Executive Summary

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 from a questioned hair sample. Such investigative leads could include a suspect's age, sex, race, region-of-origin, genetic disorders, and disease state(s) or body mass index, among other traits. Here, we used isotope ratio mass spectrometry (IRMS)—which is already in use in many government forensic laboratories—to analyze hair samples from a cohort of >200 subjects across the US and Jordan.

We contributed 100 data points to the publication of a new global database of bulk carbon and nitrogen isotope ratios in human hair containing more than 3500 analysis results, which can be used by future investigators to provide statistical basis for inclusion or exclusion of evidence. In separate work, we used LC–IRMS to measure 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 three potential sources of non-dietary factors influencing the carbon isotope ratio values of the amino acids in human hair: body mass index (BMI), age and sex. We could also discriminate 20 US subjects from 20 Jordanian subjects with 75% accuracy (leave-one-out cross validation). 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.

By quantifying the amount of amino acids in human hair, we were able to perform statistical comparisons between donors with different characteristic traits, including sex, age and region of origin. Using leave-one-individual-out cross-validation on more than 80 hair donors, the fuzzy rule-building expert system (FuRES) classification rate was 94% for sex, 83% for age group, and 61% for the region of origin. In addition, we used the amino acid profiles of scalp hair of 27 Jordanian subjects (15 diabetes mellitus (DM) type 2 patients and 12 control subjects) to predict diabetes from hair analysis. Using a FuRES, we were able to classify the amino acid profiles into diabetic and control groups with 100% sensitivity and specificity using leave-one-individual-out cross-validation. The areas under the receiver operative characteristics (ROC) curves were 1.0, which represents a highly sensitive and specific diabetes test. The associations between the abundance of amino acids of human hair and health status may have clinical applications in providing diagnostic indicator or predicting other chronic or acute diseases.

This project has the ability to provide significant advances in the scientific understanding of factors that influence the isotopic composition of amino acids in human hair. This basic understanding can benefit the criminal justice system by offering a scientific, biochemical basis for the comparison of human hairs and for the provision of investigative leads for suspects. Although the primary intended benefit of this research project is for the criminal justice system, the methods and results of this work has broader impacts that could directly benefit other fields of study such as archaeological applications—involving human ancestral habits/traits, ecological applications—involving migration and dietary habits, and disease diagnosis and health monitoring.

Contents

1 Introduction

1.1 Statement of Problem

There are a limited number of approaches that currently exist for the forensic examination of questioned hair samples and their comparison to known samples. The most common method, microscopy, received harsh criticism in the 2009 NAS Report on *Strengthening Forensic Science in the US*, mostly because of its subjectivity but also because there are no widely accepted statistics regarding the frequency with which particular characteristics of hair are distributed among the population. Nuclear or mitochondrial DNA analysis can provide a high degree of confidence in matching questioned hairs with known samples, but forensic DNA analysis is only useful in the presence of comparison samples. The criminal justice system could benefit enormously from an objective, instrumental method of analysis of human hair that could provide investigative leads such as the age, sex, race, dietary habits, region-of-origin, genetic disorders, disease state(s) or body mass index of the donor, in the absence of a comparison sample. Such forensic hair analysis would be of even more value if the technique in question were already widely validated and used in scientific disciplines outside of forensic science, and were commercially available. Such a technique does exist and it is compound specific isotope analysis (CSIA). Until now, CSIA has not been applied to the determination of biometrics from human hair in a forensic context.

The *purpose* of our study is to investigate the potential of compound specific isotope analysis of amino acids in human hair for providing a novel forensic tool for biometric information about donors. Such a capability could ultimately provide an objective and statistical approach for investigative leads. Our *goal* is to analyze the carbon isotope ratios of more than 14 amino acids in the hair samples from approximately 150 human subjects across the US. We will then perform multivariate statistical analyses including canonical discriminant analysis (a form of principal component analysis) to optimize the classification of individuals into arbitrary classification groups. The grouping factors will be determined from biometric information collected from detailed questionnaires that each volunteer subject will complete. The ultimate *objective* is to be able to link the amino acid-specific isotope ratios (aka biomarkers) to relevant biochemical processes in the body that are intrinsic to each group and therefore provide a scientific, biochemical basis for discriminating between groups. Examples of the ability to classify groups according to amino acid isotope ratios already exist in clinical studies and include the ability to identify patients with liver cirrhosis from the general population. The main discriminating amino acid in this previous case was the nitrogen isotope ratio of phenylalanine in the subjects' hair.[1] An added benefit of our proposed research is that our analysis methods and data interpretation methods could be applicable to the characterization and comparison of other forms of trace evidence, such as any proteinaceous fibers (e.g. silk and wool), or to areas outside of forensic science—such as clinical studies and non-invasive long-term health monitoring. Many government agencies around the world already have isotope ratio mass spectrometers and would be able to employ our methods, if successful. This basic forensic science study has the potential to benefit other areas of forensic science and other scientific disciplines.

1.2 Literature Citations and Review

Hair is an ideal medium for forensic and clinical analyses and stable isotope ratio analysis for several reasons.[2-4] First, hair fibers are a very common but underutilized form of trace evidence.[5, 6] Second, in contrast to most other body tissues, hair can be noninvasively sampled (by cutting) or minimally invasively sampled (by plucking), making it possible to perform sample collection outside of a clinical setting. Third, the protein in hair (keratin) is so stable that it is

essentially inert after it is synthesized.[7, 8] Fourth, unlike DNA profiles, which are static,[9] each hair shaft contains a chronological record of dietary and metabolic activities of the donor, thus providing insight into the biochemical processes in the body as a function of time/distance. Although this time/distance variability can complicate interpretation, this same variability also enables the ability to track the geospatial movements of individuals.[10-14] This investigative lead capability has enabled stable isotope analysis to solve several cases involving previously unidentified individuals.[3]

A typical hair shaft mainly consists of the persistent protein structure keratin, which can preserve the isotopic information of component amino acids for thousands of years.[8] It is this intrinsic stability that has enabled archaeologists and paleoarcheologists to determine dietary trends and changes in our human and prehistoric ancestors.[15-17] Hair from almost any source can be used for diet reconstruction. Hair has been shown to be isotopically representative of the body's amino acid pool, and its isotopic composition is found to reflect those of the dietary protein sources in addition to metabolic and environment influences.[10, 17-23]

1.2.1 *Bulk IRMS of Human Hair*

As early as 1939, Schoenheimer and Rittenberg reported using nitrogen isotope ratios to study protein metabolism.[24] They provided the theory of a dynamic equilibrium in proteins and lipids that proved using stable isotope ratios as tracers in nutrition and biomedicine.[24, 25] However, since this seminal work, only a few isotopic studies at natural stable isotope abundance have examined metabolic states and diseases in humans. Fuller et al. were the first to demonstrate that the $\delta^{15}N$ values in bulk human hair protein can be used to track changes in nitrogen balance during pregnancy and morning sickness.[26, 27] There was no significant change in the $\delta^{13}C$ results, but all the subjects showed a significant changed in the $\delta^{15}N$ by approximately 0.5–1‰ during periods of weight loss and/or restricted weight gain associated with morning sickness. Stable isotopes have been used as a diagnostic technique for tracking eating disorders, disease states, and nitrogen balance in archaeological, medical studies, and to understand metabolic pathways in humans.

In the past 30 years, IRMS has been used quite extensively used in areas such as forensic science,[3, 12, 28, 29] food analysis,[30, 31] drugs and pharmaceuticals,[32] geology,[33] biology,[34] environmental chemistry,[35] athletics and doping.[36] Most studies have focused on bulk (not compound specific) isotope analysis. Recently, multi-collector inductively coupled plasma mass spectrometry (MC-ICP-MS) has become a potential alternative method to IRMS for rapid carbon and sulfur isotope ratio determinations, but the high background levels for carbon pose a potential problem for widespread adoption at the present time.[37, 38]

The most common approach for introducing samples into an isotope ratio mass spectrometer is via an elemental analyzer (EA). In an EA system, samples are typically combusted at around 1050 ºC for bulk $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ measurements or pyrolized at around 1400 ºC for the determination of bulk $\delta^{2}H$ and $\delta^{18}O$ values. With these two approaches, one can obtain values for a maximum of five variables (elements) with which to compare samples, assuming these five elements are all present in high enough abundance in the sample. Such bulk isotope ratios have been used in multiple forensic cases involving provenancing humans and tracking geographic movements.[11, 39-41] Of more interest, and even greater impact, is the ability to look beyond bulk analysis to the compound-specific isotope ratios. Assuming each compound is independently variable (not necessarily valid), there is a major statistical advantage for measuring as many variables/compounds as possible, as is the case with compound-specific isotope ratio analysis,

1.2.2 *Compound-Specific Isotope Analysis (CSIA)*

In addition to the more common GC methods of CSIA, liquid chromatography (LC)-IRMS has been undergoing many recent developments for studying isotope ratios in a variety of biological molecules. Unlike GC-IRMS, LC-IRMS typically does not require derivatization and best realizes the belief that "the best derivatization for isotopic analysis is no derivatization at all".[34] In 2004, Krummen et al. reported the wet oxidation interface which was ultimately commercialized by Thermo (LC IsoLink®, Thermo Electron, Bremen, Germany).[42] Today, there are two commercial wet oxidation interfaces enabling the coupling of HPLC with IRMS, the Isolink® (Thermo Scientific, Bremen, Germany) and the Liquiface® (Isoprime Ltd., Cheadle Hulme, UK).[42, 43] The wet chemical oxidation interface converts organic molecules in aqueous solution to CO_2 gas using oxidizing reagents such as sodium persulfate ($Na_2S_2O_8$) and a catalyst such as phosphoric acid and silver nitrate.[34] A membrane exchanger separates CO_2 gases from the other gases (water vapor, oxygen, argon, etc.) that originate from the liquid phase. The CO_2 is then transferred through a gas permeable membrane[44] into a counterflow of helium. Finally, the helium stream enriched in CO_2 is partially dried in an online gas drying semi permeable membrane (Nafion®) and is then introduced, via an open split, into the IRMS ion source.

Several groups have reported the precision, accuracy and linearity of LC-IRMS in various applications.[45-51] LC-IRMS has provided a novel tool used in wide range of fields including chemical archaeology, biochemistry, food adulteration, medicine and forensics.[45-51] The analysis of underivatized amino acids using LC-IRMS has been of interest in many areas such as animal physiology, diet, metabolism and palaeodietary studies.[21, 52-56] In 2005, Godin and coworkers were the first to analyze 15 underivatized amino acids.[57] A strong cation-exchange column was used in series with a reversed-phase column, creating a mixed-mode chromatographic (MMC) approach, which was applied to the analysis amino acids by McCullagh et al. in 2006.[58] So began the development of other chromatographic methods providing increased resolution between the amino acids.[34, 51, 59, 60] More recently, Schierbeek, McCullagh and Godin and their coworkers have published new methodological approaches for amino acid ^{13}C analysis, [60-62] and Smith and co-workers have published a method using a mixed-phase column enabling baseline resolution between all amino acids (except leucine and isoleucine).[51] This recent developments in chromatography have advanced LC-IRMS to be a strong competitor with GC/C/IRMS both for the number of amino acids baseline resolved and for the precision and accuracy of measured $\delta^{13}C$ values.[43, 63, 64] HPLC run times of several hours can be forgiven because there is no derivatization necessary, unlike GC-IRMS.

1.2.3 *Human Identification*

As alluded to earlier, the use of stable isotopes as a tool in human identification is an important application of IRMS that is rapidly gaining momentum, acceptance and credibility within the forensic community.[65] In cases where DNA is not available, isotope ratio analysis can provide an alternative perspective. Analysis of hair and nails is often preferable to the analysis of bone and teeth because hair and nail samples can be obtained in a non-invasive manner and because the IRMS can be applied as easily to the living as to the dead.[39] Ehleringer et al. investigated the links between bulk stable isotope ratios of O and H in hair of US residents and geographic provenance.[13] Fraser et al. reported using profiling methods for hair and nails using bulk $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ and $\delta^{18}O$.[39, 66] Fraser et al. conducted studies yielding information about the intra- and inter-variability of individuals within a geographical area[39] and have demonstrated that information regarding recent geographical whereabouts can be obtained from bulk $\delta^{2}H$ isotope

profiling of hair and nail samples.[66] In 2008, Meier-Augenstein and Fraser reported the first use of bulk isotope analysis in aiding the identification of an unknown victim.[40] They examined hair and nail samples from a dismembered body. Data on bulk $\delta^{13}C$ and $\delta^{15}N$ isotopic composition of human tissue provided information on the person's diet and, hence life style or life circumstance and changes therein. Using data obtained from their previous longitudinal study,[39, 66] as well as data from the global network for isotopes in precipitation, the authors were able to provide sufficient information about the likely region-of-origin of the victim and approximate time period of entry into the country. This result provided investigators justification for DNA analysis that, remarkably, resulted in ultimate identification of the victim. IRMS has already proved the ability to provide investigative leads where all other techniques are ineffective.

1.3 Rationale for Research

The ability to use isotope ratio measurements at the amino acid level in human hair to classify subject donors into selected groups has been successfully applied to patients with liver cirrhosis.[1] Whereas this result is of limited value in a forensic setting, the result indicates that biochemical and metabolic processes in organs remote from the hair follicle can result in a measureable difference in isotope ratios in the growing hair shaft. We hypothesize that there are other hard and soft biometric traits[67] about individuals that influence the isotope ratios of amino acids to the extent that they will be observable in the hair shaft regardless of dietary fluctuations. Certain biometric traits such as eye color and height would not be expected to influence metabolism in such a way, but genetic disorders, metabolic rate, hormone levels, age, sex, and weight certainly could. The research plan is designed to explore the possibility of classifying individuals based on self-reported traits, and to provide insight into the biochemical factors influencing any potential classification.

2 Methods

2.1.1 *Recruitment and Selection of Human Subjects*

Hair and nail samples were collected from 94 Jordanian subjects and 97 US subjects, which is fewer than the original target value of 150 US subjects. Volunteers from the US were be recruited through various conventional and social media venues and include 69 female and 28 male volunteers from 22 states. Each participant has completed a thorough questionnaire on a variety of topics, including biometrics, nutritional habits, lifestyle, health status, region-of-origin and hair treatments. All 94 Jordanian samples were analyzed for bulk IRMS and 26 US subjects were analyzed for bulk IRMS. A total of 20 Jordanian and 20 US hair samples were analyzed for compound-specific IRMS.

The collection of hair samples for this study was approved by the Ohio University institution review board (IRB# 12X029), King Abdullah University hospital institution review board (IRB# 10/215/2444), and WVU review board (IRB# 1312146430).

2.1.2 *Sample Preparation*

All hair samples have been washed and stored according to standard protocols used for several years in our laboratory. In short, external contaminants such as lipids and hair treatments were removed by vortexing the hair in methanol: acetone: chloroform (1:1:1) for 30 min and then sonicating twice (30 min each) in Milli-Q water. The samples were then dried in a vacuum oven at 50 °C overnight.

The hair samples were pulverized by placing an aliquot of the hair into replicate reinforced 2 mL polypropylene tubes with four 3.2 mm chrome steel beads in an 8-plex-bead beater (Biospec Products Inc., Bartlesville, OK) for 5 min at a setting of 3450 rpm. Precisely weighed aliquots of each powdered sample was analyzed at the bulk level using the EA-IRMS to determine $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values. Additional precisely weighed aliquots were subjected to acid hydrolysis and analyzed at the amino acid level using LC-IRMS for $\delta^{13}C$ analysis.

For hydrolysis, approximately 40 mg of pulverized hair was hydrolyzed in 6 M hydrochloric acid for 24 h at 110 °C before evaporating to dryness at 30 °C in a vacuum evaporator (Mivac, Genevac, Ipswich, UK). The dry residue was redissolved in 1 mL Milli-Q water and filtered using a 0.45 μm syringe filter. The filtrates were stored in a 1.5 mL vial in a freezer until required for isotopic analysis. According to a published study,[16] and our own in-house validation, protein hydrolysis under such conditions does not significantly affect the $\delta^{13}C$ values of the recovered amino acids.

2.2 Reagents and Methods

The reagents were of analytical grade or higher purity from Sigma-Aldrich (St. Louis, MO, USA); sodium persulfate (99%), orthophosphoric acid (>85%), sulfuric acid (>95%), L-amino acids (98-99%). Sodium hydroxide (high purity, 50% solution) was purchased from Thermo Scientific. Isotope standards for $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ measurements were purchased from the US Geological Service (USGS) and the International Atomic Energy Agency (IAEA). Reference gases of CO_2 (99.999%), N_2 (99.997%), SO_2 (99.98%), and cylinders of He (99.9999%) and O_2 (99.999%) were purchased from Airgas (Morgantown, WV) and Matheson (Fairmont, WV). Organic solvents for the washing steps including methanol, acetone and chloroform were ACS or HPLC-MS grade. The acids, including HCl and H_2SO_4 were ICP-MS grade. The 18 MΩ Milli-Q water and HPLC mobile phases were be degassed in an ultrasonic vacuum degasser and sparged with an inert gas (UHP He) each morning and during use. Rigorous degassing helps reduce the dissolved CO_2 in the mobile phases and provides the lowest possible background levels in the IRMS.

2.2.1 *EA-IRMS*

Bulk carbon isotope measurements were made on precisely weighed sub-samples of approximately 0.5 mg that were placed in tin capsules in a Flash elemental analyzer (EA, Thermo Scientific, Waltham, MA, USA). The EA was coupled via a Conflo IV interface (Thermo Scientific) to the Delta V Advantage isotope ratio mass spectrometer (Thermo Scientific). Data acquisition was carried out using Isodat 3.0 Software (Thermo Scientific). During both the LC and EA, analyses used ultra high purity gases described in section 2.2. Guidelines for the selection of working standards and a review of strategies to institute universal isotopic referencing procedures have been reported by Werner et al.[68] International standards are available through the National Institute of Standards and Technology (NIST), and through the International Atomic Energy Agency (IAEA) in Vienna, Austria.[28] These standards are Vienna Pee Dee Belemnite (VPDB) for carbon, Vienna Canyon Diablo Troilite meteorite (V-CDT) for sulfur and laboratory air for nitrogen.[69] Because primary standards are expensive and are becoming environmentally depleted, secondary standards are often used in their place, as detailed by Valkiers et al.[70]

After manual verification of appropriate peak integration, peak areas were converted to isotope ratio values on a per mil (‰) scale according to conventional correction methods in the

Isodat software. For example, carbon isotope ratios are reported in per mill (‰) relative to the international standard VPDB (Vienna Pee Dee Belemnite) according to the following equation:

$$\delta\ (‰) = 1000(R_{sample} - R_{standard}) / R_{standard} \quad (1)$$

All measurements were measured relative to reference compressed gasses and converted to the relevant scales using at least two-point calibration curves. The reference cylinder of CO_2 gas was calibrated using USGS40 (L-glutamic acid, $\delta^{13}C$ = -26.389‰, 95% CI = 0.05‰), USGS41 (L-glutamic acid, $\delta^{13}C$ = 37.63‰, 95% CI = 0.05‰), USGS42 (Tibetan human hair, $\delta^{13}C$ = -21.09‰, 95% CI = 0.05‰) and USGS43 (Indian human hair, $\delta^{13}C$ = -21.28‰, 95% CI = 0.05‰) (NIST, Gaithersburg, MA). The SO_2 cylinder was calibrated against silver sulfide isotope standards IAEA-S-1 ($\delta^{34}S$ = -0.3‰, SD = 0.2‰) and IAEA-S-3 ($\delta^{34}S$ = -32.3‰, SD = 0.2‰). The N_2 cylinder was calibrated against USGS42 ($\delta^{15}N$ = 8.05‰, 95% CI = 0.05‰) and USGS41 ($\delta^{15}N$ = 47.57‰, 95% CI = 0.05‰). Although we had originally planned to run IAEA isotope standard of caffeine (IAEA-600, $\delta^{13}C$ = -27.771‰) to ensure the relative and absolute accuracy of the two measurement systems, this was not conducted because caffeine was found to undergo fractionation in the wet chemical oxidation step.

2.2.2 *LC-IRMS*

The LC-IRMS system was a Dionex ICS5000 ion chromatography system (ICS) (Dionex, Sunnyvale, CA, USA) coupled through an interface (LC Isolink, Thermo Scientific, Bremen, Germany) to the IRMS. The LC system was operated under the same conditions as before.[60, 63] Isotope measurements were measured relative to tank CO_2, and normalized relative to a two-point calbibration curve based on USGS41 and USGS42 (L-glutamic acids, described above in 2.2.1). the Underivatized amino acids were separated on a mixed-mode column (Primesep A, 2.1 x 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 followed by a gradient to 0.03 M sulfuric acid. The flow rate of the mobile phase was 260 µL/min. The eluent from the HPLC column passed into the LC Isolink where all the carbon-containing compounds were quantitatively oxidized to carbon dioxide (CO_2). Oxidation takes 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 quantitatively from the cooled solution by a membrane and transferred to the IRMS system via a split flow for analysis. An example of

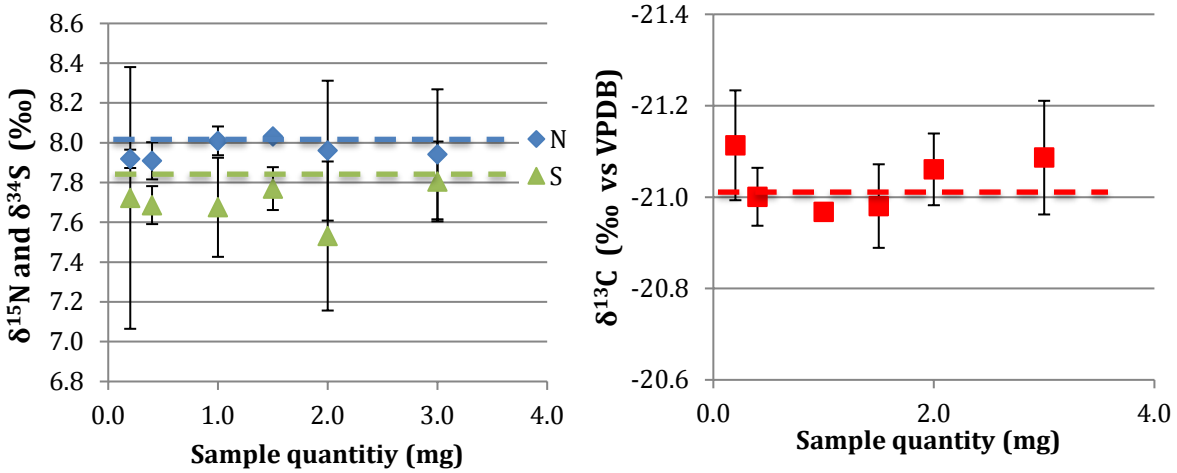

Figure 1. Measured delta values for different quantities (0.2–3 mg) of USGS42, Tibetan human hair ($\delta^{15}N$ = 8.05‰, $\delta^{13}C$ = -21.09‰, $\delta^{32}S$ = 7.84‰), measured on the EA-IRMS. Error bars show 1 s.d., N=5.

the resulting LC-IRMS chromatogram is shown in *Figure 2*. All amino acids except Leu/Ile were baseline resolved.

3 Results

3.1 Bulk IRMS: Contribution to a new global database of hair and nail isotope ratios for carbon and nitrogen

In the first stages of this work, we contributed our existing data on the bulk isotope ratios of Jordanian hair and included the new bulk isotope ratios for the US subjects. Our bulk data was combined with existing and other new EA-IRMS data for the US population and then incorporated into the global database.[71] This database of approximately 3500 individual samples was a collaboration with many different groups and was spearheaded by Dr. Hülsemann in Germany. Approximately 58% of the samples in the global database are from Europe and North America (Table 1). About one fourth of the samples originate from the USA. In contrast, the amount of data for Asia and Africa is considerably less. Approximately 60% of the world population is located in Asia, but only 17% of the isotope data are from Asian people.

Table 1. Continental distribution of available carbon and nitrogen isotope data for hair and nails. Table reproduced from reference 71.

Continent	Population *	$\delta^{13}C$		$\delta^{15}N$	
	%	n	(%)	n	(%)
Europe	10	1221	(31)	1200	(30)
North America	8	1114	(28)	1082	(27)
South America	6	813	(20)	809	(20)
Asia	60	686	(17)	728	(18)
Africa	16	58	(1)	69	(2)
Australia	1	108	(3)	101	(3)
total		4000		3989	

* Population percentage calculated from [22]

Our data contributed to this part of the global database

3.1.1 *NIJ-Funded Contribution to Global database*

Our contribution to the study included the bulk isotope analysis of hair from a cohort of 84 subjects from different regions in Jordan. The hair was processed according to the procedure in section 2.2.1, above. A total of 422 measurements (average of 4.5 measurements per subject) was compiled in a variety of ways, as shown in Tables 2, 3, and 4, and the results were consolidated with other data by Dr. Hülsemann and our team.

Table 2. Bulk IRMS measurements of 97 Jordanian hair samples. (average of 4.5 measurements per subject).

Descriptive Statistics

	N	Minimum	Maximum	**Mean**		Std. Deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
Delta^{15}N	422	6.96	10.15	**8.29**	.022	.44
Delta^{13}C	422	-22.06	-15.72	**-19.87**	.052	1.06
Delta^{34}S	422	3.64	12.69	**7.63**	.064	1.31
Valid N (listwise)	422					

Table 3. Bulk IRMS measurements of 97 Jordanian hair samples categorized by sex: 19 Males, 78 measurements (average of 4.1 measurements per subject); 75 females, 344 measurements (average of 4.6 measurements per subject).

Sex		Delta^{15}N	Delta^{13}C	Delta^{34}S
	Mean	**8.36**	**-19.59781**	**7.22838**
Male	N	78	78	78
	Std. Deviation	0.39	0.884107	1.291052
	Mean	**8.28**	**-19.93**	**7.72**
Female	N	344	344	344
	Std. Deviation	0.45	1.09	1.30
	Mean	8.29	-19.87	7.63
Total	N	422	422	422
	Std. Deviation	0.44	1.06	1.3

Table 4. Bulk IRMS measurements of 97 Jordanian hair samples categorized by region of origin* within Jordan: 64 subjects from the Mountain region, 17 subjects from the Valley Region, 8 subjects from the Desert Region, 5 subjects from unknown region within Jordan.

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Region		Delta^{15}N	Delta^{13}C	Delta^{34}S
Mountains	Mean	**8.27**	**-20.13**	**7.82**
	N	302	302	302
	Std. Deviation	0.42	1.0	1.2
Jordan Valley	Mean	**8.34**	**-19.04**	**7.18**
	N	68	68	68
	Std. Deviation	0.61	0.86	1.46
Eastern Desert	Mean	**8.41**	**-19.63**	**6.88**
	N	32	32	32
	Std. Deviation	0.25	0.71	1.40
Total	Mean	8.29[$]	-19.91[$]	7.64[$]
	N	402	402	402
	Std. Deviation	.45	1.06	1.32

[$]this mean may be different from actual total mean provided above because 5 subjects are excluded from this total because of their unknown residence within Jordan.
*see http://www.kinghussein.gov.jo/geo_env1.html for details

In addition to the large Jordanian data set, we also provided data from the first 6 US subjects to the global database. We did not have time to include more data from US subjects before the manuscript was submitted for publication.

Table 4. Bulk IRMS measurements of 6 US hair samples (from Southeastern Ohio). Total of 24 measurements (4 per person). For this data set, ratios were normalized to international scale with 2-point calibration curve using USGS 40 & 41.

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
^{13}C	24	-21.864	-17.784	-19.88	1.57
^{15}N	24	10.310	12.872	11.25	.52
Valid N (listwise)	24				

The global database is summarized in map-format can be seen in Figures 2 and 3, which are taken from the published manuscript. The countries without a colour code (i.e. white) do not have any public available information about contemporary human isotope data. Not every country shown in colour had a sufficient a number of experimental samples, which is why we created country clusters to be able to incorporate such samples into the data set. For Africa, for example, so few samples have been analysed that only four-country clusters were created, which stretch across huge regions. It is debatable if such large regions are reasonable for creating a geographically distinct data set, but these were the smallest geographic areas that could be formed

from the aggregate data to provide a reasonable degree of reliability. A similar situation is present for large countries like Russia, the USA, Canada, and China. There are large differences in the isotopic data of humans from different locations, and it is reasonable to assume that that there are geospatial differences within these countries, too.

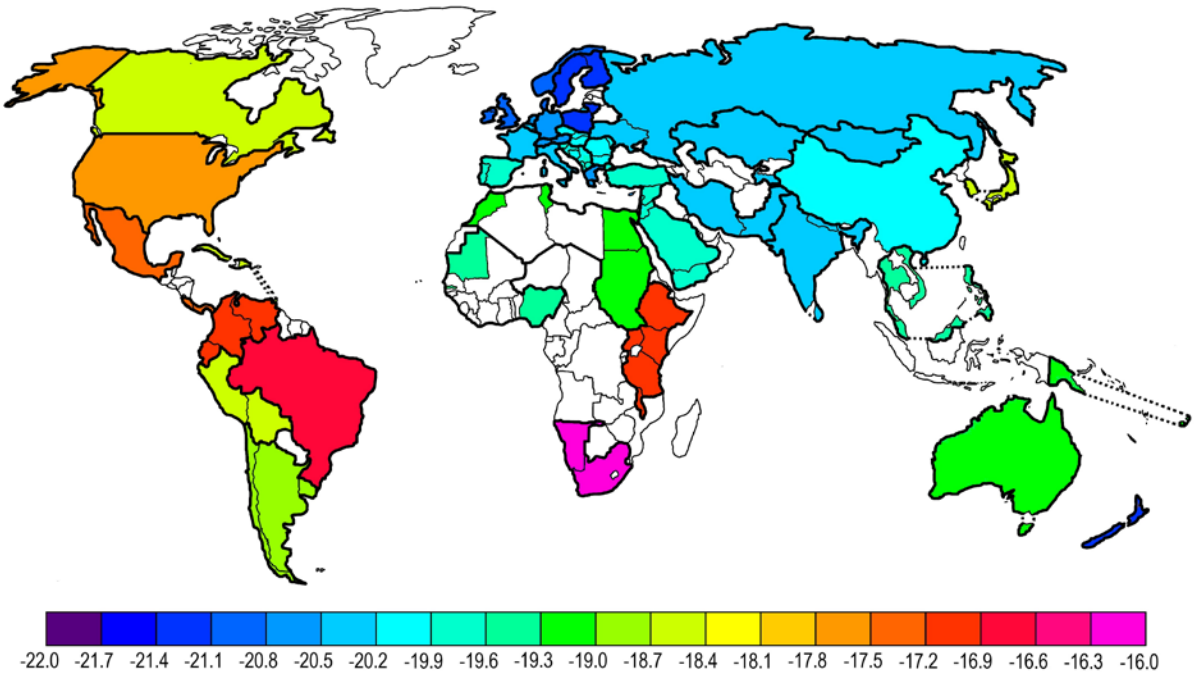


Figure. 2. Global spatial distribution of natural stable carbon isotope ratios of contemporary human hair and nails. For countries marked white no data are available. Solid black country borders indicate individual countries and country cluster for which isotope data were summarized. Figure reproduced from reference 71.

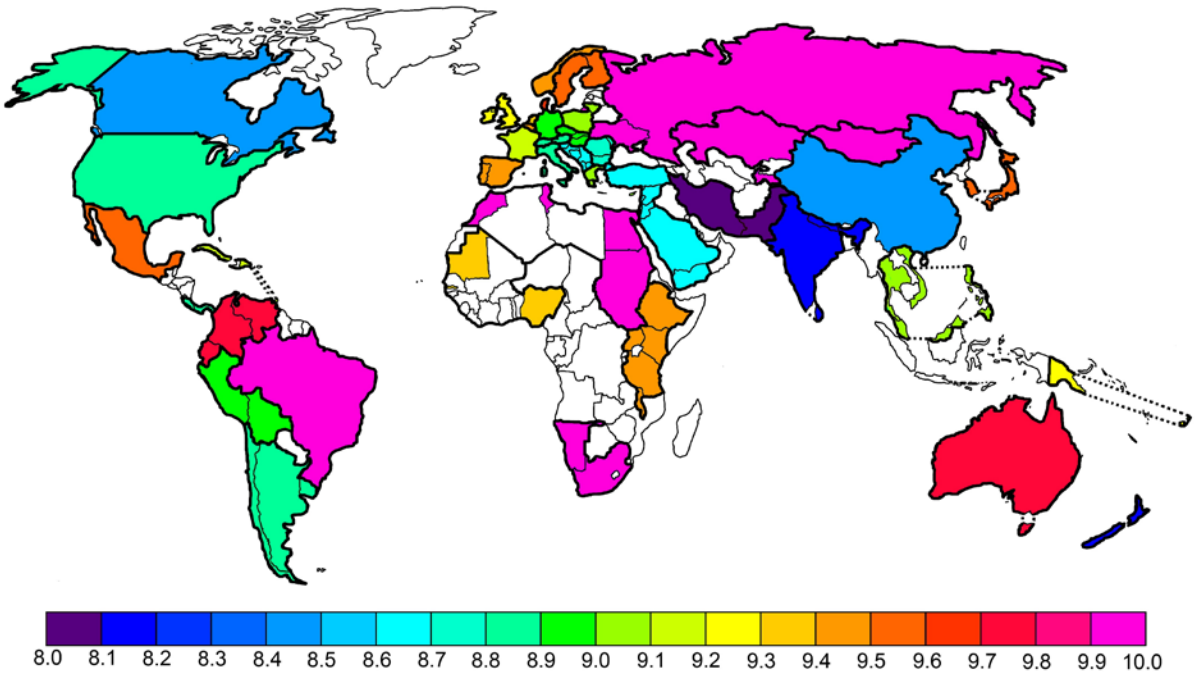


Figure 3. Global spatial distribution of natural stable nitrogen isotope ratios of contemporary human hair and nails. For countries marked white no data are available. Solid black country borders indicate individual countries and country cluster for which isotope data were summarized. Figure reproduced from reference 71.

For human identification or other purposes, human hair and nail samples are used for stable isotope analysis among other matrices like teeth or bone. In order to compare $\delta^{13}C$ and $\delta^{15}N$ analytical results for different matrices, isotopic differences between these matrices have to be taken into account. Our results confirm the fact that $\delta^{13}C$ values of human nail are lower compared to hair and nail $\delta^{15}N$ values are higher compared to hair.[19,20] The differences in isotope ratios between hair and nail are attributed to differing amino acid compositions as well as to potentially different amino acid turnover in hair follicle and the nail matrix, although the exact mechanisms remain unclear.[19] Because most of the published carbon and nitrogen isotope data on contemporary ('alive') humans are from the analysis of hair, the data set of this study was referenced to human hair isotope ratios. Any nail isotope data was corrected by constant factors (described in the manuscript) for inclusion. If this data set should be used for the interpretation of $\delta^{13}C$ and $\delta^{15}N$ values of other human matrices like bone, blood cells, etc. different correction factors would have to be applied.

3.1.2 *Spatial distribution of isotope ratios*

It has been reported for smaller data sets that modern human carbon isotope ratios change significantly with latitude,[17] which is related to the decrease in C_4 vegetation with increasing latitude.[48] This can be observed for our compiled dataset, wherein the $\delta^{13}C$ country mean values typically decrease (i.e. more negative) nearer the poles (Fig. 2). Clearly, latitude alone is not the only variable for explaining the trend in modern human $\delta^{13}C$ values. Regions like the USA or Southern Africa show larger (less negative) $\delta^{13}C$ values than other countries on the same latitude.

The highest $\delta^{13}C$ values are found for regions like Southern Africa and Brazil, which are influenced by the dominant use of C_4 plants as dietary source for humans and animals. Besides the influence of dietary C_4 plants, the consumption of maritime food leads to increased $\delta^{13}C$ values in modern humans.[6,14,48] This has been demonstrated by the analysis of fingernails and hair of indigenous populations in Northern Greenland and Southwest Alaska consuming high amounts of maritime food.[14,49] However, such high amounts of maritime food in the modern human diet are exceptional and the observed spatial distribution of human $\delta^{13}C$ values in this data set can primarily attributed to the proportion of C_3 and C_4 plants in the human diet.

Several factors have been identified to influence human $\delta^{15}N$ values: the amount of marine food, terrestrial meat, and animal products in the diet,[6,9-14,24] physiological situations[26,27] and disease, illness or malnutrition.[29,50,51] As the $\delta^{15}N$ value of human hair might be influenced by such a multitude of factors, generalised assumptions explaining the spatial distribution of human nitrogen isotope ratios are difficult. There is no correlation between latitude and country $\delta^{15}N$ mean values; enriched $\delta^{15}N$ values are found near the equator as well as in higher latitudes. Small amounts of fish and meat and also the consumption of pulses might be the cause for the ^{15}N depleted hair samples from the Indian subcontinent, including Iran and Pakistan. However this is not the case for other countries, like Africa, with typically scant meat and fish consumption but ^{15}N enriched values. Additional factors known to influence human dietary $\delta^{15}N$ values are climate and the use of agricultural fertilizers.

Temperature and precipitation also have an influence on plant $\delta^{15}N$ values, with enriched $\delta^{15}N$ values for plants in hot and arid climates and depleted $\delta^{15}N$ values for cold and humid regions.[52] Fertilization is another factor with a huge influence on plant $\delta^{15}N$ values: fertilization with animal derived manure leads to highly enriched $\delta^{15}N$ values for plants and crops, whereas the use of synthetic fertilizer leads to depleted $\delta^{15}N$ values of the agricultural product.[53,54] As a conclusion, human hair $\delta^{15}N$ values seem to be a result of different influences: dietary preferences and food supply, physiology and metabolism, climate and fertilization methods.

3.2 Biometric Traits from the bulk isotope ratio analysis of human hair (Jordanian hair)

For a subset of the Jordanian hairs samples, we had extensive questionnaire data that enabled us to investigate the ability to classify the hair donors into groups according to biometric traits. The results of this work have appeared in *Science and Justice*.[72] A summary of those findings is provided, below. A summary of the subject volunteers is provided in Table 4.

Table 4. 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 (65 female, 14 female)	Mean	27	25.2	8.29	-19.87	7.63
	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	Mean	34.1	24.8	8.04	-21.00	8.23

20 female subjects	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

Tables 5 and 6 provide summary bulk isotope data and MANOVA results, respectively, using body mass index as the fixed factor. The results show that when the hair donors are categorized into 4 broad BMI groups, the within group variance for $\partial^{13}C$ and $\partial^{15}N$ are significantly smaller than the between-group variance, which indicates that bulk isotopes may offer some degree of classification. $\partial^{34}S$ ratios were not significantly different between different BMI groups. Table 7 shows the results of the attempt to classify the subjects into BMI groups, but the classification rate was only successful 47% (biased prediction) or 44% (unbiased, leave-one-out cross-validation) of the time.

Table 5. $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ isotope ratio values of bulk hair samples from twenty female subjects in the Jordanian database using EA-IRMS (n=5 for each subject, so N=25 implies 5 subjects). The inclusion of replicate measurements for each individual does not constitute true independent measures of the variables with respect to each grouping factor, but captures the variance within the individuals.

	BMI	**N**	**Mean**	**Std. Dev.**
∂^{15}**N**	<= 20	25	8.023	0.24
	20-23	30	8.090	0.30
	23-30	20	8.273	0.35
	30+	25	7.833	0.47
	Total	100	8.045	0.37
∂^{13}**C**	<= 20	25	-20.821	0.36
	20-23	30	-20.951	0.37
	23-30	20	-21.279	0.27
	30+	25	-20.998	0.55
	Total	100	-20.996	0.43
∂^{34}**S**	<= 20	25	8.174	0.96
	20-23	30	8.280	0.50
	23-30	20	8.076	0.43
	30+	25	8.370	0.60
	Total	100	8.235	0.65

Table 6. MANOVA results of the same data in Table 5.

		Sum of Squares	df	Mean Square	F	Sig.
∂^{15}N * BMI	Between Groups	2.2	3	0.74	6.18	.001
	Within Groups	11.5	96	0.12		
	Total	13.8	99			
∂^{13}C * BMI	Between Groups	2.42	3	0.80	4.91	.003
	Within Groups	15.7	96	0.16		
	Total	18.2	99			
∂^{34}S * BMI	Between Groups	1.1	3	0.37	0.86	.463
	Within Groups	41.2	96	0.43		
	Total	42.3	99			

Table 7. Results of discriminant analysis (classification) of subjects according to BMI groups for twenty female subjects from the Jordanian database. The inclusion of replicate measurements for each individual does not constitute true independent measures of the variables with respect to each grouping factor, but does capture the measurement variance.[a,b]

		BMI (Binned)	Predicted Group Membership				
			<=20	20-23	23-30	>30	Total
Original	Count	<= 20.00	12	2	4	7	25
		20.01 - 23.00	5	11	8	6	30
		23.01 - 30.00	0	1	15	4	20
		30.01+	8	8	0	9	25
	%	<= 20.00	48.0	8.0	16.0	28.0	100.0
		20.01 - 23.00	16.7	36.7	26.7	20.0	100.0
		23.01 - 30.00	0.0	5.0	75.0	20.0	100.0
		30.01+	32.0	32.0	0.0	36.0	100.0
Cross-validated[c]	Count	<= 20.00	11	3	4	7	25
		20.01 - 23.00	5	10	8	7	30
		23.01 - 30.00	0	2	14	4	20
		30.01+	8	8	0	9	25
	%	<= 20.00	44.0	12.0	16.0	28.0	100.0
		20.01 - 23.00	16.7	33.3	26.7	23.3	100.0
		23.01 - 30.00	0.0	10.0	70.0	20.0	100.0
		30.01+	32.0	32.0	0.0	36.0	100.0

a. 47.0% of original grouped cases correctly classified.
b. 44.0% of cross-validated grouped cases correctly classified.
c. 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.

Our publication in *Science and Justice* highlights the limitations of bulk IRMS measurements, which is why we originally proposed to investigate amino-acid isotope ratio measurements, as described below.

3.3 Biometric Traits from the compound-specific isotope ratio analysis of human hair (Jordanian hair)

Figure 4A shows the different isotopes of CO_2 measured as a function of time during an LC separation when a standard mixture of amino acids was separated using our optimized method. Ref CO_2 Figure 4B is a chromatogram of a human hair hydrolysate using the same conditions as LC as the standard amino acid mixture in 4A. Although Leu/Ile cannot be baseline resolved in either chromatogram, the isotope values derived from the co-eluting Leu/Ile peaks was 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.

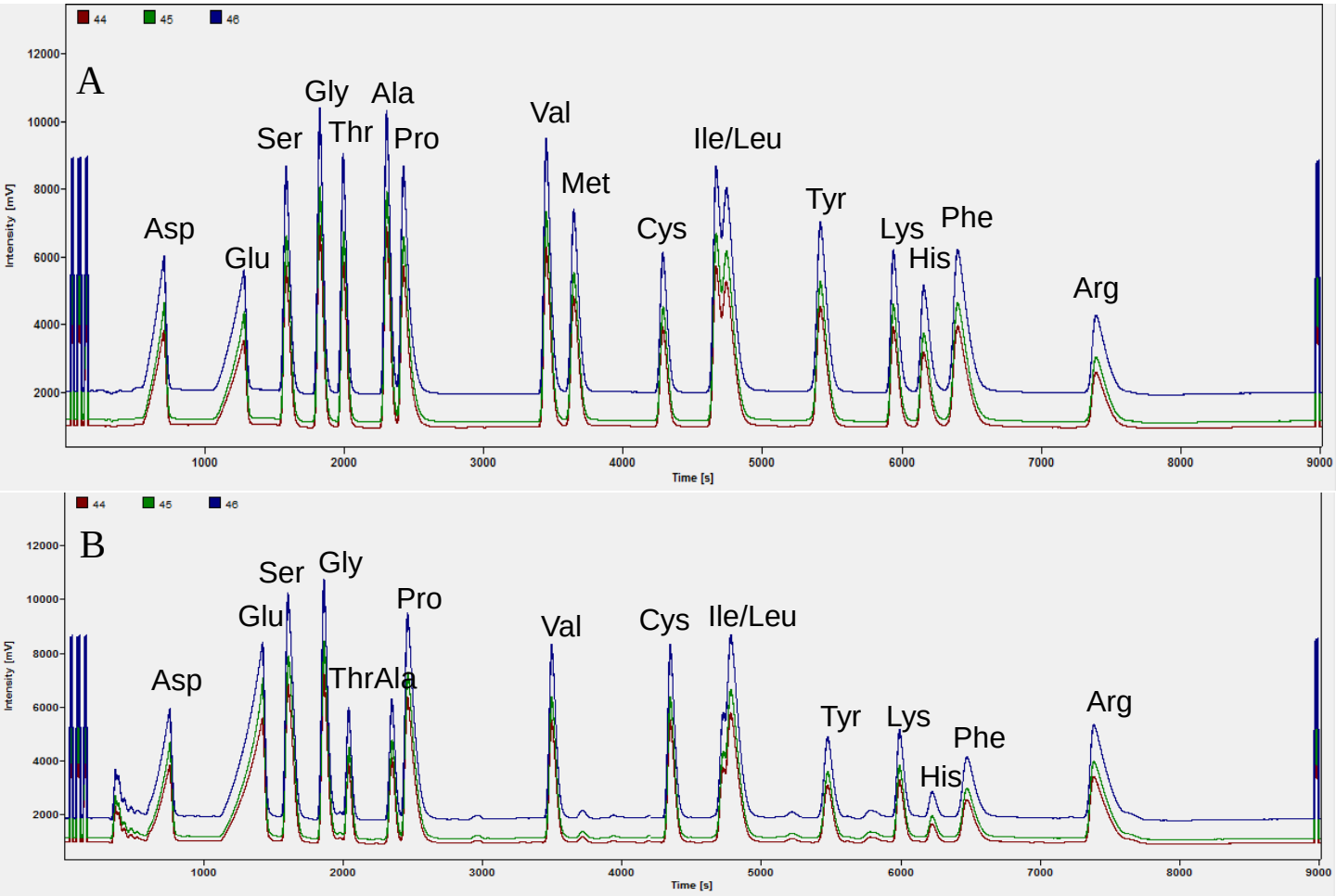


Figure 4. Example of ion chromatograms of 16 amino acids acquired on the LC-IRMS system using a mixed-phase Primesep A column and no organic modifiers: A) Standard free amino acids; B) From a hair hydrolysate. Only Leu and Ile were not baseline resolved. Although Met was resolved, it was of too low abundance in hair hydrolysates to be useful.

Exploratory data analysis of the amino-acid specific isotope ratios was performed using principal component analysis (PCA) and MANOVA. Details are provided in Table 8 and Figure 5.

Table 8. One-way ANOVA results of four different groupings of people using body mass index (BMI) as the fixed factor. All twenty subjects were females from Jordan and were grouped the same as the Tables S.6, S.7, and S.8. N=3 for each subject.

	F	Sig.
Asp	2.22	9.5×10^{-2}
Glu	3.00	3.8×10^{-2}
Ser	0.58	6.3×10^{-1}
Thr	14.51	4.1×10^{-7}
Gly	6.18	1.0×10^{-3}
Ala	8.27	1.2×10^{-4}
Pro	9.32	4.3×10^{-5}
Val	2.79	4.9×10^{-2}
Cys	17.13	5.2×10^{-8}
Leu/Ile	5.21	3.0×10^{-3}
Tyr	4.62	5.9×10^{-3}
Lys	3.80	1.5×10^{-2}
His	5.85	1.5×10^{-3}
Phe	3.03	3.7×10^{-2}
Arg	1.72	1.7×10^{-2}

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.

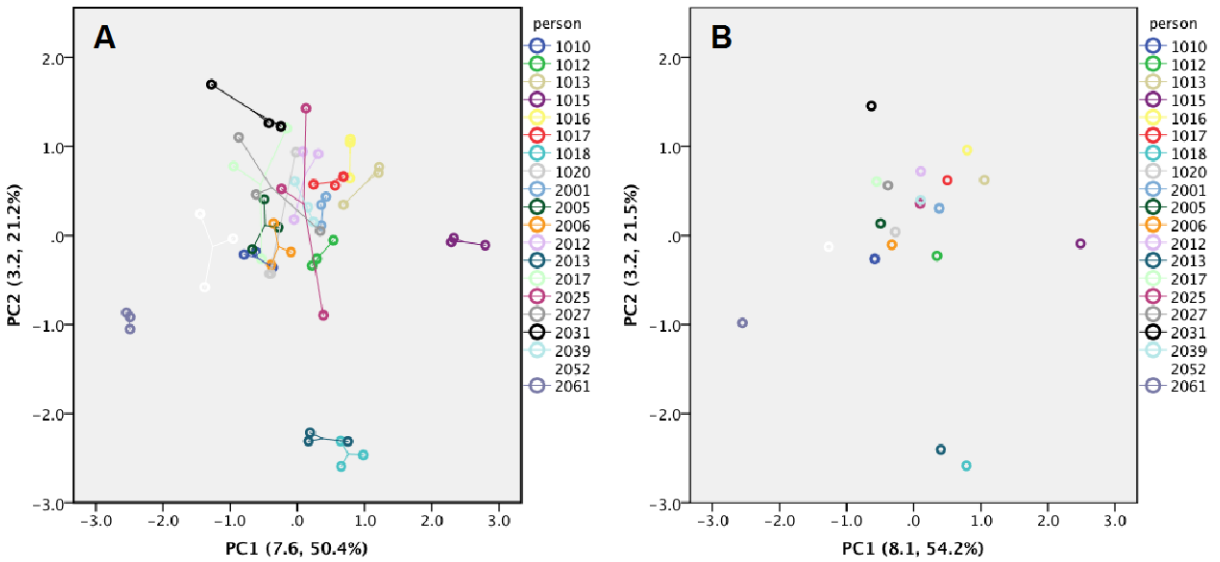


Figure 5. Principal component analysis (PCA) plots for twenty female subjects using isotope ratios of sixteen peaks (representing seventeen amino acids) as input data: A) used triplicate measurements for each person; B) used averaged-within-person data. The absolute and percent variance explained by the first two components with Eigenvalues >1 are shown in the axis titles.

The results of PCA analysis of seventeen amino acids (in sixteen peaks) are shown in Figure 5. 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 or 76% of the total variance, respectively. The Varimax rotated component coefficients shown in Tables 9 and 10 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 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).

Table 9. Rotated Component Matrix for not-averaged data.[a] (N=60 for 20 persons)

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

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

Table 10. 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	
[b]Tyr		.972
[b]Lys		.954
[b]His		.938
[b]Phe		.923
[b]Leu/Ile	.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

3.3.1 *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. Thr and Cys gave the largest F values ($p = 4x10^{-7}$ and $5x10^{-8}$, respectively), suggesting that they may be the most useful in terms of classifying according to BMI. 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 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 ±0.66‰) was different from BMI below 20 group ($\delta^{13}C$ = -8.40 ±0.86‰), BMI 20-23.99 group ($\delta^{13}C$ = -8.52 ±0.34‰) and BMI 24-30 group ($\delta^{13}C$ = -8.67 ±0.90‰) at the 99.9% CI ($p = 6.10x10^{-5}$; $4.41x10^{-6}$; $4.36x10^{-6}$ 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 11 and graphically in Figure 6 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.

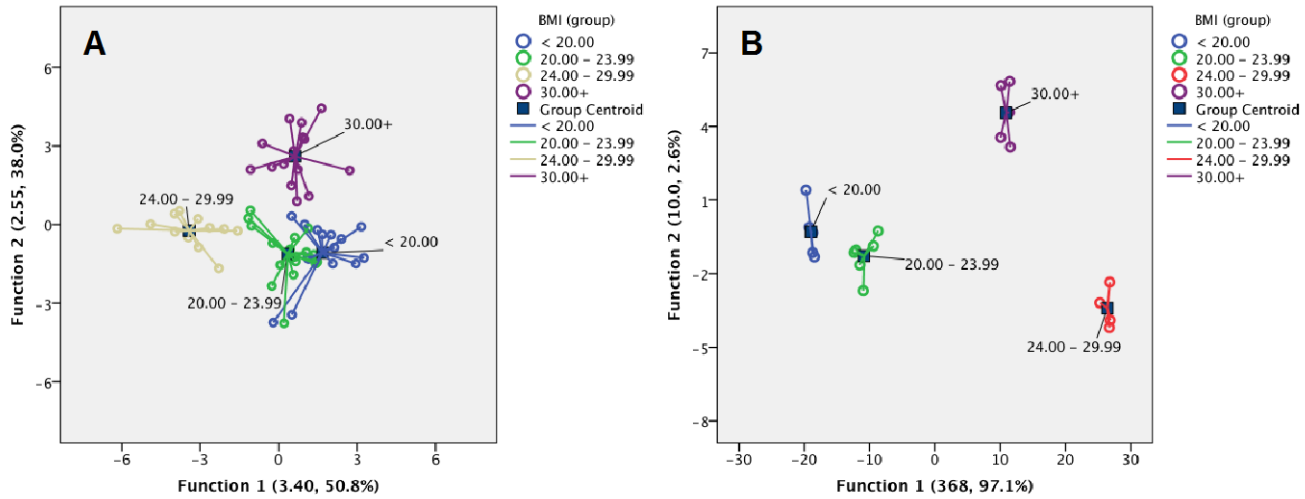


Figure 6. Canonical discriminant analysis (CDA) plots for twenty female subjects using isotope ratios of sixteen peaks (representing seventeen amino acids) as input data and BMI group as the classification factor: A) triplicate measurements for each person; B) used averaged-within-person data. The absolute and percent variance explained by the first two functions with Eigenvalues >1 are shown in the axis titles.

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. 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.

Amino acid specific $\delta^{13}C$ values were determined using the proposed LC-IRMS method for triplicate measurements of 20 female test subjects from Jordan (summary results are shown in the supporting data section). One-way ANOVA was performed for each amino acid to assess the within-group variance to between-group variance with body mass index (BMI) as the fixed factor (group). Cysteine and threonine had the largest F values and are the most discriminating amino acids, and all the amino acids except for serine provided significantly different F values. Post-hoc pair-wise comparisons using Tukey's HSD showed that the $\delta^{13}C$ values for threonine in subjects with BMI>30 were always significantly different (less negative) than subjects from the other

groups. The mean $\delta^{13}C$ value for the BMI>30 group was -7.16±0.66‰, whereas the means for the other groups were $\delta^{13}C_{(BMI<20)}$ = -8.40±0.86‰, $\delta^{13}C_{(20<BMI<24)}$ = -8.52±0.34‰, $\delta^{13}C_{(24<BMI<30)}$ = -8.67±0.90‰. These results show that for this small test group, we can measure significant differences in the $\delta^{13}C$ values of amino acids between severely obese subjects and the general population.

Table 11. Classification results for CDA of twenty female subjects into four broad body mass index (BMI) groups. The original data is the average of three replicate measurements and comprises sixteen isotope ratio values of seventeen amino acids and three replicates from each individual.[a,b]

		BMI (group)	Predicted Group Membership				Total
			< 20.00	20.00 - 23.99	24.00 - 29.99	30.00+	
Original	Count	< 20.00	5	0	0	0	5
		20.00 - 23.99	0	6	0	0	6
		24.00 - 29.99	0	0	4	0	4
		30.00+	0	0	0	5	5
	%	< 20.00	100.0	.0	.0	.0	100.0
		20.00 - 23.99	.0	100.0	.0	.0	100.0
		24.00 - 29.99	.0	.0	100.0	.0	100.0
		30.00+	.0	.0	.0	100.0	100.0
Cross-validated[c]	Count	< 20.00	3	2	0	0	5
		20.00 - 23.99	1	5	0	0	6
		24.00 - 29.99	0	0	3	1	4
		30.00+	0	0	0	5	5
	%	< 20.00	60.0	40.0	.0	.0	100.0
		20.00 - 23.99	16.7	83.3	.0	.0	100.0
		24.00 - 29.99	.0	.0	75.0	25.0	100.0
		30.00+	.0	.0	.0	100.0	100.0

a. 100.0% of original grouped cases correctly classified.
b. 80.0% of cross-validated grouped cases correctly classified.
c. 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.

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 Figure 7 and also provided in the supplemental material (Tables 12 and 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 of ~30 measurements per group (N~90) would resolve this question.

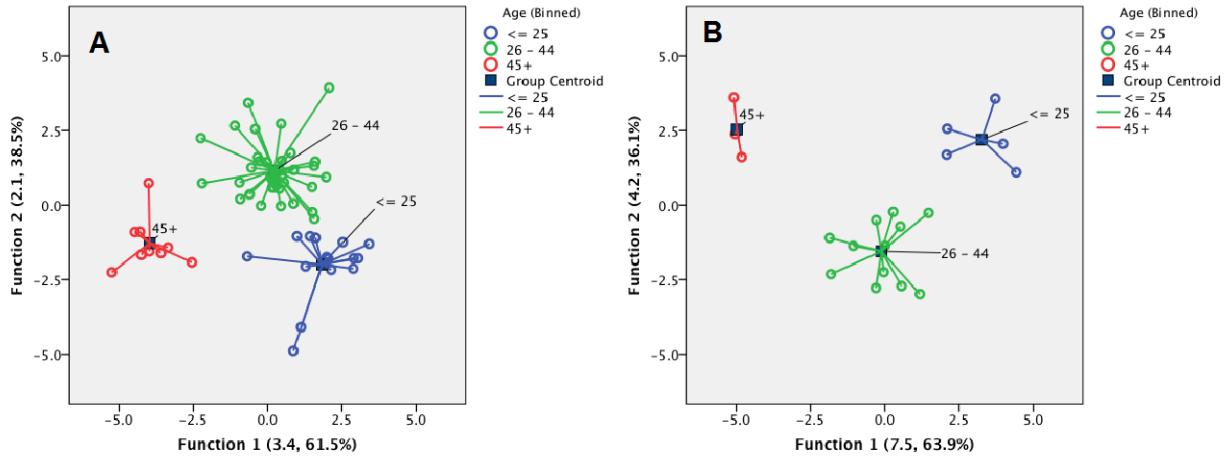


Figure 7. Canonical discriminant analysis (CDA) plots for twenty female subjects using isotope ratios of sixteen peaks (representing seventeen amino acids) as input data and age group as the classification factor: A) triplicate measurements for each person; B) used averaged-within-person data. The absolute and percent variance explained by the first two functions with Eigenvalues >1 are shown in the axis titles.

Table 12. Classification results for CDA of twenty female subjects into three broad age groups groups. The original data includes three replicate measurements for each subject and comprises sixteen isotope ratio values of seventeen amino acids and three replicates from each individual.[a,b]

Age (Binned)			Predicted Group Membership			Total
			<= 25	26 - 44	45+	
Original	Count	<= 25	15	0	0	15
		26 - 44	2	34	0	36
		45+	0	0	9	9
	%	<= 25	100.0	.0	.0	100.0
		26 - 44	5.6	94.4	.0	100.0
		45+	.0	.0	100.0	100.0
Cross-validated[c]	Count	<= 25	0	1	14	15
		26 - 44	1	6	29	36
		45+	0	9	0	9
	%	<= 25	.0	6.7	93.3	100.0
		26 - 44	2.8	16.7	80.6	100.0
		45+	.0	100.0	.0	100.0

a. 96.7% of original grouped cases correctly classified.
b. 10.0% of cross-validated grouped cases correctly classified.
c. 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.

Table 13. Classification results for CDA of twenty female subjects into three broad age groups groups. The original data includes the average of three replicate measurements for each subject and comprises sixteen isotope ratio values of seventeen amino acids.[a,b]

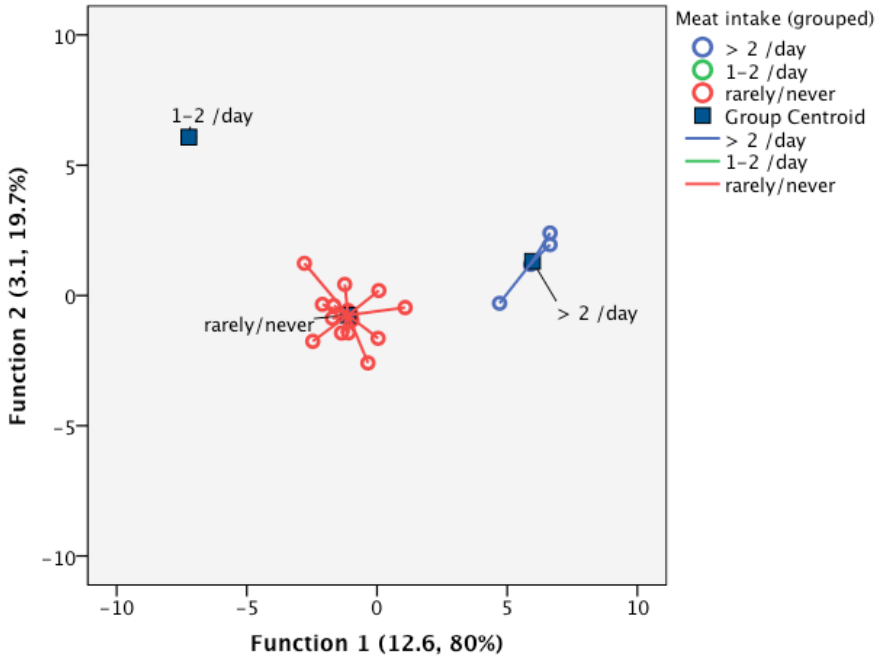
Age (Binned)			Predicted Group Membership			Total
			<= 25	26 - 44	45+	
Original	Count	<= 25	5	0	0	5
		26 - 44	0	12	0	12
		45+	0	0	3	3
	%	<= 25	100.0	.0	.0	100.0
		26 - 44	.0	100.0	.0	100.0
		45+	.0	.0	100.0	100.0
Cross-validated[c]	Count	<= 25	1	1	3	5
		26 - 44	0	2	10	12
		45+	1	0	2	3
	%	<= 25	20.0	20.0	60.0	100.0
		26 - 44	.0	16.7	83.3	100.0
		45+	33.3	.0	66.7	100.0

a. 100.0% of original grouped cases correctly classified.
b. 25.0% of cross-validated grouped cases correctly classified.
c. 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.

3.3.3 *Classification by diet*

Figure 8 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 non-averaged data provided leave-one-out cross-validation success rate of 85%, which is considerably better. 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.

Figure 8. Classification results for CDA of twenty female subjects into groups according to meat consumption The original data includes the average of three replicate measurements for each subject and comprises sixteen isotope ratio values of seventeen amino acids.[b,c]



Classification Results[a,c]

		Meat (grouped)	Predicted Group Membership > 2 /day	1-2 /day	rarely/never	Total
Original	Count	> 2 /day	4	0	0	4
		1-2 /day	0	1	0	1
		rarely/never	0	0	15	15
	%	> 2 /day	100.0	.0	.0	100.0
		1-2 /day	.0	100.0	.0	100.0
		rarely/never	.0	.0	100.0	100.0
Cross-validated[b]	Count	> 2 /day	2	0	2	4
		1-2 /day	0	0	1	1
		rarely/never	4	5	6	15
	%	> 2 /day	50.0	.0	50.0	100.0
		1-2 /day	.0	.0	100.0	100.0
		rarely/never	26.7	33.3	40.0	100.0

a. 100.0% of original grouped cases correctly classified.

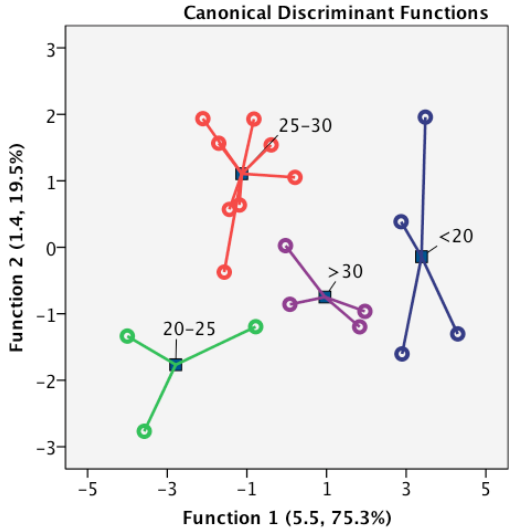
b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

c. 40.0% of cross-validated grouped cases correctly classified.

3.4 Compound-specific isotope ratio analysis of amino acids in human hair (US hair)

We have so far only been able to acquire and work-up the compound-specific IRMS data on the first 20 US subjects. These included 10 male subjects and 10 female subjects. We have performed a variety of multivariate statistics to evaluate and interpret the results, and we have compared the 20 US subjects to our original database of 20 Jordanian subjects. However, the preliminary results are very promising.

Initially, we ran one-way ANOVA followed by Tukey's HSD for each classification factor to reveal which amino acids are most significant for each factor. We then applied linear discriminant analysis (LDA) as a classification tool. Based on original rules (a biased approach), US subjects were correctly classified with probabilities of 100% (BMI, sex and age) using all the measured amino acids. However, based on an unbiased (leave-one-out cross-validation) approach, classification results were significantly reduced, as one might expect for a relatively small number of samples per class. See Figure 9 for details.



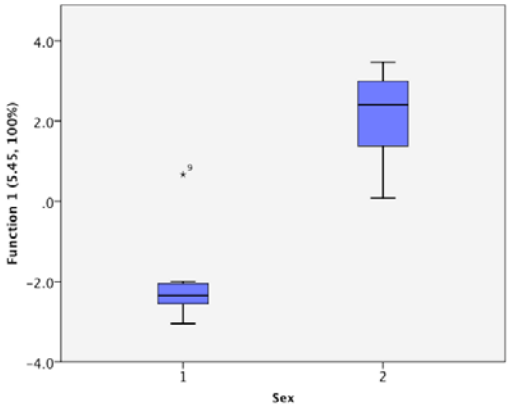
Classification Results[a,c]

		BMI group	Predicted Group Membership <20	20-25	25-30	>30	Total
Original	Count	<20	4	0	0	0	4
		20-25	0	3	0	0	3
		25-30	0	0	8	0	8
		>30	0	0	0	4	4
	%	<20	100.0	.0	.0	.0	100.0
		20-25	.0	100.0	.0	.0	100.0
		25-30	.0	.0	100.0	.0	100.0
		>30	.0	.0	.0	100.0	100.0
Cross-validated[b]	Count	<20	1	0	1	2	4
		20-25	0	2	1	0	3
		25-30	0	2	5	1	8
		>30	2	0	2	0	4
	%	<20	25.0	.0	25.0	50.0	100.0
		20-25	.0	66.7	33.3	.0	100.0
		25-30	.0	25.0	62.5	12.5	100.0
		>30	50.0	.0	50.0	.0	100.0

a. 100.0% 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. 42.1% of cross-validated grouped cases correctly classified.

Figure 9. Discriminant analysis results for classifying US subjects into groups according to self-reported body mass indexes (BMI) using the $\delta^{13}C$ values of eight amino acids, ASP, GLU, THR, ALA, VAL, HIS, PHE, and ARG.

Using ANOVA to reject the least discriminating amino acids, leave-one-out cross validation (unbiased) and original rules (biased) both determine sex group with 90% accuracy (Figure 10). In a similar approach, USA hair could be discriminated from Jordanian hair with more than 75% certainty using leave-one-out cross-validation (Figure 11). These results demonstrate that compound-specific isotope ratio analysis combined with multivariate statistics provides a potential forensic tool to provide soft biometric traits and investigative leads beyond diet.



Classification Results[a,c]

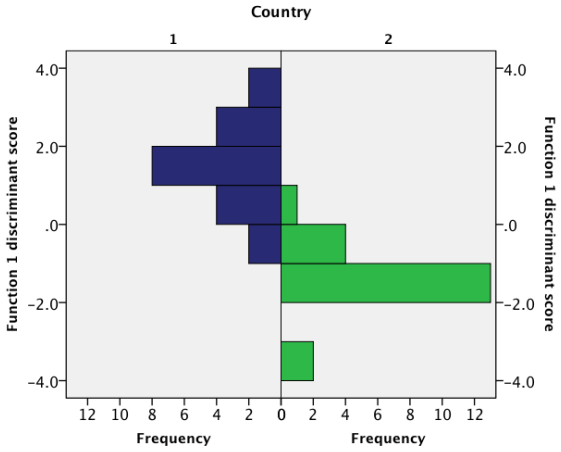
		Sex	Predicted Group Membership 1	2	Total
Original	Count	1	9	1	10
		2	1	9	10
	%	1	90.0	10.0	100.0
		2	10.0	90.0	100.0
Cross-validated[b]	Count	1	9	1	10
		2	1	9	10
	%	1	90.0	10.0	100.0
		2	10.0	90.0	100.0

a. 90.0% 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. 90.0% of cross-validated grouped cases correctly classified.

Figure 10. Discriminant analysis results for classifying US subjects into groups by sex using the δ^{13}C values of eight amino acids, ASP, GLU, VAL, ILE/LEU, TYR, HIS, PHE, and ARG.



Classification Results[a,c]

		Country	Predicted Group Membership 1	2	Total
Original	Count	1	17	3	20
		2	1	19	20
		Ungrouped cases	0	1	1
	%	1	85.0	15.0	100.0
		2	5.0	95.0	100.0
		Ungrouped cases	.0	100.0	100.0
Cross-validated[b]	Count	1	13	7	20
		2	2	18	20
	%	1	65.0	35.0	100.0
		2	10.0	90.0	100.0

a. 90.0% 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. 77.5% of cross-validated grouped cases correctly classified.

Figure 11. Discriminant analysis results for classifying subjects into country of origin (US or Jordan) using the δ^{13}C values of nine amino acids, ASP, SER, THR, ALA, ILE/LEU, TYR, LYS, HIS, and ARG.

Our larger US study will require the use of single factor ANOVA to assess significant differences for a single factor (amino acid), multiple factor ANOVA to assess interactions (covariance) between factors (groups, according to questionnaire responses) and multivariate ANOVA (MANOVA) to search for interactions between different response variables (amino acids). In addition to exploratory data analysis using ANOVA, we will also use discriminant analysis (DA) to help classify the subjects into prior groups. Unlike principal component analysis, which maximizes the total variance between all points, discriminant analysis maximizes the ratio of the within-group variance to between group variance for prior groups. Linear discriminant analysis (LDA) works well for classifying two groups at a time, but canonical discriminant analysis (CDA) is most efficient for multiple groups.

Table 14 shows that the sex of a person can be predicted with better than 89% accuracy (using unbiased, leave-one-out cross-validation) using the δ^{13}C values of the amino acids Asp, Glu, Ser, Thr, Gly, Val, Ile-Leu, Lys, His, Phe, Arg.

Table 14. CDA of not-averaged δ13C values from USA hair samples after CSIA. Sample size needs to be increased for a more successful classification based on sex. Sex: 1 = male, 2 = female. AAs = Asp, Glu, Ser, Thr, Gly, Val, Ile-Leu, Lys, His, Phe, Arg.

Classification Results[a,c]

			Predicted Group Membership		
		Sex	1	2	Total
Original	Count	1	10	0	10
		2	0	9	9
	%	1	100.0	.0	100.0
		2	.0	100.0	100.0
Cross-validated[b]	Count	1	9	1	10
		2	1	8	9
	%	1	90.0	10.0	100.0
		2	11.1	88.9	100.0

a. 100.0% 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. 89.5% of cross-validated grouped cases correctly classified.

Table 15 shows that the country of origin (USA or Jordan) of a person can be predicted with approximately 5% accuracy (using unbiased, leave-one-out cross-validation) using the δ^{13}C values of the amino acids Asp, Glu, Ser, Thr, Gly, Val, Ile-Leu, Lys, His, Phe, Arg. The database comprised of 40 individuals, 20 from each country.

Table 15. CDA of not-averaged δ^{13}C values from USA hair samples after CSIA. Sample size needs to be increased for a more successful classification based on country of origin. Country:1= USA, 2= Jordan. AAs = Asp, Glu, Ser, Thr, Gly, Ala, Val, Ile-Leu, Tyr, Lys, His, Phe, Arg.

Classification Results[a,c]

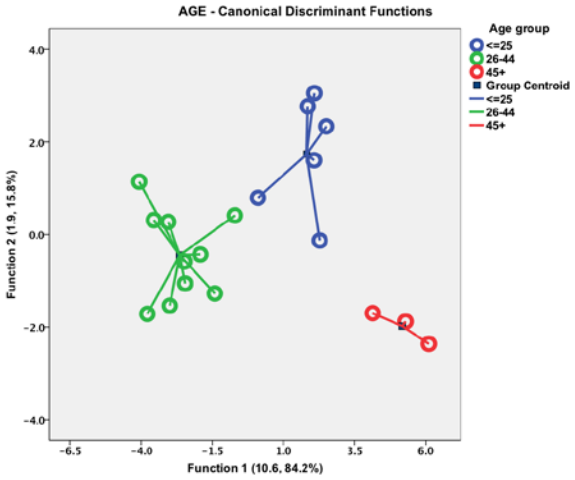
			Predicted Group Membership		
		Country	1	2	Total
Original	Count	1	19	1	20
		2	0	20	20
		Ungrouped cases	0	1	1
	%	1	95.0	5.0	100.0
		2	.0	100.0	100.0
		Ungrouped cases	.0	100.0	100.0
Cross-validated[b]	Count	1	11	9	20
		2	1	19	20
	%	1	55.0	45.0	100.0
		2	5.0	95.0	100.0

a. 97.5% 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. 75.0% of cross-validated grouped cases correctly classified.

Figure 12 shows the ability to predict age group of individuals from the US. Leave-one-out cross-validation success is not as reliable in this case (58% accuracy), unlike the biased or original rules, which have 100% accuracy.



Classification Results[a,c]

		Age group	Predicted Group Membership <=25	26-44	45+	Total
Original	Count	<=25	6	0	0	6
		26-44	0	10	0	10
		45+	0	0	3	3
	%	<=25	100.0	.0	.0	100.0
		26-44	.0	100.0	.0	100.0
		45+	.0	.0	100.0	100.0
Cross-validated[b]	Count	<=25	3	1	2	6
		26-44	2	7	1	10
		45+	2	0	1	3
	%	<=25	50.0	16.7	33.3	100.0
		26-44	20.0	70.0	10.0	100.0
		45+	66.7	.0	33.3	100.0

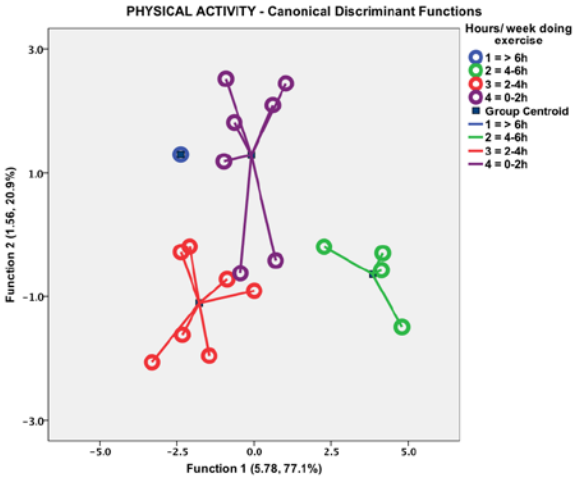
a. 100.0% of original grouped cases correctly classified.

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. 57.9% of cross-validated grouped cases correctly classified.

Figure 12. CDA of not-averaged δ^{13}C values from USA hair samples after CSIA. Sample size needs to be increased for a more successful classification based on age. Age group: <=25 (blue), 26-44 (green), 45+ (red). AAs = Asp, Glu, Ser, Thr, Gly, Ala, Val, Met-Cyt, Ile-Leu, Lys, His, Arg.

Figure 13 shows the ability to predict physical activity level of a subject from the δ^{13}C values of the amino acids Glu, Ser, Thr, Gly, Ala, Val, Met-Cyt, Ile-Leu, Lys, His and Arg. Similar to age prediction, the leave-one-out cross validation prediction rates are worse then the prediction rates using all the data, which indicates that at least twice as many samples are needed in the database.



Classification Results[a,c]

		Exercise	Predicted Group Membership 1	2	3	4	Total
Original	Count	1	1	0	0	0	1
		2	0	4	0	0	4
		3	0	0	7	0	7
		4	0	0	1	6	7
	%	1	100.0	.0	.0	.0	100.0
		2	.0	100.0	.0	.0	100.0
		3	.0	.0	100.0	.0	100.0
		4	.0	.0	14.3	85.7	100.0
Cross-validated[b]	Count	1	0	0	0	1	1
		2	0	3	0	1	4
		3	4	1	1	1	7
		4	3	1	1	2	7
	%	1	.0	.0	.0	100.0	100.0
		2	.0	75.0	.0	25.0	100.0
		3	57.1	14.3	14.3	14.3	100.0
		4	42.9	14.3	14.3	28.6	100.0

a. 94.7% 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. 31.6% of cross-validated grouped cases correctly classified.

Figure 13. CDA of not-averaged $\delta^{13}C$ values from USA hair samples after CSIA. Sample size needs to be increased for a more successful classification based on weekly exercise routine. AAs = Glu, Ser, Thr, Gly, Ala, Val, Met-Cyt, Ile-Leu, Lys, His, Arg.

3.4.1 *Predicting Type II Diabetes from the amino acids in human hair*

As described in our publication,[73] we applied a slightly different, and complementary approach to amino acid analysis of human hair, which was based on the relative quantity of each amino acid,[74] instead of the isotope ratios. The method used herein for the amino acid determination in hair included keratin protein acid hydrolysis using 6 M hydrochloric acid (HCl), followed by amino acids derivatization using N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and the determination of derivatized amino acids by gas chromatography/mass spectrometry (GC/MS). Amino acid profiles of scalp hair of 27 Jordanian subjects (15 diabetes mellitus (DM) type 2 patients and 12 control subjects) were analyzed. A fuzzy rule-building expert system (FuRES) classified the amino acid profiles into diabetic and control groups based on multivariate analyses of the abundance of 14 amino acids, as shown in Figure 14.

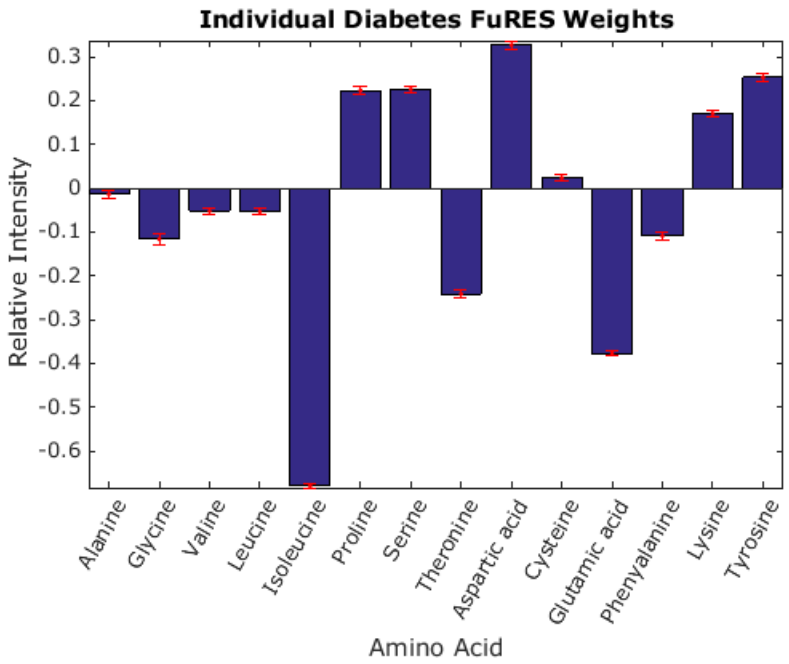


Figure 14. FuRES average variable loadings of the 14 amino acids of diabetes classification with 95% confidence intervals. Negative loadings are for diabetic subjects.

The sensitivity and specificity were 100% for diabetes detection using leave-one-individual-out cross-validation. The areas under the receiver operative characteristics (ROC) curves were 1.0, which represents a highly sensitive and specific diabetes test. The nonessential amino acids Gly and Glu, and the essential amino acid Ile were more abundant in the scalp hair of diabetic patients compared to the hair of control subjects. The associations between the abundance of amino acids of human hair and health status may have clinical applications in providing diagnostic indicator or predicting other chronic or acute diseases.

3.4.2 *Amino acid composition of human scalp hair as a biometric classifier and investigative lead*

In this sub-project, we analyzed the amino acid composition of scalp hair of 64 Jordanian subjects (33 males and 31 females) with ages ranging from 1 and 77 years.[75] Statistical comparisons between classification groups were based on the abundance of 14 abundant and acid-resistant amino acids,[74] and included classification of hairs with a fuzzy rule building system (FuRES). Using leave-one-individual-out cross validation, the FuRES classification rate was 94% for sex, 83% for age group, and 61% for the region of origin, as summarized in Figure 15. For predicting sex from amino acid concentration in hair, the essential amino acids Phe and Thr gave the most significant differences with respect to their *F* statistic (i.e., ratio of between-group to within-group variation), so they are the most discriminating for sex. Based on the same hair analyses, the non-essential amino acids Gly and Ala provide the largest loading scores classifying the subjects into two arbitrary age groups, <44 and >44 yrs. For region of origin classification, the amino acids Cys and Tyr had the highest loading scores in the classification rules and were therefore most discriminating. The techniques developed through this paper could complement the current methods of hair analysis, which include physical examinations and genomic or mitochondrial DNA analysis.

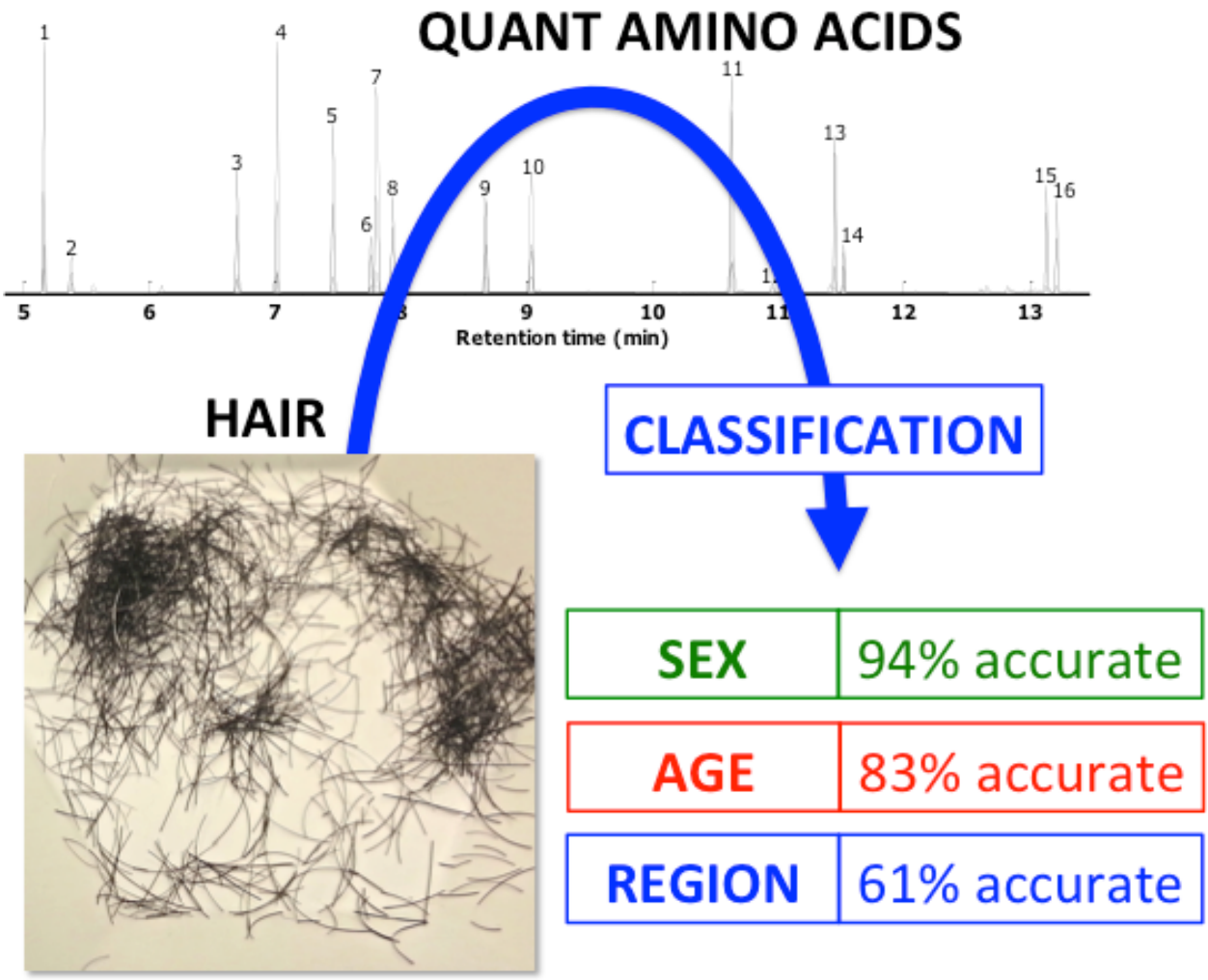


Figure 15. Schematic showing how FuRES classification tree was used to predict certain biometric traits from the abundance of 14 amino acids in human hair. The success rates are for leave-one-out cross-validation.

4 Conclusions

4.1.1 *Conclusions of global database of bulk hair isotope data*

In this work, new experimental $\delta^{13}C$ and $\delta^{15}N$ data of more than 400 hair and nail samples of contemporary humans living all over the world were summarised and added to the present public accessible data set. We contributed more than 100 of these new data points. However, there is still a lack of data on modern humans for huge regions, especially Africa. Our data on human $\delta^{13}C$ values support the established interpretation that the global spatial distribution of human carbon isotopic composition is strongly related to the amount of C_4 plant in the diet. The influence of a marine carbon isotope signal to modern human isotope ratios seems to be lower. The global spatial distribution of human $\delta^{15}N$ values cannot be directly related to dietary or environmental factors. Nevertheless, the analysis of carbon—and to lesser extent nitrogen—isotope ratios of post-mortem human remains may help in geo-location and subsequent identification of human victims.

4.1.2 *Conclusions for CSIA results on Jordanian hair*

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 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.

4.1.3 *Conclusions for CSIA results on Jordanian hair*

At present, the CSIA of US hair has supported the earlier findings using Jordanian hair. That is, that metabolic factors provide a sufficiently large and consistent fractionation effect on certain amino acids to enable subjects to be classified into different soft biometric groups. In addition, we can distinguish between subjects from the US and Jordan using the CSIA of amino acids in the hair. We are still in the process of examining more hair samples and examining the canonical discriminant function coefficients to determine the amino acids that most influence the ability to classify the subjects according to the different grouping variables. We have shown the ability 1) to collect reproducible and reliable amino acid-specific $\delta^{13}C$ values from hair, 2) to perform statistical comparisons and data reduction to classify individuals into biometric groups, and 3) to extract specific amino acid biochemistry that could point to biochemical reasons for the classification. However, to make the results more useful to the US criminal justice system, we need to continue to analyze the existing hair samples from our larger cohort of US subjects.

4.2 Implications for policy and practice

At this time, we do not expect these fundamental research findings to affect public policy or routine casework in crime laboratories. However, we have made significant strides towards the development of novel investigative leads that law enforcement could use in the future to help solve crimes. More importantly, we are building upon a body of work that is enabling objective chemical measurements of human hair that can provide statistical models for the probability of inclusion of exclusion. Such objective and metric-based analyses are the future of forensic science. Of course, we have a long way to go, but we have made a small step in the direction of statistical-based determinations of hair comparisons.

4.3 Implications for future research

The implications here are more obvious. In addition to working up the remaining data for the US subjects, we must establish more rigorous multivariate approaches to data analysis. We need to better understand the biochemical process affecting fractionation, and better understand how to compensate for different factors, when we know they are present. In future work, the carbon-based CSIA of this work could be combined with other stable isotope data, such as oxygen,

hydrogen, nitrogen and strontium, to provide even more independent variables into objective hair comparisons.

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6 Dissemination of research findings

6.1 Publications

Since commencing this grant, we have published the following 7 peer-reviewed articles related to this project:

1) A. H. B. Rashaid, **G. P. Jackson**, Peter B. Harrington, "Quantitation of Amino Acids in Human Hair by Trimethylsilyl Derivatization Gas Chromatography/Mass Spectrometry" *Enliven*: *Bio Anal. Techniques,* **2014**, 1(1) 1-12. (http://enlivenarchive.org/bioanalytical-002.html)

Abstract: The distribution of amino acids in hair can divulge information regarding the health (e.g., diabetes) and provide a means for detecting the history of the disease by segmentation of the hair as well as attributes of an individual (e.g., sex and age). Therefore, an nonenzymatic method of hair digestion and profiling is required. In addition to optimizing and validating a method for measuring the distribution of amino acids in human hair, a robust and comprehensive approach to objectively compare the most effective means of extracting and manipulating chromatographic data to obtain the best limits of detection, linearity, and sensitivity are provided. Data comparisons were made by operating the mass spectrometer in a mode that rapidly switches between total ion current (TIC) and selected ion monitoring (SIM) modes during each sample injection. In this way, any external confounding factors were negated that may otherwise influence the comparison of the linearity and sensitivity between the two modes of operation. The use of SIM, peak areas, and an internal standard provided significantly better sensitivity and limits of detection than using peak heights, TICs, or no internal standard. The sample preparation steps included protein acid hydrolysis using hydrochloric acid and trimethylsilyl (TMS) derivatization using N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). The optimal derivatization conditions were acetonitrile as reaction solvent, temperature of 100°C, and a reaction time of 30 min.

The method was validated by measuring the amino acid content of myoglobin. This validation was accurate for nine of the fourteen amino acids found in myoglobin and gave detection limits in the range of 0.04–0.1 μmol/L, quantitation limits in the range of 0.1–0.5 μmol/L, recoveries between 80% and 110%, and linear models with coefficients of determination (R2) greater than 0.99 in the tested range from 1 to 300 μmol/L. The remaining five amino acids of myoglobin were deleteriously affected by acid

2) A. H. B. Rashaid, P. B. Harrington and **G. P. Jackson** "Amino Acid Composition of Human Scalp Hair as a Biometric Classifier and Investigative Lead" *Anal. Methods*, ***2014,*** 7, 1707-1718. doi:10.1039/C4AY02588A

Abstract: Hair shaft analysis is becoming increasingly important in several applications of forensic science. Keratin is the key component of human scalp hair and is composed of all 21 known amino acids, albeit in very different proportions. The method for the amino acid determination included protein acid hydrolysis followed by trimethylsilyl (TMS) derivatization of the amino acids, and the subsequent quantitation using gas chromatography/mass spectrometry (GC/MS). The amino acid composition of scalp hair of 64 Jordanian subjects (33 males and 31 females) with ages ranging from 1 to 77 years have been analyzed. Statistical comparisons between classification groups were based on the abundance of 14 abundant and acid-resistant amino acids, and included classification of hairs with a fuzzy rule building system (FuRES). Using leave-one-

individual-out cross-validation, the FuRES classification rate was 94% for sex, 83% for age group, and 61% for the region of origin. For predicting sex from amino acid concentration in hair, the essential amino acids Phe and Thr gave the most significant differences with respect to their *F* statistic (*i.e.*, ratio of between-group to within-group variation), so they are the most discriminating for sex. Based on the same hair analyses, the non-essential amino acids Gly and Ala provide the largest loading scores classifying the subjects into two arbitrary age groups, <49 and >49 years. For region of origin classification, the amino acids Cys and Tyr had the highest loading scores in the classification rules and were therefore most discriminating. The techniques developed through this paper could complement the current methods of hair analysis, which include physical examinations and genomic or mitochondrial DNA analysis.

3) **G. P. Jackson**, Y. An. K. I. Konstantynova, A. H. B. Rashaid "Biometrics from the Carbon Isotope Ratio Analysis of Amino Acids in Human Hair" *Sci. Justice* (*FIRMS 2013 Special Edition),* **2015**, 55, 43-50. doi:10.1016/j.scijus.2014.07.002

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.

4) J. R. Almirall, **G. P. Jackson**, "Review: 27th ASMS Sanibel Conference on Mass Spectrometry—Security and Forensic Applications" *J. Am. Soc. Mass Spectrom*. **2015**, 26, 695-698. *(This report does not specifically acknowledge NIJ sponsorship)*

Abstract: The 27th Annual ASMS Sanibel Conference on Security and Forensic Applications of Mass Spectrometry was held from January 22 to 25, 2015 at the Hilton Clearwater Hotel in Clearwater Beach, FL. The co-organizers were José R. Almirall of Florida International University and Glen P. Jackson of West Virginia University. The conference brought together leading researchers and practitioners from universities, research institutions, and forensic laboratories to discuss the application of mass spectrometry to issues of national security and forensic science. Attendees represented many corners of the world, including Australia, Brazil, Canada, China, Czech Republic, Denmark, France, Germany, Sweden, United Kingdom, and the United States. Although the weather was very windy and cold by Florida standards, the enthusiastic spirit of the attendees was unaffected. Graham Cooks of Purdue University gave the conference a boost by

providing NSF funds to support student travel. The grant (CHE-1262145) titled Strengthening Forensic Science through Connections with the Analytical Sciences^ provided 20 travel awards for students to attend the conference. ASMS provided an additional 20 awards, all of which were filled. The organizers and the students remain very grateful for the exposure to cutting-edge research and researchers in forensic mass spectrometry. The conference attracted a total of 150 registrants (Figure 1) and more than 56 poster presentations. In addition, five attendees signed up to receive two continuing education units (CEU) from West Virginia University.

5) W. D. Hoffmann and **G. P. Jackson** "Forensic Mass Spectrometry" *Ann. Rev. Anal. Chem*. **2015,** 8, 419-440. free eprint: DOI: 10.1146/annurev-anchem-071114-040335.

Abstract: Developments in forensic mass spectrometry tend to follow, rather than lead, the developments in other disciplines. Techniques of great forensic utility or potential born independently of forensic applications include ambient ionization, imaging mass spectrometry, isotope ratio mass spectrometry, portable mass spectrometers, and hyphenated chromatography–mass spectrometry instruments, to name a few. Forensic science has the potential to benefit enormously from developments that are funded by other means, if only the infrastructure and personnel existed to adopt, validate, and implement the new technologies into casework. Perhaps one unique area in which forensic science is at the cutting edge is in the area of chemometrics and the determination of likelihood ratios for the evaluation of the weight of evidence. Such statistical techniques have been developed most extensively for ignitable-liquid residue analyses and isotope ratio analysis. This review attempts to capture the trends, motivating forces, and likely impact of developing areas of forensic mass spectrometry, with the caveat that none of this research is likely to have any real impact unless: (**a**) The instruments developed are turned into robust black boxes with red and green lights for positives and negatives, respectively, or (**b**) there are PhD graduates in the workforce who can help adopt these sophisticated techniques.

6) A. H. B. Rashaid, P. B. Harrington, **G. P. Jackson** "Profiling Amino Acids of Jordanian Scalp Hair as a Tool for Diabetes Mellitus Diagnosis: A Pilot Study" *Anal. Chem*. **2015**, 87, 7078-7084

Abstract: Hair analysis is an area of increasing interest in the fields of medical and forensic sciences. Human scalp hair has attractive features in clinical studies because hair can be sampled easily and noninvasively from human subjects, and unlike blood and urine samples, it contains a chronological record of medication use. Keratin protein is the major component of scalp hair shaft material and it is composed of 21 amino acids. The method used herein for the amino acid determination in hair included keratin protein acid hydrolysis using 6 M hydrochloric acid (HCl), followed by amino acids derivatization using N ,O bis(trimethylsilyl)trifluoroacetamide (BSTFA), and the determination of derivatized amino acids by gas chromatography/mass spectrometry (GC/MS). Amino acid profiles of scalp hair of 27 Jordanian subjects (15 diabetes mellitus (DM) type 2 patients and 12 control subjects) were analyzed. A fuzzy rule-building expert system (FuRES) classified the amino acid profiles into diabetic and control groups based on multivariate analyses of the abundance of 14 amino acids. The sensitivity and specificity were 100% for diabetes detection using leave-one-individual-out cross-validation. The areas under the receiver operative characteristics (ROC) curves were 1.0, which represents a highly sensitive and specific diabetes test. The nonessential amino acids Gly and Glu, and the essential amino acid Ile were more abundant in the scalp hair of diabetic patients compared to the hair of control subjects. The

associations between the abundance of amino acids of human hair and health status may have clinical applications in providing diagnostic indicator or predicting other chronic or acute diseases.

7) F. Huelsemann, C. Lehn, S. Schneiders, **G. P. Jackson**, S. Hill, U. Flenker, "Global Spatial Distributions of Nitrogen and Carbon Stable Isotope Ratios of Modern Human Hair" *Rapid Commun. Mass Spectrom.* **2015,** 29, 2111-2121.

Abstract: RATIONALE: Natural stable carbon (δ^{13}C) and nitrogen isotope ratios (δ^{15}N) of humans are related to individual dietary habits and environmental and physiological factors. In forensic science stable isotope ratios of human remains like hair and nail are used for geographical allocation. Thus the knowledge of the global spatial distribution of human δ^{13}C and δ^{15}N values is an essential component in the interpretation of stable isotope analytical results.
METHODS: Up to now no substantial global data sets of human stable isotope ratios are available, although the amount of available (published) data has increased within the last years. We hereby summarized published data on human global δ^{13}C and δ^{15}N values (around 4000 samples) and added experimental values of more than 400 additional worldwide human hair and nail samples. In order to summarize isotope ratios for hair and nail samples correction factors were determined.
RESULTS: The current available data set is biased towards Europe and North America with only limited data for countries in Africa, Central and South America and Southeast Asia. The global spatial distribution of carbon isotopes is related to latitude and supports the fact that human δ^{13}C values are dominated by the amount of C4 plant in the diet, either due to direct ingestion as plant food, or by the use as animal feed. In contrast, the global spatial distribution of human δ^{15}N values is apparently not exclusively related to the amount of fish or meat ingested, but also to environmental factors influencing agricultural production.
CONCLUSIONS: There are still a large proportion of countries, especially in Africa, where there is no existing data for human carbon and nitrogen isotope ratios. Whereas the interpretation of modern human carbon isotope ratios at the global scale is quite possible, and correlates with the latitude, the potential influences of extrinsic and/or intrinsic factors on human nitrogen isotope ratios have to be taken into consideration.

6.2 Presentations

Our group has presented the results of our work in the form of invited oral presentations at more than 15 universities and conferences since commencing this grant. The presentations based on this project from Jan 2014-present are listed below:

6.2.1 *Non-conference venues*

University of South Carolina, Columbia, SC (Nov 2016).
Genetics and Developmental Biology Program, WVU, Morgantown, WV (Oct 2015)
Food and Drug Administration (FDA), Washington, DC (March 2015)
Roads Scholar Academy (WVU), Naples, FL (Feb 2015)
Duquesne University, Pittsburgh, PA (Feb 2015)
Georgetown University, Washington, DC (Sept 2014)
Society for Applied Spectroscopy (SAS) Speaker Tour, John Carroll University, Cleveland, OH (March 2014)
Department of Chemistry, Purdue University, West Lafayette, IN (April 2014)
Department of Chemistry, Indiana University, Bloomington, IN (April 2014)

6.2.2 *Conferences*

Mayara, P. V. de Matos, Glen P. Jackson, “Compound-Specific Isotope Analyses of Hair Samples: Discrimination that goes Beyond Dietary Factors”, Forensic Isotope Ratio Mass Spectrometry Conference” Auckland, NZ, September 2016.

Glen P. Jackson, “The Future of Forensic Instrumental Methods of Analysis”, AAFS Meeting, Las Vegas, NV, Feb 2016. (Oral)

G. P. Jackson, K. I. Konstantynova, M. P. V. de Matos, R. Mohr "Forensic Source Attribution using Stable Isotopes: Hairs to Humans and Insects to Carrion”, Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, New Orleans, LA, Mar 2015. (Oral).

G. P. Jackson, K. I. Konstantynova, M. P. V. de Matos, R. Mohr "Forensic Source Attribution using Stable Isotopes: Hairs to Humans and Insects to Carrion”, 67th Meeting of the American Academy of Forensic Sciences, Orlando, FL, Feb 2015. (Oral).

*G. P. Jackson "Linking Insects to Humans and Hair to Human Phenotypes using Stable Isotopes”, ASMS Sanibel Conference on Security and Forensic Applications of Mass Spectrometry, Clearwater Beach, FL, Jan 2015. (Oral).

*G. P. Jackson, R. Mohr, K. I. Konstantynova "Forensic attribution using stable isotopes: hairs to humans and insects to carrion”, SciX Conference, Reno, NV, Oct 2014. (Oral).

The following presentations have also been scheduled, or submitted for consideration

Glen P. Jackson, “Forensic Attribution using stable isotopes: Hairs to Humans and insects to carrion”, at the Spring meeting of the American Chemical Society, San Francisco, CA, April 2017.