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The use of stable isotopes in postconflict forensic identification

Lesley A. Chesson | **Gregory E. Berg**

Defense POW/MIA Accounting Agency (DPAA) Laboratory, Joint Base Pearl Harbor-Hickam, Hawaii, USA

Correspondence
Lesley A. Chesson, Defense POW/MIA Accounting Agency (DPAA) Laboratory, Joint Base Pearl Harbor-Hickam, HI, USA.
Email: lesley.a.chesson.civ@mail.mil

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Abstract

Stable isotope ratio analysis is becoming an extremely important tool for the forensic investigator. It can be particularly useful in missing persons investigations to help establish an unknown's identity by potentially reconstructing an individual's geolocational history, determining the number of individuals present in a case, and linking/separating remains from commingled assemblages. In this review article, we explore several main themes related to the use of stable isotopes in postconflict forensic identification. First, we describe what stable isotopes are and how global isotopic variation arises in human tissues via natural processes and cultural practices. Next, we discuss the selection of appropriate human tissue samples for examination based on testable hypotheses. We provide guidance on the options that exist for isotope sample preparation, analysis, and data handling; and finally, we examine which sample quality measures and quality assurance approaches should be used in isotope data reporting and interpretation. These themes are primarily presented to help the isotope data consumer understand the benefits and limitations of the technique that might not be apparent when sending samples to and receiving results from a high throughput, contract isotope testing laboratory. Real world examples of forensic stable isotope ratio analysis are provided within the article to highlight many of the concepts, particularly in regard to identification applications.

This article is categorized under:

Forensic Anthropology > Anthropology in Mass Disaster & War Crime Contexts
Forensic Biology > Interpretation of Biological Evidence
Forensic Chemistry and Trace Evidence > Emerging Technologies and Methods

KEYWORDS

forensic anthropology, human identification, isoscapes, stable isotope ratio analysis, taphonomy

1 | INTRODUCTION

It is impossible to know exactly how many individuals go missing worldwide every year as a result of violent crimes, human trafficking, and involuntary disappearances. In recent memory, the numbers of missing persons are immense. Within the United States, the National Missing and Unidentified Persons System (NamUs) currently tracks ca. 20,000

missing persons and an additional 13,000 unidentified individuals. Numbers related specifically to armed conflicts are staggeringly large. The International Commission on Missing Persons is in the process of accounting for ca. 40,000 persons who went missing between 1991 and 1996 in the former Yugoslavia (Huffine et al., 2001). In Colombia, between 68,000 and 78,000 persons are missing as a result of socio-political conflict that has spanned more than 50 years (Eck et al., 2019). During the reign of Saddam Hussein, an estimated 29,000 persons went missing in Iraq (Baraybar et al., 2007). Sadly, the examples can continue. The effect each missing person has on the community is tremendous, as eloquently stated by the International Committee of the Red Cross [quoted by Cardia (2016, p. 82)]:

> Disappearances are a tragedy not just for the individual but also for families, who are left in the dark. Not knowing what has become of a husband or wife, child, father, mother, brother or sister is a source of terrible anguish for countless families affected by armed conflict or internal violence all over the world.

When remains are recovered postconflict, linking those to a missing person is no easy feat—especially when there is commingling (Jin et al., 2014; Megyesi, 2019; Morild et al., 2015; Parsons et al., 2019). Postconflict forensic identification in the U.S. is the primary mission of the Defense Prisoner of War (POW)/Missing in Action (MIA) Accounting Agency[1] (DPAA; Holland et al., 2008), an agency tasked to "provide the fullest possible accounting" of the nation's missing Service Personnel. At present, over 81,700 missing U.S. Americans are under the Agency's purview, spanning the conflicts of World War II, the Korean War, the Vietnam War, the Cold War, and so on. The DPAA Laboratory utilizes a number of investigative and identification tools, including historical and geospatial analyses, forensic anthropology and odontology, materials analysis, chest radiographic comparisons, and DNA analyses. Indeed, innovative work by scientists at the DPAA and its partners have markedly improved the utility of some techniques for forensic identification, particularly DNA analyses (e.g., Edson et al., 2019; Edson & Christensen, 2015; Edson & McMahon, 2016; Marshall et al., 2019, 2020), chest radiographic comparisons (Isa et al., 2017; Stephan et al., 2011, 2018), and materials analysis, such as eyeglass comparisons (Berg & Collins, 2007; Berg & Ta'ala, 2009; Collins & Berg, 2008).

The DPAA's most recent addition to its investigative toolbox is stable isotope ratio analysis (Bartelink, Berg, et al., 2014). Not long after it use was pioneered in archaeology in the late 1970s (van der Merwe, 1982), anthropologists advocated for its application in forensic settings (Katzenberg & Krouse, 1989). Forensic stable isotope ratio analysis has become increasingly prevalent from the 1990s through the present (Benson et al., 2006; Cerling et al., 2016; Gentile et al., 2015; Meier-Augenstein, 2018) and applications in postconflict settings have been occurring for almost a decade (Bartelink, Berg, et al., 2014; Chesson et al., 2020; Holland et al., 2012). Three major questions are logically answerable using isotope data for forensic identification: (1) Where did the remains originate geographically; (2) Do the remains belong to a single individual; and (3) If remains are found in a commingled context, can they be separated/linked?

As we will show, the forensic application of stable isotope ratio analysis capitalizes on the fact that certain isotopes (see Box 1) act as "nature's recorders" (West et al., 2006) and the isotopic composition of human tissues reflects the isotopic composition of what the individual ingested. In this article, we describe how food and water vary isotopically across landscapes; therefore, the interpretation of the isotope records in human tissues can help to reconstruct life history, including diet, geographic provenance, and travel movements before death. We review recent reference databases and applications of isotope testing for forensic identification, geolocation, and remains sorting. To inform the reader of the benefits and limitations of isotope testing, we briefly summarize sample selection (e.g., what tissues are useful given the hypotheses to be tested), sample preparation processes, and analytical testing procedures. Finally, we discuss the practical details of data handling, data comparability, and quality assurance, so that the consumer can be informed on the appropriate use of isotope test results *prior to* forming any conclusions.

2 | STABLE ISOTOPES IN HUMANS

2.1 | You are what you eat

The stable isotopes of the chemical elements carbon (C), nitrogen, (N), and sulfur (S) are recorders of an individual's food choices because diet is the source of these bio-elements to the body. In essence, you are what you eat, from an

BOX 1 What are stable isotopes?

Isotopes are variants of a chemical element that have the same atomic number (defined by proton number) but differ in mass due to changes in neutron number. As an example, there are two stable isotopes of carbon (C)—the more common form with six protons, six electrons, and six neutrons; and a rarer variant with seven neutrons. The shorthand for the common variant is ^{12}C, the sum of subatomic particles in the nucleus. Similarly, the shorthand for the form with one additional neutron is ^{13}C. As the name suggests, stable isotopes do not undergo radioactive decay. (There is a radioactive isotope of carbon, ^{14}C, with eight neutrons; this is the isotope used in radiocarbon dating applications.)

Chemical elements found in nature represent a mix of isotopes. Stable isotope abundances of the "bio-elements"—C, plus hydrogen (H), nitrogen (N), oxygen (O), and sulfur (S)—are typically expressed in delta, or δ, notation, as a "relative difference of isotope ratios" (Coplen, 2011) where the ratio, R, of the heavier-to-lighter isotope (e.g., $^{13}C/^{12}C$) of a sample is compared to that of an internationally agreed standard: $\delta = (R_{sample}/R_{standard}) - 1$. For carbon, stable isotope abundances would thus be reported as $\delta^{13}C$ values. Isotope δ values are frequently presented for convenience in parts per thousand, or "per mil" (‰). A notable exception is found in the expression of stable isotope abundances of trace metals, such as strontium (Sr) or lead (Pb), which are often simply reported as R (e.g., the $^{87}Sr/^{86}Sr$ ratio).

isotopic perspective—plus or minus a few ‰ (DeNiro & Epstein, 1976). For the "bio-elements," the isotopic composition of body tissues reflects that of diet, but not exactly due to fractionation processes (see below). Food is also a source of the trace metals strontium and lead to the body; however, for these elements, fractionation is generally limited and there is typically no difference between the isotopic composition of an individual's tissues and the stable isotopes incorporated from diet.

The elements C, N, and S are incorporated from food into proteinaceous tissues, such as the keratin that makes up hair/nail and the collagen that gives bone its flexibility. Bone is composed of two fractions, the proteinaceous component collagen—although there are other proteins present than just collagen (Ambrose, 1990)—and the mineral component bioapatite. Collagen makes up ca. 25% of fresh human bone while bioapatite makes up ca. 65% (Kendall et al., 2018); the remainder is comprised of water and fats. The isotopic composition of collagen reflects protein in the diet while bioapatite reflects the whole diet (Ambrose & Norr, 1993; Tieszen & Fagre, 1993).

Variation in the $\delta^{13}C$ values of dietary food items is ultimately linked to photosynthesis, when plants synthesize carbohydrates from atmospheric CO_2 (plus water) using carbon assimilation, or fixation. Plants using a process called C_3 carbon fixation have lower carbon isotope δ values in their tissues than plants using C_4 carbon fixation; C_3 plants are characterized by $\delta^{13}C$ values of ca. −35 to −20‰ while C_4 plants have ranges of ca. −14 to −10‰ (Tipple & Pagani, 2007). The C_4 photosynthetic pathway is adapted to hotter, drier environmental conditions and C_4 plants are thus typically found at lower latitudes (Still & Powell, 2010). Surveys of human hair have shown that higher $\delta^{13}C$ values are observed at lower latitudes (Hülsemann et al., 2015; Valenzuela et al., 2012), as expected based on crop availability and resource use.

As noted above, the spatial patterning of crops—and the historic importance of different food items in populations around the world—is reflected in the geographic distribution of carbon isotope δ values of humans. Most plant types consumed by humans and their livestock use C_3 photosynthesis, including oats, potatoes, rice, soy, wheat, and almost all fruits and vegetables. Traditionally, many populations relied almost exclusively on C_3 crops for sustenance. However, several C_4 crops are major components of modern human diet, including maize (corn) and sugarcane, plus millet and sorghum to a lesser extent.[2] Western populations are frequently heavily reliant on C_4 crops (e.g., corn and sugarcane), whereas Eastern populations tend to be more reliant on C_3 crops (e.g., rice, soy). This is reflected in the $\delta^{13}C$ values of bone collagen; for instance, the mean $\delta^{13}C$ value for an individual from Central America/Mexico is ca. −12.8‰, whereas an individual from Southeast Asia has a mean bone collagen $\delta^{13}C$ value of ca. −19.7‰ (data from Isolocate [Berg & Kenyhercz, 2017]).

Marine-derived food items also impact the carbon isotopic composition of an individual's diet, as plants and animals from the ocean generally have higher $\delta^{13}C$ values than terrestrial C_3 plants (and the animals that eat them). This is because phytoplankton synthesizes carbohydrates from bicarbonate, which has a higher $\delta^{13}C$ value (by ca. 9‰) than

atmospheric CO_2 (Fry, 2006; O'Brien, 2015). An individual that consumes large amounts of marine fish may have tissue $\delta^{13}C$ values similar to someone with large quantities of C_4 plants in their diet. The further analysis of nitrogen isotopes can help distinguish the source of carbon—terrestrial vs. marine—in an individual's diet as nitrogen is a tracer of ingested protein.

Nitrogen isotope ratios reflect the trophic level within a food web, with higher $\delta^{15}N$ values observed at higher levels (Fry, 2006; McCutchan et al., 2003; Post, 2002). The isotopic difference between an organism and its diet is known as the trophic shift and can be estimated at roughly 3‰ for a variety of food webs (Post, 2002).[3] In other words, for each trophic level or "step" up a food web, a shift of about +3‰ occurs for $\delta^{15}N$ values. Due to the length of their webs, marine-derived foods typically have higher $\delta^{15}N$ values than those that are terrestrially derived. The $\delta^{15}N$ values of human tissues reflect an individual's position within a food web. For example, the hair of vegans has been found to have lower $\delta^{15}N$ values than that of individuals eating meat or even vegetarians (Ellegård et al., 2019; O'Connell & Hedges, 1999) since vegans have reduced the number of steps in their web. Likewise, the hair of infants has been found to have higher $\delta^{15}N$ values than that of their mothers (De Luca et al., 2012) due to nursing. Further, an individual's metabolic condition can also affect the $\delta^{15}N$ values recorded in their tissues, as demonstrated by studies on disease (De Luca et al., 2014; Petzke et al., 2006), eating disorders (Hatch et al., 2006; Mekota et al., 2006), malnutrition (Dailey-Chwalibóg et al., 2020), and pregnancy (Fuller et al., 2004, 2005).

Global variations in sulfur isotope δ values ($\delta^{34}S$) are related to environmental processes in the geosphere and hydrosphere and cover wide ranges. For instance, $\delta^{34}S$ values of −20‰ (and even lower) to +30‰ (and even higher) have been observed for rocks and freshwaters, while ocean water has a relatively constant value of +20.3‰ (Nehlich, 2015). The variability of $\delta^{34}S$ values in food items from different environments—for instance, freshwater fish vs. marine fish—has helped archeologists determine how historic populations used resources (e.g., Privat et al., 2007). Interpretation of $\delta^{34}S$ values in human tissues is most often done in tandem with $\delta^{13}C$ and/or $\delta^{15}N$ values to provide a broad picture of dietary inputs. There has been little exploration into the utility of sulfur isotope ratios for dietary reconstruction of humans (O'Brien, 2015) and sulfur thus represents a relatively newer avenue of isotope research.

We note that not every tissue in the human body will have the same isotopic "signature" due to a variety of metabolic factors that affect the incorporation of bio-element isotopes from the diet. For example, the amino acid composition of keratin differs from that of collagen and, as a result, lower $\delta^{13}C$ and $\delta^{15}N$ values have been measured for hair than bone collagen from the same individual (O'Connell et al., 2001). Isotopic variation, most notably observed in $\delta^{15}N$ values, has been measured in collagen extracted from different bones of a single skeleton (Chenery et al., 2012; Fahy et al., 2017;Olsen et al., 2014; Pollard et al., 2012) and even within a single bone (Curto et al., 2020; Katzenberg & Lovell, 1999; Olsen et al., 2014). Intraskeletal and intrabone variations are hypothesized to reflect differences in turnover rate (Gineyts et al., 2000; Hedges et al., 2007) and the incorporation of different dietary isotopes over different periods of time. These processes are not yet clearly understood. Variation in $\delta^{13}C$ values often is limited, smaller than analytical error, or statistically insignificant (Olsen et al., 2014; Pollard et al., 2012); in contrast, disease and fracture repair can have a significant impact on $\delta^{15}N$ values in human bone (Olsen et al., 2014).

Recently, intraskeletal isotopic limits for determining when two bones are from different individuals have been built using the isotopic composition of collagen (Berg et al., 2019). The study used a large sample of approximately 25 individuals from two geographically distinct populations (5–6 long bones tested per individual). In forensic applications, the intraperson limits can be used to determine if two long bones (1) *are probably* from different individuals, at 0.75‰ for $\delta^{13}C$ values and 1.05‰ for $\delta^{15}N$ values; or (2) *are* from different individuals, at 0.95‰ for $\delta^{13}C$ values and 1.30‰ for $\delta^{15}N$ values. These intraperson isotopic ranges observed for modern humans are similar to previous studies of Medieval human skeletons (Fahy et al., 2017; Olsen et al., 2014). Intraskeletal isotopic limits have proven to be useful for sorting theoretical assemblages of commingled human remains when isotopic data and skeletal osteometric data are combined (McCormick et al., 2020).

2.2 | There's no place like home

Hydrogen (H), oxygen (O), strontium (Sr), and lead (Pb) can provide information about the geographic residence of an individual, as the stable isotope abundances of these chemical elements vary spatially in generally predictable ways. Isotopically, you are where you live.

Global variations in the isotope ratios of hydrogen (δ^2H) and oxygen (δ^{18}O) are essentially controlled by the hydrological cycle and the processes of evaporation and condensation (Gat, 1996). Water molecules containing the heavier isotope ^{2}H or ^{18}O evaporate more slowly and condense more readily than water molecules containing the lighter isotope ^{1}H or ^{16}O (Figure 1). This results in a systematic patterning of isotope δ values in water across the planet, where higher δ^2H and δ^{18}O values are found in warmer, coastal, low latitude areas and lower δ^2H and δ^{18}O values are found in colder, inland, high latitude areas (Bowen et al., 2007; Bowen & Revenaugh, 2003). An example isotope landscape, also known as an isoscape (West et al., 2010), is shown in Figure 2 for tap water from Mexico. The expected isotopic distribution of water across the country can clearly be seen in the lower δ^{18}O values in mountainous regions (interior and southwestern coast), and the higher δ^{18}O values in the eastern coast and drier desert regions.

Animals, including humans, record the isotopic composition of ingested water into their tissues, as demonstrated for hair keratin (Ehleringer et al., 2008) and bioapatite from tooth enamel and bone (Daux et al., 2008). However, the conversion from water to tissue is not one-to-one for either δ^2H or δ^{18}O values due to a process called isotopic partitioning, or fractionation. Although all isotopes of a bio-element form the same types of bonds, the mass difference between isotopes means each will behave a little differently in biological, chemical, and physical reactions; this leads to fractionation between reactants and products. (The trophic shift described above for δ^{15}N values is an example of isotopic fractionation and relates to the relative ease ^{15}N vs. ^{14}N is incorporated from dietary protein into an individual's body tissues. The spatially driven variations in the δ^{18}O values of water shown in Figure 1 are another example of isotopic fractionation.)

The geographic provenance, or geolocation, of an individual can be predicted using models of isotopic fractionation between water/tissue when compared to an isoscape. The models for a variety of human tissues were recently reviewed by Chesson et al. (2017)[4] and include regression-based methods for predicting region-of-origin using the δ^{18}O values of hair (e.g., Ehleringer et al., 2008) and bone/teeth (e.g., Chenery et al., 2012; Daux et al., 2008). For bone and teeth, in particular, there are multiple methods available for converting between tissue and drinking water (Chenery et al., 2012; Daux et al., 2008; Dotsika, 2020; Levinson et al., 1987; Longinelli, 1984; Luz et al., 1984) and there is no consensus on which may be the "best." At least one study has suggested that it may be better to build tissue-specific (human) reference databases for geolocation (Pollard et al., 2011) as opposed to predicting provenance using models based on water. Because there are relatively few databases of isotope test results for modern human bone and teeth, isoscapes based on human tissues are unavailable for most world regions. One notable exception is the publication of Pb and O tooth enamel isoscapes for the continental U.S. (Keller et al., 2016; Regan et al., 2021). There are some additional tissue isotope reference data sets available; they are discussed in Section 3.

The global distributions of the isotopes of the trace metal strontium (^{87}Sr/^{86}Sr) are impacted by geology (Hoogewerff et al., 2019). The isotope ^{87}Sr is formed from the radioactive decay of rubidium, ^{87}Rb, and the concentration of ^{87}Sr is therefore a product of bedrock age plus its initial concentration of rubidium. Higher ^{87}Sr/^{86}Sr ratios are expected for

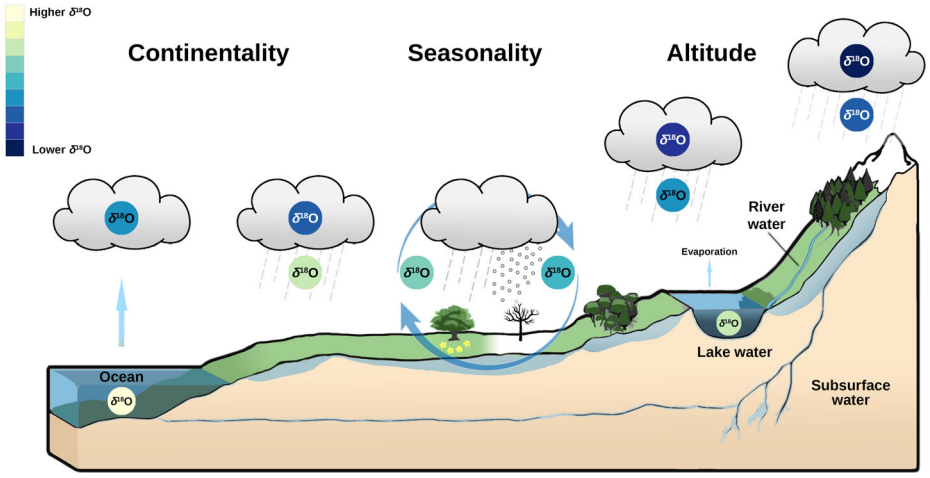

FIGURE 1 Spatial variations in the δ^{18}O values of water are an effect of the hydrological cycle and the processes of evaporation and condensation. Water molecules containing the heavier isotope ^{18}O evaporate more slowly and condense more readily than water molecules containing the lighter isotope ^{16}O, leading to higher δ^{18}O values in warmer, coastal areas and lower δ^{18}O values in colder, inland areas. Illustration reprinted from *Earth-Science Reviews*, Vol. 188, Sarah Pederzani and Kate Britton, Oxygen isotopes in bioarchaeology: Principles and applications, challenges and opportunities, 77–107, Copyright (2019), with permission from Elsevier

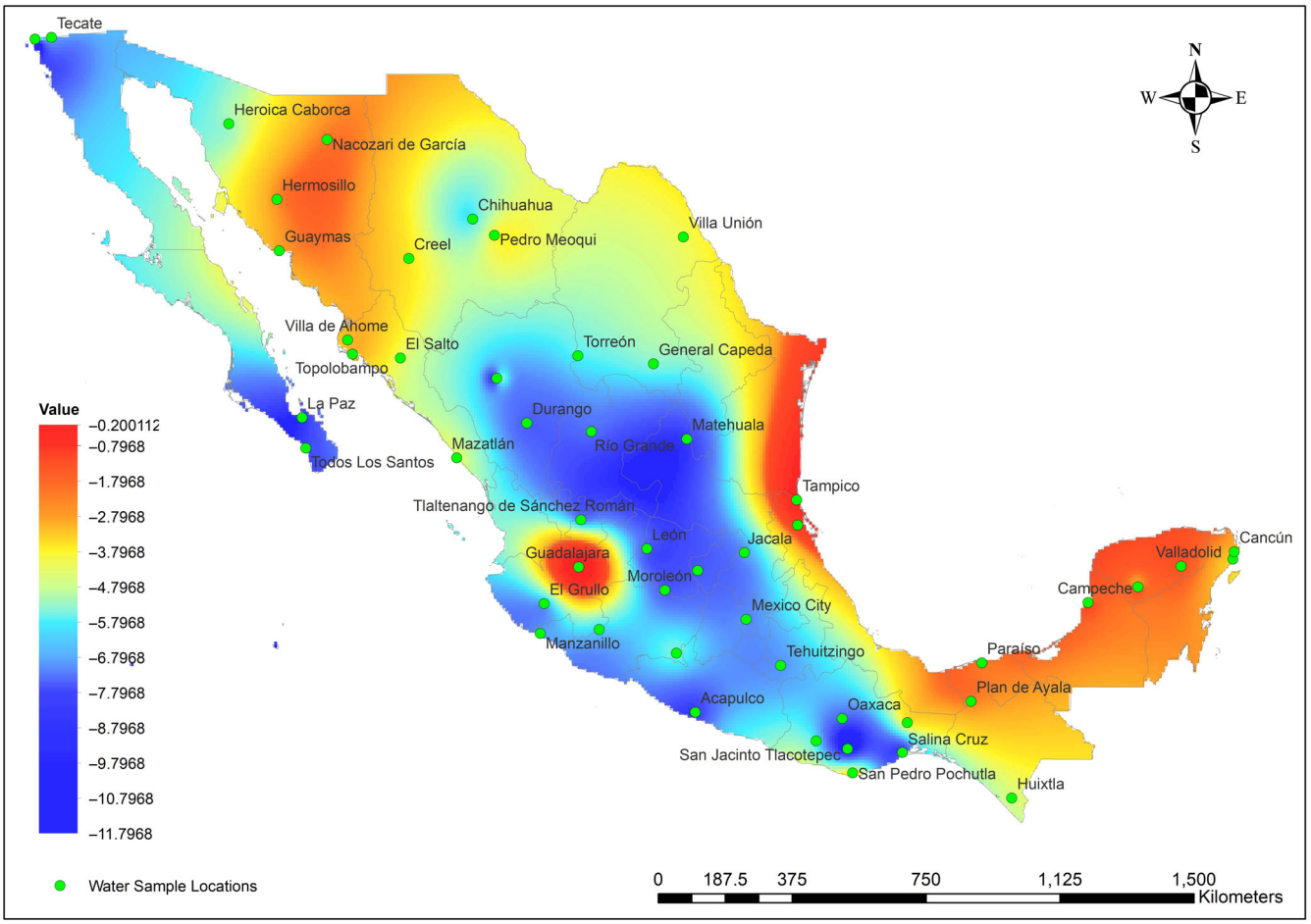

FIGURE 2 An isotope landscape (isoscape) map of tap water $\delta^{18}O$ values, given in parts per thousand (‰) vs. the internationally agreed standard Vienna Standard Mean Ocean Water (VSMOW). The isoscape shows spatial variation across Mexico, with lower $\delta^{18}O$ values observed in mountainous regions (interior and southwestern coast), and higher $\delta^{18}O$ values observed in eastern coastal and drier desert regions. Map reprinted from Ammer, Bartelink, et al. (2020), with permission

older, Rb-rich rocks (Bataille & Bowen, 2012; Beard & Johnson, 2000). From bedrock, Sr is mobilized into water and then into local food webs. Strontium is metabolically incorporated into human bone and teeth from food and water ingestion, where it can be substituted in lieu of calcium. As noted previously, there is no isotopic fractionation for Sr (or lead, see below) between source and tissue. Interestingly, Sr in bathing/washing water has a significant impact on the $^{87}Sr/^{86}Sr$ ratios of hair and nail (Hu et al., 2020; Mancuso & Ehleringer, 2018; Tipple et al., 2019), indicating it can also be incorporated into some body tissues via direct absorption.

Since spatial variations in $\delta^{18}O$ values and $^{87}Sr/^{86}Sr$ ratios are impacted by fundamentally different processes (hydrological cycle vs. geological features, respectively), a dual-isotope approach to geolocation can be very powerful. As a case example, consider the isotopic variation expected in the Circum-Caribbean region for tooth enamel (Laffoon et al., 2017), which is presented in Figure 3. Individuals who were born and raised in the three indicated locations would have recorded distinct—and different—combinations of O and Sr isotope ratios in their enamel. The combinations are neither unique nor so discrete they could be considered an exclusive fingerprint of geolocation; that is, they are not "zip code" specific. However, they can be used to eliminate many potential regions-of-origin from consideration and provide investigative leads into the source of an unidentified decedent (Chesson et al., 2018; Font, van der Peijl, et al., 2015; Kamenov & Curtis, 2017). This is demonstrated in Figure 4, where the isotope test results of a deciduous molar from an individual born in Caracas, Venezuela were used for geolocation; the prediction included both the natal origin and other potential sources (e.g., portions of Central America). Often stable isotope ratio analysis can be combined with other types of forensic analyses to assist in generating leads and steering identification efforts (i.e., Ambers et al., 2021; Trammell et al., 2018).

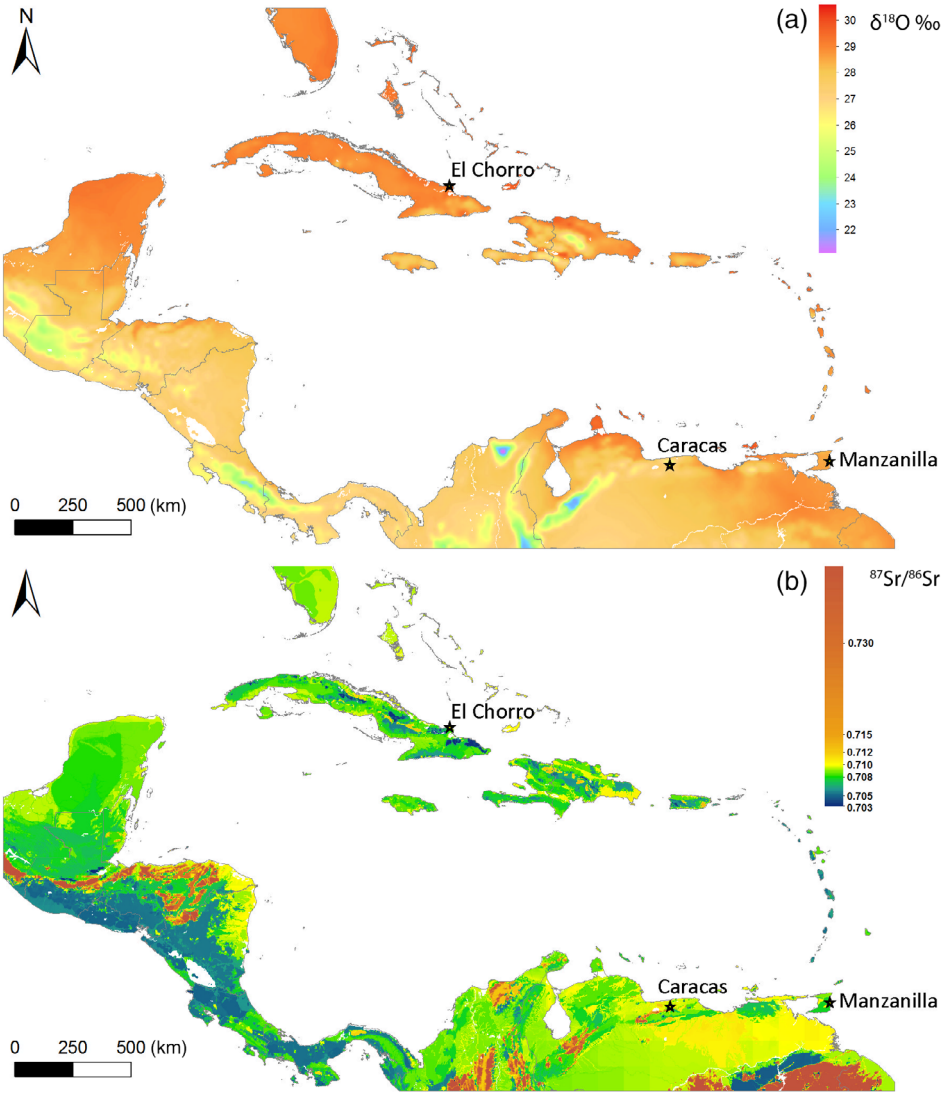

FIGURE 3 Isoscapes of $\delta^{18}O$ values (a) and $^{87}Sr/^{86}Sr$ ratios (b) expected for human tooth enamel in the Circum-Caribbean region. Individuals born and raised in the three indicated locations would have recorded different combinations of O and Sr isotopic signatures in their enamel. While not necessarily unique or "zip code" specific, the signatures can be used to eliminate many potential regions-of-origin from consideration and provide investigative leads into the source of an individual. Image © 2017 Laffoon et al. and reprinted under the terms of the Creative Commons Attribution License

Finally, some studies have found lead isotope ratios to be potentially useful for geolocation and thus human identification (Kamenov & Curtis, 2017; Keller et al., 2016; Regan et al., 2021). Similar to Sr, Pb can substitute for calcium in bone and teeth. Global variations in the abundances of the natural stable isotope ^{204}Pb plus the isotopes ^{206}Pb (formed from the radioactive decay of ^{238}U), ^{207}Pb (from ^{235}U), and ^{208}Pb (from ^{232}Th) are related to bedrock age and the initial concentrations of uranium and thorium. From local dust and soil, Pb moves into food and water and thus into the body (Kamenov, 2008). However, naturally occurring spatial patterns in Pb isotope abundances are easily impacted by anthropogenic factors—for example, the widespread use of leaded gasoline in the 20th Century and the use of coal in power stations and for domestic heating. Generally, Pb is used less often than H, O, and Sr for geolocation.

3 | DATABASES AND FORENSIC APPLICATIONS

We now turn to applications of isotope testing in postconflict forensic casework from the 20th Century to the present day. These cases involved modern human remains and could in all likelihood have led to an identification for an

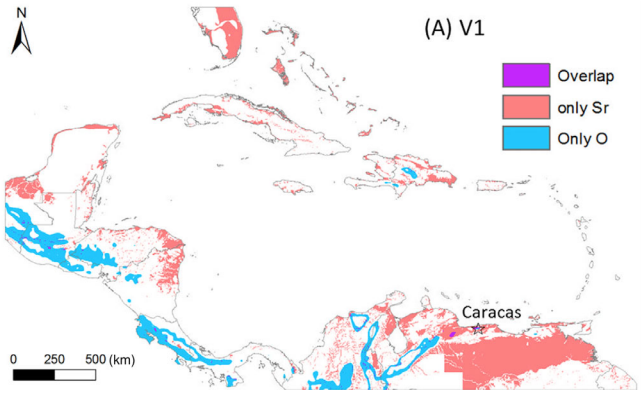

FIGURE 4 The predicted origin of an individual from Caracas, Venezuela, (A) V1, based on the measurement of O and Sr isotope ratios in tooth enamel. Measured $\delta^{18}O$ values and $^{87}Sr/^{86}Sr$ ratios were plotted on separate maps and combined, using a given margin of error. The resulting prediction included the known natal origin of the individual (starred). Image © 2017 Laffoon et al. and reprinted under the terms of the Creative Commons Attribution License

unknown individual. While not covered here, there are a few examples of stable isotope ratio analysis applied to more historic conflicts (i.e., Emery et al., 2015, 2017; Grupe et al., 2012; Raynor & Kennett, 2008).

3.1 | Military conflicts

To the best of our knowledge, the first work focused exclusively on the use of stable isotopes for postconflict forensic identification and wartime losses was the dissertation of Laura Regan. In her research, Regan (2006) built reference databases of C, O, Sr, and Pb isotope ratios of teeth from U.S. Americans ($n = 228$) and East Asians ($n = 61$) and showed that the two populations could be distinguished isotopically. Most notably, C "was the most discriminatory of the four [chemical elements] tested" (Regan, 2006, p. 166). Teeth collected from the U.S. population had a higher mean $\delta^{13}C$ value than those collected from the Asian population, reflecting the greater proportion of C_4 foods in the U.S. diet (see Section 2.1). In 2012, Holland et al. (2012) presented a paper on the use of these databases to help identify a missing U.S. pilot from the Vietnam War. In that case, the only biological material recovered from an F-100D crash site in the Lao People's Democratic Republic was a tooth crown (tooth #11) missing the root (and thus precluding DNA analyses). Material evidence found during the archeological recovery indicated that the pilot was in the aircraft at the time of impact. The $\delta^{13}C$ and $\delta^{18}O$ values from the sampled tooth were compared to the Regan (2006) data, and were found to differ significantly from East Asians, thus eliminating the supposition that the tooth crown was of a local national. The sum of the evidence in the case allowed for the identification of the aircrewman and eventual return of his remains to his family.

A decade after Regan's dissertation, Someda et al. (2016) used her data to investigate the isotopic discrimination of Japanese ($n = 62$) from Americans ($n = 202$) for the purpose of potentially segregating World War II soldiers. The authors stated that discrimination was "100% accurate" (Someda et al., 2016, p. 166.e3) and the distributions of $\delta^{13}C$ and $\delta^{18}O$ values of tooth enamel shown in Figure 5 suggest that most U.S. American and Japanese samples would correctly group into population of origin. However, an assessment of data comparability between the Regan and Someda databases would provide users with more confidence in the overall discrimination between the populations (Chesson et al., 2019; see Section 6.2).

A survey of human bone by Bartelink, Berg, et al. (2014) further promoted the systematic use of isotope data to answer questions of origin, determining if unknown remains were more likely of a missing U.S. service member or local national (Asian). This study used remains from 24 individuals of known origin from losses in World War II, the Korean War, and the Vietnam War; samples from the remains ($n = 30$) were prepared and analyzed in the blind. Custom linear discriminant functions were built using the isotope test results compared to population groupings, which correctly classified 96.7% of all study samples. The technique was recommended "to screen human remains cases that may be of U.S. versus Asian origin" (Bartelink, Berg, et al., 2014). Following this, Berg and Kenyhercz (2017) built a free, web-based graphical user interface (GUI) called Isolocate that can be used to determine the origin of unknown individuals

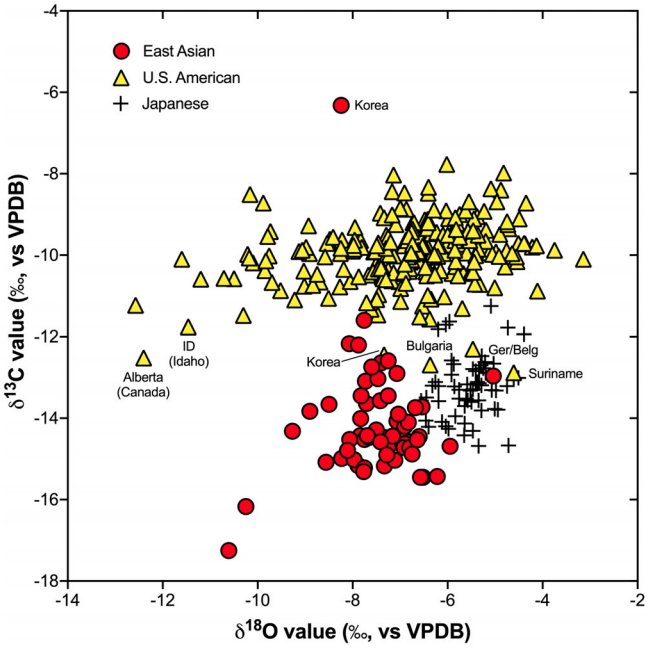

FIGURE 5 Distribution of $\delta^{13}C$ and $\delta^{18}O$ values of tooth enamel carbonate for East Asian (red circles), U.S. American (yellow triangles), and Japanese (plus signs) individuals, demonstrating that populations can isotopically differ. Data are compiled from Regan (2006) and Someda et al. (2016) and redrawn for this article. Labels for select East Asian and U.S. American data points reflect information provided in fig. 5-1 of Regan (2006)

using isotope δ values measured for bone and teeth.[5] Currently, Isolocate includes the following populations: U.S., Southeast Asia, Northeast Asia, Japan, and Unidentified Border Crossers (UBCs; Mexico/Central America/South America countries). The GUI utilizes two discrimination statistics, linear discriminant function analysis and mixture discriminant function analysis, and also produces likelihood ratios. In its nascence, Isolocate aided in the 2018 identification of a pilot who had been declared missing in action during the Vietnam War (Chesson et al., 2020, p. 295), and now is regularly used as part of DPAA casework. The GUI can be accessed via www.anthropologyapps.com and is free for use.

Recently, researchers in The Netherlands used stable isotopes to investigate the origin of two sets of remains recovered in 2010 and 2012 that were associated with the Battle for the Kapelsche Veer in World War II (Font, Jonker, et al., 2015). After dental records and DNA provided no conclusive evidence as to their identity, teeth from both individuals were submitted for stable isotope ratio analysis. Considering the Allied Nations that fought in the battle, the measured $\delta^{18}O$ values and $^{87}Sr/^{86}Sr$ ratios suggested the individuals likely came from the United Kingdom. The later recovery of a fragmented Royal Navy canvas money belt from the remains location supported this conclusion (Font, Jonker, et al., 2015, p. 15). Unfortunately, there has been no update on the identification efforts since 2015. Regardless, these surveys, databases, and applications demonstrate that human tissues can vary sufficiently for isotopic discrimination between population groups, and stable isotope ratio analysis is useful to investigate the geolocation of an unknown individual.

3.2 | Humanitarian crises

Similar to armed conflicts, humanitarian crises can also lead to large numbers of missing and unidentified persons. For instance, at least 15,000 individuals are known to have died at European Union borders in the past decade as a result of the migrant or refugee crisis faced by Mediterranean countries (Olivieri et al., 2018; Robins, 2019). Along the southern U.S. border with Mexico, the remains of over 7000 UBCs, have been recovered since 1998 and an untold number may never be found due to the desolate environments used by migrants for crossing the border (Ammer, Bartelink, et al., 2020).

Application of stable isotope ratio analysis is in its infancy in relation to the UBC situation at the U.S.-Mexico border. Initial efforts have focused on the isotopic characterization of individuals from Mexico and Central America. The

first reference database for the region was published by Juarez (2008); she measured $^{87}Sr/^{86}Sr$ ratios of teeth ($n = 19$) collected from current residents of Mexico. More recently, Ammer, Kootker, et al. (2020) measured $^{87}Sr/^{86}Sr$ ratios of hair ($n = 101$) and tap water collected in Mexico and found that they were strongly correlated. This has led investigators to use Sr in combination with C and/or O to predict the likely origin of recovered UBC remains (e.g., Bartelink et al., 2018, 2020; Bartelink & Chesson, 2019; Kramer et al., 2020). Mapping products (isoscapes) needed for these geolocation efforts are being rapidly created—that is, for Mexico (Ammer, Kootker, et al., 2020; Juarez et al., 2018; see Figure 2), and the circum-Caribbean region (Bataille et al., 2012; Laffoon et al., 2017; see Figure 3) as well as other parts of the world (Adams et al., 2019; Bataille et al., 2018, 2020; Daux et al., 2021; Gautam et al., 2020; Shin et al., 2020; Wang & Tang, 2020; Zieliński et al., 2021). These products, in conjunction with more regular use of isotope testing, should help foster more widespread application of the technique in the coming years.

An additional large humanitarian crisis inside the U.S. is that of missing and unidentified individuals, as tracked by the NamUS program. As noted earlier, more than 20,000 missing persons and another 13,000 unidentified individuals fall within the scope of NamUS. (We note that, a portion of these cases are suspected UBCs.) Isotope testing has been applied in a few instances (e.g., Chesson et al., 2018; Kamenov et al., 2014; Kimmerle & Kamenov, 2015; Remien et al., 2014) but to date there has been no large-scale use of stable isotope ratio analysis for NamUs cases. Problematically, NamUs is only a clearinghouse of information; individual cases are under the jurisdiction of individual states, counties, or municipalities. Therefore, scaled efforts to use isotope testing in the U.S. for forensic identification in a humanitarian context are dependent on individual practitioners or agencies.

Globally there are a handful of example applications of isotope testing in unidentified decedent casework (e.g., Fauberteau et al., 2020; Font, van der Peijl, et al., 2015; Kealy et al., 2014; Lehn et al., 2015; Meier-Augenstein, 2018; Meier-Augenstein & Fraser, 2008; Rauch et al., 2007). The major focus has been on "cold cases" in an attempt to provide new investigative leads for either a single set or small groups of remains. However, on a larger scale, researchers have recently started exploring the use of stable isotopes to identify individuals in large groups of remains that are associated with long-term socio-political conflict (e.g., in Colombia [Eck et al., 2019; Gutiérrez et al., 2020]).

4 | SAMPLE CHOICE AND PREPARATION

The choice to prepare one tissue type versus another for stable isotope ratio analysis is driven by three factors: (1) testable hypothesis (e.g., dietary reconstruction, geolocation, sorting commingling, etc.); (2) material availability (i.e., what tissues are present, based on case circumstances); (3) and time period of interest (days/weeks/months before death). The testable hypothesis relates back to the tenants discussed in Section 2: you are what you eat and there's no place like home. (As a corollary, "you are what you eat" is in many ways a subset of "you are where you live," including socially/culturally and environmentally/geologically.) Therefore, if you ask, "Did this person travel before death, and if so, where did s/he originate?" you should choose tissues from among those available that would provide useful answers. Long hair would be a worthwhile material to test—as the hair has been grown over the course of several months before death—while dental structures would not provide data for the most relevant time periods or time scales since these isotope ratios have not changed since childhood/adolescence. If you are testing whether multiple individuals may be present in a group of remains, bone or teeth would likely be the best choice of material for testing.

Bone, teeth, and hair/nail are the tissues most commonly encountered in forensic settings. However, each tissue is susceptible to a variety of postmortem degradation factors (see Box 2) and the availability of material can vary. Teeth may be unavailable in the elderly (edentulism) or may be unsuitable in younger individuals due to the presence of dental work or caries. Hair can be detached from skeletal remains, thus eliminating the direct link to a specific person (especially if more than one person is recovered in a group). If remains were burned, the isotopic signatures in the bones and teeth may have been altered (Robinson & Kingston, 2020; Snoeck et al., 2016). Finally, all sampling decisions are predicated on what materials can be released for destructive analysis, per the investigating authority.

Addressing the aforementioned third factor, the slow, but constant, turnover of bone means this tissue provides a time-averaged signal representing several years to a decade or more before death (Hedges et al., 2007; Meier-Augenstein & Fraser, 2008; Stenhouse & Baxter, 1979). Teeth represent a "snapshot" of time in an individual's life during the period of enamel formation, which ranges from childhood to adolescence depending on the tooth (Anderson et al., 1976); once formed, enamel does not remodel. The steady, continuous growth of hair and nail in life (Wilson & Gilbert, 2007) make these tissues serial recorders of the days, weeks, or months prior to an individual's death.

BOX 2 Postmortem alterations to remains

During the peri- and postmortem period, remains can be physically and chemically altered due to a variety of biological and/or environmental factors. *Taphonomy*, as a term, was coined by Efremov (1940) to describe "the study of the transition (in all its details) of animal remains from the biosphere into the lithosphere." Forensic taphonomy refers to processes that affect remains or their surroundings via activities that are either natural (e.g., biological, chemical, or physical systems) or manmade (e.g., human behavior), from the perimortem period until discovery (Haglund & Sorg, 1997). *Diagenesis* generally refers to processes that alter both the proportion and structure of collagen and bioapatite in bone and teeth. Common diagenetic factors include groundwater exposure, microorganism activity, soil pH, and temperature (Hedges, 2002; Kendall et al., 2018). Due to differences in bioapatite content and crystal structure, early research suggested that teeth were more resistant to diagenetic alteration than bone (Schoeninger & DeNiro, 1982; Wang & Cerling, 1994).

When both are recovered, the decision to analyze hair vs. nail should consider the following. As noted previously the δ values of hair and nail from the same individual are not the same (Fraser et al., 2006; Hülsemann et al., 2015; Lehn et al., 2015; O'Connell et al., 2001). This is true even when adjusting for different growth rates of the tissues, with 1 month represented by ca. 10 mm of scalp hair and ca. 3 mm of fingernail development (Chesson et al., 2020). Consequently, analysis of hair is recommended over analysis of nail when both tissues are available as the growth rate of hair is faster (Fraser et al., 2006) and it has "an isotopic composition which is more consistent with dietary uptake than is provided by nail" (Lehn et al., 2011, p. 704). Since the growth rate of hair is relatively well-characterized, strands can be sampled in short segments that represent discrete periods of time to sequentially reconstruct an individual's travel history (Fauberteau et al., 2020; Remien et al., 2014).

4.1 | Bone preparation

This and the following sections briefly describe the preparation of human tissues for stable isotope ratio analysis of the bio-elements H, C, N, O, and S. Preparation of remains for analysis of Sr and Pb isotope ratios is covered in Section 5.2 (analysis) since the preparation process is relatively straightforward and can sometimes partially take place in-line with the analysis process.

Cortical bone is preferred for extraction (Ambrose, 1993). Prior to the extraction of either collagen or bioapatite, the sample should first be cleaned. This can be done by abrading the external and internal surfaces with a rotary tool and then solvent cleaning in an ultrasonic bath to remove abrasion debris and other surface contaminants. Common solvents are 95–100% ethanol and distilled or deionized water (Bartelink, Berry, et al., 2014; Chesson et al., 2021). For wet or fresh bone ("green" remains), the sample can be additionally treated with a mixture of chloroform and methanol to remove lipids (Ambrose, 1993).

4.1.1 | Bone collagen

A straightforward method to extract collagen from bone is the "chunk" method described by Sealy et al. (2014). A small piece of whole bone is demineralized at room temperature using a weak solution of hydrochloric acid (e.g., 0.2–0.6 M HCl [Pestle et al., 2014][6]) to remove the bioapatite component. The exact acid concentration is not important, as experimentally demonstrated by Pestle (2010). The acid is changed every few days until demineralization is complete. A demineralized bone chunk will be flexible or spongy and there will be no hard spots found by probing with a dental pick. The resultant collagen pseudomorph is treated with a sodium hydroxide solution (commonly 0.125 M NaOH) for 24 h to remove base-soluble contaminants, such as humic acids.

Sealy et al. (2014) recommended drying the pseudomorph after humic acid treatment, but it is not uncommon for collagen preparation methods to include an additional gelatinization step. In this step, the collagen pseudomorph is treated with acidic water (ca. pH 3, or 0.001–0.01 M HCl [Pestle et al., 2014]) and heat (65–100°C) to solubilize and

homogenize the collagen, which is collected as gelatine via pour-off. An additional filtration step can be added to remove smaller molecules from the gelatine (Brown et al., 1988; Sealy et al., 2014). For collagen extracted from modern skeletal remains in good condition, filtration is infrequently used.

4.1.2 | Bone bioapatite

The mineral component bioapatite is extracted from bone for analysis of either its carbonate ions or phosphate ions (see below). The bone sample must first be powdered using a drill, mill, or mortar and pestle. Analysts then face a choice of solvents for bleaching (Pestle et al., 2014): sodium hypochlorite (i.e., a 1–3% solution of NaOCl) or hydrogen peroxide (30% H_2O_2). Snoeck and Pellegrini (2015) found that H_2O_2 did not remove all organic material from bone while NaOCl did; however, NaOCl also caused the precipitation of exogenous carbonates. Crowley and Wheatley (2014) instead advocated for 30% H_2O_2, followed by a treatment with 1 M buffered solution of acetic acid (CH_3COOH) to remove exogenous carbonates. This mirrored earlier recommendations by Koch et al. (1997) and Yoder and Bartelink (2010) on the use of buffered acetic acid solutions to remove carbonates that are diagenetic in origin (see Box 2).

Regardless of the bleaching solvent selected, powdered bone is treated for 24–72 h at room temperature (Pestle et al., 2014), typically at a volume of 0.04 ml/mg of sample (Koch et al., 1997). This step removes organics—that is, the collagen component of bone. The sample is next treated with acetic acid for ≤24 h at room temperature to remove labile, or exogenous, carbonates, again at a volume of 0.04 ml/mg of sample.

4.2 | Teeth

Preparation of teeth for stable isotope ratio analysis of enamel carbonate is similar to the method used for bone bioapatite extraction. Briefly, tooth enamel is powdered, often with a dental drill, and then treated to remove organics and labile carbonates. As enamel contains very little collagen (i.e., ≤1%, Lee-Thorp, 2002), the bleaching step can be of shorter duration using more dilute NaOCl/H_2O_2 than used during bone bioapatite extraction (e.g., Chesson et al., 2019; Kusaka & Nakano, 2014). Some researchers suggest skipping this treatment altogether as any organics present in the sample should not react with the phosphoric acid used during analysis to produce CO_2 (Ventresca Miller et al., 2018). In this case, a weak (ca. 0.1 M) acetic acid solution would be the only pretreatment required to prepare tooth enamel for stable isotope ratio analysis. Again, this treatment is of shorter duration (i.e., ≤30 min) than that used when preparing bone bioapatite (e.g., Chesson et al., 2019; Kusaka & Nakano, 2014; Ventresca Miller et al., 2018).

4.3 | Hair and nail

Hair and nail are composed almost entirely of the protein keratin, which does not need to be extracted prior to stable isotope ratio analysis. Instead, preparation of these keratinous tissues focuses on cleaning and then, if needed, sequential sampling (Mancuso & Ehleringer, 2019b; O'Connell et al., 2001; O'Connell & Hedges, 1999). Cleaning starts with a solvent wash, typically using a mixture of chloroform and methanol (Gordon et al., 2018; Kootker et al., 2020; Mancuso & Ehleringer, 2019b; O'Connell & Hedges, 1999), and then samples are dried at room temperature. For tissues recovered from forensic settings, physical debris can be first removed using forceps and low-lint laboratory wipes (Gordon et al., 2018); a blast of high pressure N_2 gas (Kootker et al., 2020); or a drop of mild detergent, such as dish soap, in water (Saul et al., in press). Hair and nail prepared for measurement of hydrogen isotopes *must* either be equilibrated with (1) local laboratory atmosphere, as per guidance for the reference materials USGS42 and USGS43 (Coplen & Qi, 2012, 2016); (2) humidity within a sealed chamber, which can provide more consistent conditions and reproducible results; or (3) an online preparation system that can reduce equilibration time from days to hours (Soto et al., 2017; Wassenaar et al., 2015). Samples must be equilibrated simultaneously with matrix-matched reference materials; equilibrated samples and reference materials should then be thoroughly dried before being analyzed. In this way, the effect of exchangeable H atoms and adsorbed water on total measured δ^2H values can be controlled/corrected.

5 | SAMPLE ANALYSIS

The most common stable isotope ratio analysis techniques involve specialized mass spectrometers operated in continuous flow mode that are attached to peripherals for the automated introduction of samples. Stable isotopes of the bioelements H, C, N, O, and S are typically measured using isotope ratio mass spectrometry (IRMS; Muccio & Jackson, 2009) while those of trace metals, such as Sr and Pb, can be measured using thermal ionization mass spectrometry (TIMS) or multi-collector inductively-coupled plasma mass spectrometry (MC-ICP-MS).

5.1 | IRMS

In IRMS, gas molecules are ionized and then passed through a magnetic field where molecules containing different stable isotopes are separated based on their mass-to-charge ratio. Helium is used as a carrier gas. Isotope ratio mass spectrometers are designed to make precise measurements of very small differences[7] in isotope abundances; that is, they are not designed to measure absolute abundances of stable isotopes. During analysis, the ratio of the heavier-to-lighter stable isotopes in a sample is compared to that of a working gas introduced just before or after the sample. This means the δ values reported by the instrument software following analysis are scaled to the working gas and *not* to an internationally agreed upon standard. Postanalysis data handling is therefore required to report δ values on the appropriate accepted international scale (see Section 6 and Dunn & Carter, 2018).

5.1.1 | Elemental analyzer IRMS

Using an elemental analyzer (EA) attached to an isotope ratio mass spectrometer, material is combusted in the presence of oxygen to form the gases N_2 (following the reduction of NO_x), CO_2, and SO_2, plus water (H_2O). Water is trapped and the other gases are separated via gas chromatography (GC) before being introduced to the mass spectrometer one-by-one for measurement of $\delta^{15}N$, $\delta^{13}C$, and $\delta^{34}S$ values, respectively. Traditionally, continuous flow EA-IRMS methods were dual-gas and measured N_2 and CO_2 from the combustion of one sample aliquot; measurement of SO_2 required combustion of another sample aliquot. However, instrument advances have made triple-gas measurements more common (Fry, 2007; Hansen et al., 2009; Sayle et al., 2019). It is possible to measure the carbon isotopic composition of carbonate-containing materials via EA-IRMS (Skrzypek & Paul, 2006) although the carbonate portion of bioapatite is more commonly measured as described next.

5.1.2 | Carbonate analysis Vis IRMS

In contrast to its analog hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], bioapatite is a carbonate-rich, hydroxyl-deficient mineral in which carbonate ions (—CO_3) replace hydroxyl ions (—OH) and even phosphate ions (—PO_4) (Wopenka & Pasteris, 2005). As mentioned previously, either the carbonate ions or phosphate ions can be analyzed. The stable isotope ratio analysis of carbonate is both easier and simpler: bioapatite is acidified using phosphoric acid and the $\delta^{13}C$ and $\delta^{18}O$ values of the resultant CO_2 gas are measured. Before the availability of continuous flow IRMS instrumentation, a single carbonate sample was acidified in a sealed reaction vessel and the CO_2 collected off-line for introduction to the mass spectrometer one-by-one (Passey et al., 2007; Swart et al., 1991). The first continuous-flow measurements of carbonate utilized a common acid bath in which samples were dropped one after another into a pool of phosphoric acid; the resultant CO_2 was swept in a helium stream to the mass spectrometer. Today, carbonate samples are once again acidified in separate vessels, but the CO_2 is collected automatically for analysis and multiple samples can be analyzed in one "run" (Paul & Skrzypek, 2006; Tobin et al., 2011).

It is important to understand that O atoms in —CO_3 are separated into two pools during the acidification reaction: CO_2 and H_2O. There are isotopic fractionation factors associated with this separation, which are temperature dependent. In short, the temperature used for acidification will impact the $\delta^{18}O$ values measured for a carbonate sample and should be corrected for (Kusaka & Nakano, 2014; Passey et al., 2007). Additionally, research has found that the particle size of bioapatite can impact measured $\delta^{13}C$ and $\delta^{18}O$ values, potentially due to reaction differences during sample preparation (Moloughney et al., 2020). An alternative approach to measuring the $\delta^{18}O$ values of bioapatite involves

analysis of the O atoms in phosphate (—PO_4), using a high temperature conversion (HTC) elemental analyzer attached to an isotope ratio mass spectrometer.

5.1.3 | HTC elemental analyzer IRMS

Using a high temperature conversion elemental analyzer (HTC or TC/EA) attached to an isotope ratio mass spectrometer, material is pyrolysed in an oxygen-free environment to produce H_2 and CO gases that are separated via GC for the measurement of δ^2H and δ^{18}O values, respectively. In forensic isotope testing, HTC-IRMS is most often used for the analysis of hair and nail (e.g., Ehleringer et al., 2008; Fraser et al., 2006; Mancuso & Ehleringer, 2019a), although bone collagen can also be analyzed (e.g., Lehn et al., 2020). Not all H atoms are converted into H_2 during the HTC-IRMS analysis of N-rich materials, which could lead to erroneous measurements of δ^2H values for organic samples. In these cases, a modified reactor packed with chromium is recommended for measurement of hydrogen δ values (Gehre et al., 2015; Nair et al., 2015; Reynard & Tuross, 2016) with oxygen δ values measured separately using another sample aliquot. For measurement of δ^{18}O values, a longer GC column and additional dilution with helium can help mitigate the interference of NO with CO (Hunsinger & Stern, 2012; Qi et al., 2011).

The O atoms in the phosphate ions of bioapatite can be analyzed using HTC-IRMS, but samples must first be prepared as silver phosphate. While straightforward methods for producing silver phosphate free of organic contamination have been recently published (Pederzani et al., 2020; Shabaga et al., 2018), they require the use of hydrofluoric acid (HF) to dissolve samples. HF is an extremely corrosive acid that can cause deep, painful burns or death; many laboratories avoid its use for health and safety reasons.

5.2 | TIMS and MC-ICP-MS

In both TIMS and MC-ICP-MS analysis, trace metal elements (e.g., Sr or Pb) are vaporized and then ionized before being moved into a mass analyzer that separates isotopes based on their mass-to-charge ratios. TIMS instruments use a filament and resistive heating for ionization (Aggarwal, 2016) while high-temperature plasma discharge is used for ionization in MC-ICP-MS systems (Thomas, 2001); argon is the carrier gas. Traditionally, analysis via TIMS was considered the "gold standard" (Aggarwal, 2016, p. 943) but the (relative) ease with which MC-ICP-MS instrumentation can be operated means the latter technique is arguably used more often today for the measurement of Sr and Pb isotope ratios.

Regardless of the technique used, elements must be in a "chemically pure form" for analysis (Aggarwal, 2016, p. 944), which requires the separation of the element of interest from other elements in the sample. Material is typically digested using acids, heat, and/or pressure; the resultant digest is then purified using anion exchange resin(s) and column chromatography (e.g., Retzmann et al., 2017; Romaniello et al., 2015). This separation step can be completed offline but in-line separation is possible during MC-ICP-MS analysis, as demonstrated for water samples (Chesson et al., 2012). Because the chemical elements analyzed using TIMS or MC-ICP-MS are found in trace amounts, sample digestion and element separation ideally take place in a clean lab setting to avoid contamination. Additionally, sample preparation prior to digestion is kept to a minimum. For example, bone submitted for measurement of Sr and Pb isotopes is not typically extracted to separate collagen or bioapatite. Instead, a piece of whole bone would be digested.

6 | DATA HANDLING

As described earlier, the δ values exported by IRMS instrument software must be linked to an internationally accepted scale for reporting. This linking process—known as calibration, normalization, or scaling—requires at least two reference materials with known (or, accepted, certified, or true) δ values to meet current best practice guidelines (Dunn & Carter, 2018; Meier-Augenstein & Schimmelmann, 2019). These materials act as scale anchors or endpoints for calculating the slope (stretch) and intercept (shift) between the measured, or "raw," δ values and the accepted, or "true," δ values. Ideally, the reference materials bracket the expected δ values of samples (Schimmelmann et al., 2016) and, if possible, match the sample matrix. The measured δ values of samples analyzed alongside the reference materials are then corrected using stretch- and shift-factors calculated from the raw data (Paul et al., 2007; Skrzypek, 2013).

The process of calculating stretch and shift factors is illustrated in Figure 6. At the top, the (hypothetical) measured δ values of two reference materials are given. A stretch-factor (m) is calculated to stretch (or alternatively compress) the measured δ values so the difference between them matches the accepted difference between the two reference materials. Next, a shift-factor (b) is added to the stretched data to bring the measured δ values of the reference materials into agreement with their accepted values (Sharp, 2017). These calculated factors are finally used to stretch and shift the measured δ values of samples so that all data are calibrated to the accepted isotope scale: $\delta_{\text{accepted}} = \text{m} * \delta_{\text{measured}} + \text{b}$.

Prior to stretching and shifting, other corrections can be applied to raw δ values to deal with analytical effects—that is, blank, drift, and/or linearity (peak area). These corrections are calculated from the raw data and applied as needed; they are described in detail by Carter and Fry (2013) and Dunn and Carter (2018). In general, blank correction accounts for the contribution of gas either evolved from the container holding the sample and/or introduced by the instrument's autosampler. Drift correction adjusts for changes in measured δ values as a function of the time of analysis. Linearity correction addresses variation in δ values with sample size and/or peak intensity. As a rule, it is better to control for the effects prior to or during analysis rather than correcting for them postanalysis. For example, linearity can be controlled through careful weighing and allowing for minimal variations in peak heights (gas amounts) sample-to-sample and between reference materials and samples.

Since the isotope test results of trace elements are typically reported as ratios (e.g., $^{87}Sr/^{86}Sr$ and $^{206}Pb/^{204}Pb$), the raw data from TIMS or MC-ICP-MS analysis are not scaled in the same way raw δ values are scaled. Instead, the data are blank corrected if needed. The measurement results for the reference materials analyzed alongside samples are then used to assess instrument performance.

6.1 | Data reporting

When reporting δ values, details of data handling should be presented, including the identity of reference materials used for scaling. An example paragraph is available from Szpak et al. (2017).

Reports of isotope test results from IRMS analysis should also include an estimation of measurement uncertainty. While there is no single best method of calculating uncertainty, guidance has been provided by the Forensic Isotope Ratio Mass Spectrometry Network in the *Good Practice Guide for Isotope Ratio Mass Spectrometry* (Dunn & Carter, 2018). An additional resource is Szpak et al. (2017), who provides a Microsoft Excel workbook that can be used for calculation of δ-value uncertainties.

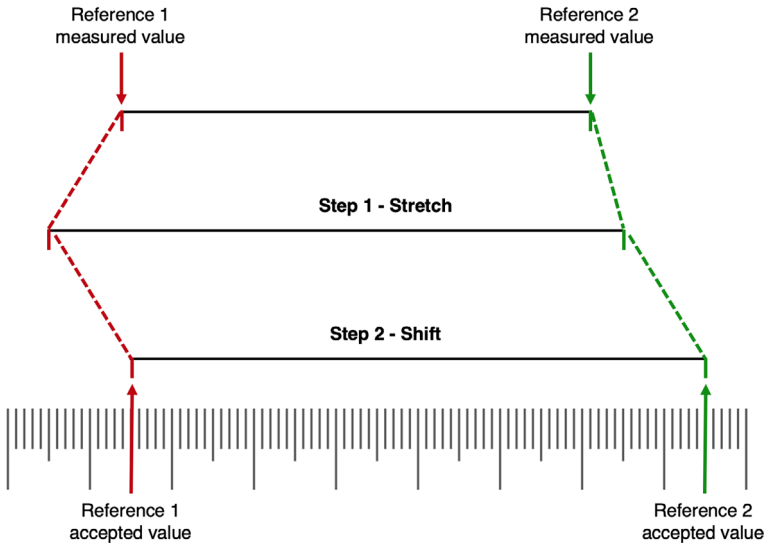

FIGURE 6 The process of stretching and shifting raw δ values to the accepted isotope scale is presented graphically, redrawn from (Sharp, 2017). First, the measured difference between two reference materials is stretched (or compressed) to match the accepted difference between the materials. Then, the stretched (or compressed) data are shifted so that the measured δ values of the reference material agree with their accepted δ values. The factors calculated in this process are finally applied to the raw δ values of samples to scale them

6.2 | Data comparability

Variations in isotope measurement results for samples prepared and/or analyzed at multiple locations should be evaluated. In other words, when combining data sets or using isotope data from multiple different sources, researchers need to directly assess—and report on—the comparability of the test results. Figure 5 illustrates the potential problem in ignoring isotope data comparability: How do we know the population differences between U.S. Americans and Japanese are accurate and not an artifact of variations in sample preparation or sample analysis methods? (We do not, unless/until we complete an interlaboratory comparison.)

The first large scale interlaboratory comparison (ILC) designed to quantify isotopic variability in the measurement of human remains found that approximately half the variation was due to sample preparation while the other half was due to sample analysis (Pestle et al., 2014). The differences between laboratories could be substantial: for example, 1.8‰ for δ^{13}C values and 1.9‰ for δ^{15}N values of collagen, and 3.5‰ for δ^{13}C values and 6.7‰ for δ^{18}O values of bioapatite. The ILC results were used to build a "minimum meaningful difference" (MMD) metric for determining when δ values were bona fide different from each other—that is, when they exceeded the MMD values. The MMD values were set at 0.6 and 0.9‰ for collagen δ^{13}C and δ^{15}N values, respectively, and 1.2 and 3.1‰ for bioapatite δ^{13}C and δ^{18}O values, respectively.

The MMD approach built by Pestle et al. (2014) quantified the isotopic effects of both preparation and analysis into one metric. While it was an important first assessment of isotope data comparability, the number of participating laboratories (ca. 20) and sample replicates (up to 5 per laboratory, per bone component) made it impractical to repeat for verification. The ILC was time consuming and resource intensive; therefore, other materials (e.g., tooth enamel, hair/nail, etc.) have not undergone similar evaluation.

In response, Chesson et al. (2019) developed a more straightforward "real interpretative difference" (RID) metric for assessing comparability between two sources of isotope data; it was a two-step process. First, a subset of samples previously prepared/analyzed at one location (or using one method) were prepared/analyzed again at another location (or using another method). The pairwise differences between the samples were used to construct RID limits. Second, the RID values were tested using a separate, independent subset of samples that had prepared/analyzed either at both locations or with both methods. The number of pairwise differences that exceeded the RID values in the test thus provided an estimate of the inherent error rate in combining isotope data from the two sources.

Two recent publications demonstrated the RID approach for comparability of sample preparation, sample analysis, and both preparation and analysis, using bone collagen. In Edwards et al. (in press) bone samples previously prepared at California State University, Chico (Bartelink, Berg, et al., 2014) were prepared again at the DPAA Laboratory in Hawaii; all samples were analyzed at the same location, the Stable Isotope Facility at the University of California, Davis. The isotope data were used to calculate RID_{prep} that was 0.43 and 0.75‰ for collagen δ^{13}C and δ^{15}N values, respectively. A test of these RID_{prep} values found an inherent error rate of 1.7% in combining test results and demonstrated that the data generated from samples prepared at one laboratory could be reliably compared to data generated from samples prepared at the other. The bone collagen samples were then analyzed at the DPAA Laboratory to construct $RID_{analysis}$; this was calculated as 0.40 and 0.51‰ for collagen δ^{13}C and δ^{15}N values, respectively (Chesson et al., in press). Finally, all data were used to construct $RID_{combined}$ values. As would be expected, these were higher at 0.59‰ for δ^{13}C values and 0.91‰ for δ^{15}N values. It is interesting to note that the $RID_{combined}$ values were similar to the earlier and respective bone collagen MMD values of 0.6 and 0.9‰ (Pestle et al., 2014), but the $RID_{combined}$ values were much easier to calculate/test and they did not require a large ILC to generate (Chesson et al., in press).

Whenever isotope data are compiled from tissues prepared and analyzed at different laboratories, it is critical that scientists directly address comparability between the data sets. We return again to the isotope data presented in Figure 5. Using the RID approach demonstrated by Chesson et al. (in press), it would be relatively easy to conduct a small assessment of the Someda et al. (2016) test results to demonstrate their comparability with the data from Regan (2006). This would give follow-on users and researchers confidence in the published databases and subsequent categorization of Japanese or other military personnel based on stable isotope ratio analysis of tooth enamel.

7 | QUALITY ASSURANCE

Quality assurance methods fall into two categories: preanalysis (i.e., those measured during sample preparation) and postanalysis. Generally, there are more established postanalysis checks than there are preanalysis checks. For samples that fail quality checks during preparation, follow-on stable isotope ratio analysis is not recommended.

7.1 | Preanalysis checks

Common preanalysis checks assess material condition. It is good practice when reporting isotope test results to provide information on sample quality, especially in cases where taphonomic alteration of remains is a concern. Roberts et al. (2018, table 1) provides a succinct summary of some common checks of bone and teeth prepared for stable isotope ratio analysis of the bio-elements. Some of these checks are described in this section and the next; they should be utilized where appropriate for assessing the condition of collagen and bioapatite.

Bone in good condition will have a collagen yield, calculated as a percentage, between 3% and 28% (Ambrose, 1990; Brock et al., 2007; Chesson et al., 2021). Chesson et al. (2021) recently investigated the use of bioapatite yield for quality control (QC), suggesting a range of 21%–63% as the least conservative limits for assessing bone condition. In addition, the molecular structure of bioapatite can be examined using histology, spectroscopy, and/or x-ray diffraction (Hedges et al., 1995; Smith et al., 2007). Fourier transform infrared spectroscopy in particular has been useful to measure the infrared splitting factor and carbonate-to-phosphate ratio of bioapatite, which are both semi-quantitative metrics of crystal structure and molecular composition (Beasley et al., 2014; France et al., 2020).

For samples prepared for stable isotope ratio analysis of the trace metals Sr and Pb, the elemental concentration of digest can be checked via inductively coupled plasma mass spectrometry. Elemental concentration measurements are often necessary to determine the proper dilution scheme to achieve sample signal intensities that are within the intensity range of bracketing standard reference materials used during MC-ICP-MS and/or TIMS analysis. Samples with elemental concentrations that fall below levels of detection are not submitted to follow-on stable isotope ratio analysis.

An internal control standard can be prepared alongside samples and its test results can be compared to an expected outcome. At the DPAA Laboratory, a small piece of bovine femur is included in each batch of casework samples submitted for collagen extraction. The % collagen yield and, eventually, the isotope δ values measured for the bovine femur are compared to long-term records to ensure nothing in the preparation process for that batch appeared "out of control" (Dunn & Carter, 2018, p. 60). In instances where the % collagen yield of the internal control sample is unexpected, the entire batch should be considered suspect, and the samples prepared again. Likewise, if the internal control isotope δ values are unexpected, the entire run should be carefully evaluated and perhaps even rejected.

7.2 | Postanalysis checks

Common postanalysis checks assess material content. For collagen, weight %C and weight %N—plus the atomic C:N ratio calculated from these—are measured during EA-IRMS analysis. An atomic C:N ratio between 3.1 and 3.5 is indicative of good condition collagen (van Klinken, 1999). Similarly, atomic C:N ratios can be used as a quality check for the EA-IRMS analysis of hair; for modern hair samples, a range of 2.9–3.8 has been previously observed (O'Connell & Hedges, 1999). The isotope data of samples that fall outside of these ranges should not be reported, or if they are, data interpretation should be withheld, citing a failure of quality assurance.

The outcome of data handling processes should be verified using a dedicated QC material (Schimmelmann et al., 2016). This material, or materials, should have an expected δ value, which may or may not be officially certified. Aliquots of the QC material are treated as samples during stable isotope ratio analysis and scaling. In other words, the raw δ values of the QC material(s) are not used in the calculation of stretch or shift. After scaling, the reported δ values of the QC material are compared to expected δ values to ensure they agree (Dunn & Carter, 2018); if they do not, the entire run should be rejected. Isotope data handling postanalysis therefore requires two types of reference materials: endpoints for scaling, plus something additional for QC.

A variety of certified collagen (Schimmelmann et al., 2020) and hair (e.g., Coplen & Qi, 2012, 2016) reference materials is available for purchase. However, no certified bioapatite reference materials are currently available; instead, only "pure" carbonate and "pure" phosphate reference materials have been produced to date (e.g., Assonov et al., 2021; Halas et al., 2011; Qi et al., 2021). It is worth noting that these may behave differently during analysis than the lower concentration carbonates found in bone and teeth bioapatite. Finally, there are two reference materials of particular importance for analysis via either TIMS or MC-ICP-MS: SRM 987, strontium carbonate isotopic standard, and SRM 981, common lead isotopic standard.

8 | CONCLUSION

The forensic applications of stable isotope ratio analysis have expanded tremendously since the first use of the technique in the 1970s to characterize the explosive TNT (Nissenbaum, 1975). Today, the power of the technique lies in providing answers to difficult questions in forensic identification—namely, who might this person(s) be? In the realm of postconflict identification, or during a humanitarian crisis, any investigative leads on the identity of a decedent are better than "unknown." Isotope test results can provide a variety of answers: Did this person travel before death? Were they local to the region of recovery? Are there multiple people involved in the incident? Can we separate the remains into individuals without using DNA? While these are worthy questions, the interpretation of isotope data in the context of forensic identification has room for research and development; in many ways, the technique is still taking small steps forward, with the occasional giant leap.

It is easy to see from this review the areas of intense growth in forensic stable isotope ratio analysis. They cluster into several main themes: new isotopes, isoscapes, postmortem alteration of remains, and data surety. Within the scope of "new" isotopes, it is clear that the chemical element sulfur might be of significant interest in the future, particularly given its broad range of δ values. If sulfur isotopic variation correlates to different populations or groups of people, analysis of bone collagen for $\delta^{34}S$ values could become a routine test for sample classification. That said, the addition of the trace metal strontium, in combination with the bio-element oxygen, can also be very powerful for forensic identification, particularly in geolocation. The use of Sr is not as widespread today as C, N, or O isotope analyses, likely due to specialized analytical instrumentation and testing environment required for measurement. If costs for equipment and analysis can decrease, perhaps strontium isotopes will become more commonplace in identification efforts in the future. More isoscapes, reflecting the modern, global isotopic variation of O, Sr, and Pb, are desperately needed. As noted previously, there is no consensus on map construction methods and there are relatively few tissue-specific isoscapes built. In order to advance the use of stable isotopes in postconflict forensic identification, more world coverage is needed, and we should strive to move away from the requirement to translate data (i.e., from water/rock to tissue, using fractionation factors); in this endeavor, isoscapes built directly from *all* human tissues—tooth enamel, bone collagen and bioapatite, hair/nail—would be enhancements to the state of the art.

Finally, isoscapes, and the data sets that they are (potentially) built upon, must be confirmed for use in the broader spectrum of forensic stable isotope ratio analysis. This can only be done through comprehensive and concise data comparability efforts (e.g., Magozzi et al., 2021), as exemplified by Pestle et al. (2014) and Chesson et al. (in press). Using RID values to compare between published isotope databases of human tissues will give confidence in the ultimate outcomes of predictions for geolocation. Standardization of methodology—in sample preparation, sample analysis, and data handling/reporting—is expected to grow as well. For the forensic investigator and the Trier of Fact (e.g., Justice departments worldwide), this likely will become mandatory in the future. Even with these improvements, it will be crucially important to understand the role that taphonomy (or diagenesis) plays for each tissue type, in both common and novel recovery contexts. For instance, if the isotope abundances in this bone sample were changed due to burning, can they be reliably "mapped" to a geolocation? The answer to this query, and many more like it, should challenge the isotope researcher today, and we hope the answers will be forthcoming in the future.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ORCID

Lesley A. Chesson https://orcid.org/0000-0002-8457-9341
Gregory E. Berg https://orcid.org/0000-0003-4225-1783

ENDNOTES

[1] The DPAA was formed in January 2015 following the consolidation of the Joint POW/MIA Accounting Command (JPAC) with the Defense Prisoner of War/Missing Personnel Office (DPMO) and the U.S. Air Force's Life Sciences Equipment Laboratory.

[2] Succulents, such as agave, cactus, and pineapple, use Crassulacean acid metabolism (CAM) photosynthesis, and have $\delta^{13}C$ values the span the range of C_4 plants (O'Leary, 1988). However, CAM plants are not usually large components of an individual's diet.

[3] For both carbon and sulfur isotopes, the trophic shift is typically ≤1‰ (McCutchan et al., 2003).

[4] See also Appendix A of Pederzani & Britton (2019).

[5] Data from Regan (2006) are included in Isolocate.

[6] For examples of the bone preparation methods used by different of laboratories, see supporting information for Pestle et al. (2014), particularly Tables S1 and S2.

[7] The letter δ is the first letter in the Greek word for difference \(diaforá).

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