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RESEARCH ARTICLE

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Examination of amino acid hydrogen isotope measurements of scalp hair for region-of-origin studies

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Rationale: Hydrogen isotope (δ^2H) analysis of keratinaceous bulk tissues has been used in forensic science to reconstruct an individual's travel history or determine their region-of-origin. Here, we use a compound-specific approach to examine patterns of individual amino acid δ^2H values in relation to those of local tap water, bulk scalp hair tissues, and region-of-origin.

Methods: We measured δ^2H values of amino acids in anonymously collected scalp hair ($n = 67$) and tap water from 28 locations in the United States. Samples were hydrolyzed into their constituent amino acids, derivatized alongside in-house reference materials, and analyzed in triplicate using a GC-C-IRMS system.

Results: Non-essential amino acid (AA_{NESS}) δ^2H values and their corresponding tap water samples varied systematically across continental regions. Hydrogen isotope values of alanine, glutamic acid, and glycine were significantly correlated with tap water and an estimated 42%–51% of the hydrogen atoms in these AA_{NESS} were derived from tap water. We used linear discriminate analysis (LDA) to explore regional patterns in scalp hair bulk tissue and amino acid δ^2H values. For the model that included AA_{NESS} data, 87% of the variance was explained by the first linear discriminant axis (LD1), and was driven by bulk hair tissue, alanine, and proline. This model had an overall 72% successful reclassification with samples from the south and northwest regions reclassifying correctly 92% and 78% of the time, respectively. For the model that included AA_{ESS} data, LD1 explained 81% of the variation and was driven bulk hair, threonine, valine, phenylalanine, and isoleucine. The overall reclassification rate for the model that included AA_{ESS} was 70%.

Conclusions: Our findings suggest that δ^2H analyses of AA_{NESS} and AA_{ESS} could help improve geolocation models for human and wildlife forensics by simultaneously providing information about both dietary and tap water inputs of hydrogen to tissue synthesis.

1 | INTRODUCTION

Stable isotope analysis of human remains has become a widely used analytical tool in forensic science over the past two decades.[1–6] The principles, mechanisms, and analytical techniques that are applied by forensic researchers were developed in the biological and geological disciplines. Forensic applications of this tool have exploited variation in the natural abundance of isotopes of carbon (δ^{13}C), nitrogen (δ^{15}N), oxygen (δ^{18}O), and hydrogen (δ^2H) to explore questions surrounding the origins of illicit drugs, human movement and migration, and food fraud.[7] Stable isotope analysis has also been an extremely useful tool for reconstructing travel history and determining the region-of-origin of unidentified human remains, especially where traditional identification methods such as facial reconstruction and DNA analysis have been unsuccessful.[7,8] For example, analysis of tissues such as bone, teeth, or hair has provided law enforcement agents with

information related to an individual's birthplace, residency during the last decade of their life, and more recent geographic location from weeks to months prior to death.[9]

Isotopic analysis of human keratin tissue (fingernails or scalp hair) can be used to differentiate broad geographic locations of human populations or determine an individual's region-of-origin. Carbon isotope analysis of keratin tissues have been used to broadly classify individuals into geographic regions based on the direct (consumption of crops) or indirect (consumption by livestock) consumption of C_3 or C_4 foods. Valenzuela et al. found scalp hair $\delta^{13}C$ values of people in the United States were higher in comparison to Europeans due to the increased use of corn and corn-based agricultural products in North America[10] Similarly, Hülsemann et al. reported latitudinal gradients in hair and fingernail $\delta^{13}C$ values, which decreased with increasing distance from the equator; however, they noted that there were other confounding factors as the samples from the United States and Southern Africa had higher $\delta^{13}C$ values than other countries at temperate latitudes.[11] While $\delta^{13}C$ measurements of keratin tissues have been used to broadly differentiate geographic regions, $\delta^{2}H$ and $\delta^{18}O$ have been predominantly used for these studies. Our work has focused on $\delta^{2}H$ measurements because there is (1) positive correlation in $\delta^{2}H$ values of tissues and tap water or precipitation[1,3,12] and (2) predictable variation in tap water or precipitation $\delta^{2}H$ values across regional or continental scales.[13–19] The $\delta^{2}H$ composition of local or regional tap waters that are sourced from rivers, lakes, or groundwater is often significantly correlated with that of local precipitation,[3,20,21] which varies across geographic scales due to environmental factors such as temperature, proximity to the coastline, and elevation.[22,23] Such spatial variation in precipitation $\delta^{2}H$ values has been exploited for forensic studies aimed at determining an individual's region of origin or travel history reconstruction.[4,24–27]

Approximately 27%–30% of the hydrogen atoms in human keratin tissues are derived from ingested water, with the remainder sourced from food.[3,19,20,28] Hydrogen isotope values of foods are also linked to local precipitation because water is the only source of hydrogen available to plants. Irrigation water for crops is largely derived from local sources and is therefore tightly correlated with the $\delta^{2}H$ isoscapes of precipitation.[13,29] Whereas locally grown or raised foods are expected to reflect local or regional $\delta^{2}H$ precipitation values, the industrially processed foods that dominate supermarket shelves are typically not local and may reflect water sources from far away.[18,30] Much of this is underscored by the "supermarket" hypothesis, which claims that the isotopic composition of supermarket foods in developed countries reflects a nonlocal or continental signal that tends to dampen the local geographic variations in $\delta^{2}H$ values of food that otherwise might be contributing to our diets.[31,32]

The potential impact of diet on $\delta^{2}H$ composition has not been well-studied in human populations; however, studies using bulk tissue $\delta^{2}H$ to track avian movement have hypothesized that variation in diet rather than drinking water sources could explain the large degree of variation in $\delta^{2}H$ values among individual birds from the same location.[33] Further research on the impact of dietary inputs of hydrogen in human populations could benefit cases where there is poor correlation between tap water and scalp hair $\delta^{2}H$ values. For example, Juarez et al. found a correlation ($r^2 = 0.34$) between the $\delta^{2}H$ of human hair and local tap water in Mexico, which they attributed to the prevalence of bottled water consumption. While this problem may be exaggerated in Mexico, other studies based in the United States highlight the complexities of tap water systems, including imported versus local tap water and seasonal shifts in water sources (e.g., groundwater vs. reservoir water)[21,34,35] that could confound the relationship between tap water and scalp hair $\delta^{2}H$ values. Overall, a better understanding of how dietary sources of hydrogen influence spatial patterns in the $\delta^{2}H$ values of human tissues could help refine geographic assignment while broadening the potential applications of stable isotope analysis in forensic sciences. Hydrogen isotope analysis of individual compounds (e.g., fatty or amino acids) may provide additional information on both the dietary and tap water inputs that are essential to better constrain region of origin and travel history.

The amino acids (AA) that form the basis of proteins in human hair consist of two types––essential (AA_{ESS}) and nonessential (AA_{NESS})––based on our ability to synthesize them. Humans, like other mammals, are unable to synthesize their own AA_{ESS} and must acquire them directly from their food.[36,37] As a result of direct assimilation (routing), AA_{ESS} undergo minimal isotopic alteration or discrimination between diet and consumer tissue[36,38,39] and by extension provide information about the source(s) of protein consumed by animals. In contrast, AA_{NESS} can be routed directly from dietary proteins or can be synthesized de novo from nonprotein dietary macromolecules such as carbohydrates and lipids.[40,41] While de novo synthesis of AA_{NESS} is possible, direct routing from dietary protein is preferred because it saves the energy otherwise required for the de novo synthesis using carbon derived from carbohydrates or lipids.[42,43]

While $\delta^{2}H$ analysis of individual amino acids may be a valuable approach for studying animal movement and diet,[44] few studies have used this approach to date.[45–47] Fogel et al. used amino acid $\delta^{2}H$ analysis to examine how hydrogen from growth media and environmental waters was incorporated into the bacteria *Escherichia coli*. The protein content of the growth media significantly influenced whether *E. coli* directly routed hydrogen from media or synthesized amino acids de novo. The latter mechanism would result in a greater contribution of hydrogen from environmental water to AA_{NESS} $\delta^{2}H$ values. These results were extended to house mice (*Mus musculus*) by Newsome et al., suggesting that with a single sample it was possible to assess the $\delta^{2}H$ of both drinking water via analysis of AA_{NESS} and food (dietary protein) via analysis of AA_{ESS}.

To explore the utility of amino acid $\delta^{2}H$ analysis of human tissues for region-of-origin studies, we utilized a set of scalp hair and tap water samples collected from mostly rural localities and towns within the contiguous USA. Using a subset of hair samples ($n = 67$) that were analyzed for bulk tissue $\delta^{2}H$ by Ehleringer et al., we examined if patterns in individual amino acid $\delta^{2}H$ values were consistent with latitudinal trends in bulk tissue $\delta^{2}H$ values across the continent. Additionally, we examined if there were specific AA_{ESS} and AA_{NESS}

that incorporated a greater proportion of hydrogen from drinking water than bulk hair tissue. Based on our understanding of amino acid synthesis and incorporation into keratin tissues, we predicted that latitudinal relationships among amino acid δ^2H would persist due to regional drinking water isotope variation across the region of interest. Additionally, AA_{NESS} would be a more faithful proxy for region of origin because de novo amino acid synthesis would utilize hydrogen from the body water pool, which is primarily derived from drinking water in obligate drinkers like humans.[48,49] In contrast, we predicted that δ^2H values of AA_{ESS} were likely to be more variable depending on the consumption of local versus continental sources of food.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Human scalp hair samples were originally collected and described by Ehleringer et al.[3] We analyzed a subset of these samples ($n = 67$) from 28 locations distributed across 13 states in the United States; sample size was limited to one to five individuals per location. Hair samples were collected as discarded trash clippings from the floor or garbage containers in salons or barbershops. Sample collections were anonymous and therefore no information regarding the identification or origin of the individual was recorded. To increase the likelihood that samples were sourced from residents, discarded hair clippings were mostly collected in cities or towns with populations of <100 000.[3] In each of these locations, at least three 25-ml vials of tap water were also collected from at least three different locations within the city or town and the tap water δ^2H data we report for each locality represents the mean δ^2H value of these collections (Table 1).

2.2 | Bulk tissue and tap water analysis

Hair samples were prepared and analyzed for δ^2H at the University of Utah Stable Isotope Ratio Facility for Environmental Research (SIRFER) (http://sirfer.utah.edu); details on sample preparation and analysis are described in Ehleringer et al.[3] Briefly, ~20–40 strands of hair were cleaned via successive washes with a 2:1 chloroform: methanol solvent solution, dried for ~24 h to remove excess solvent,[50] and finally ground into a homogenous fine powder. All hair samples and reference materials, for which the δ^2H of nonexchangeable hydrogen had been previously determined, were analyzed together using the principle of identical treatment.[51] Samples and reference materials were allowed to equilibrate with water vapor in the laboratory atmosphere and desiccated under vacuum for 7 days. Approximately 0.150 mg of each hair sample was weighed into silver capsules. The hydrogen isotope values of hair samples and tap waters were measured using a Thermo Scientific temperature conversion elemental analyzer (TCEA) coupled to a Thermo Scientific Delta Plus XL isotope ratio mass spectrometer (IRMS). For tap water analysis, a 400-µl subsample was placed into a glass autosampler vial. The reported δ^2H values represent the mean of quadruplicate injections (10 µl) into the TCEA IRMS.[3] δ^2H analysis of scalp hair and tap water samples was conducted using the best practices recommended by the Forensic Isotope Ratio Mass Spectrometry (FIRMS) Network.[52]

All δ^2H data represent the δ^2H composition of the nonexchangeable hydrogen in each sample and are reported on the Vienna Standard Mean Ocean Water (VSMOW) scale. Hydrogen isotope data is reported in standard δ notation relative to an international standard in units of per million (‰) using the following: $\delta X = (R_{sample}/R_{standard} - 1) \times 1000$, where X is the isotope of interest, and R_{sample} and $R_{standard}$ are the molar ratios of the heavy to the light isotopes (e.g., $^2H/^1H$) of the sample and international standard, respectively. Hair samples were analyzed using two in-house laboratory reference materials, human hair from Florida, with a nonexchangeable δ^2H value of −76‰, and from Utah, with a nonexchangeable δ^2H value of −142‰, that were calibrated to the VSMOW scale. Raw δ^2H values for unknown hair samples were corrected to the VSMOW scale using a two-point correction with the two reference keratin materials. The average within-run standard deviation of δ^2H values of the reference materials was 4‰.[3] Tap water samples were analyzed alongside a set of two laboratory reference waters previously calibrated to the VSMOW scale. The δ^2H values of the reference materials were −123‰ for deionized water and −0.1‰ for zero enrichment when calibrated against the international standards (GISP, SLAP, and VSMOW). Raw δ^2H values for unknown samples were corrected to the VSMOW scale using a two-point correction with the two reference waters. The average within-run standard deviation of δ^2H values of the water reference materials was 1‰.[3]

2.3 | Amino acid δ^2H analysis

Hair samples were prepared for amino acid δ^2H analysis at the University of New Mexico Center for Stable Isotopes (Albuquerque, NM). Hair samples were cleaned using the same methods described above and then ~3–4 mg of the sample was hydrolyzed to constituent amino acids in 1 ml of 6 N HCl at 110°C for 20 h. Tubes were flushed with N_2 gas before sealing to prevent oxidation during hydrolysis. Amino acids were subsequently derivatized with 2-isopropanol and trifluororacetic acid,[53] and analyzed in triplicate for δ^2H to assess accuracy and precision. Hydrogen isotope measurements were made on a Thermo Scientific Delta Plus IRMS after samples were separated on a 60 m BPX5 column (SGE Analytical Science) in a Thermo Scientific Trace 1,310 gas chromatograph (GC) and underwent thermal decomposition to H_2 in a ceramic reactor set at 1420°C using a Thermo Scientific IsoLink II. Trace 1,310 GC ramping procedure and conditions are reported in Table S1. δ^2H analysis of scalp hair amino acids was conducted using the best practices recommended by the FIRMS Network.[52]

For amino acid δ^2H measurements, we used a mixture of commercially available powdered amino acids (Sigma Aldrich) as a

TABLE 1 Summary table of location, geographic region, sample sizes, and mean (± SD) δ^2H values of bulk scalp hair and tap water samples

Location	Region	Scalp hair mean (± SD) δ^2H, ‰	N	Tap water mean (± SD) δ^2H, ‰	n
Alamosa, CO	Southwest	−108 ± 3.2	3	−111 ± 0.8	3
Alexandria, LA	Southern	−80 ± 2.2	3	−22 ± 3.0	4
Big Spring, TX	Southern	−79 ± 1.6	2	−6 ± 0.6	3
Bryon, IL	Midwest	−90 ± 3.7	3	−52 ± 2.2	3
Casper, WY	Northwest	−112 ± 3.7	3	−114 ± 0.8	5
Chicago, IL	Midwest	−100 ± 5.2	2	−44 ± 0.2	3
Conway, AR	Southern	−84 ± 0.8	2	−20 ± 0.7	3
Cut Bank, MT	Northwest	−119 ± 1.8	2	−132 ± 1.2	3
Daluth, MN	Midwest	−91 ± 7.1	2	−66 ± 3.2	4
Dillon, MT	Northwest	−125	1	−133 ± 0.8	3
Evanston, WY	Northwest	−111 ± 7.1	2	−125 ± 3.3	6
Fort Smith, Ar	Southern	−85	1	−31 ± 1.3	3
Lincoln, NE	Midwest	−95 ± 5.7	3	−58 ± 0.6	3
Lusk, WY	Northwest	−114 ± 0.2	2	−131 ± 1.4	3
Mahomet, IL	Midwest	−95 ± 0.2	2	−42 ± 3.0	3
Monroe, LA	Southern	−85 ± 5.9	3	−17 ± 0.7	3
Monticello, UT	Southwest	−103 ± 2.2	3	−94 ± 1.0	3
Muskogee, OK	Southern	−81	1	−23 ± 1.9	3
Paduach, KY	Midwest	−88 ± 1.6	3	−32 ± 1.9	3
Pecos, TX	Southern	−88 ± 0.9	2	−54 ± 2.0	3
Price, UT	Southwest	−115 ± 3.8	3	−116 ± 2.6	3
Rifle, CO	Southwest	−108 ± 1.7	4	−120 ± .8	3
Roosevelt, UT	Southwest	−119.0	1	−113 ± 3.9	3
Roswell, NM	Southwest	−94 ± 0.3	2	−58 ± 1.2	3
Valentine, NE	Midwest	−103 ± 7.3	5	−80 ± 1.0	3
Vaughn, NM	Southwest	−99.0	1	−78 ± 1.9	3
Vernal, UT	Southwest	−93 ± 0.6	3	−113 ± 2.5	4
Wykoff, MN	Midwest	−103 ± 7.5	3	−63 ± 2.7	5

Note: Colors assigned to geographic region coordinate with assignments used in linear discriminant analyses (Figures 3 and 4).

reference material that were derivatized and analyzed alongside each batch of unknown samples All reference materials and unknown samples were processed and analyzed using the principle of identical treatment, meaning that they were prepared in batches at the same time using the same reagents, and subject to the same derivatization protocol. Hydrogen isotope values for each underivatized amino acid were previously measured[46] (Table S2) using the comparative equilibration method[54] and analyzed using a Thermo Scientific TCEA coupled to a Thermo Scientific Delta V Plus IRMS. Like bulk tissue analysis, results are reported using the standard δ notation and reported using the VSMOW scale. We measured δ^2H values of 11 amino acids with this method, including five considered essential (threonine [Thr], valine [Val], leucine [Leu], isoleucine [Ile], and phenylalanine [Phe]) and six considered nonessential (alanine [Ala], glycine [Gly], serine [Ser], aspartate/asparagine [Asx], glutamate/glutamine [Glx], and proline [Pro]) for most eukaryotes. The average within-run standard deviation of δ^2H values of the in-house amino acid reference material ranged from 2‰ (Phe) to 5‰ (Ser). See Table S2 for more details about the amino acid reference materials.

Fogel et al. developed methods to assess the isotopic impact of exchangeable hydrogen during the amino acid derivatization process and found that this process changed the δ^2H value of most amino acids by only 5‰ on average.[46] Following the principle of identical treatment, the amount of potential hydrogen exchange that occurs at the individual amino acid level in the derivatization process is applied to both the unknown samples and the reference materials. Hydrogen isotope measurements of individual amino acids were calculated from three separate analyses by mass balance with corrections made for hydrogen from reagents (isopropanol and trifluoroacetic acid anhydride) that were added or removed during derivatization[46] for both the reference material and unknown samples. By derivatizing and analyzing reference materials alongside unknown samples, we can calculate the δ^2H value of the intrinsic AA (δXAA_{sample}) using the following equation: $\delta XAA_{sample} = \delta XAA_{dsa} - \delta XAA_{dst} + \delta XAAstd$

$(p_{std})/p_{std}$, where δX is the isotope of interest (δ^2H), δXAA_{dsa} and δXAA_{dst} refer to the derivatized sample and standard respectively, δXAA_{std} refers to the underivatized standard, and p_{std} is equal to the proportion of the hydrogen derivative that was sourced from the amino acid.[37]

2.4 | Statistical analysis

Statistical tests and graphic output were generated using Prism v 9.0 (GraphPad Software) and R.[55] Data were summarized as mean ± standard deviation (SD). Normality of amino acid distributions was assessed using the Shapiro–Wilks test. The relationship between the δ^2H values of tap water and bulk tissue, AA_{ESS}, or AA_{NESS} were analyzed using a linear regression analysis. We used a linear discriminant analysis (LDA) in R package mass v7.3–54.[56] We examined the relationship between δ^2H values of bulk hair and individual AA_{NESS} and AA_{ESS} for samples from the four regions. We used leave-one-out cross-validation to assess whether samples could be assigned to their respective geographic region. Differences were statistically significant at the $p < 0.05$ level.

3 | RESULTS

The paired δ^2H values for tap water and human scalp hair (bulk tissue) are reported by locality in Table 1. The northwest region had the most negative average tap water δ^2H values (−127 ± 7.9‰, mean ± SD) followed by the southwest (−100 ± 22‰), midwest (−55 ± 15.3‰), and southern (−25 ± 14.9‰) regions.

We discovered similar significant correlations between the bulk scalp hair and tap water δ^2H values for the subset of samples as those observed in the larger Ehleringer et al. dataset (Figure 1), with approximately 27% of the hydrogen atoms coming from the tap water (Table S3). Hydrogen isotope values of many AA_{NESS} (e.g., Ala, Glx, and Gly; Figure 2A–C) explained a larger proportion of the variance with the local tap water when compared to the δ^2H values for AA_{ESS} (e.g., Leu, Ile, and Phe; Figure 2D–F). Specifically, the AA_{NESS} Ala, Glx, and Gly δ^2H values reflected the largest estimated contribution of hydrogen atoms as coming from tap water (~42–51%) compared to other AA_{NESS} (Pro, Asx, and Ser) δ^2H values that ranged from 23% to 38% (Table S3). In general, tap water contributed fewer hydrogen atoms to AA_{ESS}, with proportional estimates ranging from 0% to 22% (Table S3). The estimates for the proportion of water derived from tap water for bulk tissue (27%) and individual amino acids align with predictions based on stoichiometry because approximately two-thirds of the amino acids (by weight) in scalp hair are nonessential.[57–59]

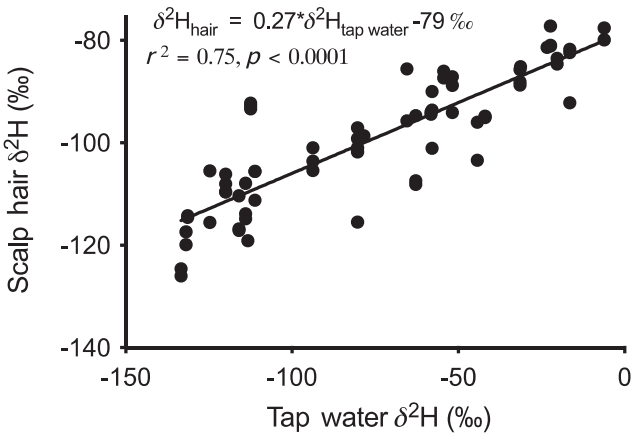

FIGURE 1 Hydrogen isotope values of water and scalp hair samples ($n = 67$) are positively and significantly correlated, with ~27% of hydrogen atoms in hair being derived from local tap water

To examine region-of-origin applications where tap water δ^2H values would be unknown, we excluded tap water δ^2H values from our list of variables in the LDAs. We were interested in assessing how well the LDA would allow us to predict regional group membership among samples. Our first model examined the covariance of δ^2H values for six AA_{NESS} (Ala, Gly, Ser, Asx, Glx, Pro) and bulk scalp hair tissue (Figure 3). We found that most of the variance (87.4%) was explained by linear discriminate one (LD1), which was driven by bulk tissue, Ala, and Pro, while LD2 explained only 6.9% of the variance and was driven by bulk hair tissue, Gly, Glx, and Ser (Figure 3 and Table 2). There was no overlap between samples from the south and northwest or southwest regions, and very little overlap between the midwest and northwest regions. Samples were correctly reclassified 72% of the time with reclassification rates varying by region from a high of 92% (south) to a low of 53% (southwest).

Our second LDA examined the covariance of δ^2H values for five AA_{ESS} (Thr, Val, Leu, Ile, Phe) and bulk scalp hair (Figure 4). We found that the LD1 explained 81% of the variation, which was driven by bulk hair tissue, Thr, Val, Phe, and Ile, while LD2 explained 16.8% of the variation and was driven by bulk hair tissue and Leu (Figure 4 and Table 3). Similar to results for the first model that included data for AA_{NESS}, there was no overlap among samples from the northwest and south or midwest regions, while samples from the southwest region had the highest degree of variance and overlapped to various degrees with the other three regions. Samples were correctly classified 70% of the time with reclassification rates varying by region from a high of 79% (midwest) to a low of 53% (southwest).

4 | DISCUSSION

Here we provide the first evaluation of amino acid δ^2H values of human tissues and show that they vary systematically over a regional gradient in a similar fashion as δ^2H values for bulk scalp hair tissue (Figure 2). Overall, the patterns in our dataset suggest that individuals from rural locations may route both AA_{NESS} and AA_{ESS} directly from local or regional sources of dietary proteins (meat and dairy products) that reflect local/regional precipitation δ^2H patterns. Specifically, we found that there were strong correlations between local tap water values that often serve as a proxy for latitude and mean δ^2H values of both AA_{ESS} and AA_{NESS} (Figure 2). The LDA results for models that

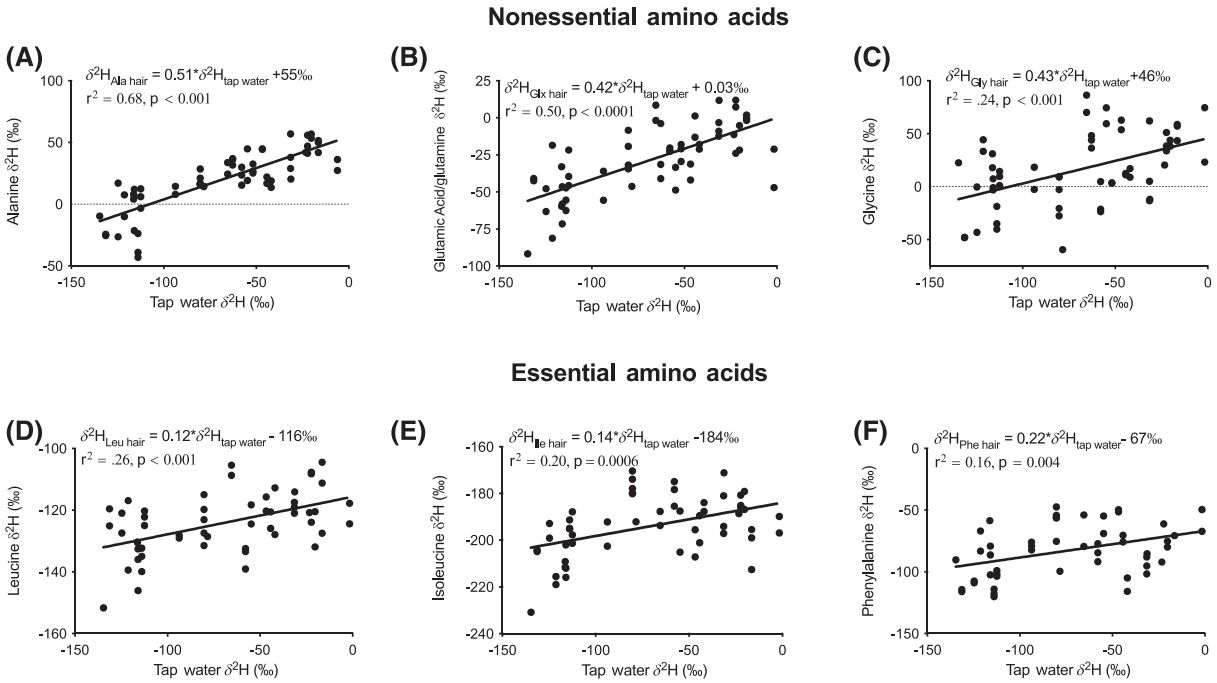

FIGURE 2 The δ^2H values of (A–C) nonessential (alanine, glutamic acid, glycine) and (D–F) essential (leucine, isoleucine, phenylalanine) amino acids are positively and significantly correlated with tap water. Regressions indicate that ~42%–50% and 12%–22% of the hydrogen atoms in AAN_{ESS} and AA_{ESS}, respectively, are derived from tap water

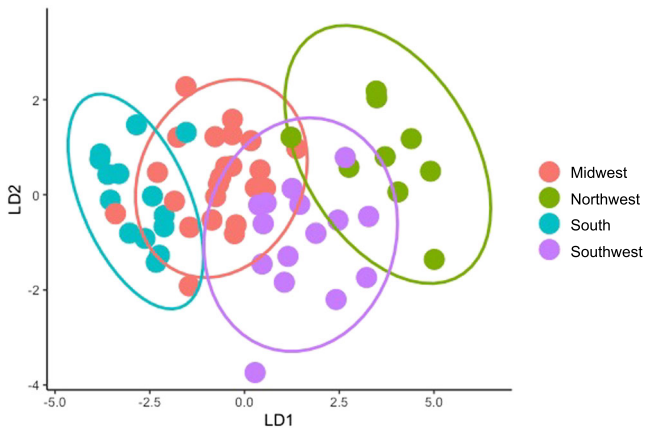

FIGURE 3 Linear discriminant analysis was performed using the δ^2H values of bulk scalp hair tissue and six nonessential amino acids: alanine (Ala), glycine (Gly), serine (Ser), aspartic acid/asparagine (Asx), glutamic acid/glutamine (Glx), and proline (Pro). Solid lines represent 95% confidence intervals. Linear discriminant 1 (LD1) explains 87.4% of the variation among bulk scalp hair and nonessential amino acids, and LD2 explains 6.9%. The overall successful reclassification rate was 72% [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Linear discriminate analysis (LDA) coefficients of linear discriminants of δ^2H values of bulk scalp hair and six nonessential amino acids: alanine (Ala), glycine (Gly), serine (Ser), aspartic acid/asparagine (Asx), glutamic acid/glutamine (Glx), and proline (Pro)

	LD1	LD2	LD3
Bulk hydrogen	−0.07980	−0.05935	0.05069
Ala	−0.05815	0.01662	−0.03204
Gly	−0.00372	−0.03779	−0.00108
Ser	0.00761	0.02267	−0.02775
Asx	0.00083	0.01453	0.02191
Glx	−0.00871	0.02927	−0.01881
Pro	−0.01046	0.00400	0.00269

included either AA_{NESS} or AA_{ESS} data were similar, showing regional differences and no to low amount of overlap in samples from the south versus the northwest or southwest (AA_{NESS}) or between the northwest versus the south or midwest (AA_{ESS}). Lastly, the significant correlations between hair AA_{ESS} δ^2H values and latitude (Figure 2D–F) are also intriguing because some have hypothesized that a supermarket diet would homogenize the isotopic composition of dietary hydrogen inputs, which would result in poor relationships between these amino acids and local tap water. Below, we discuss the influence of tap water on individual amino acid δ^2H values, mechanistic processes that control the isotopic composition of amino acid, and geographic assignment using a compound-specific approach. We also discuss the influence of food on amino acid δ^2H values and its potential impact on geographic assignment models.

We found significant and positive correlations between δ^2H values of both AA_{ESS} and AA_{NESS} and local tap water (Figure 2 and Table S3). Linear regressions showed that some AA_{NESS} (Ala, Gly, and Glx) incorporated approximately 42%–51% of their hydrogen atoms from tap water. When combined with results for Pro, these four

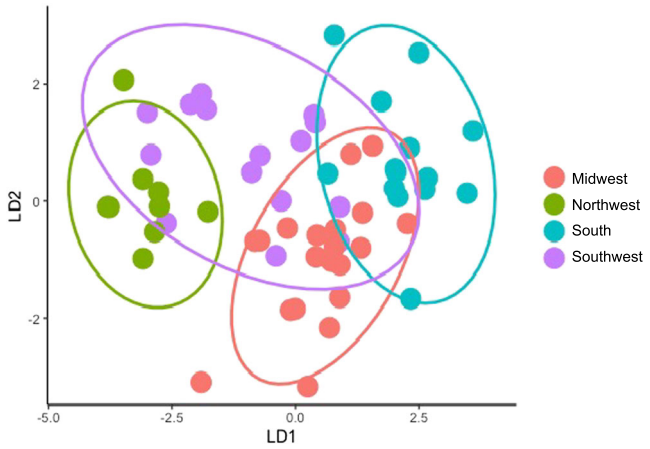

FIGURE 4 Linear discriminant analysis was performed using the δ^2H values of bulk scalp hair tissue and five essential amino acids; threonine (Thr), valine (Val), leucine (Leu), isoleucine (Ile), and phenylalanine (Phe). Solid lines represent 95% confidence intervals. Linear discriminant 1 (LD1) explains 81% of the variation among bulk scalp hair and nonessential amino acids and LD2 explains 16.8%. The overall successful reclassification rate was 70% [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Linear discriminate analysis (LDA) coefficients of linear discriminants of bulk scalp hair and five essential amino acids: threonine (Thr), valine (Val), leucine (Leu), isoleucine (Ile), and phenylalanine (Phe)

	LD1	LD2	LD3
Bulk hydrogen	0.14012	0.07481	−0.00848
Thr	−0.02420	0.01009	0.00208
Val	0.01387	0.01187	0.00484
Leu	0.00892	−0.03870	0.02497
Ile	0.01168	−0.10362	0.02825
Phe	0.01211	−0.01168	−0.03687

AA_{NESS} contributed to the coefficients of linear discriminants of the LDA and the clustering and successful reclassification rates of samples into regional groups (Figure 3). Biochemical considerations predict that a larger proportion of the hydrogen in AA_{NESS} should be sourced from drinking water in comparison to AA_{ESS} for two primary reasons. First, AA_{NESS} can be synthesized de novo from a combination of nonprotein dietary macromolecules (e.g., carbohydrates) and body water, which in humans is primarily derived from tap water.[38,49] Second, AA_{NESS} are structurally simpler than AA_{ESS}, and a larger proportion of the hydrogen atoms in these molecules can freely exchange with body water during metabolism.[9,20,60]

Our findings were consistent with these assumptions and mirrored similar relationships between AA_{NESS} and media or drinking water observed in both bacteria and mice.[45,46] Alanine and Gly are structurally simple AA_{NESS} that can be efficiently synthesized from intermediaries in glycolysis without using ATP. These AA_{NESS} may also be more likely to experience hydrogen exchange with body water because more of the hydrogen atoms are bound to oxygen in the carboxyl and nitrogen in the amine group.[9,20] Specifically, 43% and 60% of the hydrogen in Ala and Gly, respectively, are bound to oxygen or nitrogen and are readily exchangeable with body water at ambient conditions.[61] In contrast, only 23% and 27% of the hydrogen in the more complex AA_{ESS} Leu and Phe is exchangeable with body water at ambient conditions. In comparison to glycolytic AA_{NESS}, Glx more structurally complex and is synthesized from α-ketoglutarate in the tricarboxylic acid (TCA) cycle. The strong correlation between drinking water and Glx δ^2H could be the result of the frequency of trans/de-amination reactions that this metabolically active amino acid experiences as the hub of nitrogen metabolism.[38,45] More importantly the generality in patterns between AA_{NESS} and tap/media water observed among taxa from bacteria to mammals strongly suggests that these patterns are driven by ubiquitous biochemical mechanisms associated with glycolysis and the TCA cycle and as such should be reflected in other organisms.[45,46]

Relative to patterns in AA_{NESS}, we observed a small (~12%–22%) but measurable contribution of hydrogen atoms from tap water to AA_{ESS} in scalp hair that could be the result of several factors. As mentioned above, a lower proportion of the hydrogen atoms in AA_{ESS} are exchangeable with body water relative to AA_{NESS}. Second, AA_{ESS} are more likely to be directly routed with minimal isotopic alteration from food sources because they cannot be synthesized de novo by humans. Note that our dataset is largely derived from rural locations, and it is possible that people living in such contexts may consume more local sources of dietary protein that reflect the δ^2H values of local precipitation. In contrast, urban populations that consume protein from regional or continental sources[15,29,62] available in supermarket chains may have AA_{ESS} δ^2H values that would have a weak or even insignificant relationship with that of local drinking water. To date, no studies have compared amino acid isotope values of individuals living in rural and urban localities, but this analysis could be critical in understanding the impacts of diet on bulk tissues and/or amino acid δ^2H values to assess geographic origins in human populations.

Examination of the δ^2H values of AA_{NESS} and bulk scalp hair using multivariate analysis (LDA) allowed us to visualize and quantify which variables had the greatest influence on observed the regional clustering and allowed us to assess whether samples could be assigned to their respective region with accuracy. Combined, LD1 and LD2 explained 94.3% of the variation in the dataset and plots of these two variables showed the clustering of samples based on geographic region (Figure 3). The overall reclassification rate among our samples using δ^2H values of AA_{NESS} and bulk scalp hair is promising, with 72% of samples being correctly classified. The regional reclassification rates were the highest among the south (92%) and northwest (78%) regions, which was not surprising based on differences in latitude, elevation, and overall geographic distance.[22] Although we had tap water δ^2H data for the different sampling locations, we did not include this in our multivariate analysis as we wanted to assess samples using similar types of information that would be available with human remains of unknown origin where δ^2H data for potential

tap water inputs would be unknown. We predict that if tap water δ^2H values were incorporated into the LDA we would observe similar or even improved regional clustering due to the strong correlation between drinking water and bulk scalp hair δ^2H values.[1,3,20]

The LDA of δ^2H values of AA_{ESS} and bulk scalp hair were nearly as distinct as the model that included AA_{NESS} data, but patterns in regional clustering were slightly different. Samples from the northwest versus south or midwest were distinct, while samples from the southwest showed the greatest among of variation and overlapped with the other three regions to various degrees. It is possible that individuals in the northwest and south or midwest are consuming greater amounts of a local source of proteins (livestock or game meats) and are less reliant on continental (supermarket) sources of protein, which would be consistent with the δ^2H values of their AA_{ESS} and the latitudinal relationships in Figure 2. A second potential explanation is that supermarkets in the northwest obtain protein foodstuffs from a different geographic region than those in the south or midwest. At this time, we can only speculate as to what is driving these patterns as scalp hair samples were collected anonymously and no dietary records are available. Unfortunately, few bulk tissue and no amino acid δ^2H datasets exist for food in the United States, and only a few of them include isotope data for local tap water. Chesson and colleagues have shown significant correlations between ground beef purchased in supermarkets,[32] fast food hamburgers,[62] and milk sampled from dairies[15] with local tap water, suggesting that there may be a stronger regional food component to individual diets.

Most work to date has primarily focused on analyzing tap water and bulk tissue isotope values through regression analyses; however, we have found that using a multivariate analysis of 11 amino acids to examine variation (clustering) among regions and prediction models could provide for a new analytical approach to region-of-origin studies. We have demonstrated that analyses of δ^2H values of bulk scalp hair and its constituent amino acids may provide improved geographic prediction model resolution compared to a single bulk tissue measurement or compound tracer alone. The relationships we observed between the multivariate LDA and regression analyses between tap water and AA_{ESS} δ^2H values highlight the small, but significant impact that food may have on region-of-origin studies. These patterns suggest that certain AA_{ESS} (Ile, Leu, and Phe) appear to be more faithful indicators of diet than tap water compared to the other AA_{ESS} we analyzed. It is possible that dietary sources of hydrogen in rural areas may be influenced by δ^2H of local/regional precipitation to a larger degree than in urban areas where food is not locally grown. Overall, our data suggest that the consumption of local/regional versus continentally sourced foods could playing a larger role in human tissue δ^2H composition than previously assumed; however, further research needs to be conducted on this topic. Essential amino acid δ^2H analysis could be useful for geolocation studies in contexts where tap water is not locally sourced[34,63,64] because these compounds may more faithfully represent regional or local sources of food than hydrogen in bulk tissues.

Our results are promising for forensic applications and geolocation studies, and add a new dimension to prior laboratory-based experiments exploring the relationships between amino acid δ^2H values and the sources of hydrogen available to organisms for tissue synthesis. Specifically, the similarity among patterns in AA_{NESS} δ^2H (Ala, Gly, and Glx) and media/drinking water across taxa (bacteria, house mice, and humans) suggests that similar patters should be present in other species and could be useful for wildlife forensic studies of endangered animals. We acknowledge that due to the anonymous nature of specimen collection, no specific information on the diet composition, physical activity, or recent travel activity was available for the individuals included in this study. Diet, travel, and physical activity may also contribute to the isotopic variation observed here, and future studies should consider collecting these types of data from volunteers to provide enhanced insights on the patterns in amino acid δ^2H values.

DISCLAIMER

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AUTHOR CONTRIBUTIONS

C.J. Mancuso: Conceptualization, data curation, formal analysis, resources, investigation, methodology, project administration, visualization, roles/writing – original draft, writing – review and editing. J.R. Ehleringer: Conceptualization, data curation, resources, methodology, project administration, writing – review and editing. S.D. Newsome: Conceptualization; data curation; formal analysis, resources, methodology, roles/writing – original draft, writing – review and editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Summarized data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

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