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NEW METHODS FOR NITROGEN STABLE ISOTOPE MEASUREMENTS OF AMINO ACIDS: APPLICATIONS TO MARINE ECOLOGICAL STUDIES

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ABSTRACT

NEW METHODS FOR NITROGEN STABLE ISOTOPE MEASUREMENTS OF AMINO ACIDS: APPLICATIONS TO MARINE ECOLOGICAL STUDIES

Compound specific isotope analysis of individual amino acids (CSI-AA) is a powerful 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 baseline $\delta^{15}N$ values, with particular promise for paleoceanographic applications. Further, multiple studies have shown that $\delta^{15}N$ values of Phe and glutamic acid (Glu) can be coupled to provide precise estimates of trophic position (TP), while simultaneously decoupling baseline $\delta^{15}N$ values from the effects of trophic transfer. However, current derivatization/gas chromatography (GC) methods require expensive and relatively uncommon instrumentation, and have relatively low precision, posing significant issues for many potential applications. Here we present a new approach to CSI-AA based on a high-pressure liquid chromatography (HPLC) purification of underivatized amino acids, followed by offline elemental analysis-isotope ratio mass spectrometry (EA-IRMS). The utility of this approach is demonstrated in two different applications using natural materials: 1) the construction of a high precision paleoceanographic record from the $\delta^{15}N_{Phe}$ in proteinaceous coral tissue and 2) the TP estimation of five marine organisms using the coupled $\delta^{15}N$ values of Glu and Phe. Both demonstrations were compared to values measured by an established GC based method. In all cases both methods produced equivalent values, however, values generated by the new HPLC/EA-IRMS

approach had higher average precision in all cases. These results demonstrate that this approach represents a viable alternative to traditional GC based methods for $\delta^{15}N_{AA}$ measurement and may therefore allow significant expansion of CSI-AA applications, requiring only commonly available instrumentation to produce high precision $\delta^{15}N_{AA}$ values.

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Taylor A. B. Broek, Brett D. Walker, Dyke H. Andreasen and Matthew D. McCarthy. 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. *Rapid Communications in Mass Spectrometry*. Volume 27, Issue 21, 15 November 2013, Pages: 2327–2337, Article published online: 1 OCT 2013, DOI: 10.1002/rcm.6695.

Taylor A. B. Broek and Matthew D. McCarthy. A New Approach to $\delta^{15}N$ Compound-Specific Amino Acid Trophic Position Measurements: Preparative HPLC Technique for Purifying Amino Acids from Proteinaceous Samples for Stable Isotope Analysis. In review for publication in *Limnology & Oceanography: Methods.*

The co-author(s) listed in these publications directed and supervised the research which forms the basis for the thesis.

Now that the official stuff is out of the way…

I'll keep this brief and save my real decree of appreciation for the next one…
stand by for Taylor Broek's PhD dissertation….
coming to a dusty shelf near you Summer 2017 !?!?
(fingers crossed, do a dance for the instrument gods)

Lily… I love you more than anything.
Bear with me, I promise I won't be in school forever.

Mom and Dad… you made the boy who wrote this!
I literally couldn't have done it without you. Thank you for that (and so much more).

Fellow Grad Students… Dungeon Mates… our first year of hidden candles and mystery cups was one of the best of my life. Thank you for always keeping me safe.

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I cannot begin to thank you for the opportunities you have given me. When Ken first introduced us I had no idea how much it would change my life. Thank you for taking a chance on me. I'm proud to call myself a member of the McCarthy Lab for a few more years.

INTRODUCTION

Amino acids (AAs) are the most abundant identifiable class of nitrogen (N)-containing organic compounds in the marine environment, and therefore represent one of the most important molecular level organic measurements available to biogeochemists. Different types of AA measurements have been widely used to study diverse aspects of the flow of carbon (C) and N through the marine system. Relative AA molar percentages (AA Mol%) have been used to infer organic matter (OM) source (e.g., Ittekkot et al. 1984; Cowie and Hedges 1992; Ingalls et al. 2006), the degree of OM degradation, and overall OM "quality" (e.g., Cowie and Hedges 1994; Dauwe and Middelburg 1998). Enatiomeric ratios of protein AAs (i.e. D/L ratio) have been used to infer age of ancient organic material based on abiotic racemization rates (e.g., Bada and Lee 1977; Goodfriend 1997), and specific D-AAs have been developed as markers for bacterial input to actively cycling OM pools (e.g., McCarthy et al. 1998; Kaiser and Benner 2008). Most recently, the stable isotope analysis of individual amino acids (CSI-AA) has emerged as a powerful tool in ecological and biogeochemical studies, showing great promise for understanding the trophic structure and organic matter sources of ecosystems, providing new paleo N proxies, and overall providing a new level of detail for the biogeochemical cycling of organic C and N (e.g., McClelland and Montoya 2002; McCarthy et al. 2004; Popp et al. 2007; McCarthy et al. 2007; Chikaraishi et al. 2009).

Molecular level AA analysis requires chromatographic separation in order to purify individual AAs for subsequent measurement. The majority of regularly used

AA chromatography methods rely on gas phase chromatography to achieve separation, and almost all methods rely on upstream organic derivatization to render AAs volatile, and so amenable to this type of separation. The common use of gas phase separation methods for stable isotope measurements of AAs emerged alongside the development of continuous-flow isotope ratio monitoring mass spectrometers (IRMS) and in response to a number of inherent issues that arose during initial evaluation of AA isotopic values generated using large scale liquid chromatography (LC). Early IRMS systems required large relative quantities of material in order to generate accurate isotopic ratios. This requirement necessitated the use of large-scale preparative LC columns in order to generate enough AA material to make a single measurement (e.g., Serban et al. 1988; Hare et al. 1991). In addition to the research limiting sample requirements, a strong chromatographic fractionation effect was observed, whereby AA material eluting from the columns would have a gradient of isotopic value due to variable retention of molecules with different atomic masses. This fractionation effect added a number of analytical challenges to the generation of accurate CSI-AA values. Much of this early work was abandoned in favor of emerging gas-phase methods (e.g., Silfer et al. 1991; Merritt and Hayes 1994) and multiple AA derivatization procedures were developed, however, all presented a number of new inherent drawbacks. One common issue in coupled derivatization-chromatography AA approaches is that the wide functional diversity of AA side chains typically prevents any single derivatization approach from detecting all major AAs. In addition to excluding certain AAs from detection, separations based on

derivatized molecules by definition represent final values from modified (i.e. non-native) molecular structures. This is a particular issue when molecular-level isotopic ratios are the main interest, requiring significant corrections to measured isotopic values in order to account for added functional groups, ultimately limiting the achievable accuracy and precision of CSI-AA values (e.g., Silfer et al. 1991; Corr et al. 2007). Additionally, the high cost of gas chromatography – isotope ration mass spectrometry (GC-IRMS) instrumentation has limited the availability of these analyses within the scientific community.

New advances in LC column chemistry and IRMS instrumentation has opened up the possibility of a return to a combined preparative LC / offline IRMS method for CSI-AA measurements, avoiding the inherent issues of GC-IRMS based approaches without the limitations present in early LC based CSI-AA work. New "mixed-phase" LC columns allow for the complete separation of large quantities of unmodified (i.e. underivatized) -AA material, which, combined with the use of automated fraction collection could limit or prevent a number of the issues with early preparative LC columns. In addition, isotope ratio monitoring instrumentation has become nearly ubiquitous and has seen a significant reduction in required sample size. Therefore an offline coupling of high pressure liquid chromatography (HPLC) purification and elemental analyzer – isotope ratio mass spectrometry (EA-IRMS) measurements presents a viable alternative to GC-IRMS with a number of potential benefits over the established methods. In this thesis, I present my efforts to develop and evaluate an analytical method based on these advances capable of making the same

measurements as current methods, providing a complimentary analysis approach that selectively eliminates some of the inherent drawbacks of previously established methods.

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CHAPTER I:

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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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),

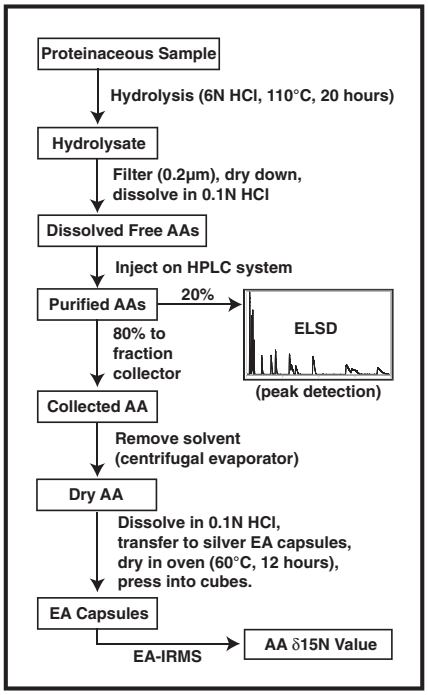

Figure 1. Flow chart showing sample preparation and analysis for the High-Pressure Liquid Chromatography/Elemental Analyzer–Isotope Ratio Mass Spectrometry method (HPLC/EA-IRMS). AA: amino acids; ELSD: Evaporative Light Scattering Detector, EA: Elemental Analyzer.

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.

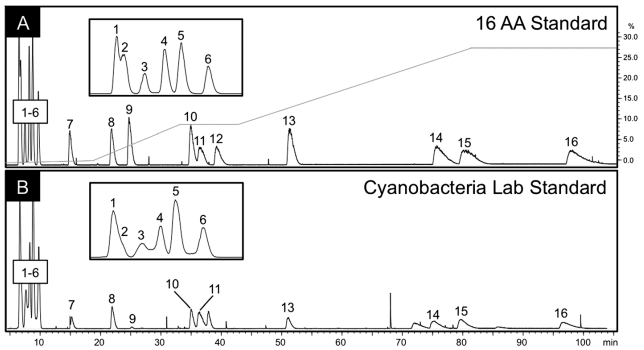

Figure 2. Representative HPLC-ELSD chromatograms. (A) 16 AA isotopic standard mixture. Each peak represents 300 nmol AA injected on-column. Light-grey line indicates % organic solvent (binary solvent program). Baseline resolution was achieved for 10 AAs; significant coelution is seen only for Asp/Ser, 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.

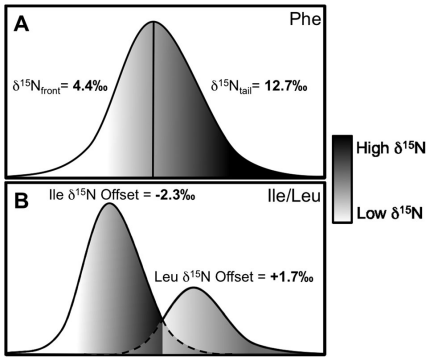

Figure 3. Effects of chromatographic fractionation on AA $\delta^{15}N$ value. Color gradient emphasizes the isotopic gradient caused by chromatographic fractionation, while numbers reflect directly measured values. (A) Graphical representation of a single Phe peak, with measured isotopic values of front 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.

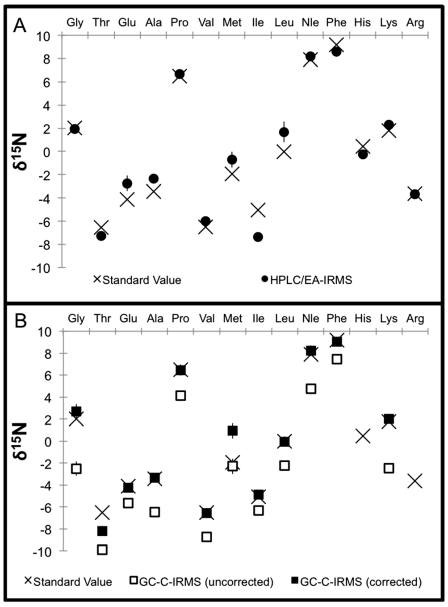

Figure 4. (A) Comparison of offline EA-IRMS reference values (X) versus HPLC/EA-IRMS results (circles) for AA standards. For HPLC/EA-IRMS, values represent averages for duplicate EA-IRMS measurements for four combined HPLC fraction collections (total of 10–14 μgN). Error bars represent mean deviation. (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

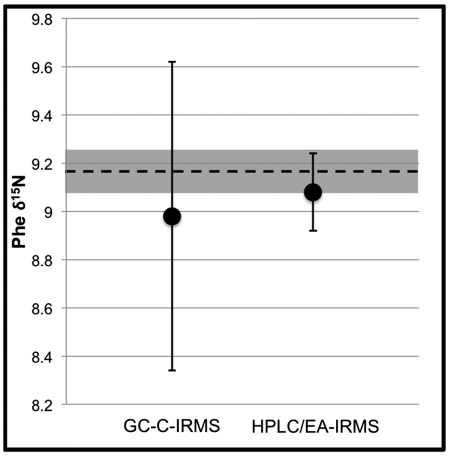

Figure 5. Accuracy and relative error for Phe standard $\delta^{15}N$ values by GC/C/IRMS versus HPLC/EA-IRMS. Dotted line represents offline EA-IRMS Phe $\delta^{15}N$ value; shaded region represents 1SD analytical error (9.17 ± 0.11‰). Measured Phe $\delta^{15}N$ values for GC/C/IRMS: 8.98 ± 0.64‰ ($n = 4$); 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

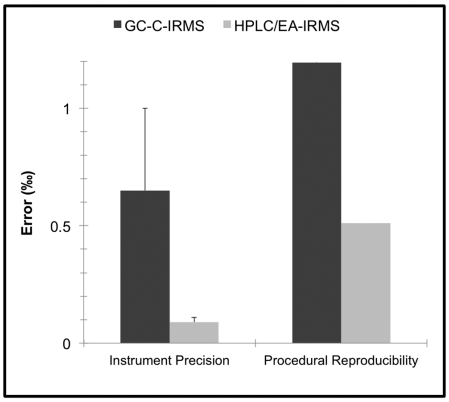

Figure 6. Comparison of instrument (analytical) precision and procedural reproducibility for GC/C/IRMS (dark bars) vs HPLC/EA-IRMS (light bars). Instrument precision: Average standard deviation for $\delta^{15}N$ Phe by GC/IRMS is 0.64 ± 0.35‰. Average standard deviation for $\delta^{15}N$ Phe by EA-IRMS is 0.09 ± 0.02‰. Procedural reproducibility: Standard deviation of all corrected GC/IRMS cyanobacteria $\delta^{15}N$ Phe (for lab standard material replicates) is ±1.19‰ (n = 5). Standard deviation for $\delta^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{15}N$ data. While our intent here is not to attempt interpretation of past changes in the Monterey

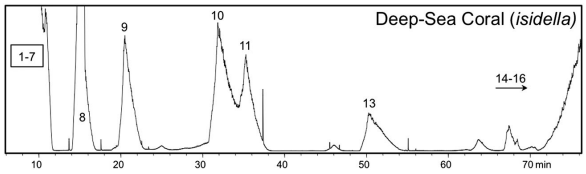

Figure 7. Representative HPLC-ELSD chromatogram of deep-sea coral gorgonin tissue. Peaks represent an overloaded injection of ~4 mg of hydrolyzed dry mass, dissolved in 80 µL 0.1 N HCl. AA peak identifications: 1. Asp, 2. Ser, 3. Gly, 4. Thr, 5. Glu, 6. Ala, 7. Pro, 8. Val, 9. Met, 10. Ile, 11. Leu,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

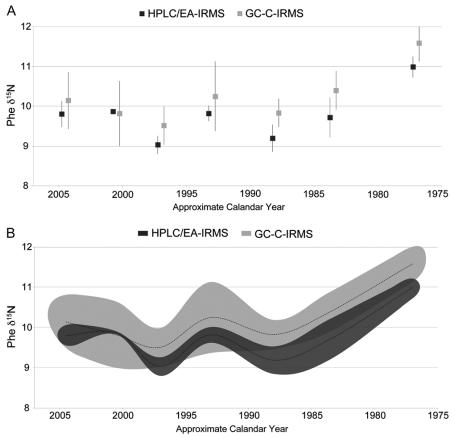

Figure 8. Comparison of $\delta^{15}N$ Phe record from a Monterey Bay deep-sea proteinaceous coral by GC/C/IRMS (light symbols and shading) vs HPLC/EA-IRMS (dark symbols and shading). (A) GC/C/IRMS average values represent four replicate injections, HPLC/EA-IRMS values represent three independent HPLC 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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CHAPTER II:

A New Approach to $\delta^{15}N$ Compound-Specific Amino Acid Trophic Position Measurements: Preparative HPLC Technique for Purifying Amino Acids from Proteinaceous Samples for Stable Isotope Analysis. Limnology & Oceanography: Methods.

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Abstract:

Compound specific isotope analysis of amino acids (CSI-AA) has emerged as an important new method for investigating trophic dynamics in both aquatic and terrestrial systems. Multiple studies have shown that $\delta^{15}N$ values of glutamic acid (Glu) and phenylalanine (Phe) can be coupled to provide precise estimates of trophic position (TP), while simultaneously decoupling baseline $\delta^{15}N$ values from the effects of trophic transfer. However, the current standard gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) approach is limited by high expense, limited availability, and relatively low precision. We present a new method for making TP estimates in biological samples by CSI-AA (TP_{CSIA}), based on a high-pressure liquid chromatography (HPLC) purification of underivatized amino acids, followed by offline elemental analysis-isotope ratio mass spectrometry (EA-IRMS). We compare results from our new HPLC/EA-IRMS method versus GC-C-IRMS in both standard and natural materials. Nitrogen isotopic values of purified Glu and Phe standards were identical within error for both methods. In five widely different marine organisms, the $\delta^{15}N$ values of Glu and Phe were also indistinguishable within error between the two approaches; however, the $\delta^{15}N$ values produced by the HPLC/EA-IRMS approach had higher average precision (average SD = 0.3±0.2‰) than the GC-C-IRMS measurements (average SD = 0.45 ± 0.15‰). The resulting TP_{CSIA} estimates were statistically indistinguishable ($t < 1.2$, df = 6, $p > 0.3$) between the two methods for all organisms examined. Or HPLC/EA-IRMS method may

therefore allow significant expansion of TP_{CSIA} applications, requiring only commonly available instrumentation to produce high precision TP_{CSIA} values.

Introduction:

The stable isotope analysis of individual amino acids (CSI-AA) is rapidly developing as a powerful new tool in ecological and biogeochemical studies, showing great promise for understanding the trophic structure of ecosystems, providing new paleo N proxies, and overall providing a new level of detail for the biogeochemical cycling of organic nitrogen (recently reviewed by McMahon et al. 2014). To date the fastest growing, and arguably most important, CSI-AA application has been as a new approach to calculating precise estimates of an organism's trophic position (TP). McClelland and Montoya first showed that two specific groups of individual AAs undergo very different ^{15}N fractionation with trophic transfer (McClelland and Montoya 2002). One group of AAs (now termed the "trophic" AAs; after Popp et al. 2007) undergoes large and predictable isotopic fractionation with each trophic transfer, while a second group ("source" AAs) maintain relatively unchanged $\delta^{15}N$ values. Within these two broad groupings, changes in the $\delta^{15}N$ value of glutamic acid ($\delta^{15}N_{Glu}$) has been found to most reproducibly indicate the extent of trophic transfer, while the $\delta^{15}N$ value of phenylalanine ($\delta^{15}N_{Phe}$) has been shown to be the most stable, therefore providing a direct estimate of the original $\delta^{15}N$ isotopic value of primary production (McClelland and Montoya 2002; Chikaraishi et al. 2009). Therefore measuring $\delta^{15}N$ values of Glu and Phe together allows a decoupling, for the first time,

of the influence of baseline $\delta^{15}N$ values (i.e., variation in inorganic-N sources, extent of N-fixation, etc.) from trophic changes. CSI-AA based TP estimates (TP_{CSIA}) therefore overcome a basic problem in the interpretation of bulk $\delta^{15}N$ data, because this approach requires no assumptions about baseline $\delta^{15}N$ values. TP_{CSIA} are therefore typically more accurate than those based on bulk $\delta^{15}N$ data alone (McMahon et al. 2013; Germain et al. 2013), and have now been widely applied in studies of trophic interactions in many ecosystems (e.g., McClelland and Montoya 2002; McCarthy et al. 2004; Chikaraishi et al. 2007; Popp et al. 2007; McCarthy et al. 2007; Chikaraishi et al. 2009; Hannides et al. 2009; Lorrain et al. 2009; Chikaraishi et al. 2011; Choy et al. 2012; McCarthy et al. 2013). In addition, these internally normalized TP_{CSIA} estimates have also provided a key parameter for paleoceanographic studies, as a proxy for the ecosystem structure of the overlying water column (e.g., Sherwood et al. 2011; Broek et al. 2013; Sherwood et al. 2014).

All published TP_{CSIA} work has so far been determined based on $\delta^{15}N$ values measured after organic derivatization, using coupled gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) approaches. However, there are a number of inherent restrictions to the GC-C-IRMS approach, which together limit accuracy, precision, and ultimately the extent of possible interpretations. First, the combined effects of the derivatization process and instrument components leads to a relatively low order of precision for GC-C-IRMS CSI-AA data (typically ±1.0‰; e.g., McClelland and Montoya 2002; McCarthy et al. 2007; Sherwood et al. 2011; Broek et al. 2013; Calleja et al. 2013), approximately an

order of magnitude less than is typical for bulk stable isotope measurements (±0.1‰) via standard elemental analyzer-isotope ratio mass spectrometry (EA-IRMS). The lower precision inherent in the GC-C-IRMS system limits the potential precision of TP_{CSIA} estimates, since these ultimately derive from the propagated analytical error of individual AA $\delta^{15}N$ measurements. In addition, the need for volatile derivatives means that GC-C-IRMS by definition requires isotopic measurements to be made on modified (i.e., non-native) molecular structures. Unlike $\delta^{13}C$ measurements, where specific corrections are commonly made directly for added C (e.g., Silfer et al. 1991; Corr et al. 2007), for $\delta^{15}N$ measurements any error introduced due to derivatization can only be inferred by comparison to standard AA materials with well defined $\delta^{15}N$ values. The magnitude of these offset corrections are sometimes large relative to the typically reported analytical precision (e.g., Broek et al. 2013; McCarthy et al. 2013), further limiting the ultimate certainty of isotopic measurements. Finally, an overarching practical consideration is that GC-C-IRMS instrumentation is quite expensive, and is therefore not currently widely available to most laboratories. In addition, extensive analytical organic chemistry experience has shown to be necessary to maintain GC-C-IRMS systems capable of producing high precision measurements, a direct contrast to many ecological TP_{CSIA} applications which derive from non-chemically oriented research groups. Ultimately, an alternative to GC-C-IRMS, which could produce accurate and precise TP_{CSIA} estimates, could have a number of significant applications.

Offline AA isolation and purification using high-pressure liquid

chromatography (HPLC), followed by offline isotopic analysis of individual compounds represents one such alternate approach to CSI-AA measurement, with significant potential advantages. These advantages include the elimination of chemical derivatization, higher inherent precision of offline stable isotope measurements, and the potential for simultaneous $\delta^{15}N$ and $\delta^{13}C$ measurements on any single compound. Previous work has explored the isolation of non-derivatized AAs by HPLC methods for subsequent isotopic analysis, although primarily focused on determining $\Delta^{14}C$ values. For example, a number of approaches have been developed for the preparative isolation of AAs, primarily hydroxyproline, from archeological bone collagen for $\Delta^{14}C$ analysis (e.g., Tripp et al. 2006; McCullagh et al. 2010). For $\delta^{13}C$ values of AAs, recent methods are now also available that utilize a commercially available LC-IRMS interface (HPLC-IsoLink-IRMS; e.g., Choy et al. 2010; Dunn et al. 2011), however the wet chemical oxidation required by this instrument precludes the measurement of $\delta^{15}N$ values, and these measurements also remain dependent on specialized and expensive instrumentation. A small number of past studies have reported $\delta^{15}N$ values using offline HPLC purification, however these have typically only resolved a limited suite of AAs, have required complex sample purification procedures (e.g. multiple column separations), and produce values with low relative accuracy and precision compared to established GC method (e.g., Tripp et al. 2006). Recently, Broek at al. (2013) demonstrated a single column, analytical scale HPLC approach for the measurement of $\delta^{15}N_{Phe}$, focused specifically on $\delta^{15}N_{Phe}$ as a key new proxy in paleoceanographic studies. While this prior method

could not resolve Glu (nor a number of other common AAs), it clearly showed the feasibility of an offline approach, including a demonstration that higher accuracy and precision is possible compared to standard GC-C-IRMS.

Here we present a new offline method for making TP_{CSIA} measurements, coupling HPLC purification with offline EA isotopic measurement (HPLC-EA/IRMS). We have focused on optimizing our method for $\delta^{15}N$ values for Glu and Phe, because the offset in $\delta^{15}N$ values for these two AAs is currently the most widely used basis for TP_{CSIA} estimates in biological materials. First we present our optimized chromatographic separation of AA standard mixtures, and subsequently evaluate the accuracy and precision of Glu and Phe $\delta^{15}N$ measurements using standards of known isotopic composition. We then compare these results to $\delta^{15}N$ values produced via GC-C-IRMS analysis to directly compare the relative precision and accuracy of the two methods with pure standards. Finally, we evaluate the chromatographic separation of AAs from a range of natural samples, including different tissue types with a range of complex organic matrices. A key goal here was to both assess method performance in actual biological materials, but also to evaluate any potential for unknown N-containing compounds to affect $\delta^{15}N$ values of Glu and Phe, and therefore potentially influence resulting TP estimates. To do this, we directly compared Glu and Phe $\delta^{15}N$ values, and also the resulting TP_{CSIA} estimates, by our new method and standard GC-C-IRMS with a particular focus on their relative analytical precision. Finally, as a secondary goal, we sought to achieve sufficient separation of large suite of proteinaceous AAs for accurate isotopic measurements, providing versatility in future

CSI-AA applications.

Materials and Procedures:

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 (0.05 M), which were then combined as an equimolar mixture of 16 individual AAs ("16 AA Standard") for developing separations. The 16 AA 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 (Popp et al. 2007; McCarthy et al. 2013). The $\delta^{15}N$ and $\delta^{13}C$ values for dry standards were determined by standard EA-IRMS at the University of California, Santa Cruz Stable Isotope Laboratory (UCSC-SIL) following standard protocols (http://es.ucsc.edu/~silab). Average precision of EA-IRMS $\delta^{15}N$ standard values was 0.11 ± 0.07‰. Additionally, a commercially available equimolar AA standard mixture "Pierce Amino Acid Standard H" (Pierce H) (Thermo Scientific) containing the same AAs as the "16 AA Standard" with the exception of the non-protein AA Nle and addition of the proteinaceous AAs cysteine (Cys) and tyrosine (Tyr) was used to construct individual ELSD calibration curves, so as to verify relative molar abundance of

individual AAs in natural samples (chromatogram in supplementary materials).

Proteinaceous Sample Preparation:

The cyanobacteria sample (*Spirulina Sp.)* was obtained as a bulk commercial dry powder (Spirulina Pacifica®, Nutrex Hawaii, Kailua-Kona, HI). This same sample has been used previously as a McCarthy laboratory internal quality control standard, and its CSI-AA values have been measured repeatedly by GC-C-IRMS, allowing an investigation of the long-term accuracy and precision of the GC-C-IRMS instrument. Coastal mussel (*Myilitus Califorianus)* sample was collected in 2012 from Santa Cruz, CA. The mussel was previously dissected, and the adductor muscle tissue removed and lyophilized prior to storage. We used a subsample of adductor muscle collected for a prior study (Vokhshoori and McCarthy 2014) hydrolyzing the bulk lyophilized adductor muscle tissue directly without lipid extraction. The 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) (Hill, *pers. comm.* 2011). A proteinaceous node was separated from the calcium carbonate skeleton and oven dried (60 °C, 24 h). White sea bass muscle tissue was subsampled from an incidental recreational catch in 2007, landed from Santa Cruz Island, Channel Islands, CA (J. Patterson, *pers. comm.* 2007). Fish muscle tissue was also lyophilized prior to hydrolysis. Harbor seal blood was collected in May-June 2007 from a wild animal in Tomales Bay, CA (38°13.9 ’N, 122°58.1 ’W) under NMFS Research Permit no. 555-1565. Blood serum was purified, lipid extracted, and lyophilized prior to hydrolysis,

as described previously (Germain et al. 2011).

For all sample types, proteinaceous material was hydrolyzed by adding 40-50 mg of bulk dry sample to an 8 mL glass vial, followed by 5 mL of 6 N hydrochloric acid (HCl) at room temperature. The vials were flushed with nitrogen gas, sealed, and allowed to hydrolyze under standard conditions (110 ºC, 20 h). Hydrolysis under acidic conditions quantitatively deaminates asparagine (Asn) to aspartic acid, and glutamine (Gln) to glutamic acid (Barrett 1985). Therefore, in this protocol (and all others based on acid hydrolysis), measured Glu in fact represents Gln+Glu, and measured Asp represents Asp+Asn. We note that while the abbreviations Glx and Asx are sometimes used to denote these combined Gln+Glu and Asp+Asn fractions, we have elected to simply use Asp and Glu as abbreviations, as defined above, in order to correspond better with prior TP_{CSIA} literature. Additionally, acid hydrolysis is known to destroy cysteine (Cys), precluding it from analysis (Barrett 1985). 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/ 100 μL 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.

GC-C-IRMS Analysis:

Trifluoroacetyl isopropyl ester (TFA-IP) AA derivatives were prepared using standardized lab protocols, as described previously (McCarthy et al. 2013). Briefly,

hydrolyzed samples were esterified in 300 μL 1:5 mixture of acetyl chloride:2-propanol (110 °C, 60 minutes). The resulting amino acid isopropyl esters were then acylated in 350 μL 1:3 mixture of dichloromethane (DCM):trifluoroacetic acid anhydride (100 °C, 15 minutes). 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 (Thermo Fisher Scientific, West Palm Beach, FL, USA) coupled via a Thermo GC IsoLink to a ThermoFinnigan Delta^{Plus} XP isotope ratio monitoring mass spectrometer (Thermo Fisher Scientific). Derivatives (1 μL) were injected (injector temp. 250 °C constant) onto an Agilent DB-5 column (50 m x 0.32 mm ID x 0.52 μm film thickness, Agilent Technologies, Inc., Santa Clara, CA, USA), with a He carrier 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. Values are typically obtained for Gly, Ala, Glu, Ile, Leu, Phe, Pro, Ser, Thr, Val, Nle, and Lys. Values for Met, His and Arg are obtained only in some samples, depending on concentration and derivatization efficiency. For $\delta^{15}N$ AA values, samples were analyzed in quadruplicate (n=4) with bracketed lab AA isotopic standard mix for subsequent standard offset and drift corrections. Corrections based on authentic external standards were applied using previously published protocols (McCarthy et al. 2013).

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 Figure 1. Liquid chromatographic separations were conducted using a Shimadzu HPLC system (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) equipped with: system controller (SCL-10A vp), degasser (DGU-20A5), 2 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 ~15% of the flow to a SEDERE (Alfortville, France) evaporative light scattering detector (ELSD-LT II, Sedex 85LT) for peak detection and quantitation. A semi-preparative scale SiELC Primesep A column (10 x 250 mm, 100 Å pore size, 5 μm particle size; SiELC Technologies Ltd., Prospect Heights, IL, USA) was used for amino acid purification. The Primesep A column used here is a reverse-phase semi-preparative scale column embedded with strong acidic ion-pairing groups. Such mixed phase columns have been developed specifically for the separation of charged organic compounds as the acidic sites in the stationary phase interact with the charged functional groups and provide additional retention mechanisms to increase chromatographic separation potential. For a more detailed description of the retention mechanisms of the Primesep A column see (McCullagh et al. 2006; 2010).

Typically, 75-100 μL of sample solution was loaded 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). The final solvent ramp program used for optimal separation was as follows: starting with 100% aqueous / 0% organic; increased from 0 to 0.5% organic from 0-30 minutes; increased to 15% organic from 30-35 minutes; increased to 22.5% from 35-70 minutes; increased to 30% from 70-95 minutes; held at 30% until 140 minutes. The column was then cleaned and equilibrated by increasing to 100% and holding for 20 minutes; then decreasing to 50% and holding for an additional 15 minutes; then decreasing to 0% and holding until the method ends at 180 minutes. A flow rate ramp is also employed in which the total flow rate is held at 2.5 mL/minute for 0-30 minutes; increased to 4.5 mL/minute from 30-35 minutes; held at 4.5 mL/min from 35-170 minutes; then decreased back to 2.5 mL/minute from 170-175 minutes and held until the completion of the analysis.

Purified AAs were collected into 3.5 mL tubes via the automated fraction collector using time-based collections, and then transferred to 20 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 (~30 μL) of 0.1 N HCl, transferred into pre-ashed tin (Sn) EA capsules, and dried to completion in a 60 °C oven for 12 hours. Capsules were then pressed into cubes and analyzed for $\delta^{15}N$ and $\delta^{13}C$ values by EA-IRMS. EA-IRMS analysis was conduced in the UCSC shared Stable Isotope

Laboratory facility (UCSC-SIL), using an EA-IRMS analyzer dedicated to smaller samples. This system uses a Carlo Erba CHNS-O EA1108-Elemental Analyzer, interfaced via a Thermo Finnigan Gasbench II device to a Thermo Finnigan Delta Plus XP isotope ratio mass spectrometer (Thermo Fisher Scientific), configured after Polissar et al. (2009). For AAs in this study, we found that ≤ 100 nmol quantities of purified AA material could be routinely measured using this instrument, although as discussed below a standard EA configuration could also equally be used. Raw EA-IRMS $\delta^{15}N$ and $\delta^{13}C$ values were corrected for instrument drift and size effects using AA isotopic standards and standard correction protocols used by the UCSC-SIL (http://es.ucsc.edu/~silab).

Trophic Position Calculations:

The resulting $\delta^{15}N$ values of Glu and Phe generated by each method were used to calculate TP for each organism using Glu/Phe based equations described previously (e.g., Chikaraishi et al. 2009; Germain et al. 2013). TP values for the cyanobacteria, mussel, coral, and fish samples were calculated using the equation for marine organisms, $TP_{Glu/Phe} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 3.4) / 7.6 + 1$, proposed by Chikaraishi et al. (2009). Harbor seal TP values were calculated using the recently proposed equation for urea excreting marine mammals, $TP_{Glu/Phe} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 7.7) / 7.6 + 2$, formulated by Germain et al. (2013. The standard deviation of TP values was calculate by propagating the error of both Glu (SD1) and Phe (SD2) $\delta^{15}N$ values using the equation: $TP_{Glu/Phe} = \sqrt{\boldsymbol{SD1^2 + SD2^2}} / 7.6$.

Assessment:

Chromatographic Optimization and Sample Capacity:

Chromatographic separation of AAs was developed beginning with the solvent system and stationary phase described previously by Broek et al. (2013) for the separation and stable isotopic analysis of Phe as a paleoproxy. Because this prior study was focused only on Phe, many AAs (including Glu) were not well separated, making its use for calculation of TP_{CSIA} impossible. Optimization of column type, as well as solvent program, including flow rates and pressures, were used together to achieve full separation of Glu and Phe, and simultaneously separate all other proteinaceous AAs. First, a semi-preparative scale column was used to increase capacity (allowing single- peak collections for EA-IRMS; *see discussion below*), while simultaneously reducing relative sample loadings. The semi-preparative scale column also had the somewhat unexpected effect of producing substantially better chromatographic resolution for most AA, in comparison to separations achieved with an analytical scale column (Broek et al. 2013). A series of trials were therefore conducted to optimize the solvent program, and system flow rate and pressure conditions, for a semi-preparative scale separation. The most challenging separations fell within an early region of the chromatogram where Glu elutes within a group of closely spaced AA peaks (Asp, Ser, Gly, Thr, Glu, Ala; Fig. 2). In combination with flow rate changes (*described below*), we found that a slow, near-isocratic ($\leq 0.5\%$ increase over 30 minutes) organic solvent ramp was required for full baseline

separation of Glu, and other AAs in this group. Similarly, isocratic conditions later in the method were required for baseline resolution His and Lys. Conversely, rapid organic solvent gradients were used in regions of well-separated peaks in order to minimize the overall analysis time.

System flow rate and pressure conditions were also found to have an important effect on separation. A flow rate of 4.5 mL/min was used for most of the analysis, to correspond to the larger column ID, which also resulted in an approximate match with system pressures used with analytical scale columns (>2500 psi; Broek et al. 2013). However, a reduced flow rate of 2.5 mL/min was found to yield significant improvement in the separation of Glu, as well as other AAs within the closely spaced group discussed above, despite a significantly reduced system pressure (1200-1500 psi). The reduced flow rate, however, did not improve separation later in the analysis, and resulted in greatly increased retention times of His, Lys, and Arg. Ultimately, an optimized separation was achieved by including a flow rate ramp program, which increased the system flow from 2.5 to 4.5 mL/min after the elution of the first group of 6 AAs. Using our final protocol (*detailed in methods*), complete baseline resolution was achieved for all 16 AAs in our working isotopic standard (Fig. 2).

The maximum sample loading (i.e., the maximum amount of compound injected on the column while maintaining baseline resolution) is also an important consideration for an offline isotopic measurement approach. Sample requirements for purified AAs are ultimately determined by EA-IRMS sensitivity. However, while

final AA sample size can be increased far beyond column capacity by collecting and combining peaks from multiple HPLC separations, Broek et al. (2013) showed that final precision of AA $\delta^{15}N$ values is greatly improved by single peak collection. The authors hypothesized that this was likely due to the combined effects of increased sample handling and variability in retention time between analyses resulting in incomplete compound collection. Therefore, another major advantage of a larger scale approach is the greatly increased chromatographic loading capacity, which allows for collection of sufficient quantities of AA for EA-IRMS analysis from a single HPLC injection. If multiple HPLC injections and collections are conducted, these then represent independent replicates for the entire protocol, including isolation, collection, and isotopic analyses.

While column capacity is related to total compound mass, final EA-IRMS analysis is limited by nitrogen content, so nmol of N per peak is the most useful measure of the potential for ultimate $\delta^{15}N$ analysis. Using the semi-preparative scale protocol presented here, the approximate maximum loading for baseline separation of *all* AAs was found to be approximately 150 nmol of N per peak (in an equimolar mixture). Above this limit, Asp and Ser first begin to lose baseline resolution (Figure 2). In addition, analysis of the commercial Pierce H AA mixture showed that a coelution of Leu and Cys also begins near this range, however acid hydrolysis results in the complete destruction of Cys, and therefore this coelution would not pose an issue in real sample analyses (Barrett 1985). However, for all other AA (including Glu and Phe, and so TP_{CSIA} calculation), full baseline resolution was still maintained

at the highest injection loadings that we were able to test here (approximately 825 nmol N/compound; based on AA solubility). This amount of N represents approximately 1.5-7.5 μmol of C (or 60-100 μg of compound), depending on the C:N ratio of the AA. Our semi-preparative scale separation would therefore allow for individual isotopic analysis of 14 of 16 AAs (including Glu and Phe) using both standard EA-IRMS (e.g., Broek at al, 2013), as well as the small sample EA-IRMS we applied here.

δ^{15}N Accuracy and Precision: Standard Materials:

In order to assess the relative accuracy and precision of this HPLC/EA-IRMS method for TP_{CSIA} calculations, versus the standard GC-C-IRMS approach, we first compared Glu and Phe δ^{15}N values from standard AA materials measured independently by both methods (Figure 3). We note that these two compounds also represent contrasting chromatographic conditions in several ways: Glu elutes in close proximity to other AAs within a primarily aqueous solvent mixture, while Phe is widely separated from all other peaks, and elutes in a solvent mixture with higher organic (acetonitrile) content.

HPLC/EA-IRMS δ^{15}N values for both Glu and Phe were equivalent to expected isotopic standard values within error (offset < 1 SD; Fig. 3) with an average standard deviation of ± 0.3‰ (n = 3). As noted above, since these are single peak collections, this represent true replication of the entire purification and isotope measurement protocol. We note that this analytical precision for Phe is somewhat

lower than that reported in Broek et al. 2013 (± 0.16‰) for $\delta^{15}N_{Phe}$ values, also produced from single HPLC collections. The slightly lower precision is likely due to use of the small sample EA-IRMS system for these analyses, and is consistent with decreased precision of standard materials analyzed by this system (average SD of $\delta^{15}N_{Phe}$ standard values analyzed by small sample EA without prior HPLC purification = 0.4 ± 0.2 ‰), which is typically linked simply to total sample size. As noted above, while our separation scheme is sufficient to use standard EA-IRMS, we chose to use this small sample EA system in this study to reduce the ultimate biological sample sizes required. These results suggest however, that this reduced sample requirement comes at the price of a small decrease in precision.

The GC-C-IRMS method also produced $\delta^{15}N$ values for Glu and Phe that were in reasonable agreement with expected standard values, with a measurement precision similar to HPLC/EA-IRMS (average SD = ± 0.2 ‰, n = 3). We would note, however, that this level of precision is not typical for $\delta^{15}N$ values via GC-C-IRMS, but rather is at the highest end of what is typically achieved for pure AAs analyzed using our GC-C-IRMS system. For example, the long-term average SD for these AAs (i.e., measured in a long term control standard mixture, using the same instrument) is 0.7 ± 0.3‰ (n = 14) This long term precision for standards is substantially closer to typical GC-C-IRMS precision for Glu and Phe in real (environmental) samples (± 1 ‰; e.g., McClelland and Montoya 2002; McCarthy et al. 2007; Sherwood et al. 2011; Calleja et al. 2013).

The accuracy of the GC-C-IRMS results is also more complex to interpret

than those for HPLC/EA-IRMS, because it depends substantially on an external standard isotopic correction (Fig. 3). As noted above (*methods*) GC-C-IRMS $\delta^{15}N$ values are corrected by applying an offset to a measured AA $\delta^{15}N$ value, based on the $\delta^{15}N$ value of bracketing AA isotopic standards. The average GC-C-IRMS value for Phe, following correction, was equivalent to the expected standard value within error. In contrast, the Glu value following correction is actually offset from the expected value by 0.5‰ (> 1 SD). Prior to correction, the GC-C-IRMS $\delta^{15}N$ value for Glu was actually closer to the expected value. Overall, however, the average mean offset of both Glu and Phe decreases based on the correction (from 0.6‰ to 0.4‰) showing that this approach did improve data quality despite the decreased accuracy of Glu. Similarly, the accuracy of a TP_{CSIA} value generated from these known values of Glu and Phe would also improve following correction (TP offset decrease from 0.14 to 0.03, when the offset is based on theoretical TP values generated from known standard values). These GC-C-IRMS results therefore are consistent with prior work in supporting the importance of data correction to compound specific standards for $\delta^{15}N_{AA}$ analysis, (e.g., Broek at al, 2013; McCarthy et al, 2013), however they also demonstrate possible drawbacks of this approach.

Most broadly, the dependence of $\delta^{15}N$ value on a correction routine likely demonstrates the inherent limitations in the ultimate accuracy of $\delta^{15}N_{AA}$ and therefore TP_{CSIA} values by GC-C-IRMS, and may also hint at potential sources of error. In this experiment, the $\delta^{15}N$ values for Glu and Phe standards made by GC-C-IRMS were produced by simultaneously derivatizing two separate aliquots of the *same* standard

mixture in parallel, and then treating one as a "sample" and one as the "standard" (i.e., one standard set was used to produce correction factors for the other standard set). The magnitude of the offset correction (average correction for both Glu and Phe = 0.9‰; Figure 3) therefore gives an indication of the variability in $\delta^{15}N$ value due to derivatization, at least in this experiment (i.e., these standards were analyzed identically, but derivatized independently). The comparison therefore suggests that the overall GC-C-IRMS accuracy may ultimately be limited in part by the consistency of the derivatization procedure rather than the instrumentation alone, a complication that the HPLC/EA-IRMS method eliminates. This would also suggest that the reproducibility of GC-C-IRMS values from multiple derivatization campaigns is significantly lower than the attainable precision of replicate GC-C-IRMS analyses of the same derivatized sample; and underscores that the maximum precision that can be attained by GC-C-IRMS for $\delta^{15}N$ AA measurement is likely poorer than is possible HPLC/EA-IRMS. Finally, it is important to note that in environmental samples analyzed by GC-C-IRMS, errors of this magnitude would likely not be detected, simply because the offsets in measured values shown in figure 3 are similar to GC-C-IRMS instrument reproducibility typically reported for $\delta^{15}N_{AA}$ of biological or geochemical materials (i.e., $\sim \pm 1$‰; e.g., McClelland and Montoya 2002; McCarthy et al. 2007; Sherwood et al. 2011; Calleja et al. 2013).

Chromatographic Separation in Natural Biological Samples:

A critical aspect of an HPLC-based approach is to assess the chromatographic

separation of AAs in real biological tissues. Because entire, underivatized peaks are collected for isotopic analysis, any coelution with non-AA nitrogenous materials that might be commonly present in biological samples could bias the result. We therefore tested five marine proteinaceous tissue types to examine both chromatographic separation, and to comparative final TP_{CSIA} values. The samples were chosen to represent a wide range of tissue types, including structural protein, blood serum, animal muscle tissue, and whole bacterial cells. In addition, the tissues represent a phylogenetically diverse group of organisms (algae, invertebrates, including mussel and deep sea coral, fish, and marine mammal), which span a wide range of expected TPs, allowing us to test our measurements over a realistic range of $\delta^{15}N$ values.

Figure 4 shows representative chromatograms for each sample type. Variability in the relative abundance of AAs in these diverse samples leads to decreased resolution in some cases. However, with a few exceptions (Asp, Ser, Gly), all AAs present in our standard mixture could also be separated in biological samples, with sufficient resolution for isotopic analysis. Most importantly, in all 5 organisms tested, our semi-preparative scale separation easily resolved both Glu and Phe with baseline separation, at loadings high enough for subsequent isotopic analysis by either standard or small sample EA-IRMS from single HPLC peak collections.

In addition to known AAs, there were also a number of unknown peaks present in the biological sample chromatograms (Figure 4). Some of these 'unknowns' are present in all or most samples (e.g., peaks at 24, 28, 58, and 60 minutes), while others are specific only to certain tissue/organism types. However,

there is neither chromatographic nor isotopic evidence for any coelution of Glu or Phe with 'unknown' peaks that might influence $\delta^{15}N$ values. For example, in three samples (cyanobacteria, mussel, and fish) a small, unknown peak elutes in close proximity to Glu. This peak was therefore collected and directly analyzed for $\delta^{13}C$, $\delta^{15}N$, and C:N ratio, to assess its potential contribution to measured values of Glu, in the case of any undetected partial coelution. Based on the high C:N ratio (~15), it was determined that it is unlikely that the partial unintentional collection of this compound with Glu could significantly affect the measured isotopic values of Glu.

Further, we also investigated the retention time of amino sugars, compounds with similar functional chemistry to AAs that are often present in complex organic matrices of marine samples. We found that common amino sugars (d-glucosamine (GlcN), d-galactosamine (GalN), and d-mannosamine (ManN)) all elute within the same narrow time window, coeluting with the AA Thr (figure in Supplemental Material). Amino sugars can exist in high concentrations in marine bacteria (Benner and Kaiser 2003) but are uncommon in other proteinaceous materials, therefore we hypothesize that this coelution would only pose an issue if a sample has a substantial bacterial contribution, and only for the analysis of Thr. None of the amino sugars tested interfered with Glu or Phe, suggesting that these compounds would have no effect on calculated TP values.

Accuracy and Precision: Relative TP estimates from $\delta^{15}N_{Glu/Phe}$ in Natural Samples:

The separations achieved with our method appear more than sufficient for producing accurate and precise $\delta^{15}N$ values for Glu and Phe across a range of biological samples. However, the most unambiguous assessment would be to directly examine the accuracy of $\delta^{15}N$ values for Glu and Phe from HPLC/EA-IRMS in diverse biological samples. While this is not strictly possible (since no reference materials for these compound values exist), a comparison of $\delta^{15}N$ values from HPLC/EA-IRMS and the established GC-C-IRMS method can largely achieve this same goal. Since GC-C-IRMS $\delta^{15}N$ values derive only from derivatized AAs, analyzed via a completely independent instrumental system, a close match between the two methods would rule out the significant influence of hidden coelutions, or any other contamination, on isotopic values. Further, a close match in $\delta^{15}N$ values from the two methods would address our primary goal of creating a viable alternative to GC-C-IRMS for TP_{CSIA} calculations, and represent an independent measure of the accuracy of both approaches.

The $\delta^{15}N$ values for Glu and Phe produced by both HPLC and GC-C based methods were equivalent within error ($p > 0.1$), for all except a single value ($\delta^{15}N_{Phe}$ of the harbor seal sample, $t = 5.0$, df = 6, $p = 0.002$*;* Fig. 5). The average *offset* of mean $\delta^{15}N$ values, across values for both AAs and between the two methods, was 0.4‰. However, when the harbor seal value is excluded, the average offset for all other samples drops to 0.25‰, essentially identical to our EA-IRMS analytical precision. The average precision (1 SD) for values measured by HPLC/EA-IRMS alone was 0.4 ± 0.2 ‰ (n = 5 samples, n = 3 replicates). The average SD of GC-C-

IRMS values was higher, although not significantly so, at 0.5 ± 0.2 ‰ in the biological samples (n = 5 samples, n = 3 replicates). As noted above, this range is comparable with precision typically reported for $\delta^{15}N_{AA}$ in other environmental samples (±1.0‰). While we cannot definitively assign any cause to the larger offset in the single harbor seal $\delta^{15}N_{Phe}$ value, a comparison with independent TP data (*discussed below*) suggests that the HPLC/EA-IRMS value is likely more accurate.

Finally, the TP of each of the 5 organisms was calculated using the Glu and Phe values generated by both methods (Fig. 6). As noted above (*methods*) we used two different TP_{CSIA} equations. For all except the harbor seal, TP_{CSIA} values were calculated using the current standard equation most commonly applied for marine organisms (Chikaraishi et al. 2009). However, Germain et al. (2013) recently showed that this equation produces TP values too low in seals (and likely all marine mammals), so the harbor seal TP values were calculated using a new multi-TEF equation for marine mammals, as proposed by these authors (Germain et al. 2013). Of course, the exact formula used does not influence a comparison of TP results derived from the same set of Glu and Phe $\delta^{15}N$ values, however these two formulas represent the most accurate current approaches to calculating TP_{CSIA}. Finally, the relative precision of TP_{CSIA} estimates from each method (Figure 6) were derived from propagating analytical error, as described above (*methods*).

The resulting calculated TP_{CSIA} values were equivalent within error ($t < 1.2$, df = 6, $p > 0.1$) for all 5 organisms. The average SD of TP_{CSIA} values was < 0.1 for both methods, although the GC-C-IRMS values had a slightly higher mean SD. The small

offsets in mean TP values (0.04 to 0.16 trophic levels for all but harbor seal) are likely negligible in terms of any practical ecological interpretation. The single exception may be the TP value for the harbor seal where the offset in mean TP_{CSIA} value between the two methods is the largest of any sample (0.23 trophic levels), almost entirely due to the difference in $\delta^{15}N_{Phe}$ values measured between the two methods: i.e., while the Glu $\delta^{15}N$ values are statistically equivalent between the two methods ($t = 0.3$, df = 6, p = 0.7), the Phe values are not (Figure 5). Although the harbor seal TP_{CSIA} values are statistically equivalent ($t = 1.1$, df = 6, p = 0.29), we hypothesize that the mean HPLC/EA-IRMS result is likely closer to the true value, with a previous study using this same harbor seal specimen (Germain et al, 2011) providing independent support for this conclusion. In this prior work, a combination of bulk isotopic, scatological, and stomach content results suggested that this seal should have a TP > 3. While not definitive, this strongly suggests that the HPLC/EA-IRMS estimate of 3.1 is the more accurate value, in comparison with the lower GC-C-IRMS estimate of 2.8 (Germain et al. 2011). While we cannot definitively identify a cause, we hypothesize the offset in Phe $\delta^{15}N$ values may be related to the requirement to correct GC-C-IRMS $\delta^{15}N$ data using external standards, as discussed above. In any case, this offset underscores the potential for additional sources of error that can occur when analyzing derivatized compounds.

Discussion:

We have developed a new method for the calculation of TP_{CSIA} based on

commonly available HPLC instrumentation, and subsequent offline EA-IRMS analysis. This method targets Glu and Phe $\delta^{15}N$ values, as they form the basis for most common formulas for TP_{CSIA} calculation. Our tests in both standard and natural biological materials have demonstrated that HPLC/EA-IRMS can fully achieve our main goal of developing a viable alternative to the current GC-C-IRMS approach to TP_{CSIA} measurements, producing equivalent values in all cases. Further, our results suggest that the HPLC/EA-IRMS method will likely produce $\delta^{15}N$ values of Glu and Phe with higher precision than typical GC-C-IRMS analyses in most cases. This is made possible by the elimination of many of the primary sources of error in GC-C-IRMS analysis, specifically the requirement for derivatization; the on-line coupling of purification and combustion; and perhaps most important, the application of offset corrections. In addition to removing a number of primary sources of error, the elimination of the need for GC-C-IRMS instrumentation can also provide a significant cost reduction, leading to a method that can be performed with significantly lower startup costs. Overall, we conclude that our new method meets or exceeds the primary objective of our study, to develop an alternate and potentially more widely available approach to TP_{CSIA} estimation.

Many of the analytical advantages of our method derive from being able to directly measure native (underivatized) molecular structures. We have hypothesized that this underlies the increases in both precisions and accuracy for isotopic values we have observed. This suggests that the increased sample handling in the HPLC/EA-IRMS approach introduces less relative error than the derivatization required by the

GC-C-IRMS method, leading to precision that is either equivalent or better in all cases. Measurement of underivatized structures has a number of additional advantages other than increased precision, potentially allowing for a wide set of additional applications of this approach.

First, while we have focused here on $\delta^{15}N$ values, this approach simultaneously generates $\delta^{15}N$ and $\delta^{13}C$ values for all measured AAs. This presents a significant potential advantage over GC-C-IRMS, which only allows for the measurement on one element per analysis. As noted below (*comments and recommendations*) sample handling considerations likely make measuring simultaneous isotopic values for all protein AAs at once very labor intensive, however the ability to precisely measure both C and N isotope values for any non-derivatized AA could prove particularly useful for investigations of specific AAs that are not quantified by GC methods. For example, Arg and His are not typically amenable to derivatization by many protocols used for GC-C-IRMS. As one practical example, the $\delta^{15}N_{AA}$ and $\delta^{13}C_{AA}$ values of *Gerardia* and similar deep-sea proteinaceous corals have enormous promise for paleoceanographic studies (Broek et al. 2013; Sherwood et al. 2014). However, the proteinaceous skeleton of these corals is often approximately 30% histidine (e.g., Goodfriend et al. 1997). Therefore, it is impossible to investigate the molecular basis for bulk protein skeleton $d^{15}N$ or $d^{13}C$ values without evaluating the relative isotopic value of this specific AA. Finally, as would be expected, this method also allows for the simultaneous and accurate determination of the relative molar abundance of amino acids (mol% AA;

supplementary material). Again, a key advantage here is direct measurement of non-derivatized structures via ELSD detection, such that the full suite of protein AAs present are quantified, removing the analytical limitation that is typical for a derivatization-based approach.

Overall, we suggest that the main impact of this new method will be to expand the potential to make highly accurate CSI-AA based estimates of both TP and baseline $\delta^{15}N$ values. As noted above, typical GC-C-IRMS instrumentation is expensive, relatively rare in most laboratories, and requires significant specialized expertise. In contrast, HPLC systems are one of the most common and widely distributed analytical instruments, common in laboratories across many fields. EA-IRMS is now also commonly available in shared facilities, or on an external recharge basis. We have shown here how coupling these two instruments can be used to produce TP_{CSIA} measurements equivalent to, or better than, those that can be achieved by GC-C-IRMS. This method could therefore ultimately stimulate substantial new research in a wide breadth of fields currently interested in applying CSI-AA approaches, including ecology, modern and paleo oceanography, marine and terrestrial biogeochemical cycles, archeology, and more.

Comments and Recommendations:

While this method clearly achieves our main goals for TP_{CSIA} calculation, at the same time there are a number of additional considerations (versus the standard GC-C-IRMS approach) that would influence its suitability for additional applications

in other environments, or with other sample types (i.e., apart from pure biological tissue samples). For example, the need to collect and process each AA individually would involve significant time requirements in order to measure a full suite of AAs. In contrast, the CG-C-IRMS method can simultaneously analyze 14 to 16 AA isotope values from a single sample injection. Therefore, the HPLC based approach is likely less suitable for any application where many AAs must be measured simultaneously, such as phylogenetic fingerprinting (Larsen et al., 2009) or organic-N diagenetic investigations (McCarthy et al. 2007). As a result, we suggest this new method is most appropriate for applications where high precision and accuracy measurements of only selected AAs are required, such as for TP or isotopic baseline determinations that are currently the major area of CSI-AA interest in many fields.

In addition, the amount of sample required is also a consideration for any specific application. As discussed previously, sample sizes required for this method are ultimately determined by EA-IRMS sensitivity, and therefore minimum sample sizes required for HPLC/EA-IRMS will likely always be larger than those for GC-C-IRMS. Therefore, this method would likely not be suitable for applications where sample amounts are extremely limited. For our primary goal here (TP estimation in biological materials) this is not typically an issue, since many biological tissues (e.g., fish muscle) are available in essentially unlimited quantities, in relation to the milligram range of tissue required. We showed here that the application of modified EA-IRMS instrumentation could lower the sample requirements to near the same order as GC-C-IRMS when less material is available (e.g., coral peels), although this

would likely limit the possibility for replication. For most biological sample types, however, use of larger samples with standard EA-IRMS would increase precision even further.

Finally, while we have demonstrated the accuracy of this method for TP_{CSIA} in a range of marine biological materials, many geochemical samples, in particular those in which organic matter is degraded (e.g., sediments, deep ocean suspended particles, dissolved organic matter, and colloids) typically have far more complex matrixes. The result is that after hydrolysis, extremely complex chromatograms can result, containing multiple unknown compounds, even following derivatization procedures specific for primary amines (e.g., McCarthy 2004, 2007). Because the purity of isolated AA peaks is essential for an offline approach, we suggest that the application of this method to non-biological sample types would need to be evaluated further, and likely assessed on a sample-by-sample basis.

Supplemental Material:

Relative Amino Acid Molar Abundance (mol% AA):

We also tested the ability of this method to make accurate measurements of the relative molar abundance of amino acids (mol% AA) in proteinaceous samples. A certified AA standard (pierce H) with equimolar concentrations of 17 AAs was used to construct individual ELSD calibration curves for each AA (Supplemental Figure 1). In order to assess the accuracy and precision of this method in making these measurements, a pure bovine beta-casein protein sample was hydrolyzed and

measured. This allowed us to assess the potential effects of hydrolysis without the presence of a complex organic matrix. Additionally, this material is well studied and the AA residue abundance is known, allowing for a direct assessment of accuracy.

Mol% AA determinations of the hydrolyzed bovine beta-casein produced accurate percentage values with an average absolute offset of 1.0 ± 0.6% from expected values based on the know protein structure. This is comparable to the accuracy of measurements made by GC-C-IRMS, which produced values with an average absolute offset of 1.1 ± 1.3% (Supplemental Figure 2).

As discussed previously there is a coelution of three amino sugars (d-glucosamine, d-galactosamine, and d-mannosamine) with the AA Thr (supplemental Figure 3). This suggests that the mol% abundance of Thr cannot be accurately assessed in the presence of these amino sugars. In order to assess this hypothesis in a real sample, we examined the chromatography and resulting mol% AA values in a cyanobacteria sample, expected to have a major amino sugar component. In this sample, Thr appears to be present in nearly twice the abundance of the measured GC-C-IRMS percentage value (Supplemental Figure 4A). This is particularly problematic because of the relative, normalized nature of mol% AA calculations; where an overestimation of one compound's abundance will lead to an underestimation of the remaining compounds. If Thr is excluded from mol% AA values of the cyanobacteria sample the average offset between HPLC-ELSD and GC-C-IRMS decreases from 1.9±2.1% to 1.2±1.0% (Supplemental Figure 4B). The substantially increased accuracy of mol% AA values following the removal of Thr from the calculations

demonstrates that the coelution of Thr with amino sugars is a main source of error in these measurements, but also suggests that this issue will only be present in samples with a significant microbial influence.

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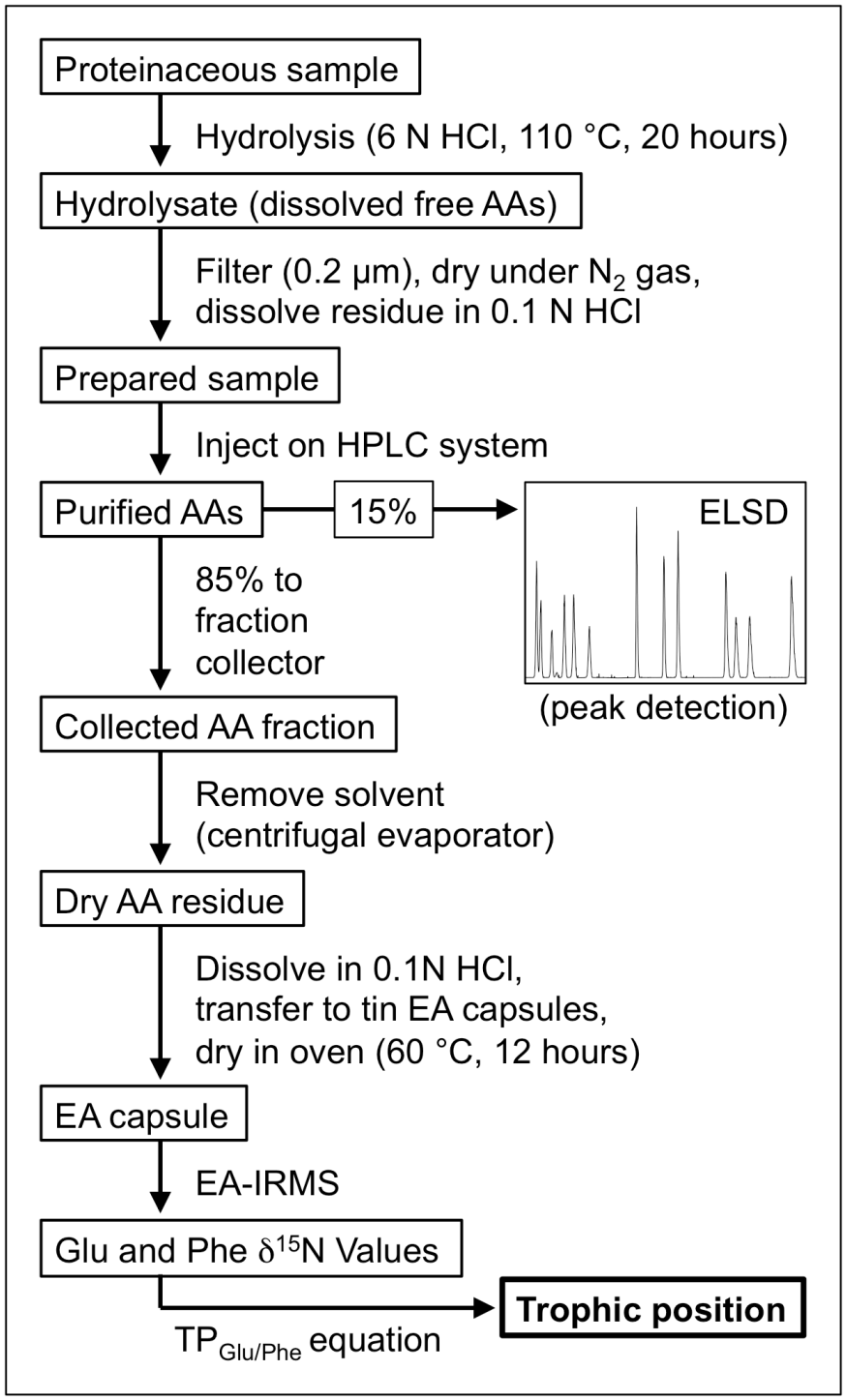


Figure 1. Flowchart showing sample preparation and analysis steps using our HPLC/EA-IRMS method. Abbreviations as defined in text.

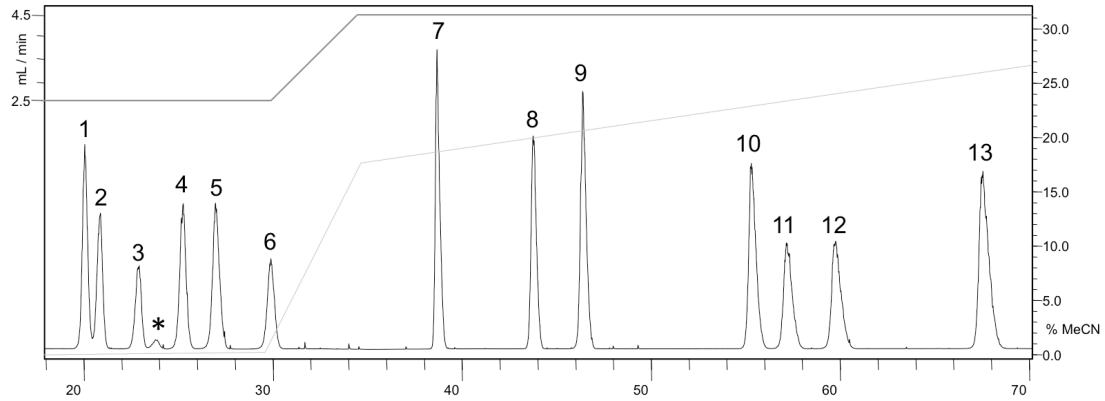


Figure 2. Representative HPLC-ELSD chromatogram section including the first 13 eluting peaks of the 16 AA isotopic standard mixture. Window shows minutes 17 - 70 in order to highlight the separation of Glu and Phe. Each peak represents 300 nmol AA injected on-column. Light-grey line indicates % organic solvent (binary solvent program). Dark-grey line indicates flow rate. Baseline resolution was achieved for 14 of 16 AAs; a slight coelution is seen only for Asp/Ser. 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. Starred (*) peak represents a contaminant in our standard mixture of unknown composition.

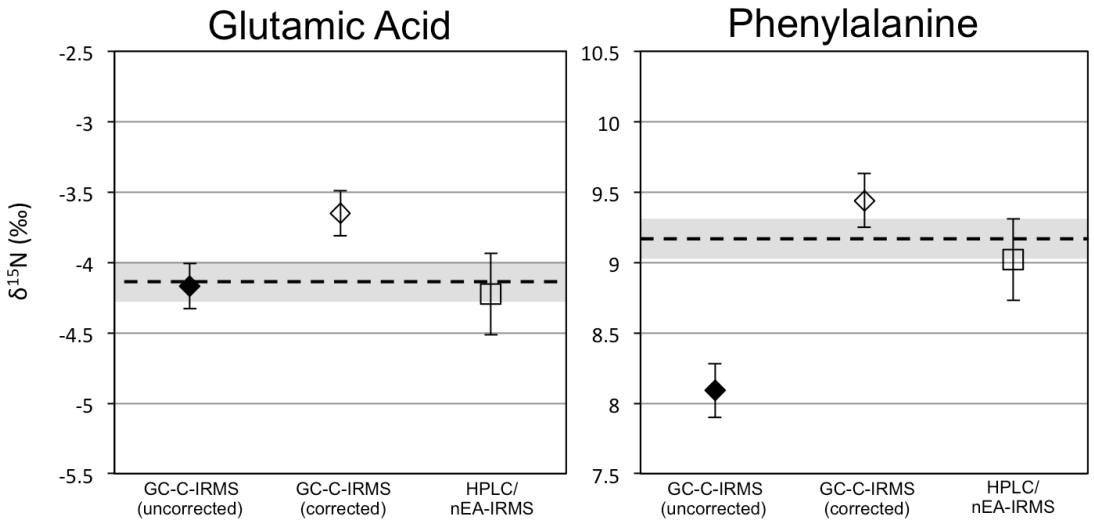


Figure 3. Comparison of $\delta^{15}N$ values of isotopic Phe and Glu standards analyzed by GC-C-IRMS (diamonds) and HPLC/EA-IRMS (squares). Error bars represent +/- 1SD. Dotted line represents known value of isotopic standard (measured by standard EA-IRMS) and the shaded region the error of that value. Solid diamonds represent GC-C-IRMS values before correction and open diamonds represent values following a standard offset correction using bracketing compound specific isotopic standards as described in text.

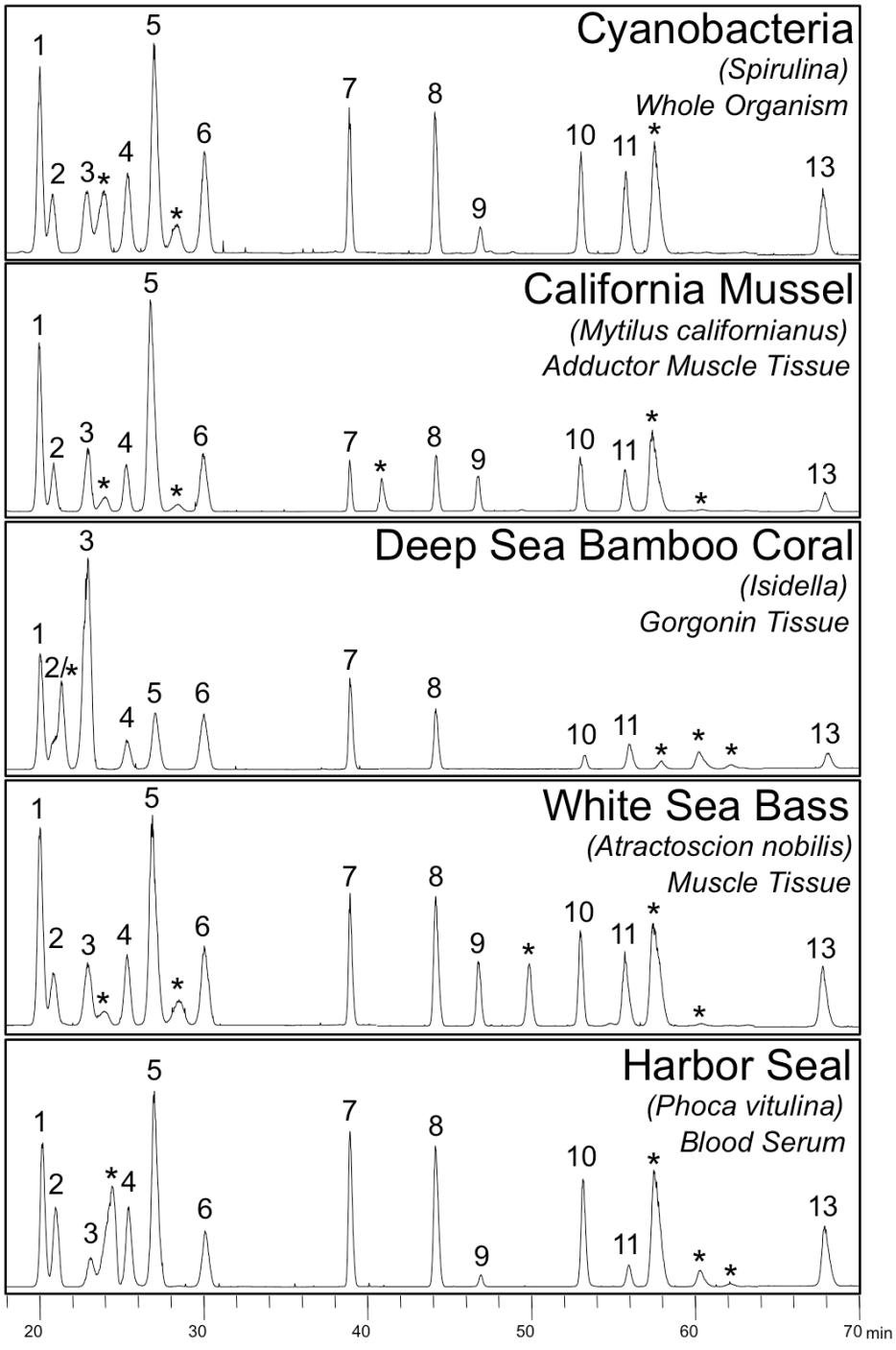


Figure 4. Representative HPLC-ELSD chromatograms of 5 samples of hydrolyzed tissue. AA peak identifications: 1. Asp, 2. Ser, 3. Gly, 4. Thr, 5. Glu, 6. Ala, 7. Pro, 8. Val, 9. Met, 10. Ile, 11. Leu, 12. Nle, 13. Phe. Stars (*) represent peaks of unknown composition.

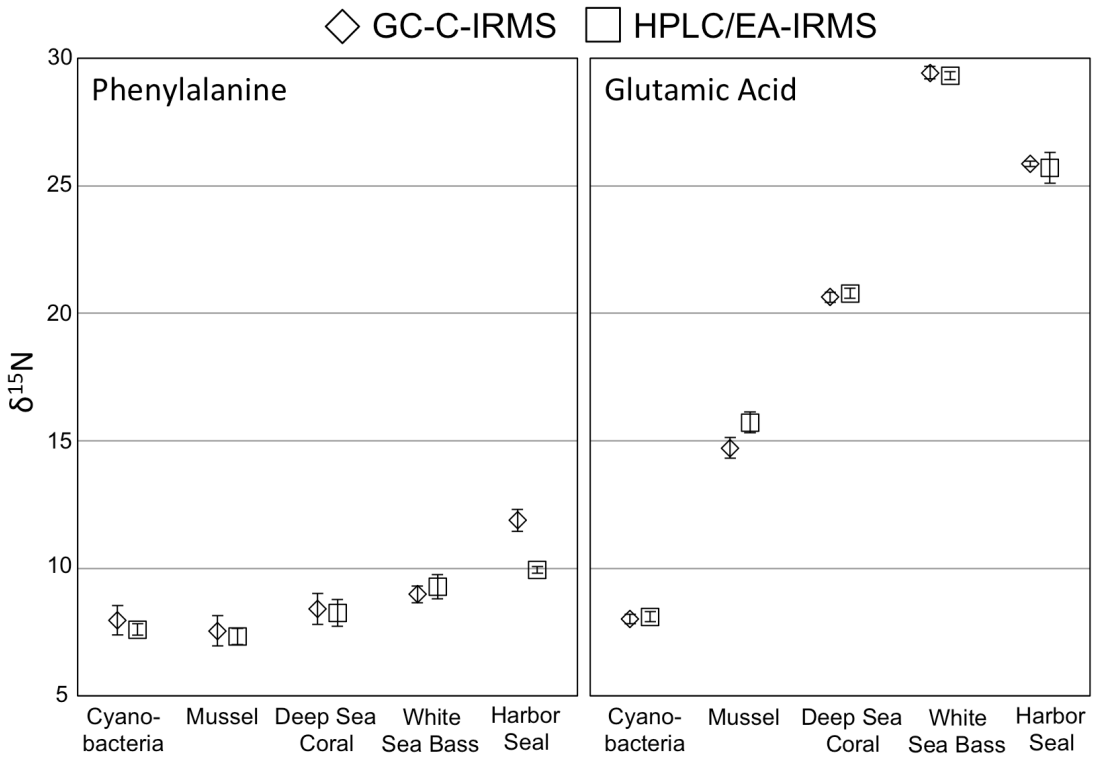


Figure 5. Comparison of $\delta^{15}N$ values of Glu and Phe extracted from 5 marine organisms by GC-C-IRMS (diamonds) and HPLC/EA-IRMS (squares). Error bars on GC-C-IRMS measurements represents +/- 1SD of n=3 replicate injections of the same derivatized material, HPLC/EA-IRMS error bars represent +/- 1SD of n=3 analyses from the same hydrolysate.

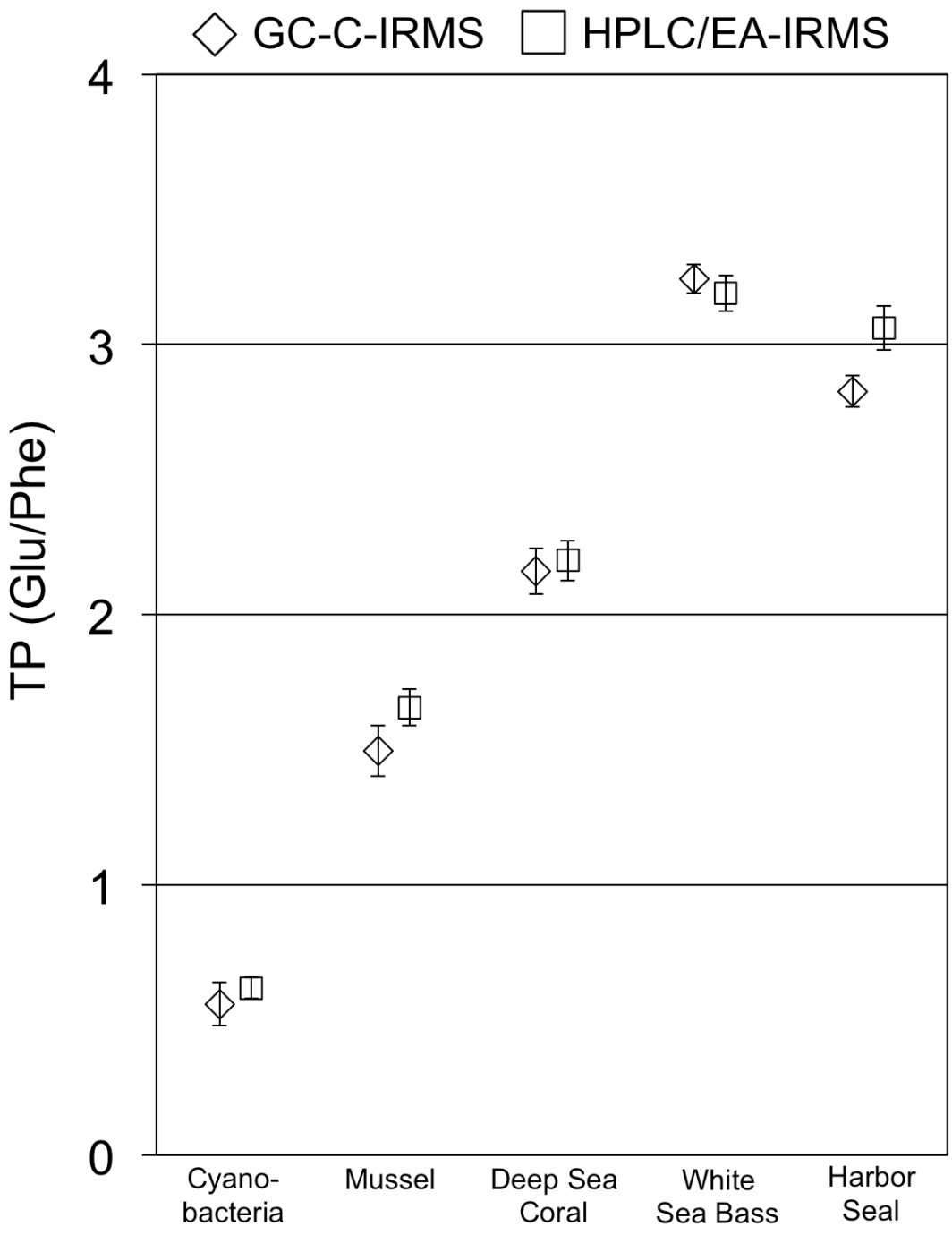
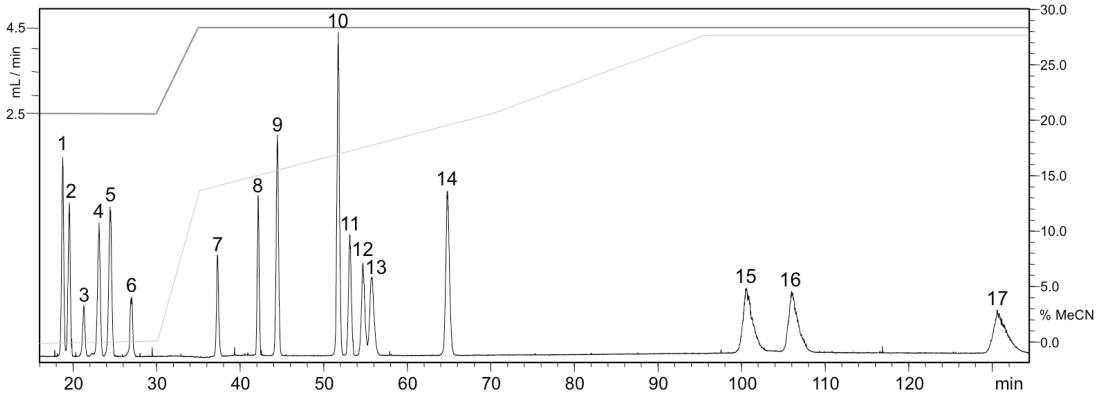
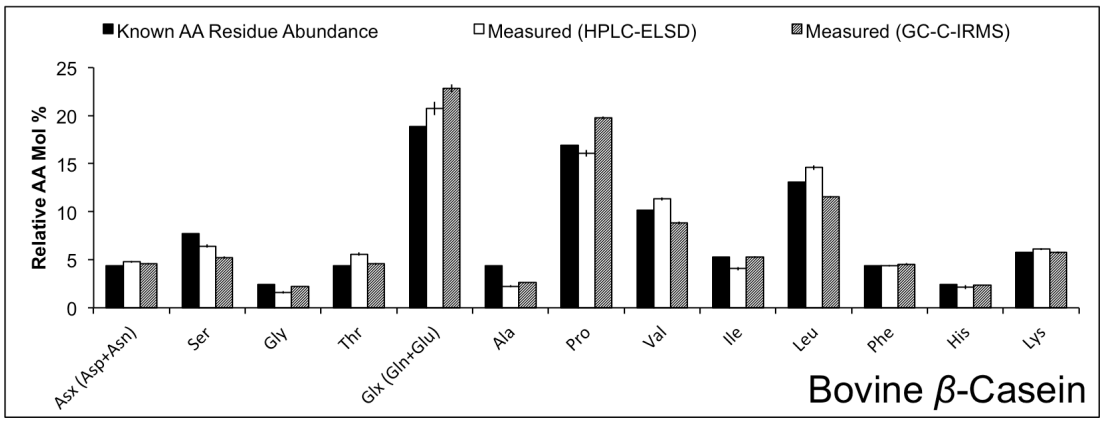


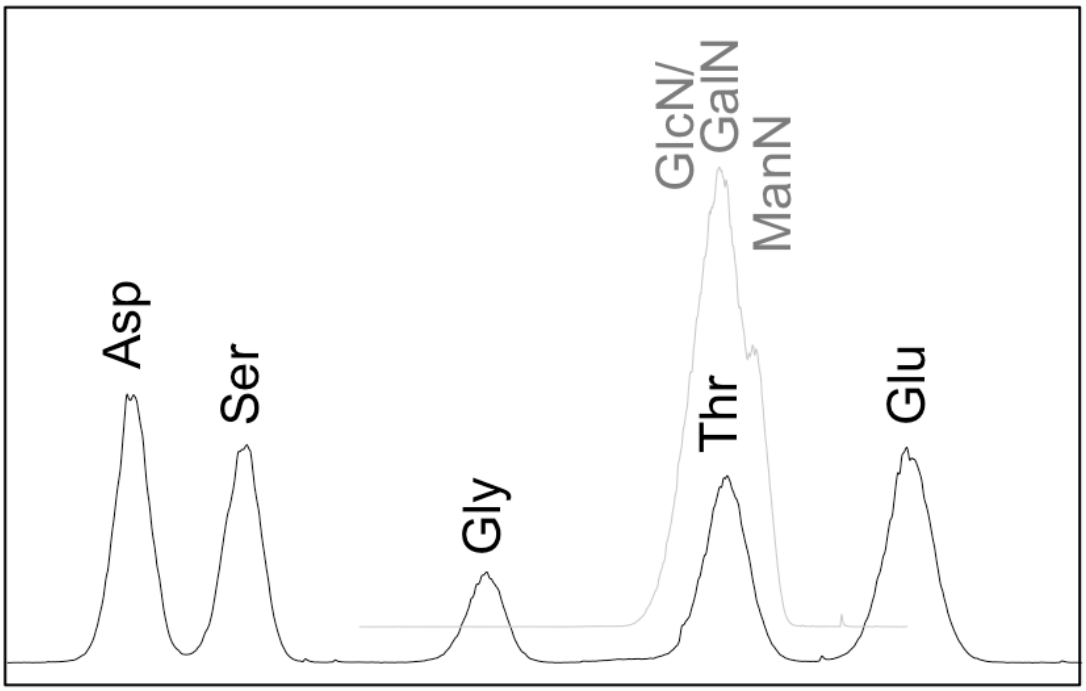
Figure 6. Comparison of trophic position estimates based on $\delta^{15}N$ values of Glu and Phe extracted from 5 marine organisms by GC-C-IRMS (diamonds) and HPLC/EA-IRMS (squares). Trophic position calculated using the internally normalized equations described in text. Error bars represent the propagated SD from n=3 analyses.



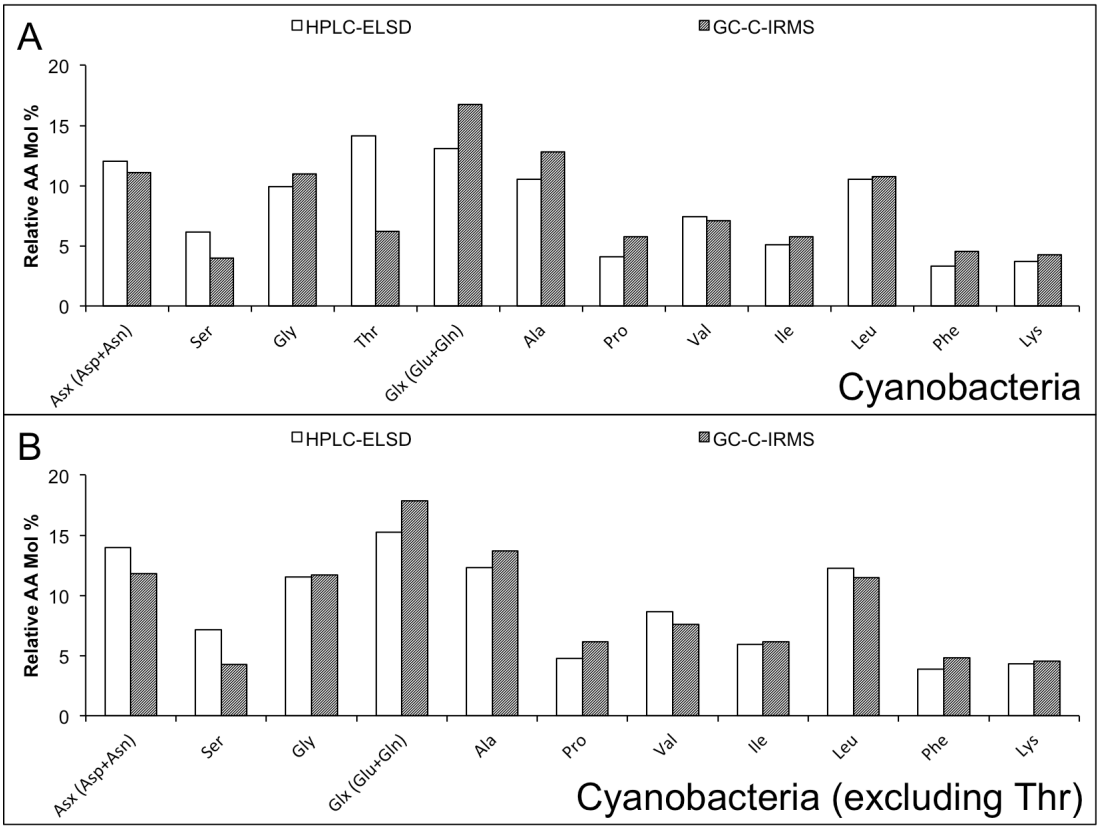
Supplemental Figure 1. Representative HPLC-ELSD chromatogram of the Pierce H AA standard mixture. Each peak represents 300 nmol AA injected on-column. Light-grey line indicates % organic solvent (binary solvent program). Dark-grey line indicates flow rate. Baseline resolution was achieved for 13 of 17 AAs; a slight coelution is seen only for Asp/Ser and Leu/Cys. AA peak identifications: 1. Asp, 2. Ser, 3. Gly, 4. Thr, 5. Glu, 6. Ala, 7. Pro, 8. Val, 9. Met, 10. Tyr, 11. Ile, 12. Leu, 13. Cys, 14. Phe, 15. His, 16. Lys, 17. Arg.



Supplemental Figure 2. Comparison of measured relative AA abundance by HPLC-ELSD (white bars) and GC-C-IRMS (hashed bars) with know AA residue abundance (black bars) of hydrolyzed beta-casein standard material.



Supplemental Figure 3. HPLC-ELSD chromatogram showing the first 5 peaks of the 16 AA Standard mixture (black line) overlaid with an HPLC-ELSD chromatogram of a three amino sugar mixture (grey line), indicating a coelution of Thr with GlcN, GalN, and ManN



Supplemental Figure 4. Comparison of measured relative AA abundance by HPLC-ELSD (white bars) and GC-C-IRMS (hashed bars) of cyanobacteria material A) including Thr, B) excluding Thr.

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