on the scientific methodologies used in organic residue analysis and provide an in-depth explanation of how several ORA technologies are being applied in the field of archaeology. Chapter Three will focus on how to properly conduct experiments and research design, based on hard science concepts. To illustrate these concepts, two examples of archaeological research will be examined highlighting both good experimental design and potential pitfalls researchers can stumble into. Chapter Four presents eight case studies to further illustrate the importance of proper research and experiment design. Finally, Chapter Five provides a critique and discussion of all the information previously discussed and this author's thoughts on the future of organic residue analysis in archaeological research on ancient alcohol.

Chapter 2: Scientific Methodologies Used in Organic Residue Analysis

To begin this chapter, it is essential to define what is meant by both organic residues and organic residue analysis. “The term ‘organic residues’ is used widely in archaeology to describe a range of amorphous organic remains from diverse natural sources associated with a variety of artefacts found at archaeological sites” (Historic England 2017: 1; see also Barnard et al. 2007, Polla and Springer 2022). Due to their amorphous character, there are no discernible structures or morphological features that would allow archaeologists to characterize the substances adhering to various containers, unlike other biological materials that survive in the archaeological record such as bone, wood, plant matter, and a variety of other items. Initially, analyses focused mainly on organic residues in metal vessels because the copper salts and iron oxides in bronze and iron artifacts tend to preserve organic material much better than ceramics (Barnard and Eerkens. 2007; Historic England 2017; Polla and Springer 2022). However, as technology has advanced it has become possible to recover organic residues within ceramic pottery even in the absence of visible remains. This is important because pottery vessels are much more common and widespread than metal ones and provide a greater range of possible functions and contexts than metal wares (Barnard et al. 2007; Historic England 2017).

There are three primary ways that organic residues survive within pottery. The substance can be found in either a solidified or liquid state *in situ*, in the form of encrustations adhering to the interior and exterior of the ceramic vessel (like carbonized or calcified residues), or most commonly, preserved within the vessel’s wall in the form of an adsorbed material. As mentioned previously, amorphous residues do not have characteristics allowing visual identification of the material, so to identify the vessel contents, archaeologists must rely on appropriate chemical methodologies. These chemical analyses in turn rely on identifying biomolecules, more

commonly known as biomarkers (Reber 2021). Biomarkers consist of a wide variety of molecules including but not limited to lipids, nucleic acids, proteins, amino acids, and carbohydrates. These biomarkers have specific characteristics, including how they decay over time into different molecules, that allow archaeologists to identify the material once contained within or on the surface of the artifact. The chemical methodologies used to identify these archaeological biomarkers are known as organic residue analysis, or ORA (Polla and Springer 2022).

An important feature of ORA is that it is not a one size fits all approach. There are many different methodologies that can be applied with various degrees of success, each with its own potential advantages and drawbacks. These methods include Gas Chromatography Mass Spectrometry (GC-MS), Liquid Chromatography Mass Spectrometry (LC-MS), Fourier Transform Infrared Spectroscopy (FTIR), Proteomics, Electrophoresis, and others. The following sections will be dedicated to discussing a few of these methodologies and their function.

Gas Chromatography Mass Spectrometry (GC-MS)

Gas Chromatography Mass Spectrometry, commonly abbreviated as GC-MS, is a combination of two different analytical methods. The first component of this analytical method, gas chromatography, provides a chromatogram, the visible output of different compounds usually represented in the form of various peaks or bands of the separated compounds, while the mass spectrometry provides the characteristic mass spectrum of each identifiable compound (Karasek and Clement 1988). GC-MS is useful in the study of ancient alcohol because it can take a mixture of materials and identify each component within the mixture. In fact, it can analyze complex mixtures containing up to 300 unique compounds and identify each compound based on

its unique chromatogram and mass spectrum. Furthermore, compounds can be detected down to a miniscule amount such as 10^{-12} grams, more commonly called a picogram.

As mentioned previously, GC-MS consists of two components, gas chromatography and mass spectrometry. The former analyzes compounds based on two phases, the mobile phase and the stationary phase. During the mobile phase, an inert gas such as helium, hydrogen, or nitrogen is used in conjunction with a GC column and the sample as well as the gas is placed in a temperature-controlled sample injection device. This device injects the gas and sample into the analyzer at a given temperature to ensure vaporization of the samples. From here the inert gas acts as a carrier and moves the vaporized compound along the column into the stationary phase. The stationary phase involves a stationary plate that is covered with a liquid film. As the compounds move along the stationary phase their solubility in relation to the liquid film impacts how quickly they move through the stationary phase. This partitions or separates the compounds based on their solubility, which is known as their retention time. GC columns containing the stationary phase are broken down into two main groups, packed columns and wall coated open tubular columns, or WCOT. Each of these has benefits as well as weaknesses that can impact the work conducted. For example, packed columns are not as efficient as WCOT, nor do they have the separating capabilities of WCOT. However, they have a much higher capacity, which in turn allows for a simpler sample introduction by allowing a larger amount of sample to be put into the detector.

Wall-coated open tubular columns, or WCOT, tend to dominate the field of GC-MS due to their efficiency as well as their increased commercial availability compared to packed columns. Initially made of metal in 1956 when first introduced, it proved difficult to produce the appropriate liquid film coating on WCOT needed for the stationary phases; there were issues

with eliminating the active sites on the metal as well. This caused the initial columns to perform poorly. However, the development of fused silica tubes between 1975 and 1980 drastically changed the field of chromatography. These columns are made of pure silicon dioxide (SiO_2) and are extremely durable when an external coating of polyimide polymer is applied. This in turn allows for more effective application of the liquid film needed for the stationary phase. Furthermore, advances in WCOT columns now include both polar and nonpolar stationary phases, which in turn increases the efficiency of and analytical effectiveness of these columns making them the column of choice for GC in general. Figure 1.1 below shows the basic schematic of a GC analyzer (Karasek and Clement 1988).

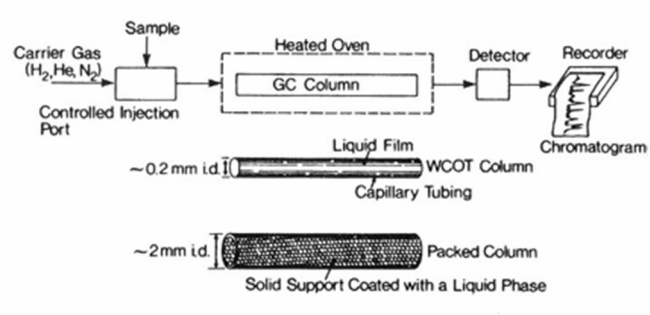

Figure 1.1: Schematic of a GC analyzer.
(Karasek and Clement 1988: 2.2).

The second part of this type of analysis is mass spectrometry. “When a molecule is ionized in a vacuum, a characteristic group of ions of different masses are formed. When these ions are separated, the plot of their relative abundance versus mass constitutes a mass spectrum” (Karasek and Clement 1988: 41). Mass spectrometry breaks down into two separate processes “ionization and the mass separation and recording of the ions formed” (Karasek and Clement 1988: 41).

Ionization can take various forms, including electrical ionization or chemical ionization. In electrical ionization, the ionization chamber is composed of several key components. There is an entrance for sample injection, a hot wire filament on one end, and an electrode at the opposite end of the chamber. Initially the chamber starts at a low pressure, roughly 10^{-8} Torr. Electrons produced from the hot wire filament are attracted to the electrode at the opposite end of the chamber. The sample is then introduced into the ionization chamber until the pressure of the chamber increases to roughly 10^{-5} Torr (Karasek and Clement 1988: 41). At this point, collisions between the electrons being produced by the hot wire filament and the injected sample cause fragmentation reactions to occur. This fragmentation creates positive ions that break down the bonds within the molecules of the sample creating ions that behave independently and do not collide with one another. From this point on the molecules of the sample continue to decompose through a series of reactions that continue to produce ions and other neutral products (Karasek and Clement 1988: 41). The relative abundance of the ions formed during these reactions creates the mass spectrum which can then be compared to published mass spectra of known substances in order to identify the unknown substance. One note of importance is that the temperature at which these reactions occur directly relates to the number of reactions that occur and therefore, this temperature must be noted and tracked carefully during analysis (Karasek and Clement 1988).

Chemical ionization is another method that can be used to complement electron ionization. During chemical ionization mass spectrometry (CIMS), a reagent gas is introduced to the ion source, and the chamber is maintained at high pressure. The reagent gas is ionized by the electrons which then interact with the sample molecules and inhibit direct ionization. The reactions that occur are ion-molecule reactions, which are low energy reactions, causing simpler

fragmentation patterns as well as more abundant molecular ions. These are then the components that create the mass spectrum, which can be compared to published mass spectra in order to identify the compound (Karasek and Clement 1988: 43).

Given the differences between the various methods of ionization, do they produce different results? The answer to this is yes. Electron ionization generates information regarding the structure of the molecules, as well as relative abundance, whereas chemical ionization helps to determine the molecular weight of the sample molecules. Furthermore, certain compounds and molecules, such as alcohols, straight chain alkanes, and alkenes do not produce stable molecular ions, and have such a small relative abundance that they cannot be detected or identified using electron ionization (Karasek and Clement 1988: 43). Therefore, it is imperative to choose the appropriate method of ionization depending on the types of compounds being sought. GC-MS is not the only choice for ORA using chromatography in tandem with mass spectrometry, however. There is also Liquid Chromatography Mass Spectrometry, more commonly referred to as LC-MS. Figure 1.2 below shows the schematic for a GC-MS analyzer.

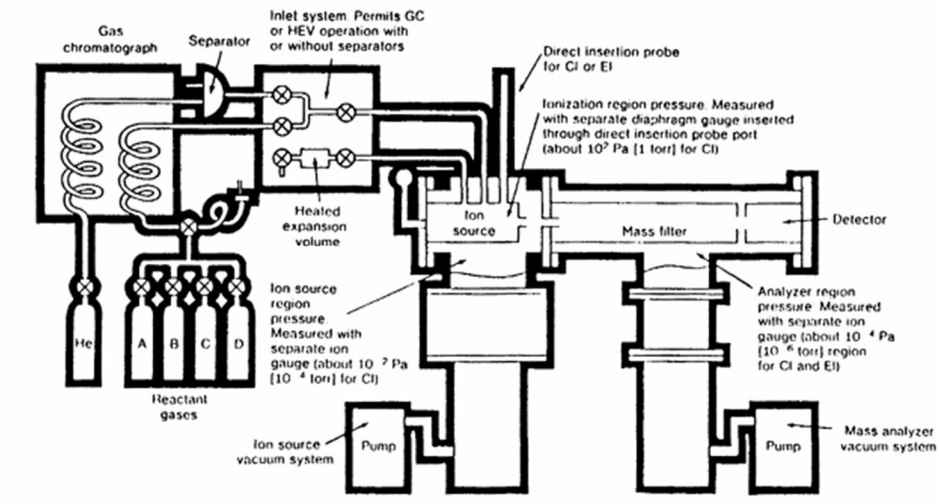

Figure 1.2: GC-MS Schematic (Karasek and Clement 1988: 4.43).

GC-MS can be particularly useful for the detection of fatty acids of various foodstuffs in the form of methyl ester derivatives (Malainey 2007) While these molecules cannot be absolutely identified, these fatty acids can be screened and categorized rapidly (Malainey 2007).

Liquid Chromatography Mass Spectrometry

Liquid chromatography mass spectrometry, or LC-MS, is another form of chromatography working in tandem with mass spectrometry to identify unknown compounds including those found in ancient alcoholic beverages. While the mass spectrometry component functions like it would within GC-MS, the liquid chromatography functions in a slightly different manner to achieve similar results to that of gas chromatography.

Ionization can be achieved in three main ways. The first source is known as electrospray ionization. In this method, a liquid sample is pumped through a metal capillary tube. This tube is maintained at a voltage of 3-5 kV, or kilovolts. It is then nebulized at the tip of the capillary system to create "a fine spray of charged droplets" (Pitt 2009:20). These droplets are then rapidly evaporated using heat and nitrogen gas, causing the residual charge of the droplets to be directly transferred to the analyte in question. These ionized analytes are then transferred to the mass spectrometer for analysis. To minimize contamination, the capillary is usually either off set or positioned at a right angle from the mass spectrometer intake.

ESI, much like chemical ionization, is considered a soft ionization technique, meaning that there is little energy transferred to the analytes, reducing the amount of fragmentation that occurs. Due to this, ESI can be useful for detecting metabolites, peptides, and xenobiotics.

The second source of ionization comes from atmospheric pressure chemical ionization, or APCI. As in ESI, APCI pumps a liquid sample through a capillary which is then nebulized at the

capillary tip. The difference between the two is that a corona discharge is created near the tip. This in turn ionizes the gas, as well as the solvent molecules. These ions then interact with the analytes and transfer the charge to the analyte. APCI is particularly useful for small molecules that are thermally stable such as steroids, lipids, and fat-soluble vitamins (Pitt 2009: 20).

Finally, atmospheric pressure photon ionization, or APPI, uses photons, particles of light, to excite and ionize particles after nebulization. Using photons minimizes concurrent ionization of solvents and the ion source gases, as well as creating singly charged ions, making it particularly useful for detecting neutral compounds such as steroids (Pitt 2009). Figure 2shows a basic schematic for LC-MS (Crawford Scientific Chromacademy MassSpectrometry Fundamental LC-MS Introduction 2004).

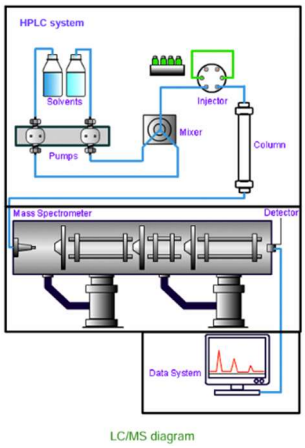

Figure 1.3: LC/MS Diagram (Diagram taken from Crawford Scientific Chromacademy MassSpectrometry Fundamental LC-MS Introduction)

Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy, commonly abbreviated as FTIR, varies slightly from the previous methods mentioned. To begin, FTIR infrared radiation, or IR radiation is passed through a sample. Some of that radiation is absorbed by the sample whereas the rest of it passes through it or is transmitted (Thomas et al. 2017). This creates a spectrum that represents

the molecular absorbance and transmission of radiation. This in turn acts like a fingerprint for each material as no two materials will have an identical transmission and absorbance spectrum. This allows FTIR to provide positive identifications of different materials. FTIR is generally preferred over other methods of IR spectroscopy because it is nondestructive, it provides measurement without the need for external calibration, its rapid and sensitive, has a greater throughput than comparable approaches, and is a mechanically simple instrument, allowing for a greater number of individuals to be able to use it (Thomas et al. 2017: 75).

FTIR, as mentioned previously, is a simple analyzer that is composed of three components: a source of radiation, an interferometer, and a detector. A source of radiation is self-explanatory, as is the role of the detector, but the interferometer's role needs explanation. The interferometer takes the IR beam coming from the source of radiation and separates it into two optical beams using a beam splitter (Thomas et al. 2017: 73). The beam splitter consists of a flat mirror that is fixed in place and a second mirror that is allowed to move a very small distance, usually only a few millimeters, away from the beam splitter. This in turn creates a signal, caused by the two beams interfering with each other that exit the interferometer, known as an interferogram. These IR beams then pass through the sample and are absorbed or transmitted. This information is then analyzed by the detector, which is specifically designed to measure the interferogram signal. Finally, all this information is digitized via an attached computer, and the IR intensity versus frequency spectrum is presented to the user for interpretation (Thomas et al. 2017). Figure 1.4 below shows an FT-IR analyzer schematic (Thomas et al. 2017: 73).

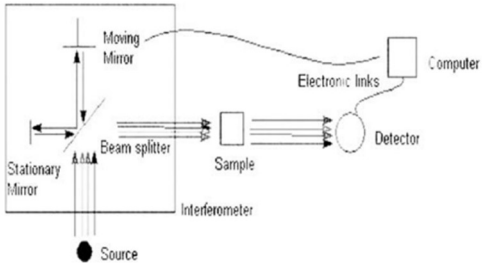

Figure 1.4: FT-IR Analyzer Schematic (Thomas et al. 2017: 73)

Data produced by FTIR can tell researchers two main things. The first is that it can help to determine the structure of the molecule, and it can be used to identify different compounds within a sample. The structure of the molecule can be determined because functional groups within complex molecules have very specific frequencies of IR that they can absorb, regardless of how they interact with other parts of the molecule. These absorbances can then be used to determine the position of functional groups and in turn the structure of the molecule. Components of a sample can be identified because each molecule has a unique spectrum. These spectra can then be compared with known reference spectra to identify unknown compounds (Thomas et al. 2017). Finally, FT-IR can be used as an appropriate screening method due to its speed and lack of complexity. This can allow ceramics from an archaeological site to be screened to determine which may be best for further analysis via GC-MS, LC-MS, or some other form of ORA, helping to streamline the process (Shillito et al. 2009). It is important to remember that these results are qualitative as opposed to quantitative. Qualitative results can allow for researchers to have a better understanding of underlying principles and can create insights into the problem and help develop hypothesis for testing, whereas quantitative results produce hard data that can be quantified using statistics. Qualitative results should be used in conjunction with other quantitative data when possible, to further support the conclusions made during interpretation.

Proteomics

Proteomics is an analytical methodology that focuses on "the systematic study of the many and diverse properties of proteins in a parallel manner with the aim of providing detailed descriptions of the structure, function and control of biological systems" (Patterson and Aebersold 2003: 311). Proteomics initially arose from the science of protein chemistry, which

sought to take observed and/or functional phenotypes and move towards understanding how the genes that cause the phenotypical products function. As this science expanded a key factor was the development of more sensitive and reliable methods for protein sequencing. This, in conjunction with gene sequencing technologies, led to the development of what is now known as proteomics, a conjunction of protein sciences and sequencing and the analysis of the genetic material encoding for the proteins and their functions. Furthermore, proteomics is not one specific type of analytical methodology, but a collection of methodologies such as electrophoresis, complex mixture analyses like GC-MS or LC-MS/MS, ICAT, ELISA, etc. (Aslam et al. 2017).

The basic structure of proteomics breaks down into five main stages: sampling, pre-fractionation, separation, sample processing, and interpretation of bioinformatics. Sampling is self-explanatory and after sampling, pre-fractionation breaks down, or fractionates, the biological samples, usually through a method such as some form of chromatography. Afterwards these fractions are separated based on weight, charge, or both through methodologies such as 2D electrophoresis, SDS-PAGE Electrophoresis, or other forms of multidimensional protein identification technology. Finally, these data are analyzed and integrated into bioinformatic databanks for others to use (Cho 2007). Proteomics can be used to determine a wide variety of useful information, however there are unique challenges posed during proteomic analysis. To begin, proteins are more difficult to work with than DNA as they can have both secondary and tertiary structures and these structures must be maintained during the analysis in order to be able to accurately identify the particular protein. Furthermore, the conformation of the structure can be damaged, commonly known as being denatured, by factors such as enzymes, a special category of proteins, heat, light, or even by aggressive physical actions and other post-

translational events (Cho 2007). Furthermore, unlike DNA, proteins cannot be amplified, meaning that there is no way to increase the amount of available protein in the sampled residue. Therefore, proteins that are found in less abundant concentrations can be more difficult to detect, making the choice of analytical methodology ever more important. Finally, some proteins have a low solubility, making them more difficult to analyze (Cho 2007).

The limitations of proteomic analysis were illustrated in a study by Barker et al. (2018), who conducted an experiment artificially aging ceramic bound proteins over a twelve-month period. To do this, researchers took unglazed bisqueware and baked them to 800 degrees Celsius to remove potential sources of contamination. Next, five purified protein powders were mixed with 600mL of MQ water (purified water using the Milli-Q^{TM} water purifier). This mixture was then placed in the ceramics and baked at 95 degrees Celsius for 5 days, before the ceramic was rinsed with MQ water, quartered, and buried ten centimeters deep in the sandy loams of Denton, Texas (Barker et al. 2018). Samples were then recovered after one month, six months, and twelve months, and were treated as if they were real samples, meaning they were excavated, minimally handled, not washed, and wrapped in foil for transport. These samples were analyzed via TOC or total organic carbon analysis, GC-MS analysis for fatty acid compounds, with reference specimens wrapped in foil and stored and -80 degrees Celsius for simultaneous LC-MS analysis at the end of the experiment (Barker et al. 2018).

These experimental samples were also compared to gray and white ceramics found at an archaeological site in southern Colorado and blank ceramic samples were used as controls. TOC results of the archaeological samples produced low and variable results between 0.25 and 2.6 μg of carbon per 1 mg of ceramic sample. This variability between ceramic samples was attributed to the original organic material that the ceramic held. The artificially aged sample showed a

dramatic decline in detectable carbon in the TOC analysis with the one-month sample having 1.0 μg/mg, with that value dropping quickly between the ages of one and six months with a value around 0.6 μg/mg. However, after six months the carbon decay reached some sort of equilibrium, and the value did not change much between six months and twelve months (Barker et al. 2018). GC-MS analysis of all samples showed an increase fatty acid content when compared to the ceramic blanks but only seven of the samples met the criteria established for a positive match (the requirements being all targeted fatty acids were recovered and the C18:0 values were ten times greater than what was seen in the ceramic blanks). LC-MS analysis of the samples produced positive protein matches for only four of the seventeen archaeological sherds tested. The researchers, however, disregarded all four positive results for the following reasons. The first positive result was linked to *Citrobacter koseri*, a bacteria found within animal digestive tracks, but due to the low values recovered the researchers concluded that these results were archaeologically meaningless. The second sample had a positive match to porcine trypsin, which is a digestive enzyme, but when the sample was rerun to confirm the positive match, the result came back negative. The final two samples were rejected upon closer inspection of the positive matches as they were more closely associated with non-food bacterial sources. For the experimental sherds, out of the five proteins injected into the ceramics, only two of the proteins were consistently recovered.

The researchers identified several possible reasons for why they were unable to detect all five protein compounds, including lower spike rates or lower binding activity of the protein compounds, variations in how susceptible protein compounds are to degradation, causing some to degrade faster than others, post-translational modifications of the various protein molecules, and finally potential limitations of the methods and instrumentation used (Barker et al. 2018).

Ultimately, this experiment helps to show the limitations of proteomics. As the researchers concluded: "Our results, derived from several lines of evidence, suggest that under typical, sub-optimal conditions for preservation—and when non-targeted approaches are employed—negative results for ceramic bound protein residues should currently be the norm rather than the exception" (Barker et al. 2018: 41). While these researchers focused on targeting protein molecules for recovery, and the targeted biomarkers for alcohol production can be significantly different, it is still imperative to remember that just like proteins, alcohol biomarkers have limitations, degrade at various rates, and can interact with other environmental compounds making detection difficult. This research demonstrates the importance of employing a targeted methodology, robust research, and experiment design, which will be discussed later in this chapter. While there are limitations to all methods of proteomics, electrophoresis—a method capable of detecting DNA, RNA, and proteins—has been widely used in ORA of alcohol.

Electrophoresis

Another method used in ORA is electrophoresis, a broad overarching term for several different analytical methodologies that employ the same basic principles. Most biomolecules have some sort of positive or negative charge (Magdeldin 2012). These charged molecules, when placed in an electric field, will migrate, or move, towards the electrode with the opposing charge. However, due to the size, structure, and other qualities of these molecules, they travel towards the electrode at different rates of speed. This in turn separates the biomolecules and allows them to be identified. Electrophoresis employs a gel medium that acts as a matrix to aid in the separation of these molecules. These molecules can be separated based on charge, molecular weight, or both. The gel plates contain sample wells for the prepared sample, which is mixed with a loading buffer that allows it to fall into the wells of the gel plate (Magdeldin 2012). The

gel is then placed in the electrophoresis machine with a running buffer, which helps to create the electric field and conduct electrical currents from one end of the device to the other. Once the samples have been incubated and allowed to migrate for an appropriate amount of time, the gel is removed from the electrophoresis chamber, and stained. This allows the various sized biomolecules to become visible within the gel matrix and compared to the reference standard to determine their molecular weight, and/or charge of the biomolecule, for identification (Magdeldin 2012). Figure 4 shows a completed SDS-PAGE gel, used to identify proteins. Notice how the first and last lanes (columns) of the gel are identical. These are the standard ladders, molecules of a known molecular weight, that are used to determine the molecular weights of the unknown protein compounds.

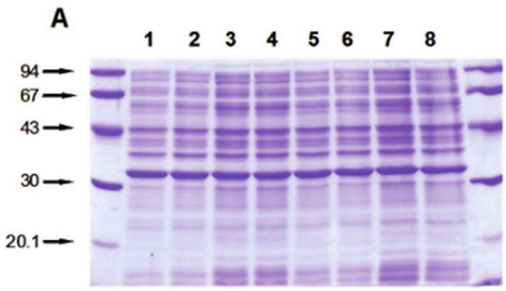

Figure 1.5: A completed SDS-PAGE gel, showing an unknown compound and the Standard Ladders used to identify molecular weight. (Magdeldin 2012: 1)

Electrophoresis is particularly useful in identifying proteins as well as DNA and RNA. While this may seem not as useful for detecting biomarkers for alcohol, electrophoresis can be useful as a screening tool, allowing archaeologists to identify proteins produced by particular plant and animal matter, as well as in the identification of genetic material from plant remains or microbial remains such as yeast. This, in conjunction with other analytical methodologies, as well as other evidence directly from the archaeological site, can help to determine the source of the organic

residue as well as help to guide further research on archaeological sites and artifacts (Magdeldin 2012).

Enzyme Linked Immunosorbent Assay (ELISA)

Finally, one other proteomic methodology to touch on is Enzyme-Linked Immunosorbent Assay, more commonly known as ELISA. Most individuals are familiar with ELISAs without actually knowing that it is an ELISA. Over-the-counter pregnancy tests are ELISAs. Initially developed for medical testing, ELISA has been applied to detect mineral-bound proteins. The general structure of an ELISA is as follows: A surface is coated with either an antigen, or some other form of bound protein. A sample is then incubated with these bound proteins and if the appropriate antibody, or other protein that is complementary to the bound protein is present, it will interact and bind to the antigen. The next incubation includes a secondary antibody that will bind to the target protein. Finally, an enzyme linked substrate is incubated with the secondary antibody and bound proteins. If the bound proteins are present, then the enzyme will metabolize the substrate, causing a color change whose optical density can be measured for a quantitative result (Craig and Collins 2000).

In an archaeological setting ELISA has been used to increase the amount of extracted amino acids in mineral-bound proteins specifically through the use of hydrofluoric acid when applied to ceramics. Furthermore, the use of hydrofluoric acid does not damage the epitopes needed for antibody recognition, allowing these proteins to be detected by ELISA (Craig and Collins 2000). Furthermore, ELISA testing using hydrofluoric acid can also prove beneficial to environmentally exposed samples, which can damage and alter the proteins, making them more difficult to analyze. Extracting using hydrofluoric acid increase the chances of recovery (Craig and Collins 2002).

These are just a few different methodologies available within the field of ORA. There are a great many other methodologies that can be applied to identify organic residues, each with potentials and pitfalls (Table 2.1). This is why it is important for archaeologists to know and understand how technology works as well as where potential pitfalls could occur. However, technological pitfalls are not the only potential problems facing archaeologists studying ancient alcohol using ORA. Poor research and experimental design can be just as problematic as not having a proper understanding of the technology being used for the analysis, however prior to this, a brief history of ORA and its application in the field of archaeology is warranted.

Table 2.1: ORA Methods: Key Points, Advantages and Disadvantages

Methodology	Key Points	Advantages	Disadvantages
GC-MS GC-MS/MS	Combination of two analytical methodologies gas chromatography (GC) and mass spectrometry (MS), GC contains a mobile and stationary phase, compounds separated by retention time.	Produces quantitative results, can detect compounds to a minute volume, can analyze and identify mixtures with up to 300 compounds Packed columns allow for more sample and are easier to use, WCOT excellent as separating compound.	Analytes must be volatile (extremely reactive), destructive analysis Packed columns not as good at detection and separation as WCOT, substance must be converted to a gas for analysis.
LC-MS HPLC-MS LC-MS/MS	Also composed of two analytical methodologies liquid chromatography, (LC) and mass spectrometry, Multiple methods of ionization including ESI, APCI and APPI.	Produces quantitative results, can identify complex compounds that cannot be analyzed by GC-MS, can be used to detect a wide variety of molecules including neutral and low polarity molecules, lipids, fat soluble vitamins, and steroids.	Destructive analysis, uses a liquid source, ionization methods have impacts on source material.
FT-IR	Three components: Source of radiation, interferometer, detector sample is bombarded with IR. IR is absorbed and passes through the sample. The interferometer beam splitter creates a second signal that interferes with the first, detector interprets this information.	Easy to use, produces results rapidly, can sample solids, liquids and gasses, high sensitivity with low noise compared to other forms of spectroscopy, user does not need to calibrate, good screening tool for determining structure and functional groups of the analyte, nondestructive.	Produces qualitative rather than quantitative results, uses a single beam as opposed to double beams of other dispersive instruments.
Electrophoresis	Sample is placed into a well of a gel medium. Electrical current is run through the gel and buffer. Current allows the samples to migrate towards the opposite polarity. Molecular weight separates out the compounds due to how fast they can travel through the gel.	Can identify DNA, RNA and proteins, useful as a screening tool.	Cannot identify direct biomarkers for alcohol, needs to be monitored to ensure sample does not run off the gel medium.
ELISA	Capture protein binds to antigen in sample, secondary antibody binds to appropriate antigen, enzyme-linked substrate binds to antibody, and produces a color change.	Produces quantitative results, can be used when proteins are exposed to acid or degraded due to environmental factors.	Can only be used to detect proteins. Can only detect a single protein per well. Human error can cause failure of results.

A Brief History of Organic Residue Analysis in Archaeology

Organic residue analysis began to emerge as a disciplinary field in the mid-20th century with the development of new analytical chemical methodologies such as infrared spectroscopy, nuclear magnetic resonance, and Raman spectroscopies (Evershed 2008). As these technologies evolved, so did the discipline of ORA, which fully matured during the 1970s with the publication of Thornton et al. (1970), who investigated "bog butters" via gas chromatography. From there many papers have been written focusing on ORA and delving further into what has become known as the archaeological biomarker concept, which focuses on human activity and can be used with any type of biomolecule, such as DNA, RNA, proteins, etc. One important feature of the archaeological biomarker concept is that it requires that the analytical techniques that are employed achieve a molecular level resolution. Since compounds are often found as amorphous mixtures with no discernible identifiers, the analytical techniques need to be able to discern and separate each compound found within the mixture at the molecular level.

ORA has been applied historically in archaeological excavations in several different ways. Archaeologists have used amino acid analysis, specifically amino acid racemization (AAR) dating, to investigate the biological origin of artifacts and other objects. By looking at the different ratios of amino acids from different biological sources, it is possible to determine the taxonomic origin of the biological material. This is because different taxa can have different compositions of amino acids. An example of this method's usefulness is seen in a study conducted by Demarchi et al. (2014), who examined the amino acid composition of shell beads found in a funerary context at the site of Great Cornard, Suffolk. The amino acid analysis indicated that the shell beads came from either *Nucella* or *Anatalis*, which are two shellfish

species found locally at the site, indicating that these beads were produced locally, as opposed to being imported from another region (Hendy 2021).

Some of the earliest studies involving ORA, and more specifically paleoproteomics, involved protein survival and degradation. These studies helped to provide insights into how biomarkers degrade over time, such as proteins undergoing a process known as diagenesis, where longer peptide chains break down into smaller and smaller peptide fragments (Hendy 2021). These smaller fragments can in turn cause issues with analysis, as seen in the Barker et al. (2018) experiment above.

One of the first uses of GC-MS as an analytical method in archaeology was a study by Condamin et al. (1976), who analyzed Roman amphorae for the presence of biomarkers of olive oil to see how this commodity was stored. Another example was an analysis of fatty acid ratios from pottery sherds in the western Great Basin of North America, where researchers applied GC-MS and discovered that all but seven of the sherds had biomarkers for lipids. More interestingly, the researchers noted that the highest percentage of sherds (roughly 35%) came from vessels used only for seeds, whereas most of the remaining sherds had lipids from a combination of seeds, roots, meat, and greens. Finally, sherds with biomarkers for only plants or only meat made up the smallest composition. This indicated to the researchers that the residues were the remains of food processed within these ceramics, and while the ethnographic studies of the region indicated that rabbit was the most common meat source exploited, the biomarkers identified via ORA indicated that ruminants like antelope, deer, and sheep were more common (Eerkens 2005).

One of the earlier applications of proteomics involved analysis of Iñupiat ceramics from Point Barrow, Alaska, dating to 1200-1400 CE. Potsherds from a vessel assumed to be used for cooking were subjected to protein extraction and enzymatic hydrolysis and the contents

identified using protein databases. In addition to these ceramic samples, muscle and blubber samples from harbor, gray, and ringed seals, as well as beluga whale, were gathered and used for confirmation of the archaeological results after being subjected to the same ORA methodologies. The potsherds yielded four myoglobin peptides associated with harbor and gray seals. Furthermore, one of the four peptides identified specific terminal sequences that are only associated with pinnipeds (seals and sea lions) and were confirmed by the peptide sequencing of the modern-day muscle and blubber samples. One of the other four contained a peptide sequence associated with both pinnipeds and cetaceans (whales and dolphins). This was the first study to identify proteins in an archaeological context via peptide sequencing (Solazzo et al. 2008).

Additionally, early experiments looking at thermal and oxidative degradation of fatty acids indicated that human activities such as cooking changed the composition of medium and long chain saturated fatty acids as well as reducing the number of polyunsaturated fatty acid chains through peroxidation (Malainey et al. 1996). However, these experiments also showed that differences in fatty acid composition of degraded residues can be used to distinguish the original contents of a particular vessel Malainey et al. 1996).

Other examples of the application of ORA in archaeological samples include a study from the Iberian Peninsula. Breu et al. (2021) used ORA to identify lipid compounds and dairy proteins found in Neolithic Cardial and Epicardial ceramics of the Iberian Peninsula (Breu et al. 2021). Similarly, Oudemans (2007) applied ORA to ceramic vessels from a variety of contexts as a method to determine the actual function of the vessels versus the intended function or interpreted function by archaeologist. Furthermore, by conducting this analysis Oudemans was able to gain insight into diets and changes in subsistence practices of the vessel producers (Oudemans 2007).

Additional examples include studies by Drieu et al. (2020), who used ORA to detect the presence of malvidin, the main anthrocyanin released from the skins and seeds of grapes that gives wine its red color, in conjunction with the presence of tartaric acid to identify the presence of wine in ceramic vessels (Drieu et al. 2020). Estriecher (2013) conducted ORA at the Roman site of Los Palacios in Madrid, Spain. Structures found at the site indicated that they may have been used to process grapes for wine, so ORA was conducted on what was believed to be a *lacus* or winepress. The analysis indicated the presence of tartaric acid and other biomarkers for grapes, leading the researchers to conclude that wine was indeed produced and stored at this site (Estreicher 2013). Estreicher (2013) also studied the Roman site of Carranque located in Spain, another prime example of ORA being used to identify the production of alcohol. The poor preservation of the site left very few clues for researchers indicating the presence or production of alcohol, however structures found at the site again indicated the potential for the production of wine. ORA was conducted on a set of double vats, as well as the floor of the site. The floor of the site indicated the presence of lipids from animal fats mixed with the substance used for plaster indicating that the floor had been waterproofed. ORA of the vats in conjunction with carbonized *Vitis domestica* pips and the waterproofed floor all indicated that this site was used to produce wine (Estreicher 2013). In another example, Beeston et al. (2006) focused on the site of Chrysokamino in Greece where researchers analyzed pottery sherds associated with Early Minoan III and Minoan Ia sites. ORA indicated biomarkers for wine, in addition to several other plants like coriander, as well as citrus. Researchers concluded that the plant additives were potentially used to created flavored herbal wines. They also noted that the evidence for citrus plants was likely due to contamination as citrus plants were not introduced to the area until the Middle Ages (Beeston et al. 2006).

Finally, Patrick McGovern has published several books for a general audience that outline the history of ORA as applied to the study of ancient alcohol, including *Uncorking the Past: The Quest for Wine, Beer, and Other Alcoholic Beverages* (2009), *Ancient Brews: Rediscovered and Recreated* (2017), and *Ancient Wine: The Search for the Origins of Viniculture* (2003). These volumes provide an overview of a wide range of ORA studies on the production, storage and consumption of alcohol conducted by McGovern and other biochemists from a wide variety of time periods and contexts, including China, Northern Europe, Italy, and the Near East.

These are but a few examples of past archaeological studies using ORA. Early research primarily focused on protein discovery, as well as lipid biomarkers, as those have the best chance of survival due to their hydrophobic nature. However, it does help to illustrate the necessity of proper experiment and research design. The next chapter of this thesis will discuss the importance of experiment and research design by means of several examples of well-designed alcohol research studies. A poorly designed project will be presented for comparison.

Chapter 3: Experiment and Research Design

Now that I have explored the concepts of scientific revolutions as well as the various methodologies employed for organic residue analysis, in this chapter I will consider the concepts surrounding experiment design. While the field of archaeology may not run into the same types of problems that other scientific fields have to contend with, because archaeologists explore the world of material culture through the lens of scientific technology and analyses, the potential for problems remains the same as in other fields that apply the scientific method. Therefore, it is imperative that archaeologists have a good understanding of the foundations of appropriate experiment and research design conducted by other scientific disciplines.

Scientists, like archaeologists, attempt to answer research questions, and while they may be focused on answering questions about the natural world using laboratory science techniques, archaeologists ask questions related to human history and culture. Although the nature of these questions might have different components, the core concepts, structures, and methods remain the same. There are four core concepts in scientific experiment design: experiments must (1) be empirical, (2) gather measurements, (3) be replicable by others, and (4) answer the research question(s) in an objective way (Field and Hole 2014: 54). The question at issue for us is how these concepts can be applied to the archaeological study of ancient alcohol, or if it is even possible to apply them to the questions that archaeologists wish to answer. However, as one delves further into experimental design, it is clear that these concepts not only can but should be applied to archaeology, especially in the case of ORA. The discussion that follows will break down the core concepts of experimental design and how they can be applied to ORA as it pertains to the study of ancient alcohol in archaeological contexts. When one considers organic residue analyses, it is easy to see how the core structure of experiment design can be applied to

its use in archaeology. When archaeologists encounter an unknown residue contained within a vessel, they take note of the vessel's context, its type, and whether it is ceramic or metal; they may also measure any visible residue, including how much area the residue covers in the vessel as well as its depth. When the residue is sampled, the amount taken is also measured and documented. Finally, the type of analysis conducted, instruments and analyzers used, as well as the specific parameters regarding sampling and analyzing the unknown substance are all documented. By doing this, archaeologists are being objective and gathering measurements that would allow other teams of researchers to replicate the analysis; they are therefore following the procedures of empirical investigation. Therefore, since these concepts are already being employed it is important to examine what constitutes appropriate experiment design.

"No matter how ingenious or important an idea for an experiment might be, if the study is badly designed, it's worthless" (Field and Hole 2014: 54). This statement holds true not only for the hard sciences, but also for archaeologists studying ancient alcohol through organic residue analysis and must always be kept in the back of the researcher's mind. The question before us is what constitutes a well-designed versus a poorly designed study. Well-designed research studies focus on three aims: (1) reliability, (2) validity, and (3) generalizable findings (i.e., the results of the experiment have a wider application than just that of the initial experiment) (Field and Hole 2014). The reliability of an experiment is closely tied to the accuracy and precision of the recorded measurements. This makes it possible for subsequent studies that might attempt to replicate the findings to achieve similar results by having an objective definition of what is being measured. For instance, when conducting organic residue analysis, one might state that in order to determine whether a particular compound is present in a sample it must meet a specific percentage of relative abundance. This objective definition allows archaeologists to say with

certainty that because these compounds are present in high enough quantities, they can be considered a product of the unknown residue as opposed to some other environmental factor. Furthermore, such an approach establishes cutoffs and criteria for replication (Field and Hole 2014). Establishing such cutoffs for each biomarker is challenging and requires appropriate experimentation. For example, establishing an appropriate cutoff for tartaric acid to indicate the presence of grapes would need to consider certain factors such as regional plant populations. Areas such as Europe and the Near East will have a different cutoff as grapes are some of the only plants that could produce tartaric acid within these regions, while other areas such as China not only have varietals of grapes growing within the region but other plants such as hawthorn whose fruit also produces tartaric acid. Furthermore, microbial activity in different soil compositions also impacts how much of an appropriate biomarker remains for sampling. Therefore, robust experimentation to establish and standardize cutoffs is warranted.

The next aim of a well-designed study is validity, specifically regarding what is being measured. In the hard sciences, when conducting an experiment, one manipulates a singular variable to see how that variable impacts the results. When the manipulation is the only factor that impacts the results, the experiment is considered valid. However, if other factors impact the results aside from the manipulated variable, the experiment lacks what is known as internal validity. While many of the threats to internal validity may not apply to ORA in archaeology, there is one major threat to internal validity that archaeologists must be vigilant about, and that is changes in the instruments used in the analysis.

Organic residue analysis relies on analyzers to reach conclusions. These instruments have many components and parts that can be very sensitive to change, which can lead to invalid results. To help identify and combat potential errors or changes within the instrumentation, there

are a few tactics that can be employed. The first is to ensure that all instruments and reagents are appropriately calibrated prior to analysis. Calibrating the instrument prior to analysis demonstrates that the instrument can determine the appropriate values as well as alerting researchers prior to analysis if there is a potential problem within the system. The next precaution archaeologists can employ during their analysis is to run appropriate positive and negative controls. The role of positive and negative controls is to show that the instrument can detect the substances it is intended to detect. Furthermore, it also allows researchers to determine whether an issue or some other error occurred during the preparation or analysis process. For instance, if the positive control does not yield a positive reaction, this could indicate that the instrument's calibration has shifted. It could also potentially alert the researcher to an issue with the preparation of specimens. The same would hold true for the negative control. If the negative control gives a positive reaction, researchers may wish to recalibrate the instrument to see if that is the cause of the issue or investigate to see if there is a potential source of contamination that could impact the results of the unknown residue. Therefore, it is important to ensure that all instrumentation is properly calibrated and working appropriately prior to conducting an analysis, as well as running appropriate controls to determine if an error has occurred during analysis.

Finally, the results of an experiment should be generalizable, meaning that the results gathered from one experiment should have a wider application than just the specific experiment conducted. Being able to apply the results from one experiment to another allows for a better understanding of the world. Within the field of archaeology and the production of ancient alcohol, as archaeologists gather additional information on biomarkers indicative of a wide variety of alcoholic beverages, past studies can be referenced in future studies. This will further

help to lend validity and reliability to future studies, as well as develop an extensive library of biomarkers relevant to the production of ancient alcoholic beverages.

A hypothetical example may be used to highlight possible issues that could occur along the way (see Table 3.1). In this example, archaeologists discover a buried vessel, which upon excavation exhibits evidence of a residue. Archaeologists wish to analyze this residue to determine what was stored within it. What possible pitfalls might arise? To begin, when the vessel is discovered at the site, soil samples should be taken from the area surrounding the vessel, as well as from within it. These can then be analyzed later for evidence of environmental contamination. Secondly, gloves, specifically cotton gloves, should always be worn when handling the vessel, as well as collecting environmental samples (Perruchini et al. 2018: 182). This will prevent contamination by material on an individual's hands, such as skin cells, food remains, oils, or microbes from interacting with the samples. Finally, storage and transport of the sample should be done in an appropriate sterile vessel. This again prevents microbial contamination of the vessel, and the residue within it. Additionally, the type of material used to store and transport the artifact could leach into the artifact due to being exposed to environmental factors such as heat. Temperature should also be controlled during transport. For example, when archaeological samples are being transported, they should be wrapped in aluminum foil that has been heated to 450°C for at least eight hours to eliminate microbial and other contamination (Perruchini et al. 2018;182). Once the sample reaches the laboratory, issues can arise from improperly calibrated instrumentation, cross contamination of specimens due to a lack of sterility protocols, as well as improper sampling of the residue. This is presented in condensed form in Table 3.1 below.

Table 3.1: ORA Sampling Pitfalls and Solutions

Location:	During Excavation	Storage and Transportation	Laboratory Analysis
Potential Problems:	Environmental contamination, human contamination	Chemical leaching, microbial contamination	False positive/false negative results, cross-contamination
Preventative Actions:	Take soil samples from interior/exterior of vessel, wear gloves when handling samples.	Wrap/store artifact in appropriate material based on temperature/humidity/weather. Store in sterile container. Transport in a temperature-controlled environment.	Check that instruments have been calibrated properly. Run positive/negative controls. Wear gloves and maintain appropriate sterility protocols.

Now that these concepts have been discussed, we will turn to the best methodology for designing an appropriate experiment within the field of archaeology. Field and Hole (2014) discuss three primary methods for conducting experiments: (1) observational, (2) quasi-experimental, and (3) true experimental. Observational studies look at phenomena "in a systematic and scientifically rigorous way" (Field and Hole 2014: 62). Many sciences, including biology, chemistry, and physics, often start with observations of a phenomenon in a systematic and rigorous way, allowing for a general understanding of the phenomenon to be generated based on the data followed by a phase of experimentation during which variables are controlled. A good example is the story of Sir Isaac Newton and the apple, from which he developed his three laws of gravity by applying observation of the phenomena followed by experimentation to determine why apples always fall straight down to the ground. Observational methods are useful for allowing researchers to understand how humans or animals as well as other natural phenomena behave and interact with the world (Field and Hole 2014). Unfortunately, the observational method is limited when it comes to the field of archaeology, as material culture is

usually all that remains from which to infer behavior and we cannot travel back in time to observe people making, transporting, or using the objects recovered. However, with residue analysis, observations may be beneficial and allow researchers to better direct future experiments utilizing ORA. For example, microscopic analysis and observations of the residue might allow potential materials contained within the residue to be identified, allowing focused sampling and analysis to be carried out (Field and Hole 2014).

The second method discussed by Field and Hole (2014:64) is the quasi-experimental design, in which "the experimenter does not have complete control over manipulation of the independent variable." This type of design can be beneficial for certain real-world scenarios, where ethical or other factors prevent researchers from conducting what is considered a true experimental design. One important factor to remember when using a quasi-experimental design is that while there is a chance that differences in variation could be caused by what the researchers are attempting to prove, it is also possible that a host of other factors or variables might affect the results.

To better understand the quasi-experimental method, Field and Hole (2014: 66-67) proposed a hypothetical experiment. Suppose that researchers wanted to see whether the use of a headlight during the day made motorcyclists more visible to other drivers. In a true experimental method, researchers would take a group of motorcyclists and randomly assign them to one of two groups of cyclists: one that used their headlights during the day and one that did not. Then the researchers would have these motorcyclists ride for a set period and then compare the number of accidents each group got into. Obviously, this experiment raises some ethical concerns as it puts the human subject participants into hazardous, even lethal situations. This in turn could prevent researchers from finding enough willing participants. Therefore, a quasi-experimental design is

warranted, where researchers can select motorcyclists who prefer to drive during the daytime with their headlights on to create one test group, and another test group of motorcyclists who prefer to drive during the daytime with their headlights off. By doing this, the researchers are not placing the participants at undue risk. However, because individuals were not randomly assigned, interpretation of results must be conducted with caution. If in this hypothetical situation the group driving with their headlights on during the daytime were involved in fewer accidents, it is possible that the headlight made the individuals more detectable to other motorists. However, it is also possible that other factors, such as the potential that the group driving with their headlights on during the day are more safety oriented, could be the variable that is causing the difference (Field and Hole 2014: 66).

Since archaeologists deal with the material culture of humanity's past, there are many variables that are well outside researchers' control, and therefore quasi-experimental designs may be beneficial, especially when conducting organic residue analyses. For example, one could design an experiment to recreate a particular ancient, fermented beverage using the methods assumed to have been available at the time, then artificially age the sample in order to create a residue (Driscoll and Damm 2023). This could then be tested through various ORA methods and compared to other residues discovered at archaeological sites. Furthermore, quasi-experimental design can be beneficial when looking at ORA because there are several extenuating environmental factors that cannot be controlled by archaeologists. By using a quasi-experimental design, one can gain some valuable insight into the residue, as well as environmental impact on the sample. On the other hand, a quasi-experimental design cannot work in every situation, nor can it be used as a universal model for all archaeological investigations. There is one other design form to consider, and that is the true experimental design (Field and Hole 2014).

The true experimental design, as defined by Field and Hole (2014: 67), is very similar to the quasi-experimental design with the exception that all variables have been controlled by the researchers. This is what people traditionally think of when picturing experimental design. One important component in controlling external variables, depending on the type of study being conducted or the subjects being studied, is randomization. Randomization helps to prevent extraneous factors from impacting the experiment (Field and Hole 2014: 70). Take as an example a hypothetical experiment regarding infants and Mozart's music. Researchers hypothesize that playing Mozart's music to infants has an impact on their intelligence later in life. So, if playing Mozart's music has any impact on intelligence it should impact all infants in the same way. However, due to external factors outside of researchers direct control, the exposure to music will have a varied magnitude of effect on each individual infant even though in general it would be expected that the group exposed to the music will score slightly higher when intelligence is measured than the control group that was not exposed as infants (Fields and Hole 2014: 71-73). This systematic variation in performance is how one can determine whether the variable being manipulated impacts the result of the experiment or not. Now, as previously mentioned in this hypothetical experiment, each infant will be exposed to slight extraneous factors that will always be outside of the control of the researchers. To make sure that those extraneous factors do not cause systematic variation, the participants are placed into the control and experimental groups randomly. By doing this, any slight variation between the participants will be negated. Further importance is placed on randomization because if one were to fail to randomize the participants or conditions, the results would become uninterpretable, leading to the need to repeat the experiment (Field and Hole 2014: 73).

Clearly there is no one method of experiment design that is completely suitable for archaeologists conducting ORA. However, there are components of each type of design that can be applied to ORA research to produce more reliable results. For example, we can use an observational method and take note of any floral and faunal remains, as well as any evidence of chemical manipulation (i.e., cooked or charred remains), as well as the artifact assemblages within the site where the object being analyzed was found, to help guide the next steps of the analysis.

Using a quasi-experimental design, archaeologists can attempt to create and artificially age a residue to be used as a comparison against an actual unknown residue being studied. These two samples can then be compared and if the sample results are similar, this can lend support to the argument the archaeologists are attempting to make. Furthermore, this same quasi-experimental design can be used to create an unknown organic residue that can be sent to various institutions and tested using a variety of residue analyses to help illustrate the strengths and potential weaknesses of ORA, similar to proficiency testing found in other scientific fields.

Finally, while they are unlikely to be able to control for every possible variable that could impact the experiment, archaeologists can apply the concept of control of variables using positive and negative controls, which will ensure that the results delivered by the instrument are valid and legitimate and not due to false positives or false negatives caused by contamination or human error.

As archaeologists continue to use hard science techniques, methodologies, and concepts of scientific exploration, it is imperative to remember to implement the concepts discussed above to ensure that the results uncovered are legitimate and valid. A comparison of studies conducted

applying ORA to the study of ancient alcohol is presented below to help illustrate the importance of these concepts.

Examples of Research and Experiment Design

The first study we will use to demonstrate the importance of understanding the potential variables involved in an ORA study is the work conducted by Correa-Ascencio et al. (2014) on pulque production in the city of Teotihuacan. The researchers wanted to determine whether pulque, a fermented beverage made from the sap of the agave, also known as the maguey, plant was used as a dietary supplement by individuals living in the city of Teotihuacan between 150 BCE and 650 CE (Correa-Ascencio et al. 2014: 14223). Teotihuacan is a major Pre-Classic city in central Mexico, a semiarid highland region with low rainfall and limited access to groundwater. These conditions make cultivating plants such as maize, a staple crop of the region, challenging. Despite the difficult growing conditions, the city grew to cover close to twenty square kilometers, with a total population of around 100,000 inhabitants at its peak (Correa-Ascencio et al. 2014: 14223). Although maize, the staple food of the inhabitants of Teotihuacan, is a high calorie crop, it lacks several important micronutrients as well as the amino acids necessary for human survival, growth, and development. The researchers noted that osteoarcheological investigations show evidence for nutritional stress, particularly among lower status households (Correa-Ascencio et al. 2014: 14224). However, the researchers also noted that at Teotihuacan there is evidence for vessels that resemble ceramics used to store and serve pulque in other Mesoamerican regions, as well as murals and other artistic renditions depicting both maguey plants and pulque consumption. This led the researchers to believe that pulque might have held a similar position of importance in the city of Teotihuacan as in these other contexts (Correa-Ascencio et al. 2014:14224). Maguey plants can withstand droughts and frosts

that other crops such as maize would not be able to survive. Furthermore, the sap is high in calories and unlike maize it contains most of the macro- and micronutrients needed by humans. When converted from a sap to the alcoholic beverage known as pulque, it also confers probiotic properties due to the presence of lactic acid bacteria that aid in the production of alcohol (Correa-Ascencio et al. 2014:14224). Therefore, the researchers wanted to determine whether pulque was used in Teotihuacan as a dietary supplement by the inhabitants of the city.

One of the crucial pitfalls that the researchers pointed out is that most of the chemical markers that indicate the presence of an alcoholic beverage are water soluble. This means that the survival of these chemical indicators to present times is very limited as environmental conditions can significantly impact the degradation of these chemical markers. (Correa-Ascencio et al. 2014: 14225). Therefore, the researchers decided to target a chemical marker that was more robust and would have a better chance of surviving environmental degradation. Since pulque is fermented not only by yeast, but by a species of bacteria as well, the researchers decided to look at a lipid biomarker that is indicative of the presence of *Zymomonas mobilis*, the species of bacteria associated with the production of pulque in the Americas, as well as palm wines in Africa (Correa-Ascencio et al. 2014: 14225). This bacterium consumes sugars, and like yeast, produces waste products of ethanol (alcohol) and carbon dioxide. These waste products are hazardous to bacteria and yeast as they change the pH of the surrounding liquid in addition to being lethal to these microorganisms in high enough concentrations (Correa-Ascencio et al. 2014: 14225). To survive in the harsh environment created by the production of ethanol and CO_2, *Z. mobilis* has evolved a membrane that contains some of the highest concentrations of hopanoid molecules of any bacterium. Hopanoids are a molecular compound used by bacteria to increase the rigidity and reduce the porousness of their membranes to allow them to survive in harsher

environments. This molecule functions in a similar manner to the way sterol molecules work within eukaryotic cells (those with a nucleus) (Correa-Ascencio et al. 2014:12446). The researchers noted that while hopanoids are used as biomarkers to study both extant and extinct bacteria in sediments, the use of hopanoids as a biomarker of an alcoholic beverage had not been tested until this study. While there are inherent risks associated with choosing a novel biomarker to determine the presence of an alcoholic beverage, by identifying specific molecules associated with this bacterium that aid in the fermentation of pulque, the researchers were able to select a chemical biomarker that would have a higher likelihood of survival in an archaeological residue, allowing them to determine whether or not pulque played an important role as a dietary supplement for the residents of Teotihuacan (Correa-Ascencio et al. 2014: 12446).

The researchers' experimental design was organized into two parts. The first part involved conducting organic residue analysis on 313 potsherds sampled from three different localities within Teotihuacan to determine the presence of these bacteriohopanoids, which would indicate that pulque was processed, stored, or consumed using those vessels. The second portion of this experiment was a bacteriohopanepolyols aging experiment to determine how well these biomarkers would survive within the archaeological record. Results of the first portion of the experiment show that around 70% of the potsherds contained detectable lipids, primarily *n*-alkanes, and *n*-alkanols. Furthermore, the distribution of these long-chain fatty acids confirms the presence of plant oils or waxes, along with the biomarker for maize (a C_{32} long-chain *n*-alkanol), which is consistent with palaeobotanical evidence of the region (Correa-Ascencio et al. 2014: 14223). On the other hand, 14 potsherds contained an unusual lipid distribution. All 14 potsherds came from the same locality within Teotihuacan, and 10 of them came from pottery vessels suspected to have been used for pulque production and transportation. All 14 of these

sherds yielded chemical indicators of pine resins (Correa-Ascencio et al. 2014). This evidence, when found in the archaeological record, has been associated with waterproofing of ceramics to allow them to better store liquids. Further GC-MS analysis indicated the presence of hopanes, which were only detected on the potsherds of resin-containing vessels (Correa-Ascencio et al. 2014:14226), which the researchers interpreted to mean that these vessels most likely contained pulque. The bacteriohopanoids that *Z. mobilis* produces were transformed and broken down into simpler components due to vessel use, microbial actions, or potentially being catalyzed by the clay used to create these vessels (Correa-Ascencio et al. 2014:14226). One possible way to interpret the evidence is that this biomarker is not as reliable as the researchers had previously believed; however, this is where the bacteriohopanepolyols aging experiment comes into play.

To conduct this experiment, the researchers took around 20 grams of alumina and a replica ceramic that was crushed into a powder and then allowed this to soak in 30 mL of pulque until the material was saturated. After saturation the material was incubated at 100 degrees Celsius (212 degrees Fahrenheit) for a two-week period. After this artificial aging process, lipids were extracted from the material and subjected to GC-MS (Correa-Ascencio et al. 2014:14226). The distribution of the hopane biomarkers of this artificial aging process was comparable to the hopane distribution found within the 14 ceramic sherds at Teotihuacan, which supports the idea that the ceramic matrix can catalyze and transform bacteriohopanoids associated with *Z. mobilis*. This suggests the sherds tested contained pulque and that the hopane biomarkers found are consistent with the catalyzation and transformation of bacteriohopanoids (Correa-Ascencio et al. 2014:14226). This evidence, in conjunction with the GC-MS studies conducted on the pottery sherds from Teotihuacan, indicate that not only was pulque produced at Teotihuacan during the

height of its population growth, but that it is possible to use bacterial biomarkers to determine the production of certain alcoholic beverages.

This study is a prime example of how to develop and implement proper experimental design to answer a specific question related to vessel contents. The researchers chose a biomarker target that had a higher chance of survival in the archaeological record, and then not only used organic residue analysis and GC-MS to determine whether the potsherds contained a proxy of these specific bacteriohopanoids but took the study one step further. To establish that the results obtained were due to the production, storage, and transportation of pulque, and not due to environmental contamination, the researchers performed an artificial aging experiment that confirmed the likely source of these chemical signatures (Correa-Ascencio et al. 2014). The design of this experiment and research should serve as a model for archaeologists because it combines information from the archaeological record with contemporary knowledge of how alcohol is produced, and what microorganisms are used for the production of a specific type of alcohol, to develop more reliable ways to identify the production of alcoholic beverages within the archaeological record using ORA. Furthermore, the work done by Correa-Ascensio et al. (2014) can be replicated by other researchers, which helps to strengthen the results of this study. Replication is one of the key components of scientific research, and the fact that this archaeological research was conducted and designed so that it can be replicated allows others to follow the same procedures and generate independent results. As hard science intersects more frequently with archaeology, experimental designs such as this one will become even more critical.

One important thing to note is that bacteriohopanoid compounds, while present within *Z. mobilis*, are not exclusive to *Z. mobilis*. This means that other microorganisms can produce the

same compounds, so understanding the microenvironment, including the available plants, in conjunction with additional archaeological evidence supports the claims of pulque production. This is why it is important that archaeologists learn the fundamentals of experimental design to ensure that as further ORA research is carried out in archaeological contexts, the evidence presented will be robust and stand up to scrutiny.

Driscoll's (2023) study of the shelf life of Iron Age beer products is another prime example of experimental archaeology that exhibits the qualities of a well-designed experiment. Traditionally, Iron Age scholars have believed that wine held more value for elites within European Iron Age society, as it had a longer shelf life than beer. Therefore, with the ability to import wine from the Mediterranean, it was assumed that this allowed the elite to shift from smaller feasts and social gatherings to gatherings that involved larger groups within the region. However, this assumption had never been tested prior to this experiment. Therefore, Driscoll set out to test the shelf-life and stability of ancient beers supported by both historical and ethnographic sources. These beers were made using six row barley as well as distilled water with minerals added to approximate the profile of water in southwest Germany, where much of the paleobotanical evidence for Iron Age beer, mead and mixed beverages has been found (Arnold and Driscoll 2021). The malted barley was mixed with the water and then transferred to the fermentation vessels without boiling, as the literature supports the notion of not boiling unhopped beverages. These mixtures were then allowed to ferment with a top fermenting strain of brewer's yeast, as well as other strains of yeast and bacteria strains and allowed to reach an ABV of 5%. Fifteen experimental batches were fermented in various containers of different oxygen permeability. Two batches were fermented in oak barrels, and six were fermented in low-fired coil built ceramic vessels corroborated through historical and archaeological data. Two of

the vessels were left unsealed, two were sealed with beeswax, and two were sealed with a pitch made from pine resin. The final batches were stored in modern brewing equipment with known oxygen permeabilities to establish a baseline. The beer was then tested for spoilage on a regular basis, looking at changes in pH, specific gravity, and titratable acidity measurements as well as by smelling and tasting the beverages.

Results of the experiments showed that most of the forms of storage allowed the beer to remain unspoiled for a minimum of 24 months, with several of them remaining drinkable at 26 months and still going strong at the time of publication. Beer in the oak barrels, which would have been available in the Iron Age, remained unspoiled after 24 months, indicating that beer could have been produced and stored for an extended period. Driscoll (2023: 178) did note that the ceramic vessels spoiled rapidly, however upon inspection of the vessels, the wax and pitch used to seal the vessels had bubbles, compromising their ability to seal the vessel. This in turn caused the beer to leak through the ceramic vessel walls, causing mold to grow both internally and externally. These data were excluded from the study as the issue was attributed to human error as opposed to a defect in the Iron Age technology.

This study has several qualities of a well-designed experiment. One aspect of the experiment is the use of repetition. Each vessel being tested has at least one other replication. Repetition can help to either eliminate data values that are caused by errors, or if entire groups of replicates give unusual results, that data can be analyzed to check for statistical significance, or as in this experiment, can alert those conducting the experiment to sources of human error.

Second, the experiment outlines its process for how the beer was produced, what yeast and bacteria were used during fermentation, how vessels were sealed, as well as what approach

was used to establish background values. This would make it possible for research teams to conduct this same experiment, allowing for independent corroboration of the results.

However, while the experiment was well explained, the explanation could be improved upon. For example, it was stated that the beer was tested on a regular schedule, however, the schedule was not elaborated on, which might cause future researchers attempting to conduct this experiment to test their samples at different intervals, potentially impacting the results. Furthermore, temperatures were not mentioned in this experiment, or whether the batches of beer were in a climate-controlled environment. The absence of these data could also change the results of future research. Additionally, listing the change in pH, specific gravity, and titrated acidity each time the samples were tested could also provide beneficial information to future researchers, as well as allowing for a comparison to see if different storage methods had any sort of impact on those particular variables. Ultimately, this experiment helps illustrate key points of good experiment and research design and demonstrated that beer, especially sour beer, can be stored for extended periods even in semi-permeable containers (Driscoll 2023).

While the previous studies are prime examples of how experimental design can yield results that are robust and can stand up to scrutiny, there are other case studies in the literature that demonstrate the importance of experimental design by revealing the potential pitfalls researchers may unknowingly fall into without a thorough understanding of how to design an appropriate experiment. This is exemplified by the study conducted by Barnard et al. (2007), which presents the results of a symposium entitled “Theory and Practice of Archaeological Residue Analysis.” Participants in this symposium were given the opportunity to conduct research and report their own analysis of a foodstuff residue that was recently cooked in a new unglazed, unseasoned ceramic vessel (Barnard et al. 2007). Eleven participants agreed to take on

this challenge and were sent a piece of the ceramic vessel in question. A ceramic bowl was purchased in Luxor, Egypt, and after purchase, fresh camel milk and mineral water were placed in it. This bowl was then wrapped in aluminum foil and left to sit for 24 hours (Barnard et al. 2007:28), after which it was placed in a gas oven with its contents, heated to 200 degrees Celsius (392 degrees Fahrenheit) for one hour, and left to cool for four hours. After the cooling period the vessel was once again heated for one hour and then left at room temperature for another 24 hours. Afterwards the contents of the vessel were discarded, and the vessel was rinsed with water and left in the air to dry for 10 days. Fungus that had grown during this 10-day period was rinsed out with cold water again and the vessel was allowed to air dry for 24 hours before being sealed in a plastic bag and transported to Los Angeles where it was cut into 12 pieces (Barnard et al. 2007: 28), 11 of which were sent to the participating laboratories. Immediately it becomes evident that the participating laboratories were facing a much more significant challenge than archaeologists typically would, as "provenance, shape and date of a pot usually offer clues to its former use and contents" (Barnard et al. 2007: 28). Due to this increased level of challenge, the importance of designing an appropriate experiment to give participants the best possible chance of identifying the unknown material is especially important. Seven of the 11 laboratories reported their findings prior to the symposium at which the vessel contents were revealed (Barnard et al. 2007: 28).

Ultimately, none of the labs were able to correctly identify the source of the material, and while the results of these lab tests will be discussed in more detail later in this thesis, one lab study will be discussed in detail here because it helps to illustrate the importance of properly designing an experiment, especially when one is faced with a challenge such as this. Laboratory A in this "round robin" challenge chose to look for proteins within the ceramic matrix. To do so,

they took the sherd and placed it in a plastic dish with 0.5 mL of a 5% concentration of ammonium hydroxide. They then sonicated the ceramic sherd for five minutes, meaning that they used ultrasonic frequencies to rapidly vibrate the dish, liquid, and ceramic sherd to extract compounds trapped within the ceramic sherd (Barnard et al. 2007: 32). Next the dish and its contents were placed in a rotating mixer and left to mix for 30 minutes to collect as much of the extracted compounds within the liquid solution as possible. This solution was then frozen at -20 degrees Celsius (-4 degrees Fahrenheit) to maintain the stability of the solution. Next 3 µL (microliters) of this solution were transferred to the well of an agarose gel, with a second well containing 3 µL of antiserum, and a positive control in a third well. After the samples were loaded into the gel a 105-volt AC current was passed through it for 45 minutes causing both the sample in question as well as the antiserum to migrate, with antigens moving towards the anode (negatively charged side) while the antibodies found in the antiserum moved towards the cathode (positively charged side) (Barnard et al. 2007: 32). During this migration if a protein present during that time matched the antiserum, there would be an antigen-antibody reaction that would cause the protein to precipitate out in a specific pattern (Barnard et al. 2007: 32).

Laboratory A used antisera that included "agave, amaranth, bear, bovine, cactus, cat, cedar, chicken, *Capparidaceae*, *Chenopodiaceae*, *Compositae*, deer, dog, guinea pig, *Graminae*, legume, pine, rabbit, rat, and sheep" (Barnard et al. 2007: 32). Furthermore, all the antisera used in this experiment were polyclonal, meaning that they recognized the epitopes of other closely related species. "For example, anti-deer serum will react positively to other members of the family *Cervidae* such as elk, moose and caribou" (Barnard et al. 2007: 32). This method of residue analysis is called crossover electrophoresis, or counter electrophoresis, commonly abbreviated as CIEP (countercurrent immunoelectrophoresis). Laboratory A did not detect any

trace of proteins within the sherd sample that was sent for analysis and interpreted this as evidence that the proteins had degraded during the cooking process (Barnard et al. 2007: 32).

To begin with, there was one very prominent flaw in the design that Laboratory A chose to conduct their organic residue analysis of this sherd. As mentioned previously, the laboratory used a wide array of antisera that targeted both plant and animal proteins. Specifically, they used ten different plant-based antisera, and ten different animal-based antisera all of which were polyclonal. While this was an appropriate choice, they omitted one key antiserum that could have potentially assisted in their analysis. This would have been any sort of antiserum related to the family *Camelidae*, which as its name suggests covers creatures such as camels, alpacas, llamas, vicuñas, and guanacos. Not including this antiserum in the experiment meant that the protein in question would almost certainly not be detected using CIEP. On top of this, the fact that the camel milk in this study was baked would also have prevented this laboratory from determining the origin of the residue. Unfortunately for Laboratory A,

> Experiments subsequent to this study have demonstrated that animal proteins, of known origin heated to 100ºC for 20 min yielded no positive readings using CIEP. This observation has important implications for the application of this particular form of residue analysis on cooking vessels or objects from hearth (Barnard et al. 2007: 32).

This shows that while organic residue analyses can be powerful tools, there are limitations that can impact the validity of the results. Heating the camel milk to 100 degrees Celsius on two separate occasions, for an hour on each occasion, would have caused sufficient degradation of the proteins, known as denaturing, that they would not have been detectable even if the appropriate antiserum had been used.

While it is unfortunate that this laboratory was not able to identify the specific protein in question, this example does help to illustrate a few important points. The first is that it is

important to understand the limitations of the technology being used. This paper specifically makes a point of reporting the negative results of two of the laboratories (Laboratory A and Laboratory H) because only publishing projects and research that is successful contributes to a biased representation of what these technologies can do within the field. In addition to this, this publication highlights the importance of using additional data to determine the unknown residue and not to rely on the chemical analysis alone (Barnard et al. 2007). Using the contextual clues within the archeological record as well as the geographic region where the site is located, in conjunction with chemical analyses, should allow archaeologists to narrow down the likely sources of any unknown organic residues. For example, if one is excavating a site in North America, camel milk would not be expected, as camels are not found in North America, just as one would not expect to find an alpaca in Mesopotamia. Understanding the geographic region, as well as the flora and fauna found in those areas, allows archaeologists to establish the range of potential plants and animals that might have been exploited by human groups in that region. Contextual clues from the vessels in question, in addition to associated material culture, can provide a better understanding of the potential uses of the artifact and identify the appropriate chemical analysis for determining the source of the unknown organic residue.

Now that the concepts of experimental design have been explored, a comparison of various studies that use organic residue analysis to explore the production of ancient alcohol can be presented. By making this comparison of various studies using the concepts that have been illustrated in previous chapters of this thesis, it will be possible to see how these hard science analyses and methodologies can be applied appropriately and how to avoid inappropriate application of these technologies and methodologies.

Chapter 4: Case Studies and Discussions

This chapter will examine several studies that have applied ORA to archaeological specimens to interpret ancient alcohol production. The purpose of this chapter is to use these case studies to discuss appropriate research and experiment design and establish a scaffolding upon which future archaeological research can be built. These case studies were chosen for two main reasons: (1) They cover both New and Old World archaeological sites and they represent a wide variety of environments that pose unique challenges regarding the recovery of evidence of not only alcohol production and consumption, but other organic residues. Examining a wide variety of research projects allows future researchers to have a better initial understanding of the potential issues that they might run into and will help guide their experiment designs to prevent common problems associated with poor research design. (2) They use a wide variety of analytical techniques, helping to highlight appropriate research design, as well as mistakes that can be made during the initial experiment design or during the interpretation of evidence. The case studies are organized based on year of publication to demonstrate how ORA research has evolved over time and how experiment and research design have changed as well.

Case Study 1: Fermented Beverages in Pre- and Proto-historic China

The first study presented in this evaluation of analytical approaches to ancient alcohol as reflected in the existing literature focuses on fermented beverages in ancient China (McGovern et al. 2004). This study included ORA on ceramic pottery sherds from the site of Jiahu, an early Neolithic village located in the Henan province. Oracle bone inscriptions from the late Shang Dynasty noted that three fermented beverages were popular in China: (1) an herbal wine known as *chang*, (2) a sweet rice or millet beverage known as *li*, and (3) *jiu*, "a fully fermented rice or millet beverage or 'wine', with an alcoholic content of 10-15% by weight" (McGovern et al.

2004: 17593). Later documents from the Zhou Dynasty mention two additional beverages, *luo* and *lao*, which were fermented beverages made from fruit or an unfiltered rice or millet beverage respectively. This textual evidence, as well as Neolithic pottery vessels with a similar vessel shape used to store and consume alcohol during the Shang Dynasty, suggest that alcoholic beverages were produced very early in Chinese history.

McGovern et al. (2004) analyzed 16 pottery sherds from the Neolithic village site of Jiahu that were radiocarbon dated to between 7000 and 5500 BCE. The vessels sampled included "perforated basins, two handled narrow-mouthed storage jars, and jars with high, flaring necks and rims, which were suited for preparing storing and serving beverages" (McGovern et al 2004: 17593). The researchers sampled base sherds, because the base is where the most liquid would have been absorbed by the ceramics and any precipitates would have settled to the bottom as well. A variety of solvents of varied polarity (such as chloroform, hexane, and methane) were used depending on the method of ORA being conducted. The sherds were added to the solvents and then either sonicated, which is the application of sound energy to agitate a sample in a liquid, or the sample was boiled twice for 20 minutes each time. Once the residue was extracted the solvent was allowed to evaporate, leaving only the dry extract from the pottery sherd. Each sherd produced between 5 to 60 milligrams of extract depending on its size and thickness. Five analytical methods, GC-MS, HPLC-MS, FT-IR, stable isotope analysis, and selective Feigl spot tests, were used to analyze the sherd extracts (McGovern et al. 2004: 17594).

Several different protocols were used by the researchers depending on the analytical methodology. For the samples subjected to GC-MS, 1 μL of the sample were extracted in chloroform and injected into a standard quadrupole instrument, which is an instrument with an ionizer, ion accelerator, and mass filter consisting of four parallel metal rods. USDA samples

were methylated and injected into a split mode for derivatization, whereas samples tested at Drexel University were injected into a splitless system and did not undergo derivatization due to the small amount of recovered extract. Highly volatile compounds were detected using a new GC-MS technique called purge and trap thermal desorption (McGovern et al. 2004: 17594). This process takes an aqueous sample and sparges it with helium, meaning the helium gas was passed through the liquid to remove and capture the volatile compounds. These compounds were then trapped on a short adsorbent column, attached to the short path thermal desorption (SPTD) accessory where the trapped material was then desorbed onto a silica GC column and eluted, that is removed from the column where the extract was trapped (McGovern et al. 2004: 17594).

Several different protocols were used for samples analyzed using HPLC-MS. The USDA protocol for the HPLC samples was to take 100 µL of extract dissolved in chloroform and run it "using a gradient normal-phase set-up with an evaporative light-scattering detector" (McGovern et al. 2004: 17595). Those extracts were then characterized even further by putting them through an isocratic normal-phase system which was interfaced into a mass spectrometer. The laboratory at the University of Pennsylvania Museum also conducted HPLC analysis. Their protocol, however, was to use methanol as the solvent to extract the residue and then inject it into an isocratic normal-phase system that was also equipped with a UV detector (McGovern et al. 2004: 17595). The results were then compared to an in-house database consisting of previously tested ancient samples, as well as modern day counterparts. These reference samples were then compared to the unknown sample from Jiahu to determine the highest probability matches for the type of residue present (McGovern et al. 2004: 17595).

The University of Pennsylvania Museum team also conducted FT-IR on the extracted samples by using one milligram of either methanol or chloroform extracted residue and testing it

at 8 cm^{-1} wavenumber. The result was then compared against commercial and in house databases to look for the most likely possible match, similar to the HPLC-MS conducted in the same laboratory (McGovern et al. 2004: 17595).

Stable isotope analysis was also conducted on the residue samples recovered at Jiahu. ^{13}C and ^{15}N isotope measurements were conducted on the pottery sherds, liquid residue samples, and the extracted residue samples. This analysis allowed the researchers to distinguish between C_3 and C_4 pathway plants. C_3 and C_4 pathway plants are distinguished by different methods of carbon fixation during photosynthesis, and C_4 plants, such as maize or corn, exhibit more efficient photosynthesis than C_3 plants such as wheat or rice. Furthermore, stable isotope analysis of nitrogen allows researchers to distinguish whether the extracted residue came from a plant or animal source, as animal sources would have a higher concentration of $\delta^{15}N$ than plant sources (McGovern et al. 2004: 17596).

Finally, the laboratory at the University of Pennsylvania Museum also conducted Feigl chemical spot tests on the methanol extracted samples. The goal of these tests was to look for tartaric acid and oxalic acid compounds. When exposed to UV light during a Feigl spot test tartaric acid fluoresces a dark green, which signals the presence of a substance containing this compound such as wine. Researchers noted that there is one interfering compound, malic acid, which produces a nonfluorescent green color and therefore can easily be distinguished from a positive tartaric acid test. The samples were tested and compared to blanks as well as acids at a low concentration (McGovern et al. 2004: 17595).

Results of this study identified several different biomarkers for honey, rice, and fruit, either grapes or hawthorn fruit. FT-IR and HPLC analysis indicated that 13 of the 16 samples present in the Jiahu ceramic collection were comparable, indicating that these vessels were used

to process or hold similar liquids. Furthermore, the FT-IR and HPLC results from the Jiahu ceramic collection were also most similar to modern rice and rice wine, resinated and nonresinated grape wine, beeswax, grape tannins and other critical biomarkers. The IR spectrum of tested samples showed strong peaks at 2920 and 2850 cm^{-1} that are most closely associated with long straight hydrocarbon chains, such as *n*-alkanes. Additional peaks at 1740 and 1720 cm^{-1} are indicative of tartaric acid being present in the extracted samples. Further FT-IR evidence for the presence of tartaric acid came in the form of a hydroxyl stretch band at 3450-3500 cm^{-1} wavelength number and the hydroxyl bending band identified at 1435 to 1445 cm^{-1} wavelength number. These hydroxyl bands support the presence of tartaric acid, which has four hydroxyl groups attached to the molecule. Therefore, the presence of not only tartaric acid peaks, but of both hydroxyl stretch and bending bands, lends support to the researchers' conclusion that the extracted residue contained tartaric acid. Positive Feigl spot tests for tartaric acid lend further support to the idea that this compound was present in the extracted residue (McGovern et al. 2004: 17596).

The GC-MS and stable isotope analysis yielded similar results. GC-MS results of the Jiahu ceramics "showed the uniform presence of an inclusive series of *n*-alkanes, C_{23}-C_{36}" (McGovern et al. 2004: 17596). Stable isotope analysis generated $\delta^{13}C$ values that are indicative of a C_3 plant such as rice or grapes rather than a C_4 plant like millet. Furthermore, low $\delta^{15}N$ values indicate that the extracted residue came from a plant rather than an animal source (McGovern et al. 2004: 17596).

Based on the results of the various tests, the researchers concluded that these ceramics most likely were used to store and drink a form of alcoholic beverage, made from rice, honey, and some sort of fruit. Researchers narrowed down the potential fruits to either grapes, possibly

the non-domesticated grapes whose seeds were recovered at the site of Jiahu, or Chinese hawthorn fruit, which not only contains tartaric acid and tartrates, but has concentrations of this biomarker four times higher than that of grapes. Seeds of this plant were also found at the site of Jiahu, lending archaeobotanical support to this claim (McGovern et al. 2004:17596).

This study provides several examples of good experimental design, as well as how to apply ORA in an appropriate manner. The first is the application of multiple forms of ORA to not only build a robust body of evidence but also to test the reliability of the results obtained. McGovern et al. (2004:17595) state: "A minimum requirement for establishing the original contents of an ancient vessel is to identify fingerprint compounds (biomarkers) for specific natural products and ingredients in its extracts." They further state: "Chemical identification and interpretation, however, is often impeded by environmental and microbial degradation, modern contamination, human processing in antiquity, and the degree to which a region's natural resources have been adequately surveyed for biomarkers" (McGovern et al. 2004: 17595). Due to the difficulties in identifying these biomarkers accurately, using multiple different chemical analyses allows researchers to be more confident that the particular compound is present in the residue being studied. Furthermore, because each of these independent analytical techniques identified the same chemical compounds, they corroborate and reinforce the results of the other analytical techniques, further legitimizing the deductions of the researchers.

For instance, the HPLC and FT-IR results indicated that the extracted residue closely matched that of rice and rice wines. Likewise, in the stable isotope analysis the $\delta^{13}C$ values indicated that the plant material used in the production of this beverage came from a C_3 plant and not a C_4 plant. This result reinforces the HPLC and FT-IR results because rice is a C_3 plant. In a similar vein, the researchers argued that this mixed alcoholic beverage was made with some sort

of fruit, potentially grapes or Chinese hawthorn fruit. HPLC results indicated that the extract, in addition to being similar to rice and rice wine, was also similar to resinated and non-resinated grape wines. GC-MS analysis, along with FT-IR, indicated the presence of tartaric acid, which is a key biomarker for grape wine. Feigl spot tests were positive for tartaric acid, helping to strengthen the FT-IR and GC-MS results and supporting the idea that grapes or hawthorn fruit were used in the production of this beverage. Finally, the archaeobotanical evidence of grape and hawthorn fruit seeds, combined with the stable isotope analysis results indicating the use of C_3 plants like grapes, all help to further support the researchers' claims.

Another example of appropriate experimental design represented by this research project is the use of several laboratories and teams testing the same material independently. There are several benefits to such an approach, in particular mitigating the impact of researcher bias. If one is determined to find a specific result one might read further into the results that are produced to fit the narrative constructed by the researchers ahead of time. However, having a second group conduct the analysis removes those potential biases when results are compared. Secondly, it also can help to catch any potentially false positive or false negative results or other errors. If one group does not detect a particular biomarker within the extracted sample but most of the others testing the same extracted compounds do, one would be able to determine that a potential source of human error caused the negative. This also allows potential contamination of a source to be detected, which further ensures the robustness of the results produced by the study

Finally, the researchers in this case study did not solely rely on chemical analyses to generate their conclusions. While the various kinds of chemical analyses make up most of their evidence and are robust enough to stand on their own, the archaeobotanical evidence discovered at the site of Jiahu, as well as the design of the ceramics discovered at the site, also informed the

conclusions generated by the team. Based on the design of the ceramics, the researchers were able to infer that the vessels most likely were used to hold or serve some form of liquid. The chemical analyses, indicating the presence of rice, honey, and fruit, are supported by the archaeobotanical evidence for grapes and hawthorn fruit at the site, as well as other archaeological evidence that indicated that rice was present.

Case Study 2: Multiple ORA Applied to a Vessel Containing a Known Foodstuff

For a recap please reference the in-depth analysis discussed in Chapter 3 on experiment and research design. This helps to illustrate two key points. The first is a check to see if there are any limitations that are known to the developers of the analytical methodology. If the limitations are not known at the time, then it would be critical to employ an additional method of ORA to act as a confirmatory test. Furthermore, by employing a second method to act as a confirmatory test, the limitations of these methodologies may be revealed, allowing researchers to adjust their studies going forward.

Case Study 3: Ancient Egyptian Herbal Wines

Another study involving ORA examined the potential medicinal role played by wine and other fermented beverages in ancient societies, in this case ancient Egypt (McGovern et al. 2009). Several different ceramic vessels were sampled to determine whether the organic residues used were “medicinal wines.” The earliest sampled vessel came from a multichambered tomb at Abydos dated to the Naqada IIIa2 period (ca. 3150 B.C.E.) (McGovern et al. 2009:7361). FT-IR HPLC and Feigl spot tests on a residue sample from Jar 156 found in this tomb all indicated the presence of tartaric acid, which as mentioned previously is a significant biomarker for grape wine. Once this biomarker was identified, researchers sought further evidence to support the idea

that the grape wine was intentionally fermented as opposed to being an accidental byproduct of the environment (McGovern et al. 2009:7362). The team identified a 256-bp DNA segment that came from a larger 840-bp segment of DNA, related to a ribosomal sequence of *Saccharomyces cerevisiae*, the main yeast used to ferment beverages such as wine. Further evidence to suggest that this was intentional fermentation was the addition of tree resins to the wine as well as its use to seal the lid of the ceramic vessel to prevent exposure to oxygen and prevent the wine from fermenting further to vinegar (McGovern et al. 2009:7362). Archaeobotanical evidence from the site in the form of grape seeds further suggests that sliced figs may have been used as an additive to sweeten the wine as well as help to jump start the fermentation process, as yeast is naturally present on the skins of fruit, likely introduced by various insects that feed on the juices escaping from splits in the skins (McGovern et al. 2009:7362).

The second sample came from a ceramic amphora from Nubia in Upper Egypt and was recovered in a tomb at Gebel Adda dating to between the 4th and 6th centuries CE. Inscriptions on the amphora indicate that it was a wine jar. Residue sampled from this jar was also tested via FT-IR, HPLC, and Feigl spot tests and, as in the previous example, all three analytical methods indicated the presence of tartaric acid and tartrates. One key difference the researchers noted is that while both samples indicated the presence of tartrates (the calcium salt of tartaric acid), only the second sample contained evidence that the acid was still present (McGovern et al. 2009:7363).

As part of the same project, researchers from the University of Barcelona conducted liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis on several ceramic vessels from the tomb of Pharoah Semerkhet of Dynasty I, and an amphora from the tomb of Pharoah Tutankhamun. Tartrates and tartaric acid were identified through multiple reaction

monitoring (MRM) of the analyzer. This further supports the idea that wine was present throughout the history of Egypt, and inspired McGovern and his team to retest the samples using LC-MS/MS, as previous testing on these samples had been conducted in the 1990s (McGovern et al. 2009:7363).

Going back to the samples from Abydos and Gebel Adda, the researchers sent samples to the Scientific Services Division of the Tax and Trade Bureau. Samples were tested for tartaric acid and tartrates via LC-MS/MS, and results indicated a peak for tartaric acid at a retention time of 0.75 min. This matched the peak and retention time of a tartaric acid standard. Furthermore, analysis of pottery blanks as well as aqueous controls that were run before and after each analysis of the residues from the Egyptian ceramic vessels gave negative results. These results all support the conclusion that at one point wine was contained in these ceramics and the results were not a result of contamination or human error.

The next step for the researchers was to identify any additives present in the residue and if these additives may have had a medicinal purpose (McGovern et al. 2009). To determine if these residues contained any additional plant additives, the researchers conducted headspace solid phase microextraction (SPME) and thermal desorption (TD) GC/MS. As mentioned in the previous case study, headspace SPME is useful for identifying volatile biomarkers because the analysis has a high sensitivity and specificity. Samples from Abydos and Gebel Adda were analyzed and identified by retention time and compared to a mass spectral library of 160,000 samples (McGovern et al. 2009:7364).

Headspace SPME also confirmed the presence of tartaric acid and tartrates in both samples analyzed. In addition to this, alcohols, esters, acids, aldehydes, fatty acid derivatives, and terpenoid compounds were identified by the headspace SPME. Three herbs were identified

in the Abydos sample, savory (*Satureja*), *Artemesia seibeni* (a member of the wormwood family), and blue tansy (*Tanacetum annum*). These herbs could account for the terpenoid compounds detected during the headspace SPME. Additionally, rosemary could account for other volatile biomarkers such as camphor, borneol, and cuminaldehyde. Volatile compounds indicative of pine resin were also detected in both residue samples. One specific compound, methyl dehydroabietate, is only produced when pine resin is exposed to heat. This is consistent with pine resin used to create a sealant for the ceramic vessel (McGovern et al. 2009:7364).

In this study there are several things that the researchers did correctly as well as some things that could have been improved. Re-analyzing the ceramic residues from Abydos and Gebel Adda, which were initially analyzed in the 1990s (the Abydos residues in 1994), was a good idea. Instead of relying on past results, newer technologies and methodologies were applied that allowed the researchers to test previous results. The researchers emphasize this when they state: "As analytical instrumentation improves, chemists and archaeologists alike, should continuously test previous results, cull out any 'false positives,' and generate data as they are made available by excavation or analytical technique" (McGovern et al. 2009: 7364-7365). This concept should always be present in the minds of archaeologists, and this study is a good example of how this goal can be achieved. Through reanalysis of older samples, archaeologists can speak with greater confidence about the origins of these residues. Additionally, as these novel technologies and methodologies are developed, new information regarding these residues and the role ancient alcohol played within ancient societies will be uncovered (McGovern et al. 2009: 7364).

One area for improvement is in the headspace SPME analysis. The researchers state: "Compounds detected in the ancient pottery blank are assumed to derive from ancient and/or

modern ‘background contaminants’ attributed to groundwater percolation or sample handling… Possibly, some of the low-boiling compounds up to hexenal were also contaminants however, it is more likely that they were preserved within the ionic clay structure” (McGovern et al. 2009: 7364). This statement indicates that something was detected in a source where it should not have been detected, yet the researchers do not discuss what sort of parameters they had in place to confirm background contamination, or contamination due to sample handling. What would have been beneficial in supporting the validity of their results would have been to collect environmental samples which could then be compared to the pottery blanks. If the blanks and environmental samples matched, then one could assume that these signal peaks were caused by environmental leaching and should be excluded from the study (McGovern et al. 2009:7364). However, if these samples did not match, then it would indicate that there was potential contamination due to the handling of the samples and that the results of this study should be questioned. Without these steps it is possible to call some of the results into question, specifically for the headspace SPME, where these potential contaminants were uncovered. One thing to keep in mind regarding this point is that these ceramics were discovered and collected during the 1990s, and gathering environmental samples to rule out contamination may not have been considered at the time. If this was indeed the case, then these facts should have been established by the researchers within the framework of the study together with a discussion of the analytical parameters to avoid additional contamination upon retesting the samples. Ultimately, this study and experiment design reveal several key points for proper development while helping to illustrate the importance of establishing proper protocols to eliminate contamination, or when working with samples previously excavated, establishing the potential for contamination from the initial excavation and protocols to prevent further contamination upon retesting the samples.

Case Study 4: Anticancer Activity of Botanical Compounds in Ancient Fermented Beverages

In another study published by McGovern and colleagues (2010), the researchers once again focus on the potential medicinal properties of resinated wines, and in this case, the possible anticancer properties of these wines. To begin with the authors discuss several different medications currently used for the treatment of diseases and their origins within the natural world, including quinine for treating malaria, which is derived from the *Cinchona* species of trees and shrubs; salicylic and acetylsalicylic acids, from willow bark (from the *Salix* genus of plants); and paclitaxel, a compound found in yew bark, now sold under the trade name TAXOL, which is used as an anticancer medication. The goal of this particular study was to test for the potential anticancer properties of the botanical compounds in ancient fermented beverages, in this case resinated wine from Egypt and herbal wine from China (McGovern et al. 2010:5).

Beginning with the wine from ancient Egypt, the researchers discuss the tomb of Scorpion I of Dynasty 0 in Egypt at the site of Abydos. This very large and rich burial contained roughly 700 ceramic jars in three rooms in the tomb. Residue found in one of the jars of this burial was analyzed via LC-MS/MS, Headspace SPME, and thermal desorption gas chromatography mass spectrometry (TD GC-MS) to determine its nature and potential additives. The spectrometry analysis indicated biomarkers for grape wine (McGovern et al. 2010: 6), while botanical evidence for the presence of grapes as well as figs was found within the vessel, supporting the evidence for grape wine. However, grapes and figs were not the only compounds discovered; Headspace SPME indicated the presence of pine and/or terebinth tree resin, as well as eight terpenoid compounds, linalool, camphor, borneol, L-menthol, α-terpineol, carvone, thymol, and geranyl acetone. Several herbs were also identified that could have been the source

of the terpenoid compounds. Finally, the compounds of luteolin and ursolic acid were also identified in the Scorpion I wine (McGovern et al. 2010:6).

Next, the researchers discuss an herbal wine from the Shang Dynasty of China. This residue came from the Changzikou Tomb in Henan province and was dated to around 1050 BCE. The liquid residue was recovered from a lidded bronze vessel and was analyzed via TD GC-MS. Analysis indicated the presence of two aromatic compounds, camphor and α-cedrene, as well as benzaldehyde, acetic acid, and short-chain alcohols. Two herbal compounds that researchers believed to be the source of these compounds are *Artemesia annua* and *Artemesia argyi* (McGovern et al. 2010:7).

After establishing that these two wines contained botanical additives, the next step was to identify the possible anticancer properties of these botanical compounds to determine whether they might have been used as a medical remedy by these two ancient civilizations. To do this the researchers decided on a novel *in vitro* approach (studies carried outside a living organism to determine the impact of a particular compound within a controlled environment such as a laboratory), analyzing several different compounds for their impact on cell proliferation of HCT116 colon adenocarcinoma cell lines, as well as the P53 luciferase activity of these cancer cell lines (McGovern et al. 2010:10). Five of the compounds tested -- artemisinin, artesunate, borneol, isoscopoletin, and ursolic acid -- impacted P53 activity and inhibited tumor growth. Isosocopoletin inhibited tumor growth better than adriamycin, which is the well-established DNA anti-damage treatment used for tumor inhibition (McGovern et al. 2010:10). Due to this discovery, the researchers proposed *in vivo* and clinical studies of these compounds to not only further test the idea that these compounds were used in medicinal treatments in the past but also to develop new therapeutic drugs to combat cancers today (McGovern et al. 2010:11).

This study does several things well. First, the ORA conducted on these samples used a wide variety of analytical techniques to corroborate and support the results, allowing the researchers to have more confidence in their conclusions. Second, conducting *in vitro* studies on the identified compounds for their potential anticancer activity instead of simply stating that these compounds could have been used as a medicinal treatment for cancer, which would not have been discernible based on the results, was an effective approach to take. By conducting the *in vitro* study, the researchers were able to show definitively that these botanical compounds do in fact have an impact on tumor growth and cell proliferation. The results of these studies support the researcher's conclusion that these botanical compounds may have been used to treat medical issues, including the possibility that they were an early cancer treatment. This again shows the importance of being able to corroborate the evidence and results through a wide variety of analytical methodologies.

Case Study 5: Roman Amphora in the Adriatic

The next case study to be presented is by Jerković et al. (2011). The goal of this study was to "determine the chemical profile of the headspace and volatile organic compounds (VOCs) of the rare resinous organic deposit obtained from an ancient Roman amphora found in the Adriatic Sea" (Jerković et al. 2011: 7937). Amphorae are a type of Mediterranean pottery vessel that, depending on their shape, were used mainly to store two specific products, olive oil and wine. Globular forms of amphorae usually stored oil products, whereas wine was stored and transported in amphorae with a distinct elongated shape. Additionally, amphorae used to carry wine were "often coated with a thin layer derived usually from pine resin or resinous wood. Due to its hydrophobic properties this resinous coat acted as a water-proofing agent sealing the inside of the amphorae and gave the wine a special flavor" (Jerković et al. 2011: 7937).

There are many techniques that the researchers could have applied to analyze these residual compounds and they discuss the specific drawbacks of each of the different forms of derivatization—a process of chemically modifying a compound to make it more suitable for GC—they might have used. Derivatization also makes the compounds more volatile, or reactive, and consists of several methods including silylation, methylation, and alkyl chloroformates reactions. The drawbacks of silylation are that it must be performed under anhydrous, or water-free, conditions and often requires the samples to be heated prior to being injected into the GC column. Methylation and alkyl chloroformate reactions cause the formation of side products, and while the ratios of the various solvent components for alkyl chloroformate reactions can be optimized to reduce the formation of these side products, they can never be eliminated completely (Jerković et al. 2011:7938). Furthermore, the researchers noted that research on organic residues primarily focused on "polar high-molecular weight compounds, neglecting the possible presence of low-molecular weight volatiles" (Jerković et al. 2011: 7937). Focusing on low-molecular weight volatile compounds allowed the researchers to analyze these compounds directly without derivatization (Jerković et al. 2011).

The analysis focused on a residue from the bottom of a "Greco-Italina amphora type Benoit Republicain-II/Lamboglia vessel" found in the Adriatic Sea near Vis Island (Jerković et al. 2011: 7946). These amphorae were produced in the Roman province of Campania from the mid-2nd century BC to the end of the 1st century AD. The sample was taken from the inner part of the residue, which measured around seven centimeters in length with a thickness of three centimeters. The reason for selecting this sample site was to avoid potential contamination found on the external surface. In addition, pine resin samples were taken from pine trees from Croatia's Adriatic coast to serve two critical purposes: (1) to help to condition the fibers used in the

headspace solid phase microextractions, and (2) to act as a control during the GC-MS analysis (Jerković et al. 2011:7946).

To analyze these compounds, the researchers conducted headspace solid phase microextractions (HS-SPME) with both divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) and polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibers. The fibers were then conditioned and equilibrated, prior to being introduced to the headspace where volatile compounds were then extracted via the fiber and then analyzed via GC-MS (Jerković et al. 2011:7941). Finally, in addition to the compounds gathered through HS-SPME, the researchers analyzed a portion of the residue found within the amphora. About 15 milligrams of the residue were dissolved in dichloromethane. This mixture was then used for GC-MS. In addition to preparing the ancient residue for analysis, the pine resin collected from the Croatian coast was also prepared identically to the residue to act as a control. All samples that underwent analysis were run in duplicate (Jerković et al. 2011:7946).

The researchers analyzed a total of 79 headspace and volatile compounds from the organic residues from the bottom of the amphorae. The individual peaks of each of the compounds was identified using a comparison of the retention indices seen within the headspace and residue samples, to the retention indices of authentic samples as well as comparing the mass spectra with the Wiley 275 MS Library and NIST02 Mass Spectral Database. The researchers discovered that the chemical profiles of the compounds found in the headspace as well as the dissolved residue differed from one another. Headspace compounds contained evidence of monoterpenes, as well as traces of compounds that give wine its distinct aroma, whereas the residue contained information regarding the waterproof coating applied to the amphorae. Monoterpenes were predominant in the headspace of the pine resins sampled off the coast of

Croatia (Jerković et al. 2011:7936). These highly volatile compounds, specifically α-pinene, were found in much higher concentrations in the resin but they were also found in even lower concentrations of the headspace. Furthermore, the headspace also contained several low-molecular weight aliphatic carboxylic acids and esters such as acetic acid, ethyl acetate, isoamyl alcohol and heptanal. In addition to these compounds, several highly volatile benzene derivatives, such as benzaldehyde, 2-phenylethanol, and others were also detected in the headspace of the amphora. However, these specific compounds were not found in the headspace of any of the pine resin samples taken, indicating that these compounds were preserved within the structure of the organic residue (Jerković et al. 2011:7945). The researchers noted that biomarkers for wine such as tartaric acid were not found within the headspace, or in the GC-MS results. However, tartaric acid is an extremely water-soluble compound, and therefore can easily leach into the environment. Another biomarker for wine, albeit less significant than tartaric acid, known as syringic acid, was also not detected. However, syringaldehyde, a compound that occurs as syringic acid breaks down, was detected within the organic residue sample (Jerković et al. 2011:7945).

GC-MS analysis of the sample revealed two high-molecular weight diterpenes, methyl dehydroabietate and retene. These two molecules occur when abietic acid, a major component of pine resin, is exposed to high temperatures and breaks down through the processes of dehydrogenation and decarboxylation, which is the removal of a hydrogen atom and carboxyl group respectively causing the abietic acid to break down into retene (Jerković et al. 2011:7945). Methyl DHA is formed when pine resin is heated in the presence of wood. Heating wood to high temperatures causes the release of methanol (CH_3OH), which then interacts with DHA to form Methyl DHA (Jerković et al. 2011:7945) The presence of both retene and methyl DHA indicates

that the pine resin used for sealing the amphora was heated in the presence of wood from pine trees. Researchers noted that certain oxygenated products seen in other ancient residues including 7-oxo-DHA, were not present in the samples from these amphorae. However, the researchers noted that due to the preservation in the Adriatic Sea, these oxygenated products would not be present as the substance had not been exposed to oxygen (Jerković et al. 2011:7945).

The conclusion the researchers came to was that these low weight molecular compounds can be important chemical tracers for unknown organic residues. Furthermore, conducting both headspace analysis and GC-MS of the sealed organic residue, in conjunction with other environmental samples, allows archaeologists to not only gain valuable insight into the material stored within amphora and other ceramic objects, but also the waterproofing method used by ancient cultures to seal otherwise permeable ceramic vessels (Jerković et al. 2011:7946).

Several aspects of this study reflect good experimental design. First and foremost, the researchers acknowledge that finding well known biomarkers for the presence of wine, such as tartaric acid, was highly unlikely due to the environment in which the amphorae were found, as well as the highly soluble nature of tartaric acid and the lack of oxygen needed to produce oxygenated biomarkers. This is another example of why it is important to understand how target biomarkers interact with the environment and how environmental preservation can impact what biomarkers will remain in the residue. This in turn directed the researchers to target novel biomarkers, in this case the low-molecular weight volatile compounds found in the headspace.

Second, the researchers conducted both the headspace and the GC and GC-MS analyses in duplicate, as well as testing potentially relevant environmental components such as the pine resins from the Adriatic coast of Croatia. By testing in duplicate, as well as testing environmental

samples, the researchers were able to confirm two critical pieces of information: (1) the results of the analysis are unlikely to be due to human error, and (2) the results are not due to environmental factors and represent the organic residue held within the amphora. This lends further support to their argument that the low-molecular weight compounds they detected were from the wine stored within the amphora and most likely contributed to the aroma of the wine that was produced, in addition to the waterproofing compounds found within the sample. Finally, the researchers included a list of instrument settings and information about where the samples were obtained. This information would allow archaeologists, chemists, and others to independently replicate the analysis discussed above, making it possible to test the original results of this analysis, lending further support to the legitimacy of these results. Ultimately, this is a good example of how to properly conduct ORA using appropriate experimental design.

Case Study 6: Pulque Production as a Dietary Supplement in Prehispanic Mesoamerica

As discussed in Chapter 3, Correa-Ascencio et al. (2014) wanted to determine whether pulque, a fermented beverage made from agave sap, was used as a dietary supplement by individuals living in the city of Teotihuacan. This is a prime example of appropriate experiment design, and application of ORA, as well as additional experimentation and data to help support and validate the results. If the researchers had only relied on the ORA of the potsherds from Teotihuacan, it would have been challenging to definitively confirm that bacteriohopanoids can be used as a biomarker to identify pulque production and storage. However, since the researchers also conducted an artificial aging experiment indicating that the ceramic matrix allows the catalysis of these bacteriohopanoid compounds into hopanes, the results of the GC-MS analysis were independently supported. Furthermore, by conducting the artificial aging experiment, the researchers gained a modicum of control over environmental

variables. Second, it helps to illustrate the importance of choosing the proper biomarker. As mentioned before, many biomarkers that indicate the presence of alcohol are water soluble, and/or can be metabolized by microorganisms within the soil. Choosing a hydrophobic biomarker increases the likelihood of the biomarker's survival.

Case Study 7: Maize Consumption in the Pre-Hispanic South-Central Andes

The next case study in this review was published by Lantos et al. (2015) and revolves around the consumption of maize in the early Andes. Maize, more commonly known as corn, was the main staple crop in the Americas, enabling human groups to establish sedentary food-producing societies. Much like other grain crops, maize can be converted into an alcoholic beverage, commonly known as chicha (Lantos et al. 2015:86). The focus of this study was the consumption of maize in the region of western Tinogasta to document "changes and continuities in maize preparation and consumption practices before and after Inka installation in order to provide insight into the foodways in Western Tinogasta" (Lantos et al. 2015: 86). Western Tinogasta is part of the south-central Andes and is located at the southwestern tip of the Catamarca province, in northwestern Argentina. The researchers selected pottery samples from several different sites dating to the first millennium AD, including La Troya LT-V50, Palo Blanco, Punta Colorada, Cardoso, Ojo del Agua, and El Zorro, among others. Organic residues were obtained from 22 different potsherds. Samples were taken from the lip, neck body and base of the pottery sherds, depending on what portion of the vessel was available, and analyzed for macroscopic evidence of residues. Once evidence of residues was identified, the ceramic potsherds were analyzed via GC-MS and stable C isotope analysis (Lantos et al. 2015:86).

Results indicated the presence of starch compounds, lipid compounds derived from maize, and long chain alcohols indicative of maize. This indicates that many of the vessels were

used at one point to process and cook maize. Evidence alcohol production was revealed by the stable C isotope analysis, which indicated the presence of both C_3 and C_4 plants. Specifically, a ceramic sample identified as A-18 had a higher concentration of $\delta^{13}C$ isotope values indicating the presence of C_4 plants, which includes maize, whereas ceramic samples designated as A-21 and A-22 had $\delta^{13}C$ values indicative of C_3 plants (Lantos et al. 2015: 96). This led the researchers to believe that A-18 was a ceramic vessel used to produce and store chicha, whereas A-21 and A-22 could have come from ceramics that were used to produce aloja, a fermented beverage made from the flour of algarroba pods, the fruit of a species of tree found in central Argentina. Spanish settlers gave the tree the name Algarroba blanco, or white carob tree, as it has features like the carob trees native to the Mediterranean. Additionally, ceramic samples A-08 and A-17 had intermediate values of $\delta^{13}C$, which the researchers interpreted as evidence that these ceramics were used to store, transport, or produce chicha and aloja (Lantos et al. 2015: 96). Furthermore, animal fatty acids were also indicated in these vessels, although the lack of soot on their surfaces indicates that they were not used for making maize stew, and that the fatty acids derived from animals were most likely the result of a waterproofing method applied to reduce seepage of the fermented beverage (Lantos et al. 2015: 96).

While this study does have some strengths, a few things could have been done better. Using multiple analytical methods supports the conclusion that the residues originated from maize. Furthermore, the researchers do well to distinguish the various types of molecular compounds present within the different residues, as well as other physical evidence, including a combination of plant and animal residues to distinguish between vessels that were used for producing maize stew versus ones that could have contained fermented beverages. Additionally, the use of stable isotope analysis to determine which vessels contained C_3 or C_4 plants to

corroborate which plant was used to produce which fermented beverage was also effective. However, the lack of corroboration between the isotope analysis and the GC-MS results to definitively confirm the presence of fermented beverages raises some questions regarding the validity of the results. The researchers could have used the starch analysis results to support the interpretation of the presence of chicha, including whether some of the starch granules showed alterations such as gelatinization of amylose and amylopectin layers of the granule. These results, in conjunction with the stable isotope analysis, would have helped to support the claim that the maize grains were processed to allow for easier fermentation. While the primary focus was to identify the changing foodways of this region, alcohol production is an important part of how people process and use excess grains, as well as extend the shelf life of perishable products and should have been considered here.

These researchers, like many others, also tend to avoid discussing the purpose of other potential compounds in the residue. Many of the studies discussed in this review automatically assume that if the compounds are not directly associated with the production of alcohol, or are a byproduct of alcohol production, they must have been used to either flavor the alcoholic beverage or to seal the vessel to prevent the loss of the alcoholic beverage. Additional investigation into these compounds may not negate the evidence for the production, storage, or consumption of alcohol, but could help further illustrate the uses of ceramic vessels, like the study discussed in Chapter 2 of this thesis. This leaves ample room for additional investigation, using newer or different methodologies, as well as an opportunity for others to attempt further organic residue analysis following the more rigorous guidelines discussed in this thesis.

Case Study 8: Revealing Invisible Brews: A New Approach to the Chemical Identification of Ancient Beer

The production and consumption of alcohol was an integral part of a wide variety of civilizations throughout the ancient world. One region where alcohol, particularly beer, was important was Mesopotamia. Beer was a nutritious dietary staple and a key component in feasting and ritual practices, which is why the region has a plethora of iconographic and textual sources related to its production. Perruchini et al. (2018) examine the production of ancient beer from the site of Khan Masi in northeastern Iraq and discuss how advances in ORA have helped to solidify evidence for the production, storage, and consumption of beer. Prior to this ORA revolution evidence of beer was primarily restricted to the presence of visual residues, i.e., beerstone (calcium oxalate) on ceramics, which was linked to production and storage vessels used in the brewing process (Perruchini et al. 2018:176). Researchers had two primary goals for this study: (1) to use ORA as a method to definitively identify vessels that were used for the serving and consumption of beer to gain insight into both daily and extravagant consumption practices, and (2) to "lay the methodological groundwork for such a study by presenting a new field-based sampling protocol for archaeological organic residue analysis that prevents the risk of sample contamination, as well as a new analytical approach using gas chromatography and compound co-occurrence for the identification of ancient beer" (Perruchini et al. 2018: 177).

Khan Masi is located along the upper region of the Sirwan/Diyala River in northeastern Iraq, between the Zagros piedmonts and the Mesopotamian lowlands. The site is composed of roughly a dozen mounds covering around 120 hectares (Perruchini et al. 2018:177). Evidence indicates that the site was occupied from the Neolithic period up until the modern period. The material culture of Khan Masi suggests a strong cultural connection with central Mesopotamia.

The site is positioned along a series of movement corridors, including the Khorasan Highway and silk routes. A magnetic gradiometer survey indicated a series of rooms roughly 30 meters by 40 meters, with the largest room centrally positioned (Perruchini et al. 2018:177). In the large central room designated as Room 1, archaeologists uncovered a large collection of ceramic vessels, potentially indicating that it was used to host events that involved significant amounts of beer consumption. These ceramic vessels were the primary sources sampled for the ORA (Perruchini et al. 2018:179).

The researchers sampled residues from 15 different ceramic vessels, representing a wide array of serving and consumption containers including Kassite goblets, small footed cups, a medium sized bowl, a namzitu, and small juglets. None of the vessels sampled showed visible evidence of residues such as beerstone, making them prime candidates for this study (Perruchini et al. 2018:181).

One major issue that the researchers attempted to avoid was the contamination of the ceramic samples. Previous studies of similar ceramics were contaminated due to several factors, including handling the sample without gloves, storing and transporting samples in plastic bags, and being exposed to chemicals such as DEET. While modern contaminants are easily excluded as there is no ancient equivalent, other potential contaminants with ancient equivalents are much more difficult to identify and exclude, making the results of previous studies unreliable in the researchers' opinion (Perruchini et al. 2018:182). Therefore, a strict protocol was implemented to reduce exogenous contamination of the 15 ceramic sherds analyzed from Khan Masi. All samples were handled with cotton gloves, and any sample manipulation was carried out using instruments sterilized with acetone. Furthermore, samples were protected during transport by being wrapped in aluminum foil that had been heated to 450°C for eight hours to eliminate any

contaminants from the foil and to protect the samples from plasticizers or other contaminants from the plastic transport bags. Finally, soil samples from the immediate area, as well as from within the vessels sampled, were collected to control for environmental contamination such as leaching. These soil samples were analyzed in the exact same manner as the ceramics (Perruchini et al. 2018:183).

The ceramic samples were analyzed via GC-MS, and due to the sampling regime used by the researchers, only a small number of modern contaminants, such as phthalates and phenols, were detected. The results of the ORA indicated the presence of several unique compounds, including carboxylic and dicarboxylic acid, benzoic acid, butanedioic acid pimelic acid, suberic acid, and azelaic acid. Furthermore, ORA indicated the presence of hydroxycinnamic and methoxycinnamic acid. These are two phenolic compounds that are often associated with pulses and cereals such as wheat and barley. Glycerols as well as long chain fatty alcohols, and midchain monosaturated and diunsaturated fatty acids, were also detected along with even chain alkanes (Perruchini et al. 2018:183). The combination of long chain fatty alcohol molecules with even chain alkane molecules is commonly associated with plant waxes. Additionally, the mass spectrometry results indicated the presence of squalene, which is a triterpene compound. This compound is commonly seen as a contaminant as it is produced from the skin sebum or oil glands, however it also has been documented and identified in germinated grains such as barley (Perruchini et al. 2018:183). Finally, two aldehyde compounds, erucylamide and 9-oxadecenal, were identified in six of the groups of ceramics sampled. One group also produced evidence of the presence of two wax esters, palmitoleate and oleyl oleate. Based on these results, the researchers concluded that the vessels were used to serve a beer most likely made from barley and other cereals (Perruchini et al. 2018:184).

This study illustrates the importance of establishing rigorous sampling methods to control for potential contamination sources. As previously mentioned, the researchers uncovered a compound known as squalene, which in previous studies had been identified as a source of human contamination. One would naturally assume that its presence in these samples would be attributed to contamination by human hands. However, based on the protocols established by the researchers, it is clear that the ceramic sherds were never touched with bare oily hands. The protocol established was to only handle the ceramics while wearing cotton gloves, as well as manipulating the samples with sterilized equipment and utensils. This means that it would be nearly impossible for the presence of squalene to come from a recent human source of contamination. Therefore, the presence of this compound now becomes archaeologically significant, as the only other source this compound could come from is plant germination, a critical step in the fermentation process.

The researchers not only established a protocol to control human contamination, but they also established a protocol to control environmental sources of contamination. As mentioned previously, samples were taken from the surrounding soil within the area as well as soil directly from the exterior of the artifact, and from the interior space of the artifact, and then these samples were subjected to ORA. The results were compared to those of the ORA conducted on the ceramics to search for contamination. None of the compounds found in the residues of the ceramic matrix were detected in any of the soil samples. Therefore, the researchers were able to consider all the residue results from the ceramics to be archaeologically significant. By using this rigorous protocol for sampling, controlling for and eliminating all potential sources of contamination, the researchers were able to provide significant results that are very difficult, if not nearly impossible, to refute. This is a prime example of how to appropriately design research

and experiments involving ORA of archaeological samples, and why it is crucial to employ rigorous protocols and controls.

Chapter 5: Critique, Discussion, and Conclusion

This thesis has discussed several case studies involving ORA and reflected on what they have done well versus what areas reveal weaknesses in the application of ORA to the study of ancient alcohol. It is important to consider critical evaluations of ORA, especially those focused on beer production, which has been subject to receive more criticism than other forms of ancient alcohol analyzed using the approaches discussed in preceding chapters (Driscoll and Damm 2023). A good example is the use of substances like calcium oxalate, a crystal structure formed from the combination of a calcium salt and oxalic acid that is more commonly known as beerstone. Since the 1990s this has been considered the main biomarker for the production of beer and its presence was considered sufficient for the identification of that beverage in ancient containers. However, while certain ORA methods allow for the direct identification of calcium oxalate other analytical methods, such as FTIR, LC-MS and HPLC, Feigl spot tests, GC-MS, and IC, are only able to detect oxalic acid or its derivatized compounds. This means that studies using these methods need additional interpretive steps to determine the presence of calcium oxalate. Furthermore, studies only using Feigl spot tests to determine the presence of calcium oxalate are on even more analytically shaky ground due to this methodology's propensity for false positive results (Driscoll and Damm 2023: 15). This again illustrates the importance of selecting the appropriate analytical methodology for the problem, as well as knowing the specific issues, like an increased rate of false positives, associated with that methodology. Furthermore, if the proposed methodology has certain limitations, then it is best to back up the results with another methodology.

The next issue with automatically associating calcium oxalate with the production of beer is related to the fact that there are many other potential sources of calcium oxalate in nature. Not only is calcium oxalate generated during the production of beer, when oxalic acids released by the cereal grains interact with the calcium in the water to form calcium oxalate, it is also found naturally in over 1000 genera of plants as a crystalline structure known as a raphide, which helps to form the structural tissues of plants. In addition to this, calcium oxalate is present in animals and soils and can be found coating rock faces (Driscoll and Damm 2023: 15). Finally, one other potential source of calcium oxalate that has not yet been explored in depth in the field of archaeology comes from human urine. Kidney stones are composed of roughly 90% inorganic crystal material, with most of those inorganic compounds made up of calcium oxalate crystals and the remaining composition consisting of several organic proteins such as Tamm-Horsfall proteins (THP), α-globulins, or γ-globulins (Kaneko et al. 2015). This shows the range of potential sources of calcium oxalate that could deceive archaeologists into believing that a certain vessel was used to produce, store or consume beer. This reinforces the need for rigorous experiment and research design, choosing the appropriate methodology to isolate and analyze the target biomarkers, as well as having additional supporting data to corroborate evidence as opposed to relying on a single data source to identify the origins of an unknown residue.

Misinterpretation and misapplication of biomarkers, particularly lipids, can also cause potential problems. As mentioned previously, certain biomarkers are ubiquitous and have multiple natural sources, making it difficult to reliably determine the origins of an unknown residue. This again illustrates the importance of selecting an appropriate biomarker in choosing an ORA application (Whelton et al. 2021). A good example is maize, a starchy grain that was a staple diet in the Americas as well as the key ingredient in the production of chicha, the most

common fermented beverage found within Central and South America. Maize however rapidly decomposes, to the point that ceramic vessels used to cook maize will only retain the lipid biomarkers for this food crop for roughly 3 months after deposition (Reber and Evershed 2004: 406). In addition, these lipids decompose rapidly when absorbed by the ceramic matrix, indicating that these biomarkers may be a poor choice for identifying the presence of maize in archaeological contexts. Finally, experiments with maize residues have demonstrated that there is no species-specific biomarker that can be used for the presence of this plant, and that the lipids from cooked maize could easily be masked by other lipids from other sources, such as meats, fish, or other plants (Reber and Evershed 2004: 407). All of this indicates that archaeologists must be very cautious when interpreting results identified through ORA. It is perfectly acceptable to be optimistic as to the origins of an unknown residue, but we should always temper our optimism with the evidence obtained and ensure that our assessment and identification of unknown residues is supported and corroborated by additional lines of the evidence. This also helps to illustrate the continued importance of interdisciplinary collaboration, to seek out new and better biomarkers unique to a single foodstuff, organism, or other consumable.

Discussion

The studies presented here have illustrated several key points regarding how archaeologists can successfully employ ORA methodologies to generate robust results that can hold up to scrutiny, as well as illustrate the potential pitfalls that researchers can stumble into when employing poorly designed research and experimental protocols. The case studies that conducted effective ORA often employed multiple ORA methodologies, established rigorous protocols for sampling to reduce and eliminate contamination, made use of control samples, and compared environmental and archaeological samples, allowing for more reliable determination

of the source of the residue. Furthermore, successful studies often relied on additional evidence, such as the shape of the vessels, presence of macro-botanical materials, potential waterproofing methods, ethnographic analogy and experimental archaeology to help build further support and scaffolding for the ORA results. Additionally, studies that ran the ORA in duplicate or triplicate also tended to produce more reliable and robust results that were less likely to be affected by contamination or human error. The studies also illustrate the importance of selecting the appropriate analytical methodology to be either targeted (looking for a specific protein or compound), or in the case of the round robin study, by using a non-targeted methodology, making identification of the mysterious residue more likely (Barker et al. 2011). Studies that had weaker results often relied on a single ORA methodology, did not employ appropriate protocols to avoid contamination, or lacked a thorough understanding of the limitations of the selected residue analysis method. These case studies provide both models and cautionary tales for future archaeologists interested in conducting organic residue analysis in the quest to uncover the role of ancient alcohol in human societies.

To continue the refinement of ORA methods, it is imperative that interdisciplinary approaches to interpreting results are incorporated into the training of future anthropologists and archaeologists as well as the chemists and other scientists who collaborate with them. Potential ways to increase this sort of collaboration could come through coursework requirements at universities, directly exposing archaeologists and anthropologists to the laboratory sciences. This would provide for a better foundation for understanding the methodologies that archaeologists may encounter, while chemists and other lab scientists should be exposed to the social sciences and humanities. This would give both groups of researchers an opportunity to explore fields outside of their main realm of study, providing members of each community to work together by

proposing and solving problems that might not be otherwise be noticed. Having different perspectives and worldviews while working together towards a common goal will continue to advance the field of ORA towards the paradigm shift and eventual revolution that appears to be imminent.

Conclusion

To conclude, this thesis has illustrated how scientific advances impact the disciplines of anthropology and archaeology, and how these methodologies and concepts can be applied in a beneficial way to archaeological research. In addition, the creation of appropriate experimental protocols was outlined and several ORA case studies analyzing ceramic vessels thought to have contained alcoholic contents were used to illustrate good as well as poor research design. ORA has the potential to push archaeology into the next paradigm shift, and revolution for the field, with the appropriate input and guidance from archaeologists working collaboratively with natural scientists. While ORA has been around for many years, there are still some issues with the technologies involved, including the fact that certain methodologies have high rates of false positives or are unable to detect specific markers. Therefore, close interdisciplinary work with chemists and other scientists to help direct the next wave of instrumentation and methodologies will be necessary if the ORA is to live up to its potential. While it may seem that the natural and social sciences are on opposite ends of the theoretical and methodological spectrum, it is possible for both research communities to gain valuable insights through interdisciplinary collaboration. It is with this interdisciplinary collaboration that archaeologists can help to set the stage for future research. We have seen that certain biomarkers are ubiquitous, so additional or new biomarkers are needed to better help identify these unknown alcohol residues. For instance, alkyl resorcinols and miliacin could serve as better biomarkers for identifying the production of beer as they are

directly related to cereal grains such as barley and millet, which were used in the production of ancient alcohols (Driscoll and Damm 2023: 24). In addition, identifying markers for the ergot fungus in residues could also prove beneficial for the identification of cereals in vessels that might have been used to produce, store or consume alcoholic beverages. Finally, isotope analysis can be used to distinguish between C3 and C4 plants, lending stronger credence to ubiquitous biomarkers suggesting the presence of the grains known to be used in the production of beer (Driscoll and Damm 2023: 25).

Through interdisciplinary collaboration archaeologists have an opportunity to work with chemists and other scientists to help direct the search for new biomarkers. By working in tandem with chemists, archaeologists can help direct experiments and technologies to better seek out compounds, as well as enhance and improve the technologies currently being used to identify these residues. Chemists, engineers, and others who work with and design these technologies are not focused on their archaeological application. Therefore, it is essential to create a bridge of communication between anthropologists and chemists, engineers and other scientists to help drive the research forward in a way that can be both beneficial to the scientific and medical community, from which these methodologies are derived, and the anthropological community. This will allow us to learn more about the past, present, and future of the human condition. The study of ancient alcohol in archaeology provides a perfect example of how aspects of these two areas of study can be implemented in a way that delivers robust results that stand up to scrutiny. Ultimately, as science advances and new technologies are developed, archaeology will continue to benefit from collaboration with the scientific community, pushing the boundaries of archaeological investigations even further and uncovering new aspects of humanity's collective past.

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