

*Human Biology Toolkit*

## Laboratory and Field Methods for Stable Isotope Analysis in Human Biology

LAURIE J. REITSEMA\*  
 Department of Anthropology, University of Georgia, Athens, Georgia 30602

**ABSTRACT:** Stable isotope analysis (SIA; carbon, hydrogen, nitrogen, sulfur, and oxygen) of human tissues offers a means for assessing diet among living humans. Stable isotope ratios of broad categories of food and drink vary systematically, and stable isotope ratios in consumer tissues represent a composite of the isotopic ratios of food and drink consumed during an individual's life. Isotopic evidence for diet is independent of errors in informant recall, and accrues during time periods when researchers are absent. Beyond diet reconstruction, tissue stable isotope ratios are sensitive to excursions from homeostasis, such as starvation and rapid growth. Because of their relationship to diet, geographic location, hydration, and nutritional status, stable isotope signatures in human tissues offer a window into human biocultural adaptations, past and present. This article describes methods for SIA that may be usefully applied in studies of living humans, with emphasis placed on carbon and nitrogen. Some of the ecological, physiological, and evolutionary applications of stable isotope data among living humans are discussed. By incorporating SIA in research, human biologists facilitate a productive dialog with bioarchaeologists, who routinely use stable isotope evidence, mingling different perspectives on human biology and behavior. *Am. J. Hum. Biol.* 27:593–604, 2015. © 2015

Wiley Periodicals, Inc.

An understanding of patterns of diet and nutrition among human populations is fundamental in the work of human biologists. A method widely used for assessing diets in archaeology and ecology is stable isotope analysis (SIA) of tissues, including bones and teeth, as well as tissues that are shed naturally (e.g., hair, nails). SIA is based on the principal that “you are what you eat”: isotopic signatures of foods consumed are recorded in consumer tissues. In general, there are patterned isotopic differences among broad categories of food; for example, plants using the C<sub>3</sub> versus the C<sub>4</sub> pathway, animals versus plants, and terrestrial versus aquatic animals. While SIA cannot replace the precision of food diaries, diet recalls, or questionnaires in recreating menus of specific food items, it improves on accuracy and record keeping while researchers are not in the field, and offers a time-average of dietary behaviors over days, weeks, months, or years, as a useful complementary dietary assessment method.

Human biologists are familiar with stable isotopes in biological systems from the doubly labeled water technique for estimating total energy expenditure, which uses foods or drinks “spiked” with artificial abundances of oxygen and hydrogen to calculate O<sub>2</sub> consumption and energy expenditure (Leonard, 2012; Schoeller, 1988, 1999). In these experimental conditions, test subjects are provisioned with a tracer that is isotopically dissimilar to other possible diet items, rendering it conspicuous and easily identified against the natural isotopic backdrop of an organism's diet as the tracer moves through body systems and is gradually excreted. Unlike experimental studies using spiked isotopic tracers, in archaeology and ecology, *natural* abundances of stable isotopes of carbon, nitrogen, sulfur, oxygen, and other elements in human tissues are assessed as a means for estimating human diet.

Widely applied to understand hominin and archaeological human dietary ecologies, SIA has seen relatively few and isolated applications among living humans (e.g., Bender et al., 2015; Ehleringer et al., 2008; Nakamura et al., 1982; Nardoto et al., 2011; Nash et al., 2012; Neuberger et al., 2013; Wilkinson et al., 2007) despite advan-

tages it offers as a complement to other methods of studying human diet and physiology. A key strength of SIA in human biology is that tissues maintain isotopic records of types of food consumed while researchers were not present, records that can extend back weeks, months, and even years preceding commencement of the research project, depending on the tissue analyzed. Stable isotope signatures in tissues are not subject to errors in informant recall. SIA of soft tissues is minimally invasive: small amounts of fingernail (e.g., one clipping), hair, excreta, or blood may be sampled for isotopic analysis to provide information about the environments and diets of human subjects. Stable isotope data, in general, do not point to individual food items, but rather, reflect consumption of general categories of food, distinguishing, for example, between diets comprising high versus low amounts of animal-derived protein, or diets comprising the C<sub>4</sub> plants sugar and maize, versus C<sub>3</sub> plants such as fruits and vegetables. Aside from offering dietary information, SIA identifies excursions from physiological homeostasis and metabolic disorders (e.g., diabetes, osteoporosis, protein stress; Reitsema, 2013).

The purpose of this article is to describe SIA for human biologists as a tool for understanding dietary habits and physiology of contemporary human populations. Primary emphasis is placed on analytical and interpretive methods for stable isotope ratios of two elements, carbon and nitrogen. Secondary emphasis is placed on sulfur, analysis of which has become more widespread in just the past dozen years thanks to improved analytical technologies (e.g.,

Additional Supporting Information may be found in the online version of this article.

\*Correspondence to: Laurie J. Reitsema, Department of Anthropology, University of Georgia, 250 Baldwin Hall, Jackson St., Athens, GA 30602. E-mail: reitsema@uga.edu

Received 1 August 2014; Revision received 31 March 2015; Accepted 22 May 2015

DOI: 10.1002/ajhb.22754  
 Published online 22 July 2015 in Wiley Online Library (wileyonlinelibrary.com).

Giesemann et al., 1994), and on oxygen, which is more commonly applied to mineralized tissue but has seen noteworthy application among soft tissues from living humans (e.g., Ehleringer et al., 2008). Soft tissues are the focus of this article, although shed or extracted deciduous or permanent teeth are other substrates for isotopic analysis and may be analyzed in the same manner as are archaeological samples (Ambrose, 1990; Garvie-Lok et al., 2004; Lee-Thorp and van der Merwe, 1987).

### PRINCIPLES OF SIA

Isotopes are atoms of an element with the same number of protons in their nuclei, but different numbers of neutrons (Fig. 1). Unlike radioactive isotopes, stable isotopes do not decay over time, making them ideal tracers in biological systems. For carbon and nitrogen, the light isotopes are the more abundant isotopes in nature:  $^{12}\text{C}$  makes up 98.9% of all carbon, and  $^{14}\text{N}$  makes up 99.6% of all nitrogen in the biosphere. Two stable isotopes of the same element have essentially the same properties, but different masses, which are detectable through mass spectrometry (Hoefs, 2004). The carbon, nitrogen, sulfur, and oxygen in human tissues are obtained chiefly or exclusively from diet, and the particular isotopic signatures of carbon and nitrogen in human tissues reflect the particular signatures of foods consumed in life.

Stable isotope values are expressed not as absolute values, but as relative values, or ratios. Carbon and nitrogen are expressed according to the equation  $\delta = (R_{\text{sample}} - R_{\text{standard}}) / R_{\text{standard}} \times 1000$ . By expressing the ratio of one isotope to another in a sample ( $R_{\text{sample}}$ ) in relation to the same ratio from a laboratory standard whose absolute isotope value is known ( $R_{\text{standard}}$ ), values from all sample types are comparable across laboratories, using the permil symbol (‰). Expressing stable isotope ratios as permil values is not analogous to expressing something as “parts per thousand,” because stable isotope ratios are not concentrations starting at zero (it is not possible for a substance to contain fewer than zero parts per thousand of something, but stable isotope ratios can be expressed as negative values). Rather, stable isotope values are *ratios* that compare a sample to a set standard, whose value is not zero but is rather set to zero while still containing both “light” and “heavy” isotopes. Positive values indicate that a sample has more of the heavy isotope than does the standard; negative values indicate a sample has fewer heavy isotopes than the standard. Using the permil expression is a matter of convenience: multiplying extremely small numbers by 1,000 makes it easier to communicate these values.

An element’s isotopes are transferred between substrates through reactions such as evaporation of  $\text{H}_2\text{O}$  from water to air, and metabolism of dietary protein to build tissues. Although stable isotopes do not decay, stable carbon and nitrogen isotope ratios differ between diet and consumer tissues due to a process referred to as “fractionation.” Fractionation occurs because bonds incorporating lighter isotopes are weaker than bonds incorporating heavier isotopes and break more readily during chemical exchanges (e.g., during biosynthesis and respiration) (Hoefs, 2004). Fractionation changes the overall isotope signatures of an initial material and its byproduct(s)—for example, atmospheric  $\text{CO}_2$  that is inspired versus the  $\text{O}_2$  that eventually binds to hemoglobin—often in predictable ways. The effect of

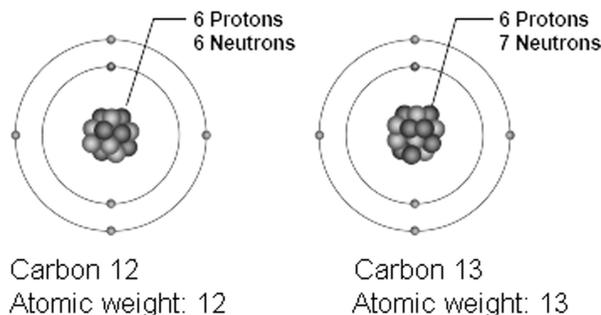


Fig. 1. Two stable isotopes of carbon are shown,  $^{12}\text{C}$  and  $^{13}\text{C}$ , having the same numbers of electrons and protons, but different numbers of neutrons, giving them “lighter” or “heavier” atomic masses.

this is referred to as a “diet-tissue space.” Different tissues are associated with different diet-tissue spaces, but applying correction factors make different tissues comparable (Supporting Information S1). Some physiological conditions affect the degree of fractionation in a consumer’s body, and, subsequently, diet-tissue spaces. For example, among smokers, the diffusion of oxygen across calloused pulmonary membranes occurs more slowly than among non-smokers, leading to more time for fractionation to occur, and ultimately, to higher diet-tissue  $\delta^{18}\text{O}$  spaces among smokers’ tissues (Epstein and Zeiri, 1988). Another example is starvation and wasting forcing an organism to recycle its own endogenous tissue reserves to meet maintenance and growth demands, which essentially increases the number of times nitrogen is subjected to fractionating steps in the body. Greater diet-tissue spaces and overall  $\delta^{15}\text{N}$  ratios in nutritionally stressed organisms are the result of this (Deschner et al., 2012; Fuller et al., 2005; Hatch et al., 2006; Hobson et al., 1993; Mekota et al., 2006; Neuberger et al., 2013; Vogel et al., 2012).

Stable isotope values are typically reported using bivariate plots, traditionally with carbon on the X axis and nitrogen, sulfur, or oxygen on the Y axis (e.g., Fig. 2). Some researchers utilize IsoSource in interpreting isotopic data, a program available online that estimates the likely proportions of foods that contributed to a consumer’s diet, based on the isotopic signatures of suspected dietary items, the known diet-tissue space relationship between foods and the consumer tissue under study, and signatures of the consumer tissue (Codron et al., 2009; Phillips and Gregg, 2003; see Useful Resources).

### INTERPRETING STABLE ISOTOPE DATA IN ANTHROPOLOGY

Different types of plants and animals have different characteristic stable isotope abundances, and these variations are passed on to consumers (Schoeninger and Moore, 1992). Some basic information about sources of variation in stable carbon, nitrogen, sulfur, and oxygen isotope ratios follows, along with illustrative examples of applications in human biology.

#### *Stable carbon isotope ratios*

Broadly speaking, three classes of plants differ in their photosynthetic strategies, producing different stable carbon isotope ranges:  $\text{C}_3$  plants, which exhibit low  $\delta^{13}\text{C}$  values

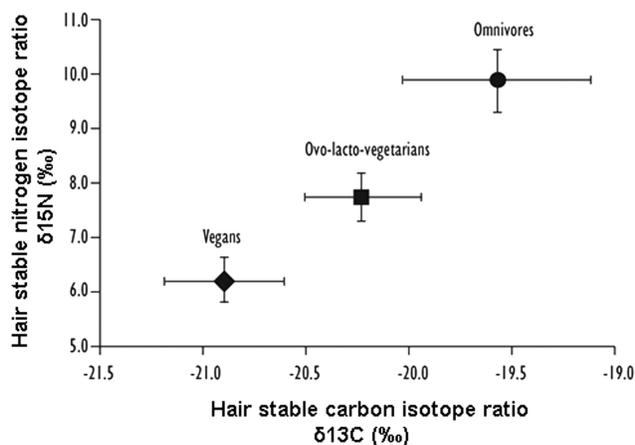


Fig. 2. Bivariate plot showing stable carbon and nitrogen isotope data from human hair, reflecting differences in dietary protein sources (modified from Petzke et al., 2005). Reproduced with permission of the publisher.

(including fruits, vegetables, and some grains such as wheat and rice),  $C_4$  plants, which exhibit comparatively high  $\delta^{13}C$  values (including maize and sugar cane), and crassulacean acid metabolism (CAM) plants, that exhibit intermediate and overlapping values (including pineapple and cactus) (Schoeninger and Moore, 1992). Marine fish, terrestrial animals, and freshwater fish typically exhibit high, intermediate, and low  $\delta^{13}C$  values, respectively, owing to variations in atmospheric and aquatic carbon sources, but with considerable variation across these categories (Chisholm et al., 1982).

Understanding differential patterns of access to plants versus animals, or  $C_3$  versus  $C_4$  plants in a population, are common applications of SIA in bioarchaeology, and, in the last decade, they have been applied among living humans as well. Among living Alaskan Natives undergoing the transition from subsistence-based to market-based diets, Wilkinson et al. (2007) and Nash et al. (2012) documented greater consumption of foods derived from sugar and corn (both  $C_4$  plants) including livestock foddered on corn, drinks sweetened with corn syrup, and sugar, among younger individuals compared with elders. Teenagers and young adults exhibited significantly higher  $\delta^{13}C$  values in red blood cells than elders. That younger community members disproportionately reflect a diet shift points to the role of young people in propelling nutrition transitions. In the Brazilian Amazon, Nardoto et al. (2011) used stable isotope evidence to document a successive increase in the consumption of  $C_4$ -derived foods—specifically in this case, frozen chicken foddered on  $C_4$  plants—between rural, town, and city inhabitants. Their isotopic evidence corroborates other indicators that urban-dwellers more acutely experience the nutrition transition from locally produced, to processed and industrialized products. In both cases,  $\delta^{13}C$  data provided a time-average of dietary decisions made by community members during weeks and months when researchers were not present, and the  $\delta^{13}C$  data generally agreed with the other dietary assessments that were implemented.

#### Stable nitrogen isotope ratios

$\delta^{15}N$  values are interpreted to understand types and amounts of animal and animal product consumption,

because with every step up the food chain,  $\delta^{15}N$  values become higher due to fractionation during nitrogen metabolism (Steele and Daniel, 1978; O'Connell et al., 2012). Whereas plants in a given ecosystem may have low  $\delta^{15}N$  values ( $\sim 1\text{--}3\text{‰}$ ), herbivores will have higher values; omnivores and carnivores will have the highest values (Fig. 2). Using hair  $\delta^{15}N$  variation from living humans in Cali, Colombia, Bender et al. (2015) documented greater animal-derived protein consumption among high socioeconomic status individuals compared with low socioeconomic status individuals. Interestingly, the isotopic evidence for diet did not closely track the 24-h diet recall evidence, which likely underscores the different time scales represented: whereas the diet recall informs a person's diet during the previous day, stable isotope ratios in hair represent at least a period of one week preceding collection, if a 1-cm segment of hair at the scalp is analyzed (see discussion of hair samples below), or several years, if a whole strand is used. Bender et al. (2015) used hair snipped from 2.5 to 5 cm below the scalp, meaning the isotopic record of diet began 2–5 months prior to the diet recall, extending back through time a variable period depending on the hairs' lengths. Rather than a cautionary tale of limitations of stable isotope evidence in diet assessment, this case study illustrates how SIA complements short-term evidence for diet toward a bigger picture.

Infants who are breastfeeding exhibit higher  $\delta^{15}N$  ratios than do their mothers. This is because breast milk has already undergone isotopic fractionation in the mother's body, and once transferred to the infant, effectively places that infant a trophic level higher than its mother. Fuller et al. (2006) collected hair and fingernail samples from mothers and infants throughout the duration of weaning, and documented variations in isotopic ratios that mapped onto breastfeeding behavior. As weaning progressed,  $\delta^{15}N$  values in infant tissues dropped. The  $\delta^{15}N$  data were capable of distinguishing between formula-fed and breast-fed infants. Detecting infant dependence on mother's milk is thus possible using SIA. It has become common practice in bioarchaeology to infer weaning ages from cross-sectional  $\delta^{15}N$  values in mortuary populations, but questions remain regarding the effects of pregnancy and lactation on  $\delta^{15}N$  values of mothers and infants. For example, a few studies of living mammals have shown significantly lower  $\delta^{15}N$  values among lactating mothers compared to nonlactating females (Koch, 1997; Kurle, 2002; Reitsem and Muir, 2015), indicating the standard practice of comparing infants to adult females in a skeletal population at large (most of whom are presumably nonlactating) may be flawed. Additional isotopic work among living humans will considerably hone bioarchaeological interpretations of weaning in the past.

Individuals who are nutritionally stressed may exhibit relatively high  $\delta^{15}N$  ratios (Fogel et al., 1989; Fuller et al., 2005, 2006; Hatch, 2012; Hobson et al., 1993; Petzke et al., 2010), owing to recycling of endogenous tissues that mimics higher trophic positions. Hatch et al. (2006) and Mekota et al. (2006) demonstrated that among individuals suffering from anorexia and bulimia, hair  $\delta^{15}N$  ratios were elevated when patients were admitted to a treatment facility, and declined as treatment successfully progressed (and BMI increased). Neuberger et al. (2013) demonstrated that among known medical cases of individuals who died after having suffered from severe undernourishment, hair  $\delta^{15}N$

ratios had become higher leading up to death. This case study demonstrated how isotopic evidence may reveal the extent and duration of nutritional stress death, which may have been unknown, having important implications in forensic cases.

#### *Stable sulfur isotope ratios*

Stable sulfur isotope ratios ( $\delta^{34}\text{S}$ ) reflect both diet and geographic place-of-residence, because  $\delta^{34}\text{S}$  values in plants vary with the isotopic values of underlying bedrock.  $\delta^{34}\text{S}$  values also help identify fish in human diet and geographic proximity to oceans, because marine water  $\delta^{34}\text{S}$  is higher than terrestrial  $\delta^{34}\text{S}$ , which is in turn higher than freshwater  $\delta^{34}\text{S}$  (Nehlich et al., 2010, 2011; Richards et al., 2001). Valenzuela et al. (2011) created a sulfur “isoscape” of North America by sampling human hair 73 cities across the United States, identifying geospatial patterning in  $\delta^{34}\text{S}$  ratios, with significantly lower values in Minnesota and North Dakota, radiating out to higher values toward the West, South, and East (i.e., toward coasts).  $\delta^{34}\text{S}$  variation in human tissues derives from dietary amino acids; thus, even if plants and animals exhibit systematic  $\delta^{34}\text{S}$  variation across geographic space, for a geographical pattern to emerge among human tissues, humans must, in theory, be eating foods produced in the same geographic vicinity. Therefore, it is surprising that Valenzuela et al. (2011) identified a geospatial pattern in modern North American  $\delta^{34}\text{S}$  values, which tempers the assumption that the typical modern North American diet does not include significant amounts of locally sourced foods. Geospatial patterning in  $\delta^{34}\text{S}$  variation could, speculatively, permit researchers to “place” people with a history of mobility and migration across an isotopically varied landscape.

#### *Stable oxygen isotope ratios*

Stable oxygen isotope ratios ( $\delta^{18}\text{O}$ ) in consumer tissues primarily reflect the  $\delta^{18}\text{O}$  of local water sources (Longinelli, 1984; Luz et al., 1984). Natural abundances of oxygen isotopes vary with latitude, humidity, and proximity to the coast (Craig, 1961), making  $\delta^{18}\text{O}$  ratios useful indicators of population movement or food sourcing (Bol et al., 2007; Bowen et al., 2009, 2010; Chesson et al., 2010; Ehleringer et al., 2008).  $\delta^{18}\text{O}$  values of waters vary depending on the extent of evaporation: when evaporation is high, as in lochs, lakes, and dams,  $^{16}\text{O}$  will be preferentially lost in evaporate, leaving behind waters with relatively high  $\delta^{18}\text{O}$  values (Darling et al., 2003). When evaporation is low, as in aquifers, rainwater, and moving water fed by rains, relatively less of the light isotope is lost, and the overall  $\delta^{18}\text{O}$  values of water is lower. Factors affecting oxygen transport through the body (e.g., smoking, anemia, increased metabolic rate) appear to affect tissue  $\delta^{18}\text{O}$  values (Epstein and Zeiri, 1988; Reitsema and Crews, 2011; Zanonato et al., 1992). Water from fruits and vegetables is isotopically higher than the meteoric waters in which the foods are grown due to evapotranspiration (Dunbar and Wilson, 1983), speculatively offering insights into human hydration strategies. The  $\delta^{18}\text{O}$  values of wine are higher than those of water and of beverages made from water, a principle used in one bioarchaeological analysis to infer that King Richard III enjoyed more wine in his diet following his coronation (Lamb et al., 2014). Water that has been boiled is isotopically heavier due to

preferential loss of  $^{16}\text{O}$  in steam (Brettell et al., 2012). Body water is derived from drinking water, water from food, and water derived from atmospheric oxygen, and the greater the proportional contribution of drinking water to total body water, the more closely body water  $\delta^{18}\text{O}$  values (and thus, tissue  $\delta^{18}\text{O}$  values) will represent the  $\delta^{18}\text{O}$  values of drinking water. O’Grady et al. (2010) demonstrated this principle among diabetic mice, whose physiological condition causes them to drink more water, tissue  $\delta^{18}\text{O}$  values approached drinking water  $\delta^{18}\text{O}$  values.  $\delta^{18}\text{O}$  evidence has been widely applied in archaeology to inform breastfeeding and weaning, as the  $\delta^{18}\text{O}$  ratios of breastmilk are higher than those of water (e.g., Wright and Schwarcz, 1998).

#### EQUIPMENT FOR SIA

In the field, little specialized equipment is required to collect samples for SIA. The chief concern in collecting and preserving samples in the field is not that the isotopic ratios will change over time, but rather that bacterial or fungal growth may affect the carbon and nitrogen in the sample. Common-sense steps can be taken to reduce this risk, such as refrigerating, freezing ( $-17$  to  $-50^\circ\text{C}$ ), or drying ( $60^\circ\text{C}$ ) samples within approximately 10 h of collection or as soon as possible, and storing samples in clean, dry containers. Preservation and pre-processing methods that should be avoided include use of formalin and ethanol (but see Hobson et al., 1997), and preprocessing steps involved with hormonal analyses (Barrow et al., 2008). Although at least two exceptions are reported (Barrow et al., 2008; Feuchtmayr and Grey, 2003), freezing is a common and likely reliable means of preserving samples for later analysis (see discussion in Barrow et al., 2008; Gloutney and Hobson, 1998).

Final processing of samples requires some specialized equipment, which is usually available in the laboratory that will undertake the isotopic analysis. Researchers should communicate with laboratories to learn which processing steps the laboratory staff are willing to undertake, and how much these steps will cost. Before analysis, samples will need to be dried: oven-drying at no more than  $60^\circ\text{C}$ , air-drying (depending on the climate), storing samples in vials or tubes with silica beads, using solar food dehydrators, and freeze-drying are “safe” strategies. Blood, urine, and feces must be homogenized, which thoroughly mixes the isotopic signal of the sample. Homogenization can be accomplished by hand in a glass, stainless steel, or ideally, non-porous 5 cm agate mortar and pestle, or in a ball/shaker mill (e.g., by Wig-L-Bug) or freezer mill (e.g., by Spex). Another step requiring special equipment is weighing samples with high precision (to the nearest 0.001 mg) into tin weigh boats using a microbalance immediately prior to analysis by mass spectrometry, which may be undertaken by laboratory personnel (Fig. 3; see Useful Notes).

Stable isotope facilities may be found in earth science, ecology, chemistry and other departments in academic institutions. There are also commercial facilities, such as the Center for Applied Isotope Studies in Athens, Georgia, that accept contract samples. Two pieces of equipment are required for stable carbon and nitrogen isotopic analysis of organic samples: an elemental analyzer and a light stable isotope mass spectrometer (Fig. 4). The elemental analyzer (EA) combusts the sample to produce a gas (carbon is measured from  $\text{CO}_2$  and nitrogen from  $\text{N}_2$ ) and its

thermal conductivity detector measures the amount of carbon and nitrogen in a sample. A mass spectrometer measures the ratios of different stable isotopes in the  $\text{CO}_2$  and  $\text{N}_2$  gases. Stable carbon and nitrogen data and carbon and nitrogen content from one sample are usually produced in the same analytical run, and are frequently billed as one analysis. Some labs analyze carbon, nitrogen and sulfur simultaneously.

Laboratories differ widely in their pricing for bulk SIA with prices ranging from no charge, to as much as \$75 or more per sample for  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  data. A range of \$10-\$25 per sample is common.

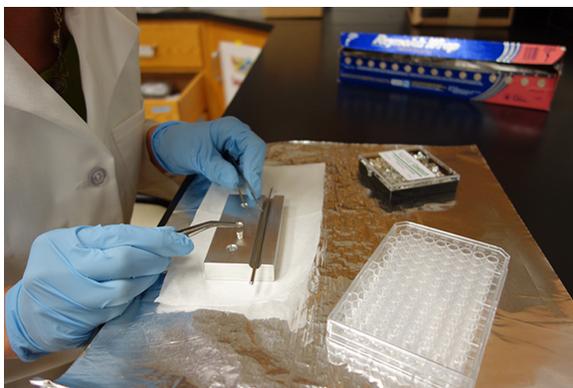


Fig. 3. All samples must be meted and sealed into tin capsules prior to combustion and analysis by mass spectrometry. Special tools are required to compress tin capsules while preventing any contamination from other particulates, as the tin capsule is combusted along with the sample to produce the gas that is ultimately analyzed. Pictured here are the sample preparation plate and spatula, forceps, tin capsules (back right and atop preparation plate), and preparation tray (front right) from Costech. If measurements of the carbon and nitrogen content are desired alongside the elemental isotopic ratios, these tin capsules are also used as weigh boats, and samples are weighed to the nearest 0.001 mg in a microbalance. Photo: D. Chasteen.

## APPLICATION

### Choosing a tissue for analysis

Because the stable isotope signatures of different tissues reflect different “snapshots” of time, the appropriate tissue for isotopic analysis depends on the research question, and whether a time-average of data or a precise window are desired. Feces, urine and blood plasma provide stable isotope information from the preceding days, whereas red blood cells, muscle, hair, and nails provide information about preceding weeks, months and/or years. Depending on their lengths, hair and nails can record incremental records of several years (Table 1). Collecting “short-term” samples longitudinally (e.g., plasma every month for a year) can lend short-term resolution to a much broader sample collection schedule. The choice of tissue for isotopic analysis also may be influenced by what tissues are already under study; for example, stable isotope data can be collected from blood samples already collected in the field.

Most stable isotope research in anthropology is conducted using bulk tissues – for example, bulk protein in either collagen or keratin. There is growing interest in the analysis of individual amino acid constituents in bulk protein, which involves specialized preparation and analytical methods. The elution of amino acids or fatty acids from a bulk protein requires gas or liquid chromatography prior to mass spectrometry. For a more detailed overview of compound-specific stable isotope mass spectrometry and specifically, analysis of individual amino acids from bulk keratin, see Petzke and Fuller (2012).

### Methods for collection and preparation of different tissue types

The following sections outline collection and preparation methods for stable carbon and nitrogen isotope analysis of hair, nails and blood. In all cases, tools used for collection and preparation should be cleaned using alcohol, such that cross-contamination between samples is prevented. Typical tools for the following collection and

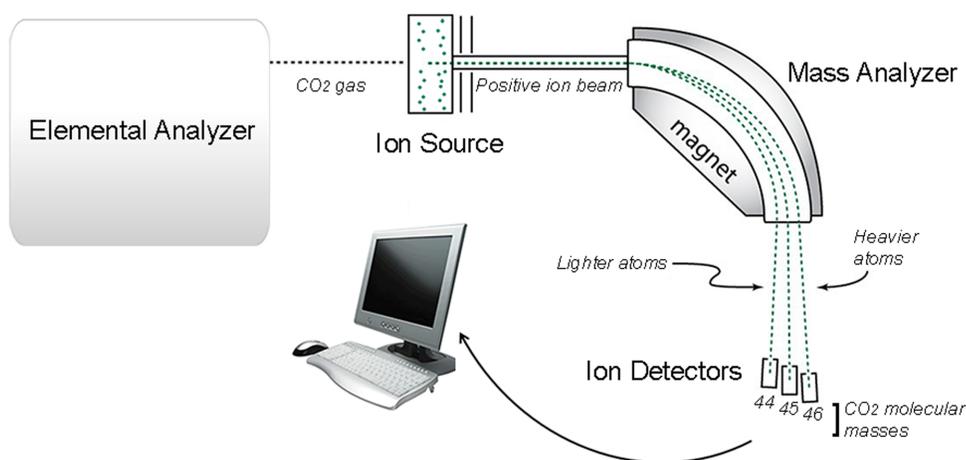


Fig. 4. Schematic showing an elemental analyzer coupled to a stable isotope ratio mass spectrometer. Tin capsules containing samples are combusted in the elemental analyzer, and ensuing gasses (in this case,  $\text{CO}_2$ ) are delivered to the mass spectrometer. The mass spectrometer comprises the ion source, where atoms/isotopes in the gas stream are ionized, a mass analyzer, where isotopes of different atomic weights are separated into a mass spectrum, and ion detectors, spaced appropriately to collect the separated ion beams. Analog current intensity information from the detectors is converted to digital data and sent to the computer.

TABLE 1. Time frames represented by isotopic ratios in soft tissues

Sample	Time period represented			
	Days	Weeks	Months	Years
Urine	X			
Feces	X			
Blood plasma	X			
Blood cells		X		
Nails		X	X	
Hair		X	X	X
Teeth (dentine; enamel)				X

preparation procedures include forceps, 50 ml glass beakers or plastic centrifuge tubes, glass sample collection vials and zip-lock bags. Target weight ranges guiding how much of a sample should be collected are given in the following sections, but more details are provided in Supporting Information S2.

It is important to wear gloves and to clean tools and surfaces between samples with solvents such as acetone, isopropyl alcohol or methanol and to use lint-free tissues such as Kimwipes®, because introducing skin cells to a sample – or anything else containing nitrogen and/or carbon – causes contamination. Also for this reason, air dusters are advised during cleaning to eliminate lingering dust, skin cells, or lint from tools and surfaces that may contact the samples. Lining surfaces with tin foil and replacing it periodically is a good way to reduce risk of cross-contamination.

**Hair.** As hair grows, keratin protein is not resorbed or recycled and its isotopic signatures do not change; thus, hair forms a static longitudinal record of stable isotope signatures. Isotopic signatures from hair have been shown to accurately reflect diet among humans (Macko et al., 1999; O'Connell and Hedges, 1999) and drinking water source among humans (Ehleringer et al., 2008). Isotopic signatures of hair are fixed as the keratin is produced at the scalp, and newly erupting hair contains a composite signal of the preceding 6-12 days of hair creation (Nakamura et al., 1982; Saitoh et al., 1964; Sponheimer et al., 2003a). Sections of hair may be subsampled to obtain a longitudinal record of isotopic variation. 1 cm sections represent approximately one month, and 2.5 mm sections have been taken to estimate weekly intervals (Mekota et al., 2006; O'Connell et al., 2001).

Hair undergoes different phases of growing and resting, and at any given time, 10-20% of scalp hairs are not actively growing (Mekota et al., 2006). That is, not all hairs follow the same clock, and two hairs from the same head do not necessarily reflect identical time periods. This problem can be minimized by collecting and analyzing multiple hairs from the same individual. Hairs that are naturally shed at the same time are in the same stage of growth.

Hairs may be tweezed or trimmed at the root, or collected during brushing or combing. Grey hairs do not differ isotopically from others. In the field, hair may be folded into tin foil or anchored in micropore tape to keep roots and ends apart, and transferred to pre-labeled plastic sample bags. Samples can be stored thus at room temperature indefinitely, although refrigerating, freezing, or storing samples in a vial with silica, or in a desiccator cab-



Fig. 5. For incremental sampling, hairs may be laid root to end beside a ruler and sectioned uniformly. Here, a measuring tape and a sample of eight hairs are secured to a glass cutting board with micropore tape (pictured in background), and cut with a scalpel into 1 cm increments. Hairs may then be placed in folded wax paper and stored in dry conditions, or compressed immediately into tin capsules (capsules pictured in background) for analysis. Photo: D. Chasteen.

inet will not alter the stable isotope signatures and may inhibit undesired bacterial or fungal activity.

Residues such as shampoos and oils must be removed from hairs prior to analysis. This can be accomplished by soaking in 3 ml acetone for 2 hr and drying for 1 hr in a 60-80°C oven to volatilize residual solvent. If sectioning of hairs is desired for the sake of gathering longitudinal data from strands, individual hairs should next be laid, root to end, alongside a ruler and sectioned precisely with a razor (Fig. 5). Hairs or hair sections can be stored in foil, folded wax paper, or plastic sample bags (see Useful Notes).

The amount of hair to sample depends on whether hair will be sectioned (for a longitudinal record) or analyzed as a single strand. 6-12 hairs are advised if 1 cm sections will be made, and as many as 20 if even smaller sections will be made. For  $\delta^{18}\text{O}$ , at least 0.150 mg is needed. For  $\delta^{34}\text{S}$ , approximately 0.900 mg is required (Petzke and Fuller, 2012). Hairs should be dried before analysis, by storing samples in a 60°C oven or in a desiccator cabinet.

Hair is the most commonly studied tissue for SIA in field studies among living humans (Bender et al., 2015; Bol et al., 2007; Bol and Pflieger, 2002; de Luca et al., 2012; Fuller et al., 2006; Hatch et al., 2006; Mekota et al., 2006; O'Connell and Hedges, 1999; Petzke et al., 2006; Petzke et al., 2010; Romek et al., 2013; Valenzuela et al., 2012; Valenzuela et al., 2011). One of the primary advantages of using hair in isotopic studies is the longitudinal record of isotopic data it contains. Within a single individual, dietary variation associated with seasons, nutrition transitions, migration, weaning, and stress may be assessed. An excellent review of applications of hair SIA among humans is provided by Petzke et al. (2010).

**Nails.** The stable isotope signatures of fingernails are fixed as keratin is produced in the nail bed, and do not change as the nail grows out. Adult fingernails grow at a rate of approximately 3.5 mm/month (Yaemsiri et al., 2010), and the distal ends of nails contain an isotopic signal from approximately 4-6 months in the past (Runne

and Orfanos, 1981). Toenails grow more slowly, at around 1.7 mm/month (Yaemsiri et al., 2010). Infant nails grow slightly faster, taking 2-3 months to grow out, which means nail trimmings taken until 3 months of infant age may have been synthesized in utero, offering a glimpse into the prenatal environment (Fogel et al., 1989).

Like hair, nails do not require elaborate collection or preparation techniques. Unlike hair, distal nail clippings do not include a long record of incremental data and must be collected at intervals if longitudinal data are desired. Distal ends of nails may be trimmed with a small scissors or nail clipper, and transferred individually to a pre-labeled storage bag or vial. Usually one fingernail clipping is sufficient; approximately 0.400-0.600 mg of nail tissue is needed for analysis. Fingernail clippings may be stored at room temperature in plastic bags for long periods of time, although as with hair, storage in a desiccator cabinet or refrigerator discourages bacterial or fungal growth.

To prepare nail samples in the laboratory, visible dirt should be washed or wiped off using water or alcohol pads. As with hair, nails can be soaked in 3 ml acetone for 2 hr and oven-dried at 60-80°C to volatilize residual solvent and dry the sample. Clean nails can then be cut into small pieces, and an amount weighed into a 1 cm square of tin (not aluminum) foil or tin capsule for analysis, as advised by the mass spectrometry laboratory.

Fingernail samples have been used in human biology to monitor infants' dietary dependence on breast milk during the weaning process (Fogel et al., 1989; Fuller et al., 2006; Millard, 2000). Fingernail data from individuals living in rural, small town, and urban settings in Amazonas State, Brazil, show isotopic support for a nutrition transition from subsistence diets comprising manioc flour and fish to diets increasingly composed of sugar and corn-fed beef (Nardoto et al., 2011). In 2000, 2003 and 2006 the United States Geological Survey conducted open houses during which researchers analyzed the fingernails of 1107 visitors. In general, results of these analyses illustrate the importance of animal protein, corn and sugar in North American foodwebs (see Useful Resources).

**Blood.** Blood comprises blood cells, serum and plasma which may be isotopically analyzed individually, or in bulk. Blood components have slightly different turnover times and thus represent different time slices. Whereas plasma and serum are replaced after approximately 1-2 weeks, red blood cells remain in the body for 2-3 months (Hildebrand et al., 1996; Kurle, 2002). This means that, from a single blood sample, two different chronological snapshots may be obtained (Table 1).

Many researchers collect blood into EDTA tubes: these are acceptable for isotopic analysis, and do not alter isotopic signatures (Wilkinson et al., 2007). Blood may be stored frozen, or dried for SIA. If "bulk" blood is to be used as the substrate for analysis, it may be frozen or dried on glass fiber filter papers immediately upon collection (see McDade, 2014 for more information on collecting dried blood spots). It is crucial to use glass fiber filter papers, as carbon from typical filter papers will contaminate the  $\delta^{13}\text{C}$  signal of the sample. If plasma is to be isolated, whole blood should be collected into heparinized tubes. Sodium heparin does not alter the isotopic signatures of the samples (Hobson et al., 1997; Kurle, 2002). To collect serum, test tubes containing no additives should be used,

and whole blood should be allowed to coagulate at room temperature for 15-30 minutes. To separate the red blood cells from either the plasma or the serum, which in the serum tubes will be clotted and in the plasma tubes will be unclotted, centrifuge the samples under refrigeration at 1,000-2,000 rpm for 10 minutes. It is possible to further divide plasma into lipid, protein and carbohydrate fractions (e.g., Schoeller et al., 1986). The isotopic signatures of each fraction offer details about dietary sources of different macronutrients.

After centrifugation, at least 200  $\mu\text{l}$  of supernatant (either serum or plasma) is needed for analysis, pipetted from tubes. If a freezer and freeze dryer are readily available, these samples should be transferred to a glass scintillation vial, frozen (including for storage) and lyophilized. If only an oven is available to dry samples, a pipette may be used to distribute at least 200  $\mu\text{l}$  of sample onto glass fiber filter circles, which may be frozen for storage or dried at 50°C (Barrow et al., 2008) and then stored in a freezer. Ethanol may be used to preserve samples if absolutely necessary, but Barrow et al. (2008) summarize possible ill effects of utilizing ethanol. Blood cell clots can be collected from tubes with a spatula or stir rod and either placed in a scintillation vial for freezing and freeze drying, or wiped onto glass fiber filter papers for oven or air drying. Entire filter circles can be placed in storage vessels (petri dishes or small sample bags) and dried (unsealed) in a 50°C oven for 24 hours. Dried filter circles bearing samples should be stored in airtight containers at room temperature or in a refrigerator until analysis. Additional details on blood sample collection and preparation for SIA can be found elsewhere (Barrow et al., 2008; Crowley, 2012; Hobson et al., 1997; Kurle, 2002).

The remaining steps should take place in the lab where samples are to be analyzed. Use a micro spoon or micro spatula to collect approximately 1.0-1.4 mg of plasma or serum and 0.7-0.9 mg of red blood cells from the scintillation vial or glass fiber filter papers and transfer into tin capsules for analysis.

Blood draws are already part of many study protocol, and researchers may identify a wide range of applications of stable isotope data to supplement the types of data already being obtained from blood (e.g., hormonal data). Blood stable isotope ratios reflect aspects of diet, and, as with the other tissues described here, preserve a record of categories of foods consumed (Nash et al., 2012; Schoeller et al., 1986; Wilkinson et al., 2007). Blood and blood fractions have seen a number of applications in studies of non-human animal weaning (Habran et al., 2010; Jenkins et al., 2001; Kurle, 2002; Polischuk et al., 2001; Reitsema and Muir, 2015). Such studies have explored mobilization of body reserves to meet nutritional requirements during fasting, hibernation, lactation, gestation, and infant growth using blood and blood fractions. Borrowing from these applications, human biologists may find use in stable isotope data as a means to learn more about biological variations in how humans mobilize endogenous and exogenous reserves to meet energetic requirements at different stages during the lifespan (e.g., growth, lactation, senescence), or about interindividual and interpopulation variation in the timing of weaning events, without complete dependence on observation or informant recall.

**Feces.** Feces comprise undigested food particles, gut microbes, nondigestible food waste, and exfoliated epithelial

cells. Along with urine, feces are a main means of nitrogen efflux (Sponheimer et al., 2003b). In terms of dietary information through SIA, feces represent both what foods are “used” by humans (sloughed tissues), and what foods are “unused” (undigested particles). Previous research has indicated fecal stable isotope data are a reliable indicator of diet and dietary transitions (Codron and Codron, 2009; Reitsema, 2012). An advantage of feces as a substrate for SIA is that feces are synthesized quickly, offering a refined snapshot of diet, the length of which depends on a species transit/retention times. Another advantage is that, unlike proteinaceous tissues such as hair, nails, or collagen, which are formed preferentially from dietary protein, feces are more sensitive to intermediate amounts of animal-based protein in the diet, and are thus less likely to over-represent animal-based protein.

Two to five ml (~2–5 g) of sample uncontaminated by extraneous substances, may be stored in a refrigerator or freezer, but must eventually be dried. Previous fecal stable isotopic research among non-human animals has utilized feces deposited at unknown times in field settings and thus not immediately frozen and dried and still yielded meaningful results (e.g., Codron and Codron, 2009). Samples may be desiccated by including silica beads in the collection or storage tubes, covered by a wad of glass wool, followed by the sample and the cap, or by the variety of other drying means previously discussed. After drying, feces should be homogenized in a mortar and pestle to thoroughly mix the sample. Homogenization reduces the problem of undigested food particles, which, if only opportunistically removed, create unwanted inter-sample variation when the time comes to subsample feces for analysis. Approximately 1.0–1.5 mg of powdered sample is needed for analysis.

Few applications of fecal SIA exist among humans, although some have been conducted among other animals (Codron and Codron, 2009; Codron et al., 2005; Focken, 2001; Sponheimer et al., 2003b; Sponheimer et al., 2003c). Rikimaru et al. (1985) used  $^{15}\text{N}$  in feces as a tracer among Papua New Guinea highlanders to measure urea nitrogen recovery and utilization as a strategy for coping with protein stress. Among non-human primates, fecal stable isotope data have been used to show that the weaning process among captive *Trachypithecus francoisi* is more protracted than what was expected based on observation alone (Reitsema, 2012) and to compare resource use by *Papio ursinis* inhabiting different environments (Codron et al., 2006).

**Urine.** Urine has the most rapid turnover of any of the substrates for SIA discussed here, making it the most sensitive indicator of changes in diet or physiological status.

Urine samples should be collected into sterilized plastic containers with screw-caps and frozen. Approximately 1–2 ml is sufficient for analysis. Samples should be freeze-dried or oven-dried, homogenized, weighed, and pressed into tin capsules for analysis (Fuller, 2003). Approximately 1–2 mg of dried sample is recommended for analysis.

Urinary  $\delta^{15}\text{N}$  signatures from humans have shown a local adaptation to urea metabolism among New Guinea highlanders, reflecting chronically low-protein diets (Rikimaru et al., 1985). Among non-human primates,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$

signatures from urine have demonstrated that under conditions of nutritional stress, either experimentally induced or in the natural environment, urine is enriched in  $^{15}\text{N}$  compared to under conditions of sufficient protein, reflecting catabolism of endogenous tissues to meet nutritional demands of the body (Deschner et al., 2012; Vogel et al., 2012). Importantly, increased efflux of  $^{15}\text{N}$  in urine is potentially a more sensitive and earlier detection method of protein stress than is weight loss.

**Foods.** Stable isotope ratios of foods fall into more or less predictable ranges, but cannot be precisely assumed, due to natural and anthropogenic influences on soil stable isotope ratios that are passed up the food chain (c.f., Szpak, 2014; Tieszen, 1991). A sense of the variation of modern foods is given by Nardoto et al. (2006) and Schoeller et al. (1986). In light of the tendency for foods to vary isotopically, researchers who will utilize stable isotope data from human tissues can analyze available foodstuffs to confidently establish an interpretive baseline.

To prepare bulk foods for isotopic analysis, parts of foods which are eaten—for example, muscle, not bone; fruits, not stems—should be selected. Food items may be desiccated in a food dehydrator, oven, or freeze dryer and homogenized by grinding. If desired, protein, lipid, and carbohydrate fractions of foods may be separated for individual isotopic analysis: guidelines for individually analyzing these macronutrients are described by Schoeller et al. (1986).

The amount of a food to weigh into tin capsules for analysis depends on the carbon and nitrogen content of the food (see Supporting Information S2), and test runs are advised. For many plants, which contain much carbon (35–45%) but little nitrogen (1–6%), 1.0–2.0 mg of dried, homogenized/powdered sample is a likely range. For muscle which is not so nitrogen-poor, weights of 0.500–0.700 mg may be appropriate.

#### MAJOR ADVANTAGES AND LIMITATIONS

A comparison of the major advantages and major limitations of SIA in human biology compared to other dietary assessment methods (WHO/FAO, 1996) is presented in Table 2. An important advantage of SIA in comparison to many other methods is that isotopic data are true to *amounts* of types foods consumed. Whereas food frequency data may record the number of meals that included a food item instead of the amount of the food item consumed at a meal, stable isotope ratios in tissues derive from all incoming food, and therefore, reflect amounts of foods consumed regardless of the timing and spacing of meals. On the other hand, stable isotope data reflect *categories* of foods, not exact food items. The classes of foods that are “isotopically relevant” (e.g., plant versus animal-derived protein,  $\text{C}_3$  versus  $\text{C}_4$  plants) may not be relevant among a researcher’s research questions. Although capable of comparing relative amounts of animal protein,  $\text{C}_4$  foods, or fish in the diets of people and populations, SIA is not, on its own, able to inform *which* animals or  $\text{C}_4$  plants were consumed, or, for example, whether milk or meat was consumed from an animal. Contextual information is required to know specific menu items, and informs the nutrient profile of the diet.

TABLE 2. Summary dietary assessment methods

Method	Major strengths	Major limitations
Stable isotope analysis	<ul style="list-style-type: none"> <li>• Does not rely on memory</li> <li>• Estimates intake amounts</li> <li>• Time-averaged information accumulates while researcher is absent</li> <li>• Hair and nails are minimally invasive to collect</li> <li>• Does not alter intake behavior</li> <li>• No literacy requirement</li> </ul>	<ul style="list-style-type: none"> <li>• Estimates food categories (e.g., animal/plant; C3/C4 plant), rather than specific foods</li> <li>• Requires laboratory analysis and fees</li> <li>• Requires knowledge of local baseline isotopic values</li> <li>• Blood, urine, and feces require some invasive collection</li> </ul>
Food record/Food diary	<ul style="list-style-type: none"> <li>• Does not rely on memory</li> <li>• Easy to quantify amounts</li> <li>• Open-ended</li> </ul>	<ul style="list-style-type: none"> <li>• High participation burden</li> <li>• Requires literacy</li> <li>• May alter intake behavior</li> </ul>
24-Hour dietary recall	<ul style="list-style-type: none"> <li>• Little respondent burden</li> <li>• No literacy requirement</li> <li>• Does not alter intake behavior</li> </ul>	<ul style="list-style-type: none"> <li>• Relies on memory</li> <li>• Requires skilled interviewer</li> <li>• Difficult to estimate amounts</li> </ul>
Food frequency questionnaire	<ul style="list-style-type: none"> <li>• Relatively inexpensive</li> <li>• Preferable method for food with high day-to-day variability</li> <li>• Does not alter intake behavior</li> <li>• Limited flexibility for describing foods</li> </ul>	<ul style="list-style-type: none"> <li>• Relies on memory</li> <li>• Requires complex calculations to estimate frequencies</li> <li>• Requires literacy</li> </ul>
Diet history (meal-based)	<ul style="list-style-type: none"> <li>• No literacy requirement</li> <li>• Does not alter intake behavior</li> <li>• Open-ended</li> </ul>	<ul style="list-style-type: none"> <li>• Relies on memory</li> <li>• Requires highly trained interviewer</li> <li>• Difficulty to estimate amounts</li> </ul>
Food habit questionnaire	<ul style="list-style-type: none"> <li>• Rapid and low cost</li> <li>• Does not alter intake behavior</li> <li>• Open-ended</li> </ul>	<ul style="list-style-type: none"> <li>• May rely on memory questionnaires</li> <li>• May require a trained interviewer</li> </ul>
Weighed food record	<ul style="list-style-type: none"> <li>• Does not rely on memory</li> <li>• Estimates intake amounts</li> <li>• No literacy requirement</li> </ul>	<ul style="list-style-type: none"> <li>• High participation burden</li> <li>• May alter intake behavior</li> <li>• May require a trained interviewer</li> <li>• Requires complex calculations</li> </ul>

Modified from WHO/FAO (1996) and reprinted with permission from the World Health Organization.

Until systematically explored under controlled or closely monitored conditions, it is unclear whether stable nitrogen isotope ratios in human tissues can discern between diets comprising, for instance, 15% versus 30% animal-derived protein. Once the amount of animal-derived protein reaches a level adequate for tissue building, the human body preferences animal-derived protein in tissue building; thus, after an unknown threshold is reached, animal-derived protein isotopically swamps the  $\delta^{15}\text{N}$  signals in proteinaceous tissues.

Stable isotope data provide a useful time-average of diet. To capture monthly, seasonal, or annual patterns in diet using questionnaire, observational, or self-recorded dietary assessments, an enormous and prolonged commitment would be required from researchers or study participants. Stable isotope data from extended time periods can be easily gathered using single cuttings of hair or fingernails during a single interview.

Because of variations in soil and plant stable isotope signatures across environments and geographic space, stable isotope data from dispersed study areas are not necessarily directly comparable. Blind assessment and comparison of stable isotope values of hair from people across ecological or geographic regions is likely to yield spurious results. This problem can be minimized by relating human stable isotope signatures to those of local plants and animals, to understand whether variation among populations is due to diet, or due to dissimilar baselines. Of course, geographic variations in stable isotope signatures can also be a boon in studies of living humans, because it may assist the characterization of mobility and migration between regions (e.g., Bol et al., 2007).

## SUMMARY

Questions in human biology about diet, mobility, and nutritional stress may be addressed using stable carbon, nitrogen, oxygen, and sulfur isotope data from human tissues. Particularly in the last decade, SIA for human diet reconstruction has expanded from paleoanthropological and bioarchaeological applications, to applications among living humans. Rather than replacing the precision obtained from observational or questionnaire-based dietary assessments, SIA is a useful complement to these methods, offering information about amounts of foods consumed during times when the researcher was absent that is free from errors in informant recall, and serving as a time- and cost-effective method, given the fact that isotopic data can preserve time-averaged information about diet over weeks, months, and/or years of food intake. Stable isotope evidence has future potential to monitor nutritional stress, allocation of endogenous versus exogenous protein during growth and development, and weaning among living humans. Future application of SIA in human biology research will expand our understanding of patterns of modern human dietary variation. Increased application of SIA by human biologists will become a useful middle-ground for bioarchaeologists, as isotopic correlates of observed/self-reported dietary behaviors and variation from living humans can map on to skeletal samples, toward understanding human dietary adaptation on much larger time scales.

## ACKNOWLEDGMENTS

The author is grateful to Yohei Matsui and Andr ea Grotoli at the Ohio State University Stable Isotope

Biogeochemistry Laboratory, Susan Tanner and Randy Culp at the University of Georgia, and Thom McDade and an anonymous reviewer for feedback on this manuscript.

#### USEFUL RESOURCES

##### *Supplies For Collecting and Preparing Soft Tissue Samples for Isotopic Analysis*

List of SIA equipment providers, with links to commercial and academic laboratories:

<http://isogechem.wikispaces.com/Isotope+Suppliers>

Description of tools used for weighing samples at the University of Ottawa lab:

<http://www.isotope.uottawa.ca/guides/guides-microbalance-en.html>

Costech Analytical Technologies catalog for purchasing materials for weighing and analyzing samples (tin capsules, weighing platforms, spatulas and sample trays):

<http://www.costechanalytical.com/>

##### *Additional Information about SIA*

A program for estimating relative contributions of “menu items” to an individual’s overall diet based on the isotopic values of the foods and the consumers (IsoSource):

<http://www.epa.gov/wed/pages/models/stableIsotopes/isosource/isosource.htm>

For more information about interpreting stable isotope data and a database of fingernail stable isotope data (United States Geological Survey):

<http://www.rcamnl.wr.usgs.gov/isoig/projects/fingernails/>

For a detailed overview of stable isotope research and information about contracting samples for analysis (Colorado Plateau Stable Isotope Laboratory):

[http://www4.nau.edu/cpsil/13c\\_15n\\_org.htm](http://www4.nau.edu/cpsil/13c_15n_org.htm)

For instructions for separating blood components:

<http://www.invitrogen.com/site/us/en/home/References/protocols/cell-and-tissue-analysis/elisa-protocol/ELISA-Sample-Preparation-Protocols/Plasma-and-Serum-Preparation.html>

#### LITERATURE CITED

- Ambrose SH. 1990. Preparation and characterization of bone and tooth collagen for isotopic analysis. *J Archaeol Sci* 17:431–451.
- Barrow LM, Bjorndal KA, Reich KJ. 2008. Effects of preservation method on stable carbon and nitrogen isotope values. *Physiol Biochem Zool* 81: 688–693.
- Bender RL, Dufour DL, Valenzuela LO, Cerling TE, Sponheimer M, Reina JC, Ehleringer JR. 2015. Stable isotopes (carbon, nitrogen, sulfur), diet, and anthropometry in urban Colombian women: investigating socioeconomic differences. *Am J Hum Biol* 27:207–218.
- Bol R, Marsh J, Heaton THE. 2007. Multiple stable isotope ( $^{18}\text{O}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$  and  $^{34}\text{S}$ ) analysis of human hair to identify the recent migrants in a rural community in SW England. *Rapid Commun Mass Spectrom* 21:2951–2954.
- Bol R, Pflieger C. 2002. Stable isotope ( $^{13}\text{C}$ ,  $^{15}\text{N}$  and  $^{34}\text{S}$ ) analysis of the hair of modern humans and their domestic animals. *Rapid Commun Mass Spectrom* 16:2195–2200.
- Bowen GJ, Chesson LA, Podlesak DW, Bowen GJ, Cerling TE, Ehleringer JR. 2010. Stable isotope analysis of modern human hair collected from Asia (China, India, Mongolia, and Pakistan). *Am J Phys Anthropol* 141: 440–451.
- Bowen GJ, Ehleringer JR, Chesson LA, Thompson AH, Podlesak DW, Cerling TE. 2009. Dietary and physiological controls on the hydrogen and oxygen isotope ratios of hair from mid-20th century indigenous populations. *Am J Phys Anthropol* 139:494–504.
- Brettell R, Montgomery J, Evans J. 2012. Brewing and stewing: the effect of culturally mediated behaviour on the oxygen isotope composition of ingested fluids and the implications for human provenance studies. *J Anal At Spectrom* 27:778–785.
- Chesson LA, Podlesak DW, Erkkila BR, Cerling TE, Ehleringer JR. 2010. Isotopic consequences of consumer food choice: hydrogen and oxygen stable isotope ratios in foods from fast food restaurants *versus* supermarkets. *Food Chem* 119:1250–1256.
- Chisholm BS, Nelson DE, Schwarcz HP. 1982. Stable-carbon isotope ratio as a measure of marine versus terrestrial protein in ancient diets. *Science* 216:1131–1132.
- Codron D, Codron J. 2009. Reliability of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  in faeces for reconstructing savanna herbivore diet. *Mamm Biol Z Säugetierkd* 74:36–48.
- Codron D, Codron J, Lee-Thorp JA, Sponheimer M, de Ruiter DJ. 2005. Animal diets in the Waterberg based on stable isotopic composition of faeces. *S Afr J Wildl Res* 35:43–52.
- Codron D, Codron J, Lee-Thorp JA, Sponheimer M, Grant CC, Brink JS. 2009. Stable isotope evidence for nutritional stress, competition, and loss of functional habitat as factors limiting recovery of rare antelope in southern Africa. *J Arid Environ* 73:449–457.
- Codron D, Lee-Thorp JA, Sponheimer M, de Ruiter D, Codron J. 2006. Inter- and intrahabitat dietary variability of Chacma baboons (*Papio ursinus*) in South African savannas based on fecal  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , and  $\% \text{N}$ . *Am J Phys Anthropol* 129:204–214.
- Crowley BE. 2012. Stable isotope techniques and applications for primatologists. *Int J Primatol* 33:673–701.
- Darling WG, Bath AH, Talbot JC. 2003. The O & H stable isotopic composition of fresh waters in the British Isles. 2. Surface waters and groundwater. *Hydrol Earth Syst Sci* 7:183–195.
- de Luca A, Boisseau N, Tea I, Louvert I, Robins RJ, Forhan A, Charles MA, Hankard R. 2012.  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  in hair from newborn infants and their mothers: a cohort study. *Pediatr Res* 71:598–604.
- Deschner T, Fuller BT, Oelze VM, Boesch C, Hublin J-J, Mundry R, Richards MP, Ortman S, Hohmann G. 2012. Identification of energy consumption and nutritional stress by isotopic and elemental analysis of urine in bonobos (*Pan paniscus*). *Rapid Commun Mass Spectrom* 26:69–77.
- Dunbar J, Wilson AT. 1983. Oxygen and hydrogen isotopes in fruits and vegetable juices. *Plant Physiol* 72:725–727.
- Ehleringer JR, Bowen GJ, Chesson LA, West AG, Podlesak DW, Cerling TE. 2008. Hydrogen and oxygen isotope ratios in human hair are related to geography. *Proc Natl Acad Sci USA* 105:2788–2793.
- Epstein S, Zeiri L. 1988. Oxygen and carbon isotopic compositions of gases respired by humans. *Proc Natl Acad Sci USA* 85:1727–1731.
- Feuchtmayr H, Grey J. 2003. Effect of preparation and preservation procedures on carbon and nitrogen stable isotope determinations from zooplankton. *Rapid Commun Mass Spectrom* 17:2605–2610.
- Focken U. 2001. Stable isotopes in animal ecology: the effect of ration size on the trophic shift of C and N isotopes between feed and carcass. *Isotopes Environ Health Stud* 37:199–211.
- Fogel ML, Tuross N, Owsley DW. 1989. Nitrogen isotope tracers of human lactation in modern and archaeological populations. *Carnegie Inst Yr Bk*, 88:111–117.
- Fuller BT. 2003. Isotopic investigation of pregnancy and breastfeeding in modern and archaeological populations. Oxford: University of Oxford. 211 p.
- Fuller BT, Fuller JL, Harris DA, Hedges REM. 2006. Detection of breastfeeding and weaning in modern human infants with carbon and nitrogen stable isotope ratios. *Am J Phys Anthropol* 129:279–298.
- Fuller BT, Fuller JL, Sage NE, Harris DA, O’Connell TC, Hedges REM. 2005. Nitrogen balance and  $\delta^{15}\text{N}$ : why you’re not what you eat during nutritional stress. *Rapid Commun Mass Spectrom* 19:2497–2506.
- Garvie-Lok SJ, Varney TL, Katzenberg MA. 2004. Preparation of bone carbonate for stable isotope analysis: the effects of treatment time and acid concentration. *J Archaeol Sci* 31:763–776.
- Giesemann A, Jager HJ, Norman AL, Krouse HR, Brand WA. 1994. On line sulphur-isotope determination using an elemental analyzer coupled to a mass spectrometer. *Anal Chem* 66:2816–2819.
- Gloutney MK, Hobson KA. 1998. Field preservation techniques for the analysis of stable carbon and nitrogen isotope ratios in eggs. *J Field Ornithol* 69:223–227.
- Habran S, Debier C, Crocker DE, Houser DS, Lepoint G, Bouquegneau JM, Das K. 2010. Assessment of gestation, lactation and fasting on stable isotope ratios in northern elephant seals (*Mirounga angustirostris*). *Mar Mamm Sci* 26:880–895.
- Hatch KA. 2012. The use and application of stable isotope analysis to the study of starvation, fasting, and nutritional stress in animals. In: McCue MD, editor. *Comparative physiology of fasting, starvation, and food limitation*. Berlin, Heidelberg: Springer. p 337–364.
- Hatch KA, Crawford MA, Kunz AW, Thomsen SR, Eggett DL, Nelson ST, Roeder BL. 2006. An objective means of diagnosing anorexia nervosa and bulimia nervosa using  $^{15}\text{N}/^{14}\text{N}$  and  $^{13}\text{C}/^{12}\text{C}$  ratios in hair. *Rapid Commun Mass Spectrom* 20:3367–3373.
- Hildebrand GV, Farley SD, Robbins CT, Hanley TA, Titus K, Servheen C. 1996. Use of stable isotopes to determine diets of living and extinct bears. *Can J Zool* 74:2080–2088.

- Hobson KA, Alisaukas RT, Clark RG. 1993. Stable-nitrogen isotope enrichment in avian tissues due to fasting and nutritional stress: implications for isotopic analysis of diet. *The Condor* 95:388–394.
- Hobson KA, Gibbs HL, Gloutney ML. 1997. Preservation of blood and tissue samples for stable carbon and stable nitrogen isotope analysis. *Can J Zool* 75:1720–1723.
- Hoefs J. 2004. *Stable isotope geochemistry*. Berlin: Springer-Verlag. 288 p.
- Jenkins SG, Stephenson TR, Robbins CT. 2001. Nitrogen and carbon isotope fractionation between mothers, neonates, and nursing offspring. *Oecologia* 129:336–341.
- Koch PL. 1997. Nitrogen isotope ecology of carnivores and herbivores. *J Vertebrate Paleontol* 17:57A.
- Kurlle CM. 2002. Stable-isotope ratios of blood components from captive northern fur seals (*Callorhinus ursinus*) and their diet: applications for studying the foraging ecology of wild otariids. *Can J Zool* 80:902–909.
- Lamb AL, Evans JE, Buckley R, Appleby J. 2014. Multi-isotope analysis demonstrates significant lifestyle changes in King Richard III. *J Archaeol Sci* 50:559–565.
- Lee-Thorp J, van der Merwe NJ. 1987. Carbon isotope analysis of fossil bone apatite. *S Afr J Sci* 83:712–715.
- Leonard WR. 2012. Laboratory and field methods for measuring human energy expenditure. *Am J Hum Biol* 24:372–384.
- Longinelli A. 1984. Oxygen isotopes in mammal bone phosphate: a new tool for paleohydrological and paleoclimatological research. *Geochim Cosmochim Acta* 48:385–390.
- Luz B, Kolodny Y, Horowitz M. 1984. Fractionation of oxygen isotopes between mammalian bone phosphate and environmental drinking-water. *Geochim Cosmochim Acta* 48:1689–1693.
- Macko SA, Engel MH, Andrusevich V, Lubec G, O'Connell TC, Hedges REM. 1999. Documenting the diet in ancient human populations through stable isotope analysis of hair. *Philos Trans R Soc Lond B Biol Sci* 354:65–75.
- McDade TW. 2014. Development and validation of assay protocols for use with dried blood spot samples. *Am J Hum Biol* 26:1–9.
- Mekota A-M, Grupe G, Ufer S, Cuntz U. 2006. Serial analysis of stable nitrogen and carbon isotopes in hair: monitoring starvation and recovery phases of patients suffering from anorexia nervosa. *Rapid Commun Mass Spectrom* 20:1604–1610.
- Millard AD. 2000. A model for the effect of weaning on nitrogen isotope ratios in humans. In: Goodfriend G, Collins M, Fogel M, Macko S, Wehmiller J, editors. *Perspectives in amino acid and protein geochemistry*. Oxford: Oxford University Press. p 51–59.
- Nakamura K, Schoeller DA, Winkler FJ, Schmidt HL. 1982. Geographical variations in the carbon isotope composition of the diet and hair in contemporary man. *Biomed Spectrom* 9:390–394.
- Nardoto GB, Silva S, Kendall C, Ehleringer JR, Chesson LA, Ferraz ES, Moreira MZ, Ometto JP, Martinelli LA. 2006. Geographical patterns of human diet derived from stable-isotope analysis of fingernails. *Am J Phys Anthropol* 131:137–146.
- Nardoto GB, Murrrieta RSS, Prates LEG, Adams C, Garavello M, Schor T, De Moraes A, Rinaldi FD, Gragnani JG, Moura EAF, Duarte-Neto PJ, Martinelli LA. 2011. Frozen chicken for wild fish: nutritional transition in the Brazilian Amazon region determined by carbon and nitrogen stable isotope ratios in fingernails. *Am J Hum Biol* 23:642–650.
- Nash SH, Bersamin A, Kristal AR, Hopkins SE, Church RS, Pasker RL, Luick BR, Mohatt GV, Boyer BB, O'Brien DM. 2012. Stable nitrogen and carbon isotope ratios indicate traditional and market food intake in an indigenous circum-polar population. *J Nutr* 142:84–90.
- Nehlich O, Boric D, Stefanovic S, Richards MP. 2010. Sulphur isotope evidence for freshwater fish consumption: a case study from the Danube Gorges, SE Europe. *J Archaeol Sci* 37:1131–1139.
- Nehlich O, Fuller BT, Jay M, Mora A, Nicholson RA, Smith CI, Richards MP. 2011. Application of sulphur isotope ratios to examine weaning patterns and freshwater fish consumption in Roman Oxfordshire, UK. *Geochim Cosmochim Acta* 75:4963–4497.
- Neuberger FM, Jopp E, Graw M, Püschel K, Grupe G. 2013. Signs of malnutrition and starvation—reconstruction of nutritional life histories by serial isotopic analyses of hair. *Forensic Sci Int* 226:22–32.
- O'Connell TC, Hedges REM. 1999. Investigation into the effect of diet on modern human hair isotopic values. *Am J Phys Anthropol* 108:409–425.
- O'Connell TC, Hedges REM, Healey MA, Simpson AHR. 2001. Isotopic comparison of hair, nail and bone: modern analyses. *J Archaeol Sci* 28:1247–1255.
- O'Connell TC, Kneale CJ, Tasevska N, Kuhnle GGC. 2012. The diet-body offset in human nitrogen isotopic values: A controlled dietary study. *Am J Phys Anthropol* 149:426–434.
- O'Grady SP, Wende AR, Remien CH, Valenzuela LO, Enright LE, Chesson LA, Abel DE, Cerling TE, Ehleringer JR. 2010. Aberrant water homeostasis detected by stable isotope analysis. *PLoS ONE* 5:1–7.
- Petzke KJ, Boeing H, Metges CC. 2005. Choice of dietary protein of vegetarians and omnivores is reflected in their hair protein  $^{13}\text{C}$  and  $^{15}\text{N}$  abundance. *Rapid Commun Mass Spectrom* 19:1392–1400.
- Petzke KJ, Feist T, Fleig WE, Metges CC. 2006. Nitrogen isotopic composition in hair protein is different in liver cirrhotic patients. *Rapid Commun Mass Spectrom* 20:2973–2978.
- Petzke KJ, Fuller BT. 2012. Stable isotope ratio analysis in human hair. In: Preedy VR, editor. *Handbook of hair in health and disease*. Wageningen: Academic Publishers. p 333–351.
- Petzke KJ, Fuller BT, Metges CC. 2010. Advances in natural stable isotope ratio analysis of human hair to determine nutritional and metabolic status. *Curr Opin Clin Nutr Metab Care* 13:532–540.
- Phillips DL, Gregg JW. 2003. Source partitioning using stable isotopes: coping with too many sources. *Oecologia* 136:261–269.
- Polischuk SC, Hobson KA, Ramsay MA. 2001. Use of stable-carbon and -nitrogen isotopes to assess weaning and fasting in female polar bears and their cubs. *Can J Zool* 79:499–511.
- Reitsema LJ. 2012. Introducing fecal stable isotope analysis in primate weaning studies. *Am J Primatol* 74:926–939.
- Reitsema LJ. 2013. Beyond diet reconstruction: stable isotope applications to human physiology, health, and nutrition. *Am J Hum Biol* 25:445–456.
- Reitsema LJ, Crews DE. 2011. Oxygen isotopes as a biomarker for sickle-cell disease? Evidence from transgenic mice expressing human hemoglobin S genes. *Am J Phys Anthropol* 145:495–498.
- Reitsema LJ, Muir AB. 2015. Brief Communication: growth velocity and weaning  $\delta^{15}\text{N}$  “dips” during ontogeny in *Macaca mulatta*. *Am J Phys Anthropol* 157:347–357.
- Richards MP, Fuller BT, Hedges REM. 2001. Sulphur isotopic variation in ancient bone collagen from Europe: implications for human palaeodiet, residence mobility, and modern pollutant studies. *Earth Planetary Sci Lett* 191:185–190.
- Rikimaru T, Fujita Y, Okuda T, Kajiwarana N, Date C, Heywood PF, Alpers MP, Koishi H. 1985. Utilization of urea nitrogen in Papua New Guinea highlanders. *J Nutr Sci Vitaminol (Tokyo)* 31:393–402.
- Romek KM, Julien M, Frasquet-Darrieux M, Tea I, Antheaume I, Hankard R, Robins RJ. 2013. Human baby hair amino acid natural abundance  $^{15}\text{N}$ -isotope values are not related to the  $^{15}\text{N}$ -isotope values of amino acids in mother's breast milk protein. *Amino Acids* 45:1365–1372.
- Runne U, Orfanos CE. 1981. The human nail: structure, growth and pathological changes. *Curr Probl Dermatol* 9:102–149.
- Saitoh M, Uzuka M, Sakamoto M, Kobori T. 1964. Rate of hair growth. In: Montagna W, Dobson RL, editors. *Hair growth: proceedings of a symposium on the biology of skin*. Oxford: Pergamon Press. p 183–201.
- Schoeller DA. 1988. Measurement of energy expenditure in free-living humans by using doubly labeled water. *J Nutr* 118:1278–1289.
- Schoeller DA. 1999. Recent advances from application of doubly labeled water to measurement of human energy expenditure. *J Nutr* 129:1765–1768.
- Schoeller DA, Minagawa M, Slater R, Kaplan IR. 1986. Stable isotopes of carbon, nitrogen and hydrogen in the contemporary North American human food web. *Ecol Food Nutr* 18:159–170.
- Schoeninger MJ, Moore K. 1992. Bone stable isotope studies in archaeology. *J World Prehist* 6:247–296.
- Sponheimer M, Robinson T, Ayliffe L, Roeder B, Hammer J, West A, Passey B, Cerling T, Dearing D, Ehleringer J. 2003a. Nitrogen isotopes in mammalian herbivores: hair  $\delta^{15}\text{N}$  values from a controlled-feeding study. *Int J Osteoarchaeol* 13:80–87.
- Sponheimer M, Robinson TF, Roeder BL, Passey BH, Ayliffe LK, Cerling TE, Dearing MD, Ehleringer JR. 2003b. An experimental study of nitrogen flux in llamas: is  $^{14}\text{N}$  preferentially excreted? *J Archaeol Sci* 30:1649–1655.
- Sponheimer M, Todd Robinson, Ayliffe L, Passey B, Roeder B, Shipley L, Lopez E, Cerling T, Dearing D, Ehleringer J. 2003c. An experimental study of carbon-isotope fractionation between diet, hair, and feces of mammalian herbivores. *Can J Zool* 81:871–876.
- Steele KW, Daniel RM. 1978. Fractionation of nitrogen isotopes by animals: a further complication to the use of variations in the natural abundance of  $^{15}\text{N}$  for tracer studies. *J Agric Sci* 90:7–9.
- Szpak P. 2014. Complexities of nitrogen isotope biogeochemistry in plant-soil systems: implications for the study of ancient agricultural and animal management practices. *Front Plant Sci* 5:288.
- Tieszen LL. 1991. Natural variations in the carbon isotope values of plants: implications for archaeology, ecology, and paleoecology. *J Archaeol Sci* 18:227–248.
- Valenzuela LO, Chesson LA, Bowen GJ, Thure E, Cerling, and Ehleringer JR. 2012. Dietary heterogeneity among Western industrialized countries reflected in the stable isotope ratios of human hair. *PLoS One* 7:1–8.
- Valenzuela LO, Chesson LA, O'Grady SP, Cerling TE, Ehleringer JR. 2011. Spatial distributions of carbon, nitrogen and sulfur isotope ratios in human hair across the central United States. *Rapid Commun Mass Spectrom* 25:861–868.

- Vogel ER, Knott CD, Crowley BE, Blakely MD, Larsen MD, Dominy NJ. 2012. Bornean orangutans on the brink of protein bankruptcy. *Biol Lett* 8:333–336.
- WHO/FAO. 1996. Preparation and use of food-based dietary guidelines. Geneva: Nutrition Programme - WHO.
- Wilkinson MJ, Youlim Y, O'Brien DM. 2007. Age-related variation in red blood cell stable isotope ratios ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) from two Yupik villages in Southwest Alaska: a pilot study. *Int J Circumpolar Health* 66:31–41.
- Wright LE, Schwarcz HP. 1998. Stable carbon and oxygen isotopes in human tooth enamel: identifying breastfeeding and weaning in prehistory. *Am J Phys Anthropol* 106:1–18.
- Yaemsiri S, Hou N, Slining MM, He K. 2010. Growth rate of human fingernails and toenails in healthy American young adults. *J Eur Acad Dermatol Venereol* 24:420–423.
- Zanconato S, Cooper DM, Armon Y, Epstein S. 1992. Effect of increased metabolic rate on oxygen isotopic fractionation. *Respir Physiol* 89:319–327.

## SUPPORTING INFORMATION

### Supplemental Material S2: Sample Amounts Required for Analysis

Different mass spectrometers have different sensitivities to carbon and nitrogen, and the best analytical precision is achieved when an optimal range amount of an element is detected. Too little CO<sub>2</sub> or N<sub>2</sub> gas will not be detected, whereas too much will flood the machine. This optimal range determines how much of a sample should be weighed (and thus, collected) for analysis. The amount (µg) of sample needed for each tissue type also depends on that tissue's carbon and nitrogen content (%C; %N). For substances that are nitrogen-rich (e.g., blood serum; %N≈10-11%), less sample is required to meet the machine's optimal range requirement for good δ<sup>15</sup>N data. For samples that are nitrogen-poor (e.g., plants; %N≈1-3%), more sample is required to meet this range. %C and %N values of many sample types are widely reported in the literature.

Machines differ in their optimal detection ranges, but in general, a range of 50-70 µg for nitrogen and 140-560 µg for carbon may yield good data (R. Culp, pers. comm., Y. Matsui, pers. comm.). For blood serum that is approximately 10% nitrogen, the optimal weight range of powdered sample to be loaded into the mass spectrometer for δ<sup>15</sup>N data is 500-700 µg, calculated according to the equation

$$R=(S \cdot E)/100$$

also expressed as  $S=(R/E) \cdot 100$ , where S is the unknown amount of sample that must be weighed (µg), E is the amount (%) of an element in the sample (in this case, 10[%] for nitrogen in serum), and R is the optimal detection range of the machine (in this example, 50-70 µg).

Serum is approximately 41% carbon. Using the same equation, the optimal amount of sample to weigh for  $\delta^{13}\text{C}$  data is 341-1,365  $\mu\text{g}$ . The overlap between the optimal carbon and the optimal nitrogen ranges – in the case of serum, 500-700  $\mu\text{g}$  – is the amount that should be weighed for the most precise  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  data. For samples with very different C and N content (e.g., breast milk), it may be necessary to weigh different amounts for each stable isotope ratio, and run each sample in two fractions. The required weight amounts for tissues in this paper are suggestions which can be confirmed by running test samples. In some cases, the laboratory will advise a different target weight range after seeing results of a test assay.