Errors associated with compound specific $\delta^{15}N$ analysis of amino acids in preserved fish samples purified by high pressure liquid chromatography

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Abstract

During the past decade compound specific nitrogen (N) isotopic analysis of amino acids (CSIA-AA) has become an increasingly used method for tracking the origin and fate of N in ecological and biogeochemical studies. CSIA-AA has the potential for resolving finer scale trophic dynamics than previously possible with bulk stable isotope analysis (SIA) and for reconstructing past food webs using historical archives of organismal samples. However, there is little information on the effects of chemical preservation used in historical archives on $\delta^{15}N_{AA}$ values, and conventional CSIA conducted on derivatized AAs using gas chromatography – combustion – isotope ratio mass spectrometry (GC-C-IRMS) has analytical errors in the range of what may be expected from chemical preservation. Here we present analytical errors across 11 underivatized AA standards analyzed by high pressure liquid chromatography followed by offline elemental analysis – IRMS (HPLC/EA-IRMS) an approach originally developed by Broek and McCarthy (2014). Using this method, we test the effects of ethanol and formaldehyde preservation (1½ and 27 years) on $\delta^{15}N_{AA}$ in Northern Anchovy (*Engraulis mordax*). We found minimal isotopic fractionation from the HPLC/EA-IRMS approach in 8 AAs and more than twice the precision (0.15 ± 0.08 ‰) typically reported for GC-C-IRMS. Preservation effects on $\delta^{15}N_{AA}$ were similar regardless of duration and type of preservative used. Although several AAs differed significantly from frozen control samples (average +1.0 ± 0.8 ‰), changes in trophic position (TP) estimates were insignificant. These results are encouraging for resolving the fine-scale natural variability expected in most low TP organisms via high precision HPLC/EA-IRMS and for the use of chemically preserved sample archives in reconstructing biogeochemical records and trophic dynamics over long time scales.

Introduction

Compound specific analysis of nitrogen (N) stable isotopes in individual amino acids (CSIA-AA) is an increasingly common analytical method for tracking the origin and fate of N in ecological and biogeochemical studies. The method has proven particularly useful in disentangling food webs by identifying both the trophic positions of analyzed species and the base N sources utilized by primary producers (McMahon and McCarthy 2016; Ohkouchi et al. 2017). For many decades bulk stable isotope analysis (SIA) has been used to address these same ecological questions, but interpretation has been confounded by the integration of variances at both the trophic and source levels. CSIA-AA solves this issue by focusing on specific groups of AAs where N isotopes fractionate at a predictable rate with each trophic transfer, labeled "trophic" AAs, and AAs that remain largely unaltered and thus reflects the inorganic N sources at the base of the food web, thus labeled "source" AAs (Popp et al. 2007). By applying appropriate trophic enrichment factors (TEFs), derived from controlled feeding experiments or in situ comparisons of diet and isotopic fractionation patterns, it is possible to estimate the trophic positions (TP) of consumer organisms (McClelland and Montoya 2002; Chikaraishi et al. 2009; Bradley et al. 2015). Over the past decade, CSIA-AA has been used to resolve trophic connections within marine food webs and to track the flows of N and its inorganic sources (e.g., Chikaraishi et al. 2007; McCarthy et al. 2007; Hannides et al. 2009; Chikaraishi et al. 2010; Choy et al. 2012; Décima et al. 2013; Sherwood et al. 2013; Mompeán et al. 2016).

In trophic ecology studies, the main advantages of a CSIA-AA approach over bulk stable isotope analysis (SIA) is the acquisition of both trophic and source N isotopic measurements from a single sample. This provides baseline estimates that only consider N sources that have actually been consumed in the studied food chain and that integrate over similar timescales as trophic estimates, thus leading to more robust TP estimates (McClelland and Montoya 2002; Chikaraishi et al. 2007). The difference between glutamic acid (Glu) and phenylalanine (Phe), the two most widely used trophic and source AAs, have a TEF approximately twice as large as the +3.4 ‰ $\delta^{15}N$ often considered in bulk SIA studies (e.g., Zanden and Rasmussen 2001; Chikaraishi et al. 2009; Bradley et al. 2015), and thus the potential for increased sensitivity and resolution of TP estimates. This approach assumes that analytical and methodological errors are similar for CSIA-AA and bulk SIA. However, Elemental Analyzer - Isotope Ratio Mass Spectrometry (EA-IRMS) used in bulk SIA typically operates with an analytical precision (0.1 -

0.2 ‰) that is substantially higher than what is reported in CSIA-AA studies (0.4 - 1.0 ‰; Bradley et al. 2016; Broek and McCarthy 2014; Broek et al. 2013; Chikaraishi et al. 2015; Hetherington et al. 2017; Nuche-Pascual et al. 2018; Ogawa et al. 2013; Ruiz-Cooley et al. 2017; Vane et al. 2018; Vokhshoori et al. 2019). This lower precision is the result of a lengthy and time-consuming analytical process inherent to the Gas Chromatography-Combustion-IRMS (GC-C-IRMS) used for almost all CSIA-AA work. For CSIA-AA, AAs first undergo derivatization that causes significant and inconsistent fractionation of N isotopes and is followed by an instrumentally long and complex sequence prior to the $\delta^{15}N$ measurement (Broek et al. 2013; Broek and McCarthy 2014). Ultimately, the propagation of errors limits the precision of TP estimates. As an alternative to GC-C-IRMS, Broek et al. (2013) developed a method using High Pressure Liquid Chromatography (HPLC) for AA purification followed by offline EA-IRMS for $\delta^{15}N$ measurement in Phe. Broek and McCarthy (2014) later optimized the method for Glu and Phe. Both studies carried out extensive comparisons to GC-C-IRMS and found that in addition to being a less expensive alternative, HPLC/EA-IRMS also had better precision and accuracy.

The CSIA-AA approach also opens up the possibility of reconstructing past food webs and N cycling processes. Extensive historic collections of various organisms currently exist within museum archives from which trophic and source AAs can be extracted. These samples have in most cases undergone some kind of chemical preservation, most commonly in formaldehyde or ethanol. Many studies have looked into the effects of such preservatives on bulk $\delta^{15}N$ measurements and found variable and sometimes significant changes across a wide range of species (Rau et al. 2003; Kelly et al. 2006; Barrow et al. 2008), occurring mainly during the first few weeks to months of preservation (Sarakinos et al. 2002; Hetherington et al. 2019). The mechanisms driving the $\delta^{15}N$ fractionation are largely unknown, but likely the result of N containing compounds being solubilized and lost from preserved tissues since neither formaldehyde nor ethanol contains any N that could be added to the tissue (Bosley and Wainright 1999; Sarakinos et al. 2002). Much of an organism's N is stored in AAs, but very little is known about the effects of chemical preservation on $\delta^{15}N$ fractionation or the impacts of multi-decadal storage. Hetherington et al. (2019) is the only comprehensive study thus far to address this issue. They investigated the short-term (2 year) effects of ethanol and formaldehyde in tuna and squid and the long-term (25 year) effects of formaldehyde on two species of

copepods. Although the study did observe substantial but variable N isotopic fractionation, the preservation effects were not significant, in line with two other studies (Hannides et al. 2009; Ogawa et al. 2013; Hetherington et al. 2019). To fully access and interpret biogeochemical and ecological information locked away in historical archives, we need to better understand how isotopic signatures are modified by preservation methods, particularly during long-term storage.

In this study we provide further evaluation of an HPLC/EA-IRMS approach to CSIA in underivatized AAs originally developed by Broek and McCarthy (2014) for Glu and Phe. We first present an optimized protocol for sample processing and chromatographic separation and purification of individual AAs followed by an assessment of the precision and accuracy of the method across 11 AA standards. The performance of the method is evaluated relative to published errors from conventional GC-C-IRMS. We then use HPLC/EA-IRMS to test the effects of short term (1½ year) preservation in ethanol and formaldehyde of white muscle tissue from adult Northern Anchovy (*Engraulis mordax*). In addition, we test the effects of long-term (27-year) preservation of anchovy larvae in formaldehyde. Based on former bulk SIA and CSIA-AA studies on marine fish, we hypothesized that the HPLC/EA-IRMS approach would demonstrate significant $\delta^{15}N$ fractionation as a result of chemical preservation. Lastly, we assess the significances of methodology and chemical preservation on the precision and accuracy of TP estimation.

Materials and procedures

Amino acid standards

Tests of nitrogen isotope fractionation during sample processing, Amino Acid (AA) purification and isotopic analysis were carried out using the liquid Pierce™ Amino Acid Standard H mix of 17 AAs. These AAs were: L-Alanine (Ala), L-Arginine (Arg), L-Aspartic Acid (Asp), L-Cystine (Cys), L-Glutamic Acid (Glu), Glycine (Gly), L-Histidine (His), L-Isoleucine (Ile), L-Leucine (Leu), L-Lysine (Lys), L-Methionine (Met), L-Phenylalanine (Phe), L-Proline (Pro), L-Serine (Ser), L-Threonine (Thr), L-Tyrosine (Tyr), L-Valine (Val). Preliminary test results using different commercially available powdered AA standards indicated that contaminants were present in some of the standards (Phe; see Supplementary Fig. S1). Therefore, 11 AAs of interest (Glu, Ala, Pro, Val, Ile, Leu, Phe, Gly, Ser, Tyr, Met) were purified from the Pierce AA mix by Liquid Chromatography (methodology described below). Following purification, half of each AA was taken as a control sample. The other halves were combined and split into four test samples. These test samples were not hydrolyzed, but were otherwise processed like fish samples and stored at -80°C for 1-4 weeks before AA re-purification (see below).

Fish sample preparation

Adult Northern Anchovy (*Engraulis mordax*) were collected during the NOAA 2015 summer coast-wide coastal pelagic species survey off the coast of central California (Zwolinski et al. 2016) using Nordic 264 rope trawl with a ~600 m^2 mouth and 8 mm mesh netting in the cod end liner that was towed at 3.5 knots, typically for 45 minutes. Within less than an hour of capture, two white muscle fillets were taken from the dorsal side of each individual fish (n = 6). One fillet was preserved in 95% tris-buffered ethanol and the other in 1.8% sodium borate-buffered formaldehyde. The remaining fish were stored at -20°C.

Larval anchovy were collected during the 1991 spring CalCOFI cruise from lines 80-83, stations 51-60 (www.calcofi.org) by oblique tows using Bongo nets of 0.71 m diameter, 0.505 mm mesh. The contents of one net were flash frozen in liquid N_2 and stored at -80°C while the other side was preserved in seawater with formaldehyde (1.3% final concentration) buffered with

sodium tetraborate. Larval anchovy of 8.5-10 mm in standard length (SL) were later sorted from the formaldehyde samples and stored at -80°C and in formaldehyde, respectively.

Adult anchovy samples for short-term preservation tests were defrosted or removed from the preservatives and a 16-51 mg dry weight (DW) sample of white muscle tissue taken for isotopic analysis. Preserved larval anchovy from long-term preservation tests were analyzed whole. For each test pair of frozen and formaldehyde-preserved samples, we ensured that the same proportion of larvae was taken from each 0.5 mm size interval and pooled 6-11 larvae to obtain enough material for isotopic analysis (0.5-2.0 mg DW).

All fish samples were frozen at -80°C and freeze-dried for 24 h. Adult tissue samples were then homogenized, and 0.5-0.8 mg subsamples taken for bulk isotopic analysis. The remaining homogenized adult tissue samples and the larval samples were stored in a desiccator until processing for CSIA. A minimum of 400 µg of fish DW was hydrolyzed in 0.5 ml of 6N HCl in capped glass tubes for 24 hours at 90°C. Samples were then dried on a Labconco centrifugal evaporator under vacuum at 60°C, re-dissolved in 0.5 ml 0.1N HCl, and filtered through an IC Nillix – LG 0.2-µm hydrophilic PTFE filter to remove particulates. The samples were then re-dried before re-dissolving in 100 µl of 0.1% trifluoroacetic acid (TFA) in Milli-Q water, transferred to glass inserts in vials, and stored at -80°C for 1-4 weeks prior to AA purification.

HPLC/EA-IRMS analysis of AAs

The AA purification methodology is modified from the method of Broek and McCarthy (2014). We used an Agilent 1200 series High Pressure Liquid Chromatography system equipped with degasser (G1322A), quaternary pump (G1311A) and autosampler (G1367B). Samples were injected onto a reverse-phase semi-preparative scale column (Primesep A, 10 × 250 mm, 100 Å pore size, 5 µm particle size, SiELC Technologies Ltd.). Downstream a 5:1 Realtek fixed flow splitter directed the flow to an analytical fraction collector (G1364C) and an Evaporative Light Scattering Detector (385-ELSD, G4261A), respectively. We found that optimal AA detection on the ELSD was achieved with a nebulizer temperature of 30°C, evaporator tube temperature of 70°C, and a nitrogen gas flow rate of 1 L min^{-1} delivered from a nitrogen generator. A 120-min

ramp solvent program with 0.1% TFA in Milli-Q water (aqueous phase) and HPLC grade acetonitrile (ACN, organic phase) was used as displayed on Fig. 1. Typically, the lifetime of the column was 200-250 runs before the chromatography deteriorated to a point where purification of Gly, Glu and/or Ala became compromised. A shorter program could be constructed, but required the system to operate at higher pressure (>250 bar), which translated into more maintenance from increased wear and tear. His, Lys and Arg can also be purified, but require a longer program (see Broek et al. 2013). A program was set up for the fraction collector to collect AAs of interest in 7 ml glass tubes at specific times based on elution times from previous runs. A steep gradient in $\delta^{15}N$ can occur across the peak of an eluting AA (Hare et al. 1991; Broek et al. 2013). Therefore, the quality of all collections was assessed by comparing the chromatogram with set collection times, and only AAs where ≥ 99% of the peak areas fit within the collection windows were accepted. Due to slight drift in AA elution timing, collection times were modified between consecutive runs. Injection volume was determined from sample DW and expected content of low concentration AAs (typically Phe and Met) based on previous runs of similar samples. The aim was to collect approximately ≥1 µg N equivalent of each AA. Here, we injected samples of 484-776 µg DW of fish biomass, but it was possible to collect sufficient Phe for isotopic analysis from 350 µg DW.

Following collection, the AA samples were dried in the centrifugal evaporator at 60°C, dissolved in 40 µl of 0.1N HCl, and transferred to tin capsules (Costech, 3.5 x 5 mm). The capsules were then dried overnight in a desiccator under vacuum. Pre-combusted borosilicate glassware with PTFE lined caps was used for all process steps, and all sample transfers were done in HPLC-grade solvents or Milli-Q water with fine glass syringes.

Isotopic analyses of the AAs were carried out at the Stable Isotope Laboratory facility at University of California, Santa Cruz (UCSC-SIL). Samples were analyzed on a Nano-EA-IRMS system designed for small sample sizes in the range of 0.8-20 µg N. The automated system is composed of a Carlo Erba CHNS-O EA1108 Elemental Analyzer connected to a Thermo Finnigan Delta Plus XP Isotope Ratio Mass Spectrometer via a Thermo Finnigan Gasbench II with a nitrogen trapping system similar to the configuration of Polissar et al. (2009). Sample $^{15}N/^{14}N$ ratios are reported using the δ notation relative to atmospheric nitrogen (N_2). Measured $\delta^{15}N$ values were corrected for size effects and instrument drift using Indiana University

acetanilide, USGS41 Glu and Phe standards and correction protocols (see https://es.ucsc.edu/~silab) based on procedures outlined in Fry et al. (1992).

EA-IRMS analysis of bulk material

Bulk stable isotope analysis of white muscle tissue from adult anchovies was carried out at the Scripps Institution of Oceanography Stable Isotope Facility. Samples were analyzed on a Costech ECS 4010 Elemental Analyzer coupled to a Thermo Finnigan Delta Plus XP Isotope Ratio Mass Spectrometer. Measured $\delta^{15}N$ values were corrected for size effects and instrument drift using acetanilide standards (Baker AO68-03, Lot A15467). Analytical precision was ≤ 0.2 ‰.

Data analysis

TP was estimated using the equation, β and trophic discrimination factor (TDF) values presented in Bradley et al. (2015) for individual trophic and source AA pairs. When calculating TP using multiple AAs, an average TP was taken of all trophic-source combinations.

Methodology effects on $\delta^{15}N$ were tested by ANOVA followed by post hoc testing using Tukey HSD. Preservation effects on $\delta^{15}N_{AA}$ and TP were tested by ANOVA followed by post hoc testing using paired two-way t-tests for each AA from frozen control and chemically preserved test material. Effects of preservation in bulk material was also done by paired two-way t-test. Before testing, data were inspected for normality of distribution followed by homogeneity of variance by the Levenes test. In the t-tests we assumed unequal variance between control and test samples and p values adjusted using a Bonferroni correction. All statistical tests were carried out in R (R_Core_Team 2014).

Assessment

Methodological induced errors on individual AAs

Our methodological procedure for purifying individual amino acids (AA) did not cause significant fractionation of $\delta^{15}N$ in most of the 11 target AAs (Fig. 2). The two exceptions were Ile and Met, which both changed significantly relative to control samples (-0.85 ‰, +2.11 ‰, $p < 0.001$). Although not significant, Tyr also showed substantial fractionation (+0.57 ‰, $p = 0.059$). For the other eight AAs, we found a high accuracy of +0.06 ‰ on average (Fig. 3). Glu, Ala and Phe showed virtually no fractionation (+0.01 - 0.04 ‰) while some insignificant fractionation was observed for Pro and Leu (+0.27 - 0.4 ‰, Fig. 2). Similar results were also seen in our preliminary testing using Glu, Ala and Pro powdered standards (+0.05 ‰, Supplementary Fig. S1).

The overall analytical precision of the EA-IRMS for all of the AAs in Fig. 1 was within the range of the long-term performance of the instrument (Fig. 3). We also found the procedural reproducibility to be comparable to the precision. This means that overall the AA purification methodology did not add additional variability to the results.

Preservation induced errors on individual AAs

Short-term (1½ year) preservation of adult anchovy white muscle fillets with ethanol and formaldehyde resulted in a significant fractionation of $\delta^{15}N$ in bulk material (+0.67 ‰, $t = 8.67$, $df = 5$, $p = 0.001$; +0.84 ‰, $t = 9.17$, $df = 5$, $p < 0.001$; Fig. 4, Table S1). However, this fractionation was not consistent across all AAs or between preservative types and durations.

Overall, short-term chemical preservation resulted in significant $\delta^{15}N_{AA}$ fractionation relative to frozen control samples ($F = 15.44$, $df = 2$, $p < 0.001$), but did not differ between ethanol and formaldehyde ($p = 0.410$). Some degree of $\delta^{15}N$ enrichment was observed for all short-term preserved AAs, with the exception of Ala which was depleted. Pairwise testing revealed that Pro (+2.00 ‰, $t = 6.07$, $df = 3$, $p = 0.027$) and Leu (+2.42 ‰, $t = 26.44$, $df = 2$, $p < 0.001$) changed significantly in ethanol preserved samples. Substantial $\delta^{15}N$ enrichment of 0.9 ‰ or more was also observed for Val, Ile, Tyr and Met, but with some variability between replicate

measurements (Fig. 4-a, Table S1). Similarly, $\delta^{15}N$ enrichment was also generally observed for all short-term formaldehyde preserved AAs with Pro (+2.56 ‰, $t = 6.45$, $df = 3$, $p = 0.023$) and Gly (+0.63 ‰, $t = 32.92$, $df = 3$, $p < 0.001$) changing significantly. Enrichment of 0.9 ‰ or more was also observed in e.g., Val, Leu, Phe and Met, but varied between replicate measurements (Fig. 4-a, Table S1). Overall, Glu and Ser were the least affected by short-term ethanol and formaldehyde preservation (+0.3 ‰ or less).

Long-term formaldehyde preservation of small 8.5-10 mm SL anchovy larvae also resulted in fractionation of $\delta\,^{15}N_{AA}$ ($F = 80.58$, $df = 1$, $p < 0.001$). Fractionation patterns differed slightly, but not significantly from that observed for short-term formaldehyde preserved adult anchovy (Fig. 4-a, Table S1, $F = 0.02$, $df = 1$, $p = 0.904$). Here, significant effects were seen for Val (+1.77 ‰, $t = 7.61$, $df = 2$, $p < 0.017$), Ile (+1.75 ‰, $t = 7.41$, $df = 2$, $p < 0.018$), Leu (+1.10 ‰, $t = 6.96$, $df = 2$, $p = 0.020$) and Ser (+0.86 ‰, $t = 5.21$, $df = 2$, $p < 0.035$). Least affected by long-term formaldehyde preservation were Ala, Phe and Gly (+0.7-0.8 ‰).

We observed similar patterns in preservation effects across a suite of AAs irrespective of the preservative used. Although formaldehyde preservation caused added variability (particularly in Ala, Val, Leu and Phe), there was no difference in average variability overall between control samples and any of the treatments (Supplementary Table S1). The slightly lower variability seen for long-term formaldehyde-preserved anchovy may have been the result of 6-11 larvae being pooled together per sample. Relative $\delta^{15}N$ change was more consistent between different AAs (Fig. 4-b), but absolute change was generally higher for trophic AAs compared to source AAs (Fig. 4-a). Unfortunately, it was not possibly to purify Glu from the anchovy larvae samples. Furthermore, Tyr could only be detected in frozen and ethanol-preserved samples.

Consequences for trophic position estimates

To evaluate the impact of using preserved samples when calculating a fish's TP, we compared different TP estimates derived from conventional trophic and source AA combinations (Fig. 5). Due to substantial fractionation of Ile, Met and Tyr during purification, we did not use these AAs in our TP estimation. We found no effect of preservative or preservative:AA interaction on TP estimates for short-term formaldehyde ($F = 0.64$, $df = 1$, $p = 0.428$; $F = 1.68$, df

= 14, p = 0.080) and ethanol-preserved (F = 3.34, df = 1, p = 0.072; F = 1.56, df = 14, p = 0.113) samples compared to frozen samples. The same was seen for long-term formaldehyde preserved samples (F = 3.94, df = 1, p = 0.053; F = 0.44, df = 11, p = 0.930). However, low p values do point to a trend in changing TP estimates as illustrated in on Fig 5. Furthermore, this TP difference varied depending on the combination of trophic and source AAs.

Discussion

The High Pressure Liquid Chromatography Elemental Analysis – Isotope Ratio Mass Spectrometry (HPLC/EA-IRMS) method successfully provided $\delta^{15}N$ measurements across a suite of standard amino acids (AAs). Sample processing procedure and purification by HPLC resulted in low isotopic fractionation of nitrogen (N) for most AAs concerned. Glu, Ala and Phe, particularly important AAs in ecological studies (e.g., Chikaraishi et al. 2009; McMahon and McCarthy 2016; Décima et al. 2017), demonstrated almost no fractionation relative to control samples and little variation between replicate measurements. Broek and McCarthy (2014) reported a mean accuracy of 0.12 ± 0.05 ‰ using Glu and Phe powdered standards, slightly below the 0.02 ± 0.02 ‰ we observed for the same AAs. The improved accuracy in our study may have resulted from pre-purification of our Pierce AA standard mix, a decision made after observing high fractionation in Phe powdered standards during preliminary testing, suggestive of N containing contaminants being present in the standard (Supplementary Fig. S1). Overall, we achieved high accuracy for most of our AA standards (0.06 ± 0.21 ‰, Fig. 3) except for Ile, Tyr and Met which underwent substantial fractionation. Tyr and Met are prone to oxidation (e.g., Sprung et al. 2009) which could explain a high $\delta^{15}N$ enrichment. These findings are largely in agreement with those of Broek et al. (2013), who also observed high fractionation in Ile and Met.

Interestingly, we found a procedural reproducibility error equal to the precision of the IRMS instrument, meaning that neither sample handling during the multiple processing steps nor purification by HPLC added any quantifiable variability to the analysis (Fig. 3). By comparison, Broek and McCarthy (2014) observed a precision of 0.3 ‰ for Glu and Phe, which was half that of their control samples. However, it is important to note that all of these tests are based on AA standards rather than organism samples. Thus, the samples did not undergo hydrolysis, a step that may impact accuracy and procedural reproducibility. Using Phe from a hydrolyzed cyanobacteria culture, Broek et al. (2013) observed a larger procedural reproducibility error (0.55 ‰) than when using Phe standards. The complex molecular structure of organismal samples relative to laboratory AA standards means that other N-containing compounds may co-elute with collected AAs adding additional analytical variability. Indeed, co-elution with an unknown compound was the reason Glu could not be purified in some of our larval anchovy samples (Supplementary Fig. S2-d).

The HPLC/EA-IRMS method performed well relative to the conventional GC-C-IRMS method generally used for CSIA-AA. Recent aquatic studies using GC-C-IRMS report average precisions in the range of 0.4 – 1.0 ‰, but with considerable variability among analyses (Broek et al. 2013; Ogawa et al. 2013; Broek and McCarthy 2014; Chikaraishi et al. 2015; e.g., Bradley et al. 2016; Hetherington et al. 2017; Ruiz-Cooley et al. 2017; Nuche-Pascual et al. 2018; Vane et al. 2018; Vokhshoori et al. 2019). In the literature, such error reporting is often based on only one to a few AA standards (Broek et al. 2013), which for the most part have not gone through any of the sample processing steps prior to derivatization. In addition, errors are often expressed as the standard deviation of replicate injections of the same derivatized standard or biological sample, thus giving only the analytical precision of the instrument, not procedural reproducibility. Nonetheless, these published errors are more than double our procedural reproducibility of 0.15 ± 0.07 ‰, and the reproducibility error of the GC-C-IRMS method is likely to be higher, e.g., from slight inconsistencies in the derivatization procedure (see Broek et al. 2013; Broek and McCarthy 2014). Derivatization of AAs can also cause significant fractionation (e.g., by 2.5 ± 1.2 ‰ in Broek and McCarthy 2014), with $\delta^{15}N$ values typically adjusted following correction protocols. Using the HPLC/EA-IRMS method, we found no significant fractionation in 8 of the 11 AAs tested, thus eliminating the need for correcting these 8 AAs.

The preservation of adult anchovy white muscle in ethanol or formaldehyde caused significant fractionation of $\delta^{15}N$ compared to frozen samples (+0.67 and +0.84 ‰, respectively). We used frozen samples as controls since freezing has been shown not to cause N fractionation in fish and is generally considered a safe method of preservation (Bosley and Wainright 1999; Kaehler and Pakhomov 2001; Sweeting et al. 2004). Past studies with a variety of aquatic organisms have found inconsistent effects of preservatives (e.g., Kelly et al. 2006; Barrow et al. 2008). However, our findings are within the range of published values (+0.6 to +1.4 ‰) for multiple species of marine fin fish (Bosley and Wainright 1999; Kaehler and Pakhomov 2001; Arrington and Winemiller 2002; Sweeting et al. 2004; Kelly et al. 2006; Hetherington et al. 2019).

We also found significant preservation effects for several individual AAs. Notably, Pro and Leu showed changes in $\delta^{15}N$ irrespective of the preservative used, and Ile, Val, Gly and Ser

also changed in the formaldehyde treatments. This is not entirely surprising given the observed isotopic changes in the bulk N, which is mainly stored in AAs. Nevertheless, of the four published studies of preservative effects on $\delta^{15}N$ in individual AAs, this is the first to observe significant differences. For two species of freshwater fishes, Ogawa et al. (2013) concluded that formaldehyde preservation had no effect. Similarly, Hetherington et al. (2019) did not find significant changes for yellowfin tuna AAs preserved in ethanol and formaldehyde despite observing significant changes in bulk values. Studies on zooplankton and squid tissue have also reported no significant change (Hannides et al. 2009; Hetherington et al. 2019). Although our different results point potentially toward species-specific differences in preservative effects, another reason why the previous studies did not find significant effects is likely due to the choice of CSIA-AA method combined with low number of replicates. These studies used the GC-C-IRMS method with an average precision of 0.5-1.0 ‰ (Hannides et al. 2009; Ogawa et al. 2013; Hetherington et al. 2019), considerably poorer than HPLC/EA-IRMS method as previously discussed. Consequently, the GC-C-IRMS would have added variability to the results, and an average $\delta^{15}N_{AA}$ change of 1 ‰ (this study; Hetherington et al. 2019) would fall within error of GC-C-IRMS, but not for HPLC/EA-IRMS.

How nitrogen is fractionated during sample preservation is not clear, though it appears to be selective for specific AAs rather than all AAs changing in proportion to bulk N isotopes. Neither ethanol nor formaldehyde contains any N that could be added to the bulk tissues or individual AAs thereby altering the N isotopic ratios. Although not expected, if proteins are partially hydrolyzed during preservation resulting in C-^{14}N bonds being preferentially cleaved, this could result in N leakage into the preservative solution as, e.g., free AAs of amines (Silfer et al. 1992; Bosley and Wainright 1999; Sarakinos et al. 2002). In bulk tissue samples, we did register a modest reduction in N content when preserved in formaldehyde (-1.5 ± 0.9 %), but for ethanol the N content increased (+1.5 ± 0.9 %) relative to frozen samples. Ethanol is an organic solvent that is well suited for extracting lipids from tissues (Kelly et al. 2006). However, lipid extraction may well have masked any N loss in the ethanol-preserved samples, although this does not explain why some AAs appear to fractionate more than others. Hetherington et al. (2019) tested whether there was a greater loss of high compared to low fractionating AAs using peak areas, but found no differences. For our analyses, however, the two AAs (Pro and Gly) that fractionated significantly in the ethanol and formaldehyde preserved adult anchovy (Fig. 4)

showed greater loss in mass relative to frozen control samples than did the two lowest fractionating AAs (Glu and Ser, $p \leq 0.05$, ANOVA, data not shown). One way to further address the effects of chemical preservatives would be to determine which compounds are lost from the tissues and which ones end up in the preservative solution. Testing how preservatives impact the hydrolysis of different proteins, if they affect how acid labile certain amine bonds are and thereby hamper our ability to recover these AAs following hydrolysis would be informative.

Another possible reason for the fractionation in preserved samples is co-elution with N-containing compounds during purification by HPLC. Indeed, we did see an unknown compound co-eluted with Ala in frozen adult anchovy samples, which may explain why Ala was the only AA depleted in $\delta^{15}N$ in the preserved samples (Supplementary Fig. S2-a). $\delta^{15}N$ gradients have been observed across chromatographic peaks of eluting compounds, with the tail ends being considerably enriched (Hare et al. 1991; Broek et al. 2013). During collection, part of the front end of the Ala peak was mixed with the tail end of the unknown compound. Assuming this compound contained N, it may well have elevated the $\delta^{15}N$ in our control samples. Broek and McCarthy (2014) also observed that amino sugars elute just prior to Glu, which was the reason we did not attempt to collect it from the frozen larval anchovy samples. However, for most other AAs, there was no indication of co-elution, indicating that poor chromatography could not have driven the general fractionation pattern that we observed. In fact, we actually observed improved chromatographic separations of AAs in both the ethanol and formaldehyde preserved samples. Hetherington et al. (2019) also suggested that co-elution was responsible for some of the observed $\delta^{15}N_{AA}$ fractionation, and that chemical preservation may have further aided the purification and improved their gas chromatography.

Overall, the effects of ethanol and formaldehyde preservation on $\delta^{15}N_{AA}$ appeared quite similar and did not increase variability between replicate measurements. Furthermore, preserving anchovy in formaldehyde for 1½ or 27 years did not significantly alter the fractionation pattern. These results are promising for comparing different samples within historical archives, where the duration of preservation can vary considerably and where different preservatives are often used. However, the substantial, and in some cases significant, enrichment in $\delta^{15}N_{AA}$ does illustrate that caution must be taken in interpreting results. This seems particularly important in studies aiming to combine or compare preserved with fresh or frozen samples, or when it is necessary to know

the true $\delta^{15}N$ value. For instance, when attempting to assess the relative contributions of N sources to the food chain of a studied organism by comparing its source AA Phe $\delta^{15}N$ signature with inorganic N isotopes in its environment, it may be necessary to correct Phe for preservation effects. Considering the uneven fractionation patterns across different AAs studies doing isotopic fingerprinting or using mixing models to determine dietary contributions of different prey may also consider correcting for preservation effects. Such correction factors may need to be species-specific (e.g., Kelly et al. 2006), given the large $\delta^{15}N_{AA}$ fractionation differences observed between various organisms (this study; Hetherington et al. 2019). Although preservation did not result in large shifts in TP estimates, the variable $\delta^{15}N_{AA}$ fractionation does highlight the need for careful consideration in the choice of AAs. Nielsen et al. (2015), amongst others, have advocated for the use of multiple trophic-source AA combinations when calculating TPs. In light of our results, this may be particularly important when working with preserved samples.

Comments and recommendations

This study has helped demonstrate the possibilities of an HPLC/EA-IRMS approach for CSIA-AA. The superior precision and accuracy of this method makes it suitable for studies attempting to resolve fine-scale variability in N sources and TPs. For instance, among primary or secondary consumers in marine pelagic systems, temporal and spatial variability in TP is usually around the order of ±0.4 TP (Hannides et al. 2009; Choy et al. 2012; Décima et al. 2013; Choy et al. 2015; Miyachi et al. 2015; Bradley et al. 2016; Laiz-Carrión et al. 2019). Even small shifts in TP can be associated with significant food web disruptions and represent considerable changes in energy transfer up to higher level consumers (Vander Zanden et al. 1999). The propagation of methodological errors reported in this study equals a ±0.04 methodological uncertainty in TP estimates using Glu and Phe, the most widely used trophic-source AA combination (TEF of 5.7, Bradley et al. 2015). In comparison, the analytical errors reported for the GC-C-IRMS approach is equivalent to a ±0.1 - 0.25 TP uncertainty (see discussion), within the range of natural variability for many lower trophic level organisms.

Before opting for the HPLC/EA-IRMS approach, there are a number of issues to consider. First and foremost, the method is only as good as the quality of the chromatography and the precision of the IRMS. Samples with complex biochemical compositions, such as degraded organic matter, can result in messy chromatograms rendering AA purification difficult or impossible (Broek and McCarthy 2014, and references therein). Although not shown here, preliminary testing on crustacean zooplankton did reveal poorer chromatographic performance than for frozen larval anchovy (Supplementary Fig. S2-d). Crustaceans notably have large amounts of polysaccharide chitin in their exoskeleton that break into several monosaccharides when hydrolyzed (e.g., GlcNAc and GlcN, Einbu and Vårum 2008). These monosaccharides can co-elute with Gly, Thr and Glu and prevent purification (present study; Broek and McCarthy 2014). For larval fish, which contain chitin in their epidermis (Tang et al. 2015) but also cartilage, this issue seems to abate with development and increasing size, but for small crustaceans, the HPLC/EA-IRMS method may not be appropriate. Due to the low concentrations of some AAs (such as Phe), the N collected from a single HPLC run may also not be within the detection range of most IRMS systems. Injecting too much sample onto the column reduces chromatographic performance, and combining multiple collections increases time and costs. One solution is to use high sensitivity Nano-EA-IRMS instrumentation as was done by Broek and

McCarthy (2014) and in the present study. This also allowed CSIA-AA to be performed on individual fish larvae of ≥350 µg DW. Lastly, although HPLC/EA-IRMS has the potential to be a relatively fast CSIA method (we routinely processed 25 or more samples per week), it can still be costly if a large suite of AAs is desired because every AA needs to be individually analyzed for $\delta^{15}N_{AA}$.

In sum, this study contributes to the growing understanding of the effects of preservatives on individual AAs. For Northern Anchovy, we showed that the duration of chemical preservation and the preservative used (ethanol or formaldehyde) within an historical archive did not result in substantial alterations of $\delta^{15}N_{AA}$. Compared to non-chemical preservation, significant $\delta^{15}N_{AA}$ alteration can be expected and may to some extent be species- or case specific. We recommend that studies involving different species or circumstances should conduct pilot testing and assess the need for correction protocols. Nonetheless, these results are encouraging for reconstructing biogeochemical records and food web trophic connections over long time scales. A vast amount of the information that is currently locked away in historical archives of preserved samples can now be accessed using CSIA-AA.

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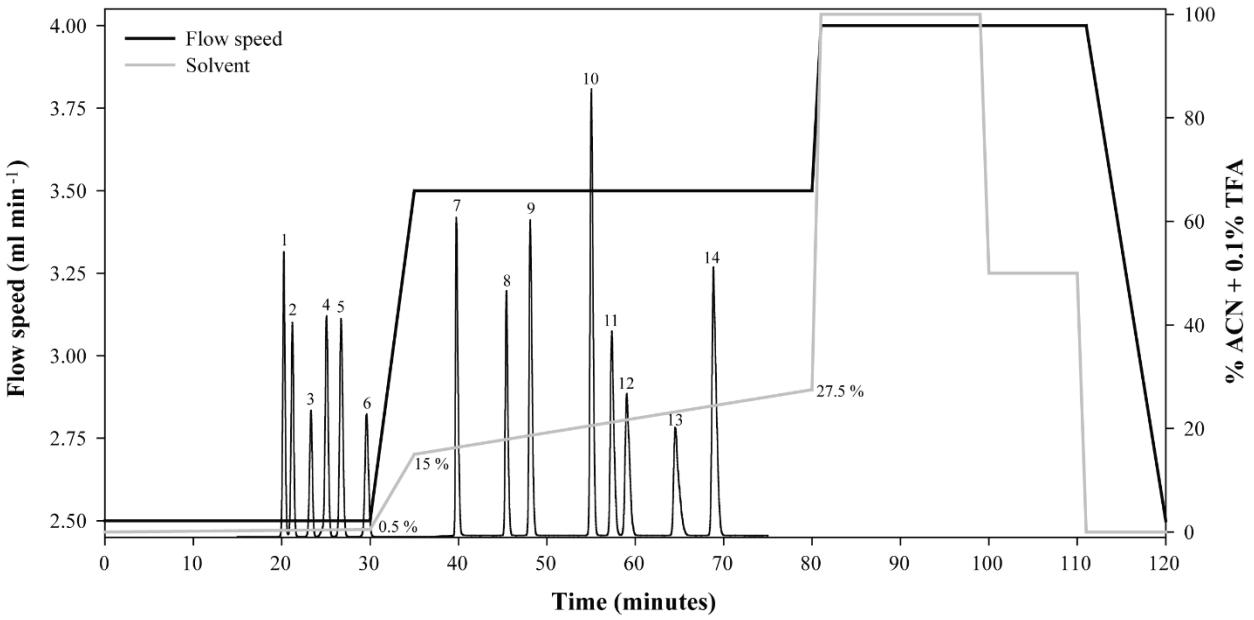

Figure 1: HPLC ramp solvent program modified from Broek and McCarthy (2014). Column cleaning and equilibration were carried out from time 80 to 120 min. System pressure typically ranged between 160 and 255 bars during a run. Background shows a chromatogram from injection of Pierce™ AA mix. Peak identities are: 1. Asp, 2. Ser, 3. Gly, 4. Thr, 5. Glu, 6. Ala, 7. Pro, 8. Val, 9. Met, 10. Tyr, 11. Ile, 12. Leu, 13. Cys, 14. Phe.

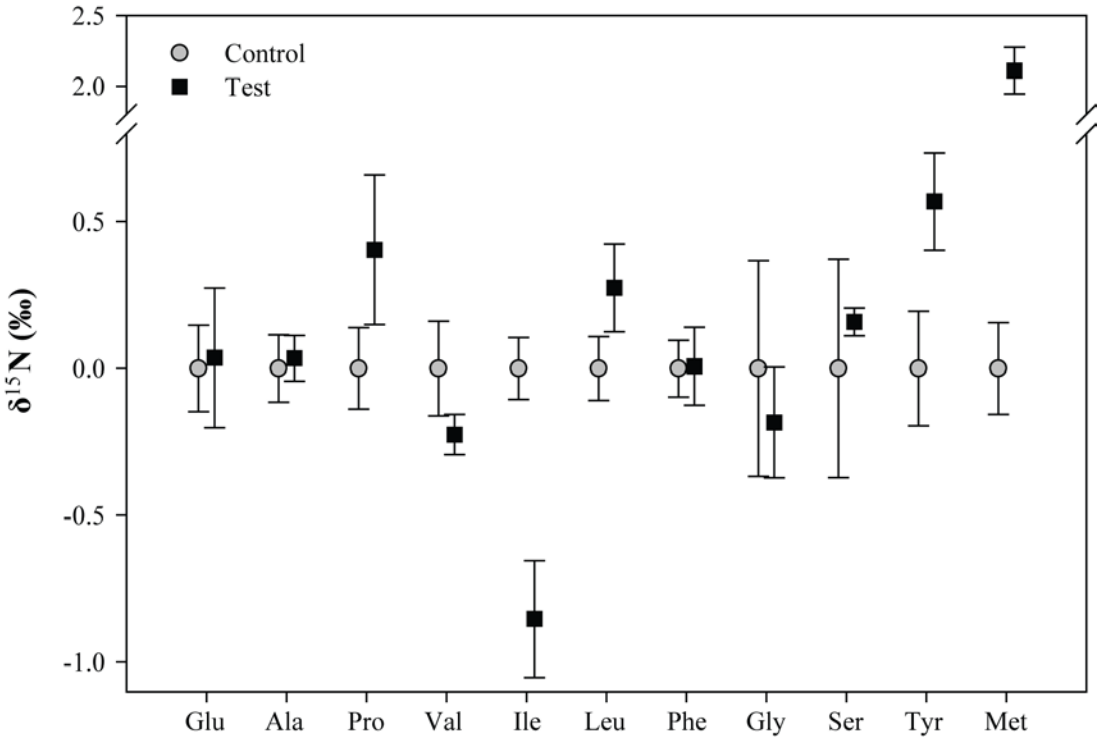

Figure 2: Change in $\delta^{15}N$ in AA standards processed and purified by HPLC relative to control samples (±1 SD; Glu, Phe and Gly, n = 4; Ala, Pro, Val, Ile, Leu, Ser, Tyr and Met, n = 3). n = minimum number of replicates per treatment.

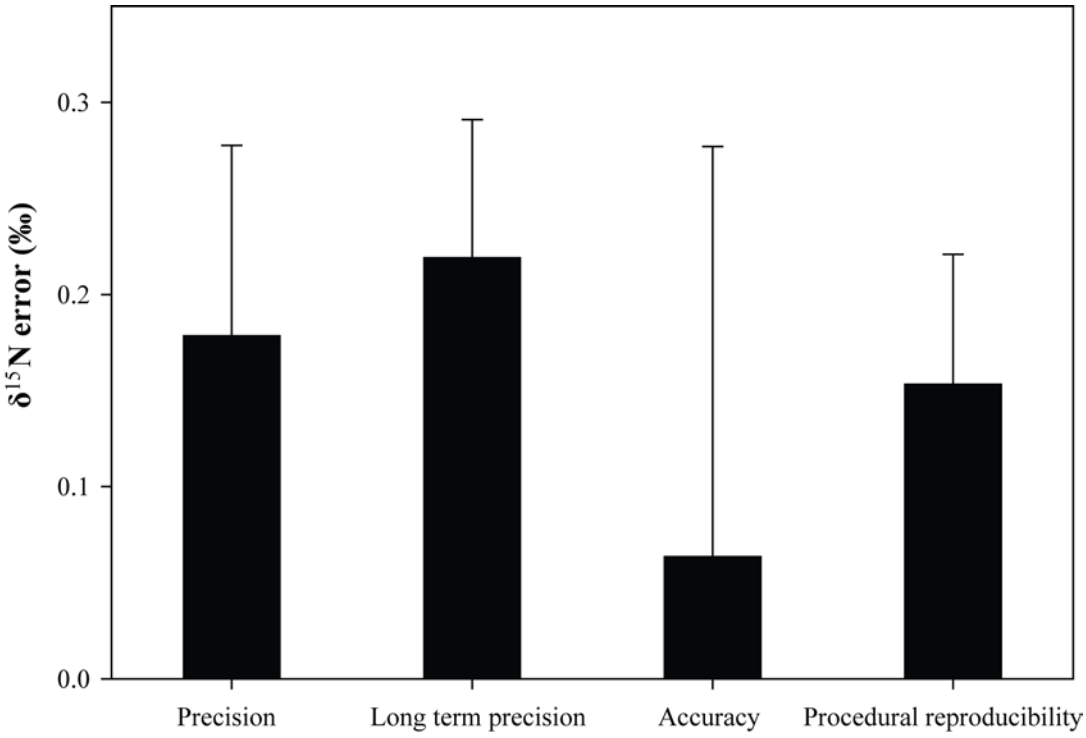

Figure 3: Errors associated with sample processing and analysis by HPLC/EA-IRMS. Analytical precision of the EA-IRMS instrument at the UCSC isotope lab calculated as the average standard deviation for all AA control samples displayed in Figure 2 (n = 11). Long-term precision of instrument is from 12 consecutive runs of powdered acetanilide and Phe standards over a one month period (n = 24). Procedural reproducibility is calculated as the average standard deviation for all AA test samples (n = 11). Accuracy of the method is calculated as the average difference between test and control samples (excluding Ile, Tyr and Met, n = 8) displayed in Figure 2. Error bars are +1 SD.

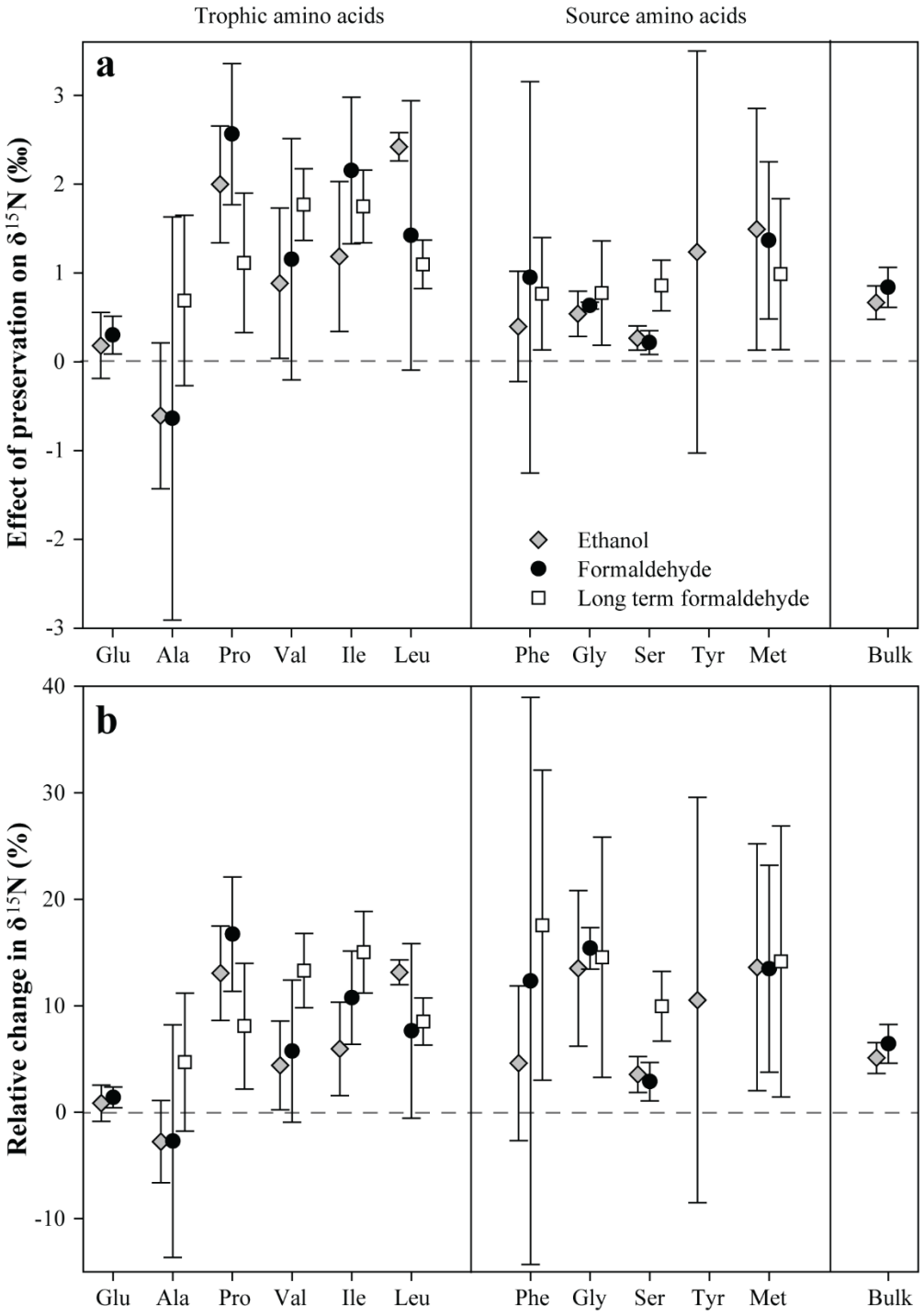

Figure 4: $\delta^{15}N$ in short-term (~1½ year) ethanol and formaldehyde preserved adult anchovy bulk material (n = 6 per treatment) and in individual AAs (Glu, Ala, Pro, Phe, Gly, n = 4; Val, Ile, Leu, Ser, Tyr, n = 3; Met, n = 2), and from long-term (27 year, n = 3 per treatment) formaldehyde-preserved larval anchovy after subtracting frozen control samples. Changes relative to controls are shown in a) ‰ and as b) relative change in $\delta^{15}N$ (±1 SD). Glu could not be purified from larval anchovy, and Tyr from formaldehyde-preserved samples. n = minimum number of replicates per treatment.

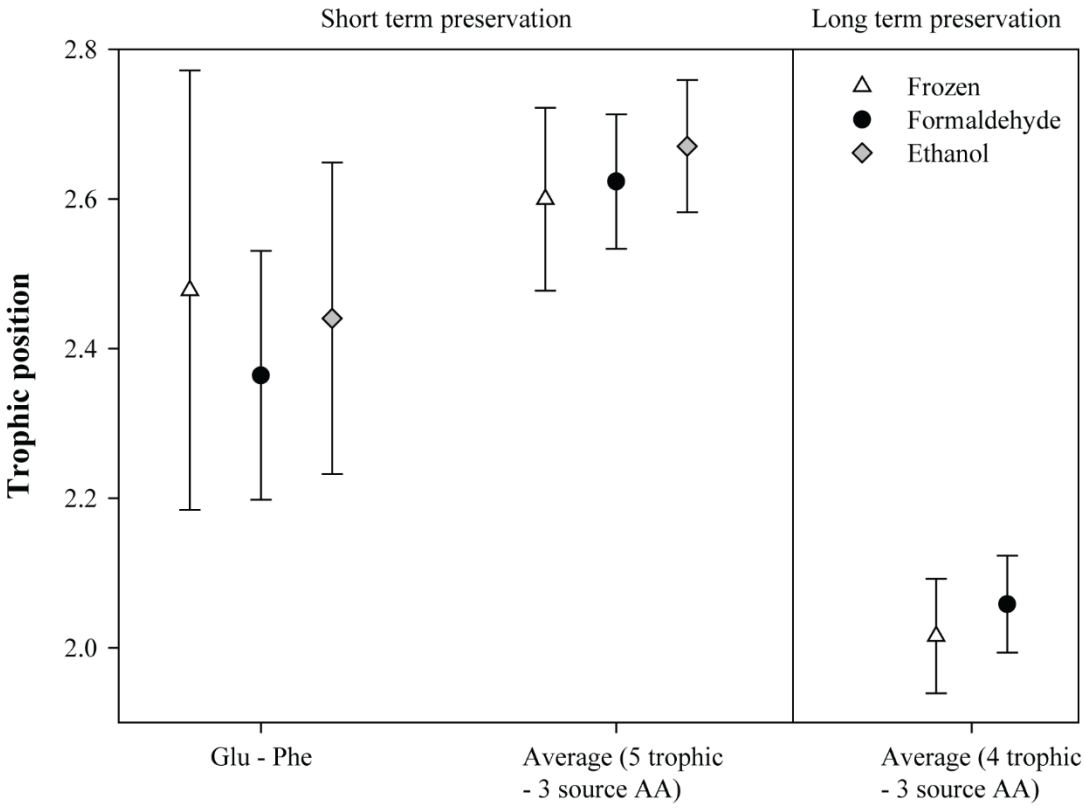

Figure 5: Comparison of TP estimates of frozen, ethanol and formaldehyde preserved samples calculated from Glu and Phe (n = 4) and as an average of all TP estimates (n = 3) based on Glu, Ala, Pro, Val, Leu, Phe, Gly and Ser (±SD). Glu could not be purified from the long-term preserved larval anchovy.