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High-precision measurement of phenylalanine $\delta^{15}N$ values for environmental samples: A new approach coupling high-pressure liquid chromatography purification and elemental analyzer isotope ratio mass spectrometry

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RATIONALE: Compound-specific isotope analysis of individual amino acids (CSI-AA) is a powerful new tool for tracing nitrogen (N) source and transformation in biogeochemical cycles. Specifically, the $\delta^{15}N$ value of phenylalanine ($\delta^{15}N_{Phe}$) represents an increasingly used proxy for source $\delta^{15}N$ signatures, with particular promise for paleoceanographic applications. However, current derivatization/gas chromatography methods require expensive and relatively uncommon instrumentation, and have relatively low precision, making many potential applications impractical.

METHODS: A new offline approach has been developed for high-precision $\delta^{15}N$ measurements of amino acids ($\delta^{15}N_{AA}$), optimized for $\delta^{15}N_{Phe}$ values. Amino acids (AAs) are first purified via high-pressure liquid chromatography (HPLC), using a mixed-phase column and automated fraction collection. The $\delta^{15}N$ values are determined via offline elemental analyzer-isotope ratio mass spectrometry (EA-IRMS).

RESULTS: The combined HPLC/EA-IRMS method separated most protein AAs with sufficient resolution to obtain accurate $\delta^{15}N$ values, despite significant intra-peak isotopic fractionation. For $\delta^{15}N_{Phe}$ values, the precision was ±0.16‰ for standards, 4× better than gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS; ±0.64‰). We also compared a $\delta^{15}N_{Phe}$ paleo-record from a deep-sea bamboo coral from Monterey Bay, CA, USA, using our method versus GC/C/IRMS. The two methods produced equivalent $\delta^{15}N_{Phe}$ values within error; however, the $\delta^{15}N_{Phe}$ values from HPLC/EA-IRMS had approximately twice the precision of GC/C/IRMS (average stdev of 0.27‰ ± 0.14‰ vs 0.60‰ ± 0.20‰, respectively).

CONCLUSIONS: These results demonstrate that offline HPLC represents a viable alternative to traditional GC/C/IMRS for $\delta^{15}N_{AA}$ measurement. HPLC/EA-IRMS is more precise and widely available, and therefore useful in applications requiring increased precision for data interpretation (e.g. $\delta^{15}N$ paleoproxies). Copyright © 2013 John Wiley & Sons, Ltd.

Compound-specific stable isotope analysis of individual amino acids (CSI-AA) has become an increasingly common tool for addressing questions regarding source, transformation, and biogeochemical cycling of nitrogen, with applications spanning trophic ecology,[1–8] the marine carbon and nitrogen cycles,[9–12] and archeology.[13] The nitrogen isotopic ratio of the amino acid phenylalanine ($\delta^{15}N_{Phe}$ value) in particular has emerged as a powerful new AA proxy. Because Phe $\delta^{15}N$ values undergo little to no fractionation during degradation and trophic transfer,[1–3,10] these values in consumer tissues or detrital materials represent an integrated record for the 'baseline' $\delta^{15}N$ values of primary production at the base of a given ecosystem.[1,4–6,10,14–16] In paleoceanographic applications in particular, $\delta^{15}N_{Phe}$ measurements of organic paleoarchives (such as sediments and deep sea corals) may therefore provide highly detailed new information about the paleo-N cycle.[17]

The measurement of $\delta^{15}N$ values of individual amino acids is currently limited by the methods available. Most published values have been determined after organic derivatization, using coupled gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) approaches. Apart from the requirement for time-consuming derivatization reactions, this approach significantly limits CSI-AA application in at least two ways. First, GC/C/IRMS instrumentation is expensive, and thus not widely available in most labs. Second, due to the combined effects of the long sequence of both chemical manipulations and instrument components upstream of the final $\delta^{15}N$ measurement, the typical $\delta^{15}N$ precision for

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GC/C/IRMS (circa ±1.0‰)[1,10,12,17] is approximately an order of magnitude less than is typical for bulk $\delta^{15}N$ measurements (±0.1‰) via standard elemental analyzer-isotope ratio mass spectrometry (EA-IRMS). While sufficient for some applications, this relatively low precision could pose significant problems for others. In particular this becomes problematic for applications where interpretation is based on $\delta^{15}N$ values for single (or a few) AAs, as opposed to proxies which combine the values of many AAs into large averages.[10,17] One example is emerging paleoceanographic applications, where the $\delta^{15}N_{Phe}$ value may be the most useful proxy, yet the entire amplitude of $\delta^{15}N$ variation linked to recent anthropogenic ocean changes may be similar in magnitude to the GC/C/IRMS precision.[17]

Offline AA isolation and purification using high-performance liquid chromatography (HPLC), followed by isotopic analysis, represents an alternate approach to CSI-AA measurement. Previous work has explored the isolation of non-derivatized AAs by HPLC methods, although primarily focused on determining $\delta^{13}C$ or $\Delta^{14}C$ values. For example, a number of approaches have been developed for the preparative isolation of AAs from archeological bone collagen for $\Delta^{14}C$ analysis.[18,19] However, to our knowledge, no study has specifically focused on AA $\delta^{15}N$ values using analytical-scale approaches, or has attempted to optimize a method for Phe in particular. In addition, the focus of this previous work on carbon (C) isotopic values required a number of methodological limitations (e.g. use of C-free solvents), which are not present in an approach focused on mainly $\delta^{15}N$ values.

The approach presented here, which we have abbreviated HPLC/EA-IRMS to emphasize the 'offline' nature of the coupling of chromatographic and spectrometric techniques, could have significant potential advantages over GC/C/IRMS and previous HPLC methods. These include elimination of chemical derivatization, single column separation, higher precision, and simultaneous $\delta^{15}N$ and $\delta^{13}C$ measurement. However, several major challenges are inherent in the HPLC separation approach. Foremost would be the large intra-peak isotopic fractionation expected during chromatography.[20] Consequently, a practical HPLC/EA-IRMS method would require sufficient baseline resolution of target compounds such that automated fraction collection could routinely isolate entire peaks. In addition, the added sample handling associated with offline collections might be expected to add new sources of error not present in standard GC/C/IRMS.

Here we describe a new 'offline' CSI-AA method, optimized specifically for $\delta^{15}N_{Phe}$ and use in paleoceanographic applications. We describe the development and validation of a method coupling HPLC with EA-IRMS, and then compare CSI-AA results versus standard GC/C/IRMS for both standards and natural samples. We first assess the chromatographic separation of Phe and other AAs, and evaluate the potential error contribution in final AA $\delta^{15}N$ values due to both chromatography and sample processing. We then evaluate the precision of our HPLC/EA-IRMS method versus traditional GC/C/IRMS, comparing AA $\delta^{15}N$ values in both pure standards and natural samples. Finally, we demonstrate the potential utility of this method by comparing $\delta^{15}N_{Phe}$ records from a proteinaceous deep-sea bamboo coral generated by both HPLC/EA-IRMS and GC/C/IRMS.

EXPERIMENTAL

Isotopic AA standards

Standard L-AA powders were purchased from Alfa Aesar (Ward Hill, MA, USA) and Acros Organics (Morris Plains, NJ, USA) and used to prepare individual liquid standards (0.05M), which were then combined as an equimolar mixture of 16 individual AAs ('16AA Standard'). The 16AA Standard contained the proteinaceous AAs: glycine (Gly), L-alanine (Ala), L-arginine (Arg), L-aspartic acid (Asp), L-glutamic acid (Glu), L-histidine (His), L-isoleucine (Ile), L-leucine (Leu), L-lysine (Lys), D/L-methionine (Met), L-phenylalanine (Phe), L-proline (Pro), L-serine (Ser), L-threonine (Thr), L-valine (Val); and non-protein AA nor-leucine (Nle), which is commonly used as an internal standard.[4,5] The $\delta^{15}N$ and $\delta^{13}C$ values for the same dry standards were determined by standard EA-IRMS at the University of California, Santa Cruz Stable Isotope Laboratory (UCSC-SIL), following standard protocols.[21] The average precision of the EA-IRMS $\delta^{15}N$ standard values was 0.11 ± 0.07‰.

GC/C/IRMS analysis

Trifluoroacetyl isopropyl ester (TFA-IP) AA derivatives were prepared using standardized lab protocols, described previously.[11] Briefly, hydrolyzed samples were esterified in 300 µL 1:5 mixture of acetyl chloride/2-propanol (110 °C, 60 min). The resulting amino acid isopropyl esters were then acylated in 350 µL of a 1:3 mixture of dichloromethane (DCM)/trifluoroacetic acid anhydride (100 °C, 15 min). The derivatized AAs were dissolved in DCM to a final ratio of 1 mg of original proteinaceous material to 50 µL DCM. Isotopic analysis was conducted on a Thermo Trace GC Ultra gas chromatograph (Thermo Fisher Scientific, West Palm Beach, FL, USA) coupled via a Thermo GC IsoLink to a ThermoFinnigan Delta^{Plus} XP isotope ratio mass spectrometer (Thermo Fisher Scientific). Derivatives (1 µL) were injected (injector temperature 250 °C) onto an Agilent DB-5 column (50 m × 0.32 mm i.d. × 0.52 µm film thickness, Agilent Technologies, Inc., Santa Clara, CA, USA), with a He carrier gas flow rate of 2 mL/min (constant-flow). Separations were achieved with a four-ramp oven program: 52 °C, 2 min hold; ramp 1 = 15 °C/min to 75 °C, hold for 2 min; ramp 2 = 4 °C/min to 185 °C, hold for 2 min; ramp 3 = 4 °C /min to 200 °C; ramp 4 = 30 °C /min to 240 °C, hold for 5 min. This method allows for the determination of 11–15 AAs depending on derivatization efficiency and instrument sensitivity: Gly, Ala, Glu, Ile, Leu, Phe, Pro, Ser, Thr, Val, Nle, and provisionally: Met, His, Lys, and Arg. Samples were analyzed in quadruplicate (n = 4) with a bracketed lab AA isotopic standard mix for subsequent standard offset and drift corrections. Corrections were applied using previously published protocols.[11]

HPLC/EA-IRMS

A schematic of the complete HPLC/EA-IRMS protocol, from sample hydrolysis to EA-IRMS determination of AA $\delta^{15}N$ values, is shown in Fig. 1. Liquid chromatographic separations were conducted using a HPLC system (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) equipped with a system controller (SCL-10A vp), degasser (DGU-20A5),

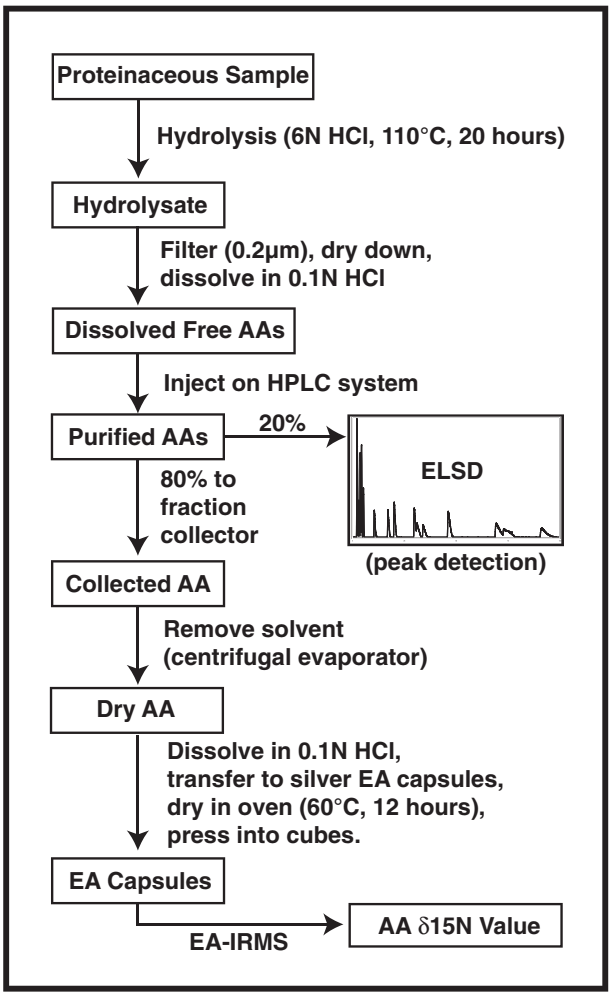

Figure 1. Flow chart showing sample preparation and analysis for the High-Pressure Liquid Chromatography/Elemental Analyzer–Isotope Ratio Mass Spectrometry method (HPLC/EA-IRMS). AA: amino acids; ELSD: Evaporative Light Scattering Detector, EA: Elemental Analyzer.

two pumps (LC-20AD), autosampler (SIL-20A) with an adjustable injection volume of 0.1–100 µL, and coupled to a Shimadzu automated fraction collector (FRC-20A). An adjustable flow splitter (Analytical Sales and Services, Inc., Pompton Plains, NJ, USA) was used inline following the chromatography column to direct ~20% of the flow to an evaporative light scattering detector (ELSD-LT II, Sedex 85LT; SEDERE, Alfortville, France) for peak detection and quantitation. A SiELC Primesep A column (4.6 × 250 mm, 100 Å pore size, 5 µm particle size; SiELC Technologies Ltd, Prospect Heights, IL, USA) was used for amino acid purification. This is a reversed-phase analytical-scale column, embedded with strong acidic ion-pairing groups. The acidic sites in the stationary phase interact with the charged functional groups and provide additional retention mechanisms to increase chromatographic separation potential for compounds with mixed functionality, such as AAs.

Typically, 75–100 µL of sample solution was injected onto the HPLC instrument. A binary solvent ramp program was used consisting of 0.1% trifluoroacetic acid (TFA) in HPLC-grade water (aqueous phase) and 0.1% TFA in acetonitrile (organic phase) at a flow rate of 1.5 mL/minute. The solvent ramp program used was as follows: starting with 100% aqueous/0% organic; increased from 0 to 1% organic from 0–15 min; increased to 9% organic from 15–30 min; held at 9% from 30–40 min; increased to 27% from 40–80 min; held at 27% until 105 min. The column was then cleaned and equilibrated by increasing to 100% and holding from 105 to 115 minutes; decreasing to 50% and holding from 115–120 min; then decreasing to 0% and holding until 125 min.

Purified AAs were collected via the automated fraction collector using time-based collections and transferred to 40 mL glass vials. The solvent was removed under vacuum using a Jouan centrifugal evaporator (Societe Jouan, Saint-Herblain, France) at a chamber temperature of 60 °C. Dry AA residues were then re-dissolved into a small volume (~150 µL) of 0.1 N HCl, transferred into pre-ashed silver (Ag) EA capsules, and dried to completion in a 60 °C oven for 12 h. The capsules were then pressed into cubes and analyzed for $\delta^{15}N$ and $\delta^{13}C$ values by EA-IRMS.

EA-IRMS analysis was conducted using a Carlo Erba CHNS-O EA1108-elemental analyzer interfaced via a ConFlo III device to a ThermoFinnigan Delta Plus XP isotope ratio mass spectrometer (Thermo Fisher Scientific). In order to accurately measure isotope ratios of small amounts (<15 µgN) of material, several modifications were made to the standard UCSC EA protocols. First, a zero-blank autosampler (Costech Analytical Technologies, Inc., Valencia, CA, USA) was used in order to reduce the atmospheric N contribution to the sample signal. The samples are then analyzed relative to three standards with varying known isotopic values and C/N ratios: acetanilide, isoleucine, and crystalized gelatin. Isoleucine EA-IRMS standards were prepared by pipetting precise volumes of liquid AA standard into silver EA capsules, and drying at 60 °C. The improved precision of this method resulted in calibration curves with higher R-values than those obtained by weighing dry standards of the same mass. The raw EA-IRMS $\delta^{15}N$ values were corrected for instrument drift and size effects using the UCSC SIL standard correction protocols.[21]

Proteinaceous coral sample preparation

A deep-sea bamboo coral (genus *isidella*) sample was previously collected in 2007 from Monterey Bay, CA, USA (36 44.6538 N, 122 2.2329 W, 870.2 m; T. Hill, personal communication, 2012). A proteinaceous node was separated from the calcium carbonate skeleton and cut into cross-sectional discs ~4mm thick. A chronological record was constructed by 'peeling' away successive layers from the outer 5 mm of a proteinaceous coral disc, following methods described by Sherwood and coauthors.[22] Seven peels of equivalent thickness averaging 0.66 mm were separated and photographed under magnification. The coral peels were oven dried (60 °C, 24 h) and 600 µg of material from each was reserved for bulk $\delta^{15}N$ analysis by EA-IRMS. The remaining material (40–50 mg) was hydrolyzed for subsequent HPLC/EA-IRMS and GC/C/IRMS analyses. Additional peels generated from the same node were analyzed for radiocarbon ($\Delta^{14}C$) age by accelerator mass spectrometry (AMS), in order to generate an age model and calculate an approximate growth rate for the coral specimen (F. C. Batista, J. T. Brown, T. P. Guilderson, unpublished work. 2012). Natural abundance $\Delta^{14}C$ analyses were conducted at

the Lawrence Livermore National Laboratories – Center for Accelerator Mass Spectrometry (Livermore, CA, USA) following standard graphitization procedures.[23] For individual AA analysis, 40–50 mg of proteinaceous coral tissue was placed in an 8 mL glass vial and dissolved in 5 mL 6 N hydrochloric acid (HCl) at room temperature. The tubes were flushed with nitrogen gas, sealed, and hydrolyzed under standard conditions (110 °C, 20 h). Acid hydrolysis quantitatively deaminates asparagine (Asn) to aspartic acid, and glutamine (Gln) to glutamic acid. While the abbreviations Glx and Asx are sometimes used to denote the combined Gln + Glu and Asn + Asp peaks, we have elected to simply use Asp and Glu abbreviations, as defined above, in order to correspond better with standard materials. The resulting hydrolysates were dried to completion under nitrogen gas and brought up in 0.1 N HCl to a final concentration of 1 mg tissue/20 µL 0.1 N HCl. Approximately 75% of each of the resulting mixtures was reserved for HPLC/EA-IRMS analysis, and the remaining material was dried to completion for derivatization and subsequent GC/C/IRMS analysis.

RESULTS AND DISCUSSION

While the ultimate goal for this work is the purification of Phe and precise $\delta^{15}N_{Phe}$ measurement for environmental, and especially paleoceanographic, applications, we first optimized our chromatographic separations for all common protein AAs. This provides a broader AA isotopic method that may be useful for other applications. In addition, focusing on AAs with a varying quality of separation allowed us to directly investigate the potential for $\delta^{15}N$ error caused by isotopic intra-peak gradients.

Method development and error evaluation: all protein AAs

Chromatographic optimization

The best overall AA separations with the Primesep A column (Fig. 2) were achieved using a binary solvent system at a flow rate of 1.5 mL/min. The use of perfluorinated carboxylic acids as ion-pairing agents in HPLC has been shown to be particularly effective at resolving polar underivatized amino acids.[24] Our optimized separation method (see Experimental section) uses TFA as an ion-pairing agent, which increases AA hydrophobicity, leading to a stronger interaction with the reverse stationary phase, increasing retention times (RTs), and improving peak resolution. Heptafluorobutyric acid (HFBA) was initially tested as an ion-pairing agent, which produced better peak separation in some cases (e.g., Asp/Ser), but also increased the degree of coelution for others peaks (e.g., Gly/Thr) leading to generally poorer overall separations (data not shown). In addition, HFBA greatly increased the RTs of late-eluting AAs, making its use impractical. In order to evaluate if a potential offset in $\delta^{13}C$ and $\delta^{15}N$ AA values might be caused by the use of TFA in the HPLC solvent, representative quantities of AAs were dissolved in similar volumes of TFA-containing solvent and dried to completion before EA-IRMS analysis. No significant offset was seen in any $\delta^{13}C$ or $\delta^{15}N$ values between pure and TFA-treated AAs (see Supplementary Fig. S1, Supporting Information). An increased flow rate of 1.5 mL/min was ultimately chosen (typical flow rate = 1 mL/min) despite decreased RTs in some cases, because the maintained system pressure of >2500 psi under these conditions improved overall peak shape and compound separations. Elution of 16 AAs for our optimized protocol required approximately 100 min and full baseline resolution was achieved for 10 AAs using pure standards (Fig. 2(a)). A near complete coelution was seen only for Asp/Ser, and partial coelutions for Ile/Leu and His/Lys.

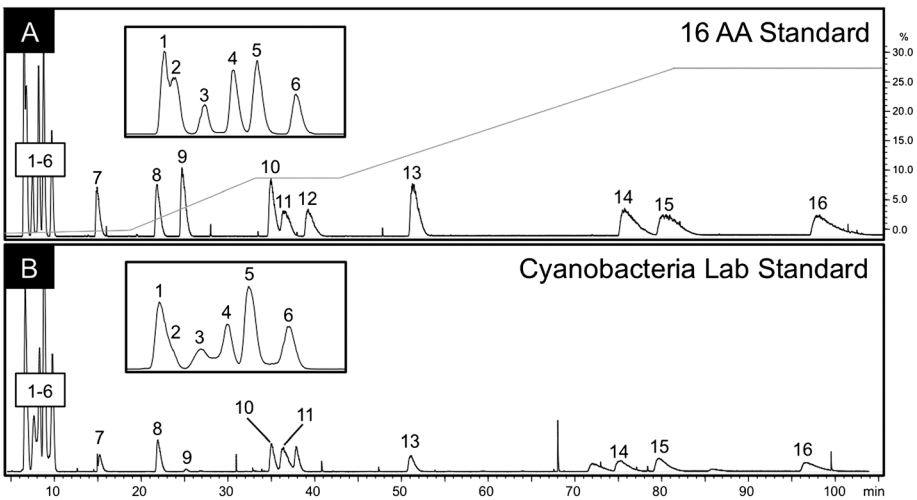

Figure 2. Representative HPLC-ELSD chromatograms. (A) 16 AA isotopic standard mixture. Each peak represents 300 nmol AA injected on-column. Light-grey line indicates % organic solvent (binary solvent program). Baseline resolution was achieved for 10 AAs; significant coelution is seen only for Asp/Ser, and minor coelutions for Ile/Leu and His/Lys. (B) Biological lab standard material (homogenized cyanobacteria). Peaks represent injection of ~1 mg of hydrolyzed dry mass, dissolved in 50 µL 0.1 N HCl. AA peak identifications: 1. Asp, 2. Ser, 3. Gly, 4. Thr, 5. Glu, 6. Ala, 7. Pro, 8. Val, 9. Met, 10. Ile, 11. Leu, 12. Nle, 13. Phe, 14. His, 15. Lys, 16. Arg.

For a natural biological reference sample, eight AAs were fully baseline resolved when analyzed at comparable peak sizes (Fig. 2(b)), due to differences in relative AA abundance and the presence of additional compounds.

Thresholds for accurate $\delta^{15}N$ measurement by EA-IRMS (7–10 μg N) largely determined sample loading. While reproducible full baseline separation for 10 AAs was possible at loadings of 300 nmol/AA, a substantially greater loading (1 μmol/AA) was required for single-peak isotopic analysis by standard EA-IRMS (i.e., without the need to combine fractions, which substantially decreased precision as discussed below). This resulted in overloaded peaks for most AAs; however, baseline separation was still achieved for a few AAs, including Phe (see Natural Paleoarchive section for overloaded sample chromatogram). A modified version of the HPLC/EA-IRMS method using a preparative-scale column would certainly reduce overloading, and might be appropriate for targeting other AAs. For our purposes, the less-expensive analytical-scale Primesep A column provides exceptional isolation of Phe (Fig. 2). Furthermore, the 'non-ideal' chromatography of some other peaks allowed us to directly constrain the isotopic effects of potential coelution.

Isotopic fractionation in HPLC

The kinetic processes that accompany the retention of a compound by a chromatography column can lead to a substantial stable isotope fractionation. For example, Hare *et al*. demonstrated strong nitrogen isotopic fractionation for AAs in large-scale, low-pressure column chromatography, reporting a $\delta^{15}N$ gradient of up to 31‰ across a single peak.[20] The kinetic isotope effect causes material collected from the leading edge of a given peak to be enriched in the light isotope (i.e., ^{15}N-depleted) compared with the bulk peak value, and, conversely, fractions near the peak tail are comparatively ^{15}N-enriched.

We directly tested the magnitude of intra-peak isotopic fractionation in our system by collecting the front and back halves (by area) of a Phe peak, chromatographed under our optimized conditions (Fig. 3(a)). The average $\delta^{15}N$ value (n = 7) of the front-end fraction was 4.4‰ versus 12.7‰ for the tail fraction, representing an average offset between the two halves of 8.3‰. A similar offset was also seen in $\delta^{13}C$ values, but (as expected) to a lesser extent (average $\delta^{13}C$ offset between front and back peak halves = 4.2‰). While not unexpected, this intra-peak fractionation for a single pure compound provides a general magnitude of possible error that could be introduced by incomplete collection of a single, well-separated peak. Overall, this result reinforces the importance of both complete peak separation and complete collection for accurate $\delta^{15}N$ values.

This predictable pattern of intra-peak fractionation can also explain the $\delta^{15}N$ error in adjacent partially coeluting peaks. Specifically, partial collection of two peaks would cause the measured $\delta^{15}N$ value in the first peak to be *low* versus the true value, due to coelution with the 'light' front of the second peak; conversely, the measured $\delta^{15}N$ value of the second peak would be *high* by coelution with the 'heavy' tail of the first peak. The magnitude of the error associated with a partial coelution would also be influenced by the isotopic offset between the true values of adjacent coeluting compounds. For example, the partial coelution of Ile and Leu (Fig. 3(b)) under our conditions produces relatively poor accuracy for both peaks (Leu: 1.67 ± 0.89‰, Ile: –7.39 ± 0.03‰) compared with the expected values of −0.01 ± 0.20‰ and −5.05 ± 0.06‰, respectively. The measured Ile $\delta^{15}N$ value (the first eluting peak) is low compared with the expected value; conversely, Leu $\delta^{15}N$ is high compared with its expected value, following predictions by intra-peak isotopic fractionation combined with partial peak mixing.

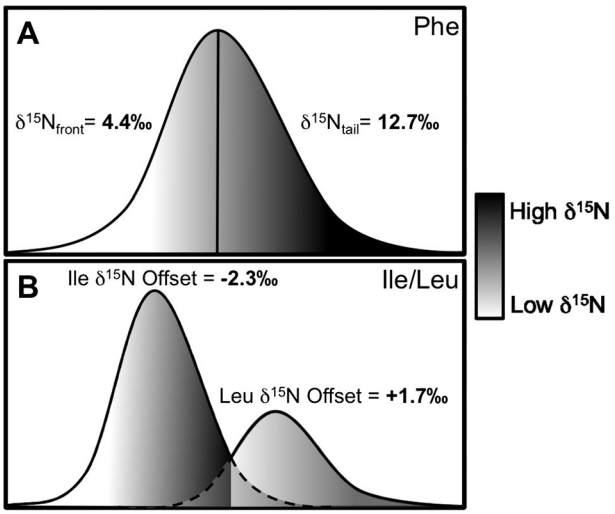

Figure 3. Effects of chromatographic fractionation on AA $\delta^{15}N$ value. Color gradient emphasizes the isotopic gradient caused by chromatographic fractionation, while numbers reflect directly measured values. (A) Graphical representation of a single Phe peak, with measured isotopic values of front and tail halves indicated. The $\delta^{15}N$ values represent the average of seven replicate collections of the front and back peak halves (by area); total offset was 8.3‰. (B) Graphical representation of a typical partial coelution, illustrated for Ile and Leu peaks (see Fig. 2(a)). The $\delta^{15}N$ value of each collected fraction is offset from the standard value due to mixing with the enriched front or depleted tail of the other peak. The $\delta^{15}N$ offsets represent the known standard value, subtracted from the HPLC/EA-IRMS average value.

Error associated with peak collection and sample handling

We also examined the effects of increased sample handling, which is inherent to the HPLC/EA-IRMS approach, on AA $\delta^{15}N$ accuracy and precision. Multiple injections with subsequent combination of individually collected fractions is a common approach in some offline isotopic methods,[25,26] as this allows improved chromatography and larger amounts of final material. However, a multiple collection approach also requires substantially more sample handing/processing, and for peaks without exceptional separation, would probably introduce variability related to minor, but unavoidable, inter-analysis shifts in RT.

We tested a series of AA isolations using replicate (3–4) injections, for which independent fractions were combined before being dried for further processing and EA-IRMS analysis. The combined fraction approach allowed isolation of substantially greater quantities of AA material (≥10 μg N) at loadings that optimized chromatographic separation. In most cases, the multi-peak collections produced AA $\delta^{15}N$ values close to the expected values, with an average offset of

0.8 ± 0.7‰ (offset magnitude range: 0.04–2.33‰; Fig. 4(a)). However, this accuracy is very low relative to offsets for single peak analysis discussed below (e.g., ~0.09‰ for Phe). The average mean deviation of replicate analysis for multi-peak collected AA standards by HPLC/EA-IRMS was also substantially higher than for single peak Phe measurements (0.36 ± 0.25‰ vs 0.16‰). We propose that the higher mean deviation and larger average offset associated with combining fractions are probably caused by a combination of three factors. Primarily, small shifts in RT can result in incomplete collection of peak fractions for which the retention 'windows' are narrow. There is also a higher risk of partial sample loss caused by the increased level of offline sample handling. Finally, it is possible that a small amount of extraneous N is present in the HPLC solvent leading to an N-blank contribution. However, in contrast to common solvent contamination issues when measuring C, the HPLC-grade reagents used here are highly unlikely to contain any significant amounts of non-volatile N-containing contaminants. This was confirmed by drying representative volumes of HPLC solvent before and after elution from the HPLC system and analyzing the resulting residues by EA-IRMS. In all cases the solvent N-blank was indistinguishable from the EA-IRMS instrument N-blank, suggesting the absence of N-containing impurities. It should be noted that the C peak associated with the dried solvent residues was also indistinguishable from the instrument blank, providing strong evidence for the complete removal of solvent during drying.

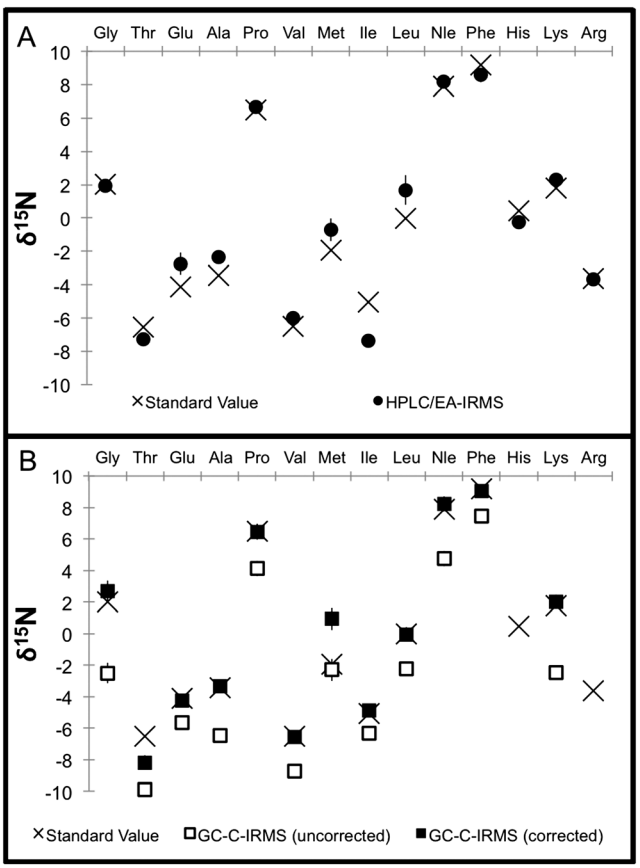

Figure 4. (A) Comparison of offline EA-IRMS reference values (X) versus HPLC/EA-IRMS results (circles) for AA standards. For HPLC/EA-IRMS, values represent averages for duplicate EA-IRMS measurements for four combined HPLC fraction collections (total of 10–14 μgN). Error bars represent mean deviation. (B) Offline EA-IRMS reference values (X) versus corrected (closed squares) and uncorrected (open squares) GC/C/IRMS values for the same standards. Average offset from references values after correction based on our external standard protocol was 0.5 ± 0.8‰; however, before correction, the average offset was substantially larger, 2.5 ± 1.2‰. AA abbreviations as defined in the text.

Although these three factors are not mutually exclusive, comparison of results for pure Phe standards is instructive, because Phe peaks are so widely separated chromatographically that coelution cannot reasonably be a factor. In addition, the allowance for very large fraction collection 'windows' should eliminate potential error caused by RT variability. The combined HPLC Phe fractions produced $\delta^{15}N$ values of 8.99 ± 0.87‰ (n = 6) compared with an expected value of 9.17‰, whereas a single peak collection experiment produced $\delta^{15}N_{Phe}$ values that were both closer to the known value (9.08‰), and with greatly improved precision (±0.16‰, n = 6). These results indicate that, despite the tradeoffs of decreased chromatographic resolution and smaller final sample amounts, even with well-separated peaks, the increased error associated with multiple fraction collections makes a single-peak collection approach superior.

Method performance for all protein AAs: GC/C/IRMS vs HPLC/EA-IRMS

While the data above shows that the HPLC/EA-IRMS method can produce good results vs known standard values, for samples having AAs with unknown values the most important comparison is how the HPLC approach compares with the widely used GC/C/IRMS method. We therefore directly compared the accuracy and precision for GC/C/IRMS $\delta^{15}N$ analyses performed on TFA/IP derivatives, versus HPLC/EA-IRMS results for the same non-derivatized AAs. A further, more detailed, comparison of relative precision and accuracy for $\delta^{15}N_{Phe}$ specifically is provided below. Following GC/C/IRMS correction protocols,[11] the values produced by the two methods were identical within error for most AAs, with an average AA $\delta^{15}N$ offset of 0.5 ± 0.8‰ (offset magnitude range: 0.02–2.85‰) across all AAs for the GC/C/IRMS method (Fig. 4(b)). This offset is lower than the typical analytical error for GC/C/IRMS. We conclude that, despite the imperfect HPLC separation for some peaks and the added error of combining multiple HPLC collections, as these methods are currently practised in our lab, GC/C/IRMS and HPLC/EA-IRMS produce comparable results for most commonly analyzed AAs.

However, one important caveat to this result involves the need for data correction in GC/C/IRMS analysis. The AA data discussed above are comparable between the two methods *only* after correcting the measured GC/C/IRMS AA $\delta^{15}N$ values based on bracketing external standard AA injections following the approaches described by McCarthy *et al.* and Calleja *et al.*[11,12] If the directly measured GC/C/IRMS data is taken instead (Fig. 4(b)) then the HPLC/EA-IRMS method produced more accurate AA $\delta^{15}N$ values overall; average GC/C/IRMS AA $\delta^{15}N$ offset for *uncorrected* values was 2.5 ± 1.2‰ (offset range: 0.4–4.5‰) compared with 0.8 ± 0.7‰ for HPLC/EA-IRMS values. This point is important for two reasons. First, most published GC/C/IRMS data report only *measured*

instrument values (based on an N_2 reference gas standard), often noting that a single internal standard was used, but without reference to any kind of systematic corrections using AA standards measured under actual analytical conditions. The measured AA $\delta^{15}N$ values can vary, sometimes widely, due to variation in GC/C oxidation/ reduction furnace conditions, and other factors.[11,27] The current comparison clearly demonstrates the necessity of making these kinds of offset corrections in $\delta^{15}N$ GC/C/IRMS analysis for AAs. Second, and perhaps more fundamentally, it demonstrates that the AA $\delta^{15}N$ values *directly* measured by the HPLC/EA-IRMS method appear to be substantially more accurate, as they are not dependent on additional non-standard secondary calibrations.

Optimized precision of phenylalanine $\delta^{15}N$ measurement

A main motivation of this work has been to develop a method for more precise $\delta^{15}N_{Phe}$ measurement since, as noted above, $\delta^{15}N_{Phe}$ records baseline $\delta^{15}N$ values,[1,4–6,10,14–16] and thus has great potential in paleoceanographic and other applications.[17] In our optimized HPLC method presented above, Phe also has the widest baseline resolution of any AA (Fig. 2). This means that sample loading has no real impact on Phe separation, allowing automated fraction collection without concern for coelution or incomplete collection, and single injections for each EA-IRMS analysis over a wide range of concentrations, which together minimize the error sources discussed above. Phe therefore represents an ideal AA to directly compare the best precision and accuracy likely to be available from HPLC/EA-IRMS versus GC/C/IRMS.

$\delta^{15}N_{Phe}$ measurement in standard materials

In order to directly assess the relative accuracy and precision of our offline HPLC/EA-IRMS method versus standard GC/C/IRMS for $\delta^{15}N_{Phe}$ values, we first compared both an isotopic standard and a hydrolysate of an internal lab standard biological material (a cyanobacteria). For the standard Phe solution (offline $\delta^{15}N$ value of 9.17 ± 0.08‰), the GC/C/IRMS analysis of TFA-IP derivatives produced a $\delta^{15}N$ value of 8.98 ± 0.64‰ ($n = 4$; following the correction routine noted above), while HPLC/EA-IRMS of the non-derivatized Phe standard yielded a $\delta^{15}N$ value of 9.08 ± 0.16‰ ($n = 6$; Fig. 5). Both methods therefore produced accurate values within error; however, the values obtained by the HPLC/EA-IRMS method were both closer to the reference offline standard value, and also had significantly greater precision than the GC/C/IRMS values (±0.16‰ vs ±0.30‰). In addition, as discussed above (Fig. 5), the GC/C/IRMS values required corrections (on average >2‰ in magnitude) to produce these final values being compared. Without this correction routine, which again is not commonly performed in many labs, the HPLC/EA-IRMS values would have been >1‰ closer to the expected standard value than the values obtained by GC/C/IRMS.

Analysis of relative precision and reproducibility

Because Phe is chromatographically very well separated in both the GC and HPLC methods, a more careful analysis of precision and reproducibility for this AA also provides an opportunity to assess the inherent 'best' performance for the two methods, and may also be able to indicate major sources

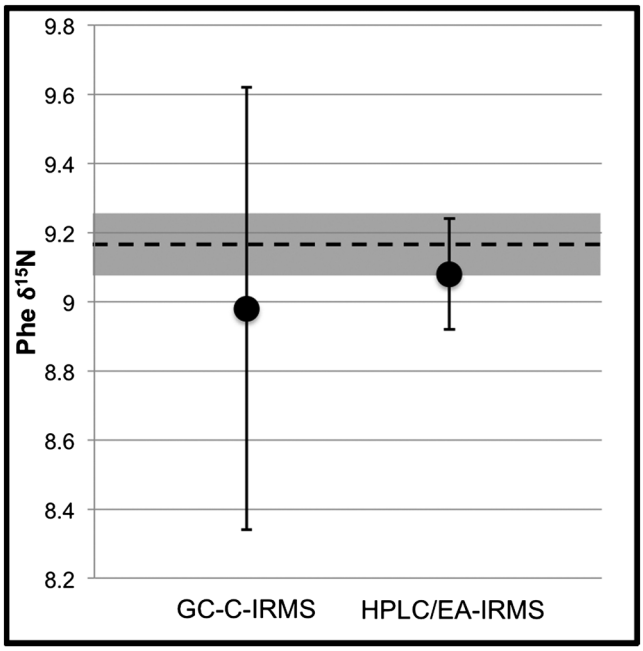

Figure 5. Accuracy and relative error for Phe standard $\delta^{15}N$ values by GC/C/IRMS versus HPLC/EA-IRMS. Dotted line represents offline EA-IRMS Phe $\delta^{15}N$ value; shaded region represents 1SD analytical error (9.17 ± 0.11‰). Measured Phe $\delta^{15}N$ values for GC/C/IRMS: 8.98 ± 0.64‰ ($n = 4$); for HPLC/EA-IRMS: 9.08 ± 0.16‰ ($n = 6$). Note that, as discussed in the text, the uncorrected GC/C/IRMS Phe value had substantially increased error.

of error in each. However, because of the substantial differences in methodology (e.g., derivatization), instrumentation (directly coupled versus offline), and sample handling between the two approaches, it is not possible to make an exact comparison of the precision of the two methods. Instead, we analyze relative precision in terms of two separate metrics: *Instrument Precision* and *Procedural Reproducibility* (Fig. 6).

Instrument Precision represents the ability of the isotope ratio mass spectrometer (and directly coupled instruments, e.g. the gas chromatograph or elemental analyzer peripherals) to measure $\delta^{15}N_{Phe}$ values reproducibly. In our HPLC/EA-IRMS method, the instrument precision is represented by the average standard deviation of Phe standard $\delta^{15}N$ values determined by EA-IRMS (0.09 ± 0.02‰). In the GC/C/IRMS approach, the instrument precision can be represented by the average standard deviation of successive replicate injections of a single derivatized Phe standard (0.64 ± 0.35‰). We note that this represents the precision of GC/C/IRMS for a TFA-derivatized compound; however, this is what is relevant for CSI-AA. The substantially higher error associated with the GC/C/IRMS instrument is typical for this analysis approach,[1,10,12,17] and is probably caused in large part by variability in oxidation/ reduction efficiency within the combustion reactors for the derivatized compound.

Procedural Reproducibility represents the ability of the entire method, from start to finish, to generate reproducible values. Unlike the instrument precision, procedural reproducibility therefore also includes error introduced through all sample preparation and handling, as well as possible matrix effects present in natural samples. In our HPLC/EA-IRMS method the procedural reproducibility can be represented by the

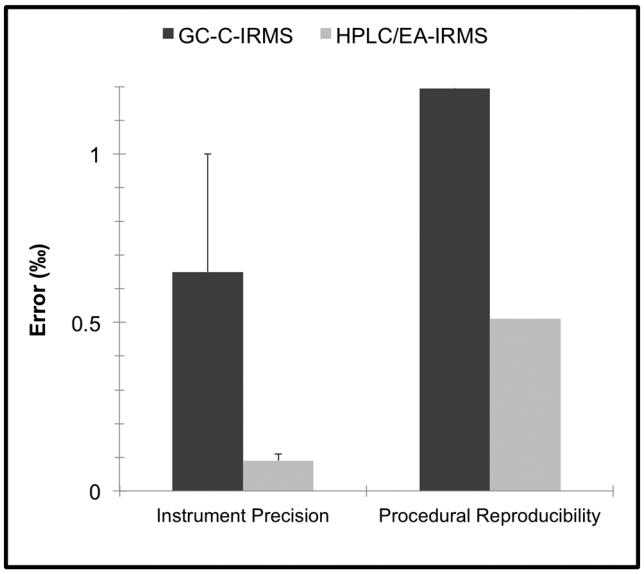

Figure 6. Comparison of instrument (analytical) precision and procedural reproducibility for GC/C/IRMS (dark bars) vs HPLC/EA-IRMS (light bars). Instrument precision: Average standard deviation for δ^{15}N Phe by GC/IRMS is 0.64 ± 0.35‰. Average standard deviation for δ^{15}N Phe by EA-IRMS is 0.09 ± 0.02‰. Procedural reproducibility: Standard deviation of all corrected GC/IRMS cyanobacteria δ^{15}N Phe (for lab standard material replicates) is ±1.19‰ (n = 5). Standard deviation for δ^{15}N Phe from cyanobacteria samples purified by HPLC and analyzed by EA-IRMS is ±0.51‰ (n = 3).

standard deviation of $\delta^{15}N_{Phe}$ values produced from replicate HPLC injections of cyanobacteria standard (±0.51‰, n = 3). The procedural reproducibility of GC/C/IRMS can similarly be expressed as the standard deviation of a number of cyanobacteria $\delta^{15}N_{Phe}$ values produced from multiple, but independent, full sample preparations (±1.19‰, n = 5).

Furthermore, a comparison of the overall instrument precision versus the procedural reproducibility within the *same method* (Fig. 6) should also approximate the relative error contributions for sample handling, preparation, and matrix effects. For example, in the HPLC/EA-IRMS method, the ~0.4‰ difference between precision and reproducibility probably represents the error associated with HPLC collections and sample handling, as well as a possible additional contribution from matrix effects typical for natural samples. The ~0.55‰ difference between these same values in the GC/C/IRMS method probably represents variability linked to matrix effects which might influence both the derivatization and the oxidation/reduction in the GC/C/IRMS interface. The larger relative difference between the two metrics (i.e., 4× difference between procedural reproducibility and instrument precision for HPLC/EA-IRMS, vs 2× for GC/C/IRMS) suggests that sample handling and matrix effects are more critical sources of error in the HPLC/EA-IRMS method. This conclusion is consistent with data discussed above suggesting that HPLC/EA-IRMS is somewhat more sensitive to error from sample handling. Overall, the greatly increased precision of the EA-IRMS instrument ultimately leads to better total reproducibility, with approximately twice the final precision of the GC approach.

$\delta^{15}N_{Phe}$ record comparison for a natural paleoarchive

The data above demonstrates that our HPLC/EA-IRMS method is generally more precise for $\delta^{15}N_{Phe}$ (and other AA) measurements in standards and reference materials. However, our ultimate goal is to assess if this approach can improve data quality from environmental samples and paleoarchives. We therefore analyzed a short record of $\delta^{15}N_{Phe}$ values in concentric proteinaceous bands of a deep-sea coral specimen (genus *Isidella*) from Monterey Bay, CA, USA (T. Hill, personal communication, 2012).

Deep-sea corals feed on freshly exported particulate organic matter (i.e. the sinking particle fraction that leaves the surface ocean), ultimately producing annual growth bands of gorgonin protein tissue.[28] As the organism lays down these concentric bands, they integrate the biogeochemical signals from the surface ocean, acting as 'living sediment traps'.[17,29] Due to the annual production of growth bands, and their very long relative lifespan (10^2–10^3 years),[28] deep-sea corals bridge a key gap between historical observations and paleoceanographic sedimentary records. These corals therefore represent a promising new tool for high-resolution records in the late Holocene where $\delta^{15}N_{Phe}$ values can represent a record of variations in export production δ^{15}N values over the time interval of a given coral specimen.[17] The gorgonin proteinaceous tissue, which makes up the banded skeletal material, remains well preserved and is highly amenable to CSI-AA because it is almost entirely composed of AAs.

We compared $\delta^{15}N_{Phe}$ records from HPLC/EA-IRMS and GC/C/IRMS for seven recent time intervals for a specimen collected live from Monterey Bay. A radiocarbon age model[22] indicates that this sample spans the last 35 years, with each value therefore representing an average of 5 years. A representative chromatogram of the overloaded HPLC injections used for Phe collection from the deep-sea coral specimen is shown in Fig. 7. After GC/C/IRMS corrections are applied, GC/C/IRMS and HPLC/EA-IRMS yielded equivalent $\delta^{15}N_{Phe}$ values for all samples (Fig. 8(a)). However, HPLC/EA-IRMS produced values with approximately twice the precision of GC/C/IRMS (0.27 ± 0.14‰ vs 0.60 ± 0.20‰). We note that the $\delta^{15}N_{Phe}$ precision for the proteinaceous gorgonin by both methods was actually better than that for the cyanobacteria lab standard material discussed above, underscoring the great potential of this specific archive for CSI-AA based paleoproxies. We also note a significant ($p < 0.01$) offset between the two records, with the average GC/C/IRMS values positively offset by 0.44 ± 0.25‰ from the HPLC/EA-IRMS values. While we cannot unequivocally demonstrate which is the correct answer for this natural record, all the data discussed above would strongly suggest that the HPLC/EA-IRMS values are more accurate. We would hypothesize that this systematic offset is caused by the offset corrections necessary for the GC/C/IRMS values. Because these corrections vary, and can at times be relatively large in magnitude (e.g., Phe standard corrections discussed above were >2‰ on average), there is an inherent danger of either over- or under-correcting values.

Finally, although individual values were equivalent within error, the increased precision for the HPLC/EA-IRMS record shows its potential to significantly improve the interpretation of paleoceanographic δ^{15}N data. While our intent here is not to attempt interpretation of past changes in the Monterey

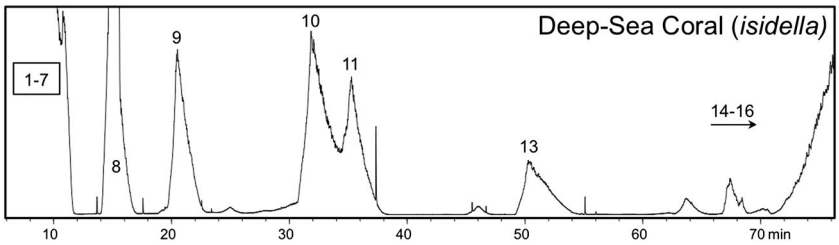

Figure 7. Representative HPLC-ELSD chromatogram of deep-sea coral gorgonin tissue. Peaks represent an overloaded injection of ~4 mg of hydrolyzed dry mass, dissolved in 80 µL 0.1 N HCl. AA peak identifications: 1. Asp, 2. Ser, 3. Gly, 4. Thr, 5. Glu, 6. Ala, 7. Pro, 8. Val, 9. Met, 10. Ile, 11. Leu, 12. Nle, 13. Phe, 14. His, 15. Lys, 16. Arg.

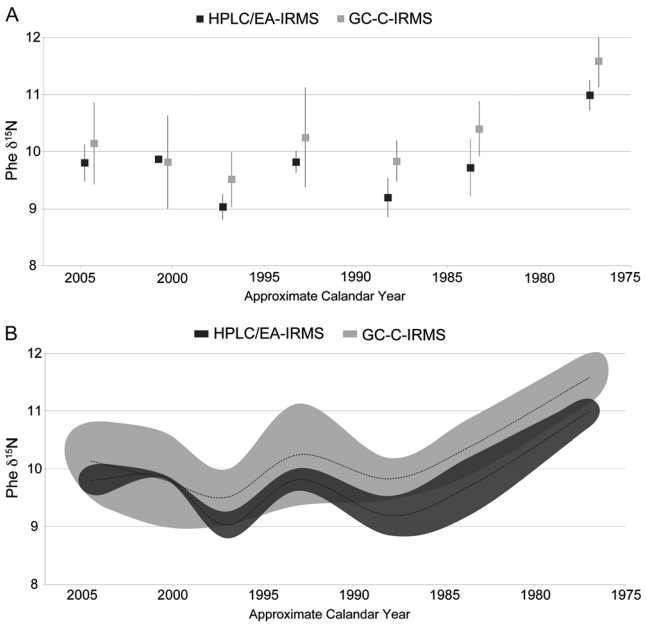

Figure 8. Comparison of $\delta^{15}N$ Phe record from a Monterey Bay deep-sea proteinaceous coral by GC/C/IRMS (light symbols and shading) vs HPLC/EA-IRMS (dark symbols and shading). (A) GC/C/IRMS average values represent four replicate injections, HPLC/EA-IRMS values represent three independent HPLC single-peak collections. (B) Same data as Fig. 7(A) is presented with shading emphasizing the relative error envelopes for the two analytical methods. The dashed lines represent a smoothed line connecting mean values for each, while the shaded envelope represents ± 1 standard deviation.

Bay ecosystem from a single specimen, we can compare potential interpretations of the replicate records produced from the two analytical procedures. Both $\delta^{15}N_{Phe}$ records indicate 1–2‰ variation in exported surface primary production, with average values of around 9.5‰ (consistent with primary production values, influenced by enriched nitrate upwelled from the California undercurrent).[30] Both records also show a marked decline in $\delta^{15}N_{Phe}$ values through the late 1970s, and suggest at least two periods of lower values in both the late 1980s and the late 1990s. Figure 8(b) shows an envelope of uncertainty for both methods and serves as a graphical representation of the relative error of the different approaches. The uncertainty in the GC/C/IRMS values typically spans 1–2‰, which is similar to or greater than both the typical natural variability indicated in this short recent record, and the magnitude of variability predicted to be caused by PDO or ENSO fluctuations in the broader California current system.[14,31,32] Therefore, these results suggest that the GC/C/IRMS approach might be unable to resolve, at least with statistical certainty, finer scale natural fluctuations linked to recent periodic forcings. For example, at the Hawaiian ocean time series site, changing stratification caused by a warming ocean has increased the strength of primary production, resulting in a decrease in $\delta^{15}N$ values of approximately 0.06‰ per year.[33] Therefore, on average a decade's worth of change would represent a 0.6‰ offset. If key processes of interest produce $\delta^{15}N_{Phe}$ offsets of this magnitude (comparable with the average precision of GC/C/IRMS measurements), this suggests that the increased precision of HPLC/EA-IRMS could be significant.

SUMMARY AND CONCLUSIONS

We have tested the viability of a new HPLC-based, 'offline' approach to making $\delta^{15}N$ measurements on individual amino acids, ultimately focused on developing a more precise method for measuring $\delta^{15}N_{Phe}$ values for potential proxy applications in paleoceanographic and other studies. We successfully developed a method capable of separating most AAs with baseline resolution. For Phe in particular, the chromatographic separation was exceptional (8 min between Phe and nearest AA peak), allowing fully automated peak collection without concern for coelution. While analysis of single peak fractions demonstrated expected large intra-peak isotopic gradients, comparison of values for authentic standards showed that for almost all AAs our chromatographic resolution was sufficient to produce accurate $\delta^{15}N$ values, which were also equivalent within error to values from GC/C/IRMS in most cases.

Direct comparisons of our HPLC/EA-IRMS approach with a now widely used GC/C/IRMS method showed that both methods produce $\delta^{15}N_{Phe}$ values for standards and natural samples, which are identical within error. However, results from the HPLC/EA-IRMS approach always had greater precision, with 4× greater precision for Phe standards (±0.16‰ vs ±0.64‰), and approximately 2× higher precision for natural samples (average standard deviation of 0.27 ± 0.14‰ vs 0.60 ± 0.20‰). Based on our analysis of the 'instrument precision' and 'analytical reproducibility' metrics, we propose that, although the non-instrument contribution to $\delta^{15}N$ standard deviation is greater in the HPLC/EA-IRMS

method, the total reproducibility is still far better than GC/C/IRMS, primarily due to the higher relative precision of the EA-IRMS instrumentation. Finally, results from the small pilot study of a deep-sea coral specimen suggest that $\delta^{15}N_{Phe}$ records from the HPLC/EA-IRMS method can provide both more information, and a higher degree of interpretability for recent and finer scale environmental change, than the standard GC/C/IRMS approach.

Overall, we conclude that our HPLC/EA-IRMS approach represents a viable alternative for $\delta^{15}N$ measurement of AAs, with substantial potential advantages over the current GC/C/IRMS method, particularly for $\delta^{15}N_{Phe}$ values. In addition, our method has a number of substantial advantages over previously published HPLC methods focused on C. These include: a single column chromatographic separation, use of analytical-scale chromatography with single injections, and vastly improved precision compared with reported $\delta^{15}N$ AA data (e.g. $\delta^{15}N_{Phe}$ $\sigma = 3.2‰$ from a two-column method).[18] The improved precision of HPLC/EA-IRMS may provide a significant benefit in interpreting $\delta^{15}N$ values in any application where the $\delta^{15}N$ variability is less than or equal to the magnitude of GC/C/IRMS error. This approach would probably be most useful for applications in which a few specific, but high precision, AAs measurements are required, because the requirement for individual AA collection and analysis significantly increases the analysis time versus that of GC/C/IRMS for a full suite of compounds. Finally, the ability of this method to make simultaneous $\delta^{15}N$ and $\delta^{13}C$ measurements suggests its potential to be modified for other applications, and possibly for additional AA isotopes. For example, future use of larger semi-preparatory or preparatory scale columns might allow the adaptation of our basic protocols for measurements having much larger sample requirements (e.g., $\Delta^{14}C$), or alternately be coupled with nano-scale EA approaches,[34] for applications that are highly sample limited.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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