Title

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Journal

Rapid Communications in Mass Spectrometry, 33(10)

ISSN

0951-4198

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Publication Date

2019-05-30

DOI

10.1002/rcm.8408

Peer reviewed

Received: 11 September 2018 | Revised: 31 December 2018 | Accepted: 31 January 2019

DOI: 10.1002/rcm.8408

RESEARCH ARTICLE

WILEY Rapid Communications in Mass Spectrometry

Effects of chemical preservation on bulk and amino acid isotope ratios of zooplankton, fish, and squid tissues

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Funding information
Frontiers of Innovation Scholarship (University of California San Diego); National Science Foundation, Grant/Award Number: OCE1041329

Rationale: It is imperative to understand how chemical preservation alters tissue isotopic compositions before using historical samples in ecological studies. Specifically, although compound-specific isotope analysis of amino acids (CSIA-AA) is becoming a widely used tool, there is little information on how preservation techniques affect amino acid $\delta^{15}N$ values.

Methods: We evaluated the effects of chemical preservatives on bulk tissue $\delta^{13}C$ and $\delta^{15}N$ and amino acid $\delta^{15}N$ values, measured by gas chromatography/isotope ratio mass spectrometry (GC/IRMS), of (a) tuna (*Thunnus albacares*) and squid (*Dosidicus gigas*) muscle tissues that were fixed in formaldehyde and stored in ethanol for 2 years and (b) two copepod species, *Calanus pacificus* and *Eucalanus californicus*, which were preserved in formaldehyde for 24–25 years.

Results: Tissues in formaldehyde-ethanol had higher bulk $\delta^{15}N$ values (+1.4, *D. gigas*; +1.6‰, *T. albacares*), higher $\delta^{13}C$ values for *D. gigas* (+0.5‰), and lower $\delta^{13}C$ values for *T. albacares* (−0.8‰) than frozen samples. The bulk $\delta^{15}N$ values from copepods were not different those from frozen samples, although the $\delta^{13}C$ values from both species were lower (−1.0‰ for *E. californicus* and −2.2‰ for *C. pacificus*) than those from frozen samples. The mean amino acid $\delta^{15}N$ values from chemically preserved tissues were largely within 1‰ of those of frozen tissues, but the phenylalanine $\delta^{15}N$ values were altered to a larger extent (range: 0.5–4.5‰).

Conclusions: The effects of preservation on bulk $\delta^{13}C$ values were variable, where the direction and magnitude of change varied among taxa. The changes in bulk $\delta^{15}N$ values associated with chemical preservation were mostly minimal, suggesting that storage in formaldehyde or ethanol will not affect the interpretation of $\delta^{15}N$ values used in ecological studies. The preservation effects on amino acid $\delta^{15}N$ values were also mostly minimal, mirroring bulk $\delta^{15}N$ trends, which is promising for future CSIA-AA studies of archived specimens. However, there were substantial differences in phenylalanine and valine $\delta^{15}N$ values, which we speculate resulted from interference in the chromatographic resolution of unknown compounds rather than alteration of tissue isotopic composition due to chemical preservation.

1 | INTRODUCTION

Evaluating the effects of chemical preservation on carbon ($\delta^{13}C$ values) and nitrogen ($\delta^{15}N$ values) stable isotope ratios from organismal tissues is essential for the best application of stable isotope data from archived samples. Stable isotope analysis has become a powerful tool for answering questions about trophic ecology, energy flow, food web dynamics,[1-3] and, more recently, to evaluate habitat use patterns of migratory animals.[4-7] Long-term tissue collections and museum specimens are extremely useful for reconstructing past food webs and addressing questions about ecological changes over time. However, samples are often preserved in chemicals such as ethanol or formaldehyde. These preservatives prevent bacterial growth and preserve the structural integrity of tissues, which allows for morphological examination of preserved organisms.[8,9] If one is interested in reconstructing the ecology of an organism using isotopic analyses from archived specimens, it is therefore imperative to understand if preservatives and long-term fixatives can alter $\delta^{13}C$ and $\delta^{15}N$ values.

In addition to the measurement of isotope ratios from bulk tissues or whole organisms (i.e., bulk isotope analysis), there is an increasing use of compound-specific isotope analysis of amino acids (CSIA-AA) in ecological studies. Using this approach, we measure the $\delta^{15}N$ values of amino acids in a consumer's tissue to obtain a proxy for $\delta^{15}N$ values at the base of the food web and estimate the trophic positions of consumers.[9] Certain amino acids (e.g., alanine, glutamic acid) are isotopically fractionated during transamination, causing a consumer's tissue to become enriched in ^{15}N relative to its prey. These are called 'trophic' amino acids, as they reflect the diet of the consumer.[10] Conversely, 'source' amino acids (e.g., phenylalanine, lysine) show little isotopic fractionation, as their primary metabolic pathway does not cleave or form nitrogen bonds. Thus, source amino acids can be used to measure the $\delta^{15}N$ values of primary producers,[10,11] which is governed by regional patterns in nitrogen cycling processes (e.g., nitrogen fixation, denitrification, nitrification, nitrate assimilation). We can interpret ecological relationships using these basic patterns in amino acids, and with some consideration of the physiological and biochemical reactions that also influence isotope ratios.[12,13]

Amino acid $\delta^{15}N$ values can therefore help determine whether variation in bulk $\delta^{15}N$ values from consumers reflect dietary differences or biogeochemical changes that occurred at the base of the food web and influenced the bulk isotopic ratios of consumers. Not only has CSIA-AA been used to successfully evaluate the trophic positions of consumers and trace nitrogen flow through ecosystems, but in recent years it has been used to reconstruct past food webs and detect environmental variability and changes in oceanographic conditions.[14,15]

Since bulk isotope analysis has been a prevalent tool in ecological studies for several decades, many previous studies have tested the effects of chemical preservation on bulk isotope ratios, particularly for tissues preserved short-term (several months to several years). However, although the application of CSIA-AA has rapidly increased in the last decade,[16] there has been little effort[17,18] to examine these effects on amino acid $\delta^{15}N$ or $\delta^{13}C$ values. Most studies use CSIA-AA as a tool to address broad ecological or biogeochemical objectives rather than to address key methodological questions that are essential for its application. CSIA-AA is also expensive and time-consuming compared with bulk isotope analysis, which may contribute to the lack of methodological studies on the effects of chemical preservation on amino acid isotope ratios.[17,18] However, such information is crucially important for future CSIA-AA studies, particularly as it becomes a widely used analytical tool.

Previous studies have identified the potential mechanisms through which chemical fixatives and preservatives can alter stable isotope ratios.[19-22] Tissues can either take up carbon or nitrogen from the fluid, or the preservative can promote the leaching of carbon or nitrogen from tissue. Since formaldehyde and ethanol do not contain nitrogen, they cannot add nitrogen to samples, although preservatives can break bonds to nitrogen atoms in tissues, which may affect stable isotope ratios.

Our study focuses on two common chemical preservatives, formaldehyde and ethanol. Formaldehyde is a non-coagulant fixing agent that reacts with proteins to form intermolecular cross-links,[8,23] which preserve the cellular organization and structure of the tissue. Cross-linking of proteins and other molecules occurs when a methylene bridge (-CH_2-) is formed by an aldehyde combining with proteins, usually by binding to nitrogen[8,9,23] (Figure 1). Since formaldehyde has a low molecular weight (30 g/mol), it can quickly penetrate and bind to tissue, although the formation of methylene bridges occurs more slowly. Once the methylene bridges are formed, they are thought to remain stable; however, if they are not formed, unfixed proteins can be denatured and coagulated by ethanol or other dehydrating solvents.[8,9,23] If carbon is added to the tissue when formaldehyde binds to protein, the direction and magnitude of change in $\delta^{13}C$ values would depend on the isotopic composition of the formaldehyde, which may vary depending on the chemical stock, relative to the $\delta^{13}C$ value of the tissue.[19,22,24] Some studies have suggested an alternative mechanism for the effects of formaldehyde on stable isotope ratios, where formaldehyde can hydrolyze proteins and promote the leaching of compounds that are enriched in ^{13}C compared with lipids, which leaves the preserved tissue relatively depleted in ^{13}C, thus altering the $\delta^{13}C$ values.

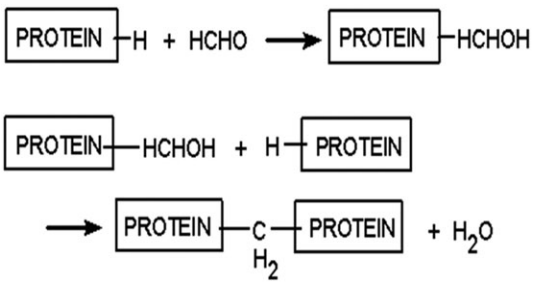

FIGURE 1 Chemical reaction of formaldehyde (HCHOOH) cross-linking proteins through the formation of a methylene bridge (=CH_2) (modified from Kiernan[23])

Ethanol is a non-cross-linking reagent, which preserves nucleic acids better than aldehydes, and is therefore a common preservation medium for samples that may be used for genetic analyses. However, ethanol may also affect the carbon content and $\delta^{13}C$ values of tissues.[9] Ethanol can extract lipids and partition them into ethanol based on their solubility.[25] Lipids are depleted in ^{13}C relative to proteins, carbohydrates, and nucleic acids that comprise animal tissues.[22,26] If ethanol removes lipids from samples, we would expect a decrease in the carbon content and C/N ratios, and an increase in the $\delta^{13}C$ values of preserved samples.[27]

Although previous studies have examined the mechanisms through which chemical preservation can alter bulk isotope ratios, the results have been somewhat inconsistent across taxa, preservation method, and length of preservation. Generally, previous research (see[19,20,22] for comprehensive reviews on preservation effects on bulk isotope ratios) demonstrates that formaldehyde or ethanol preservation has little effect on bulk $\delta^{15}N$ values (<1.5‰), but can substantially alter bulk $\delta^{13}C$ values, where the direction and magnitude of change vary across studies.[19-22,28,29] The few studies (e.g.,[18]) that have examined preservation effects on amino acid $\delta^{15}N$ values have reported no effect, but these studies have been limited in scope, preservation technique, time (~ 1 year), and taxa (fish).

The primary motivation for this study is to perform experiments that test the effects of preservation on amino acid $\delta^{15}N$ values and contribute to a better overall understanding of the effects of chemical preservation techniques on stable isotope ratios. For the bulk component of our study, we add to existing data and aim to converge on likely mechanistic explanations of preservation effects, as previous studies have reported somewhat conflicting results. In addition, we have the rare opportunity to examine the effects of longer-term (>10 years) preservation on isotope ratios. These experiments are logistically challenging, as they require sampling of paired specimens that were stored using different methods of preservation for many years. Testing the potential long-term effects of formaldehyde on stable isotope ratios is particularly important, as formaldehyde-preserved specimens are common in government, university, and museum collections. These specimens may be particularly useful, as an increasingly common objective in CSIA-AA studies is to reconstruct past trophic baselines and oceanic conditions based on source amino acid $\delta^{15}N$ values.[15]

In this study, we first evaluate the effects of formaldehyde fixation followed by storage in ethanol for up to 2 years on bulk $\delta^{15}N$ and $\delta^{13}C$ values and amino acid $\delta^{15}N$ values from tuna and squid. We also determine the $\delta^{15}N$ and $\delta^{13}C$ values from copepods preserved frozen or stored in formaldehyde for 24–25 years. Since carbon from formaldehyde can be added to tissues during the fixation process, we expect to observe higher carbon content and carbon-to-nitrogen (C/N) ratios, and a change in $\delta^{13}C$ values, in chemically preserved tissues compared with frozen samples. Based on previous studies, we hypothesize that formaldehyde and formaldehyde-ethanol preservation will have little effect on the $\delta^{15}N$ values of organismal tissues, including those of amino acids. We expect that patterns in amino acid $\delta^{15}N$ values will mirror those found in bulk $\delta^{15}N$ values, which can aid in our prediction and application of how chemical preservatives alter bulk and CSIA-AA samples.

2 | EXPERIMENTAL

2.1 | Sampling method and preservation techniques

It is often necessary to preserve samples following their collection, and freezing is a widely used method of storing samples with minimal effects on stable isotope ratios. However, freezing is often unavailable during field studies and is typically not the method used for archiving museum specimens when the preservation of anatomical features is required. To investigate effects of formaldehyde fixation-ethanol preservation on organismal isotope ratios, we compared this protocol with preservation via freezing, using three specimens each of yellowfin tuna (*Thunnus albacares*) and Humboldt squid (*Dosidicus gigas*), both of which are commercially and ecologically significant consumers in marine food webs. Tuna and squid specimens were collected on recreational fishing vessels and frozen intact until later processing. To test for differences between frozen and formaldehyde-ethanol preserved samples within each specimen, we collected a muscle tissue sample from each individual ($n = 3$ *T. albacares* and $n = 3$ *D. gigas*) and divided each sample into six, approximately 1 g subsamples. We collected samples from the dorsal side adjacent to the dorsal fin for each *T. albacares* and from the dorsal mantle muscle (with skin removed) for each *D. gigas*. We followed the protocol for preservation of tissues from Scripps Institution of Oceanography's marine vertebrate collection. All non-frozen samples were initially fixed in 3.7% formaldehyde (the deionized water to 37% formaldehyde ratio was 1:9) for approximately 48 h. To wash formalin out of the tissue, we performed rinses, where samples were placed in deionized water for 48 h and the deionized water was replaced after 24 h. The samples were then stored in 95% ethanol for specific time intervals: 1 week, 1 month, 3 months, 6 months, and 2 years.

Following the analysis of isotopic data from the formaldehyde-ethanol experiment, we separately tested the effects of formaldehyde and ethanol on tuna and squid muscle. Our sampling procedures were similar to our initial experiment, although a different bottle of formaldehyde was used, as this experiment was conducted 2 years after our initial experiment. We recognize that the $\delta^{13}C$ values of formaldehyde can vary between individual bottles and suppliers. Since the 2-year experiment indicated that the length of preservation was not a significant factor, we collected three 1-g muscle samples from new specimens (tuna: $n = 3$; squid: $n = 3$). The first sample from each specimen was frozen, the second was stored in formaldehyde for 1 month, and the third sample was stored in ethanol for 1 month. For both experiments, after samples were removed from the preservatives or thawed, they were lyophilized for 24 h, homogenized, and weighed into tin capsules for bulk stable nitrogen and carbon isotope analysis.

We also investigated the effects of longer-term storage in formaldehyde on bulk isotope ratios from marine zooplankton, as very few studies have examined these effects on specimens preserved for longer than several years. These samples were collected by the California Cooperative Oceanic Fisheries Investigations (CalCOFI; www.calcofi.org) off central California in March and April of 1991 and 1992 on lines 80 and 83 of the CalCOFI sampling grid (see calcofi.org for map of sampling locations). Zooplankton were collected by bongo tows, consisting of two nets with a 0.71-m diameter[31] that uses oblique tows (the detailed method description for bongo deployment can be found on the Southwest Fisheries Science Center website at http://swfsc.noaa.gov/textblock.aspx?Division=FRD&id=1341). Following collection, one bongo net was fixed in a 3.7% formaldehyde-seawater solution, buffered with sodium borate, and the other was frozen at −80°C until further analysis.

In 2016, we slowly thawed frozen samples and selected adult females of two copepod species, *Calanus pacificus* and *Eualanus californicus*. These species were selected for several reasons. First, *C. pacificus* and *E. californicus* are abundant zooplankton species in our collection area and are easily identifiable, even when smaller anatomical structures are obscured by the freezing and thawing process. Second, we could compare our results with those from a previous experiment that tested the effects of 11 years of preservation on the bulk isotope ratios of these same species.[30] We measured the isotope ratios of these zooplankton species from five paired bongo samples and, due to the small body size of copepods, we separately pooled 20 *C. pacificus* and 10 *E. californicus* from each sample to ensure that we had an adequate mass for each species, required for both bulk and CSIA-AA. We lyophilized the samples for 24 h, then homogenized and weighed them into tin capsules for stable isotope analyses.

To evaluate the effects of formaldehyde fixation-ethanol preservation on the amino acid δ^{15}N values, we selected samples for CSIA-AA based on the results of the bulk isotope analysis. We selected two each of the *T. albacares* and *D. gigas* specimens and analyzed matched samples that were either frozen or preserved for 2 years following the formaldehyde-ethanol protocol, and one each of the tuna and squid that were preserved for 6 months in formaldehyde-ethanol ($n = 10$). For zooplankton, we selected two frozen *C. pacificus* samples and their paired formaldehyde-preserved samples ($n = 4$) for CSIA-AA.

2.2 | Sample and data analysis

We measured δ^{15}N and δ^{13}C values, %C, %N, and the ratio of carbon to nitrogen (C/N molar ratio) at the Stable Isotope Laboratory at the University of California, Santa Cruz (Santa Cruz, CA, USA). We report stable isotope ratios in δ notation relative to PDB and atmospheric N_2 for carbon and nitrogen, respectively. The subset of *T. albacares*, *D. gigas*, and *C. pacificus* samples selected for nitrogen CSIA-AA was analyzed at the University of Hawaii Stable Isotope Biogeochemistry Laboratories (Honolulu, HI, USA). Samples were prepared for CSIA-AA by acid hydrolysis followed by derivatization of the amino acids (see[10,32] for details). Samples were hydrolyzed (6 N HCl, 150°C for 70 min), esterified (4:1 isopropanol/acetyl chloride), derivatized (3:1 methylene chloride/trifluoroacetyl anhydride), and then measured by GC/IRMS (Gas Chromatography/Isotope Ratio Mass Spectrometry) using a Trace gas chromatograph (Thermo Fisher, Waltham, MA, USA) and a Delta V Plus isotope ratio mass spectrometer (Thermo Fisher) through a GC-C III (Thermo Fisher) combustion furnace (980°C), a reduction furnace (680°C), and a liquid nitrogen cold trap. Samples were injected (split/splitless, 5:1 split ratio) with a 180°C injector temperature and a constant helium flow rate of 1.4 mL min^{-1}.

We analyzed samples for CSIA-AA in triplicate. For quality control, we co-injected each sample with internal reference compounds (norleucine and aminoadipic acid) with known δ^{15}N values and corrected the sample amino acid δ^{15}N values relative to these internal references. We also measured an amino acid suite of 12 amino acids with known δ^{15}N values, before and after each triplicate sample run to ensure that the δ^{15}N measurements were accurate and within <1‰ of their known values. Our objective was to measure the δ^{15}N values of 18 amino acids; however, some amino acids were not abundant enough in our samples to quantify their δ^{15}N values. Here, we report results from 13 amino acids grouped into three categories: metabolic, source, and trophic amino acids.[33] The analytical errors (±1 standard deviation) for bulk isotope ratios were ±0.1‰. The amino acid δ^{15}N values were based on triplicate sample analysis, where the analytical errors (±1 SD) were mostly <1.0‰ but ranged from <0.1 to 1.4‰.

We conducted data analyses using the statistical software R.[34] We tested the effects of formaldehyde-ethanol preservation on *T. albacares* and *D. gigas* muscle first by testing the potential for an interactive effect of time and species (δ^{15}N ~Time*Species and δ^{13}C ~Time*Species) to determine whether we could group samples from *T. albacares* and *D. gigas* together or analyze them independently. We then used one-way repeated measures ANOVA, using the 'Anova' function in the *car* package in R, to evaluate the isotopic differences between frozen samples and those that were chemically preserved for different lengths of time. For the long-term zooplankton formaldehyde experiment, we used paired t-tests to compare the δ^{13}C and δ^{15}N values between frozen and formaldehyde-preserved *C. pacificus* and *E. californicus*. For the amino acid δ^{15}N data, we used paired t-tests to compare isotope ratios from frozen and formaldehyde-ethanol preserved samples and used a sequential Bonferroni (Holm-Bonferroni) correction for multiple-comparisons.

3 | RESULTS

3.1 | Tuna and squid preservation experiment (bulk isotope ratios)

We found significant differences between frozen and chemically preserved tissues (Figure 2, Table 1). There was no significant interaction between time and species, so we analyzed the bulk δ^{15}N

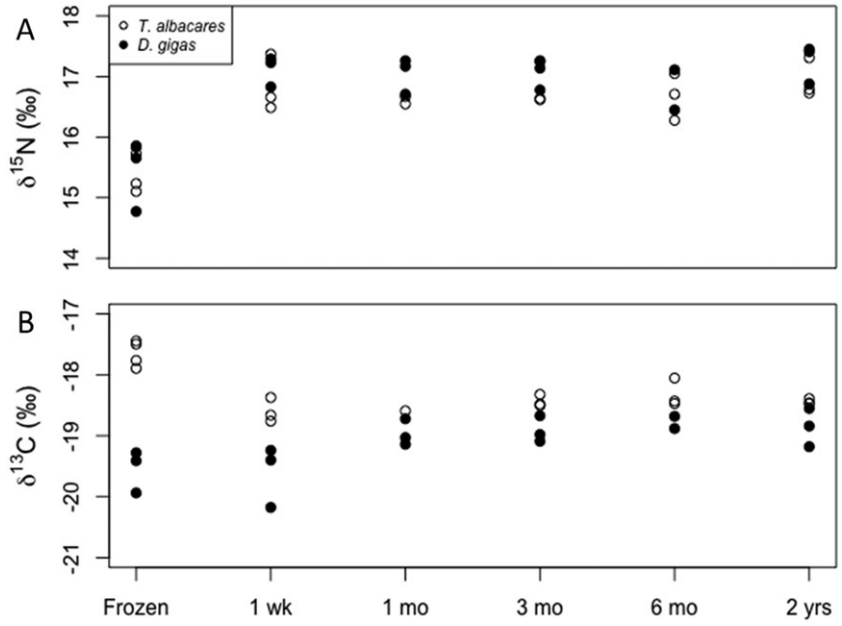

FIGURE 2 Bulk (A) $\delta^{15}N$ values and (B) $\delta^{13}C$ values from frozen tuna (*Thunnus albacares*) and squid (*Dosidicus gigas*) muscle, and muscle fixed in formaldehyde and stored in ethanol for 1 week, 1 month, 3 months, 6 months, and 2 years

TABLE 1 Mean with standard deviations (± SD) values of $\delta^{13}C$, %C, $\delta^{15}N$, %N, and C/N molar ratios of frozen versus chemically preserved tissues from three experiments. Values for *Dosidicus gigas* ($n = 3$) and *Thunnus albacares* ($n = 3$) represent means for frozen samples versus those fixed in formaldehyde and stored in ethanol (form-ethanol) for 1 week, 1 month, 3 months, 6 months, and 2 years. In the second experiment, *D. gigas* ($n = 3$) and *T. albacares* ($n = 3$) tissues were either frozen or stored in formaldehyde or ethanol for 1 month. The copepods *Calanus pacificus* ($n = 10$; five frozen, five preserved) and *Eucalanus californicus* ($n = 10$; five frozen, five preserved) were either stored in formaldehyde or frozen for 24–25 years. Δ represent the difference in values of $\delta^{13}C$, %C, $\delta^{15}N$, %N, and C/N ratios between frozen and chemically preserved samples, where arrows indicate the direction of change, and an asterisk indicates statistical significance ($p < 0.05$)

Species and treatment	$\delta^{13}C$	%C	$\delta^{15}N$	%N	C/N	$\Delta\delta^{13}C$	Δ%C	$\Delta\delta^{15}N$	Δ%N	ΔC/N
D. gigas										
Frozen	−19.5 ± 0.3	48.7 ± 9.5	15.4 ± 0.6	15.1 ± 2.8	3.8 ± 0.0					
Form-ethanol	−19.0 ± 0.3	57.5 ± 8.8	17.0 ± 0.1	18.3 ± 2.6	3.7 ± 0.1	↑0.5*	↑8.8	↑1.6*	↑3.2	↓0.1*
T. albacares										
Frozen	−17.7 ± 0.2	58.2 ± 12.4	15.4 ± 0.4	18.4 ± 3.7	3.6 ± 0.1					
Form-ethanol	−18.5 ± 0.1	53.1 ± 5.2	16.8 ± 0.3	16.5 ± 1.7	3.7 ± 0.3	↓0.8*	↓5.2	↑1.4*	↓1.8	↑0.1
D. Gigas										
Frozen	−19.0 ± 0.2	44.5 ± 1.4	11.6 ± 0.3	13.8 ± 0.7	3.8 ± 0.1					
Formaldehyde	−20.2 ± 0.6	59.7 ± 6.1	12.7 ± 0.8	15.3 ± 2.1	4.6 ± 0.1	↓1.2*	↑15.2	↑1.1	↑1.5	↑0.8*
Ethanol	−17.5 ± 0.3	65.1 ± 26.2	12.9 ± 0.7	21.0 ± 8.5	3.6 ± 0.0	↑1.5*	↑20.6	↑1.3	↑7.2	↓0.2
T. Albacares										
Frozen	−16.5 ± 0.1	51.1 ± 6.5	12.1 ± 0.1	16.4 ± 2.2	3.6 ± 0.0					
Formaldehyde	−18.0 ± 0.2	58.1 ± 5.7	13.4 ± 0.2	17.5 ± 1.7	3.9 ± 0.0	↓1.5*	↑7.0	↑1.3*	↑1.1	↑0.3*
Ethanol	−16.5 ± 0.1	61.9 ± 11.1	13.0 ± 0.1	20.4 ± 3.7	3.5 ± 0.0	↑0.1	↑10.9	↑1.1*	↑4.0	↓0.1
C. pacificus										
Frozen	−18.3 ± 0.8	34.3 ± 2.4	10.1 ± 0.5	8.2 ± 0.6	4.9 ± 0.1					
Formaldehyde	−20.5 ± 0.6	33.3 ± 3.4	10.0 ± 0.6	7.9 ± 0.9	4.9 ± 0.3	↓2.2*	↓1.0	↓0.1	↓0.3	0.0
E. californicus										
Frozen	−20.1 ± 0.9	25.9 ± 7.1	9.5 ± 0.3	4.1 ± 1.2	7.5 ± 1.8					
Formaldehyde	−21.1 ± 1.0	20.2 ± 4.4	9.7 ± 0.2	4.3 ± 1.1	5.7 ± 0.5	↓1.0*	↓5.7	↑0.2	↑0.2	↓1.8*

results of *T. albacares* and *D. gigas* together and found a consistent, significant increase in $\delta^{15}N$ values ($F_{(1,32)} = 16.94$, $p < 0.001$) with formaldehyde-ethanol preservation compared with frozen tissues (Figure 2A). The mean bulk $\delta^{15}N$ values from the formaldehyde-ethanol preserved samples of *D. gigas* and *T. albacares* were higher than those of the frozen samples by 1.5‰. Tukey's pairwise

comparisons demonstrated that the $\delta^{15}N$ values of frozen samples were significantly lower than those of formaldehyde-ethanol preserved samples (all $p < 0.0001$), but there were no differences between the $\delta^{15}N$ values of frozen tissues and tissues stored only in ethanol over the five different time periods ($p > 0.1$).

The effects of formaldehyde-ethanol preservation on the $\delta^{13}C$ values from *T. albacares* and *D. gigas* muscle were more variable. There was a significant interaction between species (Adj. $R^2 = 0.71$, $p < 0.0001$), so we measured the $\delta^{13}C$ values separately for each. We found opposite trends, where the $\delta^{13}C$ values of formaldehyde-ethanol preserved *D. gigas* muscle were significantly higher than those of frozen tissue, by a mean of 0.5‰ ($F_{(1,14)} = 19.45$, $p < 0.001$; Figure 2B; Table 1), whereas the $\delta^{13}C$ values from formaldehyde-ethanol preserved *T. albacares* tissues were lower by 0.6‰ to 0.9‰ than those of frozen tissues ($F_{(1,14)} = 5.23$, $p = 0.04$; Tukey's pairwise comparisons: $p \leq 0.02$; Figure 2B). There were no differences in the $\delta^{13}C$ values of frozen tissues from those of tissues stored in ethanol over the five different time periods ($p > 0.1$).

We found no differences in %C or %N between formaldehyde-ethanol preserved and frozen samples for *T. albacares* (%C: $F_{(1,15)} = 0.90$, $p = 0.36$; %N: $F_{(1,15)} = 0.96$, $p = 0.34$) or *D. gigas* (% C: $F_{(1,14)} = 0.56$, $p > 0.1$; % N: $F_{(1,14)} = 0.27$, $p > 0.1$). We used C/N molar ratios as a proxy for lipid content, where higher ratios indicate more lipid-rich tissues. The C/N ratios for squids and tuna were low (<4), and were fairly uniform, ranging from 3.6 to 3.8 for both species. There was no difference in C/N ratios between formaldehyde-ethanol and frozen samples for *T. albacares* ($F_{(1,15)} = 0.38$, $p > 0.1$). However, the C/N ratios of *D. gigas* were lower in formaldehyde-ethanol preserved samples than in frozen samples ($F_{(1,14)} = 9.50$, $p < 0.01$), although the change was small (0.1; Table 1).

In a subsequent experiment where we separately tested the effects of formaldehyde and ethanol on squid and tuna muscle tissue, we found that the $\delta^{15}N$ values significantly increased with preservation in both formaldehyde and ethanol by ~1‰ (Table S1, supporting information). The effects of formaldehyde or ethanol preservation on $\delta^{13}C$ values were mixed; the $\delta^{13}C$ values of formaldehyde-fixed tissues decreased in both species but those from samples preserved in ethanol increased by 1.5‰ in *D. gigas* and did not change in *T. albacares* (Table S1, supporting information). The C/N ratios of *D. gigas* and *T. albacares* increased with formaldehyde fixation but there was no change in the C/N ratios with ethanol preservation (Table 1).

3.2 | Long-term formaldehyde preservation of zooplankton (bulk isotope ratios)

The $\delta^{15}N$ values of the paired copepod samples that were preserved in buffered formaldehyde for 24–25 years were not significantly different from those from frozen copepods: *C. pacificus* (Mean ± SD: 10.1 ± 0.5 vs 10.0 ± 0.6, respectively; paired t-test: $t = 103$, df = 4, $p = 0.36$), and *E. californicus* (9.5 ± 0.3 vs 9.7 ± 0.2, respectively; paired t-test: $t = -0.80$, df = 4, $p = 0.47$; Figure 3A, Table 1). However, the $\delta^{13}C$ values of formaldehyde-preserved copepods were lower for *C. pacificus* ($t = 8.22$, df = 4, $p = 0.001$; mean difference = 2.2‰) and *E. californicus* ($t = 3.63$, df = 4, $p = 0.02$; mean difference 1.0‰; Figure 3B, Table 1) than for the frozen samples. The C/N ratios were overall higher and more variable for *E. californicus* (range: 4.9–10.5, mean ± SD: 6.6 ± 1.6) than for *C. pacificus* (range: 4.5–5.3, mean ± SD: 4.9 ± 0.2). The C/N ratios were lower in formaldehyde-preserved samples of *E. californicus* ($p < 0.01$) and there was a decrease in %C, although it was not statistically significant ($p = 0.06$). There was no difference in %N, %C, or C/N for *C. pacificus* (all $p > 0.1$) between frozen and formaldehyde-preserved samples (Table 1).

3.3 | Preservation effects on amino acid $\delta^{15}N$ values

Using paired t-tests, we found no significant differences between the amino acid $\delta^{15}N$ values from frozen and chemically preserved tissues (all $p > 0.05$). The differences in $\delta^{15}N$ values between frozen and chemically preserved samples were generally <1‰, which lies within the error of CSIA-AA (Figure 4, Table 2). However, the $\delta^{15}N$ values of several amino acids, including valine and phenylalanine, were altered by more than 1.0‰. Most notably, the $\delta^{15}N$ values of phenylalanine, the canonical source amino acid, were, on average, 3.6‰ higher in preserved tuna muscle and 1.8‰ higher in preserved *D. gigas* muscle than those from their frozen counterparts (Table 2). Our results were similar for *C. pacificus*, where 25 years of formaldehyde preservation

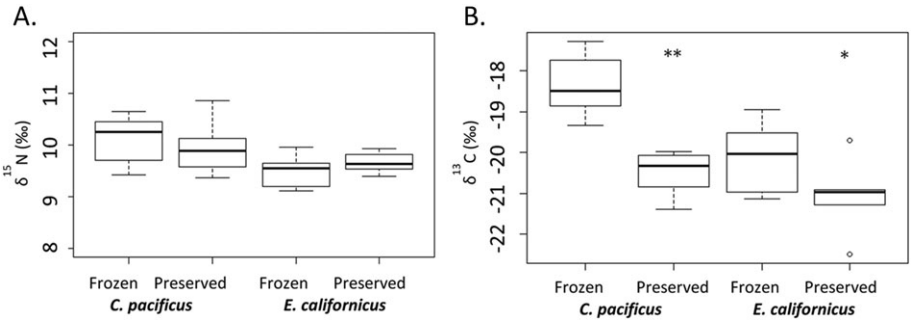

FIGURE 3 Bulk (A) $\delta^{15}N$ and (B) $\delta^{13}C$ values from the copepods *Calanus pacificus* and *Eucalanus californicus*, where paired samples were frozen or formaldehyde-preserved for 24–25 years. Mean ± 95% SD. An asterisk (*) indicates significant differences with $p < 0.05$ and ** indicates $p < 0.01$

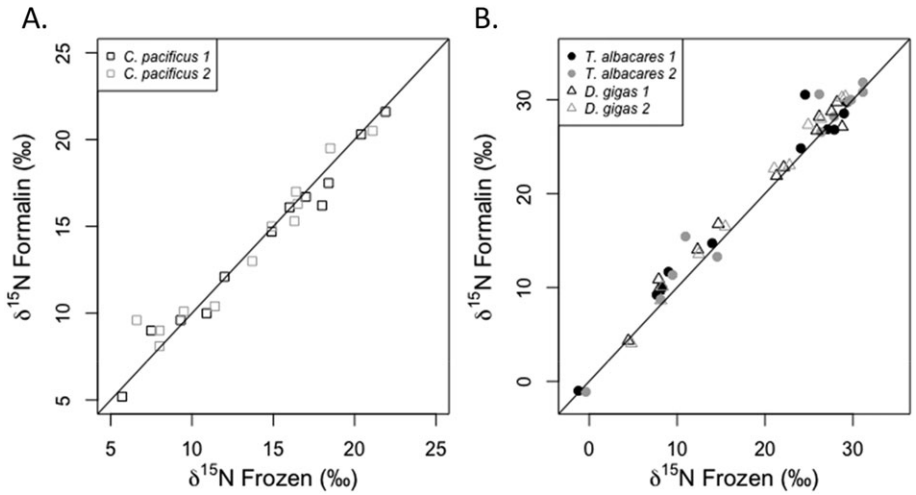

FIGURE 4 The $\delta^{15}N$ values of amino acids of frozen versus formaldehyde-preserved tissues from paired samples for (A) *Calanus pacificus* ($n = 4$), which were either frozen or stored in formaldehyde for 25 years and (B) tuna (*Thunnus albacares*; $n = 4$) and squid (*Dosidicus gigas*; $n = 4$) muscle, where the chemically preserved tissues were fixed in formaldehyde and stored for 2 years in ethanol. The black 1:1 line demonstrates where $\delta^{15}N$ values were expected to be if they were not altered by chemical preservation. The sample numbers correspond to those in Table 2

TABLE 2 Amino acid $\delta^{15}N$ values of paired *Thunnus albacares* ($n = 5$) and *Dosidicus gigas* ($n = 5$) muscle samples either frozen or initially fixed in formaldehyde then stored in ethanol (F/E) for 6 months (6 mo) or 2 years (2 yr), and paired *Calanus pacificus* samples that were either frozen or preserved in formaldehyde (F) for 25 years. The $\Delta\delta^{15}N$ values represent the differences in $\delta^{15}N$ values between frozen samples and those chemically preserved for 2 years. We categorized amino acids into metabolic, source, or trophic and we used standard three letter abbreviations for each amino acid. We report values for the 13 amino acids consistently detected on chromatographs, where Nd = not detected and NA = not applicable

		Metabolic	Source					Trophic						
Sample and treatment		Thr	Gly	Lys	Met	Phe	Ser	Ala	Asp	Glu	Leu	Iso	Pro	Val
T. albacares 1	Frozen	−22.4	−1.2	7.7	14.0	9.0	8.3	29.3	29.0	27.9	27.1	Nd	24.1	24.6
T. albacares 1	F/E (2 yr)	−22.0	−1.0	9.2	14.7	11.7	9.9	29.7	28.5	26.8	26.9	26.8	24.8	30.5
Δ $\delta^{15}N$		**0.4**	**0.2**	**1.5**	**0.7**	**2.7**	**1.6**	**0.4**	**−0.4**	**−1.1**	**−0.3**	**NA**	**0.8**	**6.0**
T. albacares 2	Frozen	−22.9	−0.4	8.1	14.6	10.9	9.5	31.1	31.1	29.8	27.8	29.5	26.4	26.2
T. albacares 2	F/E (6 mo)	−21.8	−1.8	8.4	12.3	12.3	9.7	28.9	29.1	27.9	26.4	27.9	24.9	26.1
T. albacares 2	F/E (2 yr)	−20.0	−1.1	8.7	13.3	15.4	11.3	30.8	31.8	30.0	28.2	29.9	26.5	30.6
Δ $\delta^{15}N$		**2.9**	**−0.7**	**0.6**	**−1.3**	**4.5**	**1.8**	**−0.3**	**0.7**	**0.2**	**0.4**	**0.3**	**0.2**	**4.4**
D. gigas 2	Frozen	−12.9	4.8	8.2	15.5	8.3	12.5	28.7	21.0	24.9	26.4	Nd	22.8	29.1
D. gigas 2	F/E (2 yr)	−15.5	4.0	8.6	16.5	10.1	13.6	30.2	22.7	27.3	28.0	Nd	23.0	30.3
Δ $\delta^{15}N$		**−2.6**	**−0.7**	**0.5**	**1.0**	**1.8**	**1.1**	**1.5**	**1.6**	**2.4**	**1.6**	**NA**	**0.2**	**1.2**
D. gigas 1	Frozen	−15.8	4.4	7.9	14.7	7.9	12.3	28.2	21.3	25.9	26.2	27.6	22.1	28.8
D. gigas 1	F/E (6 mo)	−17.3	2.7	10.1	17.4	11.9	12.9	27.1	20.0	24.2	25.6	25.6	20.7	26.9
D. gigas 1	F/E (2 yr)	−16.9	4.3	10.9	16.8	9.7	14.0	29.7	21.9	26.7	28.2	28.7	22.8	27.1
Δ $\delta^{15}N$		**−1.1**	**−0.1**	**3.0**	**2.1**	**1.8**	**1.7**	**1.6**	**0.6**	**0.8**	**2.0**	**1.2**	**0.7**	**−1.7**
C. pacificus 1	Frozen	7.5	12.0	9.3	Nd	5.7	10.9	21.9	18.4	20.4	14.9	16.0	17.0	18.0
C. pacificus 1	F (25 yr)	9	12.1	9.6	Nd	5.2	10.0	21.6	17.5	20.3	14.7	16.1	16.7	16.2
Δ $\delta^{15}N$		**1.5**	**−0.1**	**0.3**	**NA**	**−0.5**	**−0.9**	**−0.3**	**−0.9**	**−0.1**	**−0.2**	**0.1**	**−0.3**	**−1.8**
C. pacificus 2	Frozen	8.0	11.4	8.0	Nd	6.6	9.5	21.1	16.4	18.5	13.7	14.9	16.3	16.5
C. pacificus 2	F (25 yr)	9.0	10.4	8.1	Nd	9.6	10.1	20.5	17.0	19.5	13.0	15.0	15.3	16.3
Δ $\delta^{15}N$		**1.0**	**−1.0**	**0.1**	**NA**	**3.0**	**0.6**	**−0.6**	**0.6**	**1.0**	**−0.7**	**0.1**	**−1.0**	**−0.2**

minimally altered the $\delta^{15}N$ values of most amino acids, and paired t-tests showed no differences between formaldehyde-preserved and frozen samples (p values >0.1). However, the $\delta^{15}N$ value of phenylalanine was 3.0‰ higher in one of two formalin-preserved *C. pacificus* samples than in the two frozen samples (Figure 4, Table 2).

4 | DISCUSSION

4.1 | Bulk $\delta^{15}N$ values

Several previous studies have reported small increases (~1–1.5‰) in $\delta^{15}N$ values associated with formaldehyde or ethanol preservation.[19,21,24,26] Our two-year experiment bolsters these previous findings, as we report an increase in bulk $\delta^{15}N$ values in the tissues from both *Thunnus albacares* and *Dosidicus gigas* preserved in formaldehyde and ethanol. In addition, in the follow-up study where we independently tested the effects of formaldehyde and ethanol preservation on these species, our results were similar. The $\delta^{15}N$ values of chemically preserved tissues were also ~1‰ higher than those of frozen samples.

For the long-term (24–25 years) formaldehyde experiment on copepods, we found no differences in the $\delta^{15}N$ values between frozen and formaldehyde-preserved samples, indicating that the $\delta^{15}N$ values of copepods are minimally altered by long-term storage in formaldehyde. Our results confirm findings from a previous study with these same two species of copepods after 11 years preservation[30] and a shorter-term study of calanoid copepods stored in formaldehyde for 1 year.[24] Those previous studies focused only on bulk isotope analysis. The CSIA-AA component of our study will be useful for interpreting these bulk results and those from previous copepod preservation experiments.

Although the bulk $\delta^{15}N$ values for some species were altered by chemical preservation, the differences were consistent and relatively small compared with the ~3–5‰ changes that are typically used to detect trophic position changes in food web studies. Our data adds to previous isotope measurements in preservation studies, shows consistent trends across multiple taxa, and largely confirms previous results.

There is no easily identifiable mechanism to explain the small observed changes in $\delta^{15}N$ values that have been reported in the literature and that we observed for *T. albacares* and *D. gigas*. Since formaldehyde does not contain nitrogen, there is no mechanism by which N could be incorporated into the tissue from formaldehyde. However, formaldehyde preservation could alter $\delta^{15}N$ values if C–N bonds are broken during preservation, which is not expected as theoretically C–N bonds are not cleaved during fixation. We found no evidence of this, as there were no changes in the N content between frozen and formaldehyde-ethanol preserved samples for either species (see below). It is possible that water-soluble, N-containing compounds (e.g., free amino acids or amines) were extracted from the tissues, causing the observed increase in $\delta^{15}N$ values, although future studies are necessary to explicitly test this.

4.2 | Bulk $\delta^{13}C$ values

The effects of formaldehyde and ethanol preservation in carbon isotope ratios were variable across taxa and treatments, which makes it difficult to pinpoint one mechanism driving these observed differences. The most likely explanation is that formaldehyde can alter $\delta^{13}C$ values by adding carbon to tissues.[19,20,24] The direction and magnitude of change in $\delta^{13}C$ values depend upon the amount of carbon added to the sample and the relative difference in the $\delta^{13}C$ values of the tissue and formaldehyde.

Some studies suggest an alternative mechanism for formaldehyde altering $\delta^{13}C$ values, where compounds leach out of the tissue upon formaldehyde fixation.[26,35] If compounds enriched in ^{13}C (e.g., proteins compared with lipids) preferentially leach out of the tissue, we may observe a relative increase in ^{12}C and decrease in $\delta^{13}C$ values with formaldehyde preservation.[24]

The formaldehyde-ethanol treatment significantly altered the $\delta^{13}C$ values of both *T. albacares* and *D. gigas*. The magnitude of change was similar in both species (<1‰), but the direction of change differed, which may suggest that the $\delta^{13}C$ value of the formaldehyde was between those of the *D. gigas* and *T. albacares* tissues. We hypothesized that carbon was added from the formaldehyde to the tissue during the fixation process. However, if carbon was added to the tissues, we would expect an increase in %C and C/N ratios in the preserved tissues, which we largely did not find. Although formaldehyde can add carbon to samples, ethanol solubilizes lipids. Therefore, it is possible that long-term storage in ethanol masked an increase in C/N ratios by the removal of carbon. The mechanistic explanations are difficult to disentangle when tissues are preserved in multiple chemical preservatives that alter tissues in different ways. In future studies, one way to address this question is to examine whether lipids are added to the ethanol in which the samples were preserved.

To test the independent effects of formaldehyde and ethanol on tuna and squid muscle tissue, we separately preserved tissues in formalin and ethanol for one month. For tissues preserved in formaldehyde only, we found a decrease in $\delta^{13}C$ values and an increase in %C and C/N ratios for both *T. albacares* and *D. gigas*, which provides evidence that formaldehyde fixation adds ^{12}C-enriched carbon to the tissues. Since a different bottle of formaldehyde was used for this second experiment, we surmise that the tuna and squid tissues had $\delta^{13}C$ values greater than that of the formaldehyde used and therefore we saw consistent trends between species.

In the long-term zooplankton experiment, the $\delta^{13}C$ values of copepods also decreased with formaldehyde preservation, but the difference between frozen and chemically preserved samples was greater for *C. pacificus* than for *E. californicus*, which is consistent with a previous study.[18] The differences in the magnitude of change between *C. pacificus* and *E. californicus* can probably be explained by isotope mass balance, where the $\delta^{13}C$ values of preserved tissues converge on the $\delta^{13}C$ value of the formaldehyde used. These results generally agree with those from the tuna and squid experiment and

also bolster results from previous studies that reported a decrease in $\delta^{13}C$ values with formaldehyde preservation.[20,22,24] Specifically, one study reported a decrease of ~1‰ in the $\delta^{13}C$ values of formaldehyde-preserved copepods.[24]

If differences in $\delta^{13}C$ were attributed to proteins leaching out of the tissue from the formalin, we would expect that %N would decrease if proteins were removed from tissues, which we did not find. The most probable explanation is that formalin added carbon to our samples, which resulted in the alteration of $\delta^{13}C$ values. However, we only found significant increases in %C for some of our samples, although there may have been small changes in %C that were below our detection limit. The mechanistic driver of the observed changes in $\delta^{13}C$ values is therefore somewhat unclear. Generally, the magnitude of the change in $\delta^{13}C$ values that we observed from preserved tissues was slightly lower than the changes observed in the $\delta^{15}N$ values; however, small changes in $\delta^{13}C$ values are more likely to affect the interpretation of $\delta^{13}C$ values, as shifts of ~1‰ can represent differences in carbon sources that the consumer is relying on. Therefore, our results are in general agreement with previous studies[19,20,22] that report variable but significant changes in $\delta^{13}C$ values with chemical preservation.

4.3 | Amino acid $\delta^{15}N$ values

The isotopic compositions of C and N are largely controlled by the ratios of nucleic acids:proteins:carbohydrates:lipids in the tissues.[25] In our samples, particularly for muscle tissue that is protein-rich with relatively high N concentrations (15–20%), we expected that the bulk $\delta^{15}N$ values would be largely reflective of the amino acid $\delta^{15}N$ values because amino acids are a substantial contributor to the total N pool. The paired amino acid $\delta^{15}N$ values illustrate that 1–1.5‰ increase in bulk preservation studies may be explained by small changes (<1‰) in amino acid isotope ratios. Overall, our study illustrates that patterns in bulk isotope ratios largely mirror those of amino acid values.

The $\delta^{15}N$ values of most amino acids were minimally altered by chemical preservation, both in the formaldehyde-ethanol preserved tuna and squid samples and in copepod samples preserved in formaldehyde. These results are promising for future CSIA-AA studies and provide further evidence that formaldehyde preservation does not generally affect the $\delta^{15}N$ values of amino acids. These results agree with the few studies[17,18] that have evaluated the effects of formaldehyde on $\delta^{15}N$ values, which reported no difference between frozen and chemically preserved samples.

However, we found a surprising trend in the $\delta^{15}N$ values of valine, which were up to 6.0‰ higher in formaldehyde-preserved samples for *T. albacares*, but for *D. gigas* there was only a 1–2‰ difference between preserved and frozen samples (Table 2; Figure S2, supporting information). In addition, the $\delta^{15}N$ values of phenylalanine from preserved samples ranged from 0.5 to 4.5‰ different from those of frozen samples (see Table 2; Figure S2, supporting information). These differences were larger than the 0.5–1.0‰ differences that we generally found for most other amino acid $\delta^{15}N$ values. The results for phenylalanine are especially relevant, as it is frequently used as the canonical source amino acid to estimate the trophic positions of species and to evaluate changes in baseline $\delta^{15}N$ values. Thus, understanding how and the degree to which $\delta^{15}N$ values of phenylalanine are altered by chemical preservation is pertinent to many CSIA-AA studies.

There is no mechanism by which ^{15}N-enriched phenylalanine could be added to the sample from formaldehyde. A potential explanation for how chemical preservation could alter the $\delta^{15}N$ values of phenylalanine is through the breaking of C–N bonds. During this process, phenylalanine would be lost from the analytical pool, and thus we would expect a decrease in the peak area of phenylalanine and an enrichment of ^{15}N in the preserved samples. To evaluate this possibility, we calculated the ratios of peak areas of phenylalanine to other amino acids that were minimally altered by preservation (e.g., glutamic acid and proline). These ratios were relatively constant, which implies there was no preferential loss of phenylalanine in the formaldehyde-preserved samples and this is not a likely explanation for the difference in $\delta^{15}N$ values between chemically preserved and frozen tissues.

The discrepancy in the $\delta^{15}N$ values of phenylalanine between frozen and chemically preserved samples may reflect our inability to chromatographically separate phenylalanine from other amino acids and N-containing compounds in order to measure $\delta^{15}N$ values, rather than phenylalanine being altered by chemical preservation. Measurement of amino acid $\delta^{15}N$ values using GC/IRMS techniques requires baseline chromatographic separation of peaks of different compounds.[36,37] This is essential, as the peak areas of masses 28 and 29 are used to calculate ion-current ratios, which are then compared with those of reference materials of known isotopic composition to calculate the $^{15}N/^{14}N$ ratio of individual compounds.[36,37] Thus, to determine isotope ratios it is imperative to separate and accurately measure the entire peak without interference from fully co-eluting or partially co-eluting compounds.

In many of our samples, there were unknown N-containing compounds (e.g., natural polyamines) that appeared on the mass 28 and 29 chromatograms near phenylalanine and valine in the frozen samples, and these peaks limited our ability to accurately quantify the $\delta^{15}N$ values of these compounds. Interestingly, the unknown N-containing peaks, which eluted between glutamic acid and phenylalanine and that co-eluted with the tail of the valine peak using our derivatization method and chromatographic column, were significantly more abundant relative to phenylalanine and valine in the frozen sample and were lowest in the tissue that had been preserved in ethanol for 2 years. There was, however, no evidence in either the mass 28 or the mass 29 trace or in the 29/28 mass trace of the N-containing compound that co-eluted with valine in the chemically preserved samples. Therefore, it is possible that the 95% ethanol solution solubilized the interfering compounds and improved our ability to achieve baseline chromatographic separation of phenylalanine and valine for nitrogen isotope analysis. Unfortunately, the identification of the small interfering compounds was beyond the scope of this work.

The performance of compound-specific isotope analyses using GC/IRMS is commonly limited by chromatographic resolution of individual compounds.[16,36,38] Our results underscore the importance of the preparative chromatographic steps necessary to isolate a pure amino acid fraction from hydrolyzed tissues (e.g., see recommendations by Ohkouchi et al[16]). Although we included a solvent extraction of our hydrolysate, these results suggest that we might have further purified our tissue samples or the amino acid fraction by extraction using 95% ethanol. We hypothesize that changes in the δ^{15}N values of phenylalanine and valine can be attributed to chromatographic isolation and our ability to measure the δ^{15}N values rather than formaldehyde alteration of the δ^{15}N values of these amino acids. We recognize that amino acids other than phenylalanine and valine are not immune to co-elution in samples. It has been recognized that examination of the mass ratio trace can reveal even minor co-eluting peaks (e.g., see Figure 4 in Hayes et al[36]) and we strongly recommend careful review of all chromatograms. Future studies might focus on testing GC columns with different stationary phases, which could result in greater chromatographic separation of the interfering peaks and phenylalanine, or employ alternative or multiple derivatization techniques in samples where co-elution with amino acids occurs (see Ohkouchi et al[16]).

5 | CONCLUSIONS

The results from the bulk portion of our study largely bolster previous work. Since the δ^{13}C values were altered in inconsistent ways, using δ^{13}C values from preserved tissues should be avoided unless there is a mechanistic understanding of how the preservative specifically alters the tissue of interest. Future studies evaluating the preservation effects on amino acid δ^{13}C values would be useful to determine if and how these values are altered with preservation. Formaldehyde and ethanol can produce small changes in the δ^{15}N values of tissues.[26,29,35] However, formaldehyde by itself or in combination with 95% ethanol may promote leaching or solubilization of compounds in tissues and thus alter δ^{15}N values.[26,35] These findings generally concur with the literature, where changes in δ^{15}N values associated with formaldehyde or ethanol preservation are less than the 2 to 5‰ variation used to detect trophic level differences in food web studies.

This study provides both promise and reason for caution for future studies that aim to use CSIA-AA on frozen and chemically preserved specimens. Most measurements of amino acid δ^{15}N values from preserved tissues were within the typical 1‰ error associated with CSIA-AA. These small differences are consistent with the results from the bulk component of the study, where the δ^{15}N values were minimally altered by preservation. However, future studies that illuminate the differences that we found in some valine and phenylalanine δ^{15}N values would be useful.

We hypothesize that the differences that we found are reflective of analytical uncertainty associated with co-eluting compounds rather than preservation altering δ^{15}N values, but future studies testing this hypothesis would helpful for the best use of stable isotope data in ecological studies. Our results suggest that formaldehyde-ethanol preservation followed by rinsing of frozen (and perhaps fresh) tissues with deionized water prior to hydrolysis might remove some of the unknown N-compounds that interfered with our ability to measure the δ^{15}N values of phenylalanine and valine.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Robert J. Olson and Leanne Duffy at the Inter-American Tropical Tuna Commission for assistance with acquiring *Thunnus albacares* and *Dosidicus gigas* samples for this study and setting up the initial experiments. They would also like to thank Linsey Sala at Scripps Institution of Oceanography for assistance with proper identification of *Calanus pacificus* and *Eucalanus californicus*. Lastly, they would like to thank Natalie Wallsgrove and the University of Hawaii's Stable Isotope Biogeochemistry Laboratories with assistance with AA-CSIA. This research was partially funded by the Frontiers of Innovation Scholarship Program through the University of California San Diego, and was supported by the National Science Foundation, OCE1041329 to Brian N. Popp and Jeffrey C. Drazen. This is School of Ocean Science and Technology contribution number 10670.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Hetherington ED, Kurle CM, Ohman MD, Popp BN. Effects of chemical preservation on bulk and amino acid isotope ratios of zooplankton, fish, and squid tissues. *Rapid Commun Mass Spectrom*. 2019;33:935-945. https://doi.org/10.1002/rcm.8408