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Improved method for measuring the $\delta^{15}N$ compound-specific amino acids: Application on mesopelagic fishes in the South China Sea

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Abstract

Compound-specific stable isotope analysis of individual amino acids (CSIA-AA) has been widely used in ecological and biogeochemical studies. It has been proven to be powerful in tracing the diet sources and trophic interactions. However, assessing the N sources of mesopelagic fishes has been inconclusive because the mesopelagic fishes' unique domain (water depth ranged from 0 to 1 000 m) and unresolved nitrogen isotopes of various forms. This study proposes a new method for coupling instruments (ion chromatography and Precon-IRMS) and chemical method of oxidation-reduction of amino acids, and also combined $\delta^{15}N$ of AAs with $\delta^{13}C$ of fatty acids (FAs) to analyze the trophic interactions of mesopelagic fishes in the South China Sea (SCS). AAs were isolated by ion chromatography with high peak resolution and collected by an automated fraction collector. The chemical method then converted the AAs into N_2O with a robust oxidation yields and suitable molar ratio of NH_2OH to NO_2^-. Finally, the $\delta^{15}N$ of AAs at 20 nmol were measured with a reasonable precision (<0.6‰). With this method, this study report the first batch high precision $\delta^{15}N$ of AAs and $\delta^{13}C$ of FAs of mesopelagic fishes collected from SCS. *Diaphus luetkeni*, *Chauliodus minimus* and *Bathygadus antrodes* showed similar $\delta^{13}C$ values of 20:4n-6 (~ −28‰), while *Argyropelecus affinis* and *Stomias* had similar values (~ −32‰). These results reflect that mesopelagic fishes had complex diet sources. An increase of 4‰ in $\delta^{15}N$ of glutamic acid (Glu) was found between piscivorous and planktivorous fishes, which might suggest a trophic discrimination factor of mesopelagic fishes in the SCS. This study used $\delta^{13}C$ of 20:4n-6 to reveal the diet sources of mesopelagic fishes and $\delta^{15}N$ of Glu to clarify trophic level between piscivorous and planktivorous fishes. Thus, this combinative method could therefore ultimately be applied in a variety of deep-sea ecosystem.

Key words: $\delta^{13}C$ of fatty acids, $\delta^{15}N$ of amino acids, ion chromatography, mesopelagic fishes, Precon-IRMS

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1 Introduction

Compound-specific stable isotope analysis of individual amino acids (CSIA-AA) has become an important tool for resolving questions regarding source, transformation, and biogeochemical cycling of nitrogen (Arthur et al., 2014; McCarthy et al., 2013; McClelland and Montoya, 2002; Sherwood et al., 2011, 2014), with applications extending from archeology (Broek et al., 2013; Sherwood et al., 2014; Styring et al., 2010) to trophic ecology (Chikaraishi et al., 2009; Hetherington et al., 2017). The $\delta^{15}N$ of amino acids (AAs) in particular has emerged as a powerful new proxy of food web study. Because the AAs can be separated into two specific groups for undergoing different $\delta^{15}N$ fractionation with trophic transfer. One group of AAs maintains relatively unchanged $\delta^{15}N$ values along the trophic transfer (termed as "source" AAs), whereas the second group undergoes predictable isotope fractionation (termed as "trophic" AAs) (Chikaraishi et al., 2009; Popp et al., 2007). Within these two groups, the "trophic" AAs have been used to indicate the extent of trophic transfer, and the "source" AAs have provided the original $\delta^{15}N$ values of primary production (McMahon and McCarthy, 2016). Therefore, measuring $\delta^{15}N$ values of "source" and "trophic" AAs together overcomes a basic problem in the interpretation of bulk $\delta^{15}N$ data, because this approach requires no assumptions about baseline $\delta^{15}N$ values. Thus, CSIA-AA typically revealed more de-

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tails than bulk $\delta^{15}N$ data, and has been widely applied in the trophic interactions of fishes (Bradley et al., 2015; Chikaraishi et al., 2009; Choy et al., 2012; Gerringer et al., 2017; Hetherington et al., 2017; McMahon et al., 2015).

In recent years, more ecological studies of mesopelagic and bathyal ecosystems were conducted based on AAs and fatty acids (FAs) (Choy et al., 2015; Décima et al., 2017; Hannides et al., 2013; Wang et al., 2019). Meanwhile, new challenges also arose when these methods were applied in those ecosystems. Firstly, low temperature restricted the metabolic rates of organisms in the deep ocean, and resulted in decreasing of the $\delta^{15}N$ enrichment (Choy et al., 2012). Thus, trophic positions and relations of deep-sea organisms need to be reassessed. Secondly, the diel vertical migration (DVM) of zooplankton from deep layers also causes significant diversity of N isotopes (Bode and Hernández-León, 2018). Thirdly, the variation of heterotrophic degradation with water depth might alter $\delta^{15}N$ enrichment ("source" AAs, like phenylalanine) of suspended particles (Hannides et al., 2013). This variation might introduce mistake when we directly use the $\delta^{15}N$ of Phenylalanine (Phe) as the baseline of source AAs. Therefore, additional parameters are excepted to mirror the diet sources of deep-sea organisms. $\delta^{13}C$ of FAs have been successfully used to trace the diets of organism (Bec et al., 2011; Braeckman et al., 2015; Budge et al., 2016). For most polyunsaturated fatty acids (PUFAs), there was no discrimination between sampled fishes and their diets, suggesting that isotope fractionation did not occur during transfer of PUFAs from diet to fish tissues (Budge et al., 2016). Therefore, $\delta^{13}C$ of FAs might be an ideal substitution parameter of $\delta^{15}N$ of Phe.

Currently, gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) is still the dominant CSIA technique being used (McCarthy et al., 2007; Ohkouchi et al., 2017). Meanwhile, high-pressure liquid chromatography and offline isotopic analysis have been raised in determining $\delta^{15}N$ of AAs in recent years (Broek and McCarthy, 2014; Swalethorp et al., 2020; Takano et al., 2015). However, there are some restrictions to these methods. For example, the combined effects of the derivatization process and instrument components lead to a relatively low order of precision for GC-C-IRMS data (typically ±1.0‰) (McCarthy et al., 2007; McClelland and Montoya, 2002; Sherwood et al., 2011), which limits the potential precision of trophic position estimates. HPLC and offline isotopic analysis represent an alternate approach to GC-C-IRMS with higher inherent precision of offline stable isotope measurements and the potential of simultaneous $\delta^{15}N$ and $\delta^{13}C$ measurements by one time. However, this method could typically only resolve a limited suite of AAs and it needs a large sample size. Thus, this study tries to raise a new method which could simultaneously consider precision and sample amounts.

Here this study proposes a new method for coupling ion chromatography (IC) with Precon-IRMS and chemical method of oxidation-reduction of amino acids. The amounts of AAs are first quantified by the IC, and an adequate number of AAs are subsequently collected with an automated fraction collector. Second, this study optimizes a robust chemical method for converting the AAs into N_2O. Finally, this study measures the $\delta^{15}N$ of N_2O and compares the advantages and disadvantages with other methods. A key goal here is to both assess the method performance in fish samples, and achieve sufficient separation of most AAs. As a secondary goal, this study firstly seeks to combine $\delta^{15}N$ of AAs with $\delta^{13}C$ of FAs to analyze the trophic interactions of mesopelagic fishes in the South China Sea (SCS), shedding the light on mesopelagic ecosystem studies.

2 Experimental section

2.1 *Isotopic amino acid standards*

Standard amino acid powders were purchased from Sigma-Aldrich (Merck, USA). Based on structural and functional groups, AAs are divided into aliphatic AAs (alanine, Ala; glycine, Gly; isoleucine, Ile; leucine, Leu; valine, Val), hydroxyl AAs (threonine, Thr), sulfur-containing AAs (methionine, Met; cystine, Cys), acidic AAs (aspartic acid, Asp; glutamic acid, Glu), and aromatic AAs (phenylalanine, Phe) (Zhang et al., 2007). The $\delta^{15}N$ of AAs were determined by EA-IRMS (Finngan Delta plus XP, ThermoFisher, USA) with the precision of ±0.1‰ (Std). Additionally, two certified amino acid standards were used to test analytical method (Glu, USGS40; Gly, USGS64; the Reston Stable Isotope Laboratory of the U.S. Geological Survey). Hydrochloric acid, sodium hypochlorite, sodium bromide, sodium arsenite, sodium hydroxide and ammonium chloride were purchased from either Fisher Scientific or Lab Chem and were used in our procedures. The purities of chemicals were larger than 99.9%.

2.2 *Preparation of AAs*

A schematic of the complete protocol from sample hydrolysis to determination of AA $\delta^{15}N$ values, is shown in Fig. 1. The AAs of the samples were extracted by HCl hydrolysis (Ishikawa et al., 2018a). Briefly, the samples were hydrolyzed with 6 mol/L HCl at 110°C for 20 h. Each hydrolysate was washed with n-hexane/dichloromethane (2/1, V/V) to remove any hydrophobic constituents (e.g., lipids), subsequently dried under a vacuum system at the room temperature (Labconco, USA), and finally dissolved in deionized water (DIW) to a final volume of 5 mL and adjusted the pH to 11–12 before the IC separation (Ishikawa et al., 2018a).

2.3 *Isolation of AAs*

Amino acids separations were conducted using an IC system (Dionex, ICS-5000$^+$, Thermo Scientific, USA) equipped with a pulsed amperometric detector (PAD) (Dionex, ICS-5000$^+$ DC), two pumps (Dionex, ICS-5000$^+$ DP), autosampler (Dionex, AS-AP) with an adjustable injection volume, and coupled to an automated fraction collector (Dionex, UltiMate 3000). To separate the amino acids, the IC was equipped with an IC column (Dionex^{TM} AminoPac^{TM}PA10, 9 mm × 250 mm, Thermo Scientific, USA). The flow splitter was used inline following the column to direct 10% of the flow to the PAD for peak detection and quantification. To calculate the linear relations between concentrations and response values, the standard curves of amino acids were showed in Table 1.

The injection volume of the autosampler was set to 100 μL for each run, and every sample ran triplicate times. A solvent ramp program was used consisting of Milli-Q water (solvent A) and 1 mol/L NaOH solution (solvent B) at a flow rate of 5 mL/min. The gradient elution procedure was adopted as follows: 0 min (A: 88%, B: 12%) to 75 min (A: 75%, B: 25%), to 185 min (A: 35%, B: 65%), to 200 min (A: 0%, B: 100%), and then washed with 100% B for 10 min, followed by equilibration with 12% B for 30 min.

Purified AAs were collected into 40 mL glass vials via an automated fraction collector using time-based collections. The solvent was removed under vacuum system. Dried AA residues were then re-dissolved in 5 mL DIW and filtered through a membrane filter (Polyether sulfone, pore size: 0.22 μm, Fisher Scientific, USA) (Ishikawa et al., 2018b). The filtrates were then transformed to glass vials and adjusted to a pH around 12.

2.4 *Conversion of AAs to NO_2^-*

The protocol for oxidizing AAs to NO_2^- was modified from the

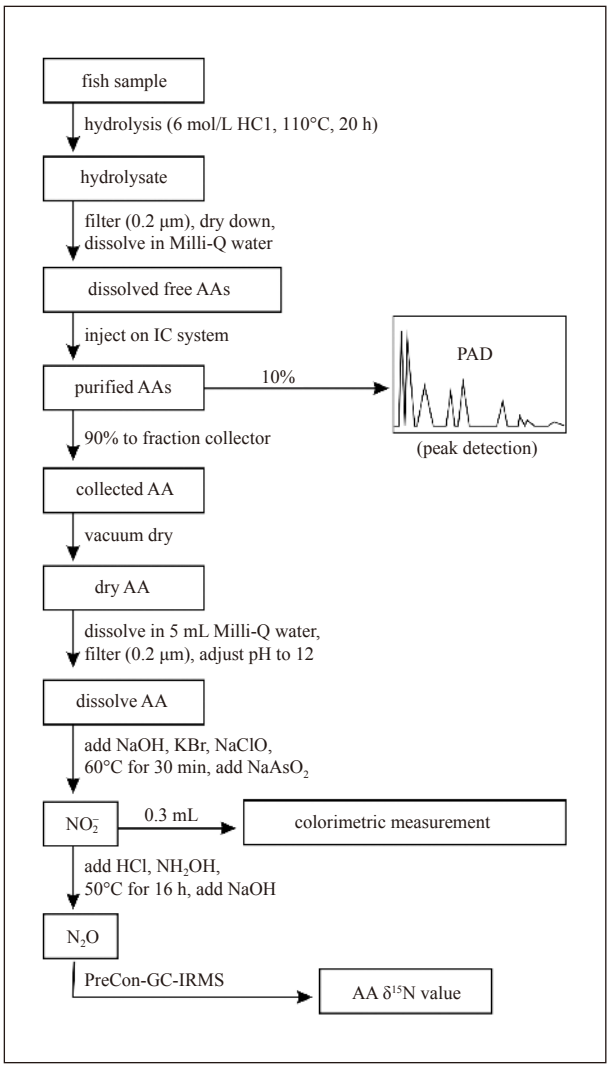

Fig. 1. Flow chart showing sample preparation and measurement for the $\delta^{15}N$ of amino acids (AA, amino acid; PAD, pulsed amperometric detector; GC-IRMS, gas chromatography-isotope ratio mass spectrometry).

"Strecker degradation" (Schonberg and Moubacher, 1952; Zhang and Altabet, 2008). Firstly, 0.1 mL of the KBr solution (50 mmol/L) was added to the vials containing about 5 mL AA samples. The pH was adjusted to 12 by addition of 0.1 mL of 10 mol/L NaOH solution. Then 0.1 mL of 30 mmol/L NaClO solution was added immediately followed by shaking. The vials were sealed with PTFE septa and placed in a water bath at 60°C for 30 min. Finally, 0.2 mL of $NaAsO_2$ solution was added to eliminate excess NaClO after the oxidation reaction. The concentration of the reaction product, NO_2^-, was measured with colorimetry to determine the yields. Oxidation yields of individual AAs were shown in Table 2.

2.5 *Conversion of NO_2^- to N_2O*

Here, the $NH_2OH{\cdot}HCl$ was used as the reductant to convert NO_2^- to N_2O (Bothner-By and Friedman, 1952; Liu et al., 2014). Hydroxylamine hydrochloride stock solution was made by every week (40 mmol/L). The working solution of $NH_2OH{\cdot}HCl$ was prepared daily by diluting stock solution. In the experiment, the NO_2^- solution was pipetted into 20 mL vials. Then 0.5 mL of 6 mol/L HCl solution was added to adjust pH to 0.2. Afterward, 0.5 mL of $NH_2OH{\cdot}HCl$ working solution (molar ratio of NH_2OH to NO_2^- was 3:1, 6:1, 10:1, 15:1 and 20:1) was added, and the vials were aluminum crimp sealed. The reaction took place at 45°C for 16 h. Finally, 0.5 mL of 5 mol/L NaOH solution was added to stop the reaction and made the solution basic to avoid remainder hydrochloric acid into the Precon-IRMS equipment when measuring the $\delta^{15}N$ of N_2O (Jin et al., 2020).

2.6 *Measure the $\delta^{15}N$ of N_2O*

The $\delta^{15}N$ of produced N_2O was analyzed by Delta V Advantage isotope ratio mass spectrometer (Thermo Fisher Scientific, Germany) coupled with a PAL autosampler (CTC Analytics AG, Switzerland) and a Finnigan PreCon Interface (Thermo Fisher Scientific, Germany) (Jin et al., 2020). A laboratory $NaNO_2$ standard was determined every 5 h to monitor the long-term consistency of the instrument (measured $\delta^{15}N$ value: – 53.0‰±0.3‰, n= 3). The content of N_2O was controlled in the range of 15–25 nmol.

2.7 *Calculate the $\delta^{15}N$ for AAs*

In theory, the slope of the relationship between the measured $\delta^{15}N$ of the produced N_2O, and the $\delta^{15}N$ of AAs should be 2 (Bothner-By and Friedman, 1952; Liu et al., 2014), since the N atoms of N_2O are verified by the 1:1 combination of AAs and NH_2OH (Bothner-By and Friedman, 1952). Thus, the calibration curve is

$$\delta^{15}N_{AA} = a \times \delta^{15}N_{N_2O} + b, \quad (1)$$

where the a (slope) and b (intercept) were gained from the linear relationship of the $\delta^{15}N$ of AA measured from the EA-IRMS and $\delta^{15}N$ measured from the N_2O produced by the standard AAs. Meanwhile, a laboratory $NaNO_2$ (–99.7‰, determined by EA-IRMS) was used to monitor the reduction efficiency of NO_2^- to N_2O and long-term consistency (the signal of 20 nmol N_2O was ca. 4 000 mV in the PreCon-IRMS).

2.8 *Mesopelagic fish preparation*

Mesopelagic fishes were collected in 2015 from the SCS (Wang et al., 2019). Based on their migrating habit and feeding behavior, they were assigned to five functional groups. The fish muscle tissue was excised from below the dorsal fin, and the tissue samples were lyophilized in a freeze-dryer (LOC-1; Christ, Germany). The dried samples were powdered using a mortar and pestle. Tissue (100 mg) was used to analyze the $\delta^{13}C$ compound-specific FAs by GC-C-IRMS (Bec et al., 2011; Wang et al., 2019). Briefly, the dried tissue was extracted by dichloromethane/methanol (2/1, V/V) to get total lipids. Then the FAs were transesterified to fatty acid methyl esters (FAMEs) in a mixture of methanol (containing 5% HCl). The compound-specific $\delta^{13}C$ FAMEs were analyzed using a Trace GC Ultra gas chromatograph coupled to a Delta V mass spectrometer via a GC combustion interface using helium as carrier gas (Thermo Scientific, Germany). To obtain the $\delta^{13}C$ of FAs, the methanol impact on the FAME isotopes should be eliminated using the following formula:

$$\delta^{13}C_{FA} = [(n+1) \times \delta^{13}C_{FAME} - \delta^{13}C_{MeOH}]/n, \quad (2)$$

where $\delta^{13}C_{FA}$ is the isotope of the FAs; $\delta^{13}C_{FAME}$ is the isotope of the FAME; $\delta^{13}C_{MeOH}$ is the isotope of the methanol derivatization reagent (–37.4‰±0.1‰); n is the number of C atoms in the FAs (Wang et al., 2019). FAs were named using a shorthand notation of *F*:*D*n-*X*, where *F* is the carbon number of FA, *D* is the number of double bonds, and *X* is the first double bond position relative

Table 1. Standard curves of amino acids by ion chromatography

	Standard concentration/(mmol·L^{-1})						Standard curve	R^2
	0.10	0.25	0.50	1.00	1.50	2.00		
$Q_{Lys}/10^{-9}$ C	1.25	3.35	6.69	16.20	22.45	27.57	$y = 0.069x+0.000\ 2$	0.99
$Q_{Ala}/10^{-9}$ C	7.95	19.47	25.90	33.00	37.60	49.98	$y = 0.049\ 7x–0.530\ 4$	0.93
$Q_{Thr}/10^{-9}$ C	37.94	89.51	182.68	311.11	398.45	492.92	$y = 0.004\ 1x–0.153\ 5$	0.98
$Q_{Gly}/10^{-9}$ C	2.12	6.97	15.62	29.21	33.85	51.93	$y = 0.039\ 7x–0.032\ 2$	0.98
$Q_{Val}/10^{-9}$ C	7.99	18.04	25.21	48.79	60.27	69.07	$y = 0.029\ 8x–0.249\ 3$	0.97
$Q_{Ile}/10^{-9}$ C	10.29	24.25	44.49	83.41	104.41	126.64	$y = 0.016x – 0.154\ 9$	0.98
$Q_{Leu}/10^{-9}$ C	14.54	34.06	65.53	119.67	151.54	187.10	$y = 0.010\ 9x–0.144\ 4$	0.98
$Q_{Met}/10^{-9}$ C	7.47	18.66	33.14	80.68	121.91	162.30	$y = 0.012\ 1x–0.039\ 1$	1.00
$Q_{His}/10^{-9}$ C	5.27	23.30	55.63	125.00	198.63	283.91	$y = 0.006\ 8x+0.102\ 2$	1.00
$Q_{Phe}/10^{-9}$ C	47.95	83.03	127.77	150.00	210.34	238.81	$y = 0.010\ 1x–0.545\ 4$	0.96
$Q_{Glu}/10^{-9}$ C	0.04	0.09	1.13	2.31	5.51	9.23	$y = 0.401x+0.574\ 2$	0.95
$Q_{Asp}/10^{-9}$ C	0.33	2.76	5.48	13.07	19.14	26.44	$y = 0.143\ 2x+0.285\ 2$	0.99
$Q_{Cys}/10^{-9}$ C	24.77	79.54	172.54	351.38	548.83	720.06	$y = 0.002\ 7x+0.033\ 2$	1.00
$Q_{Tyr}/10^{-9}$ C	21.99	58.81	140.48	278.20	326.76	428.25	$y = 0.004\ 6x–0.070\ 5$	0.97
$Q_{Trp}/10^{-9}$ C	43.41	126.53	399.92	620.98	874.43	1 241.78	$y = 0.001\ 6x+0.004\ 7$	0.99

Note: Q represents quantity of electric charge; Lys, lycine; His, histidine; Tyr, tyrosine; Trp, tryptophan.

Table 2. Oxidation efficiency (n=9) of different amino acids

Amino acid	Yield/%	Std/%
Glu	53.4	0.3
Ala	59.1	1.7
Thr	64.4	1.2
Gly	70.5	1.0
Leu	58.0	0.9
Asp	64.6	1.0
Val	65.0	1.1
Ser	55.5	1.1
Ile	67.4	1.6
Met	56.4	1.3
Phe	68.9	0.5
His	59.9	1.2

Note: Ser represents serine.

to the terminal methyl group (Budge et al., 2008).

The remaining material (~5 mg) was hydrolyzed for the $\delta^{15}N$ compound-specific amino acids. Each hydrolysate was washed with n-hexane/dichloromethane (3/2, V/V) to remove any hydrophobic constituents (e.g., lipids) (Ishikawa et al., 2018a).

3 Results and discussion

GC-C-IRMS is the conventional analytical technique for AA nitrogen isotope analysis (Ohkouchi et al., 2017). However, the lower precision involved in the lengthy GC-C-IRMS analysis constrained its application, e.g., the evaluation of chemical preservation effects on $\delta^{15}N_{AA}$ (Swalethorp et al., 2020). HPLC and offline isotopic analysis have emerged as new method in recent years but this method is limited by sample content and AA varieties (Broek et al., 2013). IC combined Dionex AminoPac column was verified to separate free amino acids without the need for derivatization (Thermo Scientific, USA). Here this study combines the IC separation and Precon-IRMS to assess underivatized AAs nitrogen isotopes with lower nitrogen content and higher precision. Meanwhile, a practical application on mesopelagic fishes in the SCS was used to evaluate this method, where the variation of $\delta^{15}N_{AA}$ might limited.

3.1 *Chromatographic optimization*

Optimization of column type, as well as solvent program were important considerations in achieving baseline separation. The best overall AA separation with the Dionex™ AminoPac™PA10 column (certified by the Thermo Scientific, USA) was achieved using a solvent system at a flow rate of 5 mL/min (Fig. 2). The use of water and 1 mol/L NaOH solution as mobile phase was showed to be particularly effective at resolving underivatized amino acids. Our optimized separation method (see experimental section) achieved almost all amino acids separation. Using the final protocol, complete baseline resolution was achieved for all AAs in mesopelagic fishes (Fig. 2).

The maximum sample loading (i.e., the maximum amount of compound injected on the column while maintaining baseline resolution) is also an important consideration for an offline isotopic measurement approach (Broek and McCarthy, 2014). Sample requirements for purified AAs were ultimately determined by oxidation yields. Considering the oxidation yields (Table 2), the collection of all AAs was found to be approximately 30–100 nmol of N per peak. Therefore, the major advantage of this method was the suitable chromatographic loading capacity, which allowed for collection of sufficient quantities of AAs from a single IC injection. If multiple IC injections and collections are conducted, these then represent independent replicates for the entire protocol, including isolation and collection.

3.2 *AA oxidation efficiency*

The second step of the $\delta^{15}N$-AAs measurement is the quantitative oxidation of AAs to NO_2^- (Zhang and Altabet, 2008). Oxidation yields were expressed as percentage of NO_2^- yield oxidized from AAs with respect to the initial amino acid amount. The oxidation efficiency ranged from 53.4% to 70.5%, in line with the results of Zhang and Altabet (2008) (Table 2).

3.3 *NO_2^- reduction efficiency*

NO_2^- reduction was conducted by NH_2OH in low pH (<0.2). In this study, the dosage of NH_2OH was evaluated. Nitrite reduction yield was highest with a molar ratio of NH_2OH to NO_2^- at 2:1 in previous study (Liu et al., 2014). However, in our experiment, the reduction efficiency was only 28% with low molar ratio of NH_2OH to NO_2^- (3:1) (Fig. 3), indicating an incomplete reduction from ni-

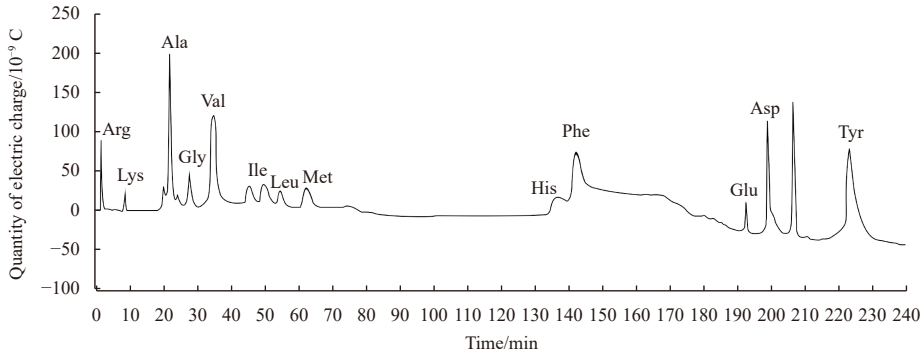

Fig. 2. Chromatogram from ion chromatogram separation of amino acids.

trite to N_2O. Hence, three comparative experiments were conducted to explore which reagents restrained the NO_2^- reduction efficiency. Zhang et al. (2007) reported that excess BrO^- should be removed after oxidation, because the remaining BrO^- likely reacted with NH_2OH in the reduction step to produce N_2O blank (Liu et al., 2014). Excess ClO^- was induced in the AA oxidation step. ClO^- is a stronger oxidant compared with BrO^-, leading to react with NH_2OH. Excess ClO^- reacted with NH_2OH in the reduction step to increase the blank of N_2O. Thus, NaClO was excluded in the comparative experiments.

In the final reaction system, $NaAsO_2$ and Na_3AsO_4 co-existed in the 20 mL vials. Thus, the comparative experiments included water, $NaAsO_2$ solution and mixture of Na_3AsO_4 and $NaAsO_2$ (produced by mixture of NaClO and excess $NaAsO_2$) to explore which reagents restrained the NO_2^- reduction efficiency. When the molar ratio of NH_2OH to NO_2^- at 3:1, the addition of $NaAsO_2$ and Na_3AsO_4 restrained the N_2O reaction, but NO_2^- reduction efficiency was also high with the addition of $NaAsO_2$ (80%, Fig. 3). The Na_3AsO_4 had more negative impact on N_2O reaction than $NaAsO_2$. Doubling the NH_2OH amount improved the reduction efficiency, and $NaAsO_2$ and Na_3AsO_4 had similar reduction efficiency. When the ratio of NH_2OH to NO_2^- larger than 10:1, the effects of $NaAsO_2$ and Na_3AsO_4 were removed and got stable reduction efficiency. Liu et al. (2014) found that nitrite reduction yield was highest with a molar ratio of NH_2OH to NO_2^- at 2:1, which was inconsistent with this study. The oxidation of amino acids needed high hypochlorite concentration (Zhang and Altabet, 2008). Thus, the differences might be the Na_3AsO_4 concentration was high in this reaction system.

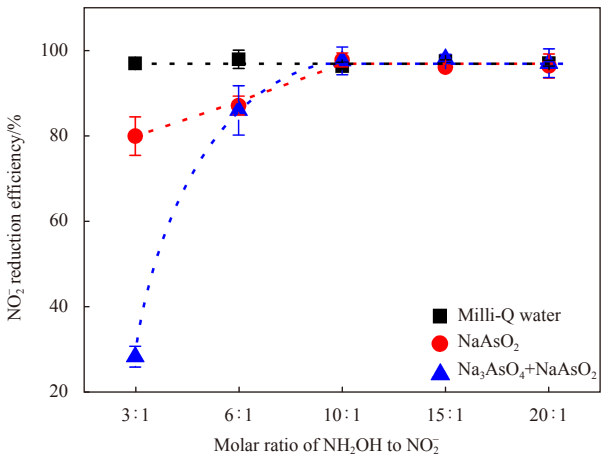

Fig. 3. Effects of molar ratio of NH_2OH to NO_2^- on the reaction of NO_2^- to N_2O.

3.4 *Isotope results of AAs*

The linear relationship between the $\delta^{15}N_{AA}$ and the $\delta^{15}N_{N_2O}$ was significant (Fig. 4). The slopes of linear relationship were 2.05 to 2.28 and had no obvious changes by structural groups of amino acids. The slopes were slightly higher than the theoretical value as predicted by other study (Bothner-By and Friedman, 1952). The reasons for the small deviation from the theoretical slope might be the blank of reagents. However, the excellent correlation of $\delta^{15}N$ demonstrated that this method was a robust and quantitative technique for measuring the $\delta^{15}N$ of AAs (Liu et al., 2014).

The intercepts of the linear regressions were distinguishable for each structural group representative (Fig. 4). Theoretically, they reflected isotopic fractionation in the procedure. Hence, it was likely that the variation was caused by the discrimination or preference for ^{15}N in the oxidation of amino acids (Schonberg and Moubacher, 1952; Zhang and Altabet, 2008). Overall, the $\delta^{15}N$ of single-N-containing AAs can be determined quantitatively and precisely by this method, and the average standard deviation in the case of standard AA was 0.6‰ (n=3)(Zhang and Altabet, 2008).

4 Comments and recommendations

In this study, the IC separation allowed fully automated peak collection without concern for coelution, and could simultaneously analyze 13 AAs from a single sample injection. Comparison of values for authentic standards showed that, for almost all AAs, our chromatographic resolution was sufficient to produce accurate $\delta^{15}N$ values, which were less than or equivalent within error to values from GC-C-IRMS in most cases (Fig. 4). The standard errors of most AAs were less than 0.6‰ (Ala, Gly, Ile, Leu, Val, Thr, Glu, Phe, Met, Cys), only Asp had high standard errors reached near 1‰. The precision of this method had raised to some extent compared with GC-C-IRMS. In addition, the amount of sample required is also a consideration for any specific application. As discussed above, sample sizes required for this method are ultimately determined by Precon-IRMS sensitivity (20 nmol), and therefore minimum sample sizes required for this method will likely always be less than those for HPLC-EA-IRMS (Broek et al., 2013; Takano et al., 2015; Tripp and McCullagh, 2012).

Nonetheless, before opting for this method, this study should consider a number of issues as this approach may not always be better than existing methods. First of all, this new method need more times than others. Considering the separating and oxidiz-

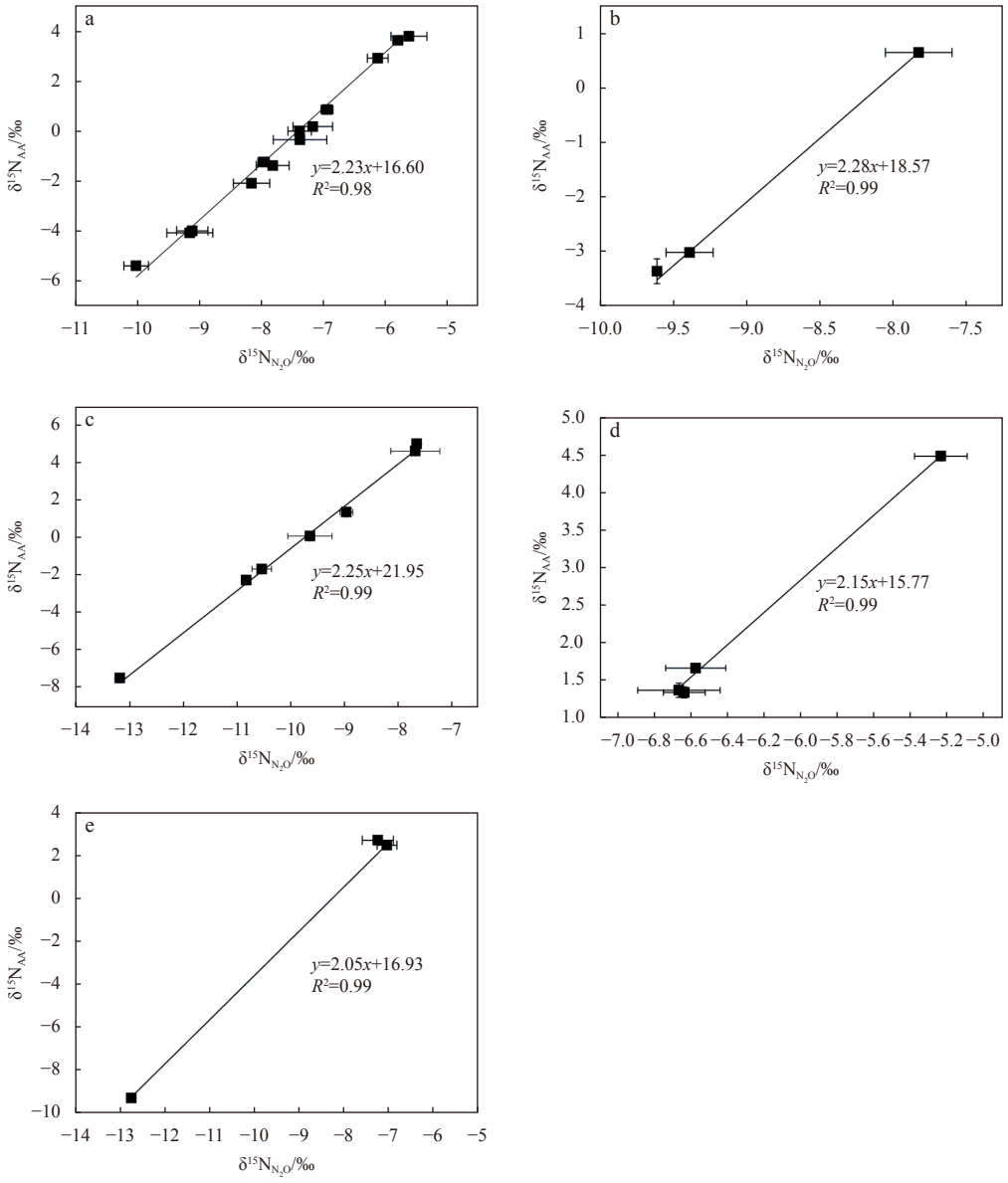

Fig. 4. Correlation between the $\delta^{15}N_{AA}$ and the $\delta^{15}N_{N_2O}$ for five groups of amino acids. a. aliphatic AA, including Gly, Ala, Val, Leu and Ile; b. hydroxyl AA, including Thr; c. acidic AA, including Asp and Glu; d. sulfur AA, including Met and Cys; e. aromatic AA, including Phe.

ing times, this study just routinely processes seven samples every week, which is much less than GC-C-IRMS and HPLC-EA-IRMS (Swalethorp et al., 2020). Because the HPLC has the potential to save times, the study group is thinking to use HPLC as the choice next. Last, the standard errors of most AAs are about 0.6‰, slightly higher than GC-C-IRMS, it can be still not better than GC-C-IRMS if a large number of samples is analyzed, since time is the critical factor. However, this study provides a new thought, especially in terms of small sample sizes, for the development of CSIA, and yet, there is still far to go.

Source amino acids (i.e., Phe, Met) fractionate $\delta^{15}N$ very little (<0.5‰) during trophic transfer, whereas trophic AAs (i.e., Glu, Ile, Leu, Val) are greatly enriched in $\delta^{15}N$ during each trophic step (Chikaraishi et al., 2009; McClelland and Montoya, 2002). The differential fractionation of these two AA groups can provide a valuable estimate of consumer trophic position that is internally indexed to the baseline $\delta^{15}N$ value of the integrated food web (McMahon and Newsome, 2019; Ohkouchi et al., 2017). However, the trophic positions of mesopelagic fishes revealed by GC-C-IRMS showed inconsistent with results derived from stomach content studies (Choy et al., 2012), which might be caused by the accuracy of GC-C-IRMS. Thus, this study tries to use the optimized method of $\delta^{15}N$-AAs to see whether it can reveal more details about the trophic interactions of mesopelagic fishes in the SCS.

The mesopelagic fishes were assigned to 5 functional groups based on their migrating habit and feeding behavior. More details were showed in Wang et al. (2019). Briefly, *Diaphus luetkeni* exhibits active DVM, feeds on plankton and is termed migrant planktivorous fishes. *Chauliodus minimus* shows passive DVM, mainly feeds on small mesopelagic fishes and is named migrant piscivorous fishes. *Argyropelecus affinis* has a non-migrating habit, feeds on plankton and is called non-migrant planktivorous fishes. *Bathygadus antrodes* has a non-migrating habit,

mainly feeds on small fishes and is called non-migrant piscivorous fishes. *Stomias nebulosus* has a semi-migrating habit, mainly preys on small fishes and is named semi-migrant piscivorous fishes. The planktivorous fishes had lower δ^{15}N of trophic AAs (Glu, Ile, Leu, Val) than piscivorous fishes (Fig. 5), which was consistent with previous studies (Bradley et al., 2015; Hetherington et al., 2017; Landry et al., 2017; McMahon et al., 2015). However, the source AAs (Phe, Met) showed different tendency: the migrant planktivorous fishes had lowest δ^{15}N of source AAs in mesopelagic fishes, the non-migrant planktivorous fishes had similar source AAs with the migrant piscivorous fishes, the non-migrant and semi-migrant piscivorous fishes had higher δ^{15}N of source AAs (Fig. 5). These results were inconsistent with most traditional studies (Gerringer et al., 2017; Hussey et al., 2014; McMahon and McCarthy, 2016), and they might be caused by many factors. Firstly, the DVM fishes mainly live in 0–450 m layers, the non-migrant fishes live in 450–600 m layers, and the semi-migrant fishes live in 450–800 m layers (Wang et al., 2019). Depth changes might cause the mesopelagic fishes being affected by the δ^{15}N of particle matter in different layers (Hannides et al., 2013). Secondly, the DVM of zooplankton from deep layers also causes significant diversity of N isotopes. Thus, the source AAs (like Phe) were not suitable to study source diets of mesopelagic fishes from different layers. Other parameters were needed to excavate the diets of mesopelagic fishes.

The compound-specific isotope analysis of FAs has been used to clarify complex trophic dynamics and produce more accurate dietary relationships that might be overlooked through stable isotopes or FAs (Bec et al., 2011; Budge et al., 2008; Gladyshev et al., 2016). The δ^{13}C of 20:4n-6 revealed that the mesopelagic fishes in the SCS might have different diets, while no data was used to reflect their trophic interactions (Wang et al., 2019). Thus, the combination of δ^{13}C values of 20:4n-6 and δ^{15}N values of Glu was firstly used to reveal their trophic relations. In Fig. 6, non-migrant planktivorous fishes (–32.8‰±0.8‰) had similar δ^{13}C of 20:4n-6 with semi-migrant piscivorous fishes (–32.3‰±0.3‰), the migrant planktivorous, migrant piscivorous and non-migrant piscivorous fishes had similar values (i.e., –28.5‰±0.6‰, –28.4‰±0.6‰ and –28.1‰±0.5‰, respectively). As described above, the piscivorous fishes had higher δ^{15}N of Glu than planktivorous fishes. Meanwhile, the difference was about 4‰ between semi-migrant piscivorous fishes (11.8‰±0.6‰) and non-migrant planktivorous fishes (7.8‰±0.6‰), between migrant and non-migrant piscivorous fishes (11.4‰±0.5‰ and 11.3‰±0.5‰, respectively) and migrant planktivorous fishes (7.2‰±0.6‰) (Fig. 6). The 4‰ enrichment was much less than previous studies (~7.6‰) (McMahon and McCarthy, 2016; Ohkouchi et al., 2017). The discrepancy might be caused by low temperature, which resulted in low locomotory abilities and low metabolic rates (Caut et al., 2009; Choy et al., 2012; Hussey et al., 2014). Therefore, the trophic discrimination factor of trophic AAs needed to be recalculated when studying the mesopelagic fishes in mesopelagic and deep waters (Bradley et al., 2015; Choy et al., 2012). The combination of the δ^{13}C of 20:4n-6 and δ^{15}N of Glu was verified as a good method to study the trophic interactions of mesopelagic fishes from the SCS in this study.

5 Summary and future research

The proposed method clearly achieved the main goals for testing the δ^{15}N of AAs in mesopelagic fishes. Furthermore, the combination of the δ^{13}C of FAs with δ^{15}N of AAs has successfully mirrored the trophic interactions of mesopelagic fishes from the SCS. Therefore, such kind of tool would likely be suitable for similar application in bathyal ecosystems. Obviously, this method can be also adopted to those less nitrogen available samples (e.g., coral) or additional applications in marine biogeochemical studies (e.g., sediments, deep ocean suspended particles and dissolved organic matter) (McCarthy et al., 2013; Sherwood et al., 2011; Yamaguchi and McCarthy, 2018). The application of the modified method could reduce the sample requirements to near the same order as GC-C-IRMS when less material is available (Broek and McCarthy, 2014). Nonetheless, this method needed more time to isolate and transform AAs than GC-C-IRMS, and had lower accuracy compared to HPLC-EA-IRMS. Therefore, reducing time and improving accuracy is the main factors to develop this new method in the future. In recent study, multi-dimensional HPLC has been used to separate AAs in biological samples (Sun et al., 2020). Therefore, this study tries some mixed mode columns to achieve a better separation. Except for chemical methods, a bacterial method for the nitrogen isotopic analysis of nitrate promises a much lower detection limit (Sigman et al., 2001). Combining these methods, this study might get a higher accuracy. Some authors also have changed the EA protocols to reduce the atmospheric N to the sample signal (Broek et al., 2013; Broek and McCarthy, 2014; Swalethorp et al., 2020). Inspired by their examples, linking IC or HPLC to IRMS to improve accuracy and reduce time can be tried in the future.

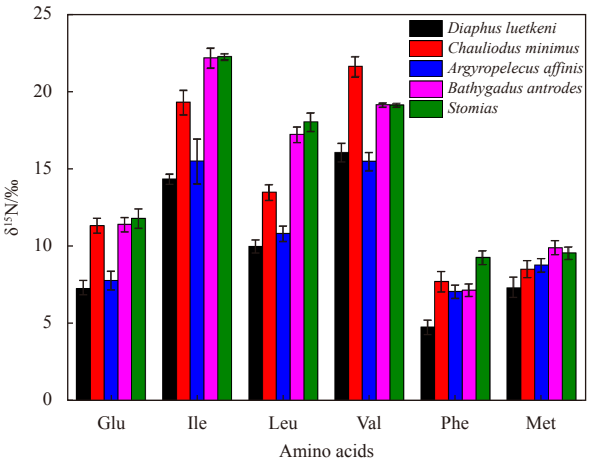

Fig. 5. δ^{15}N of amino acids in mesopelagic fishes.

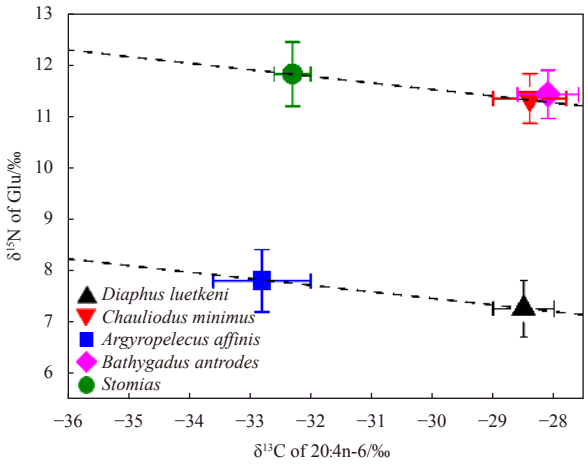

Fig. 6. δ^{15}N of Glu versus δ^{13}C of 20:4n-6 in mesopelagic fishes.

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