CHAPTER 3

Methodology:

Organic residue analysis and the identification of lipid biomarkers in pottery

Introduction

The research questions raised in the introduction concern the reasons for pottery adoption and dispersal in Alaska. They align with wider debates in archaeology that are aimed at understanding cooking traditions and the role durable containers play in those traditions. The identification of the function of pottery, but also other (rival) container technologies in the region, is vital to understanding those driving forces. Organic (lipid) residue analysis can be used for the identification of prehistoric vessel contents, which is directly connected to vessel function. What were people cooking, and what not? Did pottery have a specialized function or was it used in a more generalized way? Answering such questions has shown to provide new and interesting interpretations of pottery use throughout the prehistory of the Old World (Colonese et al., 2017; Craig et al., 2013; Dunne et al., 2012; Lucquin et al., 2016b, 2018; Outram et al., 2009), as well as the New World (Anderson et al., 2017; Colonese et al., 2014; Farrell et al., 2014; Taché and Craig, 2015), and has led to a greater understanding of prehistoric societies in general (Jordan and Gibbs, 2019; Jordan and Zvelebil, 2009).

The exploitation of aquatic resources is a well-known and well-recorded practice in Alaskan prehistory (e.g., Dumond 1998; 2016; Clark 1975; Fitzhugh 2016). In recent years the use of ceramic containers for the processing of marine and freshwater products in Alaska has been evidenced through the application of organic residue analysis (Farrell et al. 2014; Anderson et al. 2017). Indeed, the expectations for this research were to find similar signals of aquatic products in the pottery of the Southwest Alaskan Norton, Thule and Koniag

ceramic traditions. In this chapter the identification of such aquatic products by organic residue analysis will be discussed in detail. Furthermore, a short introduction into the principles of organic residue analysis, and the history of the method, will be provided.

History of the Method

A profound interest in the study of organic residues such as lipids, proteins and DNA has been present in the archaeological discipline for nearly a century. While a lack of suitable methodologies for the analysis of organic residues slowed down the progress of the biomolecular archaeological field, the possibilities of the analysis of small organic residues in archaeology was acknowledged as early as the 1930s (von Stokar, 1938). Especially the presence of ancient DNA (aDNA) in archaeological specimens such as Egyptian mummies, received considerable attention in the 1980s when such aDNA was first recovered (Pääbo, 1985). The study of lipids in archaeological residues from ceramic vessels was shaped in the early years by Richard P Evershed (Evershed, 1993; 2008; Evershed et al., 1990; 1994; 1999; 2008ab) who brought the field of biomolecular archaeology to a new level.

Today, bioarchaeological methods are a fundamental part of archaeological studies, and methods are tested and refined continuously. Determining ancient pottery vessel contents through lipid residue analysis allows to elaborate on big research topics such as the first adoption and evolution of pottery (e.g., Craig et al., 2013), the introduction of farming and pastoralism (e.g., Craig et al., 2005; Dudd and Evershed, 1998; Dunne et al., 2012; 2019; Heron et al., 2015), and, of main concern to this research, the processing of aquatic resources in pottery by pre-agricultural hunter-gatherer groups (e.g., Gibbs et al., 2017; Anderson et al., 2017; Craig et al., 2013; Farrell et al., 2014; Horiuchi et al., 2015; Lucquin et al., 2018).

Application of the method is, however, not restricted to pottery but can also be applied to other artefacts (i.e. objects and tools) in archaeology that contain organic residues. In fact, clues about the processing of aquatic resources by prehistoric humans in Alaska have been evidenced at several occasions: on stone vessels such as bowls (Admiraal et al., 2019), and lamps (Solazzo and Erhardt, 2007). But also at 'first Americans' sites of great antiquity (ca. 15.000 cal BP) in the Tanana Valley, where the soil of hearth features has been tested for residues. Results of such pioneering work are promising (Choy et al., 2016; Crass et al., 2011; Kedrowski et al., 2009). The analysis of soil in activity areas of archaeological sites is very much upcoming (Buonasera et al., 2015). Studies such as these may push the evidence of the use of aquatic resources back in time, far beyond the pottery horizon. Moreover, it can also contribute to a wider understanding of (sub)Arctic culinary practices in contexts where pottery is present by comparing different technologies (e.g., other container technologies vs. pottery), or even roasting (i.e., evidence from hearth features) vs. pottery.

The principles of organic residue analysis

During the cooking and processing of food or other organic commodities, residues get deposited on the surface of the pottery vessel as carbonized surface deposits (i.e. foodcrusts), and get absorbed into the porous ceramic walls of the pot. Entrapped in these organic (foodcrust), and mineral (ceramic) matrixes, they are protected from many degradation processes (e.g., microbial, water leaching, etc.), and can persist for thousands of years. Organic residues may refer to carbon-based substances sticking to the surface of pottery in the form of carbonized crusts, or absorbed into the porous pottery walls. Organic residues on pottery can originate from cooking, storing or manufacture of the ceramic vessel. The definition of "organic residues" encompasses a wide range of amorphous (i.e. dependent on chemical analysis for interpretation) organic remains that

includes ancient DNA, proteins and carbohydrates, here the focus is on **lipids** (Heron and Evershed, 1993).

Lipids

Lipids are hydrophobic organic molecules and compounds that occur widely in living organisms in the form of fats, oils, waxes, resins and steroids. They are mainly composed of carbon, hydrogen and oxygen, and their arrangement around the carbon core can be either linear, branched or cyclic (Heron and Evershed, 1993). The hydrophobic (i.e., water repelling) property of lipids is due to this structure and increases the chances of preservation of these molecules in the archaeological record as it makes them less soluble in water. This makes this biomolecule an attractive subject of study in biomolecular archaeology (Evershed, 1993). The term "lipids" encompasses several major molecule varieties: triacylglycerols (TAGs), fatty acids, wax esters, sterols, *n*-alkanes and *n*-alkanols, and terpenes.

Triacylglycerols, or TAGs, are the main constituents of animal fats and plant oils and are important and efficient energy stores (Brown and Brown, 2011). TAGs are made up of three fatty acid chains that are bound (esterified) to a glycerol backbone (fig. 3.2). **Fatty acids** are straight hydrocarbon chains with a carboxyl group at the end. The carbon atoms in a fatty acid chain may be connected by single bonds (i.e. saturated), or double bonds (i.e. unsaturated) (Gunstone, 2009). Naturally occurring fatty acids generally have even numbered carbon length, of which chain length 14-22 is most common, palmitic (C_{16}) and stearic acid (C_{18}) are the most common fatty acids.

Fatty acids rarely occur free in nature. They are generally part of larger compounds such as TAGs and wax esters. Bonds such as the ester bond between the TAG glycerol backbone and the fatty acids are fragile, and are therefore prone to degradation. When the ester bond

breaks, the fatty acid "tails" are released, forming free fatty acids. These free fatty acids can vary in carbon length and their characteristics may inform on the original source of the TAGs (Evershed, 2008). In some cases, when hydrolysis is incomplete this may also lead to the formation of monoacylglycerols (MAGs), with only one fatty acid chain remaining attached to the glycerol backbone; and diacylglycerols (DAGs), with two fatty acid chains remaining. These degradation markers are rare because of the fast rate of the degradation process once it is initiated (Dudd et al., 1998).

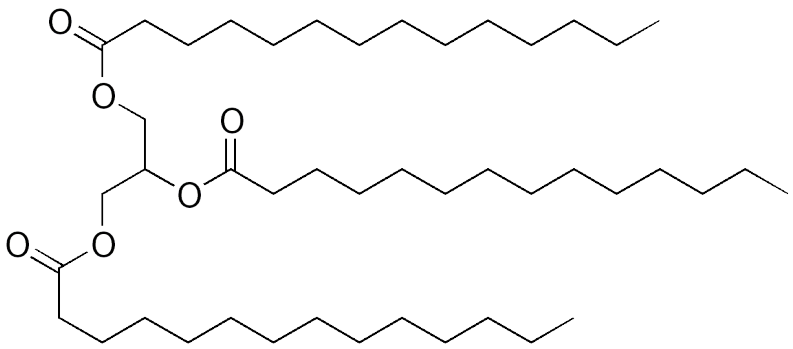


Figure 3.1: structure of a triacylglycerol showing a glycerol backbone and three fatty acid chains.

Sterols are minor components of plants and animals, and are sometimes difficult to identify in lipid samples because of their relative low abundance compared to fatty acids. Nevertheless, sterols can be used to discriminate between plant and animal sources (Heron and Evershed, 1993). **Cholesterol** is the main sterol in animal fats, including marine mammals and fish. Common phytosterols (plants) are stigmasterol, β-sitosterol and campesterol. Ergosterol is common in fungi (Gunstone 2009). In general, sterols are more resistant to degradation than fatty acids, they are however much less abundant.

Alkanes have very simple chemical structures which makes them fairly resistant to degradation (Heron and Evershed, 1993). Alkanes are comprised of an unsaturated straight hydrocarbon chain (*n*-alkanes, *n* stands for "normal"), or a hydrocarbon structure in cyclic

structure (aromatic). *n*-alkanes, of odd numbered carbon length, are degradation products of beeswax and plant waxes that are produced by hydrolyzation of the wax ester. Similarly, **linear alcohols**, or *n*-alkanols, are comprised of a straight chain with a hydroxyl functional group (OH) at the terminus. These compounds are found in tissues, and are also often part of wax esters.

Waxes often fulfill protective functions for example in the leaves of plants, or the skin, fur or feathers of animals and/or humans (e.g., wool wax, beeswax) (Berg et al., 2012; Brown and Brown, 2011; Evershed, 1993). Wax esters comprise of a fatty acid chain bound by an ester bond to a long-chain alcohol (*n*-alkanol), sugar, or sterol (fig. 3.3). Hydrolysis of the ester bond will cause the release of free fatty acids and alcohol chains, as with TAGs (Gunstone, 2009).

Terpenes are a very diverse lipid group with over 25.000 different compounds. They occur widely in higher plants and can be diagnostic of their origin. They are a major component of resins that were widely exploited throughout prehistory, such as pitches, tars and adhesives, especially pine, birch and spruce resin (e.g., abietic acid). Terpenes are made up mostly of hydrocarbons, making up a five-carbon isoprene building block $(C_5H_8)n$ (*n* stands for the number of isoprene units in the terpene). They are characterised based on the number of isoprene units in the molecule. Di- and triterpenes (20 and 30 carbon atoms respectively) are most commonly found in archaeological contexts (Harborne, 1984; Aldred et al., 2009).

General procedures of lipid residue analysis of ceramics

The method of organic (lipid) residue analysis uses various protocols to extract the preserved lipids from the ceramic pot and subsequently employs gas chromatography - mass

spectrometry ("GC-MS") to identify the individual lipids. Furthermore, bulk isotope analysis (elemental analysis-isotope ratio MS "EA-IRMS") of carbonized surface deposits (often referred to as foodcrusts), and compound specific isotope analysis of individual fatty acids $C_{16:0}$ and $C_{18:0}$ (GC-combustion-IRMS "GC-c-IRMS") of lipid extracts further specify the origin of the archaeological residues (Evershed, 1993; 2008).

Sampling

Pottery may be sampled for the presence of ancient lipid residues in two ways, depending on the character of preservation. When carbonized surface deposits (i.e. foodcrusts) are preserved, these can be collected by scraping them off using a sharp tool such as a scalpel. Due to the generally high concentration of lipids in foodcrusts, a sample size of ~20mg is usually sufficient for acid extraction (see below). This sampling method is non-destructive for the artefact. It is important to note that foodcrusts are in general more prone to contamination because of their greater exposure to human handling, as well as to contaminants from the burial environment. They are often also not as well preserved as their absorbed counterparts, that are better protected by the ceramic matrix (Heron and Craig, 2015).

In order to obtain absorbed lipid residues from the ceramic matrix a destructive method of sampling is applied, in which the ceramic sherd is drilled into, using a small handheld drill. From an area of about 1-2 cm^2 (depending on the thickness of the pottery) approximately 1-2g of ceramic powder is collected for acid extraction (see below). To avoid contamination, the top layer (>1 mm) of the ceramic is removed. In general contamination is avoided by wearing nitrile gloves during all artefact and sample handling, and all equipment used is thoroughly sterilized.

Lipid Extraction Methods

Solvent extraction

Lipids may be extracted from the archaeological sample using different methodologies. Solvent extraction is a method that uses dichloromethane (DCM) and methanol (2:1) to extract lipids. Subsequently BSTFA (N,O-Bis(trimethylsilyl)trifluoroacetamide) is used for the silylation of the carboxyl and alcohol groups, this is called derivatisation (Evershed et al., 1994; Charters et al., 1995). Through derivatization it is possible to analyse less volatile molecules in their gaseous state through GC (Rezanka et al., 2016). This method is most useful for recognizing molecules deriving from plants (Evershed et al., 1990). However, some bound compounds (e.g., diacids, dihydroxy acids) are not extractable using this method and a different procedure is necessary to extract these compounds (Hansel et al., 2011).

Acid extraction

A newer method using acidified methanol (H_2SO_4–MeOH) accomplishes this, furthermore it requires a smaller sample and has higher lipid yields. Furthermore, it is much less time-consuming. The downside of the acid extraction method is the loss of compositional information, due to the hydrolysis of more complex lipids such as acylglycerols (TAGs, DAGs and MAGs) and wax esters (Correa-Ascencio and Evershed, 2014; Craig et al., 2013; Papakosta et al., 2015). Acid extraction was chosen as the main extraction method in this research (see chapters 4-7) for the reasons stated above. Moreover, one batch of samples (10) was extracted by both extraction protocols. The solvent extracted samples yielded very little extra information as compared to acid extracts of the same ceramic sherds. Furthermore, through the derivatization of the acid extracts we were able to detect dihydroxy acids and long-chain alcohols that were not visible in the original acid extracts.

Lipid biomarker identification by GC-MS

After extraction, the lipid extracts are analysed by gas chromatography (GC) and GC-mass spectrometry (GC-MS). In short, gas chromatography allows for the separation of the complex mixture of various molecules that can be vaporized without causing decomposition. The degree of separation depends on the length of the column through which the sample is run. Mass spectrometry subsequently identifies those separated molecules by measuring the mass to charge ratio of ions (*m/z*) (Evershed, 1992). The result is a chromatogram with various peaks eluting at different retention times. Each peak represents an individual lipid molecule or compound. The mass spectrum of those peaks, displaying the different ions, can be compared to those of known references, allowing for the identification of diagnostic lipids. These diagnostic lipids are referred to as biomarkers. In essence, **the biomarker concept** (Evershed, 1993; 2008), concerns the identification of archaeological organic matter through the comparison of compounds (single, or mixtures) present, to contemporary reference materials of known plants and animals (Evershed, 1993). However, degradation processes often modify the original chemical composition, making the comparison to modern references more complicated. Below a variety of biomarkers is discussed with a particular focus on aquatic biomarkers.

Aquatic Biomarkers

Of great importance to (sub)Arctic residue analysis research is the knowledge of aquatic biomarkers. Due to the excellent preservation in the High North the value of organic residues was recognized early on. The first analysis of archaeological residues from a prehistoric site in Arctic Alaska was published in 1984 by (Morgan et al. 1984). A midden deposit at the Washout (Thule) site on Hershell Island was sampled, extracted by hexane, and subsequently analyzed by GC. Morgan et al. (1984) aimed to identify individual species by comparing the

extracted 1,000 year-old fats to modern reference values on the basis of fatty acid distributions. Additionally, conclusions were based on the absence of certain unsaturated fatty acids. For example, the absence of $C_{22:1}$ was taken to mean fish made no significant contribution to the sample, in contrast to several species of seal. In fact, mono- and polyunsaturated fatty acids are abundant in all aquatic species. And importantly, the absence of evidence is not evidence of absence. Unsaturated fatty acids are more prone to degradation than their saturated counterparts due to the presence of double bonds. This could have led to their absence in the sample.

While this was an interesting early study of a promising material, the extraction method, the assumption that the materials were not severely degraded (despite the mention of freeze thaw processes, disregarding the concept of mixing, and an incomplete knowledge of lipid degradation, led to a possible misinterpretation of the material. While it is indeed highly likely that these particular samples were rich in aquatic oils, or even marine mammal oils, as suggested by Morgan et al. (1984), it would not have been possible to determine this with the methods used. In order to extract the compounds of interest, to determine the presence of aquatic lipids, a stronger extractant is needed. oday, the use of acid extraction (as described above) greatly enhances lipid recoveries, including free and bound lipids (Correa-Ascencio and Evershed 2014; Papakosta et al. 2015; Craig et al. 2013). Furthermore, even today, without isotope analysis it is not possible to distinguish between species within the aquatic spectrum.

Over the years we have come to learn a great deal more about these topics and the characterization of aquatic fats and oils (Hansel et al. 2004; Evershed et al. 2008; Hansel and Evershed 2009; Lucquin et al. 2016). Many studies over the last decade have demonstrated

the use of pottery for the processing of aquatic resources (Anderson et al., 2017; Farrell et al., 2014; Gibbs et al., 2017; Lucquin et al., 2018; Oras et al., 2017). There are several biomarkers to securely identify the presence of aquatic organisms in a lipid sample (Cramp and Evershed, 2014; Heron and Craig, 2015).

General aquatic lipid profiles of aquatic fats and oils (fig. 3.2) include mid-chain saturated fatty acids with palmitic acid $C_{16:0}$ and stearic acid $C_{18:0}$ as the main compounds,where palmitic acid is usually the dominant fatty acid. Monounsaturated fatty acids ($C_{16:1}$, $C_{18:1}$, $C_{20:1}$, $C_{22:1}$) are abundant in these profiles, as are dicarboxylic acids (diacids) ($C_{8\text{-}11}$), which are oxidation products of the former (Evershed et al., 2008; Regert et al. 1998; Regert 2011). Polyunsaturated fatty acids ($C_{20:5}$, $C_{22:6}$) are also common components of aquatic oils and fats. While the latter are rarely preserved in the archaeological record, they are abundant in the original product.

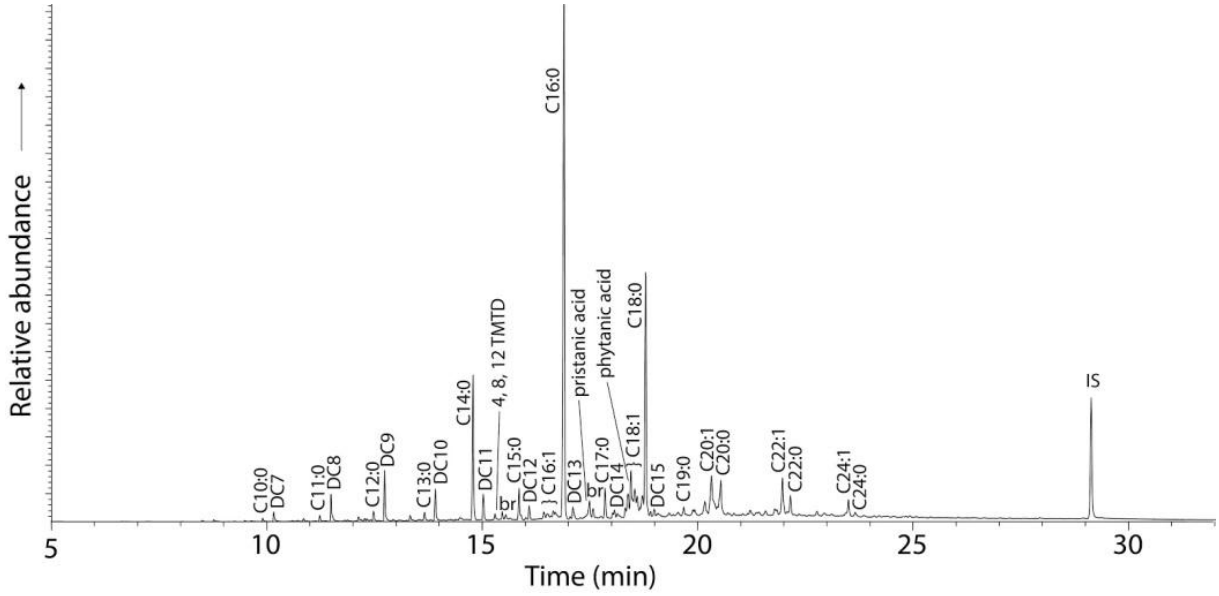


Figure 3.2: A typical total ion current of an acid/methanol extract of a stone bowl from the Margaret Bay site (UNL48-61b). A DB-5ms (5%-phenyl)-methylpolysiloxane column (30 m × 0.250 mm × 0.25 mm; J&W Scientific, Folsom, CA, USA) was used for scanning (for details on GC program see Admiraal et al., 2019). The chromatogram shows saturated fatty acids, diacids (DC), branched (br), isoprenoid acids (TMTD, pristanic, and phytanic acid), and long-chain unsaturated fatty acids (Admiraal et al., 2019).

The anthropogenic heating of these unsaturated fatty acids produces important diagnostic compounds that are not formed naturally, and may therefore not only inform us about the prehistoric vessel contents, but also of cooking practices (Hansel et al., 2004; Hansel and Evershed, 2009). These anthropogenic markers are referred to as **ω-(o-alkylphenyl) alkanoic acids** or APAAs $C_{18\text{-}22}$, and are formed during the prolonged heating (at a temperature of at least 270℃, for >4 hours) of mono- and polyunsaturated fatty acids (e.g., $C_{16:3}$, $C_{18:3}$, $C_{20:3}$, $C_{22:3}$) which occur widely in aquatic organisms (Hansel et al. 2004; Evershed et al. 2008). While these fatty acids are also present in plants and terrestrial animals (e.g., Shoda et al., 2018), those of carbon length 20-22 are only observed in significant quantities in aquatic organisms, and thus provide reliable evidence for the processing of these resources using heat (Hansel et al., 2004; Cramp and Evershed, 2014). Furthermore, the presence of these compounds eliminates the possibility of contamination from aquatic fatty substances, that may possibly be present in the burial environment, as it is highly unlikely that such fats were heated.

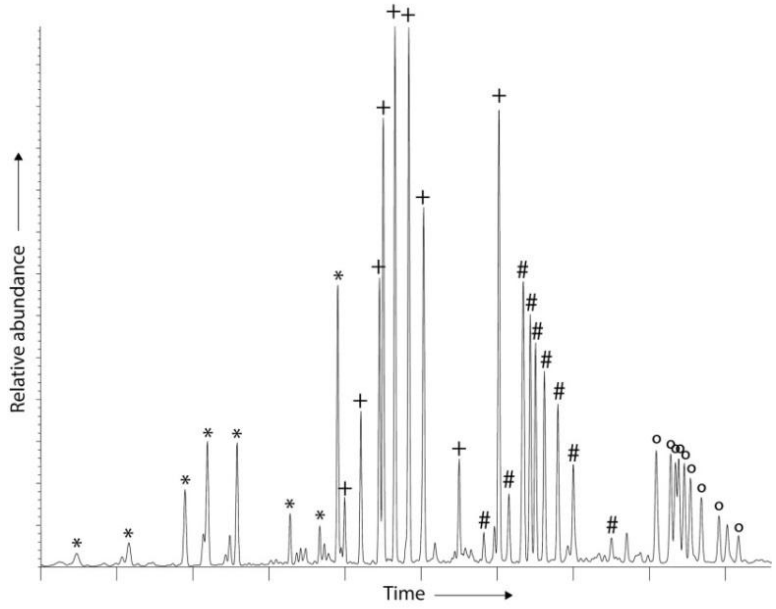
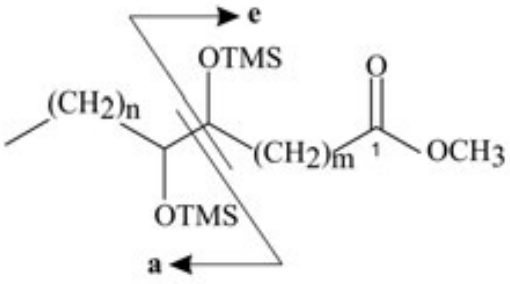


Figure 3.3: Partial summed mass chromatogram (*m/z* 105) showing ω-(o-alkylphenyl) alkanoic acid distribution in griddle stone sample AMK3-1030 run on DB-23 using the AQUASIM method. *, C16; +, C18; #, C20; °, C22 (see Admiraal et al. 2019 chapter 5 for information about the run).

Isoprenoid acids (see fig. 3.2) (4,8,12,-trimethyltridecanoic acid (4,8,12,-TMTD); 2,6,10,14-tetramethylpentadecanoic acid (pristanic acid); and 3,7,11,15- tetramethylhexadecanoic acid (phytanic acid)), are synthesized from phytol, a component of chlorophyll, which occurs widely in aquatic animals (Ackman and Hooper, 1968; Evershed et al., 2008; Cramp and Evershed, 2014). While phytanic acid is most abundant in aquatic animals, it is also present in ruminants, but in much lower quantities. Recent research by Lucquin et al. (2016a) has demonstrated significant differences in the ratio of the two diastereomers of phytanic acid (SRR:SSR) between ruminant animals and aquatic species. While the SRR% of phytanic acid diastereomers originating in modern references samples of aquatic animals is generally high (the majority plots between 70 and 90%), those of ruminant animals yield very different values (generally between 50 and 65%). This is a good means to discriminate between sources of this compound (Lucquin et al., 2016a).

As said, oxidation products of unsaturated fatty acids, such as dicarboxylic acids (or diacids), and mono- and **dihydroxy acids** are abundant in aquatic lipid profiles (Regert et al. 1998). Dihydroxy acids are oxidative degradation products of Z-monounsaturated alkenoic acids, and are also useful biomarkers ($C_{16\text{-}22}$) for the detection of aquatic products (fig. 3.4). The monounsaturated fatty acids from which they derive are distinctive due to the position of the double bond on the hydrocarbon chain (indicated by "n-"). The location of the hydroxyl group (OH) in the dihydroxy acid marks this location as it existed on the precursor fatty acid prior to oxidation. The position of this double bond can inform on the origin of the fatty acid, for example $C_{20:1}$, a major component of marine oils (Heron et al. 2010). There are some specific dihydroxy acids that are likely to originate from aquatic products, such as 9,10-dihydroxypalmitic acid ($C_{16:1}$), 9,10-dihydroxyarachidic acid ($C_{20:1}$), and 11,12-

dihydroxydocosanoic ($C_{22:1}$) acid (Hansel et al., 2011; Hansel and Evershed, 2009; Heron et al., 2010).



		m/z	
		a	**e**
7,8-dihydroxypalmitic acid	C_{16}	*215*	*231*
9,10-dihydroxypalmitic acid	C_{16}	*187*	*259*
11,12-dihydroxypalmitic acid	C_{16}	*159*	*287*
9,10-dihydroxystearic acid	C_{18}	*215*	*259*
11,12-dihydroxystearic acid	C_{18}	*187*	*287*
13-14-dihydroxystearic acid	C_{18}	*159*	*315*
9,10-dihydroxyarachidic acid	C_{20}	*243*	*259*
11,12-dihydroxyarachidic acid	C_{20}	*215*	*287*
13,14-dihydroxyarachidic acid	C_{20}	*187*	*315*
11,12-dihydroxybehenic acid	C_{22}	*243*	*287*
13,14-dihydroxybehenic acid	C_{22}	*215*	*315*

Figure 3.4: Characteristic ions of various dihydroxy acids (Hansel et al., 2011).

Terrestrial animal products

Cholesterol is a well-known compound that widely occurs in animals, and has many derivatives. Nonetheless, it is not abundantly present in archaeological pottery, possibly due to its sensitivity to degradation during heating (Evershed, 1993). Animal fats are very rich in fats, consisting mostly of triacylglycerols (**TAGs**) (fig. 3.1). It is possible to discriminate between ruminant (cattle, sheep, goat), non-ruminant (porcine, equine) and dairy fats, by examining the distribution profiles of TAGs (Dudd et al., 1999; Regert, 2011). In short, ruminants and milk products have a broad TAG distribution ($C_{42/44\text{-}54}$ for ruminants bovine/ovine, $C_{28\text{-}54}$ for fresh milk, and $C_{40\text{-}54}$ for degraded milk products), while non-ruminants have a more narrow distribution ($C_{44\text{-}54}$, with low $C_{44\text{-}46}$) (Regert, 2011). Furthermore, by looking at the fatty acid composition of TAGs Miraboud and colleagues (2007) were able to distinguish adipose fats and milk products, as well as discriminate cow and goat milk fats. Dairy products may also be recognized by their abundance of short-chain fatty acids ($C_{4\text{-}12}$). These compounds are however very prone to degradation.

While the presence of **ketones** (2-alkanones) is often described as deriving from plant epicuticular waxes, these compounds have also been shown to be the by-products of heating (>300°C) fatty acids and TAGs (Evershed et al., 1995; Raven et al., 1997). The mid-chain *iso-ketones* 14-heptacosanone and 18-pentatriacontanone particularly may derive from these sources, however, interpretation of these compounds should be done with caution. Due to the hydrolysis of TAGs, **free fatty acids** are plenty in samples containing animal adipose fats, and MAGs and DAGs may occur. The main constituents are palmitic acid ($C_{16:0}$) and stearic acid ($C_{18:0}$) (Evershed, 1993; Dudd et al., 1998). Unsaturated fatty acids, such as $C_{18:1}$ also occur. However, as said, unsaturated fatty acids are easily degraded resulting in the presence of diacids, and mono- and dihydroxy acids (Hansel et al., 2011; Copley et al., 2005; Regert, 2011).

Plant Biomarkers

Plant oils are, like most lipids, mainly comprised of TAGs (Evershed, 1993). However, their fatty acid derivatives are of little diagnostic value. Generally ***n*-alkanes** (C_{21}-C_{37}), or ***n*-alkanols** (C_{22}-C_{34}) attest to the presence of plant remains. Long-chain **wax esters** of even carbon number are also common in plants but are also often degraded (Cramp et al., 2011). **Sterols** are important biomarkers for the presence of plants, even though their identification does not provide any species specific information. The main identified phytosterols are stigmasterol, β-sitosterol and campesterol. However, β-sitosterol is also known to occur in shellfish (Steele et al., 2010). Benzoic acid is also known to occur in many plants.

There are several biomarkers that allow a higher resolution in the identification of plant species, such as several **terpenes.** Triterpenes α- and β-amyrin occur widely in plants and

possess anti-inflammatory properties (Holanda Pinto et al., 2008), as does Betulin which is common in birch (Charters et al., 1993). Friedelan-3-one (M+426) is present in oak (Sicker et al., 2019). The triterpene miliacin is one of a few species-specific biomarkers for cereal products (Heron et al., 2016). It is a biomarker for broomcorn millet, a small grain and important crop in Asia and Africa (Tafuri et al., 2009). *Levoglucosan* is a sugar-derived compound, which is a marker for the pyrolysis of carbohydrates such as starch and cellulose (Shoda et al., 2018). Long chain 2-alkanones, also known as **ketones,** are also known indicators of the processing of plant resources. Certain iso-ketones can provide diagnostic information, such as 15-nonacosanone (C_{29}) which is found in the plant epicuticular waxes of *Brassicaceae* (e.g., cabbage, broccoli, kale, turnip); and 16-hentriacontanone (C_{31}) which derives from *Allium porrum* (leek) (Evershed et al., 1991, Heron and Evershed, 1993).

Mono- and **dihydroxy** acids may form from unsaturated fatty acids in plants (Hansel and Evershed, 2009; Hansel et al, 2011), as well as dicarboxylic acids (or **diacids**) (Regert et al., 1998). Several specific plant oils may be determined by the presence of mono- and dihydroxy acids that were formed from monounsaturated fatty acids (Cramp and Evershed, 2014). For example, the presence of 13,14-dihydroxy $C_{22:0}$, 11,12-dihydroxy $C_{20:0}$ and 15,16-dihydroxy $C_{24:0}$ can identify *Brassicaceae* oils deriving from the abundantly present unsaturated fatty acids $C_{22:1}$, $C_{20:1}$, and $C_{24:1}$ (Copley et al., 2005; Regert et al., 1998). It is important to note that lipid concentrations in plants are low, especially when compared to animal fats. This may lead to an underrepresentation of the contribution of plant to the sample in the case of mixing of plant resources with more lipid-rich resources such as aquatic oils.

Resins, tars, pitches and woodsmoke

Several compounds may indicate the burning of wood. Such compounds are often found in pottery samples, possible indicators for the firing process of the pot, or cooking practices on open wood fires. These include **benzene polycarboxylic acids** (BPCAs) which are degradation products of **polycyclic aromatic hydrocarbons** (PAHs) that may also be identified in samples. These compounds are often formed during combustion of organic matter. Lower temperature fires produce low molecular weight PAHs such as anthracene (Abdel-Shafy and Mansour, 2016), which are abundantly present in samples from Southwest Alaska (see Admiraal et al., 2019b). PAHs and BPCAs may also be indicative of the charring temperature of the adhering carbonized surface residues on the ceramic, however this needs further research.

Abietic acid is a triterpene that is the primary component of coniferous (Pinaceae) resin (Brown and Brown, 2011). The presence of the abietic acid derivative **retene** is evidence for the heating of coniferous resin, which may be the result of burning wood (during firing or cooking), but it could also indicate the processing of the resin itself (Simoneit et al., 2000). However, the association of retene with ancient marine sediments (Naihuang et al., 1995) demands caution for the interpretation of the compound, when not accompanied by other abietic acid derivatives. Other common derivatives of abietic acid are **methyl-dehydroabietic acid** and **7-oxo-dehydroabietic acid (**Modugno et al., 2006). These triterpenes are the primary component of coniferous (pine) resins. Together with birch bark tar, coniferous resins are the most common plant resins identified in archaeological samples. Resins may have been used for their sealing properties in the waterproofing of pottery, or for their adhesive properties in pottery maintenance (Evershed, 1993; Oras et al., 2017; Simoneit et al., 2000).

Another interesting product recognisable in lipid samples is beeswax. Beeswax preserves exceptionally well in the archaeological record (Heron et al., 1994; 2015; Roffet-Salque et al., 2015; Regert, 2017). Furthermore, it leaves behind a clear molecular signature that is easily recognized (Tulloch and Hoffman, 1972; Tulloch, 1973). It is composed of *n*-alkanes (odd-numbered C_{21-33}), long-chain palmitate esters (C_{40-52}) and fatty acids (even-numbered C_{22-34}) (Regert et al., 2003). However, due to degradation the compounds of lowest molecular weight are often lost (Regert et al., 2001).

Isotope analysis

Identifying the prehistoric contents of pottery with the use of biomarkers alone has its limitations. Therefore, in organic residue analysis, the biomarker concept is often combined with the analysis of stable carbon and nitrogen isotope ratios. The isotopes that are measured are ^{12}C, ^{13}C, ^{14}N and ^{15}N. This allows to further differentiate between sources, as carbon and nitrogen isotopes are directly linked to the species biosynthesis and routing in different organisms (Regert, 2011). The use of stable isotope analysis allows to differentiate between several categories of products: e.g., C_3 and C_4 plants, ruminants, non-ruminants, dairy products, porcine, equine, marine species (mammals and fish), anadromous fish and freshwater fish. These distinctions are based on reference values of both modern and archaeological origin. These values may be extracted from bone or tissue (Colonese et al., 2015). This makes it a very helpful tool in the identification of prehistoric pottery contents. Without going into much detail concerning the principles of the methods, the use of isotope analysis will be shortly explained here.

Bulk isotope analysis

Stable isotope analysis is applied in two different methodologies in organic residue analysis. To gain a general understanding of the character and preservation of the organic residue bulk isotope analysis is applied to the carbonized crusts adhering to the pottery. The values of $\delta^{13}C$ and $\delta^{15}N$ are measured in bulk by elemental analysis – isotope ratio mass spectrometry (EA-IRMS). Nitrogen values increase with trophic level of the species and are especially enriched in aquatic environments (fig. 3.5). Carbon nitrogen atomic ratios can inform on the contribution of proteins against other biomolecules. For instance, high C:N ratios usually indicate higher lipid content, indicating oily substances. For example, rendered oil (of either animal or plant origin) would have fairly high C:N ratio values, while cooking dishes that contain animal tissues would lead to lower C:N ratios as they have high protein levels. (Admiraal et al., 2019). Moreover, plants are generally low on proteins, but are enriched in carbohydrates (e.g., cellulose, starch), leading to higher C:N ratios (Bondetti et al., 2019).

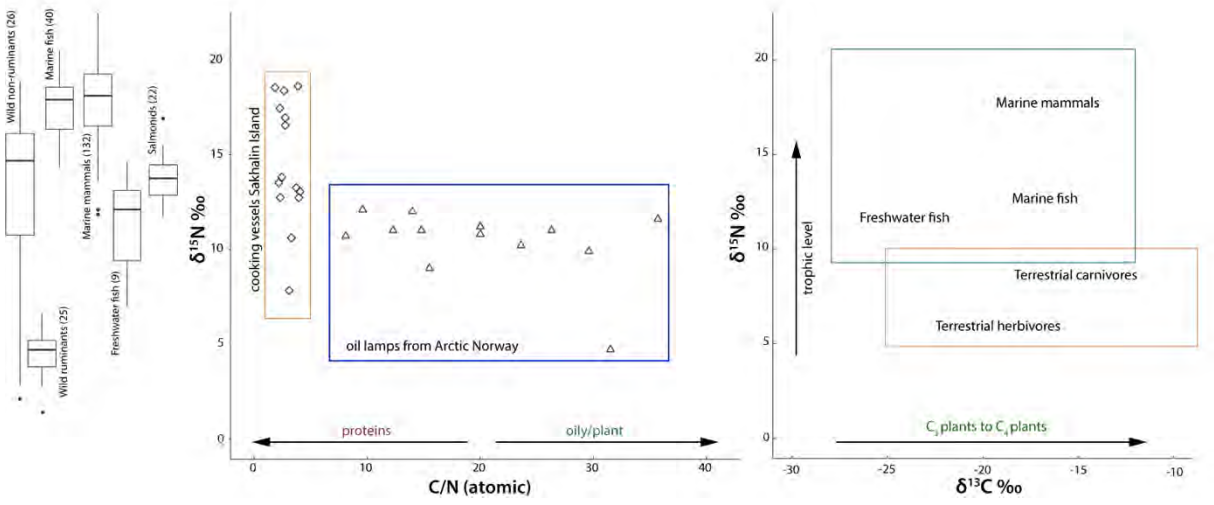


Figure 3.5: Carbon nitrogen reference bulk values of archaeological and modern references. To the left boxplots show δ15N reference data from (Admiraal et al., 2018; Britton et al., 2013; Byers et al., 2011; Coltrain et al., 2016, 2004; Misarti et al., 2009; West and France, 2015). The middle graph shows C:N ratio values of Arctic oil lamps from northern Europe (Heron et al., 2013; Oras et al., 2017; Piezonka et al., 2016), against what has been interpreted as cooking pots from Sakhalin Island, NE Asia (Gibbs et al., 2017). To the right are general reference ranges roughly plotted on a basic carbon nitrogen graph.

It is important to state that bulk isotope analysis, as the name indicates, produces mean values of the entire complex mixture of compounds that is contained within the residue, which may include both lipids and proteins (Evershed, 2009; Hastorf and DeNiro, 1985; Regert, 2011). This makes it a rather blunt tool that is nowadays mainly used to gain an initial general understanding of the residue.

Compound specific isotope analysis

In contrast to bulk isotope analysis, compound specific isotope analysis targets specific molecules in the lipid extract, and measures their $^{13}C/^{12}C$ ratio. This is achieved by gas chromatography-combustion-isotope ratio mass spectrometry (GC-c-IRMS). This method allows to identify the source of individual molecules in the lipid extract (fig. 3.4), allowing for much preciser identifications of a wide array of components. It was first applied to archaeological organic residues in the 1990s by Evershed and colleagues (Evershed et al., 1994; 1997), and since has become a standard method applied in organic residue studies (Dudd and Evershed, 1998; Dudd et al., 2003; Craig et al., 2007; Reber and Evershed, 2004).

The method is generally applied to the most common fatty acids present in lipid extracts, namely palmitic acid ($C_{16:0}$) and stearic acid ($C_{18:0}$) (fig. 3.6). The earliest studies applying GC-c-IRMS in organic residue analysis focused on the distinction between ruminant (cattle, sheep, goat) and non-ruminant species (porcine, horse) (e.g., Evershed et al., 1997; Dudd et al., 1999). In later years, the detection of dairy became an important subject of study (e.g., Dudd and Evershed, 1998; Craig et al., 2000). Another breakthrough in the application of GC-c-IRMS in organic residue analysis came with the proposed distinction between marine and freshwater species (Craig et al., 2007), which has developed into an important course of study, which this research is a part of (e.g., Gibbs et al., 2017; Shoda et al., 2017; Cramp and

Evershed, 2014, Taché and Craig, 2015). While compound specific isotope analysis generally focuses on the carbon isotope ($\delta^{13}C$), hydrogen isotopes (δD) are also of interest to discriminate between marine and terrestrial species. δD values of fatty acids $C_{16:0}$ and $C_{18:0}$ are mainly determined by the environmental water within an animals habitat, but may vary due to different digestive systems or trophic levels (Cramp and Evershed, 2014: p.326).

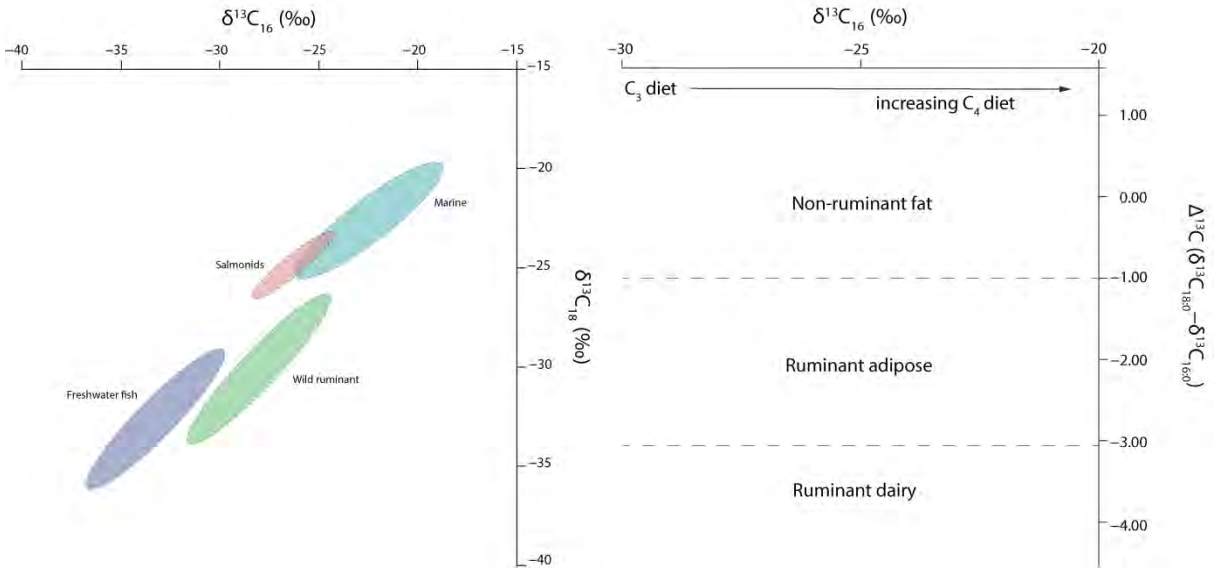


Figure 3.6: to the left: compound specific isotope reference values of $C_{16:0}$ and $C_{18:0}$ fatty acids of modern tissue and bone from the Northern Hemisphere plotted in 66,8% confidence ellipses (Choy et al. 2016; Craig et al. 2011; Horiuchi et al. 2015; Lucquin, Gibbs, et al. 2016; Paakkonen, et al. 2016; Taché and Craig 2015); to the right compound specific isotope big data differentiating between non-ruminants, ruminants and dairy fats (Dunne et al., 2018).

Together, the molecular identification of biomarkers, carbon nitrogen bulk isotope analysis, and compound specific isotope analysis allows to 1) discriminate between aquatic and terrestrial species; 2) distinguish ruminant from non-ruminant fats; 3) discriminate between marine and freshwater species; 4) differentiate between subcutaneous animal fats and dairy products; and 5) separate C_3 from C_4 plants (Cramp and Evershed, 2014; Heron and Craig, 2015; Regert, 2011). In an Arctic and Subarctic context, the approach is useful especially because organic residues are well-preserved in this cold environment, as early-on recognized

by Morgan et al. (1984), amongst others. These are great circumstances for the study of pottery function (Farrell et al. 2014; Anderson et al. 2017), but also the identification of organic residues in ancient soil and hearth features (Buonasera et al. 2015; Choy et al. 2016; Kedrowski et al. 2009). Aquatic resource processing has been an integral part of life in (sub)Arctic Alaska for thousands of years and organic residue analysis is an excellent tool to investigate these practices in more detail.

Challenges

By identifying biomarkers and interpreting isotope values of lipid and bulk samples we can learn a great deal about pottery vessel function. However, there are several challenges that need to be taken into account when interpreting results.

Contamination

While great care is taken to avoid contamination during the handling of artefacts, it is difficult to completely avoid contamination as it may derive from several sources, including from the burial environment, during excavation, storage and subsequently sampling and analysis (Evershed, 1993; 2008). Post-excavation contamination from artefact handling (e.g., skin lipids), and storing (e.g., plastics) is very common in lipid studies, and luckily easily recognizable. However, these contaminants may obscure the visibility of other, more interesting, compounds in the sample. Especially **phthalate plasticizers**, originating from plastic storage bags widely used by archaeologists, but also from plastic caps, rubber bulbs on pipettes, etc. may be substantial. Lipids originating from human skin may be introduced to the sample during artefact handling, the co-occurrence of squalene and cholesterol points to this and requires cautious interpretation of cholesterol present in the sample. **Squalene** is unlikely to be preserved from prehistoric times as it is susceptible to degradation (Evershed,

1993). To prevent such contamination it is important to store samples in aluminium foil and to use (nitrile) gloves while handling the artefacts.

A bigger threat to interpretations is cross-contamination between samples, as it is hard to detect. Therefore, strict rules apply in laboratories to avoid such contamination. Every batch of samples contains a blank "control" sample in order to make sure no contamination occurs, if it does, it is visible in this sample. Furthermore, critical examination of the results with respect to archaeological context is always advisable. Contamination may also occur in the burial environment. For this reason, it is desirable to test soil samples associated with the archaeological (pottery) samples, where possible (Heron et al., 1991). Bacterial contamination from the burial environment, or even from a humid storage environment, is hard to detect as it often produces the same compounds as animals and plants do. Therefore, biomarkers are chosen with care to exclude such overlap with bacterial lipids (Evershed, 1993).

Limitations

While the method allows for the identification of several species (groups), there are not yet biomarkers for everything, and isotope data may vary based on mixing of the original input to the sample. With compound specific isotope analysis we are able to distinguish between ruminant animals (sheep, cattle, goat), non-ruminant animals (porcine, horse), dairy products, and aquatic animal fats (marine, freshwater, and salmonids). However, interpretations must be made cautiously as the diet of animals strongly influences the isotope values. For example: a brown bear in Alaska might qualify as a non-ruminant animal. However, with a diet of salmon its isotope values may be very different from those of wild boar or domesticated pig

in other regions, furthermore isotope values will vary greatly with the season for certain species (e.g., salmon, bear).

Of major relevance to this research, are the isotope values of caribou. Caribou are designated as wild ruminant animals, but their diet is highly seasonal and consists mostly of lichen for part of the year. Lichen have enriched isotope values when compared to other sources of food of ruminant animals (e.g., grasses). This may lead to enriched isotope values for caribou in comparison to for example cattle, as demonstrated in a few studies (Taché and Craig, 2015; Paakkonen et al., 2016 referring to data presented in Paakkonen et al., in press). Paakkonen et al. (2016) showed that $\delta^{13}C$ values of reindeer may overlap with those of brackish water fish. However, $\delta^{13}C_{16:0}$ values seem to be slightly enriched as compared to aquatic (anadromous) species used for references in this study (fig. 3.6). Furthermore, the presence of biomarkers plays a big role here as it can establish the difference between these sources. Nevertheless, caution is demanded when interpreting results and future research is necessary to investigate this problem (further discussion in chapter 8).

The aquatic resource spectrum

One of the most securely identifiable resources found in pottery using organic residue analysis and isotope analysis are aquatic animal fats (Cramp and Evershed, 2014; Heron and Craig, 2015; Lucquin et al., 2016b). Biomarkers as described above leave no question about the origin of the sample. However, the aquatic spectrum is very broad and incorporates freshwater fish, anadromous fish (e.g., species that spend part of their lives in marine waters and part in freshwater environments), marine fish, and marine mammals such as seals and whales. These resources represent very diverse subsistence practices in the archaeological past, and significant economic and social changes can be linked to changes in those

subsistence practices (e.g., a transition from individual fishing to collective whaling). Therefore, it is vital to distinguish between them.

Compound specific isotope analysis allows to clearly differentiate between freshwater fish and marine species (see fig. 3.6) (Craig et al., 2007). Salmonids are anadromous species who spent part of their lives in the ocean, as well as in riverine and lacustrine environments. Their isotope values, as a result are located in between the freshwater and marine species. Samples do not always plot clearly within the boundaries determined by modern (or archaeological) reference samples. Furthermore, those reference values may differ slightly between regions. Therefore, it is important to be cautious with interpretations of samples that are plotting in regions of overlap. The inclusion of archaeological contextual information in interpretations is of great importance here (Evershed, 2008).

A challenge that is very specific to the Alaskan pottery samples analysed in this research, concerns the use of aquatic oils (e.g., marine mammal oil, or fish oil) in the manufacturing and maintenance of pottery as has been described by various ethnographic sources (see chapter 2). As the main research question concerns the function of Alaskan pottery, such practices may obscure the results of our lipid residue analysis substantially. To distinguish the possible presence of manufacture-derived lipids in the samples, an experiment was conducted successfully. The results of which are presented in chapter 4, in a paper recently published in *Archaeometry*.

Conclusion: research potentials

Despite the challenges of the method, organic residue analysis is an effective method to address the research questions raised in this PhD thesis. Identification of biomarkers form

without a doubt the most reliable method of determining the origin of a sample. While isotope values may result from the mixing of various different products, biomarkers can point to a specific origin and in some cases may even hold information about anthropogenic processes (e.g., heating). By analysing a sample using different methodologies: (i.e. lipid analysis, bulk isotope analysis, and compound specific analysis) we are now able to determine the origin of a sample with relative certainty. This is especially true of aquatic resources, making the method an ideal fit for hunter-fisher-gatherer studies.

While the differentiation between salmonids and marine species is still somewhat challenging, the method has significantly evolved throughout the past decade (Cramp and Evershed, 2014; Hansel et al., 2004; Hansel and Evershed, 2009; Lucquin et al., 2016a). As the method is constantly evolving, new biomarkers will be discovered in the future and novel methodologies may be further developed (e.g., isomer distributions of APAAs, hydrogen isotopes). This will allow for a higher resolution of sample analysis. At this moment in time, the methods applied to this research are state of the art, and this research has, in synergy with other contemporary projects at the BioArCh laboratory of the University of York, contributed to the further development of the method. In chapters 4-7 the results of this research are presented in separate papers focussing on the different research areas.