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

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ABSTRACT

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

1. Introduction

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

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

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

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

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

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

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

2. Materials and methods

2.1. Materials

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

2.2. Hair samples

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

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

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

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

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

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