Integrative assessment of amino acid nitrogen isotopic composition in biological tissue samples determined by GC/C/IRMS, LC × EA/IRMS, and LC × GC/C/IRMS

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Abstract

Compound-specific isotope analysis of nitrogen (δ^{15}N) in amino acids (CSIA-AA) has significantly contributed to environmental sciences such as anthropology, biogeochemistry, and ecology. Several methods exist for determining δ^{15}N of amino acids (AAs). Although these methods have their own strength and weakness, they have not been intercalibrated yet, especially for biological samples with matrices. To address this issue, we systematically compared AA δ^{15}N values among three methods using gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS), preparative liquid chromatography (LC) separation followed by elemental analyzer/IRMS (LC × EA/IRMS), and LC separation followed by GC/C/IRMS (LC × GC/C/IRMS). The δ^{15}N values of glutamic acid (δ^{15}N_{Glu}) and phenylalanine (δ^{15}N_{Phe}) in fish muscle, two crucial AAs for estimating the trophic positions (TPs) of organisms, were compared among methods. Although a significant difference in fish muscle δ^{15}N_{Glu} values was found among the three analytical methods, their δ^{15}N_{Glu} and δ^{15}N_{Phe} values were fairly consistent between all pairs of methods (n = 8, R^2 = 0.9968 for GC/C/IRMS vs. LC × GC/C/IRMS; 0.9936 for LC × EA/IRMS vs. LC × GC/C/IRMS; and 0.9912 for GC/C/IRMS vs. LC × EA/IRMS), which resulted in similar TP estimates among the methods. Thus, the results provide empirical validation that the CSIA-AA is comparable among different methods in interdisciplinary research fields. We also highlighted some critical features of each of the three analytical methods that can be used as a guideline for future CSIA-AA research.

Compound-specific isotope analysis of nitrogen (δ^{15}N) in amino acids (CSIA-AA) has significantly contributed to anthropological, biogeochemical, and ecological studies for the past decade (Ohkouchi et al. 2017). In particular, the δ^{15}N values of the two amino acids (AAs), glutamic acid (δ^{15}N_{Glu}) and phenylalanine (δ^{15}N_{Phe}), have been extensively used to estimate the trophic positions (TPs) of organisms, which are an important indicator of ecosystem trophic structure and energy flow, using the following equation (Chikaraishi et al. 2009a):

\[
TP = \frac{\delta^{15}N_{Glu} - \delta^{15}N_{Phe} + \beta}{TDF_{Glu} - TDF_{Phe}} + 1, \tag{1}
\]

where \(\beta\) is the initial difference (‰) between \(\delta^{15}N_{Glu}\) and \(\delta^{15}N_{Phe}\) found in the primary producers (i.e., \(-3.4 \pm 0.9\)‰ for aquatic algae and cyanobacteria). TDF_{Glu} and TDF_{Phe} are the offset of the trophic discrimination factors (‰) for \(\delta^{15}N_{Glu}\) (+8.0 ± 1.2‰) and \(\delta^{15}N_{Phe}\) (+0.4 ± 0.5‰), respectively (Chikaraishi et al. 2009a). In previous studies, gas chromatography (GC) (Chikaraishi et al. 2009a) or high-performance liquid chromatography (HPLC) (Broek and McCarthy 2014) are typically used to separate individual AAs extracted from environmental specimens for subsequent δ^{15}N measurements in each AA using isotope ratio mass spectrometry (IRMS).

In the former method, hydrolyzed AAs are derivatized by esterification of carboxyl groups and acylation of amino groups before being introduced into GC/combustion/IRMS.
(hereafter GC/C/IRMS). GC/C/IRMS can determine $^{\delta^{15}}N_{\text{Glu}}$ and $^{\delta^{15}}N_{\text{Phe}}$ and $^{\delta^{15}}N$ values for several other AAs per single injection. However, retention times of glutamic acid (Glu) and phenylalanine (Phe) derivatives (e.g., N-trifluoracetyl/isopropyl or N-pivaloyl/isopropyl derivatives) on GC chromatogram (e.g., Agilent DB-5 or Ultra 2 columns) are relatively close to each other (Metges et al. 1996, although they might be more separable using other derivatization methods and/or other GC columns; Corr et al. 2007). Suppose the tail of a large Glu peak overlapped with a small Phe peak with a lower $^{\delta^{15}}N$ value using the N-pivaloyl/isopropyl derivatization and the Agilent Ultra 2 column. In that case, $^{\delta^{15}}N_{\text{Phe}}$ may be overestimated due to partial coelution of Glu with a higher $^{\delta^{15}}N$ value, eventually resulting in underestimated TP values (Eq. 1). Therefore, the accurate determination of $^{\delta^{15}}N_{\text{Glu}}$ and $^{\delta^{15}}N_{\text{Phe}}$ values, whose peaks are completely separated at the chromatogram baseline, is critical to estimate the TPs of the focal organisms. This is particularly true for organisms in aquatic food webs where tertiary consumers (i.e., TP = 4) see a more difference of more than 30‰ between the values of $^{\delta^{15}}N_{\text{Glu}}$ and $^{\delta^{15}}N_{\text{Phe}}$ (Chikaraishi et al. 2009a).

The other method separates and isolates underivatized AAs in preparative HPLC using a reversed-phase or mixed-mode column. The recovered AAs are then introduced into elemental analyzer/IRMS (hereafter LC × EA/IRMS) (Tripp et al. 2006; Broek et al. 2013; Broek and McCarthy 2014; Swalethorp et al. 2020). Broek et al. (2013) compared $^{\delta^{15}}N_{\text{Phe}}$ values determined by LC × EA/IRMS and GC/C/IRMS and reported that the former yielded a smaller analytical error ($1\sigma \pm 0.16\%$) than the latter ($1\sigma \pm 0.64\%$). This conclusion was based on the conventional EA/IRMS system with sample amounts in the range of 7–10 $\mu$g N (Broek et al. 2013). However, when a marine apex predator (Harbor Seal) was examined using a small-scale (hereafter nano-scale, < 1.4 $\mu$g N) EA/IRMS system, its $^{\delta^{15}}N_{\text{Phe}}$ value considerably differed by 2–3‰ compared to that from GC/C/IRMS. This resulted in a 0.3-unit difference in the TP estimates between the two methods (Broek and McCarthy 2014). Therefore, it is hypothesized that the Glu and Phe fractions recovered from HPLC deliver a significant amount of exogenous nitrogen (e.g., ambient ammonia) from the experimental procedure to the nanoscale EA/IRMS system. It remains unclear on how much the LC × (nanoscale) EA/IRMS is sensitive to such exogenous nitrogen for CSIA-AA.

Furthermore, AAs hydrolyzed from biological samples often contain organic matrices, including amino sugars or nucleic acids, which are often inseparable from AAs by a single chromatography (Clarke et al. 1999). Swalethorp et al. (2020) reported that chitin coeluting with some AAs on the HPLC chromatogram makes AA purification difficult. These coeluting impurities potentially hamper the precise and accurate measurements of the $^{\delta^{15}}N$ values in AAs. Takano et al. (2015) found that the $^{\delta^{15}}N$ values of the authentic AA standard recovered from HPLC and derivatized for GC/C/IRMS measurements (hereafter LC × GC/C/IRMS) were consistent ($R^2 = 0.997$) with those without the LC treatment (i.e., GC/C/IRMS). However, differences in AA $^{\delta^{15}}N$ values for biological samples with and without the LC treatment have not been systematically compared (but see Ishikawa et al. 2018 that separated methionine from its impurity using LC × GC/C/IRMS). LC × GC/C/IRMS could provide more reliable $^{\delta^{15}}N_{\text{Glu}}$ and $^{\delta^{15}}N_{\text{Phe}}$ values than GC/C/IRMS if the coeluting impurities were significant and their $^{\delta^{15}}N$ were very different from $^{\delta^{15}}N_{\text{Glu}}$ and $^{\delta^{15}}N_{\text{Phe}}$ values. Furthermore, the LC × GC/C/IRMS approach is expected to reduce the contamination risk of underivatized blank nitrogen that can potentially affect the LC × EA/IRMS approach.

Although GC/C/IRMS, LC × EA/IRMS, and LC × GC/C/IRMS have their own strength and weakness, which is of critical importance for determination of AA $^{\delta^{15}}N$ values (Silverman et al. 2022), they have not been intercalibrated yet. To address this issue, we aimed to systematically compare $^{\delta^{15}}N_{\text{Glu}}$, $^{\delta^{15}}N_{\text{Phe}}$, and TP values, three major outcomes for biogeochemical CSIA-AA studies, among the three analytical methods using biological samples containing matrices. Although the matrix effect on AAs would be more severe in other environmental specimens such as sediments or dissolved organic matter, the muscle of four marine demersal fish species was used as representative biological samples. This is because consumers with high TP are expected to be sensitive to chromatographic separation of the $^{15}$N-enriched Glu and $^{15}$N-depleted Phe combination. The results would provide fundamental benchmark for the future CSIA-AA studies where the analytical approach is expected to be more diversified.

### Materials and procedures

#### Solvents and AA standards

Most chemical reagents used in this study (i.e., acetonitrile, ammonia water, dichloromethane, distilled water, hydrochloric acid, isopropanol, methanol, $n$-hexane, pivaloyl chloride, thionyl chloride) were purchased from Fujifilm Wako Pure Chemicals (Osaka, Japan). Nonfluoropentanoic acid (NFPA) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Seventeen AA standard powders were purchased from Sigma-Aldrich for HPLC analysis. Glutamic acid and eight AA standard powders (with known $^{\delta^{15}}N$ values as mentioned later) were purchased from Fujifilm Wako Pure Chemicals and Shoko Science (Tokyo, Japan), respectively, for GC analysis.

#### Samples

This study used four fish species: Jelly eelpout Bothrocara tanakae; Pacific cod Gadus macrocephalus; Longarm grenadier Abyssicola macrochir; and Snubnosed eel Simenchelys parasitica collected from the western North Pacific (Ohkouchi et al. 2016; Ishikawa et al. 2021). One individual per species was used for analysis. A small piece of muscle near the dorsal fin was excised, freeze-dried, and defatted with methanol and dichloromethane (Ohkouchi et al. 1997). The samples (12–13 mg dry weight) were ground into homogenous
powders using a tube mill (Tube Mill control, IKA, Staufen, Germany) and were stored at −20°C until further processing.

**AA derivatization**

AAs were extracted from the fish muscle by hydrolysis with 1 mol L⁻¹ hydrochloric acid (HCl) at 110°C for 12 h (Chikaraishi et al. 2010). Each hydrolysate was filtered through a cellulose membrane (GHP Nanosep MF, pore size: 0.45 μm, ODGHPC34, Pall Life Sciences, NY, USA) and washed with n-hexane/dichloromethane (3/2, v/v) to remove any hydrophobic constituents (e.g., lipids). After drying the samples under N₂ gas flow, the samples were split into two subsamples (Fig. 1). One AA subsample was derivatized sequentially with thionyl chloride/isopropanol (1/4, v/v) and pivaloyl chloride/dichloromethane (1/4, v/v). Prior to gas chromatographic separation, the N-pivaloyl/isopropyl (Pv/iPr) derivatives of AAs were extracted from the final fraction with n-hexane/dichloromethane (3/2, v/v).

**Separation and isolation by HPLC**

The other AA subsample was dissolved into 0.5 mL of 0.1 mol L⁻¹ HCl and injected into an HPLC system (1260 series, Agilent Technologies, CA, USA), and separated by the modified method of Takano et al. (2015) and Furuta et al. (2018). We used a porous graphite carbon column (Hypercarb, 4.6 × 150 mm, particle size 5 μm; Thermo Fisher Scientific, MA, USA) and a guard column (4.6 × 10 mm, 5 μm; Thermo Fisher Scientific) with a column cooler (Cool Pocket, Thermo Fisher Scientific) stabilized at 10.0°C. Mobile phases were distilled water with 20 mmol L⁻¹ NFPA (ion pair reagent) (solvent A) and acetonitrile (solvent B). The injection volume of the autosampler was set at 80 μL per run, and five injections were performed per sample. The solvent gradient for each run was linearly programmed as follows: 0 min (A: 100%, B: 0%) to 60 min (A: 40%, B: 60%), with a flushing time of 60–70 min using 60% B, followed by equilibration with 100% A for 30 min (Takano et al. 2015). The flow rate was maintained constant at 0.2 mL min⁻¹. The separation of Glu and Phe was monitored using a charged aerosol detector (Corona CAD Veo, Thermo Fisher Scientific). After screening the AA profile of hydrolyzed fish samples with reference to the AA standard mixture (Supporting Information Fig. S1; Fig. 2a), a fraction collector (Agilent Technologies) with a time-based trigger mode was used to separate and isolate the target Glu (retention time: 30.0–32.0 min) and Phe (retention time: 50.0–52.0 min). In addition, 2 min before and after the targets were also collected if there was a drift in the retention time. All of these fractions collected were freeze-dried to evaporate solvents, dissolved into 1 mL of 0.1 mol L⁻¹ HCl, and 1 μL injected into the HPLC to confirm that only target AAs were detected (Fraction ii for Glu and Fraction iv for Phe, Fig. 2b and c). The isolated Glu and Phe fractions for all species were each split into two subfractions. Half of the Glu subfraction was combined with the Phe subfraction for each species. The combined Glu and Phe samples (n = 4) were passed through cation exchange chromatography (CEX) using a resin (AG 50W-X8, 200–400 mesh, Bio-rad, CA, USA) following the method of Takano et al. (2010). It is reported that CEX does not affect AA δ¹⁵N measurements using GC/C/IRMS (Takano et al. 2010). We used a 10% ammonium solution to recover AAs. The samples were then dried under N₂ gas flow and derivatized as mentioned above. To test the effect of CEX on GC/C/IRMS, an aliquot of the combined Glu and Phe fractions of *G. macrocephalus* was

![Fig. 1. Schematic workflow of this study. ∗The effect of cation exchange chromatography (CEX) was examined using *Gadus macrocephalus* sample (see Supporting Information Figs. S8–S13).](image-url)
derivated without the CEX treatment. We expected that method optimization of the entire wet chemistry pretreatments could gain the benefits of analysis after CEX procedure (Evershed et al. 2007; Takano et al. 2018; Blattmann et al. 2020). The other half of the Glu and Phe subfractions were not combined, freeze-dried to evaporate solvents, and independently measured \( \delta^{15}N \) values using nanoscale EA/IRMS as described later.

\[ \delta^{15}N \] measurement by GC/C/IRMS

The \( \delta^{15}N_{\text{Glu}} \) and \( \delta^{15}N_{\text{Phe}} \) values of derivatized samples were determined using a Delta Plus XP isotope ratio mass spectrometer connected to a gas chromatograph (6890N, Agilent Technologies) via an interface (Conflo III, Thermo Finnigan) with combustion (CuO, NiO, and Pt wires in a microvolume ceramic tube) and reduction (reduced Cu wires in a microvolume ceramic tube) furnaces (Thermo Finnigan) (Chikaraishi et al. 2010). The Pt/iPr-derivatized AAs were injected with a programmable-temperature vaporizing (PTV) injector (Gerstel, Mülheim an der Ruhr, Germany). The PTV temperature program was as follows: \( 50^\circ \text{C} \) (initial temperature) for 0.3 min, heated from \( 50^\circ \text{C} \) to \( 350^\circ \text{C} \) at a rate of \( 600^\circ \text{C} \ \text{min}^{-1} \), and held at \( 350^\circ \text{C} \) for 10 min. The carrier gas (He) flow rate was controlled at a constant flow mode of 1.4 mL min\(^{-1}\). The gas chromatograph oven temperature was programmed as follows: \( 40^\circ \text{C} \) (initial temperature) for 3.0 min, heated to \( 110^\circ \text{C} \) at a rate of \( 15^\circ \text{C} \ \text{min}^{-1} \), heated to \( 150^\circ \text{C} \) at a rate of \( 3^\circ \text{C} \ \text{min}^{-1} \), heated to \( 220^\circ \text{C} \) at a rate of \( 6^\circ \text{C} \ \text{min}^{-1} \), held at \( 260^\circ \text{C} \) for 18 min, heated to \( 280^\circ \text{C} \) at a rate of \( 60^\circ \text{C} \ \text{min}^{-1} \), and held at the final temperature for 10 min. The AAs were separated on a low polar column (Ultra 2, 0.32 mm × 50 m, film thickness 0.52 \( \mu \text{m} \); Agilent Technologies) before introducing them into the IRMS through combustion (\( 950^\circ \text{C} \)) and reduction (\( 550^\circ \text{C} \)) furnaces (Chikaraishi et al. 2010). An isotopic reference mixture of nine AAs (i.e., alanine, glycine, leucine, norleucine, aspartic acid, methionine, glutamic acid, phenylalanine, and hydroxyproline), with \( \delta^{15}N \) values ranging from \( -26.6\% \) to \( +45.6\% \) (Indiana University; Shoko Science), was analyzed every 5–6 injections to confirm the reproducibility of isotope measurements. Three and two pulses of the reference \( N_2 \) gas were measured for calibration at the beginning and the end of each run, respectively (Supporting Information Fig. S7). Using Isodat 3.0 software (Thermo Finnigan), peak detection parameters were set to the following: start slope of 0.2 mV s\(^{-1}\), end slope of 0.4 mV s\(^{-1}\), minimum peak height of 50 mV, peak resolution of 95%, and maximum peak width of 500 s. The \( \delta^{15}N \) values of all samples were corrected using the regression line between the published \( \delta^{15}N \) values and the measured \( \delta^{15}N \) values for the aforementioned nine AA standards (Ohkouchi et al. 2017). The standard measurement analytical errors (1\( \sigma \)) were lower than \( \pm0.8 \% \) and covered the analyte size range of > 70 ngN. Triplicate \( \delta^{15}N \) measurements were performed per sample.

The PTV temperature program was as follows: \( 50^\circ \text{C} \) (initial temperature) for 0.3 min, heated from \( 50^\circ \text{C} \) to \( 350^\circ \text{C} \) at a rate of \( 600^\circ \text{C} \ \text{min}^{-1} \), and held at \( 350^\circ \text{C} \) for 10 min. The carrier gas (He) flow rate was controlled at a constant flow mode of 1.4 mL min\(^{-1}\). The gas chromatograph oven temperature was programmed as follows: \( 40^\circ \text{C} \) (initial temperature) for 3.0 min, heated to \( 110^\circ \text{C} \) at a rate of \( 15^\circ \text{C} \ \text{min}^{-1} \), heated to \( 150^\circ \text{C} \) at a rate of \( 3^\circ \text{C} \ \text{min}^{-1} \), heated to \( 220^\circ \text{C} \) at a rate of \( 6^\circ \text{C} \ \text{min}^{-1} \), held at \( 260^\circ \text{C} \) for 18 min, heated to \( 280^\circ \text{C} \) at a rate of \( 60^\circ \text{C} \ \text{min}^{-1} \), and held at the final temperature for 10 min. The AAs were separated on a low polar column (Ultra 2, 0.32 mm × 50 m, film thickness 0.52 \( \mu \text{m} \); Agilent Technologies) before introducing them into the IRMS through combustion (\( 950^\circ \text{C} \)) and reduction (\( 550^\circ \text{C} \)) furnaces (Chikaraishi et al. 2010). An isotopic reference mixture of nine AAs (i.e., alanine [Ala], glycine, leucine, norleucine, aspartic acid [Asp], methionine, glutamic acid, phenylalanine, and hydroxyproline), with \( \delta^{15}N \) values ranging from \( -26.6\% \) to \( +45.6\% \) (Indiana University; Shoko Science), was analyzed every 5–6 injections to confirm the reproducibility of isotope measurements. Three and two pulses of the reference \( N_2 \) gas were measured for calibration at the beginning and the end of each run, respectively (Supporting Information Fig. S7). Using Isodat 3.0 software (Thermo Finnigan), peak detection parameters were set to the following: start slope of 0.2 mV s\(^{-1}\), end slope of 0.4 mV s\(^{-1}\), minimum peak height of 50 mV, peak resolution of 95%, and maximum peak width of 500 s. The \( \delta^{15}N \) values of all samples were corrected using the regression line between the published \( \delta^{15}N \) values and the measured \( \delta^{15}N \) values for the aforementioned nine AA standards (Ohkouchi et al. 2017). The standard measurement analytical errors (1\( \sigma \)) were lower than \( \pm0.8 \% \) and covered the analyte...
size range of > 70 ngN. Triplicate δ¹⁵N measurements were performed per sample.

**GC/MS measurements**
To characterize the chemical structure of impurities found on the GC chromatogram, we used the *G. macrocephalus* sample and injected its (1) AA derivatives; (2) Glu and Phe derivatives after LC isolation with the CEX treatment; and (3) Glu and Phe derivatives after LC isolation without the CEX treatment to a gas chromatograph (6890N, Agilent Technologies). The gas chromatogram was coupled to a mass spectrometry detector (5973 MSD, Agilent Technologies) with a PTV injector (Gerstel) and a low polar column (Ultra 2, 0.32 mm × 25 m, film thickness 0.52 μm; Agilent Technologies) using the modified method of Chikaraishi et al. (2009b). The PTV temperature program was as follows: 50°C (initial temperature) for 0.3 min, heated from 50°C to 350°C at a rate of 600°C min⁻¹, and held at 350°C for 10 min. The carrier gas (He) flow rate was controlled at a constant flow mode of 1 mL min⁻¹. The gas chromatograph oven temperature was programmed as follows: 40°C (initial temperature) for 4.0 min, heated from 40°C to 220°C at a rate of 8°C min⁻¹, heated to 260°C at a rate of 30°C min⁻¹, and held at the final temperature for 2.17 min. Ionization was performed based on the electron impact method to detect m/z in the range of 40–600 at 70 eV. By comparing the mass fragmentation patterns with the standard pattern (Chikaraishi et al. 2009b), we examined whether the impurities were by-products of the Glu and Phe derivatives or whether other molecules were contaminated during the experimental process.

**δ¹⁵N measurement by EA/IRMS**
The δ¹⁵N_Glu and δ¹⁵N_Phe values of underivatized samples (i.e., Glu and Phe fractions separated by HPLC) were determined with a Flash EA1112 elemental analyzer connected to a Delta Plus XP isotope ratio mass spectrometer with a Conflo III interface (Thermo Finnigan) modified for nanoscale measurements (>0.08 μgN, Ogawa et al. 2010; Isajii et al. 2020). Samples were dissolved in approximately 20 μL of 0.1 mol L⁻¹ HCl (final concentration: 0.05–0.1 μgN μL⁻¹), placed in a smooth-wall tin capsule (pre-cleaned with methanol and dichloromethane), and completely dried on a hot plate (90°C). Triplicate or more measurements were performed per sample (n = 8, 0.5–2.7 μgN, Glu and Phe from four fish species). The dried capsule was injected by an autosampler to the oxidation (1050°C) and reduction (750°C) furnaces with a helium carrier gas (100 mL min⁻¹). Two pulses of the reference N₂ gas were discharged at the beginning of each run. The δ¹⁵N data were calibrated using five inter-laboratory determined standards in the range of -5.70‰ to +61.3‰ (BG-T, BG-P, CERKU-01, l-glutamine, and l-valine; Tayasu et al. 2011, Indiana University, Shoko Science). The analytical errors (1σ) of δ¹⁵N values obtained by the repeated measurement of BG-T were lower than ±0.3‰ (n = 12, 0.6–2.5 μgN).

**Trophic position calculations and analyses**
To systematically assess uncertainties among GC/C/IRMS, LC × GC/C/IRMS, and LC × EA/IRMS, we calculated the standard deviations (SDs, 1σ) of repeated measurements of δ¹⁵N_Glu and δ¹⁵N_Phe values for the four fish species (n = 8). TPs were then calculated using mean δ¹⁵N_Glu and δ¹⁵N_Phe values determined by each of the three analytical methods using Eq. 1. Finally, the mean and SD of TP values obtained from δ¹⁵N_Glu and δ¹⁵N_Phe repeated measurements were used to calculate the propagation of error (1σ_TP) using the following equation (Bradley et al. 2015):

\[
\sigma_{TP}^2 = \frac{\sum_{i=1}^{n} \left( \frac{\partial TP}{\partial x_i} \sigma_{x_i} \right)^2}{(TDF_{Glu} - TDF_{Phe})^2 \left( \sigma_{\delta^{15}N_{Glu}}^2 + \sigma_{\delta^{15}N_{Phe}}^2 + \sigma_{TP}^2 \right) + (\sigma_{TDF_{Glu}}^2 + \sigma_{TDF_{Phe}}^2) \left( \delta^{15}N_{Glu} - \delta^{15}N_{Phe} + \beta \right)^2}{(TDF_{Glu} - TDF_{Phe})^4},
\]

where \( x_i \) corresponds to δ¹⁵N_Glu, δ¹⁵N_Phe, \( \beta \), TDF_Glu, and TDF_Phe. One-way ANOVA was used to test differences in δ¹⁵N_Glu and δ¹⁵N_Phe values determined by GC/C/IRMS, LC × GC/C/IRMS, and LC × EA/IRMS. [Correction added on August 30, 2022, after first online publication: The word “analyis(“ has been deleted.] A Tukey HSD multiple comparison was applied when ANOVA showed significant (p < 0.05) difference. The statistical significance (p < 0.05) with and without CEX treatment was examined using the t-test. All statistics were run and the graphic was created using Matlab 2020a (Mathworks).

**Assessment**

**LC chromatograms**
Using the analytical protocol established by Takano et al. (2015), the Glu and Phe of the four fish muscle samples were successfully separated from other AAs and unidentified impurities on the LC chromatogram (Fig. 2a). Peaks around 35 and 42 min were consistently observed on chromatograms even for standard and blank injections (Fig. S1). These peaks should be incombustible inorganic ions because the carbon and nitrogen of these peaks were not detected in the EA analysis (Furuta et al. 2018). The fractions of Glu (Fraction ii, retention time 30.0–32.0 min) and Phe (Fraction v, retention time 50.0–52.0 min) were collected as single target compounds (Fig. 2b and c). The target peak was not detected by charged aerosol detector (CAD) in the fractions before Glu (Fraction i, retention time 28.0–30.0 min), after Glu (Fraction iii, retention time 32.0–34.0 min), before Phe (Fraction iv, retention time 48.0–50.0 min), and after Phe (Fraction vi, retention time 52.0–54.0 min) (Fig. 2b,c). This is important for the CSIA where the δ¹⁵N value of the focal compound often drifts from its front to tail within its peak as a consequence of chromatographic isotopic fractionation (Hayes et al. 1990; Hare
et al. 1991; Merritt and Hayes 1994; Macko et al. 1997; Filer 1999; Broek et al. 2013; Isaji et al. 2020). Here, we appreciate a pioneering statement by John Hayes and co-workers in the 1990s for the importance of chromatographic baseline resolution in AA carbon isotope analysis: “When peaks overlap, the isotopically light tail of the first component underlies the beginning of the second peak, and the isotopically heavy front of the second peak underlies the end of the first peak” (Hayes et al. 1990). This has promoted AA isotope studies by combining online and offline methods (Hare et al. 1991; Minagawa et al. 1992; Metges and Petzke 1997), as demonstrated in the present study. Our chromatograms indicate that (1) the analytical settings were sufficiently stable with no drift in the retention time of the chromatogram, and (2) the entire target compound was successfully collected in the final Glu and Phe fractions.

**GC chromatograms**

The IRMS chromatograms around the Glu and Phe peaks were comparable between GC/C/IRMS and LC × GC/C/IRMS (Fig. 3). Therefore, the GC/C/IRMS approach is likely effective for AA δ15N analysis.

**Fig. 3.** (a) GC/C/IRMS and (b) LC × GC/C/IRMS chromatograms for Pvi/iPr derivatized AAs extracted from the Gadus macrocephalus muscle sample. The first three and the last two peaks are reference N2 gas. Asterisks denote major impurities.

**Fig. 4.** Correlation plots for δ15N values of the four marine fish muscle samples among GC/C/IRMS, LC × EA/IRMS, and LC × GC/C/IRMS. Mean, 1σ standard deviation for repeated measurements, and lines for regression (solid) and 1 : 1 (dashed) are shown: (a) y = 0.9504 x + 1.277 for GC/C/IRMS vs. LC × GC/C/IRMS; (b) y = 0.9171 x + 1.597 for LC × EA/IRMS vs. LC × GC/C/IRMS; and (c) y = 1.036 x – 0.3420 for GC/C/IRMS vs. LC × EA/IRMS.
adequate, at least for the determination of δ¹⁵N⁰⁰ and δ¹⁵N⁹⁰. However, isolating Glu and Phe using HPLC before derivatization made the GC/C/IRMS chromatogram much simpler than without HPLC (Fig. 3b). This is because the separation of LC prevents compounds other than Glu and Phe from being injected into the IRMS system. Unknown peaks were found on the GC/C/IRMS and LC × GC/C/IRMS chromatograms around 25, 33, 36, 37, and 40 min (Fig. 3). Most of these peaks are assigned by GC/MS to the secondary products of the Pv/iPr derivatives of either Glu or Phe (Supporting Information Figs. S2–S7, except for Fig. S3 showing Asp Pv/iPr derivative).

δ¹⁵N and TP comparisons

A fairly good correlation was detected in δ¹⁵N⁰⁰ and δ¹⁵N⁰⁰ values (n = 8, R² = 0.9968) between GC/C/IRMS and LC × GC/C/IRMS (Fig. 4a). The impurities coeluted with the Glu and Phe peaks that could not be removed by the LC × GC, if present, are probably not important. The results confirmed that the GC/C/IRMS approach is appropriate for determining δ¹⁵N⁰⁰ and δ¹⁵N⁰⁰ values in biological samples with undesired matrices. The δ¹⁵N⁰⁰ and δ¹⁵N⁰⁰ values determined by LC × EA/IRMS were also consistent with those by the other two methods (n = 8, R² = 0.9936 for LC × EA/IRMS vs. LC × GC/C/IRMS and 0.9912 for GC/C/IRMS vs. LC × EA/IRMS) (Fig. 4b and c). One way ANOVA showed that δ¹⁵N⁰⁰ values of the four fish species were significantly different among the three methods (F > 6.84, p < 0.028). However, differences in mean δ¹⁵N⁰⁰ values among methods were not greater than the 2σ analytical error (∆1.6‰) of GC/C/IRMS. On the other hand, δ¹⁵N⁰⁰ values of the four fish species were not significantly different among the three methods (F < 2.63, p > 0.152) (Table 1). In particular, no significant difference in δ¹⁵N⁰⁰ values between GC/C/IRMS (δ¹⁵N⁰⁰ and δ¹⁵N⁰⁰ determined simultaneously) and LC × EA/IRMS (δ¹⁵N⁰⁰...
may have effectively extracted ambient ammonia during the experimental procedure, which cannot be perfectly removed during the final drying step (i.e., tin capsule heated on a hot plate at 90°C).

**Discussion**

Most previous CSIA-AA studies have been implemented by using GC/C/IRMS (after McClelland and Montoya 2002; Chikaraishi et al. 2007; McCarthy et al. 2007; Popp et al. 2007) or LC × EA/IRMS (Broek et al. 2013; Broek and McCarthy 2014; Swalethorp et al. 2020). The GC/C/IRMS has been well established as a fast and easy technique for CSIA research (Table 2). Also, its advantages include a small sample size (down to 20 ngN) and simultaneous δ¹⁵N measurements of multiple AAs. At the same time, its drawbacks are derivatization-related artifacts that can potentially generate by-products and destabilize end-products, and targets limited to derivatizable AAs (Table 2). Although the present study confirmed that it was of minor significance for biological samples, GC/C/IRMS is inherently sensitive to the effects of matrices and to a wide range in individual AA concentrations within a single sample on the chromatographic separation (Table 2). LC × (conventional) EA/IRMS with a sample amount of over 7 μgN offers a smaller analytical error (1σ ± 0.16‰) than GC/C/IRMS (1σ ± 0.64‰) (Broek et al. 2013). The analytical error of LC × (nano-scale) EA/IRMS that was used in this study (1σ ± 0.3‰ with 0.6–2.5 μgN) is still better than GC/C/IRMS. LC × EA/IRMS is free from complex derivatization and can be applied to AAs such as arginine (Arg) and histidine (His) because their Pv/iPr derivatives are nearly below detection (Ohkouchi et al. 2017, but N-acetyl/isopropyl derivatization is amenable for Arg, see Kendall and Evershed 2020) (Table 3). However, LC × EA/IRMS requires a large sample size (>50 μgN on conventional EA and >0.08 μgN on nanoscale EA) and holds the contamination risk of exogenous nitrogen (e.g., ambient ammonia), increasing with the decreasing sample size (Table 2). The exogenous nitrogen would affect δ¹⁵N of the final target AAs, as demonstrated in low reproducibility of δ¹⁵N_Phe values for G. macrocephalus and A. macrochir determined by our LC × (nanoscale) EA/IRMS.

Compared to the two methods above, LC × GC/C/IRMS is more labor-intensive and time-consuming (Fig. 1; Table 2). Similar to GC/C/IRMS, LC × GC/C/IRMS is not applicable to Pv/iPr-derivatized AAs such as Arg and His. However, multiple AAs isolated from LC can be easily recombined with arbitrary concentrations and compositions (Table 2). This is particularly useful for biological samples such as protein, where Glu is originally much more abundant than Phe. This imbalance potentially causes the Glu peak tail to overlap with the following Phe peak. LC × GC/C/IRMS has less contamination of exogenic nitrogen (e.g., ambient ammonia) than LC × EA/IRMS because the derivatization procedure eliminates all underivatized nitrogen compounds (Table 2). Although this depends on the columns, solvents, ramp programs, and derivatization methods, target AAs, which cannot be distinguished from other compounds on the LC chromatogram, may be separated on the GC chromatogram. For example, methionine and isoleucine coelute on LC but their Pv/iPr-derivatives are separable on the posterior GC (Ishikawa et al. 2018) (Figs. 2, 3). The reverse is also true: serine and threonine Pv/iPr...
Comparisons of 21 AAs that can (\(\ast\)), conditionally can (\(\ast\*,\)) measure \(\delta^{15}N\) values by the three analytical methods examined in this study. AAs are listed in LC elution order (Takano et al. 2015; Supporting Information Fig. S1). Symbols in the table correspond to supplemental footnote information.

| AAs       | Gly | Ser | Ala | Hyp | Thr | Cys | Asp | Gln | Asp | Pro | Glu | Val | Lys | Leu | Ile | Met | His | Arg | Phe | Tyr | Trp |
|-----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| GC/C/IRMS | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) |
| LC × EA/IRMS | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) |
| LC × GC/C/IRMS | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) | \(\Delta^{*}\) |

Gly: glysine; Ser: serine; Ala: alanine; Hyp: hydroxyproline; Thr: threonine; Cys: cysteine; Asp: aspartic acid; Pro: proline; Glu: glutamic acid; Val: valine; Lys: lysine; Leu: leucine; Ile: isoleucine; Met: methionine; His: histidine; Arg: arginine; Phe: phenylalanine; Tyr: tyrosine; Trp: tryptophane.

Peak coelution might be avoided by optimizing LC analytical settings.

Peak coelution might be avoided by optimizing GC analytical settings.

Peak coelution might be avoided by optimizing GC analytical settings.

Finally, the CEX treatment prior to derivatization removes impurities associated with the HPLC process (Supporting Information Fig. S8). Without CEX treatment, the peak balance of Glu and Phe changed significantly, and at least four peaks with intensities above the peaks of Glu and Phe were observed (Supporting Information Fig. S8a). The mass fragment patterns obtained from the GC/MS analysis showed that the impurities are the derivatives of their respective parent molecules (i.e., Glu and Phe Pv/iPr) (Supporting Information Figs. S9–S12). Thus, it is speculated that the isopropyl groups of Glu and Phe isolated from HPLC can be replaced with methyl groups without CEX treatment. More importantly, both the \(\delta^{15}N\) values determined by LC × GC/C/IRMS without CEX treatment were significantly (by \(\sim 6\%\)) lower than those determined by LC × GC/C/IRMS with CEX treatment (\(\delta^{15}N\) values, \(t = 14.7, p < 0.001\); \(\delta^{15}N\) values, \(t = 7.4, p = 0.002\); Supporting Information Fig. S13). The results suggest that these daughter compounds, which stemmed from the Pv/iPr derivatives of Glu and Phe, change their mother \(\delta^{15}N\) values because the isotopic shift was consistent for \(\delta^{15}N\) and \(\delta^{15}N\) values. Therefore, in future LC × GC/C/IRMS studies, it is recommended to perform CEX treatment after LC separation to accurately determine \(\delta^{15}N\) and \(\delta^{15}N\) values. It should be noted that the CEX treatment is not applicable to LC × EA/IRMS because the 10% ammonium solution that recovers AAs is not removable during the dry-up step for EA/IRMS, which delivers a significant amount of exogenous nitrogen to the sample.
Comments and recommendations

Previous studies have shown that δ15N values of AAs are consistent between LC × EA/IRMS and GC/C/IRMS (Broek and McCarthy 2014) and between LC × GC/IRMS and GC/C/IRMS (Takano et al. 2015). These results provide empirical evidence that δ15N_{Glh} and δ15N_{Phe} values reported by different studies are comparable. To our knowledge, the present study is the first to compare three analytical methods systematically for CSIA-AA using biological samples containing the matrices that potentially complicate chromatographic separation. Recently, Riekenberg et al. (2020) have examined a combined oxidation–reduction reactor on GC/C/IRMS for CSIA-AA. Furthermore, Zhang et al. (2021) have established a method using purge-and-trap continuous-flow isotope ratio mass spectrometry. In their method, the target AAs can be converted to nitrous oxide (N2O) rather than dinitrogen (N2) to determine their δ15N values. It will be crucial to compare the performance of existing and future analytical methods for CSIA-AA because several % error in δ15N values can potentially change the TP estimates. The fish TP estimates were consistent within their 1σ propagated uncertainties among the three methods tested in the present study; GC/C/IRMS, LC × EA/IRMS, and LC × GC/C/IRMS. However, as shown in Tables 2 and 3, the strengths/weaknesses of the three analytical methods and the measurable AAs are different. The best method depends on the size and type of samples being analyzed, the number of AAs targeted for measuring δ15N, and the range of analytical errors accepted by the research goal. Scientists in the future will need to take these points into account and optimize analytical conditions as needed to maximize research outcomes. This will broaden the possibilities of the CSIA-AA methodologies in interdisciplinary research fields, including anthropology, biogeochemistry, and ecology.

REFERENCES

Blattmann, T. M., D. B. Montluçon, N. Haghipour, N. F. Ishikawa, and T. I. Eglinton. 2020. Liquid chromatographic isolation of individual amino acids extracted from sediments for radiocarbon analysis. Front. Mar. Sci. 7: 174. doi: 10.3389/fmars.2020.00174
Bradley, C. J., N. J. Wallsgrove, C. A. Choy, J. C. Drazen, E. D. Hetherington, D. K. Hoen, and B. N. Popp. 2015. Trophic position estimates of marine teleosts using amino acid compound specific isotopic analysis. Limnol. Oceanogr. Methods 13: 476–493. doi: 10.1002/lom3.10041
Broek, T. A., B. D. Walker, D. H. Andreasen, and M. D. McCarthy. 2013. High-precision measurement of phenylalanine δ15N 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
Broek, T. A., and M. D. McCarthy. 2014. A new approach to δ15N compound-specific amino acid trophic position measurements: Preparative high pressure liquid chromatography technique for purifying derivatized amino acids for stable isotope analysis. Limnol. Oceanogr. Methods 12: 840–852. doi:10.4319/lom.2014.12.840
Chikaraishi, Y., Y. Kashiyma, 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
Chikaraishi, Y., N. O. Ogawa, Y. Kashiyma, Y. Takano, H. Suga, A. Tomitani, H. Miyashita, H. Kitazato, and N. Ohkouchi. 2009a. 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
Chikaraishi, Y., Y. Takano, and N. Ohkouchi. 2009b. GC/MS analysis of amino acids as pivaloyl/isopropylesters. Res. Organ. Geochem. 25: 61–70 [in Japanese with English tables and figures]. doi:10.20612/rog.25_0_61
Chikaraishi, Y., Y. Takano, N. O. Ogawa, and N. Ohkouchi. 2010. Instrumental optimization of compound-specific isotope analysis of amino acids by gas chromatography/combustion/isotope ratio mass spectrometry, p. 367–386. In N. Ohkouchi, I. Tayasu, and K. Koba [eds.], Earth, life, and isotopes. Kyoto Univ. Press.
Clarke, A. P., P. Jandik, R. D. Rocklin, Y. Liu, and N. Avdalovic. 1999. An integrated amperometry waveform for the direct, sensitive detection of amino acids and amino sugars following anion-exchange chromatography. Anal. Chem. 71: 2774–2781. doi:10.1021/acid9811524
Corr, L. T., R. Berstan, and R. P. Evershed. 2007. Optimisation of derivatisation procedures for the determination of δ13C values of amino acids by gas chromatography/combustion/isotope ratio mass spectrometry. Rapid Commun. Mass Spectrom. 21: 3759–3771. doi:10.1002/rcm.3252
Evershed, R. P., I. D. Bull, L. T. Corr, M. Z. Crossman, B. E. van Dongen, C. J. Evans, S. Jim, H. R. Mottram, A. J. Mukherjee, and R. D. Pancost. 2007. Compound-specific stable isotope analysis in ecology and paleoecology, p. 480–540. In R. Michener and K. Lajtha [eds.], Stable isotopes in ecology and environmental science. Blackwell Publishing.
Filer, C. N. 1999. Isotopic fractionation of organic compounds in chromatography. J. Label. Compd. Radiopharm. 42: 169–197. doi:10.1002/(SICI)1099-1344(199902)42:2<169::AID-JLCR178>3.0.CO;2-0
Fuortes, S., N. O. Ogawa, Y. Takano, T. Yoshimura, and N. Ohkouchi. 2018. Quantitative analysis of derivatized amino acids in the sub-to several-nanomolar range by ion-pair HPLC using a corona-charged aerosol detector (HPLC–CAD). J. Chromatogr. B 1095: 191–197. doi:10.1016/j.jchromb.2018.07.033
Hare, P. E., M. L. Fogel, T. W. Stafford Jr., A. D. Mitchell, and T. C. Hoering. 1991. The isotopic composition of carbon and nitrogen in individual amino acids isolated from modern and fossil proteins. J. Archaeol. Sci. 18: 277–292. doi: 10.1016/0305-4403(91)90066-X

Hayes, J., K. Freeman, B. Popp, and C. Hoham. 1990. Compound-specific isotopic analyses: A novel tool for reconstruction of ancient biogeochemical processes. Org. Geochem. 16: 1115–1128. doi: 10.1016/0146-6380(90)90147-R

Isaji, Y., N. O. Ogawa, C. J. Boreham, Y. Kashiyama, and N. Ohkouchi. 2020. Evaluation of δ13C and δ15N uncertainties associated with the compound-specific isotope analysis of geoporphyrins. Anal. Chem. 92: 3152–3160. doi: 10.1021/acs.analchem.9b04843

Ishikawa, N. F., Y. Chikaraishi, Y. Takano, Y. Sasaki, Y. Takizawa, M. Tsuchiya, I. Tayasu, T. Nagata, and N. Ohkouchi. 2018. A new analytical method for determination of the nitrogen isotopic composition of methionine: Its application to aquatic ecosystems with mixed resources. Limnol. Oceanogr. Methods 16: 607–620. doi: 10.1002/lom.10272

Ishikawa, N. F., N. O. Ogawa, Y. Chikaraishi, M. Yamaguchi, K. Fujikura, Y. Miyairi, Y. Yokoyama, T. Nagata, and N. Ohkouchi. 2021. Influences of ocean currents on the diets of demersal fish communities in the western North Pacific revealed by their muscle carbon and nitrogen isotopic compositions. Front. Mar. Sci. 8: 641282. doi: 10.3389/fmars.2021.641282

Kendall, I. P., and R. P. Evershed. 2020. Determination of arginine δ15N values in plant and animal proteins by gas chromatography–combustion–isotope ratio mass spectrometry. Anal. Chem. 92: 13246–13253. doi: 10.1021/acs.analchem.0c02438

Macko, S. A., M. E. Uhle, M. H. Engel, and V. Andrusевич. 1997. Stable nitrogen isotope analysis of amino acid enantiomers by gas chromatography/combustion/isotope ratio mass spectrometry. Anal. Chem. 69: 926–929. doi: 10.1021/ac960956l

McCarthy, M. D., 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

McClelland, J. W., 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

Merritt, D. A., and J. M. Hayes. 1994. Nitrogen isotopic analyses by isotope-ratio-monitoring gas chromatography/mass spectrometry. J. Am. Soc. Mass Spectrom. 5: 387–397. doi: 10.1016/1044-0305(94)85054-2

Metges, C. C., K. J. Petzke, and U. Hennig. 1996. Gas chromatography/combustion/isotope ratio mass spectrometric comparison of N-acetyl- and N-pivaloyl amino acid esters to measure 15N isotopic abundances in physiological samples: A pilot study on amino acid synthesis in the upper gastro-intestinal tract of minipigs. J. Mass Spectrom. 31: 367–376. doi: 10.1002/(SICI)1096-9888(199604)31:4<367::AID-JMS310>3.0.CO;2-V

Metges, C. C., and K. J. Petzke. 1997. Measurement of 15N/14N isotopic composition in individual plasma free amino acids of human adults at natural abundance by gas chromatography combustion isotope ratio mass spectrometry. Anal. Biochem. 247: 158–164. doi: 10.1006/abio.1997.2037

Minagawa, M., S. Egawa, Y. Kabaya, and K. Karasawa-Tsuru. 1992. Carbon and nitrogen isotope analysis for amino acids from biological sample. J. Mass Spectrom. Soc. Japan 40: 47–56. doi: 10.5702/massspec.40.47

Ogawa, N. O., T. Nagata, H. Kitazato, and N. Ohkouchi. 2010. Ultra-sensitive elemental analyzer/isotope ratio mass spectrometer for stable nitrogen and carbon isotope analyses, p. 339–353. In N. Ohkouchi, I. Tayasu, and K. Koba [eds.], Earth, life, and isotopes. Kyoto Univ. Press.

Ohkouchi, N., K. Kawamura, H. Kawahata, and A. Taira. 1997. Latitudinal distributions of terrestrial biomarkers in the sediments from the Central Pacific. Geochim. Cosmochim. Acta 61: 1911–1918. doi: 10.1016/S0016-7037(97)00040-9

Ohkouchi, N., H. Shibata, Y. Chikaraishi, H. Nomaki, N. O. Ogawa, T. Nagata, T. Goto, K. Fujikura, and H. Kitazato. 2016. A monitoring result of polychlorinated biphenyls (PCBs) in deep-sea organisms and sediments off Tohoku during 2012–2014: Temporal variation and the relationship with the trophic position. J. Oceanogr. 72: 629–639. doi: 10.1007/s10872-016-0359-z

Ohkouchi, N., Y. Chikaraishi, H. Close, B. Fry, T. Larsen, D. J. Madigan, M. D. McCarthy, K. W. McMahon, T. Nagata, Y. Naito, N. O. Ogawa, B. N. Popp, S. Steffan, Y. Takano, I. Tayasu, A. S. J. Wyatt, Y. Yamaguchi, and Y. Yokoyama. 2017. Advances in the application of amino acid nitrogen isotopic analysis in ecological and biogeochemical studies. Org. Geochem. 113: 150–174. doi: 10.1016/j.orggeochem.2017.07.009

Popp, B. N., S. B. Graham, R. J. Olson, C. C. S. Hannides, M. J. Lott, G. A. Lopez-Ibarra, F. Galvan-Magana, and B. Fry. 2007. Insight into the trophic ecology of yellowfin tuna, Thunnus albacares, from compound-specific nitrogen isotope analysis of proteinaceous amino acids, p. 173–190. In T. Dawson and R. Siegwolf [eds.], Stable isotopes as indicators of ecological change, terrestrial ecology series. Elsevier Academic Press.

Riekenberg, P. M., M. van der Meer, and S. Schouten. 2020. Practical considerations for improved reliability and precision during determination of δ15N values in amino acids using a single combined oxidation–reduction reactor. Rapid Commun. Mass Spectrom. 34: e8797. doi: 10.1002/rcm.8797
Ishikawa et al.

Silverman, S. N., A. A. Phillips, G. M. Weiss, E. B. Wilkes, J. M. Eiler, and A. L. Sessions. 2022. Practical considerations for amino acid isotope analysis. Org. Geochem. 164: 104345. doi:10.1016/j.orggeochem.2021.104345

Swalethorp, R., L. Aluwihare, A. R. Thompson, M. D. Ohman, and M. R. Landry. 2020. Errors associated with compound-specific δ15N analysis of amino acids in preserved fish samples purified by high-pressure liquid chromatography. Limnol. Oceanogr. Methods 18: 259–270. doi:10.1002/lom3.10359

Takano, Y., Y. Kashiwama, N. O. Ogawa, Y. Chikaraishi, and N. Ohkouchi. 2010. Isolation and desalting with cation-exchange chromatography for compound-specific nitrogen isotope analysis of amino acids: Application to biogeochemical samples. Rapid Commun. Mass Spectrom. 24: 2317–2323. doi:10.1002/rcm.4651

Takano, Y., Y. Chikaraishi, and N. Ohkouchi. 2015. Isolation of underivatized amino acids by ion-pair high performance liquid chromatography for precise measurement of nitrogen isotopic composition of amino acids: Development of comprehensive LC × GC/C/IRMS method. Int. J. Mass Spectrom. 379: 16–25. doi:10.1016/j.ijms.2014.11.012

Takano, Y., Y. Chikaraishi, H. Imachi, Y. Miyairi, N. O. Ogawa, M. Kaneko, Y. Yokoyama, M. Krueger, and N. Ohkouchi. 2018. Insight into anaerobic methanotrophy from 13C/12C-amino acids and 14C/12C-ANME cells in seafloor microbial ecology. Sci. Rep. 8: 14070. doi:10.1038/s41598-018-31004-5

Tayasu, I., R. Hirasawa, N. O. Ogawa, N. Ohkouchi, and K. Yamada. 2011. New organic reference materials for carbon- and nitrogen-stable isotope ratio measurements provided by Center for Ecological Research, Kyoto University, and Institute of Biogeosciences, Japan Agency for Marine-Earth Science and Technology. Limnology 12: 261–266. doi:10.1007/s10201-011-0345-5

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

Zhang, L., W. M. Lee, A. Kreider-Mueller, E. Kuhnel, J. Baca, C. Ji, and M. Altabet. 2021. High-precision measurement of phenylalanine and glutamic acid δ15N by coupling ion-exchange chromatography and purge-and-trap continuous-flow isotope ratio mass spectrometry. Rapid Commun. Mass Spectrom. 35: e9085. doi:10.1002/rcm.9085

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Conflict of interest

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AA δ15N by GC/C/IRMS, LC × EA/IRMS, and LC × GC/C/IRMS