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A new analytical method for determination of the nitrogen isotopic composition of methionine: Its application to aquatic ecosystems with mixed resources

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Abstract

Compound-specific nitrogen isotope ($\delta^{15}\text{N}$) analysis of amino acids is a powerful tool for estimating the trophic positions (TPs) of animals. The TP of an animal can be represented as a linear function of the isotopic difference between glutamic acid ($\delta^{15}\text{N}_{\text{Glu}}$) and phenylalanine ($\delta^{15}\text{N}_{\text{Phe}}$). However, the method using $\delta^{15}\text{N}_{\text{Glu}}$ and $\delta^{15}\text{N}_{\text{Phe}}$ cannot be applied to animals in mixed food webs where basal resources are derived from both terrestrial and aquatic primary producers, because the mean value of $\delta^{15}\text{N}_{\text{Phe}}$ relative to $\delta^{15}\text{N}_{\text{Glu}}$ differs greatly between terrestrial plants (+8.4‰) and aquatic algae (−3.4‰). To resolve this problem, the $\delta^{15}\text{N}$ of methionine ($\delta^{15}\text{N}_{\text{Met}}$) is useful. Because the C–N bond of methionine is not cleaved in its initial metabolic step, theoretically there should be little diversity in $\delta^{15}\text{N}_{\text{Met}}$ relative to $\delta^{15}\text{N}_{\text{Glu}}$ among primary producers and a small trophic discrimination factor for methionine in animal metabolism. We developed a dual-column-coupled GC-C-IRMS method to determine $\delta^{15}\text{N}_{\text{Met}}$. Data collected from controlled feeding experiments and wild samples demonstrated that the isotopic difference between methionine and phenylalanine in terrestrial food webs ($\Delta_{\text{Met-Phe}} = -16.5 \pm 0.5\text{‰}$) is clearly distinguishable from that in aquatic food webs ($\Delta_{\text{Met-Phe}} = -5.0 \pm 0.5\text{‰}$). This approach allowed us to determine ecologically reasonable TP values for carnivores in a stream food web, which were substantially underestimated with the conventional method. This method has potential utility in assessing TP for animals that rely on varying proportions of both terrestrial- and aquatic-derived resources, with no requirement to characterize $\delta^{15}\text{N}$ in their basal resources.

The trophic positions (TPs) of organisms have been increasingly estimated using compound-specific isotope analysis (CSIA) of nitrogen ($\delta^{15}\text{N}$) in amino acids (AAs) (e.g., McClelland and Montoya 2002; Chikaraishi et al. 2007; McCarthy et al. 2007; Popp et al. 2007; Ogawa et al. 2013; Steffan et al. 2013, 2015). The first step in animal catabolism of “trophic” amino acids such as glutamic acid (Glu) is commonly deamination (a process of transamination), which leads to the enrichment of ^{15}N at each trophic step. The extent of this

isotopic fractionation is called the “trophic discrimination factor”, hereafter referred to as TDF (e.g., $8.0 \pm 1.2\text{‰}$ for TDF_{Glu} ; Chikaraishi et al. 2009). In contrast, the dominant animal catabolism of “source” amino acids such as phenylalanine (Phe) does not start with deamination, resulting in an unchanged TDF (e.g., $0.4 \pm 0.5\text{‰}$ for TDF_{Phe} ; Chikaraishi et al. 2009). Unlike traditional $\delta^{15}\text{N}$ analyses, in which the bulk tissues of organisms are used (e.g., Minagawa and Wada 1984; Peterson and Fry 1987; Vander Zanden et al. 1999), the TP of an animal can be estimated with the following equation, without any characterization of $\delta^{15}\text{N}$ in the basal resources of the food webs studied:

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$$\text{TP}_{\text{Glu/Phe}} = \frac{\delta^{15}\text{N}_{\text{Glu}} - \delta^{15}\text{N}_{\text{Phe}} + \beta_{\text{Glu/Phe}}}{\text{TDF}_{\text{Glu}} - \text{TDF}_{\text{Phe}}} + 1 \quad (1)$$

where $\delta^{15}\text{N}_{\text{Glu}}$ and $\delta^{15}\text{N}_{\text{Phe}}$ are the $\delta^{15}\text{N}$ of Glu and Phe in the focal animal, respectively, and $\beta_{\text{Glu/Phe}}$ is the difference between $\delta^{15}\text{N}_{\text{Phe}}$ and $\delta^{15}\text{N}_{\text{Glu}}$ in a primary producer. The value of $\text{TDF}_{\text{Glu}} - \text{TDF}_{\text{Phe}}$ (hereafter $\text{TDF}_{\text{Glu/Phe}}$) was set at a standard value of 7.6‰ in this study (Chikaraishi et al. 2009), although its universality remains contentious (e.g., McMahon and McCarthy 2016).

However, Eq. 1 cannot simply be applied to the $\text{TP}_{\text{Glu/Phe}}$ estimates for animals in mixed ecosystems, such as streams, rivers, and swamps, where basal resources are potentially derived from both terrestrial and aquatic primary producers. Unlike algae, vascular plants synthesize lignin from the deamination of Phe, which is accompanied by isotopic fractionation (Fig. 1a; Ohkouchi and Takano 2014). Thus, the $\beta_{\text{Glu/Phe}}$ value for terrestrial vascular plants (i.e., $+8.4 \pm 1.6\text{‰}$) is higher than that for aquatic algae, including eukaryotic and prokaryotic photoautotrophs (i.e., $-3.4 \pm 0.9\text{‰}$) (Chikaraishi et al. 2009, 2010a, 2011). Therefore, $\beta_{\text{Glu/Phe}}$ values must be corrected for animals that rely on both terrestrial and aquatic resources, to accurately assess their $\text{TP}_{\text{Glu/Phe}}$ (Hebert et al. 2016; Choi et al. 2017).

Freshwater food webs are fueled by both terrestrial and aquatic primary production, in varying proportions (Pace et al. 2004; Cole et al. 2011). Stable and radioactive carbon isotopes ($\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$, respectively) in bulk tissues and amino acids have been extensively used in freshwater ecosystems to quantify the proportions of terrestrial and aquatic resources (e.g., Wada et al. 1993; Finlay 2001; Larsen et al. 2012, 2013; Ishikawa et al. 2014a, 2016). The $\delta^{15}\text{N}_{\text{Phe}}$ value may also be a useful proxy because it commonly differs between terrestrial and aquatic realms (Naito et al. 2013, 2016a). For instance, Ishikawa et al. (2014b) applied a two-source mixing model using $\delta^{15}\text{N}_{\text{Glu}}$ and $\delta^{15}\text{N}_{\text{Phe}}$ for both the basal resources and animals in stream food webs to estimate their $\text{TP}_{\text{Glu/Phe}}$ values. However, the very large variations in $\delta^{15}\text{N}_{\text{Glu}}$ and $\delta^{15}\text{N}_{\text{Phe}}$ that occur in both terrestrial and aquatic ecosystems frequently hinder the precise quantification of the resources consumed by animals (e.g., Naito et al. 2013). Furthermore, two-source mixing models using $\delta^{15}\text{N}_{\text{Glu}}$ and $\delta^{15}\text{N}_{\text{Phe}}$ always require an extensive isotopic assessment of the dietary sources (Naito et al. 2010), which somewhat diminishes the advantage of the CSIA-AA method.

In this study, we propose that the nitrogen isotopic composition of another source AA, methionine (Met), in combination with that of Phe, is useful in reducing the uncertainty in $\text{TP}_{\text{Glu/Phe}}$ of animals in mixed food webs. In the initial metabolic step, the C–N bond of Met is not cleaved (forming S-adenosylmethionine, Fig. 1b; Chikaraishi et al. 2007; Ohkouchi et al. 2015). Importantly, unlike Phe, which is partly used for lignin biosynthesis (Fig. 1a), the dominant anabolic/catabolic pathway of Met is identical in both terrestrial and aquatic primary producers (Ohkouchi and Takano 2014). Furthermore, Met has the second smallest TDF ($0.5 \pm 0.6\text{‰}$) of all source AAs (Chikaraishi et al. 2009), which is useful for TP estimation. Therefore, the value of $\delta^{15}\text{N}_{\text{Met}}$ minus $\delta^{15}\text{N}_{\text{Phe}}$ (hereafter, $\Delta_{\text{Met-Phe}}$) is expected to: (1) offset the background variation in

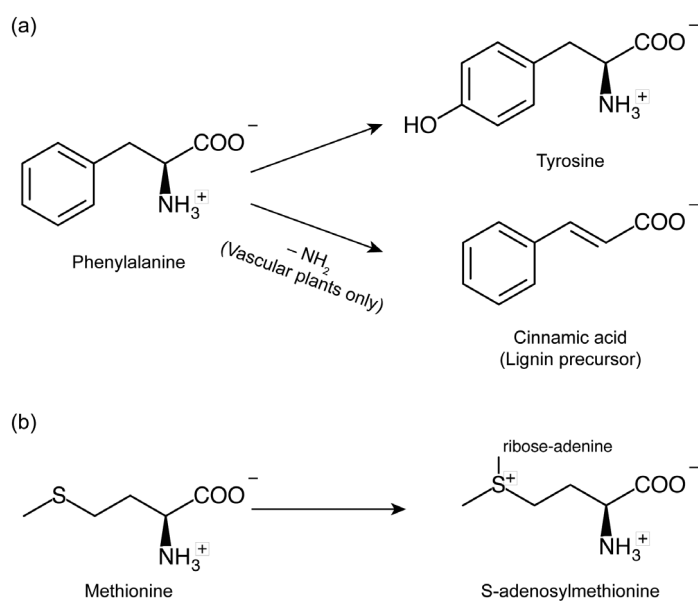


Fig. 1. Principal steps in the predominant catabolism of (a) Phe and (b) Met in autotrophs (after Chikaraishi et al. 2009; Ohkouchi and Takano 2014).

the $\delta^{15}\text{N}$ value in ecosystems, (2) behave conservatively through trophic transfer, and (3) differ between vascular plants and algae, reflecting the former's lignin biosynthesis. These are useful properties for estimating the relative contributions of terrestrial and aquatic primary producers (i.e., vascular plants and algae, respectively) to the basal resources of animals in food webs. It should be noted that aquatic vascular plants (e.g., seagrass), which synthesize lignin, have $\beta_{\text{Glu/Phe}}$ values that are nearly identical to those of terrestrial vascular plants (Vander Zanden et al. 2013; Choi et al. 2017). In this study, we will not attempt to differentiate between aquatic and terrestrial vascular plants.

The terrestrial and aquatic proportions identified by $\Delta_{\text{Met-Phe}}$ values are potentially useful to obtain a weighted estimate for $\beta_{\text{Glu/Phe}}$ in Eq. 1 for an accurate assessment of $\text{TP}_{\text{Glu/Phe}}$ in animals in mixed ecosystems. However, researchers have not used the Met approach, in part because of the technical difficulties in measuring $\delta^{15}\text{N}_{\text{Met}}$ values. Organismal proteins generally contain small amounts of Met relative to other AAs (Butler et al. 1975; Glew et al. 1997). Furthermore, in gas chromatography, Met usually elutes close to other AAs (e.g., serine for the nonpolar column or Glu for the polar column) and impurities. These factors have made it difficult to accurately determine $\delta^{15}\text{N}_{\text{Met}}$ with the conventional CSIA approach (Chikaraishi et al. 2015; McMahon et al. 2015; McMahon and McCarthy 2016). In the present study, we used dual-column coupling in gas chromatography to separate Met from other AAs and potential impurities on the chromatograms. To confirm the accuracy of the measurements, the $\delta^{15}\text{N}_{\text{Met}}$ value determined by this dual-column method was compared with the value obtained from an independent

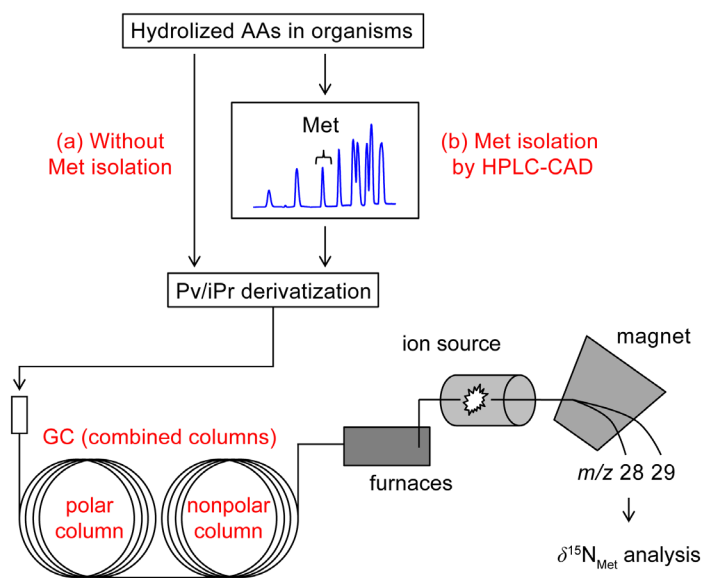


Fig. 2. Schematic diagram of GC-C-IRMS (a) without and (b) with Met isolation using HPLC-CAD for $\delta^{15}\text{N}_{\text{Met}}$ analysis. See also Fig. 4 for representative GC-C-IRMS chromatograms for (a) and (b).

approach; i.e., Met isolation using a high performance liquid chromatograph (HPLC) followed by isotope analysis with a gas chromatograph-combustion-isotope ratio mass spectrometer (GC-C-IRMS) (Fig. 2). We applied this new method to several representative samples from controlled feeding experiments and from farm (i.e., terrestrial), coastal (i.e., aquatic), and stream (i.e., mixed) ecosystems, in the first application of this $\delta^{15}\text{N}_{\text{Met}}$ analysis to food web studies.

Materials and procedures

Controlled laboratory feeding experiments and natural field samples

We reared tadpoles of the Japanese toad *Bufo japonica* by feeding them one of two different diets for 40 d, under the conditions described by Chikaraishi et al. (2015). One group of tadpoles was fed boiled pork ham purchased from markets, and the other group was fed boiled muscle tissue of the coastal gastropod *Omphalius pfeifferi*. We assumed that the pig (pork) had been fed on plant products, such as grasses, cereals, and nuts, probably on a pig farm, whereas *O. pfeifferi* predominantly fed on macroalgae in the ocean. Thus, in the feeding experiments, *B. japonica* fed on either pork or *O. pfeifferi* was considered to represent secondary consumers in terrestrial or aquatic ecosystems, respectively. After rearing, the tails of the tadpoles were collected to minimize possible contamination, cleaned with tap water, and immediately stored at -20°C . Three individuals from each experiment were used for CSIA.

We used 23 organisms collected from terrestrial (farm), aquatic (coastal marine), and mixed terrestrial-aquatic (stream) ecosystems. Seven specimens from the farm food web (leaves of the peach *Amygdalus persica* and of the Japanese hydrangea

Hydrangea macrophylla; the caterpillar *Pieris rapae*; the Japanese katydid *Gampsocleis mikado*; the wasps *Parapolybia indica* and *Polistes rothneyi*; and the hornet *Vespa ducalis*), seven specimens from the coastal marine food web (the seaweed *Ishige okamurae* and the gulfweed *Sargassum fulvellum*; the gastropod *Haliotis discus*; the sponge *Halichondria okadai*; the sea slug *Hypselodoris festiva*; the cardinal fish *Apogon semilineatus*; and the rock fish *Sebastiscus marmoratus*), and nine specimens from the stream food web (larvae of the mayfly Heptageniidae spp.; larvae of the caddisfly *Goerodes* spp. [*Lepidostoma* spp.]; larvae of the stonefly *Kamimuria tibialis*; larvae of the dragonfly Gomphidae spp.; larvae of the dobsonfly *Protohermes grandis*; the goby *Rhinogobius flumineus*; the minnow *Rhynchocypris oxycephalus juyi*; and the trout *Oncorhynchus masou ishikawae*) were selected for analysis. The samples from the farm and coastal marine food webs originated from an orange and vegetable farm, and from a stony shore in Yugawara ($35^\circ08'\text{N}$, $139^\circ07'\text{E}$), Japan, respectively (Chikaraishi et al. 2014; Takizawa et al. 2017). The samples from the stream food web originated from the Ado River ($35^\circ12'\text{N}$, $135^\circ51'\text{E}$), Japan (Ishikawa et al. 2014b).

Preparation of AAs

The AAs were extracted from all the samples with HCl hydrolysis, and then derivatized to *N*-pivaloyl-isopropyl esters (Pv/iPr) with the improved procedures described by Chikaraishi et al. (2010b). In brief, the samples were hydrolyzed with 12 M HCl at 110°C for 12 h. Each hydrolysate was washed with *n*-hexane/dichloromethane (3/2, v/v) to remove any hydrophobic constituents (e.g., lipids). After defatting and drying under an N_2 flow, derivatization was performed sequentially with thionyl chloride/2-propanol (1/4, v/v) and pivaloyl chloride/dichloromethane (1/4, v/v). The Pv/iPr derivatives of the AAs were extracted with *n*-hexane/dichloromethane (3/2, v/v) from the final fraction before GC separation.

To ensure the precision and accuracy of the $\delta^{15}\text{N}_{\text{Met}}$ measurements, we used two independent methods. In the first method, half the underivatized AA fractions from the gastropod samples (i.e., HCl-hydrolyzed soft tissue of *Haliotis discus*) were injected into an HPLC apparatus (Agilent 1260 series, Agilent Technologies, Palo Alto, California, U.S.A.) and separated with the modified method of Takano et al. (2015). We used a Hypercarb column (4.6×150 mm, particle size $5 \mu\text{m}$; Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.) and a guard column (4.6×10 mm, $5 \mu\text{m}$; Thermo Fisher Scientific) with a Cool Pocket column cooler (Thermo Fisher Scientific) stabilized at 10.0°C . The separation of underivatized Met was monitored within the range of 1–100 nmol with a charged aerosol detector (Corona CAD, Thermo Fisher Scientific). After analytical confirmation, the target Met (with partially coeluting isoleucine) was isolated using a fraction collector (Agilent Technologies) with a time-based trigger mode. The isolated Met fraction was dried and finally derivatized as mentioned

above. In the second method, the other half of the *H. discus* samples were derivatized without the HPLC isolation of Met (Fig. 2).

Dual-column coupling

Using a gas chromatograph (GC) (6890N; Agilent Technologies) connected to a mass spectrometer (MS) (5973MSD; Agilent Technologies), several combinations of columns were tested to separate the Met peak from those of other AAs (e.g., the nearest peak to Met was the Glu peak for the coupled columns) and unidentified impurities on the chromatograms at baseline. The Pv/iPr derivatives of the AAs were injected with a programmable temperature vaporizing (PTV) injector (Gerstel, Mülheim, Germany). 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 flow rate of the carrier gas (He) was controlled with a constant flow mode at 1.6 mL min⁻¹. The GC oven temperature was programmed as follows: 40°C (initial temperature) for 4.0 min, increased at 8°C min⁻¹ to 250°C, and held at the final temperature for 19.75 min. The column settings were as follows: (a) single HP-5ms (0.32 mm × 30 m, film thickness 0.25 μm; Agilent Technologies); (b) single HP-INNOWax (0.32 mm × 30 m, film thickness 0.25 μm; Agilent Technologies); (c) dual-column coupling: HP-5ms (30 m) + HP-INNOWax (30 m); (d) dual-column coupling: HP-INNOWax (30 m) + HP-5ms (30 m); and (e) dual-column coupling: HP-INNOWax (15 m) + HP-5ms (30 m) (Fig. 3). During dual-column coupling, the two columns were directly connected with an inner seal connector (GL Sciences, Tokyo, Japan). The combination of HP-INNOWax and HP-5ms was chosen because the former is a polar column and the latter is a nonpolar column, and are therefore useful in separating compounds based on their polarity.

Nitrogen isotope measurements

The $\delta^{15}\text{N}$ value of the derivatized AAs in the samples was determined with IRMS (Delta plus XP, Thermo Fisher Scientific) coupled to a GC (6890N; Agilent Technologies) via a GC-C/TC III interface with combustion (950°C) and reduction (550°C) furnaces (Thermo Fisher Scientific). The Pv/iPr derivatives of the AAs were injected with a PTV injector (Gerstel) into the coupled HP-INNOWax and HP-5ms columns (Figs. 2, 3e). The PTV temperature was programmed as for GC-MS. The carrier gas (He) flow rate was controlled with a constant flow mode at 1.4 mL min⁻¹. The GC oven temperature was programmed as follows: 40°C (initial temperature) for 3.0 min, increasing at 15°C min⁻¹ to 110°C, increasing at 3°C min⁻¹ to 150°C, increasing at 6°C min⁻¹ to 220°C, held at 220°C for 18 min, increasing at 60°C min⁻¹ to 260°C, and held at the final temperature for 5 min. The dual-column coupling method could not be used to determine the $\delta^{15}\text{N}_{\text{Glu}}$ values because Glu coeluted with an unidentified compound

on the chromatogram (Fig. 3e). Therefore, $\delta^{15}\text{N}_{\text{Glu}}$ of the samples was determined with the conventional method using a nonpolar single column (HP-Ultra 2, 0.32 mm × 50 m, film thickness 0.52 μm; Agilent Technologies) equipped for GC-C-IRMS (Chikaraishi et al. 2010b). Isotopic reference mixtures of nine AAs (i.e., alanine, glycine, leucine, norleucine, aspartic acid, methionine, glutamic acid, phenylalanine, and hydroxyproline, with $\delta^{15}\text{N}$ values ranging from -26.6‰ to +45.7‰; Indiana University, Bloomington, Indiana; SI Science, Sugitomachi, Japan) were analyzed between every 5–6 injections to confirm the reproducibility of the isotope measurements. Three and two pulses of the reference N₂ gas were discharged into the IRMS instrument at the beginning and end of each run, respectively. The $\delta^{15}\text{N}$ values of all samples were corrected using the regression line between the published $\delta^{15}\text{N}$ values and the measured $\delta^{15}\text{N}$ values for our internal AA standards. The measurement was performed once per sample, except for the *H. discus* samples. The $\delta^{15}\text{N}$ values for *H. discus* with- and without-isolation of Met averaged results from six and seven injections, respectively. The analytical errors (1 σ) of the standards were smaller than 0.5‰ for samples containing > 0.5 nmol N.

Mixing model

For any given animal, the relative contribution of nitrogen from vascular plants in terrestrial ecosystems ($0 \leq f \leq 1$) was obtained with the following equation:

$$\Delta_{\text{Met-Phe, animal}} = f (\Delta_{\text{Met-Phe, terrestrial}}) + (1 - f) (\Delta_{\text{Met-Phe, aquatic}}) \quad (2)$$

where $\Delta_{\text{Met-Phe, animal}}$, $\Delta_{\text{Met-Phe, terrestrial}}$, and $\Delta_{\text{Met-Phe, aquatic}}$ were the isotopic differences between Met and Phe (i.e., $\Delta_{\text{Met-Phe}} = \delta^{15}\text{N}_{\text{Met}} - \delta^{15}\text{N}_{\text{Phe}}$) of a focal animal, the mean value for plants and consumers in the farm food web ($N = 7$), and the mean value for algae and consumers in the coastal food web ($N = 7$), respectively. For each animal examined, $\beta_{\text{Glu/Phe}}$ in Eq. 1 was defined as follows:

$$\beta_{\text{Glu/Phe}} = f \times 8.4 + (1 - f) \times (-3.4) \quad (3)$$

$\text{TP}_{\text{Glu/Phe}}$ for animals that rely on resources derived from both terrestrial and aquatic primary production was calculated using Eq. 1, with $\beta_{\text{Glu/Phe}}$ replaced by Eq. 3, and was compared with the estimate of $\text{TP}_{\text{Glu/Phe}}$ calculated from Eq. 1 but with a fixed $\beta_{\text{Glu/Phe}}$ value (either +8.4‰ or -3.4‰).

Data analysis

The trophic shift in the $\delta^{15}\text{N}$ values in the controlled feeding experiment was examined with the Wilcoxon rank sum test. The mean $\Delta_{\text{Met-Phe}}$ values and their variations were compared between terrestrial and aquatic food webs with Student's *t*-test and the *F* test, respectively. The significance level was set at $\alpha = 0.05$.

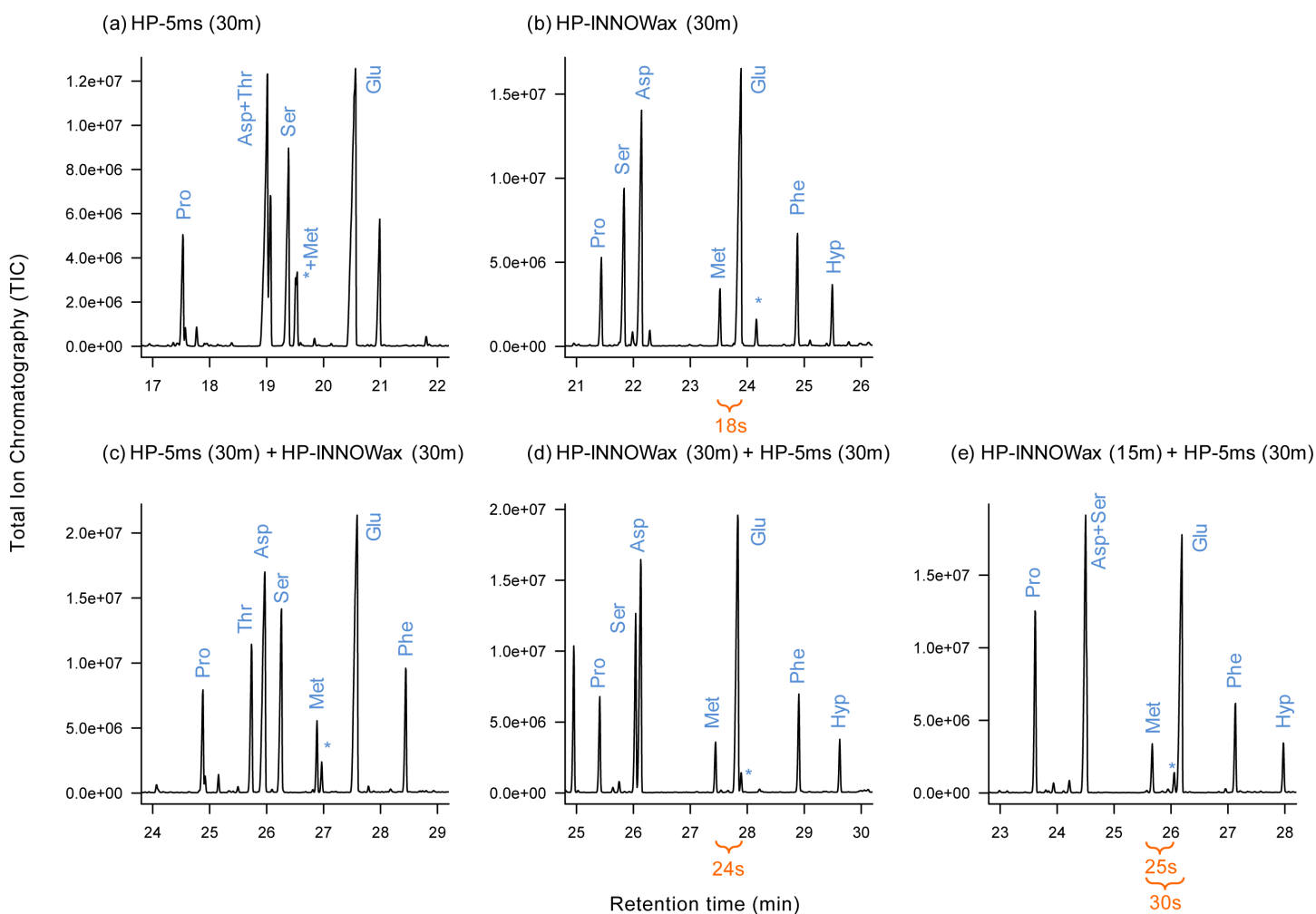


Fig. 3. Representative chromatograms of the Pv/iPr derivatives of AAs in the gastropod *Haliotis discus* with various settings of the GC column. (a) and (b) are nonpolar- and polar-single column settings, respectively. (c), (d), and (e) are combined column settings. Setting (e) offered the best separation (i.e., the longest interval between retention times) of the Met peak from Glu and impurities. Asterisks denote unidentified compounds. Asp: aspartic acid; Hyp: hydroxyproline; Pro: proline; Ser: serine; Thr: threonine.

Assessment

GC chromatograms

A single compound peak with baseline separation on the chromatogram must be obtained for the accurate analysis of $\delta^{15}\text{N}_{\text{AA}}$ with GC-C-IRMS (e.g., Sessions 2006). As shown in Fig. 3, with column settings (a) and (c), Met coeluted with an unidentified compound (indicated with an asterisk), although setting (a) has frequently been used in CISA of AAs (e.g., Chikaraishi et al. 2010b). In contrast, with column settings (b), (d), and (e), Met eluted separately from both the unidentified compound and Glu (peak intervals: 18, 24, and 30 s, respectively). In our experiments, the coupling of the HP-INNOWax (15 m) and HP-5ms (30 m) columns achieved the best separation of the Met peak from its neighboring peaks (Fig. 3e), which was never found on any single column method with various conditions in the oven temperature and

He flow rate. Using this combined column, the baseline separation of Met and the subsequently eluted Glu on the GC-C-IRMS chromatogram was clear, although they still eluted very closely (peak interval < 1 min) (Fig. 4), and the $\delta^{15}\text{N}_{\text{Met}}$ value for the gastropod without HPLC isolation of Met ($-1.6 \pm 0.4\text{‰}$, $N = 7$) was identical to that with HPLC isolation ($-1.6 \pm 0.4\text{‰}$, $N = 6$). Therefore, a 30 s interval between the Met and Glu peaks on the GC-MS chromatogram (Fig. 3e) is required for the isotope analysis. These results indicated that (1) the peak intervals shorter than ~ 30 s observed in Fig. 3a–d did not allow the separation of the Met and Glu peaks on the GC-C-IRMS chromatogram; and (2) dual-column-coupled chromatography provides a readily available method for the analysis of $\delta^{15}\text{N}_{\text{Met}}$ in biological samples. We note that isoleucine (Ile) coeluted with Met on HPLC, but that these two AAs were clearly separated with GC-C-IRMS (Fig. 4).

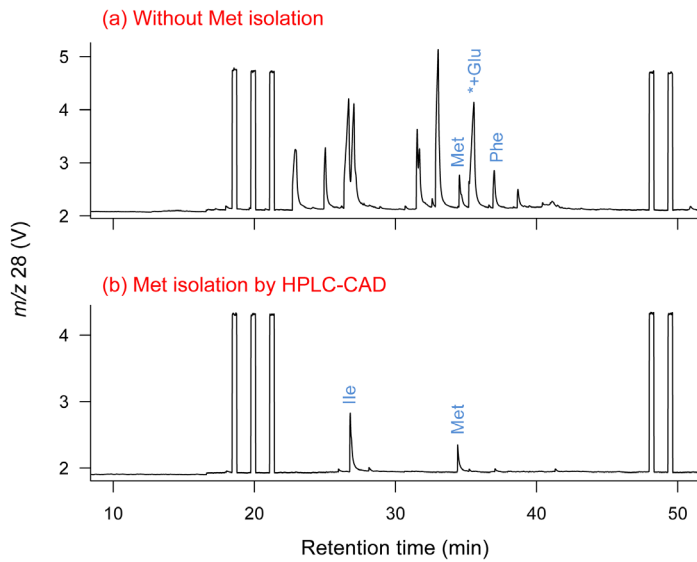


Fig. 4. Representative chromatograms (specimen: gastropod *Haliotis discus*) of dual-column-coupled GC-C-IRMS using combined HP-INNOWax and HP-5ms columns (**a**) without Met isolation, and (**b**) after Met isolation with HPLC-CAD. Asterisk denotes unidentified compound(s).

Controlled feeding experiments

The $\delta^{15}\text{N}_{\text{Phe}}$ values for the tadpole *B. japonica* (+8.5‰ to +9.5‰ for the pork-fed group and +4.5‰ to +5.1‰ for the gastropod-fed group) did not differ significantly from those for their diets (+8.7‰ to +9.3‰ for pork and +4.6‰ to +4.8‰ for gastropod) (Wilcoxon rank sum test, $W > 3$, $p > 0.7$) (Table 1). These results are consistent with expectations based on the reported TDF_{Phe} values ($0.4 \pm 0.5\%$, Chikaraishi et al. 2009). Similarly, the $\delta^{15}\text{N}_{\text{Met}}$ values for tadpoles (−5.7‰ to −5.0‰ for the pork-fed group and −1.2‰ to −0.7‰ for the gastropod-fed group) did not differ significantly from those for their diets (−5.8‰ to −4.9‰ for pork and −2.0‰ to −1.1‰ for gastropod) (Wilcoxon rank sum test, $W > 1$, $p > 0.2$) (Table 1). These deviations fell within 2σ of the reported TDF_{Met} ($0.5 \pm 0.6\%$, Chikaraishi et al. 2009). The $\Delta_{\text{Met-Phe}}$ value for pork in the feeding experiment ($-14.5 \pm 0.4\%$) differed slightly from the values for organisms in the farm food web (−17.2‰ to −15.9‰) (Table 2), perhaps because the artificial diets given to the pigs may have contained aquatic protein. Using Eqs. 2 and 3, the $\Delta_{\text{Met-Phe}}$ values for pork, *B. japonica* fed on pork, gastropod, and *B. japonica* fed on gastropod resulted in corrected $\beta_{\text{Glu/Phe}}$

Table 1. $\delta^{15}\text{N}$ (‰) of methionine (Met), glutamic acid (Glu), and phenylalanine (Phe), the fraction of terrestrial nitrogen (f), and $\text{TP}_{\text{Glu/Phe}}$ ($\beta_{\text{Glu/Phe}}$ values of +8.4‰ and −3.4‰, and the value corrected by f) in samples from the controlled feeding experiment. Means and standard deviations (SDs) within each group ($N = 3$) are also shown.

		$\delta^{15}\text{N}$ (‰)				$\text{TP}_{\text{Glu/Phe}}$			
		Met	Glu	Phe	$\Delta_{\text{Met-Phe}}$	f	$\beta_{\text{Glu/Phe}} = +8.4\%$	$\beta_{\text{Glu/Phe}} = -3.4\%$	Corrected
<i>Feeding experiment</i>									
Pork		−4.9	8.2	9.3	−14.2	0.80	2.0		1.7
		−5.8	8.7	8.7	−14.5	0.83	2.1		1.8
		−5.2	9.1	9.1	−14.3	0.81	2.1		1.8
Mean (SD)		−5.2 (0.5)	8.7 (0.5)	9.0 (0.3)	−14.3 (0.1)	0.81 (0.01)	2.1 (0.09)		1.8 (0.10)
Tadpole fed on pork		−5.7	17.7	9.2	−14.9	0.86	3.2		3.0
<i>Bufo</i>									
<i>japonica</i>		−5.3	16.9	8.5	−13.9	0.77	3.2		2.9
		−5.0	17.0	9.5	−14.5	0.82	3.1		2.8
Mean (SD)		−5.4 (0.4)	17.2 (0.5)	9.1 (0.5)	−14.4 (0.5)	0.82 (0.05)	3.2 (0.07)		2.9 (0.10)
Gastropod		−1.6	14.8	4.6	−6.1	0.10		1.9	2.1
<i>Omphalius pfeifferi</i>									
		−1.1	14.7	4.8	−5.9	0.08		1.9	2.0
		−2.0	14.1	4.6	−6.5	0.14		1.8	2.0
Mean (SD)		−1.6 (0.4)	14.5 (0.4)	4.6 (0.1)	−6.2 (0.3)	0.11 (0.03)		1.9 (0.04)	2.0 (0.04)
Tadpole fed on gastropod		−0.7	23.5	4.8	−5.5	0.04		3.0	3.1
<i>Bufo</i>									
<i>japonica</i>		−0.9	23.3	5.1	−6.0	0.09		2.9	3.1
		−1.2	22.4	4.5	−5.7	0.06		2.9	3.0
Mean (SD)		−0.9 (0.2)	23.0 (0.6)	4.8 (0.3)	−5.7 (0.3)	0.06 (0.02)		3.0 (0.1)	3.0 (0.05)

Table 2. $\delta^{15}\text{N}$ (‰) of methionine (Met), glutamic acid (Glu), and phenylalanine (Phe), the fraction of terrestrial nitrogen (f), and $\text{TP}_{\text{Glu/Phe}}$ ($\beta_{\text{Glu/Phe}}$ values of +8.4‰ and -3.4‰, and the value corrected by f) in samples from farm and coastal food webs.

		$\delta^{15}\text{N}$ (‰)				$\text{TP}_{\text{Glu/Phe}}$			
		Met	Glu	Phe	$\Delta_{\text{Met-Phe}}$	f	$\beta_{\text{Glu/Phe}} = +8.4\text{‰}$	$\beta_{\text{Glu/Phe}} = -3.4\text{‰}$	Corrected
<i>Farm food web</i>									
Peach*	<i>Amygdalus persica</i>	-21.4	-13.1	-5.0	-16.3	0.99	1.0		1.0
Japanese hydrangea*	<i>Hydrangea macrophylla</i>	-9.7	-1.0	7.5	-17.2	1.06	1.0		1.1
Caterpillar*	<i>Pieris rapae</i>	-7.5	10.6	9.5	-17.1	1.05	2.2		2.3
Japanese katydid*	<i>Gampsocleis mikado</i>	-11.4	8.8	4.9	-16.3	0.99	2.6		2.6
Wasp*	<i>Parapolybia indica</i>	-10.6	12.2	5.9	-16.5	1.00	2.9		2.9
Wasp*	<i>Polistes rothneyi</i>	-7.6	15.9	8.4	-15.9	0.95	3.1		3.0
Hornet*	<i>Vespa ducalis</i>	-8.3	21.4	7.7	-16.0	0.96	3.9		3.9
<i>Coastal food web</i>									
Seaweed	<i>Ishige okamurae</i>	-0.4	8.3	4.9	-5.3	0.02		1.0	1.0
Gulfweed	<i>Sargassum fulvellum</i>	1.3	9.5	6.4	-5.1	0.01		1.0	1.0
Gastropod*	<i>Haliotis discus</i>	-1.6	13.2	4.3	-5.9	0.08		1.7	1.8
Sponge	<i>Halichondria okadai</i>	-1.1	18.1	3.6	-4.8	-0.02		2.5	2.4
Sea slug	<i>Hypselodoris festiva</i>	-0.1	26.1	4.4	-4.5	-0.05		3.4	3.3
Cardinal fish*	<i>Apogon semilineatus</i>	0.9	29.1	6.1	-5.2	0.02		3.6	3.6
Rock fish*	<i>Sebastes marmoratus</i>	1.5	32.1	5.8	-4.3	-0.06		4.0	3.9

* $\delta^{15}\text{N}_{\text{Glu}}$ and $\delta^{15}\text{N}_{\text{Phe}}$ data are from Chikaraishi et al. (2014) or Takizawa et al. (2017).

values to $+6.2 \pm 0.1\text{‰}$, $+6.3 \pm 0.5\text{‰}$, $-2.2 \pm 0.3\text{‰}$, and $-2.7 \pm 0.3\text{‰}$, respectively. Entering these animal-specific $\beta_{\text{Glu/Phe}}$ values in Eq. 1 resulted in $\text{TP}_{\text{Glu/Phe}}$ values of 1.8 ± 0.10 , 2.9 ± 0.10 , 2.0 ± 0.04 , and 3.0 ± 0.05 for pork, gastropod, *B. japonica* fed on pork, and *B. japonica* fed on gastropod, respectively (Fig. 5a).

Farm and coastal food webs

The variations in $\delta^{15}\text{N}_{\text{Met}}$ and $\delta^{15}\text{N}_{\text{Phe}}$ were remarkably large for the organisms in the farm food web (-21.4‰ to -7.5‰ and -5.0‰ to $+9.5\text{‰}$, respectively, $N = 7$), but relatively small for the organisms in the coastal food web (-1.6‰ to $+1.5\text{‰}$ and $+3.6\text{‰}$ to $+6.4\text{‰}$, respectively, $N = 7$) (Table 2). Similar variability was reported in previous studies, and was explained by the degree of heterogeneity in the abundance and $\delta^{15}\text{N}$ of the nitrogen substrates (NH_3^+ , NO_3^- , and N_2) among different environments (Chikaraishi et al. 2014; Takizawa et al. 2017). Nevertheless, the $\Delta_{\text{Met-Phe}}$ range in the farm food web (i.e., $\Delta_{\text{Met-Phe, terrestrial}} = -16.5 \pm 0.5\text{‰}$, $N = 7$) differed significantly (Student's t -test, $t = -42.3$, $df = 12$, $p < 0.001$) from that in the coastal food web (i.e., $\Delta_{\text{Met-Phe, aquatic}} = -5.0 \pm 0.5\text{‰}$, $N = 7$), and both showed small and similar internal variations within their food webs (F test, $F = 0.82$, $p = 0.81$, Table 2). We use these $\Delta_{\text{Met-Phe}}$ values in the following discussion to estimate the relative contributions of terrestrial and aquatic resources to the organisms studied. The f values calculated with Eq. 2 (i.e., the contribution of terrestrial plants to the animals' basal resources, $0 \leq f \leq 1$) were

1.0 ± 0.04 and 0.0 ± 0.05 for the farm and coastal food webs, respectively. The error (1σ) for the f values (0.04–0.05) resulted in an error of 0.06–0.08 (1σ) for the $\text{TP}_{\text{Glu/Phe}}$ estimates, which is probably attributable to the variation in $\Delta_{\text{Met-Phe}}$ among the wild organisms and the analytical error in the $\delta^{15}\text{N}$ measurements. The $\text{TP}_{\text{Glu/Phe}}$ values corrected with the animal-specific $\beta_{\text{Glu/Phe}}$ values were 1.0–1.1 for primary producers (plant leaves and algae), 1.8–2.3 for primary consumers (caterpillar and gastropod), 2.4–3.3 for intermediate consumers (katydid, wasps, sponge, and sea slug), and 3.6–3.9 for top predators (hornet and fish). The $\text{TP}_{\text{Glu/Phe}}$ values had a consistent linear regression slope (+1.3 for both food webs) with $\Delta_{\text{Met-Phe}}$ (Table 2; Fig. 5b).

Stream food web

The animals inhabiting stream ecosystems (insect larvae and fish) displayed a large variation in $\Delta_{\text{Met-Phe}}$ (-11.2‰ to -5.7‰ , $N = 9$) that fell within the ranges of $\Delta_{\text{Met-Phe}}$ for terrestrial and aquatic organisms (Table 3; Fig. 5b). The larvae of an algal grazing mayfly showed relatively high $\Delta_{\text{Met-Phe}}$ values (i.e., low f values: 0.06 and 0.21), whereas the larvae of the leaf-shredding caddisfly and other predatory insects and fish showed relatively low $\Delta_{\text{Met-Phe}}$ values (i.e., high f values: 0.44–0.67). Based on Eqs. 2 and 3, the corrected $\text{TP}_{\text{Glu/Phe}}$ values for stream animals increased by 0.1–1.0 relative to the uncorrected $\text{TP}_{\text{Glu/Phe}}$, when the algal $\beta_{\text{Glu/Phe}}$ value (i.e., -3.4‰) was used (Table 3). In particular, the corrected $\text{TP}_{\text{Glu/Phe}}$ values were ~ 3.0 for macroinvertebrate predators,

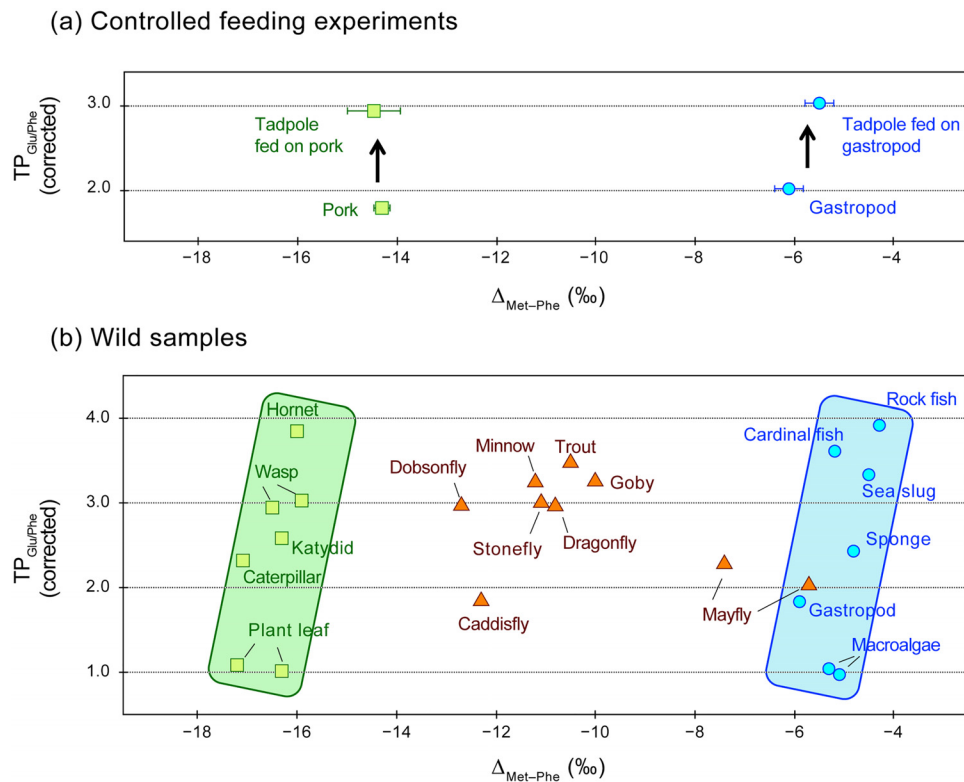


Fig. 5. Plot of $\text{TP}_{\text{Glu/Phe}}$ and $\Delta_{\text{Met-Phe}}$ in (a) controlled feeding experiments, and (b) wild samples. Green squares, orange triangles, and blue circles denote farm (surrounded by a green rectangle), stream, and coastal food webs (surrounded by a blue rectangle), respectively. Means and standard deviations ($N = 3$) are shown for tadpoles, pork, and gastropod.

Table 3. $\delta^{15}\text{N}$ (‰) of methionine (Met), glutamic acid (Glu), and phenylalanine (Phe), the fraction of terrestrial nitrogen (f), and $\text{TP}_{\text{Glu/Phe}}$ ($\beta_{\text{Glu/Phe}}$ values of +8.4‰ and -3.4‰, and the value corrected by f) in samples from the stream food web.

		$\delta^{15}\text{N}$ (‰)				$\text{TP}_{\text{Glu/Phe}}$			
		Met	Glu*	Phe*	$\Delta_{\text{Met-Phe}}$	f	$\beta_{\text{Glu/Phe}} = +8.4\text{‰}$	$\beta_{\text{Glu/Phe}} = -3.4\text{‰}$	Corrected
<i>Stream food web</i>									
Mayfly ^G	Heptageniidae spp.	-8.7	9.4	-1.3	-7.4	0.21	3.5	2.0	2.3
Mayfly ^G	Heptageniidae spp.	-4.6	11.6	1.1	-5.7	0.06	3.5	1.9	2.0
Caddisfly ^S	Goerodes spp. (<i>Lepidostoma</i> spp.)	-6.9	7.7	5.4	-12.3	0.64	2.4	0.9	1.9
Stonefly ^P	<i>Kamimuria tibialis</i>	-10.1	13.5	1.0	-11.1	0.53	3.7	2.2	3.0
Dragonfly ^P	Gomphidae spp.	-9.5	13.6	1.3	-10.8	0.51	3.7	2.2	3.0
Dobsonfly ^P	<i>Protohermes grandis</i>	-12.9	10.4	-0.2	-12.7	0.67	3.5	1.9	3.0
Goby	<i>Rhinogobius flumineus</i>	-10.0	15.4	0.1	-10.0	0.44	4.1	2.6	3.3
Minnow	<i>Rhynchocypris oxycephalus jouyi</i>	-9.0	16.4	2.2	-11.2	0.54	4.0	2.4	3.3
Trout	<i>Oncorhynchus masou ishikawae</i>	-10.0	17.1	0.5	-10.5	0.48	4.3	2.7	3.5

G, algal grazer; S, leaf shredder; P, predator; the categorization is based on Ishikawa et al. (2014b).

* $\delta^{15}\text{N}_{\text{Glu}}$ and $\delta^{15}\text{N}_{\text{Phe}}$ data are from Ishikawa et al. (2014b).

such as the larvae of the dobsonfly, dragonfly, and stonefly, and 3.3–3.5 for fish, whereas their uncorrected $\text{TP}_{\text{Glu/Phe}}$ values were < 3 when the $\beta_{\text{Glu/Phe}}$ value was set at -3.4‰ (Table 3; Fig. 5b).

Discussion

Dual-column coupled GC-C-IRMS successfully determined $\delta^{15}\text{N}_{\text{Met}}$, which was confirmed by the consistent $\delta^{15}\text{N}_{\text{Met}}$ values for the on-line and off-line analyses (Fig. 4). Many

previous CSIA studies have not reported $\delta^{15}\text{N}_{\text{Met}}$ values because the abundance of Met in biological samples is low, and/or the chromatographic separation of Met from other compounds is difficult (Chikaraishi et al. 2009; McMahon et al. 2015; McMahon and McCarthy 2016). On the other hand, a few studies reported $\delta^{15}\text{N}_{\text{Met}}$ values using a low-polar single column (e.g., Schmidt et al. 2004; Chikaraishi et al. 2007, 2009). However, $\delta^{15}\text{N}_{\text{Met}}$ values measured using a single-column method may not be simply comparable to those in the present study using the dual-column coupling method, because the former is potentially contaminated with impurities.

Animals' $\Delta_{\text{Met-Phe}}$ values are theoretically identical to those of their diets because both Met and Phe are "source" AAs (Ohkouchi et al. 2015). Furthermore, the differences in $\Delta_{\text{Met-Phe}}$ values between terrestrial and aquatic ecosystems indicate that $\Delta_{\text{Met-Phe}}$ (1) offsets background variations (i.e., inorganic nitrogen sources) in the $\delta^{15}\text{N}$ value; (2) is highly independent of the trophic isotopic discrimination in organisms; and (3) clearly distinguishes terrestrial and aquatic food webs.

Both terrestrial and aquatic resources contribute to stream food webs in varying proportions. Most stream animals displayed high f values (i.e., 0.44–0.67) in the present study, which is somewhat inconsistent with a mixing model based solely on $\delta^{15}\text{N}_{\text{Glu}}$ and $\delta^{15}\text{N}_{\text{Phe}}$ values ($f < 0.3$; Ishikawa et al. 2014b). However, such mixing models are sensitive to heterogeneity in the $\delta^{15}\text{N}_{\text{Phe}}$ values of primary producers. Indeed, the $\delta^{15}\text{N}_{\text{Phe}}$ values varied by over 5‰ for primary producers such as periphyton, even in a single stream reach (Ishikawa et al. 2014b), reflecting seasonal variability in the nitrate concentration and its $\delta^{15}\text{N}$ (Ohte et al. 2010). This large variability in $\delta^{15}\text{N}_{\text{Phe}}$ value leads to a less precise estimate of the f values of stream animals. In contrast, such variability is offset when $\Delta_{\text{Met-Phe}}$ is used to estimate f values. This provides a key advantage in quantifying the proportions of terrestrial and aquatic nitrogen resources in a food web, and for estimating the $\text{TP}_{\text{Glu/Phe}}$ values of animals.

High f values for some stream animals result in remarkable changes in their $\text{TP}_{\text{Glu/Phe}}$ estimates. For example, the corrected $\text{TP}_{\text{Glu/Phe}}$ for the dobsonfly *P. grandis* larva (3.0) indicated that it is a carnivore, whereas its uncorrected (i.e., $\beta_{\text{Glu/Phe}}$ is set at -3.4 ‰) $\text{TP}_{\text{Glu/Phe}}$ (1.9) undoubtedly indicated that it is an algivore (Table 3). Given that the *P. grandis* larva is a predator (Hayashi 1988), its ecologically unrealistic $\text{TP}_{\text{Glu/Phe}}$ value could be explained by the following three hypotheses: (1) low $\text{TDF}_{\text{Glu/Phe}}$; (2) an effect of starvation; and/or (3) an incorrect $\beta_{\text{Glu/Phe}}$ value. Although $\text{TDF}_{\text{Glu/Phe}}$ can potentially vary for a variety of diet-animal combinations (Chikaraishi et al. 2015; McMahon et al. 2015; Nielsen et al. 2015; McMahon and McCarthy 2016), Ishikawa et al. (2017) reported that the $\text{TDF}_{\text{Glu/Phe}}$ value for *P. grandis* larvae is nearly constant, even under conditions of satiation (7.1 ± 0.5 ‰) and starvation (7.3 ± 0.5 ‰) in a controlled feeding experiment. These results are inconsistent with hypotheses (1) and (2), but

support hypothesis (3) described above. It should be noted that the $\text{TDF}_{\text{Glu/Phe}}$ values for other stream animals examined in the present study are not known. However, the corrected $\text{TP}_{\text{Glu/Phe}}$ values for the insect larvae (1.9–3.0) and the fish (3.3–3.5) are consistent with their respective feeding guilds (i.e., algivores, detritivores, omnivores, or carnivores for insect larvae; and carnivores for fish) in stream food webs, whereas the uncorrected $\text{TP}_{\text{Glu/Phe}}$ values for carnivores tend to underestimate their ecologically reasonable TPs (e.g., Vander Zanden et al. 1997; Takemon 2005).

Overall, we conclude that the dual-column coupling method works for precise determination of $\delta^{15}\text{N}_{\text{Met}}$, and that this improvement helps us identify both $\text{TP}_{\text{Glu/Phe}}$ and resource use in mixed food webs.

Comments and recommendations

Methionine is generally less abundant than other AAs, such as Glu and Phe, in many organisms and their tissues. In particular, the Met concentration is extremely low in terrestrial plants ($\sim 1\%$ of total protein; Glew et al. 1997), fish scales ($\sim 1\%$; Nagai et al. 2004), and bone collagen ($\sim 1\%$; Butler et al. 1975). In such cases, even our dual-column coupling method is unable to determine $\delta^{15}\text{N}_{\text{Met}}$. Increasing the amount of AAs injected during GC-C-IRMS does not improve detection, as nearby peaks (Met and Glu) coelute and are indistinguishable on the chromatograms. The Glu peak and the level of background impurities must be reduced by with open-column chromatography before GC injection (Takano et al. 2010) or by isolating Met with HPLC followed by EA-IRMS (Broek and McCarthy 2014; Takano et al. 2015) to improve the analytical stability and sensitivity of the $\delta^{15}\text{N}_{\text{Met}}$ analysis. Alternatively, the $\beta_{\text{Glu/Phe}}$ value must be corrected with an independent approach (e.g., using fatty acid profiles; Hebert et al. 2016).

In stream food webs, the leaf-shredding caddisfly larva is thought to be a terrestrial (i.e., vascular)-plant feeder, based on the analysis of its gut contents (Takemon 2005). The $\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$ values also suggest that the carbon source of leaf shredders is derived from terrestrial resources (Finlay 2001; Ishikawa et al. 2016). However, in the present study, the f value for the shredder *Goerodes* spp. (*Lepidostoma* spp.) was lower than expected (i.e., 0.64), suggesting that one-third of the nitrogen in *Goerodes* spp. is derived from aquatic (i.e., algal) resources (Table 3; Fig. 5b). A possible explanation of this observation is that in streams, the leaf feeder assimilates nitrogen that is not only derived from high-C/N terrestrial plants, but also from other sources, such as microalgae attached to the leaf (Hall et al. 2000; Cross et al. 2003). In other words, the leaf feeder may assimilate carbon partly by digesting cellulose from leaf litter (Sinsabaugh et al. 1985) and nitrogen mainly by digesting protein from leaf-attached microalgae. If animals and their diets have very different stoichiometry (e.g., C/N), the use of different elements (e.g., bulk $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) for both the

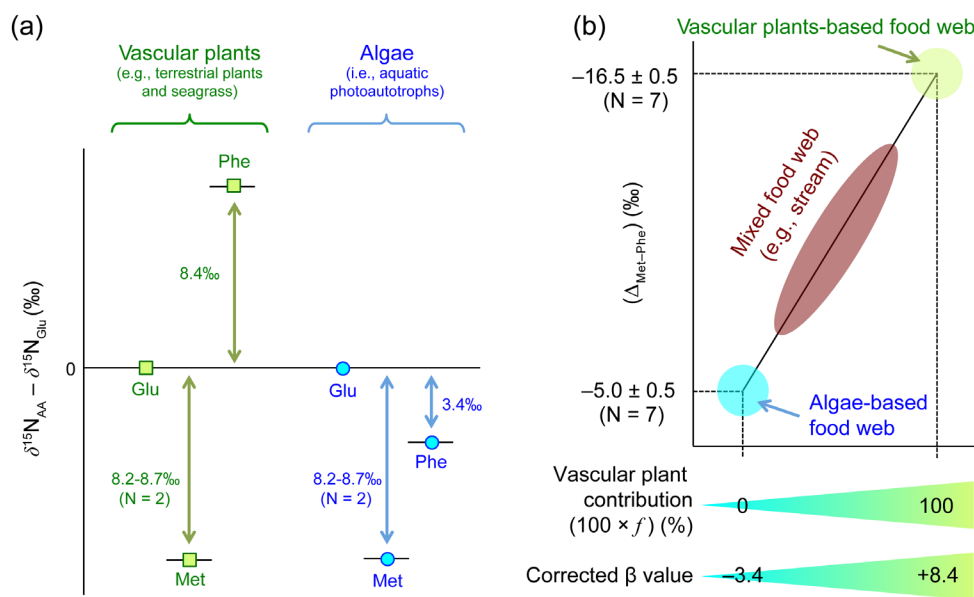


Fig. 6. (a) Relationships between $\delta^{15}\text{N}$ (normalized to the value of Glu) of Met and Phe in vascular plants and algae. (b) Two end-member mixing model using $\Delta_{\text{Met-Phe}}$ to estimate the nitrogen contribution to animals from vascular plants, and their corrected $\beta_{\text{Glu/Phe}}$ values in vascular-plant-based, alga-based, and mixed food webs.

mixing model and TP estimation is problematic (Gannes et al. 1997; Post 2002). This “routing effect” can be reduced by weighting the mixing proportion with the carbon and nitrogen concentrations of each resource (Phillips and Koch 2002). However, bulk $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analyses record mixed effects of different elements (e.g., carbon vs. nitrogen), compounds (e.g., carbon hydrate vs. amino acid), and tissues (e.g., muscle vs. liver) in the organism, all of which show unique turnover times ranging from days to years (Vander Zanden et al. 2015). On the other hand, $\delta^{15}\text{N}$ in most AAs shows similar turnover times (monthly scale) within organisms (Braun et al. 2014). Therefore, the use of $\delta^{15}\text{N}$ of AAs, possibly in combination with $\delta^{13}\text{C}$ of AAs (e.g., Larsen et al. 2013), is less sensitive to this routing effect and is useful for the simultaneous assessment of resource proportions and the TPs of animals in the mixed food webs (Ishikawa 2018).

In ocean ecosystems, the $\beta_{\text{Glu/Phe}}$ value of seagrass is close to +8.4‰, and this differs considerably from the algal value of -3.4‰, because the seagrass is a vascular plant (Vander Zanden et al. 2013; Choi et al. 2017). This is a potential pitfall that could lead to erroneous low $\text{TP}_{\text{Glu/Phe}}$ values for sea turtles and polychaetes, because these animals rely directly or indirectly on the production of seagrass (Arthur et al. 2014; Choi et al. 2017). Moreover, the contribution of seagrass to sea turtles and polychaetes will be passed on to their predators via trophic transfer, thereby increasing the uncertainty of $\text{TP}_{\text{Glu/Phe}}$ estimates in ocean food webs. The $\Delta_{\text{Met-Phe}}$ value cannot distinguish between seagrass and terrestrial vascular plants, but instead will be useful in quantifying resource inputs from both seagrass and sea algae to sea turtles and polychaetes, and will allow accurate estimates of their $\text{TP}_{\text{Glu/Phe}}$ values.

$\delta^{15}\text{N}_{\text{Met}}$ is also useful in archeological studies, allowing the quantification of dietary proteins from a variety of resources (e.g., Naito et al. 2016a). Few previous archeological studies have reported $\delta^{15}\text{N}_{\text{Met}}$ values, because bone collagen has relatively small amounts of Met compared with other AAs. Reconstructing the utilization of Met from aquatic resources by ancient human communities is difficult because of the large variation in $\delta^{15}\text{N}_{\text{Phe}}$ generally observed in their potential diets (Schwartz-Narbonne et al. 2015; Naito et al. 2016b). Further methodological refinement of the determination of the $\delta^{15}\text{N}_{\text{Met}}$ value is required to provide a robust estimate of the utilization of aquatic resources by the ancient humans.

Without considering the mixing proportions and calculating the specimen-specific $\beta_{\text{Glu/Phe}}$ values, as shown in Eq. 1, the conventional CSIA method may under- or overestimate $\text{TP}_{\text{Glu/Phe}}$ of animals, particularly in freshwater or seagrass meadow ecosystems. The $\Delta_{\text{Met-Phe}}$ value offers a unique fingerprint that can distinguish terrestrial and aquatic resources in freshwater ecosystems, or seagrass and algae in seagrass meadow ecosystems (Fig. 6). However, this approach is based on the assumption that Met and Phe are transferred in the same fashion to the focal animal from vascular plants and aquatic algae, although some symbiotic bacteria recycle Met in their metabolic processes (Sekowska et al. 2004). Therefore, this assumption must be examined critically to fully validate the utility of $\delta^{15}\text{N}_{\text{Met}}$ in the analysis of mixed food webs.

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Conflict of Interest

None declared.

GLOSSARY

AA	amino acid
$\beta_{\text{Glu/Phe}}$	mean difference in the $\delta^{15}\text{N}$ values of glutamic acid and phenylalanine in primary producers ($+8.4 \pm 1.6\text{‰}$ for vascular plants; $-3.4 \pm 0.9\text{‰}$ for eukaryotic and prokaryotic photoautotrophs; Chikaraishi et al. 2009, 2010a, 2011)
CAD	charged aerosol detector
CSIA	compound-specific isotope analysis
$\delta^{15}\text{N}_{\text{AA}}$	$\delta^{15}\text{N}$ of amino acid (‰)
$\delta^{15}\text{N}_{\text{Glu}}$	$\delta^{15}\text{N}$ of glutamic acid (‰)
$\delta^{15}\text{N}_{\text{Met}}$	$\delta^{15}\text{N}$ of methionine (‰)
$\delta^{15}\text{N}_{\text{Phe}}$	$\delta^{15}\text{N}$ of phenylalanine (‰)
$\Delta_{\text{Met-Phe}}$	difference in the $\delta^{15}\text{N}$ values of methionine and phenylalanine
f	contribution to animals of AAs derived from vascular plants ($0 \leq f \leq 1$)
GC-C-IRMS	gas chromatograph-combustion-isotope ratio mass spectrometer
Glu	glutamic acid
HPLC	high performance liquid chromatograph
Met	methionine
Phe	phenylalanine
PTV	programmable temperature vaporizing
Pv/iPr	<i>N</i> -pivaloyl/isopropyl
TDF	trophic discrimination factor
TDF_{Glu}	TDF of Glu
TDF_{Met}	TDF of Met
TDF_{Phe}	TDF of Phe
$\text{TDF}_{\text{Glu/Phe}}$	TDF_{Glu} minus TDF_{Phe}
TP	trophic position
$\text{TP}_{\text{Glu/Phe}}$	TP calculated from $\delta^{15}\text{N}_{\text{Glu}}$ and $\delta^{15}\text{N}_{\text{Phe}}$ values (see Eqs. 1–3)

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