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$\delta^{13}C$ analysis of amino acids in human hair using trimethylsilyl derivatives and gas chromatography/combustion/isotope ratio mass spectrometry

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RATIONALE: To provide a simple one-step derivatization procedure for the analysis of a wide variety of amino acids in human hair by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) derivatization is already widely used outside the IRMS community, is applicable to a variety of functional groups, and provides products that are common entries in mass spectral databases, thus simplifying compound identification.
METHODS: Method optimization and validation were performed on a mixture of ten standard amino acids found abundantly in human hair. The method was then applied to the analysis of scalp hair from six human subjects. The hair was washed, hydrolyzed with 6 M HCl, derivatized using BSTFA in acetonitrile and analyzed using gas chromatography (GC) with concurrent quadrupole and isotope ratio mass spectrometry (IRMS) detectors.
RESULTS: The reproducibility for the $\delta^{13}C$ measurements, including the derivatization procedure and GC/C/IRMS analysis, on a day-to-day comparison was between 0.19‰ and 0.35‰ (SD, N = 12), with an average standard deviation of 0.26‰. Because trimethylsilylation adds 3N carbon atoms (where N = # reactive protons) to each amino acid, the $\delta^{13}C$ values for amino acid derivatives were corrected using a mass balance correction and the measured kinetic isotope effect (KIE). The KIE values ranged from 0.984 to 1.020.
CONCLUSIONS: The procedure gave consistent $\delta^{13}C$ values with precision similar to other derivatization methods for the range of sample sizes studied: 50–1000 μg of each amino acid. The method gave $\delta^{13}C$ values consistent with the known literature values when applied to the analysis of amino acids in human hair. Copyright © 2013 John Wiley & Sons, Ltd.

The isotopic analysis of human and animal hair has been an active area of research for several decades. Hair is an important biological matrix for clinical and forensic applications and, because of its intrinsic stability, is an ideal medium for stable isotope ratio analysis.[1] For the purposes of identifying people from their hair or linking victims or suspects to a questioned hair sample from a crime scene, the gold standard is chromosomal DNA or mitochondrial (m) DNA.[2] In cases where no DNA can be recovered or when a reference sample is not available, isotope ratio analysis can provide an alternative perspective.[3] The method developed in this work provides a relatively simple procedure for the compound-specific isotopic analysis of amino acids in hair that could be applied to a variety of clinical and forensic applications.

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Isotope ratio mass spectrometry (IRMS) is a technique that is used to measure the natural isotopic abundances of $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{2}D/^{1}H$, and $^{34}S/^{32}S$ in organic and inorganic materials. Isotope ratio analysis can provide evidence that two compounds with otherwise identical chemical composition could actually have different sources or origins. IRMS is widely used in different areas such as forensic science,[4,5] food analysis,[6,7] drugs and pharmaceutics,[8] geology,[9] biology,[10] environmental chemistry,[11] and athletics and doping.[12] There have been many reports of IRMS being used as a forensic tool for hair and nails. In 2006, IRMS was used to provide information for the identification of disaster victims by using hair and nails when DNA methods had failed to yield the identification of an individual.[3] Scalp hair and fingernails have also been examined by using an elemental analyzer coupled to isotope ratio mass spectrometry (EA/IRMS) to obtain the hydrogen and oxygen isotopic ratios of the hair and mail matrices.[13] Ehleringer and coworkers have developed a model to predict the geographic origin of a person based upon the stable isotope composition of hydrogen and oxygen of the person's scalp hair.[13] Schaenzer's group has performed longitudinal bulk

stable isotope ratios analysis of human hair to determine such factors as dietary changes.[14] Petzke *et al*. have reported using bulk ^{13}C and ^{15}N values and amino acid $\delta^{13}C$ values to distinguish between vegetarians and omnivores and as a biomarker for protein intake.[15]

Human hair consists of approximately 65–95% proteins[16] of which ~12% are basic amino acids and ~17% are acidic amino acids. The amino acids that comprise the majority of the hair material are therefore an obvious choice for compound-specific isotope ratio mass spectrometry (CS-IRMS) because they will be the easiest to detect, will provide the best signal-to-noise (S/N) values and will be least affected by co-elution with minor constituents. Compared with previous time-consuming off-line methods,[1] gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) enables rapid on-line separation and carbon isotope determinations of individual compounds from complex matrices. The determination of the $\delta^{13}C$ values of individual amino acids by GC/C/IRMS enables natural isotopic signatures to be examined at a more detailed level than a bulk average. GC/C/IRMS requires that the sample be in a volatile state, whereas native amino acids are typically nonvolatile, polyfunctional compounds. Therefore, amino acids need to be derivatized or otherwise altered to make them volatile. Brenna's group showed that reducing the polar amino acids to amino alcohols was an effective way to reduce the polarity and improve the chromatographic performance of selected amino acids without affecting the carbon isotope ratios.[17] However, the approach was not universally applicable to the majority of amino acids and was only tested on relatively large samples (2 g). Derivatization is a more widespread approach, although less ideal. Because derivatization usually adds carbon atoms to the amino acids, the derivatizing agent influences the $\delta^{13}C$ value of the derivatized products and this is one of the principal challenges to the GC/C/IRMS carbon isotope analysis of amino acids.

Several derivatization procedures have been used for the GC/C/IRMS analyses of amino acids, as summarized in Table 1. The most widely used methods for amino acid derivatization for GC/C/IRMS analysis involve a stepwise procedure of esterification of the carboxylic acid groups with an acidified alcohol and acylation of the amine, hydroxyl, and thiol groups with an anhydride, forming *N*(*O*,*S*)-acyl alkyl derivatives.[17,18] Three derivatives have been used widely for the GC/C/IRMS analysis of amino acids, trifluoroacetyl isopropyl (TFA-IP) esters, *N*-acetyl-*n*-propyl (NAP) esters, and *N*-pivaloylisopropyl (NPP) esters. Evershed and coworkers recently promoted the use of *N*-acetyl, methyl ester derivatization with the major advantage of a small carbon load.[18] However, these procedures all require two derivatization steps, each with a different reagent, and therefore require multiple correction steps for mass balance and kinetic isotope effect (KIE).

Trimethylsilyl (TMS) derivatives using reagents such as *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) have been widely used for amino acids since the 1960s.[19–21] Such derivatives have the benefit of being frequently abundant in commercially available mass spectral libraries. There is evidence that under certain conditions *t*-butyl dimethylsilyl (TBDMS) derivatives can cause incomplete combustion in the catalytic oxidation chamber during continuous flow conditions.[22] However, such incomplete combustion is usually selective towards the carbon atom adjacent to the silylation group, which is the C-atom that was preferentially enriched in ^{13}C in a previously examined tracer study. For this reason, incomplete combustion at this position caused a reduced mass balance contribution from this specific position when the amino acid was converted to CO_2. The result of the incomplete combustion was biased, weighted in favor of the remaining C-atom positions, independent of any additional fractionation. However, in natural abundance studies, where the ^{13}C-atom is randomly distributed throughout the amino

Table 1. Summary of different derivatization agents used for $\delta^{13}C$ analysis of free amino acids

Reaction type	Derivatization	Number of reaction steps	Extra carbon atoms added, e.g. valine	Typical GC column used	Comments	Ref.
Esterification Acylation	*N*-Acetyl methyl ester derivative (NACME)	2	3	ZB-WAX (polar)	No major caveats. Low carbon load.	[18]
Esterification Acylation	Trifluoroacetyl isopropyl ester derivative (TFA-IP)	2	4	DB 5 MS (apolar)	Nonquantitative reaction.	[35]
Esterification Acylation	*N*-Acetyl-*n*-propyl ester derivative (NAP)	2	5	Ultra 2 (apolar)	Poor chromatographic resolution.	[32]
Esterification Acylation	*N*-Pivaloylisopropyl ester derivative (NPP)	2	8	Ultra 2 (apolar)	Quite high carbon load. No major caveats except that Val gave a double peak. Method validated for ^{13}C and ^{15}N amino acids.	[33]
Silylation	*tert*-Butyldimethylsilyl derivative (tBDMS)	1	12	CP-Sil 8 (apolar)	Excessive carbon load.	[36]
Alkoxycarbonyl alkylation	*N*(*O*,*S*)-Methoxycarbonyl methyl ester derivative	1	3	CP-Sil 19CB (apolar)	Nonquantititative reaction. Low carbon load.	[37]
Alkoxycarbonyl alkylation	*N*(*O*,*S*)-Ethoxycarbonyl ethyl ester derivative	1	5	DB 5 MS (apolar)	Poor chromatographic resolution.	[38]
Silylation	*N*,*O*-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA)	1	6	DB 5 MS (apolar)	Quite high carbon load. Ubiquitous, one-step method.	This work

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acid carbon atoms, the use of TBDMS derivatives would not be expected to cause a shift in mass balance to the same extent because any carbon retained in the combustion chamber would contain a random proportion of ^{13}C and ^{12}C. To our knowledge, such problems due to incomplete combustion for TMS derivatives, instead of TBS derivatives, at elevated or natural isotope levels have not been reported.

Here, we present using a one-step BSTFA derivatization procedure with GC/C/IRMS for the determination of $\delta^{13}C$ values of amino acids in human hair. The proposed method enables the measurement of $\delta^{13}C$ values of amino acids to a precision that is typical for conventional derivatization GC/C/IRMS. The accuracy and precision of the described method have been validated with standards and tested with a human hair sample. We have applied the technique to the hair samples from different donors and to chronological studies of individuals, the results of which are being prepared for submission in a separate manuscript.

EXPERIMENTAL

Subjects

The collection of hair samples for the following experiments was approved by the Ohio University Institution Review Board (IRB # 06X096). The test group consisted of four males and two females who were all inhabitants of the Ohio Valley region (southwest Ohio and Kentucky). The subjects were not on any special calorie-controlled diets during the period of their hair growth.

Reagents and standards

The ten standard amino acids were L-alanine, L-valine, L-leucine, L-isoleucine, L-serine, L-threonine, L-proline, L-aspartic acid, L-tyrosine, and L-cysteine, all purchased from Sigma-Aldrich (St. Louis, MO, USA). The derivatizing agent was *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) purchased from Supelco Analytical (Bellefonte, PA, USA). The solvent used was HPLC grade acetronitrile, purchased from EMD Chemicals (Gibbstown, NJ, USA). The acid hydrolysis agent was HCl, 37% reagent (ACS) grade, purchased from GPS Chemicals (Columbus, OH, USA).

A stock solution was made that contained 5.0 μmol/mL of each of the ten amino acid standards in 0.1 M HCl. A volume of 100 μL of the stock solution was added to reaction vials and the solvent was evaporated under a stream of room temperature nitrogen. A volume of 100 μL of BSTFA was added to the samples and an additional 100 μL of acetonitrile was then added. The samples were heated at 100 °C for 30 min in a sealed vial. A volume of 1 μL of the solution was then injected into the injection port of the GC/C/IRMS instrument using an AS 3000 autosampler (Thermo Scientific, Waltham, MA, USA).

Derivatization of hair samples

Each hair sample was washed twice in water and once in methanol/acetone/chloroform (1:1:1). The individual hair samples were vortexed for approximately 1 min and then dried under a stream of nitrogen to ensure that no water remained before derivatization.[23] Hair samples of approximately 12 mg were pulverized by placing the hair into a capped plastic vial with 6–8 stainless steel ball bearings, and violently agitated for 5 min using a Mini Beadbeater (Biospec Products Inc., Bartlesville, OK, USA) at 4800 rpm. The ball bearings were then removed and the fine powder was transferred into a 10 mL glass vial. Pulverization was performed to increase the surface area/volume ratio of the hair and to enhance the rate and extent of hydrolysis.

Free amino acids were obtained from the hair proteins by using strong acid hydrolysis. Each pulverized hair sample was precisely weighed out in 10 mg samples and 1 mL of 6 M HCl was added to the pulverized hair. The solution was then placed in an oven for 16 h at 110 °C. After hydrolysis, the liquid sample was filtered (0.45 μm) to remove any particulates that might remain. The filtered sample was then dried under a constant stream of nitrogen. Volumes of 100 μL of BSTFA and 100 μL of acetonitrile were added to the sample, which was then heated for 30 min at 100 °C. A pipette was used to transfer 150 μL of each sample to a glass autosampler vial with plastic insert before placing the sample vials in the autosampler carousel to await injection.

Bulk isotope ratio mass spectrometry

Bulk carbon isotope measurements were made on precisely weighed samples of approximately 1 mg that were placed in tin capsules in a ECS 4010 elemental analyzer (EA) (Costech, Winter Springs, FL, USA). The EA was coupled via a Conflo III interface (ThermoFinnigan, Waltham, MA, USA) to a Delta Plus Advantage isotope ratio mass spectrometer (ThermoFinnigan). Data acquisition was carried out using Isodat 2.0 software (ThermoFinnigan). During both the GC and EA analyses, high-purity gases from Airgas (Parkersburg, WV, USA) were used: >99.9999% He, 99.999% N_2, 99.997% CO_2, 99.999% O_2. The results of the carbon isotope ratio analyses are reported in per mill (‰) on the relative δ-scale. The $\delta^{13}C$ values refer to the international standard V-PDB (Vienna Pee Dee Belemnite) calcium carbonate according to the following equation:

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

where R is the ratio of $^{13}C/^{12}C$. The reference cylinder of CO_2 gas was calibrated using replicate analyses of USGS (Reston, VA, USA) standards USGS40 and USGS41. For quality control, an IAEA (Vienna, Austria) isotope standard of caffeine (IAEA-600, $\delta^{13}C = -27.77‰$) was run on both the EA/IRMS and the GC/C/IRMS instruments and provided $\delta^{13}C$ values of −27.74‰ (N = 4) and −27.75‰ (N = 6), respectively, thus validating the relative and absolute accuracy of the two measurement systems. The precisions of the EA/IRMS and GC/C/IRMS measurements for caffeine were both 0.14‰ (N = 4 and N = 6, respectively). Caffeine had a retention time that was significantly different from those of the amino acid derivatives and could therefore be used in future experiments as an internal isotope reference material.

Gas chromatography/mass spectrometry/isotope ratio mass spectrometry

The electron ionization (EI) mass spectrometry and C/IRMS analyses were performed concurrently using a single quadrupole mass spectrometer (HP5970B, Hewlett Packard

(now Agilent), Santa Clara, CA, USA) and a Delta Plus Advantage isotope ratio mass spectrometer (ThermoFinnigan). These instruments were used in combination with a Trace GC gas chromatograph (ThermoFinnigan) equipped with an AS3000 autosampler (ThermoFinnigan). A typical cycle time for the GC/IRMS run was 75 min and samples were placed on a 100-sample wheel, which allowed for unattended sample analysis. The GC column was a 5%-phenyl-95%-dimethyl-polysiloxane (DB-5) column (60 m × 0.25 mm × 0.25 μm; J & W Scientific, Folsom, CA, USA). The GC effluent was split using a low-dead-volume X-connector (Valco Instruments Co. Inc., Houston, TX, USA) so that approximately 10% of the effluent flowed to the single quadrupole mass spectrometer for structural elucidation and approximately 90% flowed to the IRMS instrument for concurrent isotopic analysis. The real-time EI-MS detector permitted any co-eluting peaks to be identified. No co-elution was observed in the example hair samples studied here. However, different samples might contain different potential interferences and require solid-phase extraction to clean up the amino acids prior to analysis, as is usually performed.

The GC oven temperature was programmed as follows: initial temperature 70 °C (hold 9.0 min); program rate, 3 °C/min final temperature 300 °C (hold 8.0 min). The injector temperature was set at 280 °C and the helium carrier gas flow rate was 1.0 mL/min. A volume of 1 μL was injected in splitless mode using the aforementioned autosampler. The temperature of the auxiliary transfer line to the single quadrupole was set at 280 °C. The purge valve between the gas chromatograph and the oxidation chamber remained open for the first 20 min to allow any excess derivatizing agent to vent to waste, thus protecting the lifetime of the catalyst. Meier-Augenstein recommends against using fluorinated compounds in GC/C/IRMS, not only because the fluorine liberated on combustion forms CuF_2 and reduces the oxidation efficiency of the system, but also because fluorine poisons the combustion catalyst, platinum.[24,25] However, because the excess BSTFA is vented to waste for the first few minutes of every GC run – and therefore bypasses the oxidation reactor – we have not detected any deleterious effects on our system involving more than 300 analyses of BSTFA-derivatized standards and samples.

Total ion chromatograms were recorded over the range of *m/z* 50–550 using HP Chemstation B.02.05 (Hewlett Packard (now Agilent)). The data was exported as a text file for comparison with the NIST (Gaithersburg, MD, USA) mass spectral library to confirm the identity of the individual amino acids of interest.

The combustion oven temperature for the GC interface (GC Combustion III) was held at 940 °C and the reduction oven temperature was held at 650 °C. The combustion tube was regenerated with O_2 approximately every 100 runs. Data acquisition on the IRMS instrument was carried out using Isodat 2.0 software. Each run was begun with three injections of the high-purity (99.997%) CO_2 reference gas (Airgas, Great Lakes, Independence, OH, USA), of which the second CO_2 peak – the flat-topped peak in Fig. 3 – is designated as the standard to be used for calculating the $\delta^{13}C$ values by the instrument acquisition software. The isotope standard and samples were injected, separated and analyzed under identical conditions, thereby adhering to the identical treatment (IT) principle for IRMS calculations.[26] The GC/C/IRMS standard (Chiron International Standards, Laramie, WY, USA) consisted of n-undecane (C_{11}) with a $\delta^{13}C$ value of −26.11‰, n-pentadecane (C_{15}) with a $\delta^{13}C$ value of −30.22‰, and n-eicosane (C_{20}) with a $\delta^{13}C$ value of −33.06‰, each at 0.15 mg/mL in cyclohexane. Injections of 1 μL of this standard were used to calibrate the reference CO_2 gas. The C_{11} peak in the isotope standard mixture was used to calibrate the reference cylinder to a user-defined value immediately prior to running all the samples in similar manner to Merritt *et al*.[27] The accuracy of the method was validated by using an IAEA sample of caffeine, which gave the correct isotopic value to within the error of the measurements (see previous section for details).

RESULTS

Study of amino acid derivatization reaction conditions

Although three different solvents – acetonitrile (ACN), pyridine and ethyl acetate – are commonly used in the derivatization of amino acids with BSTFA,[28] we chose to use ACN as the reaction solvent.[29] Initially, the effects of derivatization reaction temperature and time were optimized. Derivatization was performed at different temperatures (room temperature and 100 °C)[20,30] and different times (15, 30, 45, and 60 min). As shown in Fig. 1, all ten amino acids gave maximum peak areas at a reaction temperature of 100 °C and a reaction time of 30 min, which was therefore adopted for the remaining experiments.

Through the use of BSTFA as a derivatizing agent, we were able to separate the ten most abundant amino acids in hair: alanine, valine, leucine, isoleucine, serine, threonine, aspartic acid, proline, tyrosine, and cysteine (Figs. 2 and 3). If proteins or amino acids other than those studied in hair or keratin are of interest, BSTFA derivatization would still be applicable to the derivatization of these other amino acids.[28]

Precision of isotopic measurement

Because BSTFA derivatives have not been previously employed for GC/C/IRMS amino acid analysis, several steps were taken here to assess the reproducibility. First, three

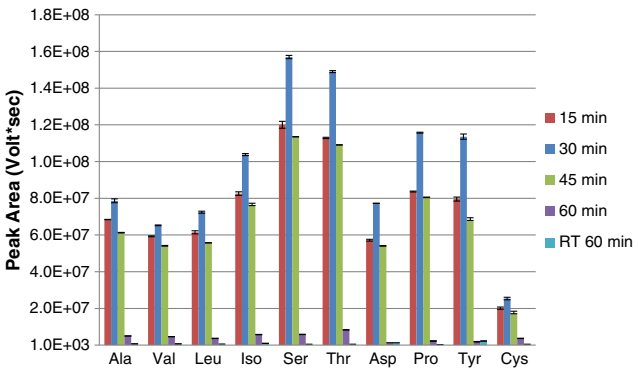

Figure 1. Effect of derivatization temperature and time on the absolute peak area of BSTFA derivatives of ten amino acids. All derivatizations were at 100 °C with the exception of room temperature (RT) 60 min. Error bars show 1 s.d., n = 3.

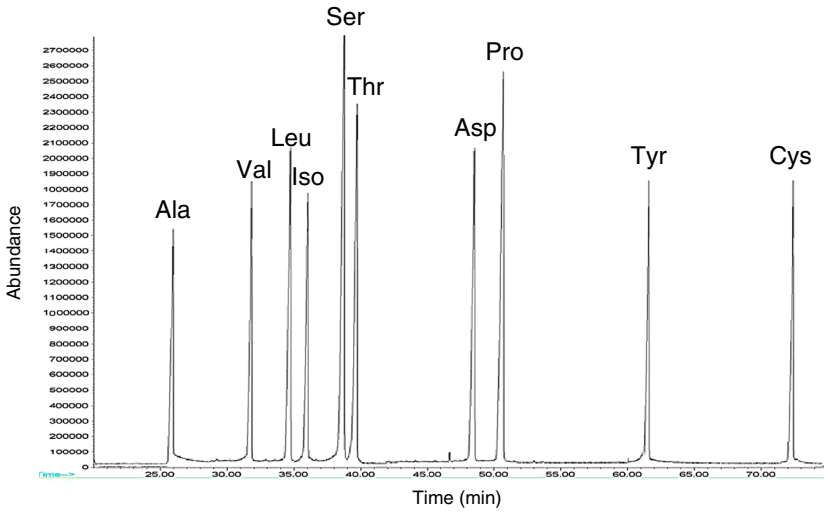

Figure 2. TIC chromatogram of BSTFA derivatives of ten amino acid standards.

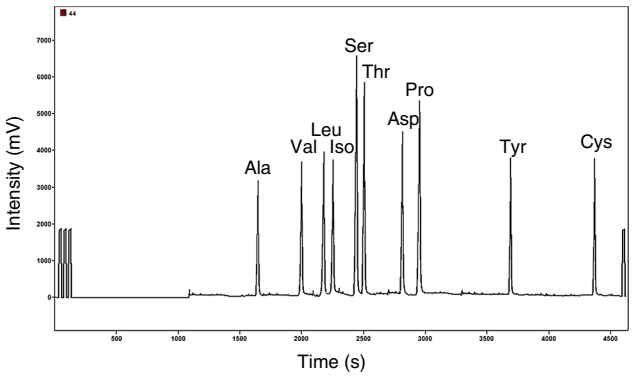

Figure 3. Concurrent IRMS output acquired for the same injectionas as shown in Fig. 2.

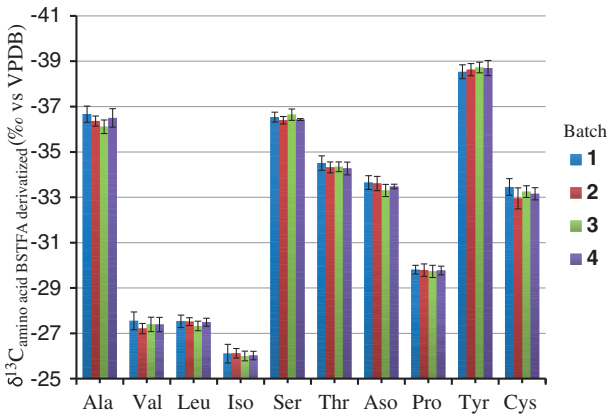

Figure 4. Reproducibility of BSTFA derivatization procedure for a standard mixture of ten different amino acids. $\delta^{13}C$ values are uncorrected for the added derivatization carbon atoms. Error bars represent 1 s.d. of the three replicates measured in each batch on four different days.

replicates of a standard of ten L-amino acids were converted into BSTFA derivatives on four different days and the $\delta^{13}C$ values of the twelve replicates were determined using GC/C/IRMS. The results are displayed in Fig. 4. The four batches produced very similar $\delta^{13}C$ values for BSTFA derivatives. The pooled standard deviations for twelve measurements ranged from 0.19‰ to 0.35‰ with an average precision of 0.26‰, which is similar to reported values.[18] One-way analysis of variance (ANOVA) shows that even at the 95% confidence level no significant differences were found between within-batch variances and between-batch variances for the four batches, thereby confirming the reproducibility of the technique when identical conditions were employed. Figure 5 shows the consistency of the measured $\delta^{13}C$ values when different starting quantities of (50–1000 µg) of each amino acid were used. Alanine and aspartic acid are shown, but the other amino acids gave similar results. Over this range of sample sizes, no significant differences were found between the mean isotope values or precision of different sample quantities. It appears that the method could be valid with sample sizes less than 50 µg, but we have not yet established the lower limits of the method. The absence of

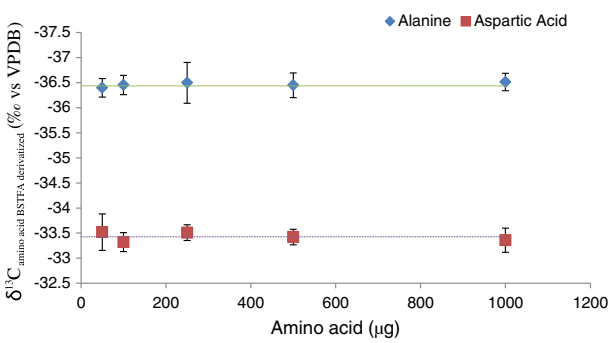

Figure 5. Variation in $\delta^{13}C$ values (uncorrected) following derivatization with BSTFA of varying amounts (50–1000 µg) of each amino acid; alanine and aspartic acid shown. The dashed lines represent the mean $\delta^{13}C$ value of each amino acid. Error bars represent the 1 s.d. of triplicate GC/C/IRMS determinations.

bias over this range of sample sizes suggests that corrections for absolute peak areas are not necessary with $\delta^{13}C$ bias related to this range.

Kinetic isotope effect (KIE)

During a chemical reaction such as derivatization, the rate-limiting step of the reaction may involve the cleavage of a C–X bond, which can be sensitive to the vibrational frequency of the bond. Such sensitivity to bond frequencies is called the kinetic isotope effect (KIE) and it can lead to enrichment or depletion of the heavier isotope, as described in detail elsewhere.[31,32]

In order to investigate the occurrence and magnitude of the KIE associated with the derivatization procedure, the $\delta^{13}C$ value of each amino acid was determined before and after derivatization (underivatized using EA/IRMS and derivatized using GC/C/IRMS). Mass balance equations were employed to determine the measured $\delta^{13}C$ values of the derivatized standard amino acids and the predicted $\delta^{13}C$ values. We therefore measured the $\delta^{13}C$ values of the BSTFA derivatization agent off-line using EA/IRMS to enable mass balance corrections, and all reactions were performed with the same batch of BSTFA. If no KIE is associated with the derivatization reaction, the exogenous carbon atoms can be corrected for using a mass balance equation[31]:

$$n_{cd}\delta^{13}C_{cd} = n_c\delta^{13}C_c + n_d\delta^{13}C_d \quad (2)$$

where n is the number of moles of carbon, subscript c represents the compound of interest, d the derivative group, and cd the derivatized compound. The KIE for both derivatization procedures was then calculated using the following equation[32]:

$$\mathrm{KIE} = 1 + \frac{\Delta n_{cd}}{1000x} \quad (3)$$

where Δ is the difference between the measured and predicted $\delta^{13}C$ values of the derivatized amino acids, n_{cd} is the total number of carbon atoms in the derivatized compound, and x is the number of the reactive protons available for silylation. Table 2 lists the KIE value calculated for each of the amino acid derivatizations with BSTFA. In this experiment, four replicate samples comprising 100 μg of each derivatized amino acid were measured three times each for a total of 12 measurements. The KIE values ranged from 0.984 to 1.020, with no obvious trends in the magnitude or sign of the KIE with respect to different functional groups.[32–34]

Application of the BSTFA derivatization method to human hair amino acids

Once the reproducibility of the method and the accuracy of the measurement had been assessed with standards, the BSTFA derivatization method was applied to human hair amino acids. In this approach, strong acid hydrolysis was first used to liberate amino acids from the protein matrix of the hair, which were then derivatized with BSTFA for GC/C/IRMS analysis. With our current procedure, ten amino acids were readily separated and analyzed, thereby providing many more data points than bulk analyses for distinguishing between the hair samples. A typical concurrent response is shown in Figs. 6 and 7. Three replicate measurements of three different sub-samples of the same batch of hair were pooled to provide a measurement error (1 s.d.) that ranged from ±0.68‰ for alanine to 0.23‰ for cysteine (n = 9). These relatively small errors were not significantly different from the precision determined for derivatization and analysis of amino acid standards. The precision of the $\delta^{13}C$ measurements of hair amino acids was also consistent with that of other published derivatization procedures and should permit longitudinal analysis or bulk comparison of within-person or between-person differences in amino acid isotopes in hair.

The results of the amino-acid-specific $\delta^{13}C$ values obtained for four replicate sub-samples of six individuals from the Ohio Valley region in the USA are shown in Table 3, along with a limited number of comparison values from the literature. With the exception of aspartic acid and serine, the compound-specific isotope ratios for the individual amino acids are in very close agreement with previously reported values for German subjects, whose average bulk $\delta^{13}C$ values for hair were similar to those found in the present study. Evershed's group has shown that aspartic acid from rat bone collagen had a $\delta^{13}C$ value around −25‰[18] (on a mostly C_3 diet), which is closer to

Table 2. Kinetic isotope effect (KIE) values of ten amino acid standards derivatized with BSTFA

Amino acid	EA/IRMS for $\delta^{13}C$ of bulk standard (n = 5) (95% CI)	GC/IRMS for BSTFA derivatized samples $\delta^{13}C$ (n = 12) (95% CI)	Predicted $\delta^{13}C$ value based on mass balance	KIE
Alanine	−19.70 (0.15)	−36.41 (0.22)	−35.29	1.005
Aspartic acid	−21.96 (0.05)	−33.51 (0.17)	−36.59	0.987
Isoleucine	−10.93 (0.30)	−26.06 (0.15)	−27.01	0.994
Leucine	−14.04 (0.05)	−27.47 (0.13)	−28.56	0.993
Proline	−11.40 (0.07)	−29.77 (0.13)	−28.69	1.006
Threonine	−26.35 (0.17)	−34.36 (0.16)	−37.94	0.984
Serine	−7.12 (0.09)	−36.50 (0.12)	−34.10	1.010
Tyrosine	−27.68 (0.14)	−38.65 (0.16)	−35.39	1.020
Valine	−11.17 (0.04)	−27.39 (0.19)	−28.58	0.993
Cysteine	−13.78 (0.12)	−33.20 (0.22)	−33.32	0.999

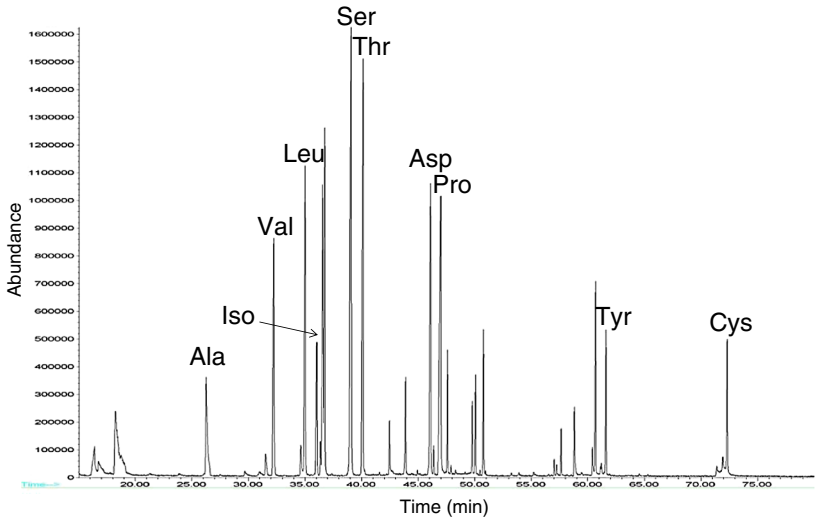

Figure 6. TIC chromatogram for a single injection of hair sample, after acid hydrolysis and derivatization, displaying individual amino acids.

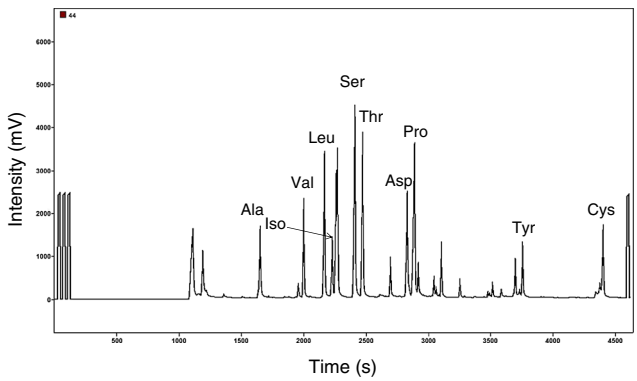

Figure 7. Concurrent IRMS output acquisition for the hair sample analyzed in Fig. 6.

our measured human hair value of −28%, but we cannot find any other examples of aspartic acid isotope ratio values for North American humans.

CONCLUSIONS

The new GC/C/IRMS method enables simultaneous detection of at least ten free amino acids using one-step BSTFA derivatization with no sample cleanup. The study was extended to the derivatization of amino acids in hair that were liberated from hair keratin through acid hydrolysis. Unfortunately, no hair standards exist that have been characterized at the amino acid level, but our corrected isotope values seem consistent with those previously reported for all amino acids except aspartic acid. The study of $\delta^{13}C$ values of amino acids in human hair is of interest in a wide variety of areas of basic and applied research and this simple derivatization method could make it easier for groups with a history of BSTFA derivatization to adopt IRMS as an alternative analysis method. The extensive spectral libraries for TMS-derivatized compounds would make it easier to identify potential interfering peaks in complex matrices.

Table 3. Example of the application of BSTFA derivatization to a small sample of volunteers (N = 6, 4 male, 2 female) from the Ohio Valley region in the Midwestern US. Reported values in this study take into account the mass balance correction and the kinetic isotope effects caused by derivatization

Component in hair	Average $\delta^{13}C$ (‰ vs. VPDB) *(95% CI in parentheses)* *EA/IRMS, n = 24 GC/IRMS, n = 54*	Typical $\delta^{13}C$ values from the literature (‰ vs. VPDB)
Bulk hair	−19.88[#] (0.66)	−16 to −20[a]
Alanine	−17.2 (1.7)	−12 to −21[b]
Aspartic acid*	−28.80 (0.70)	−14 to −18[b]
Isoleucine	−23.19 (0.74)	−22 to −27[b]
Leucine	−33.83 (0.62)	−30 to −34[b]
Proline	−19.77 (0.59)	−19 to −23[b]
Threonine	−27.31 (0.88)	−20 to −27[b], −26 to −30
Serine*	−14.13 (0.66)	−16 to −22[b], −22 to −25[c]
Tyrosine	−23.73 (0.53)	−29 to −31[b], −15 to −18[c]
Valine	−30.63 (0.68)	−20 to −25[b]
Cysteine	−20.19 (0.81)	

*Possible discrepancy, although amino-acid-specific values have not been reported for the US.
[#]Typical US average is closer to −17 per mil.[39]
[a]Valenzuela *et al.*[40]
[b]Based on typical German omnivorous diet from Petzke and Lemke.[41]
[c]Based on typical German diet between 1987 and 1988 from Petzke *et al.*[15]

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