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**Title:** Isotopic analysis of dietary patterns in northern China from the Proto-Shang Period to the Qin Dynasty
**Issue Date:** 2017-11-09
CHAPTER 2 Paleodiet Reconstruction and Stable Isotope Ratio Analysis

2.1 Introduction

Eating is one of the most common, yet one of the most fundamental and vital activities of everyday life. The choice of foods consumed by an individual is a complex process that can be dependent on a variety of factors such as: traditions, religion, economics, social factors, gender, personal preferences and food availability (Coyston et al., 1999; Sutton and Yohe, 2007; Mays, 2010). The investigation of dietary habits in the past, known as paleodiet reconstruction (Kazenberg, 2000; Twiss, 2012; Reitsema, 2013; Price, 2015), can provide important information about an individual or a human population, well beyond that of: What did ancient people eat? Thus, there are a variety of different techniques that have been developed and applied to archaeological materials in order to investigate paleodiet practices. Here, I will briefly discuss some of these and then focus on stable isotope ratio analysis, as this is the analytical technique that is the focus of this thesis work.

2.1.1 Techniques for the investigation of paleodiet

Unfortunately, the actual remains of foodstuffs are rarely found at most archaeological sites around the world. However, in cases where foods are recovered, this information can yield exceptional insights into the dietary habits of the past. For example, at the late Neolithic (c. 4000 BP) site of Lajia in Qinghai Province, millet noodles were found well preserved in an overturned bowl (Figure 2.1) (Lv et al., 2005). This site is commonly referred to as the Pompeii of China, since a large earthquake destroyed the dwellings and quickly entombed individuals and their everyday possessions. Thus, Lajia provides a unique and unprecedented view of the life and activities of individuals in northwest China during the late Neolithic period. In addition, to the actual remains of dietary items, written records and historical sources as well as art (if these items survive) can supply detailed and potentially accurate information about the types of foods consumed, cooking techniques and agricultural practices in the past. For example, in China the book Yin Shan Zheng Yao (饮膳正要), dating to the 14th century AD during the Yuan Dynasty lists abundant recipes for the preparation of meat and noodle dishes for elite individuals (Hu, 2010). However, many archaeological sites do not contain these sorts of information, so other means of investigation are required to understand the culinary habits of
our ancestors, and these are briefly summarized below.

Figure 2.1. A picture of 4000-year-old millet noodles from the site of Lajia in Qinghai Province, China (from Lv et al., 2005).

2.1.2 Archaeobotany

Archaeobotany (or paleoethnobotany) is defined as “the analysis and interpretation of archaeobotanical remains to provide information on the interactions of human populations and plants” (Hastorf, 1999). Archaeobotany was first carried out in Europe by botanists in the 1950’s at archaeological sites in Switzerland (Wright, 2010). Since this time, this discipline has greatly expanded to include a host of different methods for the recovery and identification of archaeological plant remains (Hastorf and Popper, 1989; Pearsall, 2009). Macro-plant remains, such as cereal grains, seeds and chaff are identified by morphological characteristics such as size, shape and surface features. This type of analysis provides valuable information for examining questions related to hunter-gather economies and the origins of agriculture and domestication (Gremillion, 1997; Pennington and Weber, 2010). Micro-plant remains such as starch grains, phytoliths and pollen can also be recovered and identified to the species level (Warnock, 1998; Harvey and Fuller, 2005). In addition to paleodietary information, this type
of analysis is important for the reconstruction of past climates and environmental conditions (Piperno, 2006; Pearsall and Hastorf, 2011). For example, Lv et al. (2016) extracted phytoliths and biomolecular compounds such as caffeine from ancient plant remains from two funerary sites in China: the Han Yangling Mausoleum in Xi’an (Shaanxi Province) and the Gurgyam cemetery in the Ngari District of Tibet. They found that tea was grown 2100 years ago to cater to the drinking habits of individuals of the Western Han Dynasty (207 BC-9 AD), and then carried to Central Asia by ca. 200 AD along a branch of the Silk Road, several hundred years earlier than previously thought. In another example, Jiang et al. (2006) analyzed plant remains which were found in a leather basket and wooden bowl for a tomb of a male shaman at the Yanghai cemetery, Turpan District in Xinjiang and dating to 500 BC (Figure 2.2). By comparing the morphological and anatomical characteristics with modern samples, they were able to identify the plant remains as Cannabis sativa and believe that this plant was used for ritual or medicinal purposes.

Figure 2.2. A) Leather basket B) Wooden bowl C) Cannabis sativa from the Yanghai cemetery, Xinjiang. (from Jiang et al. 2006).

2.1.3 Zooarchaeology

Zooarchaeology involves the study of animal remains from archaeological sites, to understand all aspects of past human and animal interactions (Reitz and Wing, 2008; Broderick, 2016). In most cases the remains primarily consist of the hard parts of the body such as bones and teeth, but where available, research has also been focused on hair, hides, horns and shells (O’Connor, 2004). Zooarchaeology became a sub-discipline of archaeology after World War II, and is now commonly employed at most archaeological excavations (Thomas, 1996). Techniques regularly used by zooarchaeologists involve the identification and quantification of the type of species, taphonomy (the study of the burial processes that influence bones), isotopic analysis,
DNA analysis, etc. (Peres, 2010; Albarella et al., 2016). These studies permit investigations into complex questions relating to human and animal relationships such as past hunting activities, subsistence practices, economics and paleoenvironments (Lyman, 1996; O’Day et al., 2003; Defrance, 2009). For example, Li et al. (2014) analyzed sheep and goats remains from two terminal Neolithic sites, Taosi and Xinzhai (ca. 2500-1800 BC), as well as one Early Bronze Age site Erlitou (ca. 1800-1500 BC) in Shanxi and Henan Provinces. This research revealed the complexity of sheep and goat herding practices in the Central Plains of China, and explored the use of secondary production products such as, wool and milk, by the inhabitants of these sites. In particular, the results indicate that at Erlitou, sheep were mainly exploited for meat consumption during phases II and III, but were mainly exploited for wool production during phase IV of the occupation. This work also found evidence that there were different sheep/goat usage patterns between elite and non-elite social groups as well as between urban vs. village sites.

2.1.4 Residue analysis

Residue analysis involves the isolation and identification of chemical components or biomarkers found on archaeological objects, and this technique has been used on a host of diverse materials such as bone, stone, metal and soils (Pecci, 2014). Analysis of organic residues from archaeological artifacts can provide important information about what an object was used for, as well as, the type of materials found in vessels such as cups or pots (Evershed, 1993; 2008). This type of analysis consists of techniques such as: infrared spectroscopy, Raman spectroscopy, nuclear magnetic resonance (NMR) and various forms of gas chromatography/mass spectrometry (GC/MS) (Evershed et al., 1990; Eerkens, and Barnard, 2007; Baeten, 2012). For example, in China, this method has been used to directly identify the presence of rice, honey or grape in a mixed fermented beverage from early Neolithic pottery jars at the site of Jiahu in Henan Province (McGovern et al., 2004). In addition, a recent study by Wang et al. (2016) examined pottery vessels from the Yangshao site of Mijiaya (3400-2900 BC) in Shaanxi Province (Figure 2.3). They found that the organic residues were a match for a beer recipe where broomcorn millet (Panicum miliaceum), barley (Hordeum vulgare), Job’s tears (Coix lacrymajobi) and tubers were fermented together. This indicates that people in ancient China established advanced beer brewing technology by using specialized tools to create favorable fermentation conditions over 5,000 years ago.
2.2 Introduction to Stable Isotope Ratio Analysis

Developed in the 1970s as an offshoot of radiocarbon dating (Makarewicz and Sealy, 2015), stable isotope ratio analysis can be summarized by the common phrase: “You are what you eat”. Basically, the isotopic signatures of the foods and liquids consumed by an organism are incorporated into its tissues in a relatively known and predictable manner (Lee-Thorp, 2008). Therefore, the measurement of these isotopic signatures allows an approximate reconstruction of dietary habits in the past. This technique has become a common addition to archaeological studies in the last 25 years and has been used to investigate a variety of research topics such as: subsistence practices, animal husbandry patterns, health and nutrition, social status, etc. (Kazenberg, 2000; Lee-Thorp, 2008; Schoeninger, 2011; Reitsema and Vercellotti, 2012; Reitsema, 2013). What follows in this chapter is a brief overview of the technique of stable isotope ratio analysis.

2.2.1 Definitions

An isotope is defined as an atom with the same number of protons but with a different number of neutrons (Sharp, 2007) (Figure 2.4). Isotopes were first discovered in 1913 by the English
chemist, Sir Fredrick Soddy. The word “isotope” is derived from the Greek word *iso* (same or equal) and *topos* (place), and this is because isotopes of an element occupy the same position or place in the periodic table (Platzner, 2012). Isotopes can be divided into two types, stable and unstable (radioactive), and are commonly written using the following notation $^A_X$, where $X$ represents the chemical symbol of an element and $A$ is the sum of the number of protons and neutrons. For example, three isotopes of carbon exist in nature:

$^{12}\text{C} = \text{Stable} \quad (6\text{ protons} + 6\text{ neutrons} = 12)$

$^{13}\text{C} = \text{Stable} \quad (6\text{ protons} + 7\text{ neutrons} = 13)$

$^{14}\text{C} = \text{ radioactive} \quad (6\text{ protons} + 8\text{ neutrons} = 14)$

Figure 2.4. The three naturally occurring isotopes of carbon, each having the same numbers of electrons and protons but a different numbers of neutrons. (from http://imgarcade.com/1/all-carbon-isotopes/).

Most of the natural elements (66 out of 94) exist as mixtures of two or more isotopes, but the relative abundances of these different isotopes can vary substantially (Platzner, 2012). There are approximately 300 naturally occurring stable isotopes, and 1200 radioactive isotopes that have been discovered to date. Table 2.1 shows the stable isotopes of the common elements that
are studied in archaeology, but since this thesis is only focused on the measurement of carbon, nitrogen, and sulfur isotopes, only these will be discussed in detail (Fritz and Fontes, 1986; Fry, 2006).

Table 2.1. Stable isotopes of the common elements used paleodiet reconstruction (modified from Fritz and Fontes, 1986; Fry, 2006).

<table>
<thead>
<tr>
<th>Element</th>
<th>Isotopes</th>
<th>Nature Abundance (in percent)</th>
<th>Isotope Ratio Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen</td>
<td>^1H</td>
<td>99.985</td>
<td>D/H</td>
</tr>
<tr>
<td></td>
<td>^2H or (D)</td>
<td>0.015</td>
<td></td>
</tr>
<tr>
<td>Carbon</td>
<td>^12C</td>
<td>98.89</td>
<td>^13C/^12C</td>
</tr>
<tr>
<td></td>
<td>^13C</td>
<td>1.11</td>
<td></td>
</tr>
<tr>
<td>Nitrogen</td>
<td>^14N</td>
<td>99.64</td>
<td>^15N/^14N</td>
</tr>
<tr>
<td></td>
<td>^15N</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>Oxygen</td>
<td>^16O</td>
<td>99.76</td>
<td>^18O/^16O</td>
</tr>
<tr>
<td></td>
<td>^17O</td>
<td>0.037</td>
<td></td>
</tr>
<tr>
<td></td>
<td>^18O</td>
<td>0.024</td>
<td></td>
</tr>
<tr>
<td>Sulphur</td>
<td>^32S</td>
<td>95.02</td>
<td>^34S/^32S</td>
</tr>
<tr>
<td></td>
<td>^33S</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td></td>
<td>^34S</td>
<td>4.21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>^36S</td>
<td>0.014</td>
<td></td>
</tr>
</tbody>
</table>

2.2.2 Fractionation

While possessing the same chemical properties (e.g. ^12C, ^13C and ^14C are all elements of carbon), the mass differences related to the variable number of neutrons can result in subtle yet detectable differences in the isotopes of an element. Fractionation is the term used to describe the partitioning of isotopes between two substances or two phases of the same substance (Schoeller, 1999). In general, the lighter elements (lower atomic masses) show more fractionation than the heavier elements since they tend to have chemical bonds that are more susceptible to breakage. There are two types of fractionations for isotopes: kinetic disequilibrium fractionation and thermodynamic equilibrium fractionation (Hayers, 1982). Kinetic fractionation is associated with incomplete and unidirectional processes, and this is the
main form of isotopic fractionation associated with biological systems in terms of enzymatic reactions (O’Leary, 1981). Thermodynamic fractionation is associated with physical reactions where no bonds are broken and examples include: evaporation, distillation or infrared absorption (White, 2015).

2.2.3 Notation

Because the variations between stable isotope ratios are very small, generally only a few tenths of a percent, it is common practice to express the isotopic composition of a substance as a delta “δ” value which represents the measured deviation of an isotopic ratio against a particular elemental standard (Table 2). The δ values are expressed in parts per thousand (‰) using the following notation:

$$\delta_{\text{sample}} (\text{‰}) = \left( \frac{R_{\text{sample}}}{R_{\text{st}}} - 1 \right) \times 1000$$

Where $R = \text{the isotope ratio of the heavier to the lighter element in a measured sample divided by the standard of that element.}$ For example, the equation for the stable isotope ratios of carbon is as follows:

$$\delta^{13}C = \left[ \frac{\left( \frac{^{13}C}{^{12}C} \right)_{\text{sample}}}{\left( \frac{^{13}C}{^{12}C} \right)_{\text{st}}} - 1 \right] \times 1000$$

The international reference standards for the five common elements used in paleodiet reconstruction are listed in Table 2.2. A positive δ value reflects samples that have more of the heavy isotope compared to the standard, whereas a negative δ value reflects samples that have more of the lighter isotope compared to the standard. For example, the majority materials have less $^{13}C$ than the VPDB standard, so nearly all $\delta^{13}C$ values encountered in archaeological specimens are negative.
Table 2.2. The international reference standards for the five common elements used in paleodiet reconstruction (from Hoefs, 2009).

<table>
<thead>
<tr>
<th>Element</th>
<th>International Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen</td>
<td>Vienna-Standard Mean Ocean Water (V-SMOW)</td>
</tr>
<tr>
<td>Carbon</td>
<td>Vienna-Pee Dee Belemnite (VPDB)</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>Atmospheric Nitrogen (AIR)</td>
</tr>
<tr>
<td>Oxygen</td>
<td>Vienna-Standard Mean Ocean Water (V-SMOW)</td>
</tr>
<tr>
<td>Sulphur</td>
<td>Vienna-Canyon Diablo Meteorite Troilite (VCDT)</td>
</tr>
</tbody>
</table>

2.2.4 Mass spectrometry

The most effective method of measuring the stable isotope ratios of an element is with an isotope ratio mass spectrometer (IRMS). This machine is specifically designed to measure small differences in the mixtures of naturally occurring isotopes by the manipulation of external electric and magnetic fields (Figure 2.5) (Brand, 2002).

Figure 2.5. Schematic diagram showing a generic isotope ratio mass spectrometer (modified from Hoefs, 2009).
In paleodietary studies, solid substances such as bone/dentine collagen, hair, etc. are first placed in tin (for $\delta^{13}$C and $\delta^{15}$N analysis) or silver (for $\delta$D and $\delta^{18}$O) capsules. These capsules are then introduced into an elemental analyzer and combusted to create CO$_2$ and N$_2$, and these gasses are carried to the ion source where they are transformed into electrically charged particles. The resulting ion beam is focused and directed into the mass analyzer via a flight tube. The mass analyzer contains electric and magnetic fields that are used to alter the speed and direction of the ions. The amount of deflection of the ion’s trajectory depends on its mass-to-charge ratio (m/z) with lighter ions more easily deflected than heavier ions (Fry, 2006). When the beam of ions passes from the analyzer, it is measured in the ion detectors, and the abundances of each ion are calculated. For example, in Figure 2.6 a schematic diagram of a continuous flow IRMS is presented (Carter and Barwick, 2012).

![Diagram](image_url)

**Figure 2.6.** Simple schematic diagram of continuous flow isotope ratio mass spectrometry for the determination of $\delta^{13}$C and $\delta^{15}$N (modified from Carter and Barwick, 2012).

The four main components include: 1) Elemental analyzer – combuts or thermally converts a specimen into gas; 2) Interface – where the evolved gases are introduced into the mass spectrometer; 3) IRMS – separates and detects the ionized particles; 4) Calculation – quantification of the isotope results. In addition, laboratory standards must be also be analyzed for comparison with the unknown standards. In general, duplicate or triplicate measurements should be done for each sample (where possible) to increase the precision of the results (Faure
2.3 Common Stable Isotope Ratios that are Studied in Paleodiet Research

2.3.1 Carbon (δ¹³C)

The two stable isotopes of carbon, ¹³C and ¹²C, have natural abundances of 1.1% and 98.9%, respectively (Burlingame and Schnoes, 1969). The majority of the carbon in the world is non-biological, but the largest sources of variability occur during the transfer of atmospheric carbon to and from the ocean and into biological systems (Schoeninger and Moore, 1992). As a result of diffusion, the concentration of ¹²C increases relative to ¹³C, so that plants have negative δ¹³C values compared with the source carbon (O’Leary, 1981). The δ¹³C value of modern CO₂ is about -7‰. However, before the Industrial Revolution, this value was elevated by approximately 1.5‰. As a result of the release of anthropogenic carbon into the atmosphere from deforestation and the burning of fossil fuels (Suess effect), δ¹³C values have become lower over the past few hundred years (Keeling, 1961; Bada et al., 1990). Thus, when we directly compare modern and archaeological δ¹³C samples, 1.5‰ should be added to the modern δ¹³C values.

Atmospheric carbon dioxide (CO₂) is the main carbon source for all terrestrial plants. Land plants are divided into three types according to how they biosynthesize or fix carbon during photosynthesis. The three pathways of photosynthesis are: C₃ (Calvin-Benson Cycle), C₄ (Hatch-Slack Cycle) and CAM (Crassulacean Acid Metabolism), and each of these pathways results in distinct δ¹³C values in the tissues of plants (Park and Epstein, 1961; O’Leary, 1988). Plants are called C₃, because the first product that they make during photosynthesis has a molecule with three carbon atoms (Schoeninger and Moore, 1992). The majority of the vegetation in temperate zones is composed of C₃ plants: trees, bushes, leafy plants and some grasses. The typical δ¹³C values of C₃ plants range from −24‰ to −34‰ (with an average of −26.5‰) (Smith and Epstein, 1971). In contrast, C₄ plants produce a four-carbon compound during their first step of photosynthesis, hence their name. C₄ plants usually are native to hot and arid environments and include some important cultivated crops such as: maize, millet, sorghum and sugar cane (Price et al., 1985). The δ¹³C results of C₄ plants are about 13‰ higher than C₃ plants, ranging from −16‰ to −9‰ (with an average of −12.5‰) (Smith and Epstein,
1971). Figure 2.7 shows the frequency and $\delta^{13}\text{C}$ values of C$_3$ and C$_4$ plants (from Cerling and Harris, 1999; Hoefs, 2009).

![Figure 2.7](image)

Figure 2.7. The frequency and $\delta^{13}\text{C}$ values of C$_3$ and C$_4$ plants (from Cerling and Harris, 1999; Hoefs, 2009).

CAM plants have more flexible means of photosynthesis and are able to switch between C$_3$ and C$_4$ pathways, depending on environmental conditions (Bender et al., 1973; Osmond, 1978). However, CAM plants are not the subject of frequent study in archaeology since few edible crops use this photosynthetic pathway (Price et al., 1985). Beside the principal influences of photosynthesis, some climatic effects can also lead to substantial variations in the $\delta^{13}\text{C}$ values of both C$_3$ and C$_4$ plants. These factors include but are not limited to: water and nutrient availability, temperature, altitude, light intensity and soil quality (Tieszen, 1991; van der Merwe and Medina, 1991; Lajtha and Marshall, 1994). For example, increased light and/or temperature and decreased water availability can result in an increase of the $\delta^{13}\text{C}$ values in plants of the same species (Heaton, 1999; Hedges et al., 2004).

In aquatic ecosystems, organisms can derive carbon from several sources, including terrestrial detritus (with $\delta^{13}\text{C}$ values representative of a mixture of local terrestrial plants), dissolved CO$_2$ (with $\delta^{13}\text{C}$ of atmospheric CO$_2$), and dissolved carbonic acid (with $\delta^{13}\text{C}$ values close to 0) (Schwarcz and Schoeninger, 1991). Therefore, the $\delta^{13}\text{C}$ values of aquatic plants can overlap with the values of terrestrial plants (Fry and Sherr, 1984). Marine organisms derive carbon
mainly from seawater, which is about 7-8‰ elevated in $\delta^{13}C$ values compare to atmospheric CO$_2$ (Craig, 1953; Richards and Hedges, 1999). Sea grasses have $\delta^{13}C$ values similar to C$_4$ plants, whereas some cold water plankton species have $\delta^{13}C$ values close to C$_3$ plants (see Figure 2.8).

The majority of marine species such as fish and higher carnivores such as seals and whales have $^{13}$C-enriched values near $-12\%$, and these values overlap with terrestrial C$_4$ consumers (Schwarcz and Schoeninger, 1991; Richards and Hedges, 1999). This can make the identification between the two groups difficult based on only $\delta^{13}C$ results. Freshwater plants and the fish that feed on them usually have mean $\delta^{13}C$ values near $-25\%$, which is close to C$_3$ plants, but sometimes may vary widely, reflecting the contribution of various carbon sources (Rau, 1978; Chisholm et al., 1982; Schoeninger and DeNiro, 1984).

![Figure 2.8. $\delta^{13}C$ values of different plants species (after Cerling and Harris, 1999).](image)

The $\delta^{13}C$ difference between bulk diet and collagen, the carbon trophic level effect, is estimated to be 5‰ by a number of laboratory experiments (DeNiro and Epstein, 1978; van der Merwe and Vogel, 1978; Tieszen and Fagre, 1993). In addition, there is a trophic level effect of approximately 1‰ between the protein values of herbivores, omnivores and carnivores, and this has been observed in a number of studies (e.g. Bocherens and Drucker, 2003; McCutchan et al., 2003).
2.3.2 Nitrogen ($\delta^{15}$N)

The two stable isotopes of nitrogen, $^{15}$N and $^{14}$N, have abundances of 0.4% and 99.6%, respectively. More than 99% of the exchangeable nitrogen exists as either N$_2$ in the atmosphere or as dissolved N$_2$ in the ocean (White, 2015). Plants can uptake nitrogen from two different sources: the atmosphere or the soil. For example, some legumes (such as peas and beans) and algae (such as blue or green algae) can obtain nitrogen directly from the atmosphere. The $\delta^{15}$N values of this fixed nitrogen in organic matter are generally similar to atmosphere N$_2$ (~0‰) (Michener and Lajtha, 2007). However, most plants are not able to directly uptake N$_2$, and instead absorb nitrogen from the soil by assimilating NH$_4^+$ or NO$_3^-$. Thus, terrestrial plants using these nitrates display more positive $\delta^{15}$N values than plants that use N$_2$-fixing. Most modern non-N$_2$-fixing plants have $\delta^{15}$N values that generally range between 0‰ and 6‰, although a broad range is observed (Choi et al., 2003). The $\delta^{15}$N values of terrestrial plants are strongly dependent on the soil, and other factors, such as: water availability, temperature, altitude, rainfall and the application of fertilizers (Ambrose, 1991; Schwarcz et al., 1999). Marine plants take up nitrogen from dissolved nitrates in the seawater, which are produced by bacterial denitrification, and these are more positive in $^{15}$N compared with atmospheric and soil nitrogen. Thus, marine plants have higher $\delta^{15}$N values than terrestrial plants (Wada, 1980). In addition, similar to marine systems, freshwater plants have high $\delta^{15}$N values (France, 1995; Katzenberg and Weber, 1999).

The $\delta^{15}$N values of animals and humans are related to their diets and increase by approximately 3-6‰ with each ascending step of the food chain, and this is known as the nitrogen trophic level effect (DeNiro and Epstein, 1981; Minagawa and Wada, 1984; Bocherens and Drucker, 2003; O’Connell et al., 2012). For example, in temperate environments, terrestrial herbivores have lower $\delta^{15}$N values compared to carnivores, and in marine ecosystems lower trophic level fish have lower $\delta^{15}$N values compared to higher trophic level fish and marine carnivores (Richards and Hedges, 1999; Lee-Throp, 2008) (Figure 2.9). Humans show distinct $\delta^{15}$N differences related to diet with omnivores having higher $\delta^{15}$N values compared to vegetarians and vegans (Petzke et al., 2005). Thus, the combination of $\delta^{13}$C and $\delta^{15}$N values is a valuable tool for distinguishing the types of food consumed from temperate C$_3$ and C$_4$ environments and from marine ecosystems.
2.3.3 Sulphur ($\delta^{34}S$)

The four stable isotopes of sulphur and their natural abundances are listed: $^{32}S$ (95.02%), $^{33}S$ (0.76%), $^{34}S$ (4.21%), $^{36}S$ (0.014%). The isotopes that are commonly measured are $^{32}S$ and $^{34}S$, since these are the two most abundant of the four (Hoefs, 2009). The $\delta^{34}S$ value is reported relative to the meteorite standard Vienna Canyon Diablo Troilite (VCDT) (Coplen and Krouse, 1998) by the standard equation:

$$
\delta^{34}S_{sample} = \left[ \frac{\left( \frac{^{34}S}{^{32}S} \right)_{sample}}{\left( \frac{^{34}S}{^{32}S} \right)_{VCDT}} - 1 \right] \times 1000
$$

Sulphur is an important constituent of the lithosphere, biosphere, hydrosphere, and atmosphere.
The main reservoirs of sulphur are oceanic soluble sulphates, evaporitic sulphates, and reduced pyrites, with minor reservoirs found in the atmosphere, fossil fuels and the soil (Newton and Bottrell, 2007; Nehlich, 2015). The sulphur cycle has a number of similarities to the carbon cycle, through weathering, the water cycle and tectonic movements (Bottrell and Newton, 2006). Sulfur in marine environments is mainly present as sulphate (SO$_4^{2-}$), which is $^{34}$S-enriched with a fairly uniform value of 20‰ across the global (Rees et al., 1978). Thus, marine organisms usually have δ$^{34}$S values near to 20‰. Sulphate in seawater can be transported by the wind and influence the δ$^{34}$S values of islands or coastal terrestrial regions, and this is known as the ‘sea spray effect’ (O’Dowd et al., 1997). The δ$^{34}$S values of freshwater environments have a wide range of variability, and this can be attributed to anaerobic bacteria in the sediments of rivers and lakes which can reduce the sulphate ions to hydrogen sulphide (Faure, 1997; Richards et al., 2003a). The δ$^{34}$S values for sedimentary rocks are highly variable depending on rock type and age (Faure, 1997). Terrestrial plants incorporate the majority of sulphur as sulphate (SO$_4^{2-}$) from the soil, and thus their δ$^{34}$S values generally reflect the geological δ$^{34}$S values. However, plants can also derive inorganic sulphur from the atmosphere (SO$_2$, up to 90%) (Peterson and Fry, 1987; Richards et al., 2003a). Terrestrial plants have reported δ$^{34}$S values ranging from −22 to +22‰, and this high variability is a result of local environmental and geological conditions (Figure 2.10).

Figure 2.10. Box model of the sulphur cycle in the environment (modified from Nehlich, 2015).

Sulphur is an essential nutrient for animals and humans and is found in protein, various
vitamins and cofactors (Nielsen et al., 1991). There is only a slight fractionation (approximately -1‰) for δ^{34}S values with each step of the food chain or increase in trophic level (Richards et al., 2003a). Therefore, the δ^{34}S values in human and animal tissues generally reflect the δ^{34}S values of the food consumed (Hobson, 1999). Figure 2.11 shows the theoretical ranges for δ^{13}C and δ^{34}S values for specific food web niches and environments in archaeological research (Nehlich, 2015).

![Theoretical ranges for δ^{13}C and δ^{34}S values for specific food web niches and environments in archaeological research.](adapted from Nehlich, 2015)

With the improvements in continuous flow methods, smaller amounts of bone collagen (3-10 mg) can now be analyzed for δ^{34}S values, but this is still larger than what is required for δ^{13}C and δ^{15}N measurements (Figure 2.12). Thus, combined with δ^{13}C and δ^{15}N measurements, the isotopic analysis of sulphur has become a more routine method applied to archaeological specimens.
Figure 2.12. Differences in the amount of bone collagen needed for δ\textsuperscript{13}C and δ\textsuperscript{15}N vs. δ\textsuperscript{34}S measurements.

2.4 Experimental Procedure of Stable Isotope Ratio Analysis

2.4.1 Common materials used for stable isotope analysis

The majority of paleodietary studies in archaeology are focused on bones and teeth for the simple reason that these are often the only tissues of an organism that survive the burial process (Mays, 2010). Bone is a complex tissue composed of an inorganic (mineral) fraction (approximately 55-75\% of dry weight) and an organic matrix (White and Folkens, 2005). More than 90\% of the organic component is the protein collagen, with the rest composed of other proteins such as osteocalcin (Masters, 1987; Bass, 2005). The mineral fraction in bone and tooth is mainly composed of calcium phosphate, which has the chemical formula Ca\textsubscript{10}(PO\textsubscript{4})\textsubscript{6}(OH)\textsubscript{2}. Both collagen and bioapatite contain measurable amounts of carbon, which can be used to reconstruct dietary information. The bioapatite of carbon gives a measure of the whole diet, because bone mineral carbonate is dissolved in the blood, and thus reflects the total metabolic carbon pool which originates from dietary carbonate, lipid and protein (Katzenberg, 2000). However, collagen is mainly derived from dietary protein, and thus δ\textsuperscript{13}C collagen values mainly reflect ingested protein (Krueger and Sullivan, 1984; Ambrose and Norr, 1993; Tieszen
and Fagre, 1993). While the bone collagen turnover in an adult is rather slow, about 1.5-4% every year in cortical bone, the collagen turnover in children is much more rapid and is estimated to be between 10-30% in cortical bone per year (Hedges et al., 2007). Therefore, the collagen stable isotope data from adult bones reflect a person’s average diet over the last decade or so of life.

Bone degrades over time after burial and collagen survival mainly depends on many factors including: temperature, soil moisture and pH and microbial attack (Smith et al., 2005; Mays, 2010). In certain special conditions such as cold or cave environments, collagen can survive for more than 100,000 years (e.g. Britton et al., 2012). Degraded collagen or collagen that is contaminated can result in altered isotopic compositions from the original value, and thus identifying the preservation of bone collagen is a priority for isotopic analysis. There are four main criteria that are routinely used to assess if $\delta^{13}$C and $\delta^{15}$N results from collagen are acceptable. These include: % collagen yield, %C, %N and atomic C:N (DeNiro, 1985; van Klinken, 1999). Of these four, the C:N is usually the most important and while a value of 3.2-3.3 is expected for modern collagen, values between 2.9-3.6 are considered acceptable for $\delta^{13}$C and $\delta^{15}$N measurements (DeNiro, 1985). For $\delta^{34}$S measurements, Nehlich and Richards (2009) designed quality criteria for archaeological bone samples. Measurements of samples with atomic C:S of 600 ± 300 and N:S of 200 ± 100 for mammalian collagen, and atomic C:S of 175 ± 50 and N:S ratio of 60 ± 20 for fish collagen are considered acceptable.

2.4.2 Collagen extraction

There have been several different methods developed to extract collagen from archaeological bones (see Longin, 1971; Schoeninger and DeNiro, 1984; Sealy, 1986; Brown et al., 1988; Tuross et al., 1989, Richards and Hedges, 1999). In this dissertation, collagen samples were prepared following the standard protocol detailed in Richards and Hedges (1999) with the addition of an ultrafiltration step as recommended by Brown et al. (1988) and Jørkov et al. (2007). Small bone chunks (approximately, 0.5-1.0 g) were cleaned by air abrasion with Al₂O₃ and then demineralized at 4 °C in a 0.5 M HCl solution for two weeks, with the acid changed every 2 days. Once demineralized, the samples were rinsed three times with deionized water, and then introduced to a pH=3 solution and gelatinized at 70 °C for 48 hours. The samples were first filtered with a 5μm EZEE© filter to remove the insoluble residues; then the solution was
concentrated by Amicon® ultrafilters (<30kDa), and finally the purified collagen was frozen and freeze dried for 2 days.

2.5 Application of Stable Isotope Ratios in Archaeological Research

2.5.1 Subsistence practices

The earliest applications of stable isotopes to archaeological studies were mainly focused on maize agriculture in North America (Vogel and van der Merwe, 1977; van der Merwe and Vogel, 1978; Bender et al., 1981), and many subsequent studies on this topic were published (Schwarcz and Schoeninger, 1991; Schoeninger, 1995; Lee-Thorp, 2008). For example, it was found that maize replaced previously domesticated C₃ plants at different times in different regions. Near the Lake Erie area of Canada, maize became important to diets by about 500 AD (Stothers and Bechtel, 1987) whereas it was not until after 1000 AD that maize became important to human diets in southeastern Missouri, USA (Lynott et al., 1986). In contrast to North and South America, Europe does not have widespread C₄ agriculture, and the majority of edible plants are C₃ (Lee-Thorp, 2008). As a result of this, the first isotopic studies in Europe were focused on detecting marine vs. terrestrial diets. Tauber (1981) found that Danish Mesolithic individuals had a high proportion of marine foods in their diet, however, with the introduction of farming and domestic animals during the Neolithic period there was a dramatic shift away from marine foods toward these domestic foods. Similar isotopic patterns indicative of diets shifting from marine to terrestrial foods were also observed for the Mesolithic to Neolithic transitions in Portugal (Lubell et al., 1994) and Britain (Richards et al., 2003b).

In addition, to δ¹³C and δ¹⁵N analysis, δ³⁴S measurements are increasingly applied to paleodiet studies to investigate aquatic vs. terrestrial resource consumption. For example, Nehlich et al. (2010) examined δ³⁴S values from humans and animals from five archaeological sites in Serbia along the Danube Gorges region dating from the Mesolithic to the middle Neolithic. The δ³⁴S values helped show clear evidence that both terrestrial and freshwater resources were exploited during the Mesolithic to the early Neolithic periods at these sites. However, during the later Neolithic period, fish and other aquatic foods were not consumed and diets were mainly composed of domestic animals.
2.5.2 Breastfeeding and weaning studies

An area of stable isotope analysis that has seen an increase in growth over the past 20 years is the reconstruction of breastfeeding and weaning patterns in modern and ancient humans (for a recent review see Tsutaya and Yoneda, 2015). This research is based on the fact that an infant becomes one trophic level higher than its mother during exclusive breastfeeding (e.g. Fogel et al., 1989; Fuller et al., 2006a). Thus, a 2-3‰ $^{15}$N-enrichment in the tissues of an infant permits the tracking of the duration of breastfeeding, and with the weaning process or the introduction of solid food to the diet, the $\delta^{15}$N values of an infant or child decrease due to the consumption of $^{15}$N-depleted foods. At the point when a child becomes fully weaned their $\delta^{15}$N results closely resemble maternal $\delta^{15}$N values, assuming that both the mother and child consumed similar diets. In addition, $\delta^{13}$C measurements also provide important information about breastfeeding and weaning patterns since a 1‰ increase is found between mothers and exclusively breastfed infants (Fuller et al., 2006a). The $\delta^{13}$C values decline more rapidly to maternal levels during the weaning process, and this allows an estimation of the duration of exclusive breastfeeding. Thus, the combination of both $\delta^{13}$C and $\delta^{15}$N measurements from the bones of children of different ages allows the reconstruction of breastfeeding and weaning patterns for an archaeological population. For example, Fuller et al. (2006b) conducted isotopic analysis on collagen from the Late/Sub-Roman site of Queenford Farm, UK. This study found that most children were fully weaned in a gradual process between the ages of 2-4 years old. In addition, lower $\delta^{13}$C values suggest that children had a more plant based diet compared to the adult population. Isotopic analysis has also been done using tooth dentine and enamel to investigate individual patterns of breastfeeding and weaning (e.g. Fuller et al., 2003; Dupras and Tocheri, 2007). This type of research allows the investigation not only of infant/childhood diet but can be used to look at dietary patterns over the course of the age of formation of the tooth, and this has been applied with much success recently (Guiry et al., 2016). Unfortunately, there have been no isotopic applications of breastfeeding and weaning in China, but it is hoped that this type of research will be conducted in the future.

2.5.3 Hominid evolution

Isotopic studies have played an important role in the elucidation of hominid diets (e.g. Schoeninger, 1995; Lee-Thorp, 2008). For example, enamel $\delta^{13}$C results show that
Australopithecus africanus, a ~3 million year old hominid from the Makapansgat Limeworks in South Africa, ate fruits, leaves and large amounts of $^{13}$C-enriched foods, such as grasses and sedges or animals which ate these plants (Sponheimer and Lee-Thorp, 1999). In addition, this study found that these early hominids regularly exploited relatively open environments such as woodlands or grasslands for food. The results of this study suggest that Australopithecus africanus consumed high quality foods before the development of stone tools and the origin of the genus Homo (Sponheimer and Lee-Thorp, 1999). In Europe, isotopic studies on Neanderthals have discovered that they had similar diets through time (ca. 120000 - 37000 BP) and that they were top-level carnivores, obtaining almost all of their dietary protein from large herbivores (Richards et al., 2000; Richards and Trinkaus, 2009). In contrast, early modern humans in Europe (ca. 40000-27000 BP) had more variable diets compared to the Neanderthals. Specifically, some of the European early modern humans were consuming significant amounts of aquatic (marine and freshwater) foods (Richards and Trinkaus, 2009), and these dietary differences may have been one of the reasons that modern humans survived while Neanderthals went extinct.