# A Guide to Using Compound-Specific Stable Isotope Analysis to Study the Fates of Molecules in Organisms and Ecosystems

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**Abstract:** The measurement of stable isotopes in 'bulk' animal and plant tissues (e.g., muscle or leaf) has become an important tool for studies of functional diversity from organismal to continental scales. In consumers, isotope values reflect their diet, trophic position, physiological state, and geographic location. However, interpretation of bulk tissue isotope values can be confounded by variation in primary producer baseline values and by overlapping values among potential food items. To resolve these issues, biologists increasingly use compound-specific isotope analysis (CSIA), in which the isotope values of monomers that constitute a macromolecule (e.g., amino acids in protein) are measured. In this review, we provide the theoretical underpinnings for CSIA, summarize its methodology and recent applications, and identify future research directions. The key principle is that some monomers are reliably routed directly from the diet into animal tissue, whereas others are biochemically transformed during assimilation. As a result, CSIA of consumer tissue simultaneously provides information about an animal's nutrient sources (e.g., food items or contributions from gut microbes) and its physiology (e.g., nitrogen excretion mode). In combination, these data clarify many of the confounding issues in bulk analysis and enable novel precision for tracing nutrient and energy flow within and among organisms and ecosystems.

**Keywords:** carbon; fatty acids; hydrogen; isotope ecology; nitrogen

## 1. Stable Isotope Analysis of Bulk Tissues

The measurement of the abundance of naturally-occurring stable isotopes within animal and plant tissues has proven to be a powerful and cost-effective tool for a wide range of ecological and physiological studies [1]. Here, we use δ notation to describe isotope values, defined as the measure of the heavy (e.g., $^{13}$C) to light (e.g., $^{12}$C) isotope relative to an internationally accepted standard. The key premises of isotope ecology are that: (1) carbon isotope ($\delta^{13}$C) values vary among primary producers, and this variation persists in higher trophic-level consumers [2]; (2) nitrogen isotope ($\delta^{15}$N) values increase systematically moving up a food chain with consumers having higher $\delta^{15}$N values than their food [3,4]; and (3) hydrogen ($\delta^2$H) and oxygen ($\delta^{18}$O) isotope values of consumer tissues are influenced by local precipitation as well as food, allowing them to be used to characterize animal movement [5,6]. Other isotope systems (e.g., sulfur: $\delta^{34}$S) also have ecological applications, although here we focus on carbon, nitrogen, and hydrogen. An implicit assumption behind these three key premises is that isotope values are also influenced by organismal physiology, namely the biochemical reactions that control the assimilation, synthesis, and degradation of macromolecules (protein, lipid, carbohydrate) required by producers and consumers for homeostasis. In other words, after accounting for physiology, "you are what you eat" isotopically.

$\delta^{13}$C and $\delta^{15}$N values of whole animals such as small invertebrates, or of 'bulk' animal tissues such as muscle therefore reflect their diet, trophic position, and physiological state [7]. These values can also be used to calculate dietary niche metrics of individuals and populations, as well as community measures of food chain length and food web structure [8–10]. Analysis of metabolically active tissues with different turnover rates (e.g., liver versus muscle) or inert tissues that continuously grow (e.g., whiskers, baleen) can provide inferences reflecting varying time periods [11–13]. Bulk tissue isotope analysis complements traditional methods such as gut content analysis, with the benefits of illustrating the assimilated diet integrated over time [2,7]. In addition, because tissue $\delta^2$H and $\delta^{18}$O values are influenced by drinking and food water that is ultimately sourced from local precipitation, they can be used to study habitat selection and migration at regional and continental spatial scales [6]. Although here we focus on animals, there is also a rich history of applying bulk tissue isotope analysis to plant tissue in ecological and physiological studies; see [14–16] for reviews.

Bulk tissue isotope analysis is appealing for a variety of reasons including the ease of sample preparation, low cost, and quick analysis time. Sample preparation can be as simple as drying and homogenizing a specimen and then carefully weighing it into tin or silver capsules. Some samples may need to be demineralized to remove inorganic carbon, or lipid-extracted because lipids have lower $\delta^{13}$C values than protein—the dominant macromolecule in animal tissue [17,18]—and thus are commonly removed to avoid biasing measurements. Access to stable isotope laboratories in many regions is readily available because universities or government agencies have centralized facilities for both internal and external sample submission. Sample analysis is also quick—with modern instrumentation, the $\delta^{13}$C and $\delta^{15}$N values of a sample can be measured in <10 min.

Although there are many benefits to bulk tissue isotope analysis, there are also limitations. The most prevalent is that isotope values of primary producers at the base of the food web (i.e., baseline values) can vary across space and time, confounding interpretations of the values of organisms at higher trophic levels. To account for this variation, researchers must sample potential dietary items from appropriate locations and times, after careful consideration of the turnover rates of the consumer tissue(s) being analyzed [19,20]. Another limitation is isotopic overlap among co-occurring dietary resources. For example, terrestrial $C_3$ plants and freshwater aquatic algae can have overlapping $\delta^{13}$C values [21], making it impossible to determine whether a consumer primarily relies on terrestrial-derived (allochthonous) or algal-derived (autochthonous) energy, a key question in many aquatic food web studies [22].

In addition, interpretations of bulk tissue isotope data often must assume that macromolecules in the diet are routed directly into consumer tissue; e.g., prey protein is directly incorporated into predator protein. However, some macromolecules are extensively synthesized by consumers de novo using carbon and nitrogen from alternative dietary macromolecules. For example, animals can synthesize protein components such as non-essential amino acids ($AA_{NESS}$) using carbon from dietary carbohydrates or lipids, creating differences between the $\delta^{13}$C values of protein in consumer tissues and their diet items [23]. Even if dietary macromolecules are directly routed, there can be offsets in isotope values between a consumer and its diet because of the metabolic processing of nutrients during routing. These offsets, known as trophic discrimination factors (TDF), reflect isotopic fractionation caused by physiological processes. One prominent example is that excretion of waste nitrogen often favors $^{14}$N, causing consumers to have higher $\delta^{15}$N values than their food [3,24].

## 2. Principles of Compound-Specific Isotope Analysis (CSIA)

To help reveal shifts in baseline isotope values, resolve overlapping isotope values of diet items, and explicitly address the potential routing of dietary nutrients, biologists are increasingly using compound-specific stable isotope analysis, or CSIA. In this approach, the isotope values of individual monomers that constitute a macromolecule (e.g., amino acids, or AA, in protein) are measured and interpreted in biochemical and ecological contexts. The basic premise is the same as in bulk tissue isotope analysis: after accounting for physiology, "you are what you eat" isotopically. However,

because many monomers have unique anabolic and catabolic biochemical pathways that are well understood, CSIA has the potential to provide much greater information than bulk tissue isotope analysis of the same sample.

Bulk tissue and CSIA studies both frequently focus on protein because it constitutes >50% of the organic matter in most consumers [25]. All proteins are made of the same monomers, 20 standard AA, each containing three primary functional groups: an amine ($-NH_2$), a carboxyl (-COOH), and a unique side chain that creates distinct biochemical properties (R-group; [26]). AA fall into three categories based on an organism's ability to synthesize the sequence of carbon atoms (i.e., the carbon skeleton) bonded to the $\alpha$-carbon that links these functional groups (Table 1). First, AA can be essential ($AA_{ESS}$), meaning that an organism cannot synthesize them at all, or rapidly enough to meet their basic needs. Second, AA can be conditionally essential, meaning that the rate of de novo synthesis becomes inadequate under some physiological conditions. Third, AA can be non-essential ($AA_{NESS}$), meaning that organismal needs can be fully met with de novo synthesis. All 20 standard AA are non-essential for bacteria, fungi, plants, and many protists, but some AA are essential for animals and some protists because the enzymes required for de novo synthesis were lost during their evolution [27].

**Table 1.** Classification of 15 standard amino acids (AA) for which stable isotope carbon ($\delta^{13}$C) or nitrogen ($\delta^{15}$N) values have been measured. Classifications based on data from invertebrates, fish, mammals, and birds [26,28–33].

| $^{13}$C Classification | $^{15}$N Classification | | |
| --- | --- | --- | --- |
| | Trophic AA | Source AA | Other AA |
| $AA_{ESS}$ | Isoleucine Leucine Valine | Lysine Methionine Phenylalanine Tyrosine [1] | Arginine [2] Threonine [3] |
| $AA_{NESS}$ | Aspartic acid Alanine Glutamic acid Proline | | Glycine [4] Serine [5] |

[1] Conditionally essential. [2] Conditionally essential. Not yet classified as trophic or source. [3] Value of $\delta^{15}$N is lower in consumer than in diet. [4] Exchanges N with serine but can exhibit patterns like source AA. Classified as $AA_{ESS}$ for birds but as $AA_{NESS}$ or conditionally essential for other taxa. [5] Exchanges N with glycine but can exhibit patterns like source AA.

Based on a consumer's ability to synthesize AA de novo, general relationships between the $\delta^{13}$C values of consumers and their diets ($\delta^{13}C_{Consumer} - \delta^{13}C_{Diet}$ or $\Delta^{13}C_{C-D}$) can be predicted for essential and non-essential AA (Figure 1A). For the $AA_{ESS}$ of eukaryotic consumers, $\Delta^{13}C_{C-D}$ values should be near 0‰ because these organisms must route them directly from their diet into their tissues, leading to minimal isotopic fractionation [34]. However, recent research has highlighted that $AA_{ESS}$ may be assimilated from symbiotic gut microbes, causing $\Delta^{13}C_{C-D}$ values to deviate from 0‰ ([35]; see "CSIA applications" below). For $AA_{NESS}$, $\Delta^{13}C_{C-D}$ values can differ from zero as a result of de novo synthesis because the biosynthetic steps may cause fractionation and because the carbon substrate (e.g., lipids or carbohydrates) may vary in isotopic composition [35,36]. Similar to $AA_{ESS}$, $\Delta^{13}C_{C-D}$ values close to 0‰ for $AA_{NESS}$ likely indicate direct routing of these molecules from the diet into endogenous tissue with minimal isotopic alteration. For example, gentoo penguins (*Pygoscelis papua*) eating a known diet had $\Delta^{13}C_{C-D}$ values that were ~0‰ for $AA_{ESS}$ (−0.1‰ to 0.3‰), but their $\Delta^{13}C_{C-D}$ values for $AA_{NESS}$ were larger and more variable (−0.5‰ to 2.4‰), reflecting a combination of direct routing and de novo synthesis from non-protein dietary macromolecules [34].

Predictions for $\Delta^{15}N_{C-D}$ values are not based on the essential versus non-essential classifications used for carbon, but instead rely on the categories of source versus trophic AA (Table 1; [32]) that refer to the lability of the nitrogen atom in the amine group (Figure 1B). This classification was initially empirically-derived but recent studies provide a sound biochemical basis for identifying AA as trophic

or source [31,32,37]. The amine nitrogen atom can be replaced during transamination reactions in which the AA carbon skeleton is left intact, leading to a dissociation between changes in $\delta^{13}C$ and $\delta^{15}N$ values of AA and by extension, between the classifications of essential versus non-essential and source versus trophic (Table 1). Source AA generally do not participate in reactions involving deamination and transamination, thus their $\delta^{15}N$ values remain largely unaltered during assimilation by consumers and their $\Delta^{15}N_{C-D}$ values are ~0‰ . In contrast, trophic AA frequently undergo deamination and transamination reactions which result in $^{15}N$-enrichment, reflecting preferential deamination and ultimate excretion of light nitrogen ($^{14}N$). This causes the $\Delta^{15}N_{C-D}$ values for trophic AA to be ~2–8‰ [31]. Several AA do not consistently fall into either category. Glycine and serine readily exchange nitrogen with each other but participate in few transaminations with other AA. As a result, the $\Delta^{15}N_{C-D}$ values of these two AA are frequently similar within an organism but vary widely between organisms, depending on diet and physiological status. Threonine is apparently often involved with transaminations that cause its $\delta^{15}N$ value to decrease rather than increase with each trophic level, leading to negative $\Delta^{15}N_{C-D}$ values, although the biochemical mechanism remains unclear [38]. Overall, the primary benefit of CSIA is that the measurement of trophic AA, source AA, $AA_{ESS}$, and $AA_{NESS}$ from a single sample can enable multiple, interwoven inferences about consumer physiology and ecology.
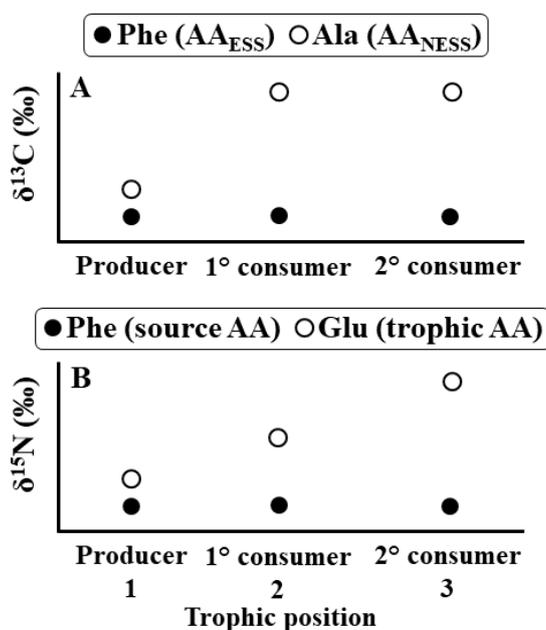


**Figure 1.** Idealized isotope values are shown for two amino acids (AA) in tissue from three different organisms, including a primary producer, a primary consumer (1°), and a secondary consumer (2°)—note that these are hypothetical data to illustrate trends. (**A**) shows carbon isotope values. For phenylalanine, an essential AA, the value of $\Delta^{13}C_{C-D}$ ($\delta^{13}C_{Consumer} - \delta^{13}C_{Diet}$) was 0‰ for both consumers. As a result, $\delta^{13}C_{Phe}$ values were the same for all organisms. For alanine, a non-essential AA, the primary consumer synthesized substantial amounts of this AA de novo, causing it to have a higher $\delta^{13}C_{Ala}$ value than its diet (i.e., the producer). In contrast, the secondary consumer extensively routed alanine from its diet (i.e., the primary consumer) directly into its tissues, causing both consumers to have the same $\delta^{13}C_{Ala}$ value. (**B**) shows nitrogen isotope values. The values of $\Delta^{15}N_{C-D}$ for phenylalanine, a source AA, were 0‰ for both consumers. As a result, $\delta^{15}N_{Phe}$ values were the same for all organisms. In contrast, the values of $\Delta^{15}N_{C-D}$ for glutamic acid, a trophic AA, were similarly positive for both consumers. As a result, $\delta^{15}N_{Glu}$ values steadily increased from the producer to each consumer.

CSIA can also be applied to fatty acids (FA) in lipids, however, thus far this approach is less common than AA isotope analysis. Values of $\Delta^{13}C_{C-D}$ for individual FA have been assessed in

controlled feeding experiments for birds [39,40] and invertebrates [41,42]. It is more difficult to predict $\Delta^{13}C_{C-D}$ for FA than for AA because of the greater diversity of FA structure, the variety of potential post-assimilation FA modifications, and the prominent dual roles of FA as structural molecules and as metabolic fuel [40]. However, these challenging dynamics also provide a wealth of potential hypotheses under which to evaluate $\Delta^{13}C_{C-D}$, thus we anticipate that the application of CSIA to FA will continue to expand.

## 3. CSIA Methods

### 3.1. Sample Collection and Storage

Following field collection, samples for AA isotopic analysis should be stored frozen at $\leq -20\,^{\circ}$C or lyophilized, while it is best to store samples for FA analysis at $\leq -80\,^{\circ}$C and possibly with antioxidants (see [43]). Oxidation is less problematic for AA than for FA analysis because of the stability of the strong peptide bonds within proteins. For samples that have been stored with liquid preservative, the effects on AA and FA isotope values have not been comprehensively studied to our knowledge. Studies are available, however, for storage effects on AA composition and bulk stable isotope values. Up to 22 years of storage of delphinid skin samples in dimethyl sulfoxide (DMSO) did not alter the tissue AA composition [44]. In contrast, six months of storage of muscle samples from elasmobranchs and marine teleosts in 95% ethanol led to small decreases in bulk $\delta^{13}$C values and C:N ratios, suggesting a loss of small AA [45,46]. However, a previous study found that eight weeks of storage in 70% ethanol did not appear to significantly influence bulk $\delta^{13}$C and $\delta^{15}$N values [47]. Until results are available for AA and FA isotopic analyses, we suggest using these and other bulk stable isotope studies as a guide for evaluating the potential effects of sample preservation techniques.

For both AA and FA analyses, plastic storage containers should be avoided because of potential contamination from plastic hydrocarbons. Best practice involves collecting, drying, and storing samples in glassware that has been pre-combusted in a muffle furnace at 500 $^{\circ}$C for $\geq$8 h to burn off any organic residue. Caps for glassware should be either Teflon or foil-lined and cleaned of surface contaminants via acid washes (typically 0.1 N HCl) followed by rinses in deionized $H_2O$. If feasible, tissues can also be stored in pre-combusted aluminum foil.

### 3.2. Chemical Preparation for AA Isotope Analysis

Methods to prepare a sample for AA $\delta^{13}$C and $\delta^{15}$N analysis vary among tissues, although three general steps apply to all samples: (1) the isolation and purification of protein from other organic compounds within a sample (e.g., lipids, carbohydrates), (2) the breakdown of the protein structure via hydrolysis to produce free AA, and (3) the derivatization of free AA to more volatile forms that can be readily separated via gas chromatography (GC), combusted or pyrolyzed to gas, and analyzed in an isotope ratio mass spectrometer (IRMS). Derivatization is the most complex process and typically involves a full day of laboratory work, although some methods require considerably less time [48]. Alternatively, liquid chromatography can also be used to separate individual AA which then undergo standard isotope analysis in an elemental analyzer interfaced to an IRMS (e.g., [49]), although this approach is less common.

For lipid-rich tissue or whole animal samples (e.g., liver or small invertebrates), fats must be removed prior to hydrolysis, typically with multi-day solvent rinses [50,51]. Samples can be soaked in a 2:1 mixture of chloroform:methanol for 72 h, with the solvent changed every 24 h. Alternatively, samples can be soaked in petroleum ether for 72–96 h—this milder solution is advantageous for delicate samples such as invertebrate tissue because more polar solvent mixtures like chloroform:methanol may leach nitrogen from whole body samples (e.g., [52]). Following the final solvent soak, samples should be rinsed 5–7 times in deionized water and then lyophilized.

To break down protein structure and obtain free AA, lipid-free samples are hydrolyzed in strong acid at high temperature. Samples are added to 0.5–1.5 mL of 6 N HCl in pre-combusted glass

vials which are then flushed with $N_2$ gas before sealing to prevent oxidation. Samples are then held at either 150 °C for 70 min [48] or 110 °C for 20–24 h [53]; this process coverts glutamine and asparagine into glutamic acid and aspartic acid respectively due to cleavage of the terminal amine group. Following hydrolysis, samples are dried down under $N_2$ gas. The remaining solids form a film on the interior of the glass vial which consists of pure AA and, if present, contaminants such as hydrolyzed carbohydrates or lipids. This film is stable and can be safely stored at −20 °C for at least six months. For carbohydrate-rich samples such as primary producers and whole invertebrates an additional purification step is required post-hydrolysis to remove carbohydrates and other contaminants, leaving isolated AA for derivatization. The most common method calls for hydrolyzed samples to be resuspended in weak acid, passed through a Dowex resin (50WX8 100–200 mesh) that retains AA while allowing other molecules to pass through, then eluting the AA with a base [54]. Samples are then dried again to a film under $N_2$ gas.

AA are not easily converted into gas phase; to be separated on a GC column, they must be transformed into more volatile forms with a low boiling point. This transformation is termed 'derivatization' and includes adding functional groups to each AA after their purification; three methods are prevalent in the AA literature. The most common method was developed by Silfer et al. [53] and it derivatizes AA into N-trifluoroacetyl isopropyl esters in two steps. First, esterification of the carboxyl terminus of each AA is achieved by adding a 4:1 isopropanol:acetyl chloride mixture to the hydrolyzed film and reacting at 110 °C for one hour. Following this, the solvent is dried down under $N_2$ gas, and the sample is rinsed twice with a neutral dichloromethane solution to remove excess isopropanol:acetyl chloride (after each rinse, the sample is again dried down under $N_2$ gas). Second, acetylation of the amine terminus is achieved by adding 1:1 trifluoroacetic anhydride:dichloromethane and reacting at 110 °C for 10 min. Following these steps, derivatized samples may be stored at −20 °C in a freezer designed to accommodate flammable or explosive reagents. However, derivatized AA slowly dissociate into their non-derivatized forms, affecting their isotopic composition [55], and thus samples should be analyzed as soon as feasible. For analysis, derivatized samples are dried under $N_2$ gas, rinsed twice with dichloromethane, then dissolved in dichloromethane for injection on a GC column.

A second method derivatizes AA to N-pivaloyl isopropyl esters [56,57]. Hydrolysates are first reacted at 110 °C for two hours with a 4:1 mixture of isopropanol:thionyl chloride, dried under $N_2$ gas and rinsed three times with dichloromethane, and then reacted again at 110 °C for two hours with 4:1 mixture of dichloromethane:pivaloyl chloride. A third technique, modified by Walsh et al. [48] from GC-MS procedures [58], involves a single-step derivatization which simultaneously modifies both the carboxyl and amine terminus. The derivatizing agent, methyl chloroformate, is added to a hydrolyzed sample which has been resuspended in 0.1 N HCl. This mixture sits for ~1–2 min before being vortexed, after which an organic layer containing derivatized AA forms and is removed for GC injection.

Each derivatization method described above adds carbon to AA and thus the measured AA $\delta^{13}C$ values reflect a combination of intrinsic AA carbon from the original molecule and reagent carbon added during derivatization. Researchers account for this by derivatizing each batch of unknown samples alongside a standard that contains AA in known concentrations and of known isotopic values. Correction equations are then used to calculate the $\delta^{13}C$ values of the intrinsic carbon alone in each AA, as described in detail in [48,53,59]. Nitrogen is not added to AA during derivatization in any of these methods, thus measured $\delta^{15}N$ values need only standard IRMS corrections (e.g., linearity or drift corrections). The N-pivaloyl isopropyl ester methods [56,57] have only been used for AA $\delta^{15}N$ analysis and thus to our knowledge, their application to $\delta^{13}C$ measurements has not been experimentally validated. Ultimately, deciding which derivatization method to use depends on the scientific question of interest, as each technique better resolves certain AA with specific GC protocols [48,53,59]. In addition, where possible, researchers should consider which technique has been used in generating previous datasets on similar questions. This is important because without

direct comparisons of materials derivatized with each method, raw AA $\delta^{13}$C and $\delta^{15}$N values are not directly comparable between methods [60].

## 3.3. Chemical Preparation for FA Isotope Analysis

As with AA analysis, individual FA must be made more volatile before GC separation and isotopic analysis, however, this process is simpler than the derivatization for AA. To extract and save lipids for FA analysis, researchers typically use variants of the methods of Folch et al. [50] or Bligh and Dyer [51]. Several grams of wet tissue are homogenized in a mixture of 2:1 chloroform:methanol. Samples are then centrifuged and rinsed with a salt solution to remove aqueous solutes. The remaining organic phase is dried under $N_2$ gas and typically resuspended in chloroform. After extraction, FA can be converted to methyl esters (FAME) by the addition of 1M acetyl chloride in methanol and reacting at 90 °C for two hours. Once this solution is dried, FAME can be resuspended in dichloromethane or hexane and injected directly onto a GC column for analysis. As with AA derivatization, producing FAME adds carbon to the original molecules, and each batch of unknown samples must be esterified and analyzed alongside a standard for which the intrinsic $\delta^{13}$C is known to account for the addition of the methyl group.

## 3.4. Isotopic Analysis of Individual AA and FA

Once samples have been chemically prepared, they can be analyzed via a GC coupled to a combustion/reduction and pyrolysis unit that is interfaced with an IRMS system (i.e., GC-C-IRMS). A small aliquot of sample (~1–5 μL) is injected into a small heated space where it is volatilized at high temperature into a gas phase, and then the sample enters the GC column. The GC temperature ramps following a protocol designed to separate the monomers (AA or FA) by mass and polarity. Individual AA or FA elute from the column at particular times based on their size and chemical properties. To separate the elements, monomers are then oxidized and reduced into $CO_2$ or $N_2$, or pyrolyzed into $H_2$ gas, in a high temperature reactor and these reaction products are delivered to the IRMS. Each AA or FA is then detected as a voltage increase, represented by a distinct peak in a chromatogram (Figure 2), from which isotopic ratios are calculated. Individual AA and FA always elute in the same order unless the GC temperature ramping protocol is changed. The final result is measurement of $\delta^{13}$C, $\delta^{15}$N or $\delta^2$H for 10–14 AA, or $\delta^{13}$C for multiple FA (depending on sample type), from a single injection of a single sample. This data richness is a main reason that CSIA is rapidly becoming a powerful tool in physiological and ecological studies.
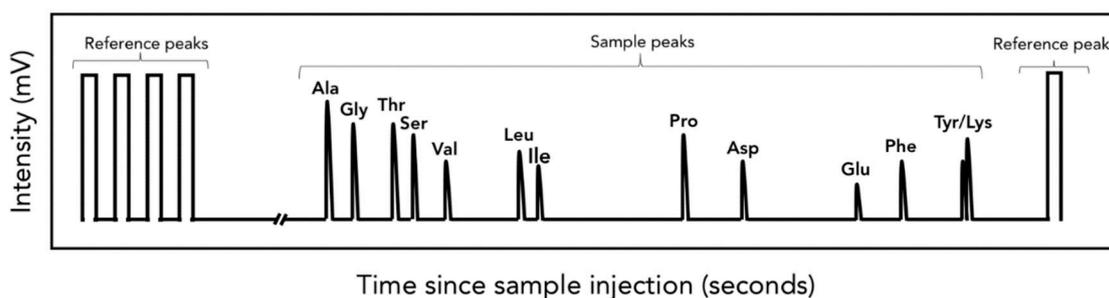


**Figure 2.** A typical amino acid (AA) chromatogram of a sample prepared with the Silfer et al. [53] derivatization method. Here the X axis has been broken for clarity; typical injections take ~1 h. Included are peaks of reference gas (square) and individual AA from the sample (rounded). AA with simpler molecular structures tend to elute earlier (e.g., Gly and Ser) whereas more complex AA require more time to pass through the GC column (e.g., Phe, Lys). Although a single line is shown for clarity, chromatograms have multiple lines representing different atomic masses that are then integrated to calculate isotopic ratios. During carbon analysis, lines represent atomic masses 44, 45, 46; during nitrogen analysis, masses 28, 29, 30; and during hydrogen analysis, masses 1 and 2.

Best GC-C-IRMS practices involve running duplicate or triplicate injections of every sample and bracketing them with standard injections, to account for any temporal drift in isotope values caused by variation in instrument performance. Quality control processes involve examining chromatograms and assessing peak shape (symmetrical peaks are ideal), baseline gas levels, and the degree of separation between AA peaks because coelution can affect isotope values. These assessments are where expertise becomes critical, because chromatograms require time and experience for accurate interpretation, producing the highest degree of data quality control.

## 4. CSIA Applications to Date

Thus far, CSIA studies have provided important insights at molecular, organismal, and ecological scales. Physiological applications have illustrated that in addition to AA classifications (essential or non-essential for carbon; source or trophic for nitrogen), expectations for $\Delta^{13}C_{C-D}$ and $\Delta^{15}N_{C-D}$ should consider factors such as diet composition (e.g., quality and content of protein), mode of nitrogen excretion, and if possible, previously published $\Delta_{C-D}$ values for species with similar physiologies [31,36,61,62]. Ecological applications have demonstrated that variation in baseline isotope values can produce misleading results when using bulk tissue isotope analysis, which can be resolved with CSIA. CSIA has also enabled the tracing of nutrient and energy flow within and among ecosystems, providing a new approach to assessing species interactions and the relative importance of different sources of primary production to consumers across trophic levels.

One prominent research focus has been the carbon sources for AA synthesis. Regarding de novo synthesis of $AA_{NESS}$, the use of carbon from non-protein dietary macromolecules (i.e., carbohydrates and lipids) can be extensive when consumers consume diets that contain inadequate amounts of the protein required for growth or homeostasis. For example, O'Brien et al. [59] showed that adult hawkmoths (*Amphion floridensis*) fed on a sugar solution synthesized ~60–95% of the $AA_{NESS}$ used to build their bodies from dietary carbohydrates; in contrast, all the $AA_{ESS}$ in their tissues were sourced from the plant diet that they consumed as larvae, which contained low but sufficient amounts of protein. Differential use of dietary nutrients to synthesize AA has also been observed in pigs (*Sus domesticus*; [63]), rats (*Rattus norvegicus*; [64]), and mice (*Mus musculus*; [36]). For example, a feeding experiment revealed that mice synthesized a significant portion of the $AA_{NESS}$ used to build their tissues from dietary lipids, even when fed diets containing as little as 10% fat by weight [36]. As expected, $AA_{NESS}$ synthesized from intermediaries in the tricarboxylic acid (TCA) cycle were more sensitive to dietary fat content than those synthesized from glycolytic intermediaries, because the TCA cycle is more closely linked to the metabolic processing of lipids. These results also call into question the benefits of removing lipids from potential food sources before bulk tissue $\delta^{13}C$ analyses, as is common in studies of foraging ecology [65]. Combined, these controlled feeding studies using CSIA show that (1) herbivorous and omnivorous animals are quite flexible in their ability to maintain $AA_{NESS}$ homeostasis when fed diets containing insufficient amounts of protein, and (2) that complete, direct routing of dietary $AA_{NESS}$ into tissues is a poor assumption when animals consume diets rich in carbohydrates and lipids that exceed energy requirements.

Regarding $AA_{ESS}$, recent studies on invertebrates [66–68] and vertebrates [35,69] show that dietary protein is not the sole source of $AA_{ESS}$ used to build and maintain animal tissues. Because eukaryotic consumers cannot synthesize $AA_{ESS}$ de novo, they should directly route dietary $AA_{ESS}$ into their tissues, resulting in $\Delta^{13}C_{C-D}$ values of ~0‰; however, this fundamental expectation has not always been met in controlled experiments. Because animals in these feeding experiments were consuming known diets with carefully measured AA isotopic composition, there are two feasible explanations. First, animals can assimilate a substantial portion of their $AA_{ESS}$ from symbiotic gut microbes (e.g., bacteria and fungi) that use carbon from dietary carbohydrates or lipids to synthesize the carbon skeletons of $AA_{ESS}$, which would result in non-zero $\Delta^{13}C_{C-D}$ values. There is evidence for this phenomenon for a limited suite of $AA_{ESS}$ using other methodologies [70]. Second, it is possible that extensive catabolism of $AA_{ESS}$ immediately upon their absorption into cells lining the gut [71] causes fractionation. This would

alter the $\delta^{13}C$ values of the remaining $AA_{ESS}$ that are incorporated into consumer tissue. This second pathway could feasibly occur without gut microbe activity; however, the relevant study [71] occurred prior to the recognition that gut microbes make appreciable contributions to the protein balance of their eukaryotic hosts. To our knowledge, isotopic fractionation related to $AA_{ESS}$ catabolism by the gut microbiome or by eukaryotic hosts has not been experimentally explored. Overall, a summary of current literature highlights the fact that while $AA_{ESS}$ are more likely than $AA_{NESS}$ to have $\Delta^{13}C_{C-D}$ values near 0‰, there is considerable variation (Figure 3; Table S1), and a principal cause is likely the increasingly-recognized role of the gut microbiome in host protein homeostasis.
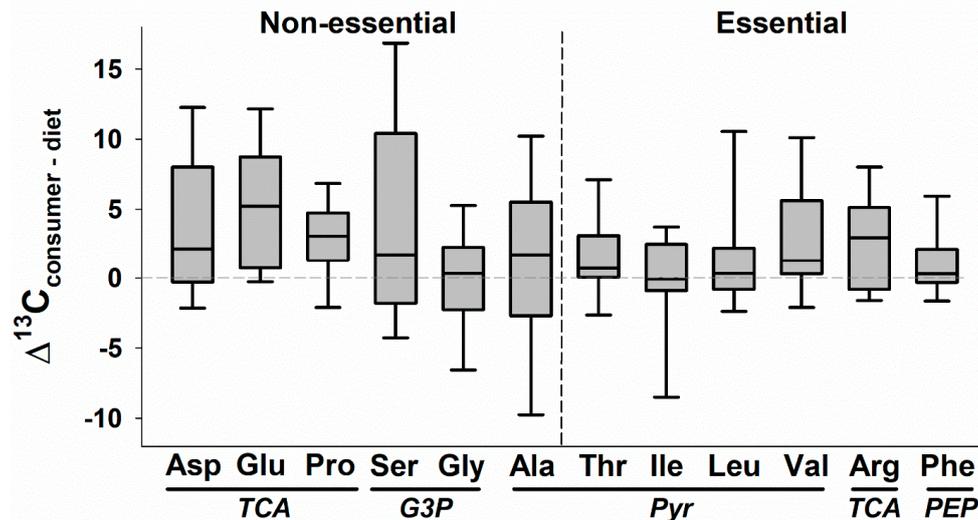


**Figure 3.** Median values (solid line; boxes and error bars represent 25th and 10th percentiles) of the carbon isotope discrimination factors ($\Delta^{13}C_{C-D}$, or $\delta^{13}C_{Consumer\ tissue} - \delta^{13}C_{Diet\ item}$) for individual amino acids (AA) across nine species of animals consuming a total of 24 different diets in controlled experiments. AA that are essential tend to have reduced variation and values closer to 0‰ in comparison to those that are non-essential. Note that Arg is conditionally essential, and Gly is essential for birds. AA are also grouped by the source of precursor molecules for biosynthesis: TCA = intermediates of the tricarboxylic acid cycle; G3P = glycerate-3-phosphate of glycolysis; Pyr = pyruvate; PEP = phosphoenolpyruvate of glycolysis. Note that of the 20 standard AA, four are excluded here because they are usually not measured (histidine, methionine, tryptophan, cysteine) and two are excluded because they have been reported in only one study of which we are aware (lysine, tyrosine; [72]). In addition, during sample preparation, glutamine is converted to glutamic acid and asparagine to aspartic acid. Studies were located by (1) searching the references cited in a recent review of AA $\Delta^{15}N_{C-D}$ [31]; (2) searching "Web of Science" electronic database for "carbon isotope" and "amino acid"; and (3) searching citations of relevant publications. Studies are listed in Table S1.

There is also substantial variation in published $\Delta^{15}N_{C-D}$ values (Figure 4); for a comprehensive review see [31]. Some of this variation can be attributed to dietary protein content and quality [31,61,73]. Consumers eating diets that are high in protein with AA compositions that closely match their requirements (i.e., high biological quality) generally have lower $\Delta^{15}N_{C-D}$ values, suggestive of little metabolic processing and extensive direct protein routing [62]. In addition, consumers that excrete nitrogen as ammonia rather than as urea or uric acid have higher $\Delta^{15}N_{C-D}$ values [74], possibly because the relatively greater toxicity of ammonia requires this molecule to be excreted rapidly, which can cause substantial fractionation [37,75]. Values of $\Delta^{15}N_{C-D}$ can also vary among tissues [76,77] with the highest values occurring in tissues with very active metabolic processing of AA, such as liver [73]. Unique physiological processes can also affect $\Delta^{15}N_{C-D}$. A recent study of captive leopard sharks (*Triakis semifasciata*) found surprisingly low $\Delta^{15}N_{C-D}$ values for trophic AA [72], likely related to the ability of elasmobranchs to retain and recycle urea nitrogen into newly-synthesized AA [31,76].

Importantly, the distinction between trophic and source AA applies to microbial consumers as well as animals [78].
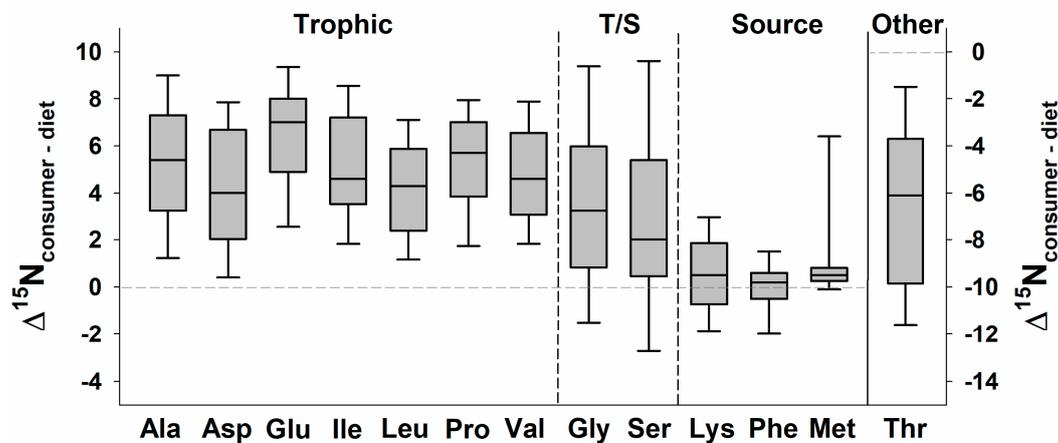


**Figure 4.** Median values (solid line; boxes and error bars represent 25th and 10th percentiles) of the nitrogen isotope discrimination factors ($\Delta^{15}N_{C\text{-}D}$, or $\delta^{15}N_{\text{Consumer tissue}} - \delta^{15}N_{\text{Diet item}}$) for individual amino acids (AA) across 58 species of consumers in controlled experiments and well-constrained field studies. AA that are trophic tend to have greater $\Delta^{15}N_{C\text{-}D}$ values in comparison to source AA. Note that T/S indicates AA that can be classified as either trophic or source, and that threonine has a separate y-axis. Of the 20 standard AA, five are excluded here because they are rarely reported (arginine, cysteine, histidine, tryptophan, tyrosine). In addition, during sample preparation, glutamine is converted to glutamic acid and asparagine to aspartic acid. Studies were located (1) in Supplement 1 of [31]; and (2) by repeating the literature search described in [31] to add more recent studies. Studies are listed in Table S2.

CSIA studies have also yielded important ecological advances, particularly for resolving resource use and trophic level for consumers. Trophic level can be estimated by comparing the $\delta^{15}N$ values between trophic and source AA [79,80]. The most common pairing in published studies is $\delta^{15}N_{\text{Glu}} - \delta^{15}N_{\text{Phe}}$ [31,32,37]. Because trophic AA experience fractionation at each trophic level, their $\delta^{15}N$ values increase proportionately with trophic steps, and consequently larger $\delta^{15}N_{\text{Glu}} - \delta^{15}N_{\text{Phe}}$ offsets indicate higher trophic positions. The inference of trophic level can also be formalized by the equation [57,81]

$$\text{Trophic Level} = 1 + [\delta^{15}N_{\text{Trophic AA}} - \delta^{15}N_{\text{SourceAA}} - \beta] \times \text{TDF}_{\text{Trophic AA}}^{-1}, \tag{1}$$

Here, $\beta$ is the offset between trophic and source $\delta^{15}N$ in primary producers at the base of the food web, thus this term assigns primary producers to trophic level 1. TDF is the trophic discrimination factor which represents the magnitude of the increase in the $\delta^{15}N$ value which occurs with a single trophic step. Recent research has emphasized that values of both $\beta$ and TDF can vary among biological systems [31], and such variability should be considered in the application of Equation (1) (see CSIA limitations below).

In several studies, CSIA revealed that ecological inferences based on bulk tissue isotope analyses were misleading. Dale et al. [82] found that when brown stingrays (*Dasyatis lata*) sampled inshore reached a body size indicative of sexual maturity, bulk muscle tissue $\delta^{15}N$ values declined, suggesting that large stingrays were feeding almost one trophic level lower than smaller individuals [4]. Values of source AA $\delta^{15}N$ were surprisingly lower for the larger stingrays, however, indicating that the cause of the decrease in their bulk tissue $\delta^{15}N$ values was not feeding at a lower trophic level, but instead feeding on prey which originated in offshore waters with lower $\delta^{15}N$ values at the base of the food web. Similarly, Seminoff et al. [83] showed that leatherback sea turtles (*Dermochelys coriacea*) departing nesting beaches in Indonesia could be divided into groups with bulk skin tissue $\delta^{15}N$ values of 13–18‰ and 10–12‰. However, rather than feeding at different trophic levels, this difference was attributed

to lower $\delta^{15}$N values for source AA resulting from varying oceanographic characteristics among foraging regions.

In addition to nitrogen isotope values, $\delta^{13}$C values are also informative for understanding animal feeding ecology. For example, Ruiz-Cooley et al. [84] reported a striking temporal trend in sperm whales (*Physeter macrocephalus*) feeding in the California Current off the western coast of North America. Bulk skin tissue $\delta^{13}$C and $\delta^{15}$N values of the whales declined by 1.1‰ and 1.7‰ from 1993 to 2005; a single sample from 1972 suggested that the decline had been occurring for decades. These trends were paralleled by decreases of similar magnitude in the $AA_{ESS}$ $\delta^{13}$C and source AA $\delta^{15}$N values. This pattern indicates that the whales likely did not change their diet over time, but rather that the California Current food web likely experienced changes in biogeochemical cycling, primary production rates, or primary producer species composition that affected the baseline stable isotope composition of the food web.

Analysis of $AA_{ESS}$ $\delta^{13}$C values have been particularly useful for studying community ecology, food web structure, and energy flow. Scott et al. [85] and Larsen et al. [86,87] found that patterns of the relative $AA_{ESS}$ $\delta^{13}$C values in producers such as plants, bacteria, and fungi consistently differed among groups defined by physiology or $CO_2$ sources. These patterns can be distilled via multivariate statistical techniques such as principal component analysis (PCA) and linear discriminant analysis (LDA), leading to the identification of distinct 'fingerprints' or patterns in $\delta^{13}$C values among individual $AA_{ESS}$. These fingerprints can provide resolution among types of primary producers that often do not differ in bulk tissue isotope values; e.g., fingerprints can distinguish between phytoplankton and understory macroalgae [88], and between seagrass and mangroves [89]. Importantly, because isotope values of $AA_{ESS}$ are shaped by biochemical processes that are conserved across taxa, these fingerprints are not thought to change with growth rates, seasons or locales, which provides a tremendous advantage over bulk tissue isotope studies [88,90,91]. Furthermore, because $AA_{ESS}$ $\delta^{13}$C values are generally not altered by consumers, $AA_{ESS}$ $\delta^{13}$C fingerprints appear to persist across trophic levels and to be identifiable in top consumers [87,88,91]. We note, however, that the previously-discussed potential contribution of $AA_{ESS}$ from the gut microbiome to host protein metabolism should be considered when interpreting $\delta^{13}$C fingerprints.

To date, fingerprinting has been primarily utilized in marine ecology. The first application assessed the relative importance of terrestrial, marine, and microbial production to consumers living in a mangrove ecosystem [89]. Fingerprinting was also used by Larsen et al. [90] to quantify the amino acid contribution from bacteria to organic carbon in ancient Peruvian marine sediments and to infer historical ecosystem processes. McMahon et al. [91] measured producer fingerprints in coral reef fishes to demonstrate that shelf vs. oceanic reefs differ in the degree to which consumers use benthic, pelagic, or recycled carbon. Most recently, Elliott Smith et al. [88] applied this approach to test for connectivity between inter- and subtidal components of a nearshore ecosystem in south central Alaska, finding differences among trophic levels in the degree of subtidal kelp-derived nutrients. Outside of marine ecosystems, this approach has been used to evaluate the importance of terrestrial versus instream primary production for freshwater fishes [92] and the sources of $AA_{ESS}$ for detritus feeders in terrestrial soils [68,93].

## 5. CSIA Limitations

To fully achieve the promise of CSIA in addressing a variety of ecological and physiological questions, further technical refinement is needed via both laboratory experiments and field studies. In particular, the values of β and TDF in Equation (1) require attention. Based on a limited amount of published data for a combination of cultivated and wild-collected primary producers, β values appear to vary among marine phytoplankton, $C_3$ plants, and $C_4$ plants [37]; however, the paucity of available data does not allow for an estimate of how β varies within these general classes of producers. Furthermore, we currently lack β values for many important groups of producers, including marine and freshwater macroalgae, seagrass, aquatic macrophytes, and CAM plants.

In regard to TDF values, some reviews have suggested that they are approximately constant. For example, $\delta^{15}N_{Glu} - \delta^{15}N_{Phe}$ for consumers in marine ecosystems were reported to be ~7.5‰ for each trophic level increase [37]. However, more recent meta-analyses that include data from a larger number of laboratory and field-based studies show that TDF values are quite variable and are correlated to consumer physiology [31,62,94], especially differences in how animals excrete nitrogen. Surprisingly, the fact that ammonia requires few steps for biosynthesis and is rapidly excreted, while urea production requires additional steps and may be retained or recycled, has been used to explain opposite patterns: that the TDF of ammonia-excreting animals are lower than urea-excreting animals in bulk tissue analyses [3], and higher for trophic AA in CSIA [74]. This inconsistency has yet to be explained; $\delta^{15}N$ values for arginine, a nitrogen-rich AA that is not commonly measured in nitrogen-focused CSIA studies, would potentially be informative. Indeed, for all CSIA studies, researchers should bear in mind that dynamics of carbon, nitrogen, and hydrogen can be influenced by AA that are not commonly measured. Some CSIA studies have also suggested that multiple pairs of trophic-source AA (e.g., Glu-Phe and Pro-Phe) should be used to address the issue of inherent variation in physiologically-mediated discrimination and to provide more tailored taxon-specific estimates of trophic level based on AA $\delta^{15}N$ analysis [74,94]. In general, CSIA data from a greater variety of species and ecosystems will enable comparative analyses that will help reveal the mechanisms driving variation in isotope values.

Similar to AA-based estimates of trophic level, AA fingerprinting requires additional data collected from wild contexts in addition to controlled feeding experiments on a wider range of taxa. Currently, not enough information is available to broadly assess the uniqueness of fingerprints across time and space among the organisms—plants, algae, bacteria, and fungi—that synthesize $AA_{ESS}$ de novo. To our knowledge, only a single study has examined $AA_{ESS}$ $\delta^{13}C$ fingerprints among primary producers at local scales [88]; other studies have used global compilations of fingerprints from various $AA_{ESS}$ synthesizers (e.g., [85,87,89,90]). It is also unclear at what taxonomic and functional scales fingerprints become indistinguishable. Furthermore, no study has validated via controlled feeding experiments the persistence of $AA_{ESS}$ fingerprints from dietary protein into consumer tissue. Eliminating these gaps in our understanding will require greater inclusion of physiological and biochemical mechanisms, which offers tremendous opportunities for refining and expanding this approach to address fundamental questions regarding energy flow in community and ecosystem ecology.

Another limitation and potential research opportunity are estimates of the isotopic incorporation rates for individual AA and FA, which are fundamental to applications of this approach to wild animal populations because it is paramount to understand the timeframe over which isotope values integrate ecological information. There is extensive literature on isotopic incorporation in bulk tissues (e.g., [12,95,96]) in which the isotopic composition of a consumer diet is changed in a feeding experiment and tissues are collected at regular intervals to quantify the incorporation rate, residence time, and half-life of isotopes. Studies of bluefin tuna (*Thunnus orientalis*; [97]), white shrimp (*Litopenaeus vannamei*; [98]), and zebra finches (*Taeniopygia guttata*; [40]) indicate that there is substantial variation among AA and FA in incorporation rates, and that rates can be altered by demands such as increased muscular activity. Additional studies should test if this variation is consistent across taxa and through time. Results will allow ecologists and physiologists to better understand the time reflected in the isotopic composition of compounds mediated by physiological processes associated with nutrient assimilation and tissue synthesis. Such information from controlled settings is essential for the physiological and ecological interpretations of CSIA data collected from wild animals.

Lastly, as CSIA becomes more widely adopted, it is important that researchers standardize sample preparation protocols and share internal reference materials. This is especially important for derivatization [53] and methylation [39] techniques which add carbon and/or hydrogen to AA and FA, and which require careful post analysis corrections and data reduction. In addition, because CSIA data for AA have been generated using multiple derivatization techniques [48], meta-analyses will

require direct comparisons of materials prepared with each technique so that differences resulting from methodology alone can be reconciled.

## 6. CSIA Future Directions

In addition to the opportunities highlighted in the section above, several recent technical advances in the application of CSIA to ecological and physiological questions have opened new avenues of research. Fogel et al. [99] were the first to report hydrogen isotope data for individual AA, using experiments on cultured *E. coli* to show that the incorporation of hydrogen from environmental water into both $AA_{NESS}$ and $AA_{ESS}$ during de novo synthesis varied with media protein content. As expected, *E. coli* used a higher proportion of environmental water to synthesize AA when supplied media did not contain protein. In the treatment containing protein, the degree of direct routing of hydrogen from media into *E. coli* was higher for $AA_{ESS}$ than for $AA_{NESS}$, even though as prokaryotes *E. coli* can synthesize all forms de novo. Lastly, the proportion of water used to synthesize alanine ($AA_{NESS}$) was high (40–50%) regardless of media treatment. Collectively, these patterns suggest that AA hydrogen isotope analysis may enable ecologists to simultaneously trace both dietary and environmental water sources of hydrogen to an individual animal.

A second technical advance that promises to broaden the use of CSIA is position-specific isotope analysis. Using a high-pressure liquid chromatography system interfaced with an IRMS, Fry et al. [100] automated a ninhydrin-based method for separating the carboxyl carbon atom and measuring its carbon isotope composition at a level of analytical precision (<0.5‰) that will likely make it very useful for tracing key biochemical reactions. While the carboxyl carbon represents a single atom in an AA skeleton containing up to nine carbons (i.e., in Phe), it is arguably the most labile carbon because it is directly involved in a number of (de)carboxylation reactions involving AA precursors in the Calvin cycle, glycolysis, and the TCA cycle [101]. This relatively high degree of mobility likely produces significant isotopic fractionation that can be now traced with position-specific isotope analysis. Additional methods are being developed for measuring the $\delta^{13}C$ of carbon in specific positions and sections of AA, and over the next decade this capability will likely open new frontiers for isotope ecology, enabling ecologists and physiologists to address novel questions.

In many respects, the development of CSIA is analogous to that of bulk tissue isotope analysis. In the early stages of widespread adoption of bulk tissue analysis in animal biology, Gannes et al. [102] predicted explosive growth in the use of this method and exhorted researchers to complement their field applications with controlled experimental studies to validate key assumptions. A decade later, Martínez del Rio et al. [7] were pleased to report that bulk tissue isotope analysis had indeed flourished and was now supported by a broader suite of experimental evidence for the mechanisms driving observed patterns. Here, we echo this prediction and exhortation for CSIA. In the last five years, there has been a dramatic increase in the number of studies using CSIA to answer biological questions (e.g., [31]) and this tool is poised to make significant contributions to ecology and physiology. In addition to novel applications, we urge researchers to continue developing theoretical and experimental validations of the processes that shape AA and FA isotope values.

**Conflicts of Interest:** The authors declare no conflict of interest.

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