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Compound-Specific Isotope Analysis of Human Hair: Predicting Behaviors and Biometrics beyond Dietary Factors

Mayara P. V. Matos and Glen P. Jackson*

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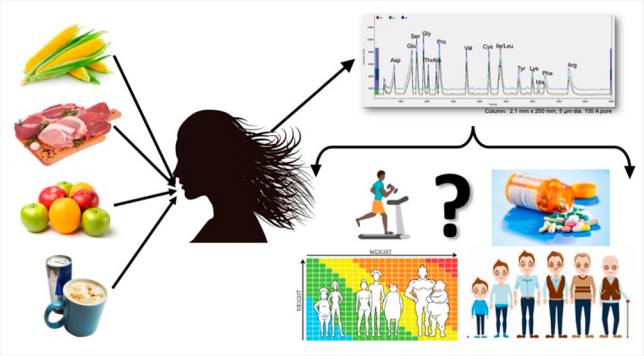
ABSTRACT: This study describes the use of amino acid quantitation and amino-acid-specific isotope ratio analysis of scalp hair of American individuals to predict soft biometric traits about the donors. The scalp hair of each donor was washed, dried, homogenized and acid hydrolyzed before analysis using liquid chromatography-isotope ratio mass spectrometry (LC-IRMS). A variety of statistical tests examined the relationships between the amino acid variables and biometric questionnaire responses, the latter of which could be assessed as continuous variables—in the case of age or body mass index (BMI)—or as categorical variables in the case of sex and alcohol consumption. Correlations between biometric factors and amino acid $\delta^{13}C$ values were more significant after controlling for the extent of ^{13}C in the subjects' diets. Multivariate analysis revealed that the sex of a donor could be correctly predicted with cross-validated accuracies of 80% using the isotope ratios and 89% using amino acid quantities. Using amino acid $\delta^{13}C$ values or quantities, the age of a subject could only be predicted with an accuracy of ±27 years (95% CI). Hair treatments, such as chemical straitening and dying, did not have any measurable effect on the isotope ratios or quantities of amino acids in the hair. Unexpectedly, the $\delta^{13}C$ values of sulfur-containing amino acids were significantly different between teetotalers and subjects who consumed alcohol daily. Further refinement of this study, including larger cohorts with controlled behavioral states or genetic factors, could provide helpful investigative leads in forensic casework.



Isotope ratio mass spectrometry (IRMS) is a useful tool in ecology[1] and for the investigation of human origins in forensic, archeological, and anthropological studies.[2−4] Most studies rely on dietary factors as the primary variable influencing the isotope ratios, but metabolic fractionation of isotope ratios has provided a source of considerable interference when interpreting stable isotope results.[5−8] Few studies have investigated whether or not isotopes are affected by some underlying biometric traits, such as sex. The goal of the present work is to provide insight into some of the ways in which metabolic differences between hair donors are reflected in the quantity or isotope ratio of amino acids in their hair. Hair is a reliable investigative material because it is robust to diagenetic changes, it is easy and noninvasive to collect, it has a quick growth cycle, and it records a chronological relationship between the time of hair growth and the distance from the root.[9−13]

Hair is mainly composed by keratin, a family of proteins that accounts for 90−95% of hair dry mass.[14] Although the AA composition of hair contains known variance,[15] cysteine, serine, glutamic acid, threonine, glycine, and leucine represent approximately 19%, 13%, 12%, 8%, and 7% of the composition of hair shaft proteins, respectively.[13] Carbon and nitrogen isotope values for hair keratin strongly relate to diet, but because the lighter isotopes are preferentially excreted during metabolism, hair is typically enriched by 1−2 ‰ and 2−3 ‰, respectively, relative to one's diet.[16−19] Human scalp hair grows at an approximate rate of 0.8 to 1.3 cm/month,[20] and each hair follicle undergoes a cycle of growing and resting stages named anagen, catagen, and telogen, as described elsewhere.[13,21,22] Hair growth and AA composition can be influenced by the isotope ratios or concentration of the AAs supplied by the circulatory blood.[23] Essential AAs can be used as a proxy to infer human dietary sources, and it is widely acknowledged that, as a general rule, the isotope ratios of nonessential amino acids follow the bulk isotope ratios of the diet.

Bulk measurements of tissues provide straightforward and robust isotope results, but the interpretation of its data is affected by the type of tissue, the physiological state of the organism, and isotopic composition of the diet.[24] Bulk IRMS is typically constrained to a handful of light elements, so it has

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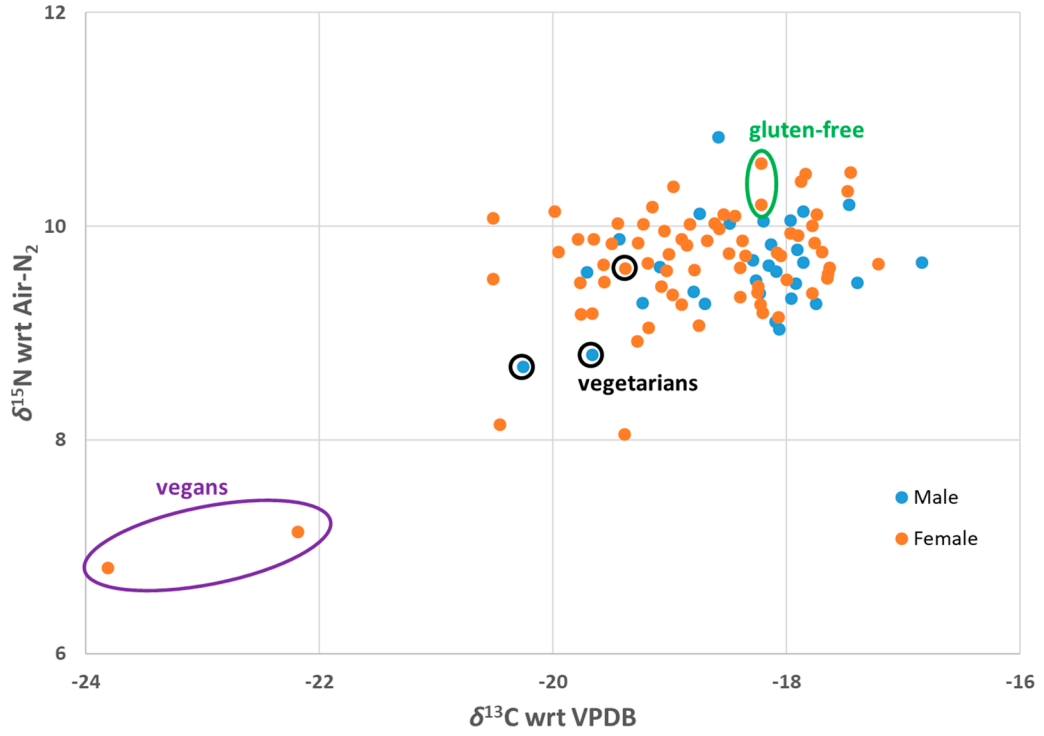

Figure 1. Carbon and nitrogen bulk isotope measurements (EA-IRMS) of 101 subjects. Circled areas identify vegan, vegetarian, and gluten-free dietary restrictions.

limited utility when studying organisms that constantly change resources and habitat,[25] such as humans. Also, the intra-individual variability in hair growth rate and physiological variances in hair growth phases affect the recorded isotopic information in scalp hair. The variance of hair growth rates must be taken into consideration when interpreting results, especially when working with segmental hair analysis.[22,26] However, the ability to analyze isotopes at the molecular level, such as through compound-specific isotope analysis (CSIA), can provide a deeper understanding of metabolism and fractionation processes.[27]

Gas-chromatography combustion isotope ratio mass spectrometry (GC/C-IRMS) is the most commonly used separation method for CSIA of AAs, but CSIA requires the AAs to be derivatized before analysis. Derivatization introduces exogenous carbons to the AA skeleton, so significant attention is required to adjust the $\delta^{13}C$ values of each AA and reduce experimental variance.[28,29] The separation of some coeluting AAs, and the recovery of glutamine (Gln) and glutamic acid (Glu), were recently reported as positive aspects of the NAIP derivatization method.[30] However, our study employs the alternative and well-stablished liquid chromatography-IRMS (LC-IRMS) method to perform CSIA of underivatized AAs from scalp hair,[31−33] which does not require any corrections caused by derivatization.

Obtaining information about individuals from their hair is of great interest to different research areas. This study explored the combination of bulk isotope analysis, compound-specific isotope analysis, and amino acid composition of human hair to predict soft biometric traits in a cohort of 101 American individuals. Briefly, diet heavily influenced the ^{13}C and ^{15}N content of the hair, especially for vegans, but the sex of the hair donors could still be predicted with better than 80% accuracy using either isotope ratio measurements or amino acid quantities. These results show that human hair shafts store a record of human behaviors and traits as a result of metabolic and degradation differences between humans, including those caused by age, alcohol, and sunlight exposure. In contrast, hair treatments did not influence the isotope ratio measurements and had a negligible effect on the quantities of amino acids.

EXPERIMENTAL SECTION

Study Participants. The recruitment of volunteers was approved by WVU Institutional Review Board (no. 1702466936R001). Volunteers represented 22 states within the continental United States. Individuals were asked to provide a minimum of 0.1 g of hair (e.g., >30 hairs of 24″ length or >60 hairs of 12″ length) cut as close as possible to the scalp. Pregnant, cognitively impaired, or incarcerated subjects were not included in this study. A detailed description about the recruitment of participants and sample collection is given in Supporting Information (SI).

Sample Preparation and Instrumental Analysis. Bulk and compound-specific isotope ratio measurements were performed on cleaned, pulverized hair samples from random portions of the provided hair samples, as described in Supporting Information. Pulverized samples are more homogeneous, easier to weigh precisely in small aliquots and faster to digest, which has been shown to lead to more reproducible results.[2] For bulk analysis, samples of ∼0.5 mg were weighed in tin capsules. For CSIA, ∼2 mg of each pulverized hair sample was hydrolyzed in 6 M HCl for 24 h at 110 °C. AA chromatographic separation was performed in a Primesep A mixed-mode column before CSIA. All isotopes were measured relative to the respective reference gas (CO_2 or N_2) and expressed in the international scale against VPDB or air, respectively, using a two-point calibration curve based on international standard reference materials. For CSIA measure-

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Anal. Chem. 2020, 92, 3014−3022

ments, one replicate was used for each subject, which is expected to provide conservative estimates of model correlations and prediction accuracies. Although the use of one replicate per subject is justified by the independence of each measurement within a grouping factor, such as sex, where n = 25 for males and n = 57 for females, the models built on single replicates include instrumental variance. Future work that uses the average of multiple replicates within each individual will decrease the effects of instrument variance and provide results that are at least as successful as the present study, if not superior. Further information on sample treatments and data analysis is given in SI.

Data Analysis. Isodat 3.0 was used as the data acquisition software, and IBM SPSS Statistics 25 was used for the statistical analyses. Information collected in the questionnaires provided by each subject allowed us to group individuals based on different biometric factors. The assumptions of normality, linearity, univariate and multivariate outliers, homogeneity of variance−covariance matrices, homoscedascity, and multicollinearity were tested prior to each statistical analysis and defined which test should be performed. Further information can be found in SI.

RESULTS AND DISCUSSION

Bulk Isotope Analysis (EA-IRMS). The hair of 101 volunteers from the US (30 males and 71 females) underwent $\delta^{13}C$ and $\delta^{15}N$ bulk isotope measurements. Some of these individuals self-reported dietary restrictions, such as vegan, ovo-lacto-vegetarian, pescatarian, paleo, or gluten-free diets. From these diet categories, the vegans ($\delta^{13}C$ = −23.00 ‰, $\delta^{15}N$ = 6.97 ‰) clustered separately from the omnivorous population ($\delta^{13}C$ = −18.61 ‰, $\delta^{15}N$ = 9.67 ‰). Self-reported vegetarians gave average isotope values of −19.53 ‰ for carbon and 9.17 ‰ for nitrogen, which fell between those of vegans and omnivorous subjects, but not distinct enough to discriminate (Figure 1). The hair of ovo-lacto-vegetarians is expected to be isotopically indistinguishable from the hair of omnivores because the consumption of any animal-derived protein other than meat, such as eggs, cheese, and milk, still reflects the isotopic signature of animal protein.[17,34,35] The same isotope signatures are observed with $\delta^{15}N$ values, which increased from the hair of vegans to omnivores[35−37] due to the correlation with meat or marine fish dietary intake.[38,39] True vegans had carbon and nitrogen values very distinct from the other groups, which is in accordance with the literature.[17,40,41] We note that we do not know the length of time that each individual has maintained their dietary lifestyle. Because entire lengths of hair within a donor were homogenized before hydrolysis, it is possible that the isotope signatures of the claimed dietary behavior are partially diluted with hair that was grown under a previous dietary behavior. Still, these imperfections are expected to be insignificant relative to the weighted contribution of hair protein grown with the claimed dietary habit.

The female subjects (n = 71) had average bulk isotope values of $\delta^{13}C$ = −18.84 ± 0.25 ‰ and $\delta^{15}N$ = 9.62 ± 0.15 ‰ (95% confidence interval (CI) of the mean). After omitting the two female vegan subjects, the remaining females (n = 69) had a bulk average of values of $\delta^{13}C$ = −18.72 ± 0.19 ‰ and $\delta^{15}N$ = 9.70 ± 0.11 ‰ (95% CI). Relative to the females, the hair of the male subjects (n = 30) was significantly enriched in ^{13}C (mean $\delta^{13}C$ = −18.37 ± 0.28 ‰, Student's t test, P = 0.045) but not significantly different in nitrogen (Student's t test, P = 0.38). These values are also expressed as mean ±95% CI of the mean. The results for carbon are consistent with those of a German cohort by Petzke et al.,[36] but their data also found the nitrogen isotopes of females to be more depleted than the males.

$\delta^{13}C$ CSIA of Amino Acids in Hair. This study included the LC-IRMS analysis of a total of 101 subjects' hair. Nineteen samples could not be used because of irregular, small, or coeluting peaks of one or more AAs. Of the remaining 82 samples, the following nine AAs were baseline resolved: aspartic acid/asparagine (Asx), serine (Ser), glycine (Gly), valine (Val), tyrosine (Tyr), lysine (Lys), histidine (His), phenylalanine (Phe), and arginine (Arg). Methionine/cystine (Met/Cyt) and isoleucine/leucine (Xle) were measured as coeluting pairs to provide a total of 11 variables from 14 AAs. The AAs glutamic acid/glutamine (Glx), threonine (Thr), alanine (Ala), and proline (Pro) have been used in previous studies,[33] but they were not used here because they either had coelution issues or were of insufficient abundance.

Quantitation was accomplished by first assuming complete oxidation in the Isolink interface. The relative peak areas of the CO_2 (m/z 44) of each AA were thereby converted to the relative moles of carbon for each AA and, using the known elemental composition of each AA, to the relative moles of each AA in the original hair sample. Methionine oxidation could have occurred in these samples because O_2 was not removed from the vials before hydrolysis,[33] so it is likely that the majority of the Met/Cyt peak is composed of Cyt. The quantitative analysis of AA composition of hair worked as a second set of variables for classification and correlation analyses.

Of the 82 samples that were deemed acceptable in quality, 25 were males and 57 were females. One 11-year-old boy had very significantly different $\delta^{13}C$ values for Phe in his hair relative to the other subjects. This subject's Phe $\delta^{13}C$ value of −37.5 ‰ was more than five standard deviations away from the mean value of −26.0 ‰. The $\delta^{13}C$ values of his remaining amino acids were in the normal range. This subject's parent reported that the boy has autism and was taking risperidone in addition to several prescription allergy medications. Although the subject did not provide specific formulation details, four of the five listed medications have optional formulations that contain aspartame, which is approximately 50% Phe by mass. One medication also contains 0.25% by mass of 2-phenylethanol, which is readily metabolized to Phe. These exogenous sources of Phe are likely to be a small fraction of the typical Phe content of a normal diet, so are unlikely to cause such depleted $\delta^{13}C$ values. However, the only other explanation for the unusual $\delta^{13}C$ value of Phe is the possibility that the subject might have an undiagnosed metabolic disorder such as phenylketonuria, which is known to be linked with developmental disorders such as autism.[42−44] Because of the unusual nature of the Phe $\delta^{13}C$ value of this subject, this subject's CSIA results were excluded from the subsequent analyses.

In the present study, the essential AAs were typically more depleted than the bulk $\delta^{13}C$ values of hair, consistent with previous findings in other cohorts.[18] In contrast, the nonessential AAs were generally more enriched than the bulk $\delta^{13}C$ values (except for Tyr, Table 1). Phe, Val, and Xle were the most depleted AAs in ^{13}C, probably because the plant enzymes involved in the biosynthesis of these essential AAs selectively enrich the ^{12}C isotope.[16,45] Mammals cannot

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Table 1. Stable Isotope Signatures of Bulk Carbon and Essential and Nonessential AAs in Human Hair[a]

bulk $\delta^{13}C$ (‰)	$\delta^{13}C$ essential amino acids (‰)		$\delta^{13}C$ nonessential amino acids (‰)	
−18.68 ± 0.23	Val	−23.82 ± 0.33	Asx	−16.40 ± 0.29
	Xle	−24.14 ± 0.28	Ser	−9.46 ± 0.34
	Lys	−17.59 ± 0.55	Gly	−11.18 ± 0.41
	His	−10.78 ± 0.81	Tyr	−23.02 ± 0.31
	Phe	−25.93 ± 0.49	Arg	−18.11 ± 0.32

[a]Values are expressed as mean ± 95% CI of the mean. n = 81.

synthesize these three AAs, so the isotopic imprint from the diet is reflected in the negligible fractionation from their ingested isotope values.[36]

Correlation analysis revealed significant positive correlations among the $\delta^{13}C$ values of different AAs (Table S1), which were as high as r = 0.707 (P < 0.001) between Val and Arg. Some of the strong correlations, such as for Val and Arg, have unidentified causes because they do not possess simple or direct metabolic relationships. For the AA quantities, correlations cannot always be positive because the quantities are normalized relative to the sum of all the quantified AAs. Ile, Leu, and Val are essential branched-chain amino acids (BCAAs) that share catabolic pathways,[46] so the strong correlation between these AAs (r = 0.826 for Xle and Val, P < 0.001) is understandable (Table S2).

Through one-way analysis of variance (ANOVA) and correlation analyses, several characteristic traits and biometric factors were found to contribute to the variance in the relative quantities and $\delta^{13}C$ values of AAs in hair. Among the factors influencing $\delta^{13}C$ values of AAs in hair, diet contributed the largest variance. Of the factors influencing relative quantities of AAs in hair, sex had one of the largest effects. Unless one accounts for the major influences of those effects, the smaller effects of other biometric factors, such as age, would be difficult to resolve. For these reasons, the quantities and $\delta^{13}C$ values were controlled for the effect of sex and diet, respectively, before assessing other factors. Among the different ways to control the AA $\delta^{13}C$ values for diet, the use of separate bulk $\delta^{13}C$ measurements of the same hair samples provided the simplest and most objective approach. In this approach, a subject's bulk $\delta^{13}C$ value was used to directly control for the $\delta^{13}C$ value of each AA, and in almost all cases controlling for diet helped improve the statistical significance of nondietary factors on $\delta^{13}C$ values.

Predicting Sex. In the original dataset, there was a small, but significant, correlation between sex and the $\delta^{13}C$ values of Gly (r = 0.355, P = 0.001), Val (r = 0.229, P = 0.038), and His (r = 0.232, P = 0.036) (Table S3). After controlling for diet, the correlations between AA $\delta^{13}C$ values and sex improved for Gly (r = 0.468, P < 0.001), Val (r = 0.457, P < 0.001), and His (r = 0.287, P = 0.009) and became significant for Ser and Arg (r = 0.338, P = 0.002 and r = 0.419, P < 0.001, respectively).

One-way between-groups multivariate analysis of variance (MANOVA) was used to assess whether or not males and females differ in their $\delta^{13}C$ values of AAs. The independent variable was sex and the dependent variables were the $\delta^{13}C$ values. No serious violations were noted when testing the validity of the test assumptions (e.g., normality). Even without controlling for diet, a linear combination of the 11 AA's carbon isotope values indicated a statistically significant difference between males and females (P < 0.001), where the sex of a donor explained 36% of the variance in the linear combination of terms. Table S4 shows that the $\delta^{13}C$ values of Gly (P = 0.001), Val (P = 0.038), and His (P = 0.036) were significantly different between males and females. The $\delta^{13}C$ value of Gly showed the most significant difference, with 13% of its variance explained by sex.

After controlling for the effect of diet using bulk $\delta^{13}C$ measurements, the sex of the donor explained 41% of the variance in the linear combination of $\delta^{13}C$ values (P < 0.001). The $\delta^{13}C$ values of Ser, Gly, Val, His, and Arg were statistically significant different between males and females at α = 0.05 (Table S5). To reduce the chance of type 1 errors, a Bonferroni adjustment (i.e., α = 0.05 divided by the number of dependent variables analyzed) was used to provide an alpha level of 4.5 × 10^{-3} as a threshold for significance.[47] The $\delta^{13}C$ values of Ser (P = 0.002), Gly (P < 0.001), Arg (P < 0.001), and Val (P < 0.001) were still significantly different between males and females (Table S5). Gly and Val had the largest effect size for sex[48] (partial eta squared η^2 = 0.219 and 0.209, respectively), with 22% and 21% of their variance explained by sex, respectively (Table S5 and Figure S1). In simple terms, partial eta square is a type of estimate used to describe how much variance in the dependent variable (e.g., $\delta^{13}C$ value) can be attributable to the independent variable (e.g., sex). The difference in the mean $\delta^{13}C$ values was between 1 and 2 ‰,

A

Classification Results[a,c]

		Sex	Predicted Group Membership 1	2	Total
Original	Count	1	14	11	25
		2	3	53	56
	%	1	56.0	44.0	100.0
		2	5.4	94.6	100.0
Cross-validated[b]	Count	1	13	12	25
		2	4	52	56
	%	1	52.0	48.0	100.0
		2	7.1	92.9	100.0

a. 82.7% of original grouped cases correctly classified.

b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

c. 80.2% of cross-validated grouped cases correctly classified.

B

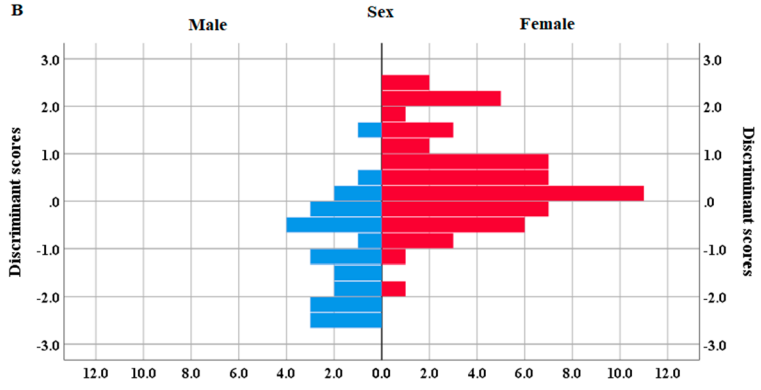

Figure 2. (A) Classification results from LDA of 81 hair samples (25 males, 56 females) according to sex. The variables were $\delta^{13}C$ values of Ser, Gly, Val, His, and Arg controlled for the effect of diet. LOOCV accuracy for predicting sex is 80%. (B) Frequency determination plot based on the discriminant scores for the LDA discriminant function.

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Anal. Chem. 2020, 92, 3014−3022

A

Classification Results[a,c]

		Sex	Predicted Group Membership 1	2	Total
Original	Count	1	20	5	25
		2	4	53	57
	%	1	80.0	20.0	100.0
		2	7.0	93.0	100.0
Cross-validated[b]	Count	1	20	5	25
		2	4	53	57
	%	1	80.0	20.0	100.0
		2	7.0	93.0	100.0

a. 89.0% of original grouped cases correctly classified.

b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

c. 89.0% of cross-validated grouped cases correctly classified.

B

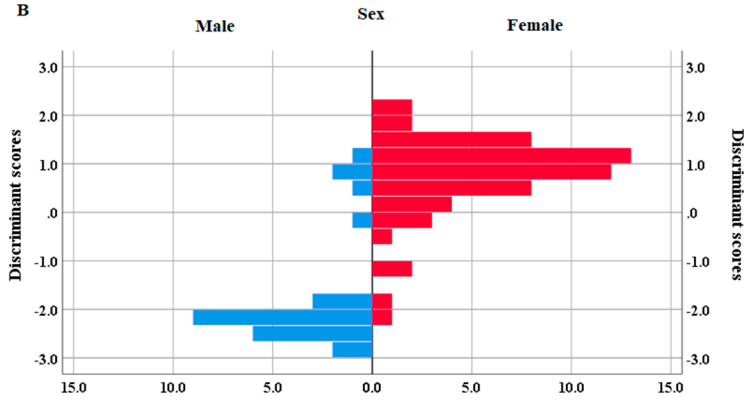

Figure 3. (A) Classification results from LDA of 81 hair samples (25 males, 57 females) according to sex. The variables were relative quantities of five AAs; Val_q, Xle_q, Tyr_q, and Lys_q. LOOCV accuracy for predicting sex is 89%. (B) Frequency determination plot based on the discriminant scores for the LDA discriminant function.

where males were more depleted in ^{13}C than females in every statistically significant AA (Table S6).

Linear discriminant analysis (LDA) helped classify individuals into males and females. Using a classification based on the LDA estimates (original rules), in which every subject is included in the generation of the classification algorithm, 81.5% of the subjects were estimated into the correct sex when using the $\delta^{13}C$ values of all 11 AA variables controlled for bulk diet. Because LDA is a supervised classifier, the approach was validated using leave-one-out cross validation (LOOCV) and obtained ~78% prediction accuracy using the same 11 variables (Table S7). As expected from the MANOVA results, Gly, Arg, and Val showed the largest discriminant function coefficients and were most useful in the classification (Table S8). Figure 2 shows that when using the five most significant AAs, the classification rate improves to 83% based on the original rules and 80% based on LOOCV.

Studies involving sex and stable isotopes are often related to dietary differences linked to social identity status in the organization of ancient societies.[49−52] The limited publications in this field suggest that sex differences do not interfere with the bulk isotope values.[12,53,54] In contrast to our findings above, Petzke et al.[36] reported no significant differences in the $\delta^{13}C$ and $\delta^{15}N$ values of individual AAs from hair of a large cohort of German subjects. Regarding the significant effect of Gly in our work, Morishita et al.[55] suggested that, in female rats, Gly is involved in the neural regulation of luteinizing hormone (LH) excretion. This hormone is directly related to male and female sex hormones, working in biochemical pathways that lead to the production and release of estrogen and testosterone. Considering that mammals share similar metabolic pathways, males could have a higher demand of Gly to regulate the excretion of LH, which could lead to the observed isotopic differences between males and females.

The relationships between AA quantities and the sex of a subject was also investigated. When referring to the relative abundance in the moles of AAs, the subscript letter "q" was included after each variable name to differentiate the relative quantities from the $\delta^{13}C$ values. The variable Met/Cyt_q was removed from multivariate classification algorithms because it violated several assumptions regarding multicollinearity. The relative quantities of Val_q and Xle_q were more abundant in females than males, whereas Tyr_q and Lys_q were more abundant in males than females (Figure S2). Val_q had the largest size effect ($\eta^2 = 0.501$), with approximately 50% of its variance explained by sex. In a previous study employing derivatization GC-MS to quantify the AAs in the hair of 64 Jordanian subjects, Rashaid et al.[56] showed that Val was also more abundant in the hair of females and that Try and Lys were more abundant in the hair of males.

LDA correctly estimated and predicted the sex of the subjects with 94% accuracy based on both the original rules and with LOOCV, respectively (Table S10). When limiting LDA to only the statistically significant variables from MANOVA (i.e., Val_q, Ile/Leu_q, Tyr_q, and Lys_q), the sex of the subjects could still be predicted with 89% accuracy (original rules and LOOCV) (Figure 3). This latter result suggests that the quantities of four amino acids are the dominant biochemical differences between the hair shaft composition of males and females.

The literature on sex as an influencing factor in the composition of AAs in human hair is rather convoluted. The FTIR spectra of hair from males and females is not significantly different in secondary structure (α-helix and β-sheet).[57] However, some authors have found higher Cys_q and Cyt_q content in the scalp hair of males than females[56,58] and others have observed no relationship between these AAs and the sex of the hair donors.[59] Others have shown male hair to have a higher concentration of Lys and Thr and lower concentration of Phe relative to female hair.[56] Sex has been shown to have a significant effect on the whole-body leucine oxidation in muscle mass during exercise, where males have greater muscle protein synthesis and oxidize more leucine than females.[60−62] Physical activity and different responses to fat and carbohydrate availabilities during endurance exercise might play a role on sexual dysmorphism.[60] Sex hormones also affect muscle protein metabolism,[63] although some have reported that muscle protein synthesis pathways are independent of sex.[64] Clearly, more research is necessary to provide a stronger biochemical understanding for the measurable differences in fractionation and incorporation of certain AAs in human hair.

Predicting Age or Age Group. The relationship between the 11 $\delta^{13}C$ variables and age was assessed with age as an ordinal variable, by grouping individuals into three arbitrary categories (≤25, 26−45, and ≥46 years old), or as a continuous variable. The only significant correlation found in the original (uncontrolled for any effects) data set was a small negative correlation between age and the $\delta^{13}C$ value of Lys ($r = -0.26$, $P = 0.02$). After controlling the 11 $\delta^{13}C$ values for diet, the correlation between the $\delta^{13}C$ value of Lys and age

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increased in magnitude to $r = -0.31$ ($P = 0.004$) (Table S12). After controlling for diet, the correlation between age and the $\delta^{13}C$ value of Xle became significant ($r = 0.25$, $P = 0.026$). Controlling for sex in addition to diet did not improve the significance of the results relative to the data controlled only for diet.

A multivariate general linear regression model (GLM) was used to predict age as a continuous dependent variable using the $\delta^{13}C$ values of AAs as multiple independent variables. The model with all the $\delta^{13}C$ variables explained approximately 26% of the variance in age ($P = 0.023$). Controlling for sex slightly improved the model, which then explained 28% of the variance ($R^2 = 0.28$, $P = 0.013$). A second, simpler model using only five variables (Asp, Val, Xle, Lys, and Arg) still explained 25% of the variance in age ($R^2 = 0.25$, $P < 0.001$), as shown in Figure S5. Lys was the strongest contributing AA (beta = −0.46, $P < 0.001$) at $\alpha = 0.05$, while Asx provided the second biggest contribution (beta = −0.21, $P = 0.053$). For the GLM model using five isotope variables, the standard deviation of the residual error of 81 age predictions was 13.5 years and the 95% CI of a predicted age was 27 years. This model does not have the precision required to be useful as an investigative lead, but the model is sufficient to discriminate between the hair of individuals that are different in age by more than 27 years.

The Kruskal−Wallis test was used to account for the lack of normality and homogeneity of samples when age was separated into categories. This test revealed a statistically significant difference in the $\delta^{13}C$ values of Xle median ranks across the three age groups (≤25, $n = 38$; 26−45, $n = 28$; ≥ 46 years old, $n = 16$; $P = 0.011$). Dunn's post hoc test followed by a Bonferroni adjustment revealed that the $\delta^{13}C$ value of Xle was statistically distinct between the age group 26−45 and ≥46 years old ($P = 0.008$). The ≥46 years-old age group gave a more enriched median $\delta^{13}C$ value of Xle (−23.36 ‰) relative to the median of the 26−45-year-old group (−24.18 ‰). The other age groups were not different after the alpha adjustment. When working with the dataset controlled for diet, a Kruskal−Wallis test confirmed the result, showing $\delta^{13}C$ values of Xle as the only statistically different AA across the three age groups ($P = 0.013$). Pairwise comparisons once again showed the difference was between the age group 26−45 and ≥46 years old ($P = 0.010$), with the same pattern of ^{13}C enrichment for the older group (−23.81 ‰) compared to the younger age group (−24.29 ‰).

Using only six of the $\delta^{13}C$ variables from the original dataset, 57% of the cases were correctly classified into the three age groups mentioned above based on the LDA estimates (original rules) and 52% after LOOCV. Using the data controlled for diet and sex and only three $\delta^{13}C$ variables (Met/Cyt, Xle and Lys), LDA predicted 55% correct classification based on the LDA estimates and 50% after LOOCV (Figure S3). Even though this classification rate exceeds guessing, which would have a classification rate of ∼33%, the prediction of age group using the $\delta^{13}C$ values of AAs in hair is currently not sufficiently reliable to serve as an investigative lead.

Associations between age and isotopic signature of mammalian tissues are found in a few studies that relied on bulk isotope measurements. The analysis of archeological human bones with ages ranging from newborn to around 60 years old indicated no effect of age on bulk $\delta^{13}C$ values.[65] Similar results were found when testing bulk carbon and bulk nitrogen values of Japanese hair.[53] In the few cases where an age effect on the isotope signatures of human samples was found, the interpretation was based on a correlation between age and dietary habits.[66,67] Jackson et al.[33] reported the first attempt to classify individuals into age groups using compound-specific isotope analysis of AAs in a small dataset of hair donors ($n = 20$). Their classification rate was 100% using a biased approach of a small dataset (in which samples in the database were also predicted), but only reached 25% accuracy using LOOCV, which was not better than guessing. The small dataset (20 samples) used in that study was apparently insufficient to draw any meaningful conclusions.

The current work also examined the relationship between age and the quantities of AAs. Significant correlations appeared between age as a continuous variable and Ser_q ($r = 0.274$, $P = 0.013$) and Gly_q ($r = 0.306$, $P = 0.005$). Significant correlations and anticorrelations also appeared with the arbitrarily binned age categories for Asx_q ($r = -0.226$, $P = 0.041$), Ser_q ($r = 0.249$, $P = 0.024$), Gly_q ($r = 0.237$, $P = 0.032$), and Xle_q ($r = -0.231$, $P = 0.037$). Given that sex has a measurable effect on the relative quantities of the AAs, the models to predict age from AA quantities were examined before and after controlling for sex. In casework samples, the ground truth for the sex of a donor may be unknown, so controlling for sex may not be a viable option. However, in applications where the sex of a donor is known, such as a mutilated victim, such controls would be feasible.

Prior to testing the 11 quantities of AAs, Met/Cyt_q was again removed from the analysis because it violated some of the test assumptions. The general linear regression model with the relative mols of 10 AA variables explained approximately 27% of the variance in age ($R^2 = 0.267$, $P = 0.010$). A simpler model using the five variables with the greatest effects (Asx_q, Ser_q, Gly_q, Xle_q, and Tyr_q) still explained 22% of the variance in age ($R^2 = 0.221$, $P = 0.002$). Tyr_q provided the largest unique contribution to the model (beta = 0.358, $P = 0.009$) at $\alpha = 0.05$. Using five variables, the mean absolute error in the predicted age of an individual was 13.8 years (Figure S6). The model was more inaccurate for subjects older than 40 years old. Attempts to classify individuals into age groups using amino acid quantities were unsuccessful (see details in SI).

Rieck reported age-associated differences in the scalp hair of three sisters between 1 and 24 years old.[14] Among other AAs, Ser concentrations increased with age whereas Asp and Gly decreased with age. The differences were explained based on hormonal changes during puberty. The same trend was observed in the present study for Asx and Ser, but not for Gly, which was slightly more concentrated in the older group. In another study, Ala, Gly, Ile, and Asp were more concentrated in subjects younger than 49 years old compared to those older than 49 years.[56] The cohort presented in our study is four times larger than the previous work and also has subjects covering a wider range of ages. Our age-dependent concentration differences were always for nonessential or conditionally essential AAs between the youngest and oldest groups, which seems to suggest metabolic or hormonal changes with age.

Among other physiological functions, BCAAs (especially leucine) are directly responsible for the stimulation of muscle protein synthesis and the reduction of protein degradation.[68,69] During sarcopenia (progressive age-dependent loss of muscle mass), aging skeletal muscle might become less sensitive to the low physiological concentrations of BCAAs.[70,71] One hypothesis to explain some of our results is that the slower recruitment of leucine to the skeleton muscle of older

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Anal. Chem. 2020, 92, 3014−3022

individuals could be preferential toward the ^{12}C, therefore leaving a more-enriched leucine carbon signature in the hair of older subjects.

Predicting BMI or BMI Group. The self-reported questionnaire data was used to assign subjects into four BMI categories: <18.5, 18.5−24.9, 25−29.9, and ≥30. These groups were divided based on the World Health Organization recommendations,[72] where the BMI value indicates if a person is underweight, normal weight, overweight, or obese, respectively. The relationship between the 11 continuous $\delta^{13}C$ variables and BMI as a continuous or ordinal variable showed that, before controlling for diet, the $\delta^{13}C$ value of Asx was the only variable that significantly correlated with BMI (r = 0.267, P = 0.015). None of the controls for diet or sex presented significant values at the 95% confidence level, but Asx and Arg were significant at the 90% confidence level (r = 0.195; P = 0.079 and r = 0.202; P = 0.068, respectively). With an R^2 = 0.128, linear regression analysis confirmed these two AAs had a very weak correlation with BMI (at α = 0.1). However, the combined model was not significant (P = 0.52). Unlike a previous work by our group, which included additional variables such as the $\delta^{13}C$ values of Thr and Glu,[33] the current model is not a reliable predictor of BMI.

Regarding the AA quantities, Tyr_q showed the only significant correlation with BMI as a continuous variable (r = 0.288, P = 0.009) or category (r = 0.219, P = 0.048). Controlling for sex did not significantly change these results. Using a GLM model, the linear combination of 11 variables explained ∼23% of the variance in BMI (R^2 = 0.234, P = 0.030). Asx_q (P = 0.003) and Tyr_q (P = 0.001) were statistically significant predictors at the 95% CI, and Tyr_q provided the highest unique contribution to the model (beta = 0.662). The significance of the model with 11 variables did not change after controlling for sex. When working with only four variables, the GLM model only explained 13% of variance in BMI (R^2 = 0.131, P = 0.027).

Only Tyr_q was statistically different across the four BMI groups at the 85% significance level in the Kruskal−Wallis test (P = 0.13). Tyr_q was more abundant in the hair of obese individuals (median = 0.684 rel mols) relative to individuals with normal BMI (median = 0.562 rel mols). Interestingly, elevated serum Tyr concentration has been reported as one of the early biomarkers for adults (25−49 years old) prone to metabolic syndrome (e.g., obesity and insulin resistance [IR]).[73] Discriminant analysis using all 11 variables reached just 52.4% success in predicting BMI group membership after LOOCV (Figure S4).

Predicting Alcohol Consumption. Among the 81 subjects with reliable CSIA values, 79 reported their alcohol drinking frequency. Subjects (number of subjects listed in parentheses) were divided into five arbitrary categories based on their self-reported information: "drink daily, heavily" (1), "drink daily, some" (7), "drink weekly" (16), "drink occasionally" (31), "never drink" (24). Surprisingly, the isotopic ratios of some AAs correlated with drinking habits. In the original data set, the $\delta^{13}C$ value of Met/Cyt gave the highest negative correlation with drinking frequency (r = −0.451, P < 0.001), followed by Ser (r = −0.364, P = 0.001) and Xle (r = −0.276, P = 0.014). After controlling $\delta^{13}C$ values for sex and diet, the correlations between alcohol consumption and the $\delta^{13}C$ values of Ser (r = −0.302, P = 0.007) and Met/Cyt (r = −0.280, P < 0.013) were confirmed.

Only one person claimed to a be a heavy alcoholic drinker, and to prevent distortion of the data, this individual's data was removed from further analyses. A Kruskal−Wallis test was used because some variables failed the parametric assumptions when working with categories. The only statistically significant difference across the four drinking habits was for the $\delta^{13}C$ value of Ser (P = 0.035). After Dunn's posthoc test and Bonferroni adjustment, the difference between individuals with a daily drinking habit and teetotalers was significant at the 90% CI (P = 0.089). The median values showed a depletion of ∼1.15 ‰ for the $\delta^{13}C$ value of Ser when the frequency of drinking changed from daily (−8.69 ‰) to none (−9.83 ‰). Unfortunately, LDA was not successful in classifying individuals into drinking frequency, providing only 50% correct classification using original rules, and ∼49% after LOOCV. None of the quantities of AAs were significantly different between the alcohol drinking frequency in any of the statistical tests.

The influence of alcohol on Ser and Gly were shown by Blum et al.[74] in an ethanol-induced animal model. Blum et al. suggested the inhibitory effect was likely due to the role these AAs have on the gastrointestinal tract, which is to retard ethanol absorption.L-Serine was also shown to be a potential candidate to treat alcoholic fatty liver.[75] In our results, the ^{13}C enrichment of Ser in the hair of daily alcohol drinkers might reflect the preferential allocation of carbon-depleted Ser to delay alcohol absorption in the stomach. Despite these interesting results, we recognize the need for further research with a large cohort of heavy alcohol drinkers and the use of independent markers of alcohol consumption to provide more conclusive evidence about the influence of alcohol consumption on isotope ratios of Ser in hair.

Other Effects. The influence of hair treatment and sunlight exposure per day on the isotope ratios and quantities of AA in hair were also evaluated in this work, but these factors minimally influenced the results (see SI for more details). In short, hair treatments had no determinable effect on the $\delta^{13}C$ values and AA quantities. For sunlight exposure, the aromatic AAs were the most significant, with the $\delta^{13}C$ values of Tyr and the quantities of Phe_q showing some significant differences with sunlight exposure, as reported elsewhere.[54]

CONCLUSIONS

The scalp hair of subjects from the US were subjected to bulk isotope analysis (n = 101) and amino-acid-specific isotope analysis (n = 82). One 11-year-old boy with autism had a $\delta^{13}C$ value of Phe depleted in ^{13}C by more than five standard deviations from the mean. The cause of Phe depletion is not known but could be related to depleted $\delta^{13}C$ values of Phe in the artificial sweeteners in the subject's medications or to an underlying metabolic disorder. After controlling for diet and using LOOCV, the $\delta^{13}C$ values of five amino acids predicted the sex of an individual with greater than 80% accuracy. Similarly, the relative quantities of four amino acids predicted sex with 89% accuracy using LOOCV. From a forensic casework perspective, predicting the sex of an individual by analyzing an unknown hair can be a valuable way to minimize the pool of potential donors and save time. Investigative leads can help identify suspects, victims, or their family members, all of which can lead to DNA evidence that can provide definitive evidence for the case.

Other soft biometric traits correlated with the analyzed variables, but with lower classification rates. For example, those

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who drank alcohol daily had $\delta^{13}C$ values of Ser enriched in ^{13}C by an average of 1.15 ‰ relative to teetotalers, but drinking frequency could only be predicted with 50% accuracy. There were no statistically significant correlations between the $\delta^{13}C$ values of AAs and respective quantities for other factors such as smoking, hair treatments, and consumption of soda or sweeteners (data not shown). Our results provide insight into the capabilities of compound specific isotope analysis in the investigation of certain human biometric traits. Further investigations would benefit from the use of larger cohorts, controlled diets, controlled behavioral states, and independent validation of questionnaire data such as the measurement of ethyl glucuronide for alcohol consumption.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.9b04085.

Extended experimental section and data analysis, including factors such as age group, hair treatment, and sunlight exposure. Figures S1−S6, Tables S1−S16, and supplementary reference citations (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Glen P. Jackson − *Department of Forensic and Investigative Science and C. Eugene Bennett Department of Chemistry, West Virginia University, Morgantown, West Virginia 26506, United States*; orcid.org/0000-0003-0803-6254; Phone: (+1) 304-293-9236; Email: glen.jackson@mail.wvu.edu

Author

Mayara P. V. Matos − *Department of Biology, West Virginia University, Morgantown, West Virginia 26506, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.analchem.9b04085

Author Contributions

Both authors contributed to the design and implementation of the study. M.P.V.M. performed all the sample analyses and data acquisitions and drafted the manuscript. M.P.V.M. and G.P.J. contributed to the data analysis, interpretation, and contextualization.

Notes

The authors declare no competing financial interest.

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