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Biometrics from the carbon isotope ratio analysis of amino acids in human hair

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ABSTRACT

This study compares and contrasts the ability to classify individuals into different grouping factors through either bulk isotope ratio analysis or amino-acid-specific isotope ratio analysis of human hair. Using LC–IRMS, we measured the isotope ratios of 14 amino acids in hair proteins independently, and leucine/isoleucine as a co-eluting pair, to provide 15 variables for classification. Multivariate analysis confirmed that the essential amino acids and non-essential amino acids were mostly independent variables in the classification rules, thereby enabling the separation of dietary factors of isotope intake from intrinsic or phenotypic factors of isotope fractionation. Multivariate analysis revealed at least two potential sources of non-dietary factors influencing the carbon isotope ratio values of the amino acids in human hair: body mass index (BMI) and age. These results provide evidence that compound-specific isotope ratio analysis has the potential to go beyond region-of-origin or geospatial movements of individuals—obtainable through bulk isotope measurements—to the provision of physical and characteristic traits about the individuals, such as age and BMI. Further development and refinement, for example to genetic, metabolic, disease and hormonal factors could ultimately be of great assistance in forensic and clinical casework.

1. Introduction

Instrumental methods of analysis can provide elemental, proteomic and isotopic information about human hair samples, which, because of their objectivity, scientific foundation, and statistical nature, offer many advantages over forensic hair microscopy. The criminal justice system could benefit greatly from an objective instrumental method of analysis that could provide investigative leads about a suspect or victim from a questioned hair sample. Such investigative leads could include a suspect's or victim's age, sex, race, region-of-origin, genetic disorders, and disease-state(s) or body mass index (BMI), among other traits. IRMS has the potential to answer these questions, is already in use in many government forensic laboratories and has passed Daubert standards for admissibility in court on many occasions [1–8].

The use of carbon isotope ratios for phenotypic analyses or disease-state diagnosis was demonstrated and promulgated at least as early as the late 1960s and early 1970s [9–11]. As a sample matrix, hair is not without its challenges [12], but has a few advantages over breath, bones and teeth for stable isotope ratio analysis [3]. For example, hair provides a chronological record of one's metabolism, is easily and non-invasively collected, and is very robust [13]. Despite the increasing applications of CSIA, the ability to use isotopic relationships at the amino acid level to classify individuals into clinical, biometric or phenotypic groups so far has not been very thoroughly addressed. The use of ^{15}N isotope ratios as a biomarker for liver cirrhosis is one exception [14]. Most ^{13}C studies focus on exogenous classification factors such as protein intake or dietary habits [15–18] rather than endogenous factors related to phenotype or disease state.

The development of LC–IRMS has included a moving wire interface [19,20] but has found better success with a wet chemical oxidation interface that has been commercially available since 2004 [21]. LC–IRMS provides a convenient way to measure the carbon isotope ratios of water-soluble organic mixtures directly from aqueous mixtures and, unlike GC–IRMS, it does not require derivatization. Derivatization requires careful and laborious interpretation because of the mass balance and kinetic isotope effects caused by the additional carbons from the derivatizing agent(s) [22–25]. LC–IRMS is now quite mature and provides a reliable tool in application areas including archeology, biochemistry, food adulteration, medicine and forensic science [26–32].

The analysis of underivatized amino acids using liquid chromatography coupled with isotope ratio mass spectrometry (LC–IRMS) is of interest in many scientific disciplines, including physiology, diet, metabolism and palaeodietary studies [16,33–37] and has been demonstrated by several groups. In 2005, Godin and coworkers were among the first to

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analyze a mixture of fifteen underivatized amino acids using LC–IRMS [38]. In 2006, McCullagh et al. used a strong cation-exchange column in series with a reversed-phase column to create a mixed-mode chromatographic (MMC) approach that was applied to the analysis of amino acids [39]. The availability of mixed-phase columns spurred a rapid improvement in the efficiency of separation of amino acids [32,40–42].

Schierbeek, McCullagh and Godin and their coworkers have published several new methodological approaches and validation studies for amino acid $\delta^{13}C$ analysis [42–44]. Smith et al. published a three-phase method in which they achieved baseline resolution between all the amino acids except leucine and isoleucine [32]. This was a significant advance and made the LC–IRMS approach a strong competitor with GC–IRMS both for the number of amino acids baseline resolved and for the precision and accuracy of $\delta^{13}C$ values. The time duration of the LC analysis was a high price to pay for this benefit, but additional focus on amino acid chromatography for LC–IRMS by the chromatography community is likely to reduce run times while maintaining baseline resolution, even with non-organic modifiers [45]. Morrison and Preston and coworkers in the UK have developed strong anion exchange liquid chromatography for the LC–IRMS analysis using the Liquiface interface (IsoPrime, UK). Their work demonstrates that anion chromatography can also be effective for the separation and IRMS analysis of amino acids and carbohydrates [46,47].

In addition to validating a relatively fast LC–IRMS method for fifteen amino acids in human hair, this manuscript presents results of bulk $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values and compound-specific $\delta^{13}C$ values of amino acids from the scalp hair of 20 female subjects. Each subject completed a thorough questionnaire covering topics of basic biometrics (sex, height, weight etc.), diet, exercise, health, genetics and hair treatments. Multivariate analysis of variance (MANOVA) shows that, for this small cohort, the LC–IRMS analysis of amino acids is able to classify the individuals into groups according to certain soft biometric traits or phenotypes with reasonable success. We apply this method to explore the possibility of using hair isotope ratios to diagnose or predict health problems and risk factors in individuals (like obesity), and the ability to use isotope ratios as an instrumental method of analysis for comparing or contrasting questioned hair samples with known sources. The significant differences between within-group and between-group variances established for this small group of subjects warrant additional development and could ultimately be of great assistance in forensic and clinical applications.

2. Materials and methods

2.1. Materials

The reagents were all of analytical grade or higher purity; sodium persulfate (99% purity), orthophosphoric acid (>85% purity), sulfuric acid (>95% purity) and amino acids (98–99% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide (high purity, 50% solution) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Isotope standards were purchased from NIST (Gaithersburg, MD, USA), USGS (Reston, VA, USA), and the IAEA (Vienna, Austria)

2.2. Hair samples

The collection of hair samples was approved by the institution review board of Ohio University (IRB# 12X029) and King Abdullah University Hospital (IRB# 10/215/2444). More than 140 volunteers were identified from a randomly selected portion of a database held by the General Civic Status Department in Jordan. Telephone calls were placed to potential volunteers to recruit participants and to schedule home visits. During the scheduled home-visits, prospective participants were provided with a summary of the project, the risks and benefits (no financial benefits) and were allowed to ask questions and decline participation. Consent forms from adults and assent forms from minors were collected prior to collecting hair and completing the questionnaires. Pregnant women and subjects reporting any chronic or acute diseases were excluded. A pencil-diameter (~1 cm) lock of hair was cut adjacent to the scalp in the posterior vertex region using surgical scissors. Extensive questionnaires regarding certain biometric information, health, hair-care and diet were provided by each participant.

Jordan was selected primarily because of the ease of access for one of the co-investigators, who collected the hair samples during an extended visit to Jordan in 2012. Jordan has three regions with distinct geography, culture and dietary habits. These include the mountain heights plateau region, which is lush and highly vegetated, the Jordan valley region, where the primary protein source is fish, and the eastern dessert or Bedouin region, where food sources are scarcer and therefore mostly animal products (e.g. from herded animals). Whereas we were able to collect hair samples from more than twenty individuals from each of the three regions—including men, women and children—time constraints dictated that a small sub-set of the samples were compared at the amino acid level in this proof-of-principle study. We therefore selected a subset of twenty female subjects from whom we had large quantities of hair to work with. The subset of twenty female subjects from the mountain region provided a range of BMI values and ages, as shown in Table 1 and the supporting information (Table S.1). All the hair was reported to be rarely or never chemically altered and none of the subjects drank alcohol. Approximately 10% of the subjects smoked, but we have not yet considered smoking, or many of the other questionnaire results, as independent variables for classification.

External contaminants such as lipids were removed from each hair sample by soaking in a mixture of methanol:acetone:chloroform (1:1:1) for 30 min and then sonicating twice (30 min each) in Milli-Q water [15]. The samples were left in a vacuum oven overnight at 50 °C to dry. The clean, dry hair was then prepared separately for bulk analysis and CSIA. For bulk analyses, hair samples were first pulverized in a 2 mL polypropylene tube in a minibead beater (Biospec Products Inc., Bartlesville, OK, USA) for 5 min at a setting of 3450 rpm with four 3.2 mm chrome steel beads before weighing precise amounts into the tin capsules. For CSIA, a 40 mg aliquot of each hair sample was similarly weighed and pulverized to aid hydrolysis. The samples were then hydrolyzed in 6 M hydrochloric acid for 24 h at 110 °C in an incubation oven (Thermo Scientific) before evaporating to dryness at 30 °C in a Mivac evaporator (Genevac, Ipswich, UK). The dry residue was reconstituted in 1 mL Milli-Q water (EMD Millipore, Billerica, MA, USA) and filtered using a 0.45 μm syringe filter to remove any unhydrolyzed melanin and other particulates. The filtrate was frozen in a 1.5 mL vial until required for isotopic analysis. Previous work has shown that protein hydrolysis under such conditions does not significantly affect the $\delta^{13}C$ values of the recovered amino acids [48].

Table 1
Summary of characteristics and isotope ratio data for bulk hair analysis of the eighty-four subjects from the Jordanian database and a subset of 20 female subjects used for the LC–IRMS experiments. N = 5 for each subject in the subset; N = 4 or 5 for the remaining subjects. Body Mass Index (BMI) is from self-reported data, which approximately 60% of 84 subjects provided.

		Age	BMI	$\delta^{15}N$[a]	$\delta^{13}C$[b]	$\delta^{34}S$[c]
84 subjects	Mean	27	25.2	8.29	−19.87	7.63
(65 female, 14 female)	Min.	1	14.7	6.96	−22.06	3.64
	Max.	77	40.0	10.14	−15.72	12.69
	Std. Dev.	17	5.2	0.44	1.06	1.31
Subset of 20 female subjects	Mean	34.1	24.8	8.04	−21.00	8.23
	Min.	17	14.7	6.96	−22.06	7.04
	Max.	50	40.0	8.66	−20.28	10.09
	Std. Dev.	9.2	6.5	0.37	0.43	0.65

[a] Versus air N_2.
[b] Versus VPDB.
[c] Versus VCDT.

Samples were not made devoid of oxygen before hydrolysis, so oxidative losses such as methionine oxidation cannot be ruled out. Under the acid hydrolysis conditions employed here, Glutamine (Gln) and asparagine (Asn) are typically de-amidated to aspartic acid (Asp) and glutamic acid (Glu), respectively, so the reported values for Glu and Asp will include a small contribution from Gln and Asn, respectively. Free cysteine residues were not observed in the hair hydrolysates, but the dimer form of the amino acid, cystine, was observed and is reported here using the label Cys.

2.3. EA–IRMS

Bulk isotope ratio measurements were made on precisely weighed samples of approximately 0.5 mg that were placed in tin capsules in a Thermo Flash HT Plus elemental analyzer (EA). The EA was coupled via a Conflo IV interface (Thermo Finnigan, Waltham, MA) to the Thermo Delta V Advantage isotope ratio mass spectrometer. Data acquisition was carried out using Isodat 3.0 Software (Thermo Scientific, Waltham, MA). During both the LC and EA analyses, high-purity gases from Airgas (Morgantown, WV) were used: >99.9999% He, 99.999% N_2, 99.997% CO_2, 99.999% O_2, and 99.98% SO_2.

For EA–IRMS analyses, samples were measured relative to the reference CO_2 gas and calibrated to the international scale relative to VPDB using a multiple point calibration curve ($R^2 > 0.999$) using replicate analyses of L-SVEC ($\delta^{13}C = -46.60‰$), NBS-19 ($\delta^{13}C = +1.950‰$) and USGS-41 ($\delta^{13}C = +37.626‰$). Sulfur isotopes were measured against a reference SO_2 gas and converted to the international scale relative to VCDT using a two-point calibration curve based on IAEA-S1 ($\delta^{34}S = -0.3‰$), and IAEA-S2 ($\delta^{34}S = +22.7‰$). Nitrogen isotope ratio values were similarly determined using a two-point calibration curve relative to air N_2 using USGS-40 ($\delta^{15}N = -4.5‰$), and USGS-41 ($\delta^{15}N = +47.6‰$). For quality control, human hair standard samples of USGS-42 or USGS-43 were run between every third or fourth sample to verify that the measured and accepted values were not significantly different at the 95% confidence interval (CI).

2.4. LC–IRMS

The LC–IRMS system consisted of a Dionex ICS5000 ion chromatography system (ICS) (Dionex, Sunnyvale, CA, USA) coupled through an interface (LC Isolink, Thermo Fisher Scientific, Bremen, Germany) to a Delta V Plus isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The ICS was operated in both the standard configuration and in direct- or flow-injection mode (FI–IRMS), wherein samples were injected directly into the interface via a six-port, three-way injection valve.

The LC system was operated under standard conditions with an electrochemical detector and followed a similar procedure to that described by McCullagh et al. [45]. Underivatized amino acids were separated on a mixed-mode column (Primesep A, 2.1 × 250 mm) and the stationary phase particle size was 5 μm, pore size 100 Å (SIELC Technologies, Prospect Heights, IL, USA). The mobile phase was initially pure Milli-Q water. Subsequently, a gradient of Milli-Q water with 0.03 M sulfuric acid was applied to provide an environment of decreasing pH (see Fig. S.2). The flow rate of the mobile phase was 260 μL/min. The eluent from the HPLC column was passed into the LC Isolink where all the carbon-containing compounds were quantitatively oxidized to carbon dioxide (CO_2) using wet chemical oxidation. Oxidation took place in the aqueous phase at 99.9 °C using sodium peroxodisulphate (200 g/L) and phosphoric acid (1.5 M) as an oxidizer and catalyst, respectively. The resulting CO_2 was removed from the cooled solution by a membrane separator and transferred to the IRMS system.

The mobile phase solvents and oxidation reagents were filtered using an Aura solvent degassing system with a 1.5 μm Teflon filter membrane (Sigma-Aldrich, St. Louis, MO, USA) and degassed under vacuum for 30 min prior to use. Reagent solutions were continuously sparged with UHP helium during use to reduce interference from extraneous CO_2. Data analysis was performed using Excel 2011 for Mac (Microsoft, Redmond, WA, USA) and SPSS 20 and 22 for Mac (IBM, Armonk, NY, USA).

For FI–IRMS and LC–IRMS analyses, carbon isotope ratios were measured relative to the reference CO_2 gas and reported relative to the international scale (VPDB) using a two point calibration curve using glutamic acid reference materials USGS-40 ($\delta^{13}C = -26.389‰$) and USGS-41 ($\delta^{13}C = +37.626‰$). For quality control, a caffeine standard of IAEA-600 ($\delta^{13}C = -27.77‰$) was run between every third or fourth sample to verify that the measured and accepted value was not significantly different at the 95% confidence interval (CI).

3. Results and discussion

3.1. EA–IRMS isotope measurements

USGS-42 Tibetan human hair standard ($\delta^{15}N = +8.05‰$, $\delta^{13}C = -21.09‰$, $\delta^{34}S = +7.84‰$) was used to test the precision of EA–IRMS isotopic measurements. Different amounts of USGS-42 hair samples from 0.2 to 3 mg in pentuplicate measurements. Fig. S.1 in the supplemental material shows the consistency of the measured $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values using EA-IRMS. The bulk $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ results are not significantly different than the accepted values within the error of the results.

Bulk (EA) IRMS measurements were made as a benchmark against which the compound-specific measurements could be compared. Bulk measurements of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ are considerably easier to make than compound specific measurements and would be the preferred approach, if it would provide the discriminating power to classify the subjects into different groups. Bulk isotope ratio analysis was performed on eighty-four of the Jordanian subjects, including the twenty female subjects for which we have compound-specific isotope ratio data.

A summary of the age, BMI and bulk stable isotope ratios of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values in the hair of eighty-four subjects and twenty female subjects are shown in Table 1. Multivariate analysis of variance (MANOVA) of bulk (i.e. time-averaged) $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values showed statistically significant F values for the within-person variance to between-person variance for the subset of twenty female subjects and for the total eighty-four subjects, thereby indicating that these isotope ratios can be used, at least partially, to discriminate between the hair of individuals (See Tables S.2–S.5 for details).

Grouping the individuals according to ordinal categories of BMI (BMI < 20, 20–23, 23–30, BMI > 30) also leads to significantly different F values for MANOVA analysis for carbon ($p = 0.003$) and nitrogen ratios ($p = 0.001$), but not sulfur (See Tables S.6–S.7 for details). We changed the BMI range to ensure a more uniform distribution of the subjects between groups due to the limitation in the number of the subjects. According to the World Health Organization [49–51], a person is considered underweight if the BMI is lower than 18.5; normal weight if the BMI is from 18.5 to 24.9; overweight if the BMI is from 25 to 30 and obese if the BMI is higher than 30.

Attempts to classify individuals into BMI groups using canonical discriminant analysis (CDA) or hierarchical cluster analysis of the bulk isotope ratio values were quite ineffective. For example, using leave-one-out cross-validation of CDA, only 39% of the subjects in the total database and 44% in the sub-database of twenty female subjects were correctly classified to their BMI group. These success rates are not much better than a random probability of approximately 25% correct. There were no obvious trends in the misclassifications, such as confusing overweight and obese cases.

In addition to BMI classifications, we performed MANOVA and sought to classify individuals in the database of eighty-four subjects using CDA according to a variety of grouping factors including geographic origin (mountain region, valley region and desert region), sex, age and dietary factors such as meat and dairy intake. Classification

success rates were typically 5–20% better than random probabilities (based on leave-one-out cross-validation), but never more accurate than ~62% (for sex determination and geographic origin). In summary, bulk isotope ratio analysis of human hair did not provide sufficiently reliable classification rates to enable class or group differentiation. The weakness in classification using only three variables (isotope ratios) is the main argument for utilizing more or different variables, such as through CSIA of the amino acids within the hair matrix.

3.2. Flow-injection and LC–IRMS isotope measurements

To test the precision and accuracy of flow-injection and LC isotope measurements, these two modes of sample introduction were compared to standard bulk (EA) analysis of the same amino acid standards. The validation steps included: 1) measurement of the individual amino acids using EA–IRMS; 2) measurement of the individual amino acids using the LC–Isolink interface in flow injection mode; and 3) measurement of a mixture of the same amino acids, separated by the Primesep A column using the LC–IRMS system. Fig. 1 is a bar graph summarizing the results of the three modes of analysis (additional information in Figs. S.3, S.4 and Table S.8). The accuracy remains consistent across the three analysis modes for all the amino acids, but the precision gets worse in the order EA (SD = 0.08‰) < Flow Injection (SD = 0.09‰) < LC (SD = 0.6‰). Flow injection was the easiest to implement, but like the EA, it only provides one average isotope value for all the carbon present in the sample—regardless of the chemical form. On the other hand, LC–IRMS has the profound advantage of being able to analyze almost all the individual amino acids in one injection, albeit with a loss of precision. Fig. 2A shows extracted ion chromatograms for the relevant CO_2 ions (m/z 44, 45 and 46) for an approximately equimolar mix of seventeen standard amino acids collected on the LC–IRMS system. All of the amino acids, with the exception of co-eluting leucine (Leu) and isoleucine (Ile), were baseline resolved in less than 2.5 h using a gradient elution of acidified water as the mobile phase.

3.3. LC–IRMS analysis of human hair samples

Fig. 2B is a chromatogram of a human hair hydrolysate using the same conditions as LC–IRMS conditions as Fig. 2A. Although Leu/Ile cannot be baseline resolved, the isotope values derived from the co-eluting Leu/Ile peaks were usually included in the data analyses because it still represents an independent measurement, or variable, for each person. Methionine (Met) gave the smallest absolute ion signals for the hair hydrolysates. This is most likely due to: 1) the fact that of the seventeen amino acids studied, it is the least abundant in hair, and 2) oxidative losses to sulphone or sulphoxide. The small Met peak for the hair hydrolysates rendered the amino acid unsuitable for inclusion at this time.

Exploratory data analysis of the amino-acid specific isotope ratios was performed using principal component analysis (PCA) and MANOVA. Details of the approach, validation and some results are provided in the supplementary information (LC–IRMS data analysis section and Table S.9). PCA was performed using the unweighted, mean-centered isotope ratio values for each subject in two different ways. In one approach, each of the three instrument replicates for each person was included in the data analysis (N = 60 for 20 subjects). Mathematically speaking, this approach incorrectly assumes that each replicate measurement of a person's hair is an independent measurement of a given grouping factor of that person. However, this approach captures more of the variance expected within each grouping factor because it captures more of the measurement variance for each person. We also performed the calculations using averaged-within-person data for each person to see if using the mean values for each person assisted in clustering and classification (N = 20 for 20 subjects). Grouping factors in the present study include age, BMI, meat intake and dairy intake.

The results of PCA analysis of seventeen amino acids (in sixteen peaks) are shown in Fig. 3. The data are grouped according to subject ID and help show the within-person variance and between-person variance. The first two principal components in each analysis (not averaged and averaged-within-person) have Eigenvalues greater than one and together explain between 72 and 76% of the total variance, respectively. The Varimax rotated component coefficients shown in Tables 2 and 3 show that in both cases (averaged-within-person and not-averaged-within-person), the amino acids clustering close to y-axis (PC2) were mainly the essential amino acids Tyr, Lys, Phe, His and Leu/Ile, whereas the amino acids clustering close to x-axis (PC1) were mainly the non-essential amino acids Asp, Glu, Gly, Cys, Ser and Ala. Although tyrosine is not strictly an essential amino acid, its carbon skeleton is derived directly from the essential amino acid Phe, so it is included here. The phenotypic non-essential amino acids (PC1) therefore explained more of the variance between subjects than the diet-derived essential amino acids (PC2).

In short, with the exception of three amino acids (Arg, Thr and Val), the isotope ratios of the amino acids naturally converged into two

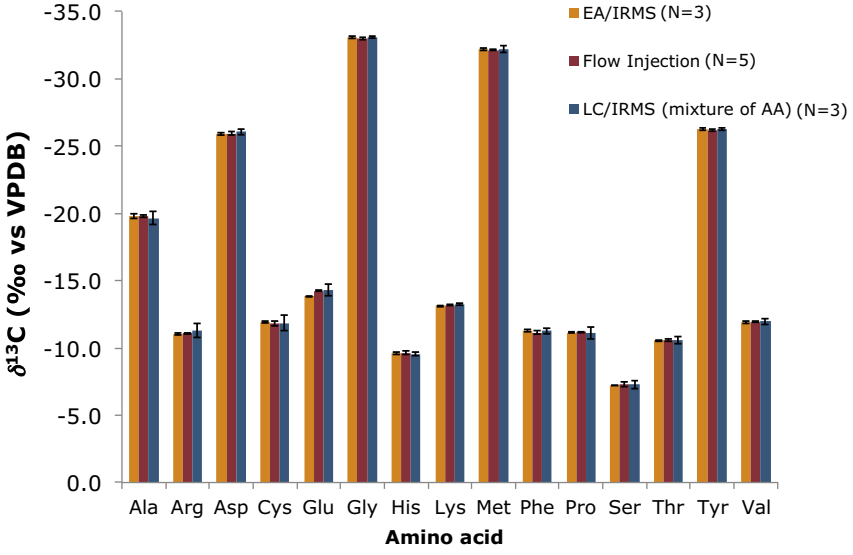

Fig. 1. Comparison of EA–IRMS, flow injection and LC–IRMS mode for the analysis of fifteen standard amino acids. Leu and Ile were also analyzed using all three introduction systems, but co-eluted on the LC–IRMS. Error bars represent one standard deviation (N = 3, 5 and 3 respectively).

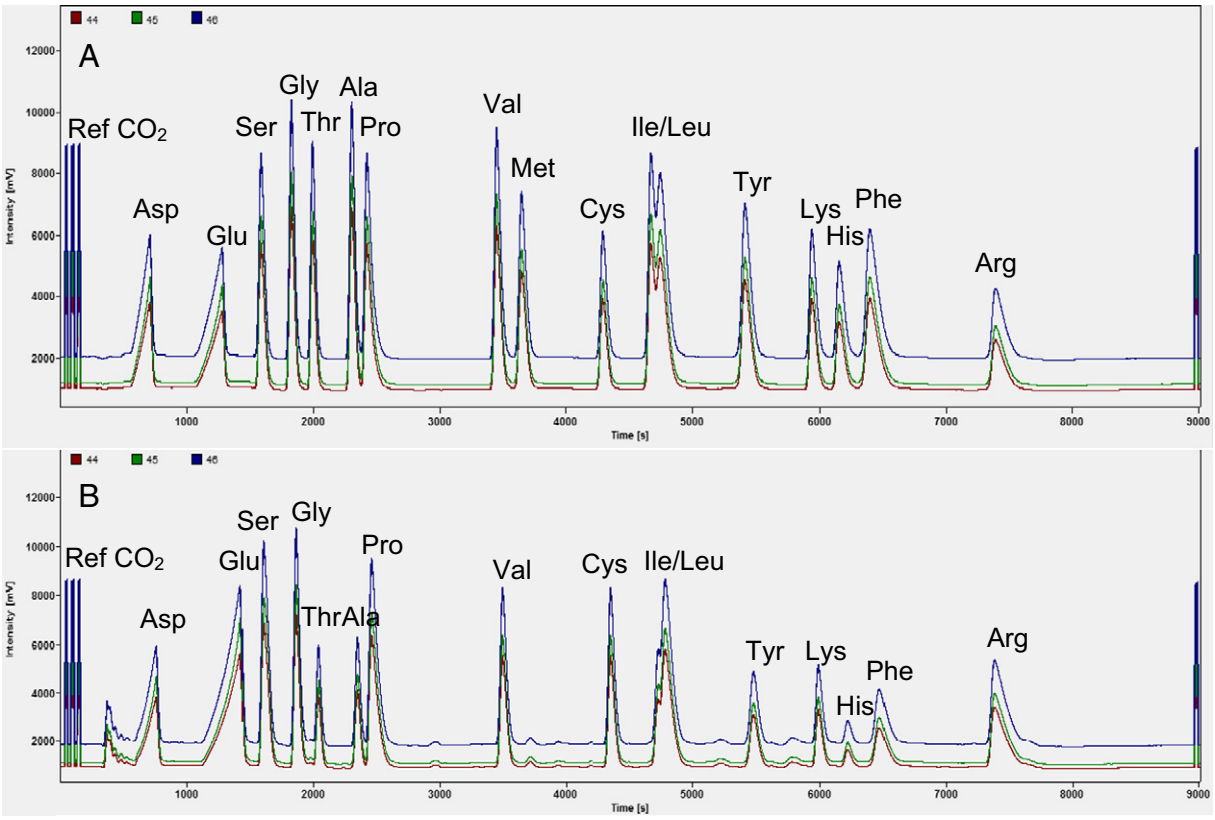

Fig. 2. Example of ion chromatograms of 16 amino acids acquired on the LC–IRMS system using a mixed-phase Primesep A column and no organic modifiers: A) standard free amino acids; B) from a hair hydrolysate. Only Leu and Ile were not baseline resolved. Although Met was resolved, it was of too low abundance in hair hydrolysates to be useful.

orthogonal (i.e. unrelated) components described respectively by the essential and non-essential amino acids. In general, dispensable (non-essential) amino acid carbon skeleton also ultimately comes from the diet with alanine reflecting dietary carbohydrates and glutamate and aspartate reflecting dietary energy. However, the orthogonal components here indicate that, regarding the ability to separate individuals, the isotope ratios of the non-essential amino acids are unrelated to the isotope ratios of the essential amino acids. This natural convergence may provide some assurance as to the validity of the present results, and to the fact that, broadly speaking; external factors and internal factors of isotope fractionation are independent, at least in this data set of twenty female subjects. The significance of this finding in a larger pool of subjects has yet to be established.

3.3.1. Classification by BMI group

After grouping the subjects into approximately equal-sized ordinal scale of BMI, MANOVA was performed using BMI as the fixed factor (See Table S.9 in supplemental material). Thr and Cys gave the largest F values ($p = 4 \times 10^{-7}$ and 5×10^{-8}, respectively), suggesting that they may be the most useful in terms of classifying according to BMI.

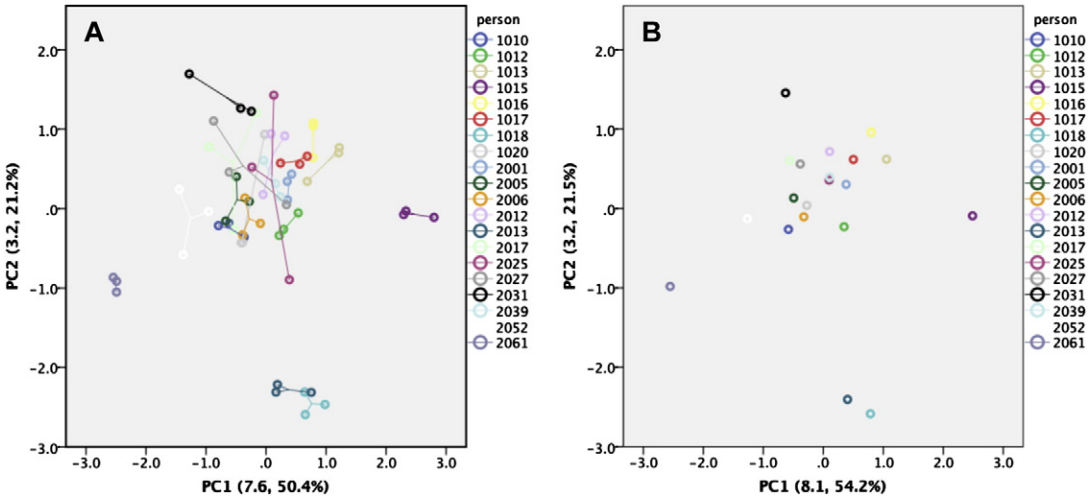

Fig. 3. Principal component analysis (PCA) plots for twenty female subjects using isotope ratios of sixteen peaks (representing seventeen amino acids) as input data: A) used triplicate measurements for each person; B) used averaged-within-person data. The absolute and percent variance explained by the first two components with Eigenvalues >1 are shown in the axis titles.

Table 2
Rotated component matrix for not-averaged data.[a] (N = 60 for 20 persons).

	Component	
	1	2
Asp	.865	
Glu	.846	
Gly	.829	
Ser	.797	
Thr[b]	.755	
Cys	.682	.450
Ala	.675	
Val[b]	.672	
Pro	.597	
Lys[b]		.960
Tyr[b]		.945
Phe[b]		.942
His[b]		.903
Arg		.770
Leu/Ile[b]	.419	.761

Extraction method: principal component analysis.
Rotation method: varimax with Kaiser normalization.
Only values > 0.4 are shown.
[a] Rotation converged in 3 iterations.
[b] Essential amino acids.

However, post-hoc comparisons using Tukey's honestly significant difference (HSD) showed that although the $\delta^{13}C$ values of Thr showed very significant differences between the obese group (BMI > 30) and all the other groups, the $\delta^{13}C$ values for Thr were not significantly different between the underweight, normal or overweight groups. Tukey's HSD provides for better control of Type 1 errors than a simple T-test, so it is less likely to find significant differences between sample means when the means are not significantly different. Therefore, Thr is only effective in identifying the obese subjects. The mean $\delta^{13}C$ value for Thr of the BMI > 30 group ($-7.16 \pm 0.66‰$) was different from BMI below 20 group ($\delta^{13}C = -8.40 \pm 0.86‰$), BMI 20–23.99 group ($\delta^{13}C = -8.52 \pm 0.34‰$) and BMI 24–30 group ($\delta^{13}C = -8.67 \pm 0.90‰$) at the 99.9% CI ($p = 6.10 \times 10^{-5}$; 4.41×10^{-6}; 4.36×10^{-6} individually). According to post-hoc Tukey's HSD tests, no single amino acid provided significant differences between more than two groups of BMI, so multivariate approaches were necessary.

Discriminant analysis was applied to classify the twenty female subjects according to the BMI groups. The results are shown in table form in supplementary Tables S.10 and S.11 and graphically in Fig. 4 for both the not-averaged-within-person and averaged-within-person data. According to the classification results, 93% of the not-averaged data points can be classified to the correct BMI group on the basis of the original discriminant rules, and 77% of the data points can be correctly classified using leave-one-out cross-validation. In leave-one-out cross-validation, each case (person or replicate from a person) is classified by the functions derived from all the cases other than that case. The result of the classification is repeated for each case to provide a more rigorous measure of the classification ability.

Classification for the averaged-within-person data was quite different than the not-averaged-within-person data because the number of data points is considerably different. The subjects cluster very tightly and most of the variance (93%) is explained in one function. The very large Eigenvalue indicates that the group means are very significantly different in the averaged-within-person data. Classification success in this case was 100%, based on the original functions, but only 80% using leave-one-out cross-validation. This is because the number of data points is somewhat inadequate for the averaged-within-person data, so one data point can influence the functions quite considerably. The function coefficients for each amino acid can be retroactively investigated to link the classification ability to certain key amino acids or sources, but we have not found any obvious trends between the amino acids and their function coefficients at this time.

Table 3
Rotated component matrix for averaged-within-person data.[a] (N = 20 for 20 persons).

	Component	
	1	2
Gly	.905	
Asp	.890	
Glu	.830	
Thr	.815	
Val	.762	
Cys	.730	.439
Ala	.684	
Tyr[b]		.972
Lys[b]		.954
H[b]is		.938
Phe[b]		.923
Leu/Ile[b]	.496	.754

Extraction method: principal component analysis.
Rotation method: Varimax with Kaiser Normalization. Only values > 0.4 are shown.
[a] Rotation converged in 3 iterations.
[b] Essential amino acids.

3.3.2. Classification by age group

A second discriminant analysis was applied to classify the subjects according to arbitrary age groups: <25, 25–45 and 45+ years old. The results are plotted in Fig. 5 and also provided in the supplemental material (Tables S.12 and S.13). Using the un-averaged data, 96.7% of the cases were correctly classified based on the original functions and yet only 10% were correctly classified in the leave-one-out cross-validation. The averaged-within-person results were similar, at 100% and 25%, respectively. The source of the poor validation success is likely due to the small Eigenvalues associated with the discriminant functions and the weaker significant difference between the group means. At this stage, it is difficult to tell if classification success would improve with a larger cohort, because of the improved sampling and lack of stochastic effects, or if classification would be worse because the captured variance may reveal that the small difference between the means is insignificant. A larger sample size would resolve this question.

3.3.3. Classification by diet

The supplemental material (Fig. S.5) includes an example of CDA using meat intake as a fixed factor. Using the averaged-within-person data, Eigenvalues are relatively large (~12) for function 1, enabling 100% classification from the original functions. However, leave-one-out cross-validation was only 40% successful, which is not significantly better than random probability. Using the not-averaged data provided leave-one-out cross-validation success rate of 85%, which is considerably better. The large differences between the classification success based on original rules versus the leave-one-out rules is an indication that the data set is not yet large enough to provide a more rigorous result. Because the original analysis of variance showed that most of the variance between the subjects can be explained by the non-essential amino acids, which fractionate in a manner that is independent of dietary source, one would expect that phenotypic factors would actually have better classification success than diet, assuming that one could identify the most influential phenotypic factors by which to classify. These questions are the basis of our ongoing work.

3.4. Conclusions

This study has compared the bulk isotope ratios of hair with compound specific isotope ratio values of amino acids from human hair hydrolysates. We were able to measure the carbon isotope ratios of 15 of the amino acids in hair independently, and Leu/Ile as a co-eluting pair. Data analysis confirmed that, regarding the differences between

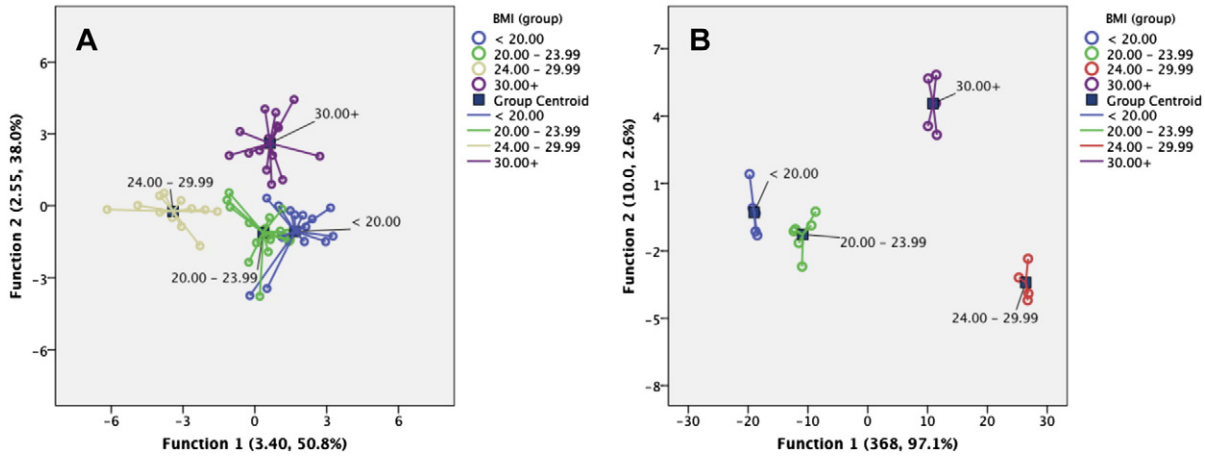

Fig. 4. Canonical discriminant analysis (CDA) plots for twenty female subjects using isotope ratios of sixteen peaks (representing seventeen amino acids) as input data and BMI group as the classification factor: A) triplicate measurements for each person; B) used averaged-within-person data. The absolute and percent variance explained by the first two functions with Eigenvalues >1 are shown in the axis titles.

individuals; most of the essential amino acids and non-essential amino acids were independently variable, thereby enabling the separation of dietary factors from intrinsic or phenotypic factors within the subjects. Multivariate analysis revealed at least two potential sources of non-dietary factors influencing the carbon isotope ratio values of the amino acids in human hair: BMI and age. We are currently in the process of increasing the number of participants and investigating other potential sources of intrinsic or phenotypic variance, such as sex, exercise habits, metabolic disorders and disease-state. We welcome communication from anyone with a reference collection of hair samples who might be interested in this approach.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scijus.2014.07.002.

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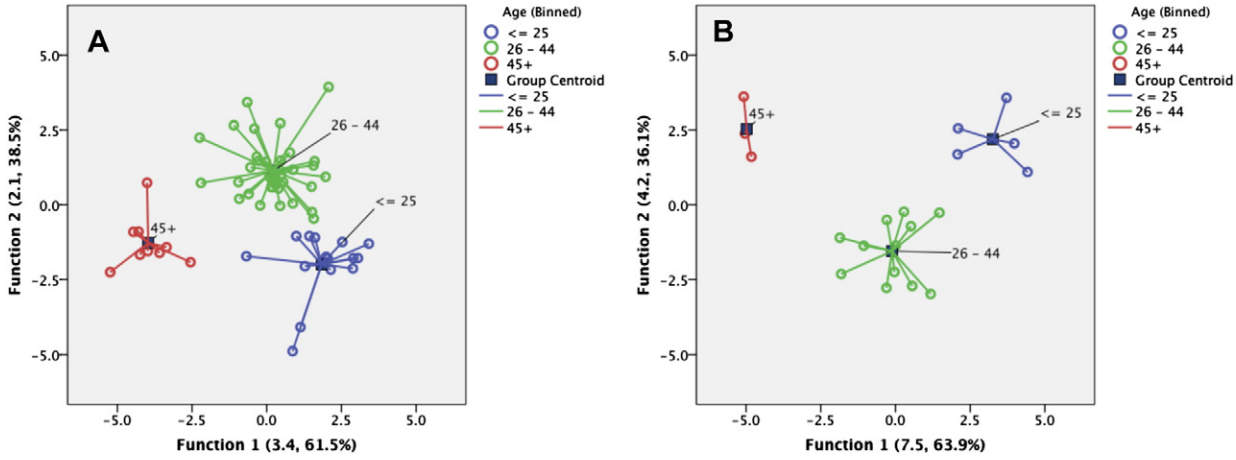

Fig. 5. Canonical discriminant analysis (CDA) plots for twenty female subjects using isotope ratios of sixteen peaks (representing seventeen amino acids) as input data and age group as the classification factor: A) triplicate measurements for each person; B) used averaged-within-person data. The absolute and percent variance explained by the first two functions with Eigenvalues >1 are shown in the axis titles.

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