

A new approach to $\delta^{15}\text{N}$ compound-specific amino acid trophic position measurements: preparative high pressure liquid chromatography technique for purifying underivatized amino acids for stable isotope analysis

Taylor A. B. Broek* and Matthew D. McCarthy

University of California, Santa Cruz. Ocean Sciences Department, 1156 High Street, Santa Cruz, CA 95064

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 $\delta^{15}\text{N}$ values of glutamic acid (Glu) and phenylalanine (Phe) can be coupled to provide precise estimates of trophic position (TP), while simultaneously decoupling baseline $\delta^{15}\text{N}$ 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 $\delta^{15}\text{N}$ values of Glu and Phe were also indistinguishable within error between the two approaches; however, the $\delta^{15}\text{N}$ 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 ($t < 1.2$, $\text{df} = 6$, $P > 0.3$) between the two methods for all organisms examined. Our 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.

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. 2013). 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 (2002) first showed that two specific groups of individual AAs undergo very different ^{15}N fractionation with trophic transfer. One group of AAs (now termed the "trophic" AAs; after Popp

et al. 2007) undergoes large and predictable isotopic fractionation with each trophic transfer, whereas a second group ("source" AAs) maintain relatively unchanged $\delta^{15}\text{N}$ values. Within these two broad groupings, changes in the $\delta^{15}\text{N}$ value of glutamic acid ($\delta^{15}\text{N}_{\text{Glu}}$) has been found to most reproducibly indicate the extent of trophic transfer, whereas the $\delta^{15}\text{N}$ value of phenylalanine ($\delta^{15}\text{N}_{\text{Phe}}$) has been shown to be the most stable, therefore providing a direct estimate of the original $\delta^{15}\text{N}$ isotopic value of primary production (McClelland and Montoya 2002; Chikaraishi et al. 2009). Therefore measuring $\delta^{15}\text{N}$ values of Glu and Phe together allows a decoupling, for the first time, of the influence of baseline $\delta^{15}\text{N}$ 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 $\delta^{15}\text{N}$ data, because this approach requires no assumptions about baseline $\delta^{15}\text{N}$ values. TP_{CSIA} are therefore typically more accurate than those based on bulk $\delta^{15}\text{N}$ data alone (McMahon et al. 2013; Germain et al. 2013), and have now been widely applied

*Corresponding author: E-mail: tborrius@ucsc.edu

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in studies of trophic interactions in many ecosystems (e.g., McClelland and Montoya 2002; McCarthy et al. 2004, 2007, 2013; Chikaraishi et al. 2007, 2009, 2011; Popp et al. 2007; Hannides et al. 2009; Lorrain et al. 2009; Choy et al. 2012). 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, 2014; Broek et al. 2013).

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 inherent restrictions to the GC-C-IRMS approach, which together limit accuracy, precision, and ultimately the extent of possible interpretations. There are a number of different derivatization schemes that can be used for this work that each have inherent advantages and disadvantages, however they all share a number of limitations. 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, because 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 $\delta^{15}N$ values can then be corrected using a number of techniques, however, the magnitude of these 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 use 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 methods (e.g., Tripp et al. 2006). Recently, Broek et 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 with 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 $\delta^{15}\text{N}$ values, and also the resulting TP_{CSIA} estimates, by our new method and a standard GC-C-IRMS approach using a fluorinated derivative system, with a particular focus on their relative analytical precision. Finally, as a secondary goal, we sought to achieve sufficient separation of a 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 and Acros Organics 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 nonprotein AA nor-leucine (Nle), which is commonly used as an internal standard (Popp et al. 2007; McCarthy et al. 2013). The $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ 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 $\delta^{15}\text{N}$ 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 nonprotein AA Nle and addition of the proteinaceous AAs cysteine (Cys) and tyrosine (Tyr) was used to construct individual calibration curves, so as to verify relative molar abundance of individual AAs in natural samples (chromatogram in Web Appendix A).

Proteinaceous sample preparation

The cyanobacteria sample (*Spirulina* sp.) was obtained as a bulk commercial dry powder (*Spirulina* Pacifica). 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 (*Mytilus Californianus*) sample was collected in 2012 from Santa Cruz, CA. The mussel was previously dissected, and the adductor muscle tissue removed and lyophilized before 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.). 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.). Fish muscle tissue was also lyophilized before 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 before hydrolysis, as described previously (Germain et al. 2011).

For all sample types, proteinaceous material was hydrolyzed by adding 40-50 mg 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°C, 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, 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 min). The resulting amino acid isopropyl esters were then acylated in 350 μL 1:3 mixture of dichloromethane (DCM):trifluoroacetic acid anhydride (100°C, 15 min). 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) 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 \times 0.32 mm ID \times 0.52 μm film thickness, Agilent Technologies Inc.), 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 Fig. 1. Liquid chromatographic separations were conducted using a Shimadzu HPLC system (Shimadzu Scientific Instruments Inc.) 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 μ L, and coupled to a Shimadzu automated fraction collector (FRC-20A). An adjustable flow splitter (Analytical Sales and Services Inc.) was used inline following the chromatography column to direct $\sim 15\%$ of the flow to a SEDERE evaporative light-scattering detector (ELSD-LT II, Sedex 85LT) for peak detection and quantitation. A semi-preparative scale SiELC Primesep A column (10 \times 250 mm, 100 \AA pore size, 5 μ m particle size; SiELC Technologies Ltd.) 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 μ L 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 min; increased to 15% organic from 30–35 min; increased to 22.5% from 35–70 min; increased to 30% from 70–95 min; held at 30% until 140 min. The column was then cleaned and equilibrated by increasing to 100% and holding for 20 min; then decreasing to 50% and holding for an additional 15 min; then decreasing to 0% and holding until the method ends at 180 min. A flow rate ramp is also employed in which the total flow rate is held at 2.5 mL/min for 0–30 min; increased to 4.5 mL/min from 30–35 min; held at 4.5 mL/min from 35–170 min; then decreased back to 2.5 mL/min from 170–175 min and held until the completion of the analysis. This HPLC method allows for the baseline separation of 16 proteinaceous

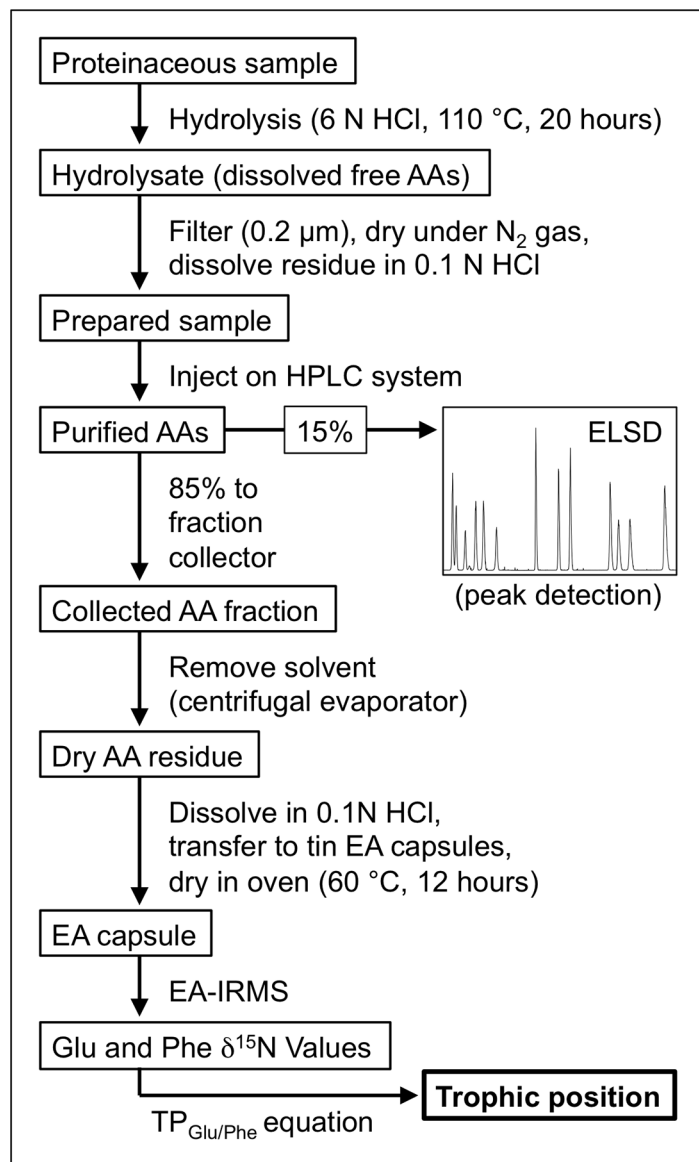


Fig. 1. Flowchart showing sample preparation and analysis steps using our HPLC/EA-IRMS method. Abbreviations as defined in text.

AAs, however if only Glu and Phe are required for the subsequent analysis, a modified method can be used that reduces the total method time. In the abridged method, the solvent ramp speed is increased after the elution of the first set of peaks with the % organic increasing from 0.5% directly to 22.5% from 30–60 min. After the elution of Phe at approximately 60 min, the column can be cleaned and equilibrated resulting in a 90 min chromatographic method.

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) at a chamber temperature of 60°C. Dry AA residues were then redissolved into a small volume (~ 30 μ L) 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 $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values by EA-IRMS. EA-IRMS analysis was conducted 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 $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ 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>) based on the procedures described by Fry et al. (1992).

Trophic position calculations

The resulting $\delta^{15}\text{N}$ 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, $\text{TP}_{\text{Glu/Phe}} = (\delta^{15}\text{N}_{\text{Glu}} - \delta^{15}\text{N}_{\text{Phe}} - 3.4)/7.6 + 1$, proposed by Chikaraishi et al. (2009). Harbor seal TP values were calculated using the recently proposed equation for urea excreting marine mammals, $\text{TP}_{\text{Glu/Phe}} = (\delta^{15}\text{N}_{\text{Glu}} - \delta^{15}\text{N}_{\text{Phe}} - 7.7)/7.6 + 2$, formulated by Germain et al. (2013). The standard deviation of TP values was calculated by propagating the error of both Glu (SD1) and Phe (SD2) $\delta^{15}\text{N}$ values using the equation: $\text{TP}_{\text{Glu/Phe}} = \sqrt{\text{SD1}^2 + \text{SD2}^2} / 7.6$. This measure of precision only addresses the error in the analytical measurement and does not account for the uncertainty in the TEF value of 7.6 (discussed in “Assessment”).

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”), 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 with 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 min) 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 of His and Lys. Conversely, rapid organic solvent gradients were used in regions of well-separated peaks 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

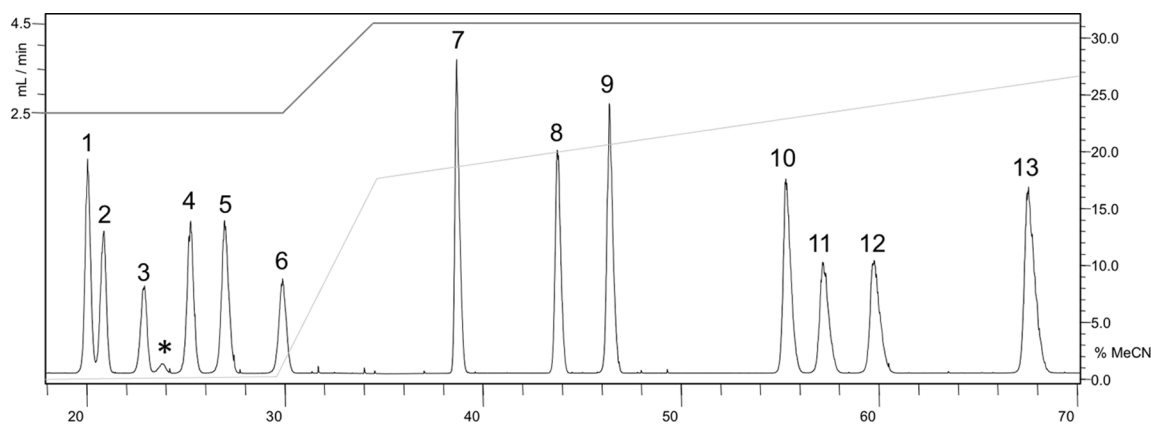


Fig. 2. Representative HPLC-ELSD chromatogram section including the first 13 eluting peaks of the 16 AA isotopic standard mixture. Window shows min 17–70 to highlight the separation of Glu and Phe. Each peak represents 300 nmol AA injected on-column. Light-gray line indicates % organic solvent (binary solvent program). Dark-gray 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.

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 chromatographic method used for this separation takes 3 hours per sample to elute all 16 AAs with baseline resolution, however if only Glu and Phe are needed (as for TP calculations), a modified method can be used (described in "Methods"), which sacrifices the resolution of some compounds but reduces the total chromatographic analysis to approximately 90 min per sample. In this abridged method both Glu and Phe retain their baseline resolution, however, separation is not achieved for Val, Met, Ile, Leu, Nle, His, Lys, or Arg.

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.

Whereas 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 (Fig. 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 μ mol of C (or 60-100 μ g 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 et al. 2013), as well as the small sample EA-IRMS that we applied here.

$\delta^{15}N$ accuracy and precision: standard materials

To assess the relative accuracy and precision of this HPLC/EA-IRMS method for TP_{CSIA} calculations, versus a standard GC-C-IRMS approach, we first compared Glu and Phe $\delta^{15}N$ values from standard AA materials measured independently by both methods (Fig. 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, whereas Phe is widely separated from all other peaks, and elutes in a solvent mixture with higher organic (acetonitrile) content.

HPLC/EA-IRMS $\delta^{15}N$ 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 represents 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

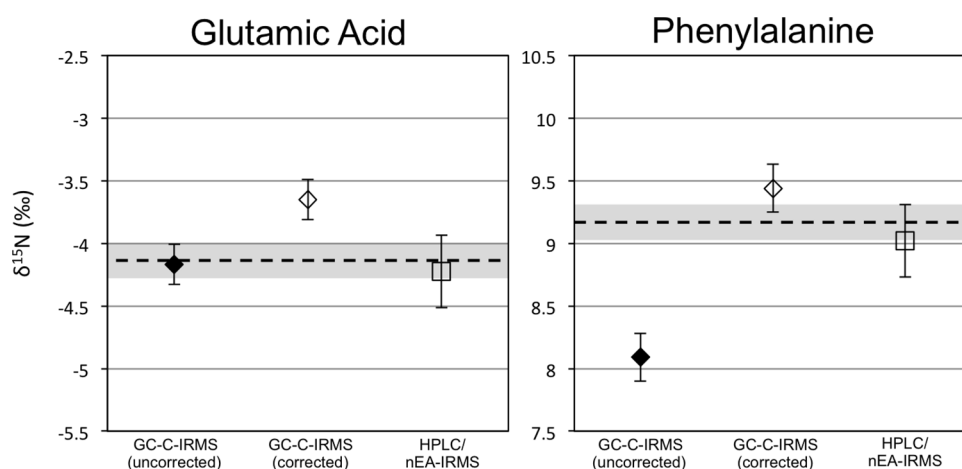


Fig. 3. Comparison of $\delta^{15}N$ values of isotopic Phe and Glu standards analyzed by GC-C-IRMS (diamonds) and HPLC/EA-IRMS (squares). Error bars represent ± 1 SD. Dotted line represents known value of isotopic standard (measured by standard EA-IRMS) and the shaded region the error of that value. 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.

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 (in “Methods”), the GC-C-IRMS $\delta^{15}N$ values were 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). Before 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 et 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 ‰; Fig. 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 by 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 Fig. 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 compare 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 $\delta^{15}\text{N}$ values.

Fig. 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 (Fig. 4). Some of these 'unknowns' are present in all or most samples (e.g., peaks at 24, 28, 58, and 60 min), 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 $\delta^{15}\text{N}$ 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 $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, 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 (Fig. A3 in Web Appendix A). 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 $\delta^{15}\text{N}_{\text{Glu/Phe}}$ in natural samples

The separations achieved with our method appear more than sufficient for producing accurate and precise $\delta^{15}\text{N}$ values for Glu and Phe across a range of biological samples. However, the most unambiguous assessment would be to directly examine the accuracy of $\delta^{15}\text{N}$ 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 $\delta^{15}\text{N}$ values from HPLC/EA-IRMS and an established GC-C-IRMS method can largely achieve this same goal. Because GC-C-IRMS $\delta^{15}\text{N}$ 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 $\delta^{15}\text{N}$ values from the two meth-

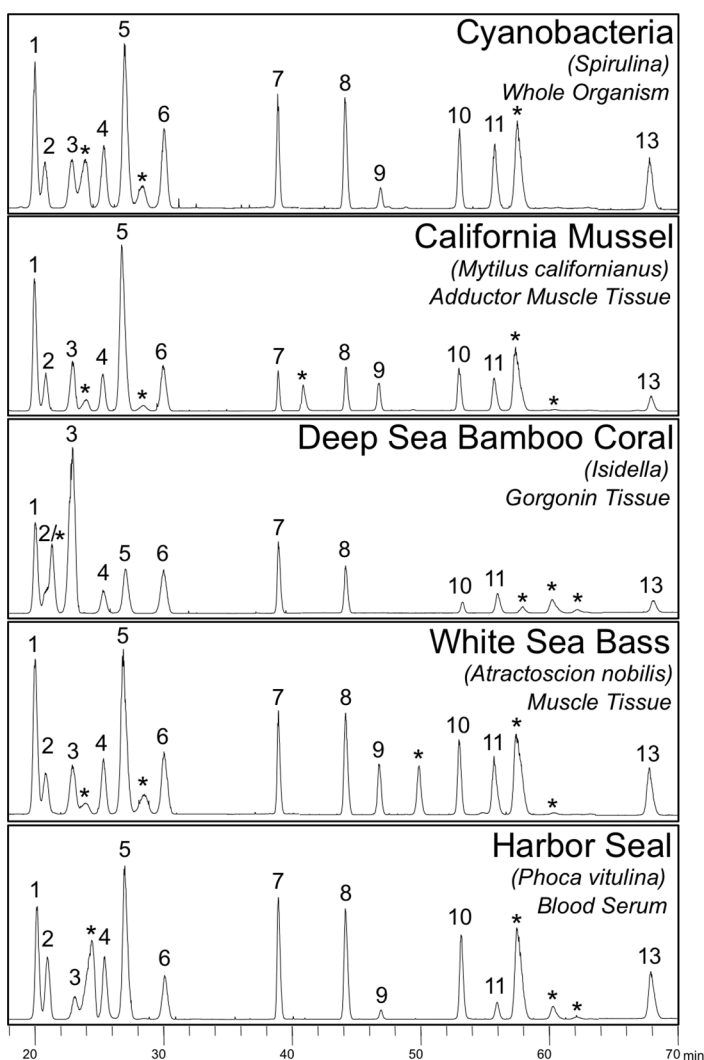


Fig. 4. Representative HPLC-ELSD chromatograms of 5 samples of hydrolyzed tissue. Each chromatogram represents an injection of approximately 1 mg dry proteinaceous material. AA peak identifications: 1. Asp, 2. Ser, 3. Gly, 4. Thr, 5. Glu, 6. Ala, 7. Pro, 8. Val, 9. Met, 10. Ile, 11. Leu, 12. Nle, 13. Phe. Asterisks (*) represent peaks of unknown composition.

ods 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 (see “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 (Fig. 6) were derived from propagating analytical error, as described above (see “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 derived values had a slightly higher mean SD. As mentioned in the “Methods” section, this SD takes into account only the uncertainty in the analytical measurements. We chose to treat the TEF as a constant value, as the variability in this value is not well constrained. Further, the ultimate goal of this work is to compare the analytical capabilities of the two methods and the added uncertainty inherent in the TP equations would not change the interpretation of the relative precision.

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 meth-

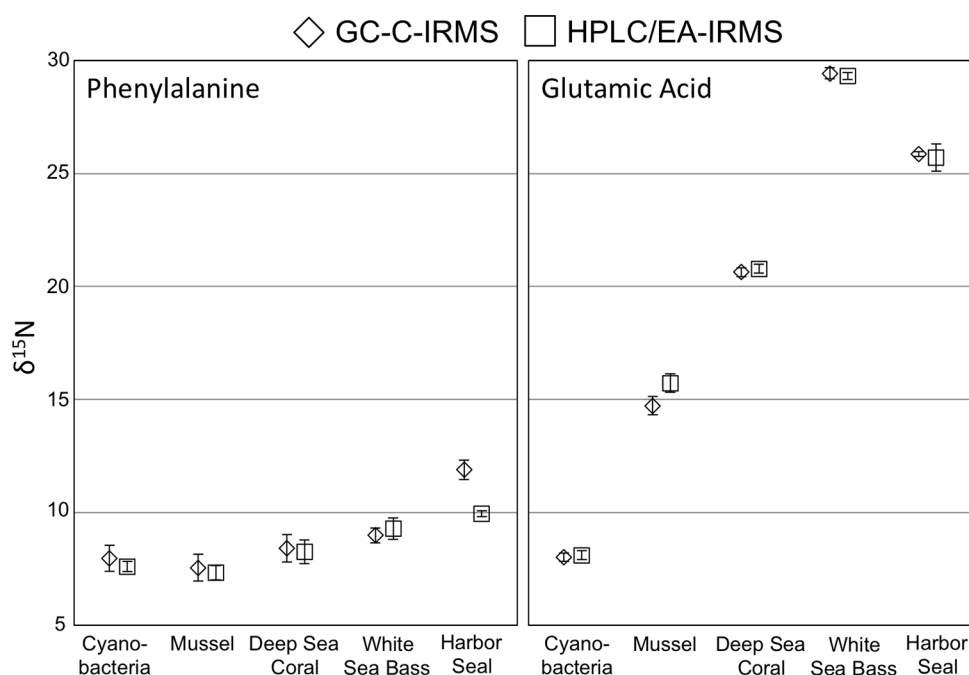


Fig. 5. Comparison of $\delta^{15}N$ values of Glu and Phe extracted from 5 marine organisms by GC-C-IRMS (diamonds) and HPLC/EA-IRMS (squares). Error bars on GC-C-IRMS and HPLC/EA-IRMS values represent ± 1 SD of $n = 3$ analyses.

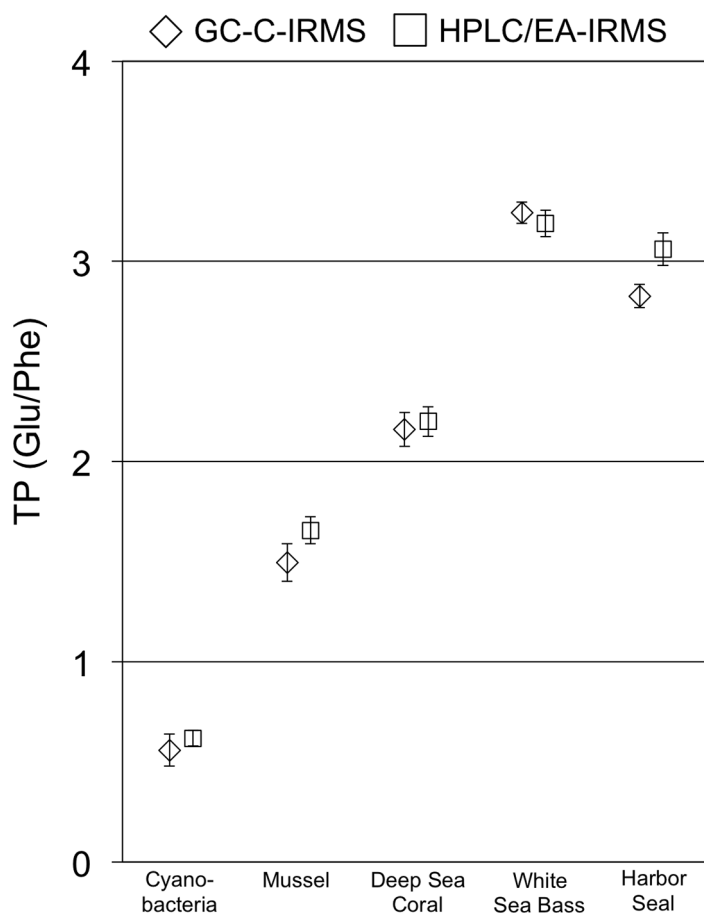


Fig. 6. Comparison of trophic position estimates based on $\delta^{15}N$ values of Glu and Phe extracted from 5 marine organisms by GC-C-IRMS (diamonds) and HPLC/EA-IRMS (squares). Trophic position calculated using the internally normalized equations described in text. Error bars represent the propagated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

ods: 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 (Fig. 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. 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, although 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 (see "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 $\delta^{15}N$ or $\delta^{13}C$ 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; Web Appendix A). 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 $\delta^{15}\text{N}$ 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 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 to measure a full suite of AAs. In contrast, the GC-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 because 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 nonbiological sample types would need to be evaluated further, and likely assessed on a sample-by-sample basis.

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References

- Barrett, G. C. 1985. Chemistry and biochemistry of the amino acids, 1st ed. Chapman and Hall [doi:10.1007/978-94-009-4832-7].
- Benner, R., and K. Kaiser. 2003. Abundance of amino sugars and peptidoglycan in marine particulate and dissolved organic matter. *Limnol. Oceanogr.* 48(1):118-128 [doi:10.4319/lm.2003.48.1.0118].
- Broek, T. A. B., B. D. Walker, D. H. Andreasen, and M. D. McCarthy. 2013. High-precision measurement of phenylalanine $\delta^{15}\text{N}$ values for environmental samples: A new approach coupling high-pressure liquid chromatography

- purification and elemental analyzer isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.* 27:2327-2337 [doi:10.1002/rcm.6695].
- Calleja, M. L., F. Batista, M. Peacock, R. Kudela, and M. D. McCarthy. 2013. Changes in compound specific $\delta^{15}\text{N}$ amino acid signatures and d/l ratios in marine dissolved organic matter induced by heterotrophic bacterial reworking. *Mar. Chem.* 149:32-44 [doi:10.1016/j.marchem.2012.12.001].
- Chikaraishi, Y., Y. Kashiyama, N. O. Ogawa, H. Kitazato, and N. Ohkouchi. 2007. Metabolic control of nitrogen isotope composition of amino acids in macroalgae and gastropods: implications for aquatic food web studies. *Mar. Ecol. Prog. Ser.* 342:85-90 [doi:10.3354/meps342085].
- , and others. 2009. Determination of aquatic food-web structure based on compound-specific nitrogen isotopic composition of amino acids. *Limnol. Oceanogr. Methods* 7:740-750 [doi:10.4319/lom.2009.7.740].
- , N. O. Ogawa, H. Doi, and N. Ohkouchi. 2011. $^{15}\text{N}/^{14}\text{N}$ ratios of amino acids as a tool for studying terrestrial food webs: a case study of terrestrial insects (bees, wasps, and hornets). *Ecol. Res.* 26:835-844 [doi:10.1007/s11284-011-0844-1].
- Choy, C. A., and others. 2012. Global trophic position comparison of two dominant mesopelagic fish families (Myctophidae, Stomiidae) using amino acid nitrogen isotopic analyses. *PLoS ONE* 7(11):e50133 [doi:10.1371/journal.pone.0050133].
- Choy, K., C. I. Smith, B. T. Fuller, and M. P. Richards. 2010. Investigation of amino acid $\delta^{13}\text{C}$ signatures in bone collagen to reconstruct human paleodiets using liquid chromatography-isotope ratio mass spectrometry. *Geochim. Cosmochim. Acta* 74:6093-6111 [doi:10.1016/j.gca.2010.07.025].
- Corr, L. T., R. Berstan, and R. P. Evershed. 2007. Optimisation of derivatization procedures for the determination of $\delta^{13}\text{C}$ values of amino acids by gas chromatography/combustion/isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.* 21:3759-3771 [doi:10.1002/rcm.3252].
- Dunn, P. J. H., N. V. Honch, and R. P. Evershed. 2011. Comparison of liquid chromatography-isotope ratio mass spectrometry (LC/IRMS) and gas chromatography-combustion-isotope ratio mass spectrometry (GC/C/IRMS) for the determination of collagen amino acid $\delta^{13}\text{C}$ values for palaeodietary and palaeoecological rec. *Rapid Commun. Mass Spectrom.* 25:2995-3011 [doi:10.1002/rcm.5174].
- Fry, B., W. Brand, F. J. Mersch, K. Tholke, and R. Garritt. 1992. Automated analysis system for coupled $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ measurements. *Anal. Chem.* 62:288-291 [doi:10.1021/ac00027a009].
- Germain, L. R., M. D. McCarthy, P. L. Koch, and J. T. Harvey. 2011. Stable carbon and nitrogen isotopes in multiple tissues of wild and captive harbor seals (*Phoca vitulina*) off the California coast. *Mar. Mammal Sci.* 28:542-560 [doi:10.1111/j.1748-7692.2011.00516.x].
- , P. L. Koch, J. Harvey, and M. D. McCarthy. 2013. Nitrogen isotope fractionation in amino acids from harbor seals: implications for compound-specific trophic position calculations. *Mar. Ecol. Prog. Ser.* 482:265-277 [doi:10.3354/meps10257].
- Goodfriend, G. A. 1997. Aspartic acid racemization and amino acid composition of the organic endoskeleton of the deep-water colonial anemone *Gerardia*: Determination of longevity from kinetic experiments. *Geochim. Cosmochim. Acta.* 61(9):19931-1939 [doi:10.1016/S0016-7037(97)00067-7].
- Hannides, C. C. S., B. N. Popp, M. R. Landry, and B. S. Graham. 2009. Quantification of zooplankton trophic position in the North Pacific Subtropical Gyre using stable nitrogen isotopes. *Limnol. Oceanogr.* 54:50-61 [doi:10.4319/lo.2009.54.1.0050].
- Larsen, T., D. L. Taylor, M. B. Leigh, and D. M. O'Brien. 2009. Stable isotope fingerprinting: a novel method for identifying plant, fungal, or bacterial origins of amino acids. *Ecology* 90:3526-3535 [doi:10.1890/08-1695.1].
- Lorrain, A., B. S. Graham, F. Ménard, B. N. Popp, S. Bouillon, P. van Breugel, and Y. Cherel. 2009. Nitrogen and carbon isotope values of individual amino acids: a tool to study foraging ecology of penguins in the Southern Ocean. *Mar. Ecol. Prog. Ser.* 391:293-306 [doi:10.3354/meps08215].
- McCarthy, M. D., R. Benner, C. Lee, J. I. Hedges, and M. L. Fogel. 2004. Amino acid carbon isotopic fractionation patterns in oceanic dissolved organic matter: an unaltered photoautotrophic source for dissolved organic nitrogen in the ocean? *Mar. Chem.* 92:123-134 [doi:10.1016/j.marchem.2004.06.021].
- , R. Benner, C. Lee, and M. L. Fogel. 2007. Amino acid nitrogen isotopic fractionation patterns as indicators of heterotrophy in plankton, particulate, and dissolved organic matter. *Geochim. Cosmochim. Acta* 71:4727-4744 [doi:10.1016/j.gca.2007.06.061].
- , J. Lehman, and R. M. Kudela. 2013. Compound-specific amino acid $\delta^{15}\text{N}$ patterns in marine algae: Tracer potential for cyanobacterial vs. eukaryotic organic nitrogen sources in the ocean. *Geochim. Cosmochim. Acta* 103:104-120 [doi:10.1016/j.gca.2012.10.037].
- McClelland, J., and J. P. Montoya. 2002. Trophic relationships and the nitrogen isotopic composition of amino acids in plankton. *Ecology* 83:2173-2180 [doi:10.1890/0012-9658(2002)083[2173:TRATNI]2.0.CO;2].
- McCullagh, J. S. O., D. Juchelka, and R. E. M. Hedges. 2006. Analysis of amino acid ^{13}C abundance from human and faunal bone collagen using liquid chromatography/isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.* 20:2761-2768 [doi:10.1002/rcm.2651].
- McCullagh, J. O., A. Marom, and R. M. Hedges. 2010. Radiocarbon dating of individual amino acids from archaeological bone collagen. *Radiocarbon* 52:620-634.

- McMahon, K. W., L. L. Hamady, S. R. Thorrold, and I. P. Smith. 2013. Ocean ecogeochemistry: a review. *Oceanogr. Mar. Biol.* 51:327-373.
- Popp, B. N., and others. 2007. Insight into the trophic ecology of Yellowfin Tuna, *Thunnus albacares*, from compound-specific nitrogen isotope analysis of proteinaceous amino acids. *Terrestr. Ecol.* 1:173-190 [doi:10.1016/S1936-7961(07)01012-3].
- Sherwood, O. A., M. F. Lehmann, C. J. Schubert, D. B. Scott, and M. D. McCarthy. 2011. Nutrient regime shift in the western North Atlantic indicated by compound-specific $\delta^{15}\text{N}$ of deep-sea gorgonian corals. *Proc. Natl. Acad. Sci. USA* 108:1011-1015 [doi:10.1073/pnas.1004904108].
- , T. P. Guilderson, F. C. Batista, J. T. Schiff, and M. D. McCarthy. 2014. Increasing subtropical North Pacific Ocean nitrogen fixation since the Little Ice Age. *Nature* 505:78-81 [doi:10.1038/nature12784].
- Silfer, J. A., M. H. Engel, S. A. Macko, and E. J. Jumeau. 1991. Stable carbon isotope analysis of amino acid enantiomers by conventional isotope ratio mass spectrometry and combined gas chromatography/isotope ratio mass spectrometry. *Anal. Chem.* 63:370-374 [doi:10.1021/ac00004a014].
- Tripp, J. A., J. S. McCullagh, and R. E. Hedges. 2006. Preparative separation of underivatized amino acids for compound-specific stable isotope analysis and radiocarbon dating of hydrolyzed bone collagen. *J. Sep. Sci.* 29:41-48 [doi:10.1002/jssc.200500247].
- Vokhshoori, N. L., and M. D. McCarthy. 2014. Compound-specific $\delta^{15}\text{N}$ amino acid measurements in littoral mussels in the California Upwelling Ecosystem: A new approach to generating baseline. *PLoS ONE*. 9(6):e98087 [doi:10.1371/journal.pone.0098087].

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