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Compound-specific isotope analysis of amino acids for aquatic systems – Problems, challenges, solutions: A review

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ABSTRACT

Compound-specific isotope analysis of individual amino acids (AAs) is a promising method for characterizing resource flow and transformation in aquatic systems, encompassing microbial domains to macroscopic consumers. This review delves into two key areas of research concerning AAs in aquatic ecosystems. Firstly, carbon isotope analysis is employed to explore carbon sources assimilated for AAs synthesis, and secondly, nitrogen isotope analysis is used to clarify ecosystem baselines and trophic dynamics of organisms. Surprisingly, literature provides limited discussions on the limitations or critical challenges faced during AAs isotope analysis over the past decade. To address this gap, this review focuses on challenges encountered in method development, sample preparation, and technical aspects, including instrumental design, and $\delta^{13}C$ and $\delta^{15}N$ analysis of AAs. Recommendations are provided to increase the accuracy and reliability. The review also highlights the significance of comprehensive understanding and detailed methodology to address challenges during AA isotope analysis in aquatic systems.

1. Introduction

1.1. Stable isotope analysis (SIA) in aquatic systems

Compound-specific isotope analysis (CSIA) has gained increasing attention among researchers in recent years, as it allows further insight into various aspects of plant and animal ecophysiology, the structure of food webs and trophic interactions within aquatic ecosystems [1], energy pathways in freshwater ecosystems and their connections to the surrounding marine and terrestrial ecosystems [2]. Insights from CSIA are particularly valuable in aquatic environments due to factors such as limited opportunities for direct observation, high spatial complexity, and multiple potential sources of nutrients, prey, and organic matter [2]. CSIA has been used to (i) study food web dynamics [3,4], (ii) topography [5],(iii) estimate dietary composition [6], (iv) investigate biogeochemical processes [7], (v) assess the field metabolic rate of aquatic organisms such as fishes [8],(vi) understand host-parasite nutritional relationships [9], (vii) examine migration patterns of aquatic organisms such as fish and marine mammals [10], (viii) evaluate the impact of wastewater on macroinvertebrate assemblages [11], (ix) identify the

Abbreviations: AA, amino acids; BSIA, bulk stable isotope analysis; CSIA, compound-specific isotope analysis; CSIA-AA, compound-specific stable isotope analysis of amino acids; DOM, dissolved organic matter; EA, elemental analyzer; EAAs, essential amino acids; GC, gas chromatography; GC-*C*-IRMS, Gas Chromatography-Combustion-Isotope Ratio monitoring Mass Spectrometry; GC-MS, Gas Chromatography- Mass Spectrometry; Glu, glutamic acid; HRMS, high-resolution mass spectrometry; IPL, polar lipids; IRMS, isotope ratio monitoring mass spectrometer; KIE, kinetic isotope effect; LC, liquid chromatography; LC-IRMS, Liquid Chromatography-Isotope Ratio monitoring Mass Spectrometry; NEAAs, non-essential amino acids; NANP, N-Acetyl-*n*-propyl; NANP, N-pivaloyl-*i*-propyl; Phe, phenylalanine; PLFAs, phospholipid fatty acids; PSIA, position-specific isotope analysis; SPM, suspended particulate matter; SIR, Stable isotope ratio; TFA-IP, trifluoroacetyl-isopropyl; TL, trophic level; TP, trophic position.

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chemistry of flowing water [12], and (x) understand the effects of urbanization on freshwater organisms [13].

Stable isotope ratios of nitrogen ($^{15}N/^{14}N$) and carbon ($^{13}C/^{12}C$) are among the most abundant biogenic elements in the environment. Biological processes are primarily driven by kinetic isotope effects that stem from the different energy requirements to break chemical bonds in molecules containing different isotopes. In such scenarios, breaking of the bonds involving the lighter isotopic variant (^{12}C) takes place at a faster pace compared to the heavier isotopic form (^{13}C) [14,15]. This divergence in bond cleavage rates leads to the emergence of differences between the two isotopic forms, a phenomenon referred to as isotopic fractionation. Consequently, when the substrate is not entirely consumed, this fractionation results in the substrate being relatively heavier compared to the biologically produced product.

Isotope ratios are generally expressed in the δ-notation. The general expression is provided in Eq. (1) [16].

$$\delta X = \left[\left(\frac{R_{sample}}{R_{std}} \right) - 1 \right] \quad \text{Eq. 1}$$

Here, X represents the heavy isotope form of an element (e.g., ^{15}N or ^{13}C), R denotes the heavy-to-light isotope ratio (e.g., $^{15}N/^{14}N$ or $^{13}C/^{12}C$), and the units are expressed in per mil (‰). For nitrogen and carbon, the international standards used are atmospheric nitrogen (AIR; $\delta^{15}N$) and Vienna Pee Dee Belemnite (VPDB; $\delta^{13}C$) [17]. A positive or negative δX value in the sample indicates that it contains a higher or lower amount of the heavy isotope compared to the corresponding international standard [18].

To accurately interpret stable isotope data, ecologists must understand several key principles that govern how stable isotopes are incorporated into organisms [3,14]. These include how organisms assimilate dietary nutrients, how compounds and nutrients are routed within the body, and the varying rates at which different tissues turn over or incorporate isotopes [19–21]. Understanding these processes is crucial, as overlooking them can lead to errors such as misidentifying diet composition, incorrectly estimating turnover rates and dietary shifts, or making inaccurate assessments of trophic position and community structure [14]. The effective use of stable isotopes for the estimation of turnover rates in animal tissues and key compounds within tissues, as well as exploring the significance of isotopic turnover estimates for nutritional and ecological studies [15] is still a subject of investigation.

The principle of "you are what you eat" holds true in a general sense for carbon and nitrogen isotopes in source amino acids, reflecting how an organism's isotopic signature closely mirrors that of its diet [22,23]. However, metabolic branching can cause isotope values in certain compounds to deviate from the diet, resulting in values that can be either more positive or more negative than those found in the diet [24]. Despite these variations, the average isotopic values typically align with the dietary baseline [25,26]. As these compounds are further metabolized, particularly trophic amino acids, a process known as trophic fractionation occurs. This process, which often involves the preferential retention of heavier isotopes, leads to an enrichment of these isotopes along the food chain. Consequently, consumers often exhibit higher $\delta^{13}C$ or $\delta^{15}N$ values than their diet due to this metabolic reworking [23,25,27]. These differences between consumer and diet isotopic values are symbolically represented as $\Delta_{con\text{-}diet}$ (as detailed in supporting information S1, Eq. (6) [28]).

In aquatic systems, samples for CSIA are comprised of a variety of matrix related compounds such as sediment, suspended particulate matter (SPM), dissolved organic matter (DOM) [29,30], and aquatic organisms [31,32]. Analysis of these samples often includes various clean-up, extraction, decalcification, and/or derivatization steps before analysis using an isotope ratio monitoring mass spectrometer (IRMS). Depending on the aims of the investigation, analysis might proceed via three possible sample introduction methods: elemental analyzer (EA) for bulk stable isotope analysis (BSIA) of biomass, SPM, sediment, or previously isolated single compounds (BSIA); gas chromatography (GC) and liquid chromatography (LC) for CSIA of individual molecules like amino acids (AAs), phospholipid fatty acids (PLFAs) or intact polar lipids (IPLs) [33]. The application of compound-specific stable isotope analysis of amino acids (CSIA-AA) in aquatic systems has been extensively reviewed [27,34,35]. However, the challenges encountered during the preparation and execution of CSIA-AA have not received extensive attention. In some instances, inclusion of detailed methodological parameters that could prove invaluable for future researchers in replicating individual studies has been overlooked. The current review aims to discuss the technical and methodological challenges accompanying CSIA-AA when studying aquatic systems, to offer suitable recommendations or solutions as far as possible, and to highlight existing research gaps.

1.2. CSIA as supplement to BSIA

Analytical techniques such as GC-*C*-IRMS (Gas Chromatography-Combustion-Isotope Ratio monitoring Mass Spectrometry) or LC-IRMS (Liquid Chromatography-Isotope Ratio monitoring Mass Spectrometry) have opened up new possibilities to enhance the specificity of dietary studies [26,36,37] and to expand the knowledge in the field of trophic ecology [3,25,31,38]. Introducing LC or GC before IRMS analysis separates target compounds (like AAs or fatty acids) from complex mixtures, in contrast to BSIA, which assesses the target element in all compounds in the sample as a weighted average. This biochemical building-block approach of CSIA enables more precise tracing of molecular exchange within the food web [39,40]. The choice of using either GC or LC depends on various intrinsic and extrinsic factors ranging from the nature of target analytes (e.g. their chemical functionality), matrix complexity, and the potential of developing suitable chromatographic LC or GC separation for the compounds of interest [41]. Despite the widespread use of BSIA in ecological studies focused on diet and food webs, the interpretation of BSIA results can be complex due to factors associated with baseline interpretation, isotopic discrimination, and routing [42]. CSIA can be usefully applied to situations where BSIA yields ambiguous results such as (a) tracing incorporation of quantitatively minor but qualitatively significant components, (b) different food sources with similar bulk isotope values or (c) isolating contributions from organisms or food sources (specifically microphytobenthos) that cannot be physically separated from each other [43]. Ecologists have viewed compound-specific isotope analysis of amino acids (CSIA-AA) as a valuable method to supplement BSIA [42]. Recent studies [34,44] emphasize the potential of CSIA-AA in conjunction with bulk stable isotope analysis (BSIA), offering insights into resource flow, trophic dynamics, and spatiotemporal shifts in basal resource use within food webs. A more detailed discussion on the importance and applications of CSIA-AA is provided in the subsequent section, " importance of CSIA of amino acids."

1.3. Importance of CSIA of amino acids

AAs can be found in various types of environmental samples and are predominately of biological origin (synthetized by living organisms). The continuous-flow isotope analysis of individual compounds, which emerged in the late 1990s, has facilitated the broader and more frequent use of AAs $\delta^{15}N$ and $\delta^{13}C$ in the field of aquatic ecology throughout the past few decades [34]. In the field of aquatic systems, two major research areas have placed significant emphasis on CSIA-AAs, (i) using carbon isotope analysis to explore the sources of carbon used in amino acid synthesis and (ii) refining the estimation of trophic position (TP) of organisms through nitrogen isotope analysis of amino acids. These research areas have contributed to a better understanding of consumer-prey relationships concerning dietary items [45,46], estimation of trophic position and food chain length [47,48], fractionation and incorporation of amino acids into consumer tissue in host-parasite

studies [49,50], exploring host-parasite dynamics [9,51,52], and examining dietary routing [26] within aquatic ecosystems.

To understand the underlying biochemical and physiological processes in these research areas, comparing isotope ratios between individual AAs is a common practice. The variations in AAs $\delta^{15}N$ (ΔN) and $\delta^{13}C$ (ΔC) values are widely examined during ecological studies [25,27]. Considerable differences between AA ΔN values have led to the classification of trophic, source and metabolic AAs (Table 1). "Trophic" AAs typically undergo a large fractionation during metabolism that leads to a large increase in $\delta^{15}N$ values (~2–8 ‰ [25]) during trophic transfer [36,53]. This larger fractionation is associated with deamination (conversion of an amine group (-NH_2) to ammonia), which is a primary metabolic pathway in case of trophic AAs. In contrast, source amino acids undergo minimal fractionation (0.4 ‰ in case of Phe [45]) in $\delta^{15}N$ values during trophic transfer and closely resemble the $\delta^{15}N$ values of their dietary sources. This is likely due to the fact that the initial metabolic reactions of source AAs are generally dominated by processes that do not involve the formation or cleavage of C–N bonds [27]. Threonine is classified as a metabolic AA with a significantly lower $\delta^{15}N$ value compared to other amino acids found within the same protein [54]. The fractionation processes involving metabolic AA are less well understood and still to be characterized. Similarly, the extent of AAs ΔC values is related to the classification of amino acids into essential amino acids (EAAs) and non-essential amino acids (NEAAs) (Table 1). Larger variation in ΔC as a result of fractionation is observed in NEAAs (0.5–2.4 ‰ [55]), which consumers can synthesize *de novo* using the carbon skeleton of other biomolecules such as fatty acids and carbohydrates. In contrast the $\delta^{13}C$ values of EAAs typically closely resemble those of their food sources, showing only minor changes (0.1–0.3 ‰ [55]) during trophic transfer [34]. The reactions involving N and C in amino acids, similar to other physiological processes, show a selective preference for the lighter stable isotope through kinetic isotope fractionation except for N in Thr, which is a strong reverse fractionation [56]. As metabolic processes occur, molecucles including the lighter isotopes (^{14}N and ^{12}C), are favored and preferentially used or removed, resulting in the relatively higher abundance of the heavier isotopes (^{15}N or ^{13}C), in the consumer's body. This phenomenon helps explain the large differences observed in trophic AA $\delta^{15}N$ values [25,27] as increased interaction with the internal Glu pool cause increased values.

CSIA-AA N values can be used to provide a baseline integrated estimate for each individual's trophic level (TL) within the food web. This estimate often uses the difference between the $\delta^{15}N$ values of glutamic acid (Glu), a trophic AA, and phenylalanine (Phe), a source AA to calculate TL using Eq. (2) [45].

$$TL_{Glu/Phe} = [(\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - \beta_{Glu/Phe})/TDF_{Glu\text{-}Phe}] + 1 \quad \text{Eq. 2}$$

Table 1
Classification of amino acids into essential and non-essential [26,49] to understand AA $\delta^{13}C$ fractionation and into source, trophic and metabolic [53,54] to understand AA $\delta^{15}N$ fractionation.

	NEAAs	EAAs	Fractionation on basis of $\delta^{15}N$
Source AAs	Glycine[a] Serine[a] Tyrosine	Phenylalanine Methionine Lysine	0–1.7 ‰ [58] 0.4 ‰ for Phe [45]
Trophic AAs	Alanine Aspartic acid Glutamic acid Proline	Isoleucine Leucine Valine	~2–8 ‰ [25]
Metabolic AA		Threonine	–
Fractionation on basis of $\delta^{13}C$	0.5–2.4 ‰ [55]	0.1–0.3 ‰ [55]	

[a] Glycine and serine exchange N with each other during biochemical processes [53].

Where $\beta_{Glu/Phe}$ represents the isotopic difference between Glu and Phe in the underlying primary producers supporting the ecosystem and $TDF_{Glu\text{-}Phe}$ represents the trophic discrimination factor indicating a 2–8 ‰ stepwise increase in the difference between Glu and Phe that occurs with each trophic level. However, a combination of multiple trophic and source AAs [57] can also be used as well as scaled equations that can better account for impacts of diet quality or larger TDF values of lower trophic level organisms (e.g. herbivores) [25].

2. GC-IRMS analysis of AAs

GC-*C*-IRMS is a valuable technique for automated online measurement of GC compatible organic compounds [59]. It can be used to measure isotope ratios of light elements, e.g. $^{13}C/^{12}C$, $^{3}H/^{2}H$, $^{18}O/^{16}O$, and $^{15}N/^{14}N$ [60] Currently, GC-IRMS is the most widely used choice for carbon and nitrogen isotope analysis of AAs [31,38,45,61,62]. Many published methods can separate around 10–14 AAs in approximately an hour, using a variety of different columns and types of derivatization reagents to make AA GC amenable to analysis. These methods usually use 30 or 60 m columns, He flow rates ranging from 1 to 2 mL/min, and GC oven temperatures ranging from 40 to 300 °C. Silverman et al. recently conducted a review on methods regarding derivatization pathways during preparation of AAs samples, column selection, and emerging technologies [63].

For $\delta^{13}C$ and $\delta^{15}N$ GC-IRMS isotope analysis of derivatized AAs, two different systems are available, one consisting of two reactors – a combustion (Fig. 1a) and a reduction reactor – or a single reactor system with a combined oxidation-reduction system (Fig. 1b). In the two-reactor system (Fig. 1), the analytes are combusted in an oxidation reactor operating at a temperature of 940–1000 °C, resulting in the formation of carbon oxides (CO_x) and nitrogen oxides (NO_x). In order to convert NO_x to N_2, an additional reduction reactor is used at 550–600 °C. A cryogenic trap is used downstream of the reduction reactor to capture CO_x prior to introduction into the IRMS to prevent interference with nitrogen isotope measurements. The oxidation reactor consists of a non-porous alumina tube with a combination of wires, such as three wires of copper (Cu), nickel (Ni), and platinum (Pt) [45,64], three wires consisting of two Cu and Pt [65], or six wires consisting of two Ni, three Cu, and Pt [32]. The reduction reactor, on the other hand, consists of a non-porous alumina tube with varying numbers of copper wires, typically ranging from three [45,59,65,66] to six [32]. In all scenarios, all wires should have an identical length and diameter. This system was initially introduced in 1994 by Merrit and Hayes [67] and has been widely used since for the analysis of amino acid derivatives.

Despite its widespread use, the conventional system exhibits poor oxidation performance, posing a greater challenge for measuring nitrogen isotope values compared to carbon isotopes, thereby resulting in systematic isotope fractionation [59]. To address this issue and to improve chromatography, precision, and accuracy in nitrogen isotope analysis while minimizing the risk of leaks due to fewer connections, Thermo Fisher Scientific (Bremen, Germany) has developed a single-reactor system [59] (Fig. 1b). This system, employed in numerous studies for analysing AAs $\delta^{15}N$ [31,62,68], features a non-porous alumina tube containing a NiO tube filled with either Cu and Ni wires [59,62] or Cu, Ni, and Pt wires [68]. This design serves as both an oxidation and reduction reactor, eliminating the need for a separate reduction reactor. The Ni tube in this reactor has a greater oxidized surface when operated at higher temperatures (1010–1030 °C), resulting in a prolonged lifetime and increased active surface area. The Ni/NiO surface acts like a reduction catalyst by withdrawing oxygen from NO_x and converting it to N_2. These surface properties have the potential to enhance the conversion of challenging target compounds e.g. amino acid derivatives, that conventional designs struggle to convert [59].

However, occasionally in published studies using CSIA-AA derivatives and analysis by GC-IRMS important methodological and

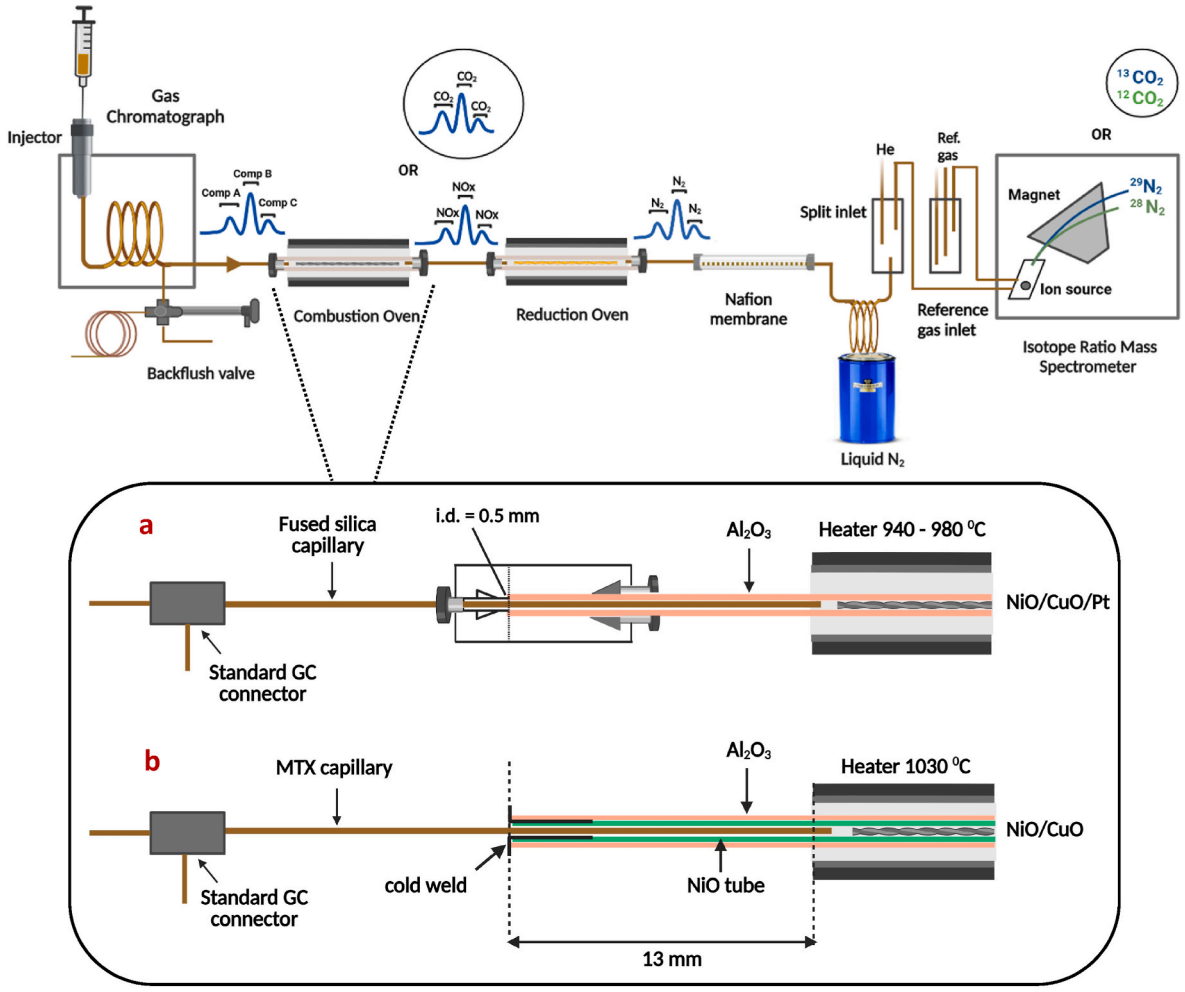

Fig. 1. Schematic representation of GC-IRMS for carbon and nitrogen isotope analysis. **a)** Conventional combustion reactor. **b)** Combined oxidation-reduction reactor.

instrumental details are missing as indicated in Table S2 in the supporting information. Detailed information is valuable for researchers to understand the specific factors and procedures involved in generating the data which might be critical for reproducibility of the results. Furthermore, in existing literature, there is little discussion regarding the specific limitations or critical challenges accompanying CSIA-AA.

2.1. Methodological and technical challenges and solutions

2.1.1. Hydrolysis

In order to release individual AAs from proteins, peptide bonds need to be broken, which is mostly done by acidic hydrolysis [32] with 6–12 M hydrochloric acid (HCl) incubated for 1 h - 1 day at 100–150 °C. AAs are typically hydrophilic, containing short carbon skeletons with functional groups like $–NH_2$, –NH, –COOH, –OH, and –SH. This hydrolysis reliably yields 13 out of 20 amino acids, including all basic and aliphatic amino acids. Adjusting the hydrolysis duration can influence the recovery of stable residues and even enhance the yield of specific amino acids [63]. For instance, extending the hydrolysis time beyond 24 h can enhance the recovery of aliphatic AAs such as leucine, isoleucine, and valine, but may decrease the recovery of other AAs [69]. On the other hand, reducing the hydrolysis time to less than 20 h can optimize the recovery of serine and threonine [70]. Furthermore, phenylalanine and glutamic acid remain stable regardless of the duration of hydrolysis, while tryptophan is unstable regardless of the hydrolysis time [63,71]. Consequently, the selection of hydrolysis duration should be tailored to the specific amino acids of interest.

After hydrolysis, hydrophobic groups (e.g., lipids) may remain or form, requiring sample cleaning before further derivatization steps, such as lipid extraction using organic solvents like n-hexane and DCM (in a 3:2 v/v ratio). Some samples may also contain interfering materials, such as aggregated microbial samples, sediments, soils and some biological tissues, which require pre-treatment before derivatization, such as solid phase extraction [27]. Several procedures are available in the literature to remove interfering materials, with cation exchange chromatography being considered as an effective method with sufficient recovery [72–74].

2.1.2. Derivatization

In order to analyze AAs by GC-IRMS, it is required to first derivatize them to reduce their polarity and increase their volatility. Derivatization involves stabilizing polar groups like amino, carboxyl, and hydroxyl groups by replacing active hydrogen atoms with nonpolar groups, which improves their chromatographic separation [27]. Over the last two decades, a wide variety of derivatization agents have been utilized for this purpose [63]. In recent years, commonly used amino acid derivatives in aquatic ecological studies include pivaloyl-isopropyl AAs ester (Pv/iPr), which can substitute at both the amino (NH_2) group and hydroxyl (OH) group [45,62,68], trifluoro acyl-isopropyl AAs ester (TFA/iPr), which also targets both the NH_2 and the OH groups for derivatization [75–78], and methoxycarbonyl (MOC) AA ester, which primarily substitutes at the NH_2 group and may also substitute at the OH group if it is present

[31,32,79,80]. During derivatization, the general chemical reaction (Fig. 2) involves esterifying the carboxyl group with an alcohol group under acidic conditions. Subsequently, the amino group undergoes acylation, followed by the acetylation of the hydroxyl group, if present in the amino acids, using an acid chloride or acid anhydride [27].

Several factors guide the selection of GC derivatives, including their applicability to common AAs, stability, ease of use, the number of atoms added by the derivatization agent, volatility of the product, by-product formation, reaction time, reaction yield, kinetic isotope effect, analyte to derivatized carbon (C) ratio, correction factors, pyrolysis or combustion efficiency, enantiomer preservation, and chromatographic resolution [63,81]. For instance, to avoid isotopic fractionation during AA reactions, sufficient derivatization reagents must be present to fully convert the substrate. However, a drawback of derivatization is its non-quantitative nature, even under appropriate conditions, leading to the formation of secondary derivatization products [82] or products formed as a result of excess derivatization reagents [32]. This effect is more pronounced in C isotope analysis of AAs compared to nitrogen (N) isotope analysis. For N isotope measurements, results can be obtained with various derivatization agents without the need for stoichiometric correction of resulting delta (‰) values. This is because the derivatization process does not introduce additional N atoms into the analyte. Therefore, the isotope effect, where isotopic composition can be altered due to changes in molecular structure, is not a concern if the analyte is quantitatively derivatized [83,84]. However, in the case of carbon, it is essential to subtract the additional C atoms added by all derivatization agents, otherwise they alter the isotope ratio values of the derivatized AAs. Furthermore, larger derivative groups may be less suitable for isotope analysis due to increased propagated uncertainty associated with calculated isotope values of the amino acid if an increasing number of atoms are added during derivatization [85].

2.1.3. Kinetic isotope effect

The KIE arises from differences in vibrational energy levels between bonds and represents the ratio of reaction rate constants between two isotopologues. Typically, lighter isotopes react faster than heavier isotopes [86] because heavier isotopes have lower vibrational energy levels due to their higher reduced mass, resulting in varying reaction rates. Within derivatization reactions, the predominant influence often arises from non-quantitative reaction involving one of the reagents. This can lead to the manifestation of a KIE at any carbon site participating in the step that determines the rate, ultimately resulting in carbon isotope fractionation at a particular position within the derivatized compound [85]. Derivatization can influence the analysis in various ways: (I) The isotopic composition of the analyte may vary due to the introduction of atoms from the derivatization agents. (II) Kinetic isotope effects (KIE) during derivatization can lead to additional isotopic fractionation in the isotope values of the newly introduced atoms. (III) In the case of non quantitative sample conversion also the atoms of the original AA backbone may be affected by isotope effects further introducing potential bias in the isotope values of the derivative [87].

An example of the second scenario is observed during the derivatization of amino acids into TFA-IP [88], NANP, and NPNP esters [89]. These reactions demonstrate KIEs associated with trifluoroacetylation, acetylation, and pivaloylation, which can influence the trueness of $\delta^{13}C$ value determinations. Although such reactions may achieve excellent precision, systematic deviation from the true $\delta^{13}C$ values of the underivatized analyte may occur due to the introduction of additional carbon atoms with distinct isotopic signatures from the derivatization reagents [85,89]. Even when the analyte's conversion is assumed to be quantitative, kinetic isotope fractionation may still occur during the acylation step. In this step, derivatization reagent molecules that are isotopically lighter than the average reagent molecule preferentially react, introducing a potential bias in the $\delta^{13}C$ value of the derivative [88]. This isotopic fractionation is due to excess derivatization reagent, where the carbon atoms in the rate-determining step come from the derivatization reagents rather than the amino acids. Consequently, the overall isotope bias stems from the derivatization reagent's unique isotope value, with additional fractionation effects introduced during the derivatization reaction [85,88,89]. Furthermore, when the derivatizing reagent is present in excess but does not fully react with the amino acids, a KIE can occur at the acyl carbonyl carbon, leading to isotopic fractionation [81]. To prevent this, it is crucial to ensure that derivatization reagents are available in sufficient quantities to complete the reaction [90]. To calculate the KIE for each derivatization procedure under conditions of excess reagent, a mass balance equation (Eq. (3)) [81] is first employed to ascertain the $\delta^{13}C$ values of the derivatized amino acid standards. This is crucial for understanding how the additional carbon from the derivatizing reagent contributes to the overall $\delta^{13}C$ value of the derivatized compound:

$$n_{cd}\,\delta^{13}C_{cd} = n_c\,\delta^{13}C_c + n_d\,\delta^{13}C_d \qquad \text{Eq. 3}$$

where n represents the number of moles of carbon, and the subscripts c, d, and cd refer to the compound of interest, the derivative group, and the derivatized compound, respectively. Next, the discrepancy between the measured and predicted $\delta^{13}C$ values of the amino acids after derivatization is defined as follows:

$$\Delta = \delta^{13}C_{cd(predicted)} - \delta^{13}C_{cd(measured)} \qquad \text{Eq. 4}$$

Finally, this information can be used to calculate the KIE (Eq. (5)) [81]:

$$KIE = 1 + \frac{\Delta \times n_{cd}}{x} \qquad \text{Eq. 5}$$

where Δ signifies the discrepancy described in Eq. (4), n_{cd} corresponds to the total count of carbon atoms present in the derivatized compound, while x denotes the quantity of functional groups accessible for acylation.

2.1.4. Chromatographic considerations

Peak broadening may arise due to several factors including incomplete oxidation, varying reaction kinetics of different analytes, oven temperature variations, and contamination of reactor components. In addition to peak broadening, poor peak resolution can be a significant problem during nitrogen isotope analysis of AAs. Compared to carbon the AAs typically contain only one nitrogen atom compared to two or more carbon atoms. For the generation of one analyte N_2 molecule, two N atoms are required in comparison to one C atom for CO_2 generation. The ionization efficiency of N_2 molecules in the IRMS ion source is around 70 % of that of CO_2 at 70 eV [84,91]. Furthermore, the natural abundance of ^{15}N (0.365) in total nitrogen is approximately three times

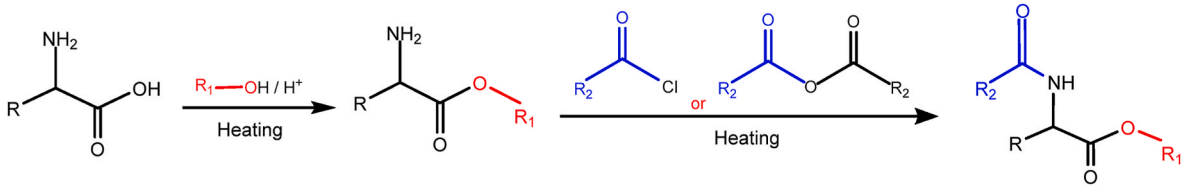

Fig. 2. General chemical reaction of derivatization of amino acids for carbon and nitrogen isotope analysis using GC-IRMS.

smaller than the abundance of ^{13}C (1.108) in carbon [17]. These factors necessitate approximately fifty times higher sample amounts for nitrogen isotope analysis to achieve the same precision as carbon isotope analysis [91]. Hence, overloading of carbon atoms of AAs is expected during the nitrogen isotope determination. For example, in the case of phenylalanine, approximately 18 times more carbon is incorporated to produce one molecule of N_2. Despite the use of high substance or carbon loads, the consequence is a decrease in peak amplitudes and a potential deterioration in chromatographic performance. Moreover, carbon overloading results in quicker and more frequent oxidation, and earlier reactor replacement to maintain combustion capacity [68,84].

Additionally, nitrogen-containing compounds tend to be more sticky, which can adversely affect chromatographic performance and peak separation due to increased sorption by active sites. Chemical conversion processes, such as combustion with metal oxides to form nitrogen oxides and subsequent reduction to N_2 in the reduction oven, along with associated plumbing, can degrade peak shape and contribute to peak broadening and tailing [92]. Other factors that can contribute to peak distortion and broadening such as a poor resolution in GC-IRMS include active sites in connectors, GC columns, and colder parts of the backflush valve, the oxidation and reduction ovens [84] (Fig. 3). The mean peak width of the peaks obtained in GC-IRMS and GC-MS is 0.83 min and 0.25 min (Table S1), respectively. Nevertheless, employing an appropriate column can also contribute significantly to enhancing the resolution for carbon and nitrogen isotope analysis of amino acids.

2.1.5. Isobaric interferences

The combustion of AAs produces H_2O, CO_x, NOx and sulfur oxides (SO_x) (e.g. in the case of methionine) as byproducts. The presence of SO_x and NO_x is problematic as they can cause corrosion to the MS. In particular, NO_x (e.g., $NO^{\cdot+}$ radical ion) can interfere with $a^{12}C^{18}O^{16}O^{\cdot+}$ radical ion at *m/z* 46 due to isobaric interference. Elimination of these ionic species is necessary and is accomplished by the reduction step, where combustion gases are passed over elemental Cu wires at 500–700 °C in the two reactor set up. This process helps remove SO_x and NO_x, but it also serves as an O_2 scrubber. Another interference can be caused by water (H_2O) which should be kept low to avoid interference in the ion source of the IRMS. Otherwise, H_2O will interfere with $^{13}C^{16}O^{\cdot+}$ by the formation of $^{12}C^{16}O^{2}H^{\cdot+}$ at *m/z* 45 [93]. H_2O is typically removed by using a countercurrent drier which is based on a sulfonated fluoropolymer Nafion permeable membrane [92]. Operation of these water driers is generally adequate at room temperature because they produce lower dew points by being more effective at colder temperatures.

The measurement of nitrogen isotopes might also be affected by the isobaric interferences from carbon monoxide (CO) (*m/z* 28) resulting from incomplete combustion. Specifically, an isotope value of ~470 ‰ indicates interference of $CO^{\cdot+}$ with N_2. $CO^{\cdot+}$ can also create interference due to its formation from CO_2^{+} ions that can decompose within the ion source, resulting in approximately 10 % of the ion current being composed of $CO^{\cdot+}$ derived from CO_2. To avoid this, it is essential to prevent CO_2 from entering the ion source using cryogenic trapping to remove CO_2 produced during combustion reactions. Moreover, in the two reactor system, achieving a balance in the operation of oxidation reactor tubes is crucial during nitrogen isotope analysis. On the one hand, sufficient oxygen must be released to ensure a complete combustion reaction of CO_2, and on the other hand, reduction reactor deactivation results in a breakthrough of NO_x in presence of excess oxygen [84,91].

2.1.6. Oxidation reactor setups for carbon and nitrogen isotope analysis

The oxidizing reagents in the combustion oven need to be regenerated, which consumes oxygen during analyte combustion and contributes to an elevated baseline during measurement. Over time, these oxidation reagents degrade and require replacement after certain sample injection. This restoration can be done by flushing the oven with a substantial amount of oxygen every two or three days for a two-reactor setup, diverting it away from IRMS. Reduced intensity of analyte peaks can indicate the need for reoxidation. Alternatively, continuous introduction of a small amount of O_2 (O_2 trickle) can also accomplish regeneration. However, the trickle amount of O_2 needs careful consideration in order to prevent damage to the IRMS filament. Typically, the flow is adjusted to produce approximately 20 nA ion current at *m/z* 32 [92].

One of the major sources responsible for peak broadening in GC-IRMS is the combustion oven, and considerable effort is required to optimize its physical design [92]. The ongoing oxidation in the combustion reactor directly impacts the reduction reactor, which serves as a barrier to prevent excess O_2 from reaching the ion source during $\delta^{13}C$ measurement and to reduce nitrogen oxides (NO_x) to N_2 during $\delta^{15}N$ measurement. Continuous reoxidation of the combustion reactor can lead to its deterioration, as identified by reduced peak intensity at *m/z* 28/29 or increased *m/z* 30 signal intensity, indicating an incomplete reduction of nitrogen oxides (NO_x). Therefore, the reducing reagents in the reduction reactor also need replacement after a specific number of sample injections. Interference from NO_x, after reoxidation of the combustion reactor, indicated by Riekenberg et al. [68], regardless of the reoxidation period, whether it is less than 2 min or more than 60 min. Various parameters play an important role for the complete reduction of NO_x to molecular nitrogen through the chemical reaction between copper and NiO, whereby the primary one is the temperature optimization. The primary method for removing nitrogen oxides involves catalytic conversion to N_2 at temperatures below 800 °C where NO_x are thermodynamically unstable [94]. Catalytic poisoning can occur due to interference from CO produced during incomplete combustion, reducing the catalytic activity of copper by competing for active sites on the catalyst. Poisoning of the combustion reactor can occur, especially when using TFA-IP amino acid derivatives. Fluorine forms stable fluorides with copper (CuF_2) and nickel (NiF_2), which irreversibly reduces the combustion efficiency of CuO/NiO systems. Additionally, fluorine also irreversibly poisons the platinum (Pt) when CuO/Pt is used as the combustion catalyst, leading to a rapid loss of platinum's catalytic activity. Combustion systems based on CuO/NiO/Pt may not immediately exhibit this effect due to the large oxygen capacity of CuO, which serves as an oxygen donor [67], but they deteriorate over time, usually after 100–150 analyte injections [87].

Researchers are now shifting to combined oxidation-reduction reactor systems to address the challenges associated with the reduction reactor, primarily M30-related issues, due to extended downtime for M30 baseline recovery after oxidation. However, optimizing the reactor performance is crucial for accurate isotope analysis. For instance, the study by Ref. [68] included an extended oxidation period at the beginning of the daily sequence before standard and sample analysis to improve performance. Their study found that a 30-min backflush following oxidation effectively restored the *m/z* 28/29 baseline, regardless of whether the oxidation period was 30 min or 2 h. Overall, the single-reactor system offers improved performance and has been beneficial, particularly for the $\delta^{15}N$ analysis of AA derivatives, as demonstrated in many studies working with samples of aquatic environments [31,62,68,78,79].

2.1.7. Leakage and memory effect

Leakage is a common issue that requires attention, particularly during nitrogen isotope analysis compared to carbon isotope analysis. Since ambient air contains a higher proportion of nitrogen ~80 % compared to CO_2 ~0.042 %, it increases the system's sensitivity to leaks and can introduce impurities into the carrier gas. The nitrogen abundance in air poses leakage problems in the GC column, connectors and in interface. Leaks often occur in connections and can develop during repeated heating cycles in GC [68]. Post-column air leaks during temperature-controlled GC runs are especially important as they result in a gradual increase in the N_2 background and partial or complete loss of analyte [91]. Therefore, ensuring a leak-free system is essential for

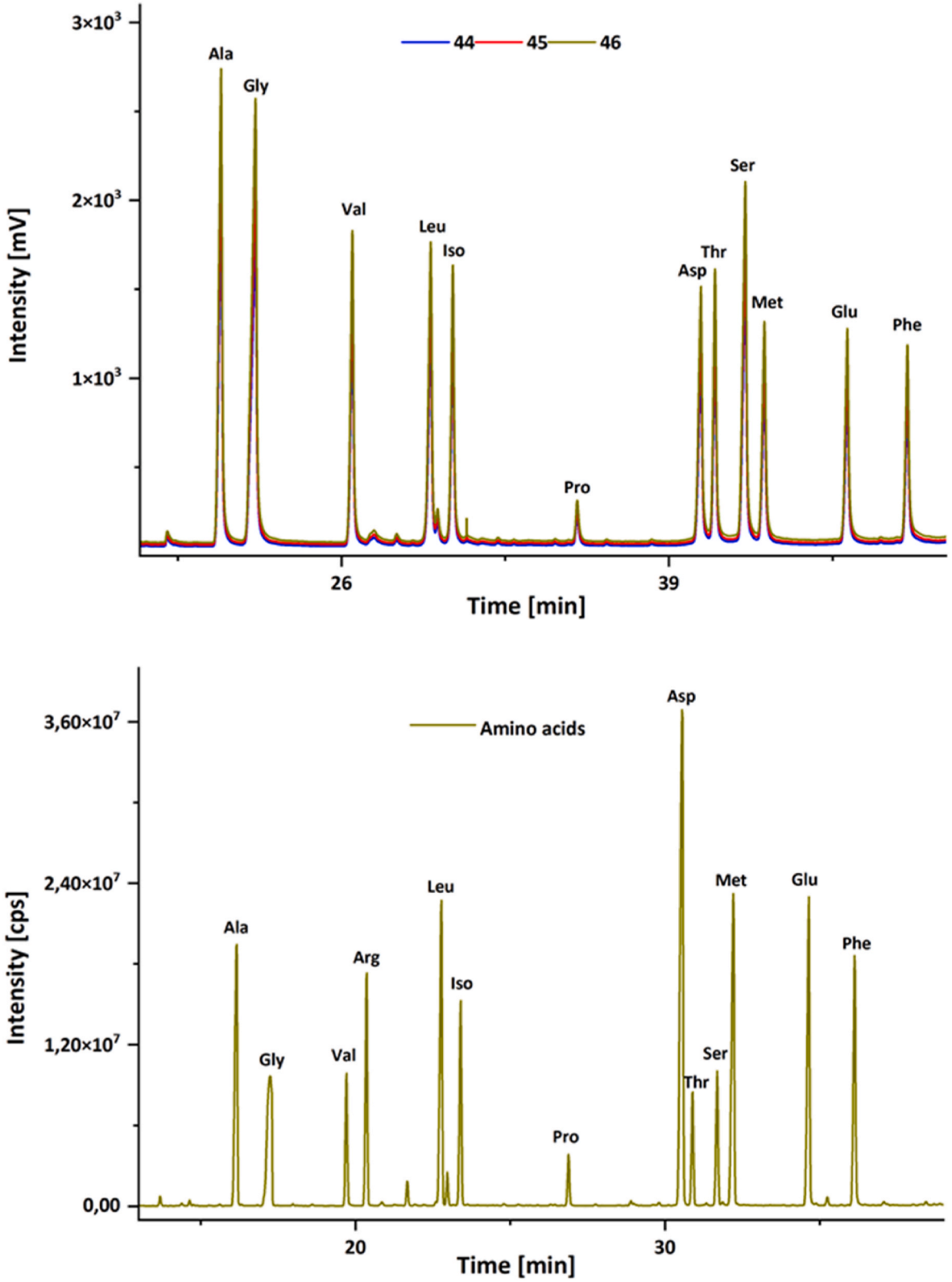

Fig. 3. Upper: $\delta^{13}C$ GC-IRMS chromatogram of (pv-ipr) derivatized amino acid standards. The chromatogram displays the detection of 12 amino acids, each highlighted individually. Three colors of peaks represent different molecular masses of measurement gas CO_2. **Lower:** GC-MS chromatogram of (pv-ipr) derivatized amino acid standards. The chromatogram shows the detection of 13 amino acids, highlighted for clarity. These chromatograms were generated as part of method development within our research group (Instrumental Analytical Chemistry, University Duisburg-Essen). Detailed methodology for both chromatograms can be found in supporting information S2 and the comparison of peak width in both techniques is given in (Supporting information, Table S1).

obtaining accurate isotope ratios, especially for $\delta^{15}N$ analysis. Regular background checks should be conducted before each analysis. Surpassing the daily background levels of argon (Ar) and nitrogen (N_2) may suggest the presence of leaks. Constant high H_2O background levels are indicative of leaks, lack of He flow (e.g. if MS valve has been closed for a while) or degradation of the column or Nafion membrane.

Another challenge encountered during isotope analysis is memory effect. When using an IRMS to analyze samples with high artificial enrichment in heavier isotopes, issues such as saturation of the Faraday cup amplifier and memory effects between samples can arise. Memory effects occur when the isotope composition of previous samples or peaks influences the measurement of subsequent samples. Various factors contribute to memory effects, including the conversion of analyte molecules into gases measured (e.g., oxidation/reduction to N_2 or oxidation to CO_2) and chromatographic separation of gases for measurement [95]. The study revealed that memory effects of NPP derivatives in measuring $\delta^{15}N$ of AAs by GC-IRMS may lead to poor reproducibility. The insufficient oxidation and reduction capacities due to the long-term use of the combustion and reduction furnaces during the analytical sequence can increase the measurement error of $\delta^{15}N$ in AAs [96].

To mitigate or prevent the bias caused by memory effects, several options can be considered. Firstly, it is advisable to separate samples with natural isotope abundance from those artificially enriched in heavier isotopes and measure them separately in distinct measurement sequences. Secondly, the influence of memory effects can be minimized by analyzing multiple sample aliquots in a series and discarding the initial aliquots' measurement results [95,97]. Thirdly, if the memory effect remains constant throughout the measurement sequence, a mathematical correction can be applied [98]. Another approach to minimize the impact of memory effects is to run samples in ascending order of isotope enrichment, but this requires pre-measuring the samples in advance, resulting in increased instrumental time and study costs [99]. Moreover, it has been proposed that in case an instrument experiences a memory effect, running a prolonged oxidation program followed by a purge program might help eliminate it [96].

3. LC-IRMS analysis of AAs

Advancements in interface design for liquid chromatography-isotope ratio mass spectrometry (LC-IRMS), have enabled precise measurement of $\delta^{13}C$ values of amino acids at natural abundance, eliminating the requirement for prior derivatization [100,101]. This advancement is particularly beneficial because derivatization can alter $\delta^{13}C$ values of amino acid derivatives, introducing additional sources of error in isotope determinations [100]. To ensure accurate isotope ratio measurements, it is crucial to achieve complete chromatographic resolution of analytes before chemical oxidation of C to CO_2 and their introduction into an IRMS instrument. The LC-IRMS instrument (Fig. 4) consists of a liquid chromatograph directly coupled to an oxidation interface, which is connected through an open split device to the ion source of the IRMS. The LC-interface comprises two pumps for oxidation agents, an oxidation reactor, a CO_2 separator unit, and an open split unit. In this system, sodium peroxydisulfate ($Na_2S_2O_8$) is used for wet chemical oxidation of eluting compounds under acidic conditions. The released CO_2 is removed in a membrane separator and, after the removal of water vapor, carried into the mass separator ion source using a constant helium flow [41,102]. Recently, The LC IsoLink II Conversion Interface by Thermo Scientific is designed for exceptional reliability, featuring a modular pull-out design and a cartridge-based reactor that allows for easy, cost-efficient maintenance without de-stacking.

LC-IRMS is generally used for the analysis of polar, non-volatile analytes, e.g., AAs, peptides, proteins, carbohydrates, and nucleic acids [41]. In aquatic ecosystems, LC-IRMS analysis of AAs is particularly valuable in exploring the relationship between dietary AAs and consumer's tissue [101,103], host-parasite interaction [51], tracing carbon fixation [40] and, a tool for studying foraging ecology [104]. However, this technique has its own limitations. One significant drawback is that LC-IRMS can only measure $\delta^{13}C/^{12}C$ isotope ratios, whereas GC-IRMS can measure multiple other elemental isotope ratios, such as $\delta^{18}O/^{16}O$, $\delta^{15}N/^{14}N$, and $\delta^{3}H/^{2}H$ [105]. This makes GC-IRMS a more versatile tool for amino acid isotope analysis compared to LC-IRMS, despite the advantages of LC-IRMS in avoiding derivatization errors.

3.1. Methodical and technical challenges and solutions

3.1.1. Chromatographic resolution

In the pursuit of accurate carbon isotope analysis of amino acids (AAs), achieving optimal chromatographic resolution becomes a significant challenge in HPLC-IRMS. Achieving complete separation of AA peaks, especially for certain AAs, is difficult [106]. To improve resolution, adjustments can be made to both the type of column used and column temperature [107]. Three different types of columns with distinct stationary phases have been employed for carbon isotope ratio determination of AAs in HPLC-IRMS: ion exchange chromatography, reverse-phase chromatography, and mixed-mode chromatography. Ion exchange chromatography, as the sole separation mechanism, struggles to provide good resolution or complete separation of certain AAs, including Ala, Thr, Gly, Val, Ser, and Pro [106]. Using this ion exchange column, 11 out of 15 AAs can be successfully separated with most AAs reaching a precision of around 0.3 ‰. While reverse-phase columns enhance resolution through hydrophobic interactions, they may still lead to co-elution of Ala, Leu, and Tyr [100]. Nevertheless, the technique involving a reversed-phase column with ion-pairing reagents facilitates the separation of 15 amino acids, with the accuracy being within 2.0 ‰ of the expected values [108]. In aquatic ecosystems, the most widely used technique for AAs isotope analysis is mixed-mode chromatography,

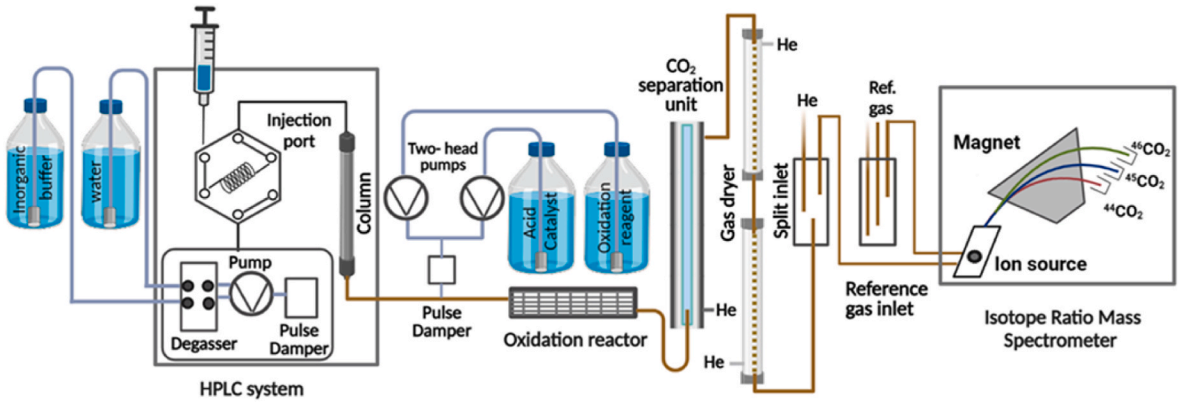

Fig. 4. Schematic representation of LC-IRMS for carbon isotope analysis.

which combines ion exchange and reverse-phase interactions for separation [40,49,51,101,104]. This approach effectively separates most AA peaks, except for Leu and Iso, from a mixture of 15 AAs and also provides standard deviation precision between 0.06 and 0.38 ‰ [109]. However, one should be aware that the elution sequence can occasionally change or even be reversed as columns age. Sometimes, regeneration of the column with an eluent specified by the manufacturer can reverse this effect. In high-temperature LC the same effect is observed if adjustments to the temperature program are made [110].

Temperature also plays a vital role in chromatographic resolution by improving performance and reducing analysis time. Generally, higher column temperatures lead to more efficient and faster separation. However, this temperature-induced decrease in retention time may not be applicable to all compounds in the same sample mixture. It can result in changes to the retention factor, potentially causing co-elution of sample compounds and variations in selectivity even with slight temperature variations. Therefore, proper optimization of column temperature is crucial for LC-IRMS separations [107].

3.1.2. Method development

While striving for optimal resolution, researchers face another challenge when developing methods for LC-IRMS. Long chromatographic run times result from the limited flow rates that must not exceed 0.7 mL/min [100], as well as the necessity to align retention and elution conditions using solely aqueous mobile phases (see below), all while maintaining baseline resolution between analytes. Addressing these constraints involves aligning the internal diameter (i.d.) of the LC column with the flow rate used and minimizing peak broadening at the interface through carrier gas (He) flow rate optimization. An illustration of this is by decreasing the column i.d. From 4.6 mm to 2.1 mm, the optimal linear flow rate can be maintained while decreasing the volumetric flow rate from around 1.0 to 0.3 mL/min (for a 250 mm column length). This reduction allows for shorter run times due to the smaller column diameter, despite the lower volumetric flow rate. Additionally, it minimizes the required amount of mobile phase and reduces equilibrium time of the column, while enhancing relative sensitivity through the reduction of peak widths [41]. However, by reducing the column i.d., column capacity is reduced so that smaller injection volumes and absolute amounts can be used. Column bleed is another crucial consideration when selecting an analytical column to avoid high or unstable backgrounds caused by the release of the strongly bonded phase of the column. Proper column regeneration and cleaning using wet oxidation agents, as recommended by the manufacturer, should be undertaken. For continuous usage, it is recommended to clean the column once every month or after a certain number of sample injections. In a study [106], cleaning of the anion exchange column involved flowing clean water for 10 min at a flow rate of 0.30 mL/min, followed by 145 mM of nitric acid (HNO_3) for 1 h at a flow rate of 0.25 mL/min, and another rinse with water for 30 min. The column was then regenerated by flowing 1 M NaOH for 4 h at a flow rate of 0.25 mL/min and finally stabilized by running 35 mM HNO_3 for half an hour at the same flow rate. Dealing with these analytical challenges, along with the necessity of baseline separation for precise compound measurement, makes method development in this field demanding.

Furthermore, when analysing closely eluting peaks, the method used for peak integration and background subtraction becomes crucial. The typical LC/IRMS chromatogram's baseline, which is significantly higher than zero, may contain contributions from column bleed, dissolved CO_2, and other background carbon sources. Subtraction of these contributions is necessary to provide accurate isotope ratios for the analyte. Software offers automatic background subtraction algorithms designed for different types of chromatograms. However, for peaks that partially co-elute or exhibit non-flat baselines, manually selecting a suitable background section away from the peak may be necessary to obtain accurate results. One should be cautious not to include the shallow tailing frequently observed immediately after eluting peaks. For example, for separation of AAs using Primesep mixed-mode stationary phases, manual selection of the background around Thr, Glu, Gly, and Ser may be required to ensure accuracy in results [111].

3.1.3. Limitations of the mobile phase

In LC-IRMS, the use of any mobile phase other than degassed water is not possible. Mobile phases containing organic solvents or organic components are strictly prohibited due to their potential interference with results by introducing organic carbon. Such interference can lead to reduced sensitivity and misinterpretation of the carbon isotope value of an analyte. However, to enhance the separation of all amino acids, acidified mobile phases can be employed by adjusting the pH of water. For example, the study conducted by Raghavan et al. [112], used a gradient of mobile phase A (pure water: pH 7) and mobile phase B (acidified water; pH 1.5) to achieve improved separation. In a study by Boschken et al. (2008) [113], basic eluent (1mmM NaOH) was used to improve the separation of carbohydrates on an ion exchange column and to reduce background noise. However, this leads to gradual loss of activity of analytical column and hence gradual decrease of retention time.

It is crucial to degas the mobile phase within 24 h before usage to eliminate any traces of CO_2, if present. This degassing process should also be applied to HPLC or MQ water to avoid any potential source of CO_2 interference during analysis. Additionally, the use inorganic buffers such as phosphate buffer is possible.

3.1.4. Oxidation based conversion

Incomplete oxidation in LC-IRMS can significantly influence the $\delta^{13}C$ values of amino acids (AAs). The use of an oxidizing reagent in the LC-IRMS system can lead to various mechanical and non-mechanical issues. Non-continuous instrument use can cause clogging in pumps and capillaries, resulting in an increase in backpressure. Monitoring the backpressure of the entire LC-IRMS system is essential, as higher backpressure indicates potential blockage that needs to be addressed before proceeding with the analysis. In cases of extended machine downtimes, it is necessary to flush out the oxidant and replace it with water. Following the flushing process, the reactor should be cooled, and the flow rates of the mobile phase and reagent pump reduced to a minimum [107].

Achieving a complete conversion of the analyte's carbon into CO_2 measurement gas is crucial, as incomplete conversion can not only affect sensitivity but also alter the $\delta^{13}C$ values. Offsets in LC-IRMS cannot be reliably corrected by identical treatment of the analyte and reference material due to the challenges posed by incomplete oxidation. In LC-IRMS, the efficiency of oxidation is highly sensitive to several factors, including the concentration of the oxidizing agent and HPLC flow rates [114]. Unlike other techniques, where such offsets can often be corrected by comparing the analyte with a standard [98], the incomplete and variable oxidation in LC-IRMS leads to inconsistent conversion of analytes. Techniques like monitoring oxygen levels and using calibration functions aim to ensure complete oxidation [100,115,116], but they often fall short because oxidation conditions are not consistently optimized for different analyte concentrations and flow rates. Lower oxidizing agent concentrations, particularly at higher flow rates, reduce oxidation efficiency, making it difficult to achieve the linearity needed for accurate offset correction [114].

The efficiency of analyte oxidation in LC-IRMS depends on various factors. One approach involves monitoring of oxygen background, as recommended by the manufacturer [100]. The commercial design of the IsoLink interface lacks a mechanism to remove unused O_2, leading to a gas mixture reaching the mass spectrometer ion source that contains CO_2 in helium along with varying levels of O_2. This presence of O_2 introduces several challenges: it can cause isobaric interference, complicating CO_2^+ ion detection, increase space charge effects that disrupt ionization, and fluctuate based on oxidation efficiency, further compromising isotope ratio measurements. Additionally, the rising O_2 levels between sample peaks cause oxidative reactions with the hot

filament, leading to ion source tuning issues, CO_2 background variability, and accelerated filament degradation. This shortens the lifespan of tungsten and tantalum filaments, increasing costs and instrument downtime, ultimately complicating the analysis of large sample batches [41,117]. It is known that filaments made of Tungsten and a Vacromium alloy, possess a longer lifetime in case of higher oxygen backgrounds and are recommended by ThrmoFisher for LC-IRMS applications.

To tackle this problem, Thermo Fisher [117] introduced an O_2-removing device into the system known as the O_2 scrubber or reduction reactor. This consists of two parallel hot Cu reduction reactors with an internal diameter of 0.8 mm and an active length of 120 mm, along with a switch-over valve positioned between them. However, it is important to note that this design may come with certain limitations in terms of precision and accuracy of the measured $\delta^{13}C$ values along with peak broadening [117].

Beyond oxygen management, Additional considerations include the residence time of the analyte in the reactor, determined by flow rates, oxidation agent, and acid. The relative concentration of the oxidation agent to the analyte is also critical. Research has demonstrated that exceeding a certain concentration threshold disrupts the linear relationship between the injected carbon amount and the IRMS peak area response. This linearity can be restored by either reducing the analyte amount or increasing the concentration of the oxidation agent [102].

3.1.5. Other technical issues

During AA isotope analysis using LC-IRMS, several technical challenges may arise. One notable issue is the regular replacement of reactors and separation or drying units, which depends on factors such as flow rates, types of mobile phases used, and the level of usage. Thus, a frequent usage and annual part replacement due to exhaustion is advantageous. Godin and McCullagh [41] indicated that an incorporation of a pressure readback feature is beneficial for the reagent pumping system to monitor flow accurately and troubleshoot any potential problems, even though it may not directly impact sample analysis. Additionally, having a moisture sensor at the interface unit's base would be advantageous as it could detect leaks and promptly cut out the oxidation reagents and mobile phase flows if necessary. Another major limitation of using LC-IRMS is that it doesnot measure isotope ratio of other elements (e.g., nitrogen), because the designed separation unit only works with CO_2.

4. Future outlook of AA isotope analysis

GC- and LC-IRMS are effective for obtaining molecular average isotope values of AA in complex mixtures, but they may obscure valuable rare isotope distribution details. This is particularly significant because various chemical and physical processes can lead to notable isotopic variations between distinct atomic sites [118]. The direct combustion of amino acids into CO_2 and N_2 during GC–IRMS erases position specific isotope information, such as the distinct ^{13}C-enrichment at the C-1 versus C-2 position in alanine. This detail, crucial for understanding its origins, cannot be discerned from the $^{13}C/^{12}C$ ratio of whole molecule combustion in CO_2 [119]. To address this limitation, position-specific isotope analysis (PSIA) methods, like online-pyrolysis-GC–IRMS and high-resolution mass spectrometry (HRMS), have been developed. Several variations of online-pyrolysis-GC–IRMS systems have been applied to analyze position-specific $^{13}C/^{12}C$ in amino acid standards [120]. ESI-Orbitrap-MS, exemplified in this case by HRMS, simplifies the assessment of intermolecular carbon isotope distribution in alanine [118]. This allows for the extraction of distinct compound signatures without requiring extensive sample preparation. In another investigation, the position-specific isotope ratio of methionine samples was determined [121]. The coupling of HPLC and GC to Orbitrap mass spectrometers presents an opportunity for on-line separation of complex mixtures and PSIA [122,123]. While these techniques hold promise for the future of amino acid isotope analysis, challenges such as insufficient fragmentation and the lack of position-specific isotopic reference materials require further research for development.

Expanding beyond the typical carbon and nitrogen isotope analysis, δ^2H and $\delta^{18}O$ analyses in amino acids present transformative opportunities to explore the biochemical processes by which organisms incorporate hydrogen and oxygen from their environment and diet. These isotopes are especially valuable for tracking processes like amino acid assimilation and animal migration [124–126]. Additionally, measuring $\delta^{34}S$ values in cysteine and methionine can help uncover metabolic pathways, providing further perspectives on ecological and biochemical patterns [127,128]. However, these analyses come with their own set of challenges. For instance, elements like H and S have lower ionization efficiency in many IRMS instruments compared to carbon, leading to smaller peak sizes and reduced analytical precision [91,129]. Sulfur isotope analysis, in particular, faces issues with incomplete combustion and SO_2 adhesion, which can corrode surfaces. While converting sulfur to SF_6 may mitigate some of these challenges, it introduces halogens that could damage the IRMS system [98,128,129]. Overcoming these technical barriers through continued methodological advancements is essential to fully realize the potential of these isotopes in addressing broader scientific questions.

5. Conclusion

Advancements in analytical techniques, such as GC-IRMS or LC-IRMS, have opened up new possibilities to enhance the specificity of dietary studies and trophic ecology in aquatic systems. Ecologists consider CSIA-AA as a valuable method to complement SIR analysis. By comparing the isotope ratios of individual AAs, researchers can gain insights into the biochemical and physiological processes that drive trophic interactions, nutrient pathways, and organismal responses to environmental changes. However, the application of CSIA-AA is accompanied by several methodological and technical challenges that must be carefully managed to ensure reliable and accurate results. Significant challenges include not only the complexity of sample preparation but also the technical difficulties related to instrument performance and precise calibration. Regular maintenance, such as timely conditioning and replacement of critical components, is essential to maintain the accuracy of the analysis. Moreover, the use of high-quality reference materials and thoughtful interpretation of isotope data are vital to overcoming these challenges and achieving reliable results. Despite these challenges, the continued development and improvement of CSIA-AA techniques hold great promise for advancing our understanding of aquatic ecosystems. Future research should focus on overcoming these limitations, improving analytical precision, and integrating CSIA-AA with other emerging technologies. By doing so, researchers can reveal the full potential of CSIA-AA, providing deeper insights into the complexities of ecological interactions and the effects of environmental changes on aquatic life.

CRediT authorship contribution statement

Shaista Khaliq: Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization. **Maik A. Jochmann:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Tobias Hesse:** Writing – review & editing. **Milen Nachev:** Writing – review & editing, Supervision, Conceptualization. **Bernd Sures:** Writing – review & editing, Supervision, Conceptualization. **Philip M. Riekenberg:** Writing – review & editing, Writing – original draft, Supervision. **Marcel T.J. van der Meer:** Writing – review & editing, Supervision. **Torsten C. Schmidt:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no competing interests. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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Data availability

No data was used for the research described in the article.

References

[1] N.E. Hussey, et al., Rescaling the trophic structure of marine food webs, Ecol. Lett. 17 (2) (2014) 239–250.
[2] J.C. Finlay, C. Kendall, Stable isotope tracing of temporal and spatial variability in organic matter sources to freshwater ecosystems, Stable isotopes in ecology and environmental science 2 (2007) 283–333.
[3] D.M. Post, Using stable isotopes to estimate trophic position: models, methods, and assumptions, Ecology 83 (3) (2002) 703–718.
[4] P.M. Glibert, et al., Stable isotope tracers: enriching our perspectives and questions on sources, fates, rates, and pathways of major elements in aquatic systems, Limnol. Oceanogr. 64 (3) (2019) 950–981.
[5] S.D. Newsome, et al., A niche for isotopic ecology, Front. Ecol. Environ. 5 (8) (2007) 429–436.
[6] W.J. Boecklen, et al., On the use of stable isotopes in trophic ecology, in: D. J. Futuyma, H.B. Shaffer, D. Simberloff (Eds.), Annual Review of Ecology, Evolution, and Systematics, vol. 42, 2011, pp. 411–440.
[7] S. Sánchez-Carrillo, M. Álvarez-Cobelas, Stable isotopes as tracers in aquatic ecosystems, Environ. Rev. 26 (1) (2018) 69–81.
[8] M.-T. Chung, et al., Otolith δ13C values as a metabolic proxy: approaches and mechanical underpinnings, Mar. Freshw. Res. 70 (12) (2019) 1747–1756.
[9] A. Born-Torrijos, et al., Parasite effects on host's trophic and isotopic niches, Trends Parasitol 39 (9) (2023) 749–759.
[10] K.A. Hobson, Tracing origins and migration of wildlife using stable isotopes: a review, Oecologia (1999) 314–326.
[11] N.J. Smucker, et al., Stable isotopes of algae and macroinvertebrates in streams respond to watershed urbanization, inform management goals, and indicate food web relationships, Ecol. Indicat. 90 (2018) 295–304.
[12] S.S. Kaushal, et al., Tracking nonpoint source nitrogen pollution in human-impacted watersheds, Environ. Sci. Technol. 45 (19) (2011) 8225–8232.
[13] J. Burbank, D.A.R. Drake, M. Power, Use of stable isotopes for assessing urbanization impacts on freshwater fishes, Front. Environ. Sci. 10 (2022).
[14] C. Martínez del Rio, et al., Isotopic ecology ten years after a call for more laboratory experiments, Biol. Rev. 84 (1) (2009) 91–111.
[15] W.A. Carter, U. Bauchinger, S.R. McWilliams, The importance of isotopic turnover for understanding key aspects of animal ecology and nutrition, Diversity-Basel 11 (5) (2019).
[16] T.B. Coplen, Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results, Rapid Commun. Mass Spectrom. 25 (17) (2011) 2538–2560.
[17] M. Ben-David, E.A. Flaherty, Stable isotopes in mammalian research: a beginner's guide, J. Mammal. 93 (2) (2012) 312–328.
[18] P.A. Sandberg, J.E. Loudon, M. Sponheimer, Stable isotope analysis in primatology: a critical review, Am. J. Primatol. 74 (11) (2012) 969–989.
[19] S. Bearhop, et al., Factors that influence assimilation rates and fractionation of nitrogen and carbon stable isotopes in avian blood and feathers, Physiol. Biochem. Zool. 75 (5) (2002) 451–458.
[20] S.D. Newsome, et al., Amino acid δ13C analysis shows flexibility in the routing of dietary protein and lipids to the tissue of an omnivore, The Society for Integrative and Comparative Biology 54 (5) (2014) 890–902.
[21] M. Davis, S. Pineda-Munoz, The temporal scale of diet and dietary proxies, Ecol. Evol. 6 (6) (2016) 1883–1897.
[22] M.J. DeNiro, S. Epstein, Influence of diet on the distribution of carbon isotopes in animals, Geochem. Cosmochim. Acta 42 (5) (1978) 495–506.
[23] M.J. DeNiro, S. Epstein, Influence of diet on the distribution of nitrogen isotopes in animals, Geochem. Cosmochim. Acta 45 (3) (1981) 341–351.
[24] J.M. Hayes, Fractionation of carbon and hydrogen isotopes in biosynthetic processes, Rev. Mineral. Geochem. 43 (2001) 225–278.
[25] K.W. McMahon, M.D. McCarthy, Embracing variability in amino acid δ15N fractionation: mechanisms, implications, and applications for trophic ecology, Ecosphere 7 (12) (2016) e01511.
[26] K.W. McMahon, et al., Carbon isotope fractionation of amino acids in fish muscle reflects biosynthesis and isotopic routing from dietary protein, J. Anim. Ecol. 79 (5) (2010) 1132–1141.
[27] N. Ohkouchi, et al., Advances in the application of amino acid nitrogen isotopic analysis in ecological and biogeochemical studies, Org. Geochem. 113 (2017) 150–174.
[28] M.A. Vanderklift, S. Ponsard, Sources of variation in consumer-diet δ15N enrichment: a meta-analysis, Oecologia 136 (2003) 169–182.
[29] P.H. Kang, et al., A model of algal organic carbon distributions in the Pearl River estuary using the amino acid carbon isotope values, Geochem. Cosmochim. Acta 294 (2021) 1–12.
[30] G. Saboret, et al., Autochthonous production sustains food webs in large perialpine lakes, independent of trophic status: evidence from amino acid stable isotopes, Freshw. Biol. 68 (5) (2023) 870–887.
[31] R. Sahm, et al., Compound-specific δ15N analyses of amino acids for trophic level estimation from indigenous and invasive freshwater amphipods, Int. Rev. Hydrobiol. 106 (1) (2021) 41–47.
[32] R.G. Walsh, S. He, C.T. Yarnes, *Compound-specific δ13C and δ15N analysis of amino acids: a rapid, chloroformate-based method for ecological studies*, Rapid Commun. Mass Spectrom. 28 (1) (2014) 96–108.
[33] J.P. Whiteman, et al., A guide to using compound-specific stable isotope analysis to study the fates of molecules in organisms and ecosystems, Diversity-Basel 11 (1) (2019).
[34] H.Y. Yun, et al., Amino acid nitrogen and carbon isotope data: potential and implications for ecological studies, Ecol. Evol. 12 (6) (2022) e8929.
[35] E.J. Won, et al., Importance of accurate trophic level determination by nitrogen isotope of amino acids for trophic magnification studies: a review, Environ. Pollut. 238 (2018) 677–690.
[36] B.N. Popp, et al., Insight into the trophic ecology of yellowfin tuna, Thunnus albacares, from compound-specific nitrogen isotope analysis of proteinaceous amino acids, Terrestrial Ecology 1 (2007) 173–190.
[37] Larsen, et al., Tracing carbon sources through aquatic and terrestrial food webs using amino acid stable isotope fingerprinting, PLoS One 8 (9) (2013) e73441.
[38] Y. Chikaraishi, et al., Metabolic control of nitrogen isotope composition of amino acids in macroalgae and gastropods: implications for aquatic food web studies, Mar. Ecol. Prog. Ser. 342 (2007) 85–90.
[39] C. Ferrier-Pages, M.C. Leal, Stable isotopes as tracers of trophic interactions in marine mutualistic symbioses, Ecol. Evol. 9 (1) (2019) 723–740.
[40] J. Grosse, P. van Breugel, H.T. Boschker, *Tracing carbon fixation in phytoplankton—compound specific and total 13C incorporation rates*, Limnol Oceanogr. Methods 13 (6) (2015) 288–302.
[41] J.P. Godin, J.S. McCullagh, Review: current applications and challenges for liquid chromatography coupled to isotope ratio mass spectrometry (LC/IRMS), Rapid Commun. Mass Spectrom. 25 (20) (2011) 3019–3028.
[42] L. Ruess, D.C. Müller-Navarra, Essential biomolecules in food webs, Frontiers in Ecology and Evolution 7 (2019) 269.
[43] M.I. Gladyshev, et al., Stable isotope composition of fatty acids in organisms of different trophic levels in the Yenisei River, PLoS One 7 (3) (2012) e34059.
[44] K. Vane, M.R. Cobain, T. Larsen, The Power and Pitfalls of Amino Acid Carbon Stable Isotopes for Tracing the Use and Fate of Basal Resources in Food Webs, 2023.
[45] Y. Chikaraishi, et al., Determination of aquatic food-web structure based on compound-specific nitrogen isotopic composition of amino acids, Limnol Oceanogr. Methods 7 (11) (2009) 740–750.
[46] J.H. Liew, et al., Quantifying terrestrial carbon in freshwater food webs using amino acid isotope analysis: case study with an endemic cavefish, Methods Ecol. Evol. 10 (9) (2019) 1594–1605.
[47] M.J. Perkins, et al., *Application of nitrogen and carbon stable isotopes (δ15N and δ13C) to quantify food chain length and trophic structure*, PLoS One 9 (3) (2014) e93281.
[48] R.E. Bowes, J.H. Thorp, Consequences of employing amino acid vs. bulk-tissue, stable isotope analysis: a laboratory trophic position experiment, Ecosphere 6 (1) (2015) 1–12.
[49] T. Hesse, et al., Insights into amino acid fractionation and incorporation by compound-specific carbon isotope analysis of three-spined sticklebacks, Sci. Rep. 12 (1) (2022) 11690.
[50] P.M. Riekenberg, et al., Stable nitrogen isotope analysis of amino acids as a new tool to clarify complex parasite–host interactions within food webs, Oikos 130 (10) (2021) 1650–1664.
[51] T. Hesse, et al., A new technique to study nutrient flow in host-parasite systems by carbon stable isotope analysis of amino acids and glucose, Sci. Rep. 13 (1) (2023) 1054.
[52] A. Sabadel, et al., Compound-specific isotope analysis of amino acids: a tool to unravel complex symbiotic trophic relationships, Food Webs 6 (2016) 9–18.
[53] T. O'Connell, 'Trophic'and 'source'amino acids in trophic estimation: a likely metabolic explanation, Oecologia 184 (2) (2017) 317–326.
[54] L.R. Germain, et al., Nitrogen isotope fractionation in amino acids from harbor seals: implications for compound-specific trophic position calculations, Mar. Ecol. Prog. Ser. 482 (2013) 265–277.

[55] K.W. McMahon, et al., Carbon and nitrogen isotope fractionation of amino acids in an avian marine predator, the gentoo penguin (Pygoscelis papua), Ecol. Evol. 5 (6) (2015) 1278–1290.
[56] C.J. Wallace, R.E. Hedges, Nitrogen isotopic discrimination in dietary amino acids: the threonine anomaly, Rapid Commun. Mass Spectrom. 30 (22) (2016) 2442–2446.
[57] T.M. Richards, T.T. Sutton, R.D. Wells, Trophic structure and sources of variation influencing the stable isotope signatures of meso-and bathypelagic micronekton fishes, Front. Mar. Sci. 7 (2020) 507992.
[58] J.W. McClelland, J.P. Montoya, Trophic relationships and the nitrogen isotopic composition of amino acids in plankton, Ecology 83 (8) (2002) 2173–2180.
[59] S. Reinnicke, et al., Gas chromatography/isotope ratio mass spectrometry of recalcitrant target compounds: performance of different combustion reactors and strategies for standardization, Rapid Commun. Mass Spectrom. 26 (9) (2012) 1053–1060.
[60] T.C. Moerdijk-Poortvliet, et al., Comparison of gas chromatography/isotope ratio mass spectrometry and liquid chromatography/isotope ratio mass spectrometry for carbon stable-isotope analysis of carbohydrates, Rapid Commun. Mass Spectrom. 29 (13) (2015) 1205–1214.
[61] Y. Takizawa, et al., A new insight into isotopic fractionation associated with decarboxylation in organisms: implications for amino acid isotope approaches in biogeoscience, Prog. Earth Planet. Sci. 7 (1) (2020).
[62] Z. Zhang, et al., A reliable compound-specific nitrogen isotope analysis of amino acids by GC-C-IRMS following derivatisation into N-pivaloyl-iso-propyl (NPIP) esters for high-resolution food webs estimation, J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. 1033–1034 (2016) 382–389.
[63] S.N. Silverman, et al., Practical considerations for amino acid isotope analysis, Org. Geochem. 164 (2022).
[64] D. Kim, et al., Environmental fate and trophic transfer of synthetic musk compounds and siloxanes in Geum River, Korea: compound-specific nitrogen isotope analysis of amino acids for accurate trophic position estimation, Environ. Int. (2022) 161.
[65] C.C. Metges, K.J. Petzke, U. Hennig, Gas chromatography/combustion/isotope ratio mass spectrometric comparison of N-acetyl-and N-pivaloyl amino acid esters to measure 15N isotopic abundances in physiological samples: a pilot study on amino acid synthesis in the upper gastro-intestinal tract of minipigs, J. Mass Spectrom. 31 (4) (1996) 367–376.
[66] Q.H.S. Chan, et al., Amino acid compositions in heated carbonaceous chondrites and their compound-specific nitrogen isotopic ratios, Earth Planets Space 68 (1) (2016).
[67] D.A. Merritt, J. Hayes, Nitrogen isotopic analyses by isotope-ratio-monitoring gas chromatography/mass spectrometry, J. Am. Soc. Mass Spectrom. 5 (5) (1994) 387–397.
[68] P.M. Riekenberg, M. van der Meer, S. Schouten, Practical considerations for improved reliability and precision during determination of δ15N values in amino acids using a single combined oxidation-reduction reactor, Rapid Commun. Mass Spectrom. 34 (14) (2020) e8797.
[69] A.J. Darragh, P.J. Moughan, The effect of hydrolysis time on amino acid analysis, J. AOAC Int. 88 (3) (2005) 888–893.
[70] D.M. Albin, J.E. Wubben, V.M. Gabert, Effect of hydrolysis time on the determination of amino acids in samples of soybean products with ion-exchange chromatography or precolumn derivatization with phenyl isothiocyanate, J. Agric. Food Chem. 48 (5) (2000) 1684–1691.
[71] S.M. Rutherfurd, G.S. Gilani, Amino acid analysis, Current protocols in protein science 58 (1) (2009), 11.9. 1-11.9. 37.
[72] P.E. Hare, et al., The isotopic composition of carbon and nitrogen in individual amino acids isolated from modern and fossil proteins, J. Archaeol. Sci. 18 (3) (1991) 277–292.
[73] C.C. Metges, K.J. Petzke, Measurement of 15N/14N isotopic Composition in individual plasma free amino Acids of human Adults at natural Abundance by gas chromatography–combustion isotope ratio mass spectrometry, Anal. Biochem. 247 (1) (1997) 158–164.
[74] Y. Takano, et al., Isolation and desalting with cation-exchange chromatography for compound-specific nitrogen isotope analysis of amino acids: application to biogeochemical samples, Rapid Commun. Mass Spectrom. 24 (16) (2010) 2317–2323.
[75] M.D. McCarthy, et al., Amino acid nitrogen isotopic fractionation patterns as indicators of heterotrophy in plankton, particulate, and dissolved organic matter, Geochem. Cosmochim. Acta 71 (19) (2007) 4727–4744.
[76] T.A.B. Broek, M.D. McCarthy, *A new approach to δ15N compound-specific amino acid trophic position measurements: preparative high pressure liquid chromatography technique for purifying underivatized amino acids for stable isotope analysis*, Limnol Oceanogr. Methods 12 (2014) 840–852.
[77] E. Eglite, et al., Strategies of amino acid supply in mesozooplankton during cyanobacteria blooms: a stable nitrogen isotope approach, Ecosphere 9 (3) (2018) e02135.
[78] M. Decima, et al., *Alanine δ15N trophic fractionation in heterotrophic protists*, Limnol. Oceanogr. 62 (5) (2017) 2308–2322.
[79] K.H. Elliott, B.M. Braune, J.E. Elliott, *Beyond bulk δ15N: Combining a suite of stable isotopic measures improves the resolution of the food webs mediating contaminant signals across space, time and communities*, Environ. Int. (2021) 148.
[80] J.J. Johnson, J.A. Olin, M.J. Polito, A multi-biomarker approach supports the use of compound-specific stable isotope analysis of amino acids to quantify basal carbon source use in a salt marsh consumer, Rapid Commun. Mass Spectrom. 33 (23) (2019) 1781–1791.
[81] L.T. Corr, R. Berstan, R.P. Evershed, *Optimisation of derivatisation procedures for the determination of δ13C values of amino acids by gas chromatography/combustion/isotope ratio mass spectrometry*, Rapid Commun. Mass Spectrom.: An International Journal Devoted to the Rapid Dissemination of Up-to-the-Minute Research in Mass Spectrometry 21 (23) (2007) 3759–3771.
[82] W.-P. Chen, et al., Microscale analysis of amino acids using gas chromatography–mass spectrometry after methyl chloroformate derivatization, J. Chromatogr. B 878 (24) (2010) 2199–2208.
[83] D. Hofmann, M. Gehre, K. Jung, *Sample preparation techniques for the determination of natural 15N/14N variations in amino acids by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS)*, Isot. Environ. Health Stud. 39 (3) (2003) 233–244.
[84] M. Elsner, et al., Current challenges in compound-specific stable isotope analysis of environmental organic contaminants, Anal. Bioanal. Chem. 403 (9) (2012) 2471–2491.
[85] G. Rieley, Derivatization of organic compounds prior to gas chromatographic–combustion–isotope ratio mass spectrometric analysis: identification of isotope fractionation processes, Analyst 119 (5) (1994) 915–919.
[86] J.M. Hayes, Practice and principles of isotopic measurements in organic geochemistry, Organic geochemistry of contemporaneous and ancient sediments 5 (1983) e5.
[87] W. Meier-Augenstein, *GC and IRMS technology for 13C and 15N analysis on organic compounds and related gases*, in: Handbook of Stable Isotope Analytical Techniques, Elsevier, 2004, pp. 153–176.
[88] J.A. Silfer, et al., Stable carbon isotope analysis of amino-acid enantiomers by conventional isotope ratio mass-spectrometry and combined gas-chromatography isotope ratio mass-spectrometry, Anal. Chem. 63 (4) (1991) 370–374.
[89] C.C. Metges, M. Daenzer, *13C gas chromatography-combustion isotope ratio mass spectrometry analysis of N-pivaloyl amino acid esters of tissue and plasma samples*, Anal. Biochem. 278 (2) (2000) 156–164.
[90] G. Docherty, V. Jones, R.P. Evershed, Practical and theoretical considerations in the gas chromatography/combustion/isotope ratio mass spectrometry δ13C analysis of small polyfunctional compounds, Rapid Commun. Mass Spectrom. 15 (9) (2001) 730–738.
[91] W. Brand, A.R. Tegtmeyer, A. Hilkert, Compound-specific isotope analysis: extending toward 15N14N and 18O16O, Org. Geochem. 21 (6–7) (1994) 585–594.
[92] A.L. Sessions, Isotope-ratio detection for gas chromatography, J. Separ. Sci. 29 (12) (2006) 1946–1961.
[93] K.J. Leckrone, J.M. Hayes, Water-induced errors in continuous-flow carbon isotope ratio mass spectrometry, Anal. Chem. 70 (13) (1998) 2737–2744.
[94] M. Shelef, Selective catalytic reduction of NOx with N-free reductants, Chem. Rev. 95 (1) (1995) 209–225.
[95] W.W. Wong, P.D. Klein, A review of techniques for the preparation of biological samples for mass-spectrometric measurements of hydrogen-2/hydrogen-1 and oxygen-18/oxygen-16 isotope ratios, Mass Spectrom. Rev. 5 (3) (1986) 313–342.
[96] K.J. Petzke, C.C. Metges, *Practical recommendations for the reduction of memory effects in compound-specific 15N/14N ratio analysis of enriched amino acids by gas chromatography/combustion/isotope ratio mass spectrometry*, Rapid Commun. Mass Spectrom. 26 (2) (2012) 195–204.
[97] P.A. De Groot, Handbook of Stable Isotope Analytical Techniques, vol. 1, Elsevier, 2004.
[98] R.A. Werner, W.A. Brand, Referencing strategies and techniques in stable isotope ratio analysis, Rapid Commun. Mass Spectrom. 15 (7) (2001) 501–519.
[99] N. Mannerheim, R.A. Werner, N. Buchmann, *Measurement precision and accuracy of high artificial enrichment 15N and 13C tracer samples*, Rapid Commun. Mass Spectrom. 33 (13) (2019) 1153–1163.
[100] J.P. Godin, et al., Isotope ratio monitoring of small molecules and macromolecules by liquid chromatography coupled to isotope ratio mass spectrometry, Rapid Commun. Mass Spectrom.: An International Journal Devoted to the Rapid Dissemination of Up-to-the-Minute Research in Mass Spectrometry 19 (18) (2005) 2689–2698.
[101] J. McCullagh, J. Gaye-Siessegger, U. Focken, *Determination of underivatized amino acid δ13C by liquid chromatography/isotope ratio mass spectrometry for nutritional studies: the effect of dietary non-essential amino acid profile on the isotopic signature of individual amino acids in fish*, Rapid Commun. Mass Spectrom.: An International Journal Devoted to the Rapid Dissemination of Up-to-the-Minute Research in Mass Spectrometry 22 (12) (2008) 1817–1822.
[102] M. Krummen, et al., A new concept for isotope ratio monitoring liquid chromatography/mass spectrometry, Rapid Commun. Mass Spectrom. 18 (19) (2004) 2260–2266.
[103] J. Gaye-Siessegger, J.S. McCullagh, U. Focken, *The effect of dietary amino acid abundance and isotopic composition on the growth rate, metabolism and tissue δ13C of rainbow trout*, Br. J. Nutr. 105 (12) (2011) 1764–1771.
[104] A. Lorrain, et al., Nitrogen and carbon isotope values of individual amino acids: a tool to study foraging ecology of penguins in the Southern Ocean, Mar. Ecol. Prog. Ser. 391 (2009) 293–306.
[105] T.C. Moerdijk-Poortvliet, L.J. Stal, H.T. Boschker, LC/IRMS analysis: a powerful technique to trace carbon flow in microphytobenthic communities in intertidal sediments, J. Sea Res. 92 (2014) 19–25.
[106] D.A. Abaye, D.J. Morrison, T. Preston, Strong anion exchange liquid chromatographic separation of protein amino acids for natural *13C*-abundance determination by isotope ratio mass spectrometry, Rapid Commun. Mass Spectrom. 25 (3) (2011) 429–435.
[107] M. Perini, L. Bontempo, Liquid chromatography coupled to isotope ratio mass spectrometry (LC-IRMS): a review, TrAC, Trends Anal. Chem. 147 (2022).

[108] J.A. Tripp, J.S. McCullagh, R.E. Hedges, Preparative separation of underivatized amino acids for compound-specific stable isotope analysis and radiocarbon dating of hydrolyzed bone collagen, J. Separ. Sci. 29 (1) (2006) 41–48.
[109] J.S. McCullagh, D. Juchelka, R.E. Hedges, Analysis of amino acid *13C* abundance from human and faunal bone collagen using liquid chromatography/isotope ratio mass spectrometry, Rapid Commun. Mass Spectrom.: An International Journal Devoted to the Rapid Dissemination of Up-to-the-Minute Research in Mass Spectrometry 20 (18) (2006) 2761–2768.
[110] L. Zhang, et al., Caffeine in your drink: natural or synthetic? Anal. Chem. 84 (6) (2012) 2805–2810.
[111] J.S. McCullagh, Mixed-mode chromatography/isotope ratio mass spectrometry, Rapid Commun. Mass Spectrom.: An International Journal Devoted to the Rapid Dissemination of Up-to-the-Minute Research in Mass Spectrometry 24 (5) (2010) 483–494.
[112] M. Raghavan, et al., *Amino acid δ13C analysis of hair proteins and bone collagen using liquid chromatography/isotope ratio mass spectrometry: Paleodietary implications from intra-individual comparisons*, Rapid Commun. Mass Spectrom.: An International Journal Devoted to the Rapid Dissemination of Up-to-the-Minute Research in Mass Spectrometry 24 (5) (2010) 541–548.
[113] H. Boschker, et al., A versatile method for stable carbon isotope analysis of carbohydrates by high-performance liquid chromatography/isotope ratio mass spectrometry, Rapid Commun. Mass Spectrom.: An International Journal Devoted to the Rapid Dissemination of Up-to-the-Minute Research in Mass Spectrometry 22 (23) (2008) 3902–3908.
[114] D. Köster, et al., New concepts for the determination of oxidation efficiencies in liquid chromatography–isotope ratio mass spectrometry, Anal. Chem. 91 (8) (2019) 5067–5073.
[115] T.F.S.L.I. Operating, Manual Revision C-1156140, Thermo Fisher Scientific Inc., Bremen, Germany, 2011.
[116] J.P. Godin, et al., *Liquid and gas chromatography coupled to isotope ratio mass spectrometry for the determination of* 13*C–valine isotopic ratios in complex biological samples*, J. Mass Spectrom. 43 (10) (2008) 1334–1343.
[117] E. Hettmann, W.A. Brand, G. Gleixner, Improved isotope ratio measurement performance in liquid chromatography/isotope ratio mass spectrometry by removing excess oxygen, Rapid Commun. Mass Spectrom.: An International Journal Devoted to the Rapid Dissemination of Up-to-the-Minute Research in Mass Spectrometry 21 (24) (2007) 4135–4141.
[118] G.M. Weiss, et al., Analysis of intramolecular carbon isotope distributions in alanine by electrospray ionization Orbitrap mass spectrometry, Int. J. Mass Spectrom. 493 (2023) 117128.
[119] L. Chimiak, et al., Carbon isotope evidence for the substrates and mechanisms of prebiotic synthesis in the early solar system, Geochem. Cosmochim. Acta 292 (2021) 188–202.
[120] A. Gilbert, The organic isotopologue frontier, Annu. Rev. Earth Planet Sci. 49 (2021) 435–464.
[121] C. Neubauer, et al., Scanning the isotopic structure of molecules by tandem mass spectrometry, Int. J. Mass Spectrom. 434 (2018) 276–286.
[122] S.S. Zeichner, et al., Methods and limitations of stable isotope measurements via direct elution of chromatographic peaks using gas chromotography-Orbitrap mass spectrometry, Int. J. Mass Spectrom. 477 (2022) 116848.
[123] E.B. Wilkes, et al., Position-specific carbon isotope analysis of serine by gas chromatography/Orbitrap mass spectrometry, and an application to plant metabolism, Rapid Commun. Mass Spectrom. 36 (18) (2022) e9347.
[124] S.D. Newsome, et al., Compound-specific δ 2 H analysis highlights the relationship between direct assimilation and de novo synthesis of amino acids from food and water in a terrestrial mammalian omnivore, Oecologia 193 (2020) 827–842.
[125] K.W. McMahon, S.D. Newsome, Amino acid isotope analysis: a new frontier in studies of animal migration and foraging ecology, in: Tracking Animal Migration with Stable Isotopes, Elsevier, 2019, pp. 173–190.
[126] M.L. Fogel, P.L. Griffin, S.D. Newsome, Hydrogen isotopes in individual amino acids reflect differentiated pools of hydrogen from food and water in Escherichia coli, Proc. Natl. Acad. Sci. USA 113 (32) (2016) E4648–E4653.
[127] M.P. Richards, B. Fuller, R.E. Hedges, Sulphur isotopic variation in ancient bone collagen from Europe: implications for human palaeodiet, residence mobility, and modern pollutant studies, Earth Planet Sci. Lett. 191 (3–4) (2001) 185–190.
[128] A.A. Phillips, F. Wu, A.L. Sessions, Sulfur isotope analysis of cysteine and methionine via preparatory liquid chromatography and elemental analyzer isotope ratio mass spectrometry, Rapid Commun. Mass Spectrom. 35 (4) (2021) e9007.
[129] T. Hansen, A. Burmeister, U. Sommer, Simultaneous δ15N, δ13C and δ34S measurements of low-biomass samples using a technically advanced high sensitivity elemental analyzer connected to an isotope ratio mass spectrometer, Rapid Commun. Mass Spectrom.: An International Journal Devoted to the Rapid Dissemination of Up-to-the-Minute Research in Mass Spectrometry 23 (21) (2009) 3387–3393.