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Efficient acid hydrolysis for compound-specific $\delta^{15}N$ analysis of amino acids for determining trophic positions

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ABSTRACT

Compound-specific isotope analysis of nitrogen in amino acids (CSIA-AA, $\delta^{15}N_{AA}$) has gained increasing popularity for elucidating energy flow within food chains and determining the trophic positions of various organisms. However, there is a lack of research on the impact of hydrolysis conditions, such as HCl concentration and hydrolysis time, on $\delta^{15}N_{AA}$ analysis in biota samples. In this study, we investigated two HCl concentrations (6 M and 12 M) and four hydrolysis times (2 h, 6 h, 12 h, and 24 h) for hydrolyzing and derivatizing AAs in reference materials (Tuna) and biological samples of little egret (n = 4), night heron (n = 4), sharpbelly (n = 4) and Algae (n = 1) using the n-pivaloyl-iso-propyl (NPIP) ester approach. A Dowex cation exchange resin was used to purify amino acids before derivatization. We then determined $\delta^{15}N_{AA}$ values using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). The results revealed no significant differences ($p > 0.05$) in $\delta^{15}N_{AA}$ values among samples treated with different HCl concentrations or hydrolysis times, particularly for $\delta^{15}N_{Glx}$ (range: 21.0–23.5‰) and $\delta^{15}N_{Phe}$ (range: 4.3–5.4‰) in Tuna (12 M). Trophic positions (TPs) calculated based on $\delta^{15}N_{AA}$ at 2 h (little egret: 2.9 ± 0.1, night heron: 2.8 ± 0.1, sharpbelly: 2.0 ± 0.1 and Algae: 1.3 ± 0.2) were consistent with those at 24 h (3.1 ± 0.1, 2.8 ± 0.1, 2.2 ± 0.1 and 1.1 ± 0.1, respectively), suggesting that a 2-h hydrolysis time and a 6 M HCl concentration are efficient pretreatment conditions for determining $\delta^{15}N_{AA}$ and estimating TP. Compared to the currently used hydrolysis conditions (24 h, 6 M), the proposed conditions (2 h, 6 M) accelerated the $\delta^{15}N_{AA}$ assay, making it faster, more convenient, and more efficient. Further research is needed to simplify the operational processes and reduce the time costs, enabling more efficient applications of CSIA-AA.

1. Introduction

Over the past two decades, compound-specific isotope analysis (CSIA) of nitrogen in amino acids (AAs), known as CSIA-AA, has been increasingly employed to elucidate the structure of aquatic food chains, the enrichment and magnification of heavy metals within food chains, and nutritional relationships (Popp et al., 2007; Endo et al., 2016; Won et al., 2020; Zhang et al., 2021; Yang et al., 2022; Jiang et al., 2023a). CSIA-AA relies on isotopic fractionation, with significant ^{15}N enrichment in glutamic acid (a typical trophic AA, $\Delta_{Glu} = 8.0 \pm 1.2$‰) and minimal variation in phenylalanine (a typical source AA, $\Delta_{Phe} = 0.4 \pm 0.5$‰) between two trophic levels (Chikaraishi et al. 2007, 2009). Compared to traditional methods such as stomach content analysis (SCA) and bulk stable isotope analysis of nitrogen (BSIA), CSIA-AA offers high precision and accuracy due to its independence from spatiotemporal scales and species (Chikaraishi et al., 2014; Yun et al., 2022). For instance, in the Eastern Tropical Pacific, the trophic position of dolphinfish (*Coryphaena hippurus*) based on BSIA ($\delta^{15}N_{bulk}$) was notably lower than that of SCA and CSIA-AA ($\delta^{15}N_{AA}$), with average estimates of 3.5, 4.3, and 4.1, respectively (Briones-Hernández et al., 2023). Chikaraishi et al. (2011) reported that $\delta^{15}N_{bulk}$ values varied significantly among different body tissues of a wasp (e.g., head of 6.4‰ and abdomen

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of 3.8‰), while the $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values differed by only 0.4‰. Therefore, CSIA-AA is now considered a powerful tool for accurately determining food chain structures.

The methods for protein hydrolysis are primarily divided into chemical and enzymatic hydrolysis methods (Fountoulakis and Lahm, 1998). Different samples require different hydrolysis methods to obtain the best recovery of amino acids. This is because no single method can completely recover all amino acids. In most studies on CSIA-AA, the most commonly used method of hydrolysis is acidic hydrolysis, particularly with hydrochloric acid (HCl) (Silverman et al., 2022; Jiang et al., 2023b). This reagent is convenient because it can evaporate relatively easily (Fountoulakis and Lahm, 1998). Quantitative analysis of AAs is primarily conducted using liquid chromatography–mass spectrometry (LC–MS) and gas chromatography–mass spectrometry (GC–MS) (Krumpochova et al., 2015). Unlike liquid chromatography (LC), gas chromatography (GC) necessitates the derivatization of certain AAs for isolation from environmental samples (Silverman et al., 2022). Gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) is the preferred technique for measuring $\delta^{15}N$ in AAs ($\delta^{15}N_{AA}$), as it can increase analysis speed and sensitivity (Godin and McCullagh, 2011).

Before conducting isotope analysis via GC-C-IRMS, amino acids need to be separated from complex mixtures and treated with derivatization to safeguard the polar groups of the amino acid molecule and lower its boiling point. Moreover, efforts should be made to minimize any unintentional isotope fractionation during the separation process. Takano et al. (2010) reported that AAs treated with a cation-exchange resin exhibited negligible nitrogen isotope fractionation, with an average recovery exceeding 94%. Methods for derivatizing nitrogen isotopes in AAs include methoxycarbonyl ester (MOC), trifluoroacetyl (TFA) ester, and n-pivaloyl-iso-propyl (NPIP) ester (Yarnes and Herszage, 2017; Zhang et al., 2016; Silverman et al., 2022). The NPIP ester method is a higher-yield, heteroatom-free approach (Zhang et al., 2016), requiring only 20 ngN of a low sample mass for nitrogen isotope determination in AAs (Zhang et al., 2016). Thus, the NPIP approach is considered a reliable and recommended method, especially for trophic position calculations.

Currently, the most commonly used hydrolysis condition for AAs in biota samples is 6 M HCl hydrolysis at 110 °C for 20–24 h (Rutherfurd and Gilani, 2009; Ohkouchi et al., 2017; Fountoulakis and Lahm, 1998; Darragh et al., 1996). This condition ensures an optimal average recovery of 93% for most AAs (Darragh et al., 1996). However, for the NPIP approach, a 24-h hydrolysis period is lengthy and inefficient (Zhang et al., 2016). While extending the hydrolysis time can improve the recovery of certain aliphatic AAs, such as valine and leucine (Darragh and Moughan, 2005), it simultaneously affects the recovery of other AAs (e.g., serine and threonine) (Albin et al., 2000). Furthermore, prolonged hydrolysis may induce isotopic fractionation, particularly for nitrogen isotopes, due to distinct mechanisms of AA hydrolysis (Bada et al., 1989). Specific studies investigating the effects of hydrolysis conditions, including hydrolysis time and HCl concentration, on $\delta^{15}N_{AA}$ analysis in biological samples are lacking.

In this study, four hydrolysis durations (2 h, 6 h, 12 h, and 24 h) and two HCl concentrations (6 M and 12 M) were explored as hydrolysis conditions to evaluate the compound-specific analysis of $\delta^{15}N_{AA}$ in biota samples. Amino acids in relevant biological samples and reference materials were purified using a cation-exchange resin and subjected to derivatization using the NPIP method. The resulting derivatives of amino acids were analyzed qualitatively using GC–MS. The $\delta^{15}N_{AA}$ values were then determined via GC-C-IRMS to assess the impact of different hydrolysis conditions on these values. Subsequently, the trophic positions of the investigated biota were estimated based on the measured $\delta^{15}N_{AA}$ data. Our study aimed to propose efficient acid hydrolysis conditions for the compound-specific analysis of $\delta^{15}N_{AA}$ in biological samples, allowing for the determination of trophic positions in food webs. These findings can also provide fundamental data for research on nitrogen isotopes in AAs.

2. Experimental

2.1. Standards, reagents, and reference materials

A standard mixed solution of AAs (AAS18, 2.5 μmol/mL) comprising alanine, glycine, valine, leucine, isoleucine, proline, aspartate, threonine, serine, methionine, glutamic acid, and phenylalanine were acquired from Sigma Aldrich (St. Louis, MO, USA). The isotope reference material USGS-41a (United States Geological Survey, Reston, Virginia, USA) and the internal standard α-aminobutyric acid (AABA) were purchased for quality assurance and quality control (QA/QC). A cation exchange resin (Dowex 50W X8 H, 200–400 mesh, Sigma–Aldrich) was utilized for purification.

The reagents for derivatization, including pivaloyl chloride, thionyl chloride, isopropyl alcohol, dichloromethane (DCM), and n-hexane, were obtained from Sigma–Aldrich. Other reagents, such as ammonia, anhydrous sodium sulfate, and hydrochloric acid (12 M, analytical grade), were obtained from Aladdin (Shanghai, China).

2.2. Sample preparation, hydrolysis, and derivatization

Muscle tissues of sharpbelly (*Hemiculter leucisculus*) (n = 4) and algae (*Ulothrix*) (n = 1) and feathers of waterbirds of little egret (*Egretta garzetta*) (n = 4) and night heron (*Nycticorax nycticorax*) (n = 4), collected from Guizhou and Yunnan Provinces, respectively, were selected for the experiment. Briefly, each sharpbelly sample was collected from four individual samples, while each feather sample of each waterbird was collected from an individual bird. The algae and sharpbelly specimens were freeze-dried and ground, and the feathers from the waterbirds were cut into small pieces using stainless steel scissors after being cleaned with ultrasound. The reference material of Tuna (GBW10029, National Institute of Metrology, China) was also processed alongside the samples for analysis.

For the hydrolysis of AAs, approximately 0.5 mL of either 6 M or 12 M HCl was added to a reaction vessel containing approximately 10 mg of sample. Hydrolysis was carried out at 110 °C for 2 h, 6 h, 12 h, or 24 h. After cooling, a filtration step was performed to remove any sediment using a 0.45 μm filter (JinTeng, China). In the liquid extraction phase, the hydrolysate was washed with a mixture of n-hexane and DCM (*v/v*: 3/2) to eliminate lipophilic compounds. Subsequently, a cation exchange resin (Dowex 50W X8, 200–400 mesh, H^+) was employed to separate AAs from sugars, organic acids, and other components before derivatization (Takano et al., 2010).

For AA derivatization, we followed the procedure reported by Zhang et al. (2016) and Corr et al. (2007). In brief, approximately 1 mL of thionyl chloride/isopropyl alcohol (*v/v*: 1/4) was added to the reaction vessel for esterification at 110 °C for 2 h. The esterified sample underwent drying using a gentle stream of N2, followed by the addition of DCM (2 × 0.25 mL), and drying once more to completely eliminate the derivative reagent. Subsequently, approximately 1 mL of pivaloyl chloride/dichloromethane solution (*v/v*: 1/4) was added to the same reaction vessel and acylated at 110 °C for 2 h to generate n-pivaloyl o-isopropanol amino acid ester. Similarly, the derivative product was dried with N2, DCM (2 × 0.25 mL) was added, and allowed to dry once more. Finally, it was dissolved in 0.5 mL of DCM and stored at −20 °C before analysis.

To evaluate $\delta^{15}N$ variations before and after cation exchange, six parallel amino acid standard solutions were derivatized as described above. Three of these solutions underwent treatment with a cationic resin and were then combined with 0.1 mL of internal standard for derivatization. The $\delta^{15}N$ value was determined under the same analytical conditions.

2.3. Isotope ratio mass spectrometry

The $\delta^{15}N_{AA}$ values were determined using GC–C-IRMS (IRMS coupled with a Trace GC and Interface III, Bremen, Germany, Thermo Scientific). The measurement conditions for isotope ratio mass spectrometry were optimized as follows:

GC–C-IRMS: Agilent DB-5 ms capillary column (30 m × 0.25 mm × 0.25 μm); helium flow rate: 1.4 mL/min; inlet temperature: 250 °C; temperature increase procedure: initially held at 40 °C for 2.5 min, then increased to 110 °C at 15 °C/min, followed by an increase to 150 °C at 3 °C/min, and finally reached 230 °C at 6 °C/min. The combustion furnace temperature was set at 1030 °C, the N_2 measurement mode was selected, and ion source parameters of m/z 28, 29, and 30 were automatically used.

The AA derivative samples were separated by GC and then introduced into the capillary to be converted into the corresponding gas. The water generated during combustion was removed by a perfluorosulfonic acid permeable membrane. The capillary connecting the combustion tube and the mass spectrometer was placed in a liquid nitrogen cold trap to trap the CO_2 produced during sample combustion. The cold trap was removed to release the trapped CO_2 and prevent capillary clogging.

The isotope ratios of nitrogen are expressed as $\delta^{15}N_{AA}$ values (‰) using the following formula:

$$\delta^{15}N_{AA} = (R_{sample}/R_{standard} - 1) * 1000\ (‰)$$

where:

AA represents various amino acids, including "trophic" AAs such as alanine (Ala), valine (Val), leucine (Leu), isoleucine (Ile), proline (Pro), aspartic acid and asparagine (Asx), glutamic acid and glutamine (Glx); and "source" AAs such as glycine (Gly), serine (Ser), methionine (Met), and phenylalanine (Phe) (McClelland and Montoya, 2002; Popp et al., 2007). R is the $^{15}N/^{14}N$ ratio, with atmospheric air (0‰) as the international standard for nitrogen.

The AA standard solution and USGS-41a were measured every 5 samples, and the combustion furnace was reactivated after each measurement of 15 samples. The analytical precision was within 1‰, and the correction procedure followed Yarnes and Herszage (2017). When three parallel samples were digested and analyzed in the same way, the $\delta^{15}N$ of most amino acids changed within 1‰.

2.4. Trophic position calculation

The trophic position (TP) is calculated using the $\delta^{15}N_{AA}$ values of Glx and Phe (Chikaraishi et al., 2009) with equation (1):

$$TP = ((Glx\text{-}Phe\text{-}\beta)/TDF_{Glx\text{-}Phe}) + 1 \quad (1)$$

where:

β represents the difference in $\delta^{15}N_{AA}$ between Glx and Phe in primary producers, which are 3.4‰, 8.4‰, and 0.4‰ for aquatic food webs, terrestrial C3 plant food webs, and terrestrial C4 plant food webs, respectively (Chikaraishi, 2010). $TDF_{Glx\text{-}Phe}$ is the trophic discrimination factor, representing the change in $\delta^{15}N$ of Glx relative to Phe at each trophic level, and it is generally 7.6‰ (Chikaraishi et al., 2009).

Since increasing evidence indicates variable $TDF_{Glx\text{-}Phe}$ values for biota with high trophic levels (e.g., Gentoo penguins and elasmobranchs) (McMahon et al., 2015), a multi-$TDF_{Glx\text{-}Phe}$ approach employed in TP calculations can generate more precise results, especially for waterbirds using feathers (Quillfeldt and Masello, 2020; Hoen et al., 2014). Hence, the TP of the little egret and night heron in the present study was calculated with equation (2):

$$TP_{feathers} = ((Glx\text{-}Phe\text{-}3.5‰\text{-}\beta)/6.2‰) + 2 \quad (2)$$

where:

β is the literature value of 3.4‰. According to McMahon et al. (2015), 3.5‰ is the TDF for seabird feathers, and 6.2‰ is the overall average TDF across a wide range of taxa (McMahon and McCarthy, 2016).

2.5. Statistical data analysis

All data analyses were conducted using Microsoft Excel 2013 (Microsoft Corporation, WA, USA) or SPSS 25 (International Business Machines Corporation, USA). We used two-way analysis of variance (ANOVA) to test for differences when the data were normal. All figures and linear regressions were generated using Origin 2023 (OriginLab Corporation, USA).

3. Results and discussion

3.1. Chromatographic separation

The ion chromatograms derived from the GC–C-IRMS analysis of 13 amino acid NPIP ester derivatives extracted from the amino acid standard solution and a biological sample of sharpbelly are depicted in Fig. 1. Background compounds were not identified in the blank sample (Fig. 1a). GC−MS was utilized for the qualitative examination of amino acids in this investigation. Additional information is available in the supplementary information (SI).

The 13 AA derivatives were effectively separated on a typically nonpolar gas chromatographic column (DB-5 ms). The elution order of AAs is related to their polarity and molecular weight (Zhang et al., 2016). In this study, nonpolar low-molecular-weight AAs (such as Ala and Gly), which do not strongly interact with the stationary phase, eluted first, while high-molecular-weight polar and aromatic AAs eluted last (Fig. 1b).

During sample hydrolysis, tryptophan and cysteine were destroyed and not detected in the standard AA solution or the biological sample of sharpbelly (Fig. 1c). Under all acid hydrolysis conditions using the NPIP approach, asparagine and glutamine (Gln) are converted to Asp and Glu, respectively (Fountoulakis and Lahm, 1998; Zhang et al., 2016). Therefore, we used Glx and Asx to represent Glu and Gln and Asp and Asn, respectively.

3.2. Verification of $\delta^{15}N$ consistency before and after cation exchange

Quantitative analysis of AAs was primarily conducted using GC−MS (Table 1). The recovery of AAs after purification with a cationic resin ranged from 75.1% to 111.8%, with the average recovery of AAs reaching 95 ± 15% (n = 3). Although there was a variable trend in the AA nitrogen isotope values after treatment with the cationic resin, no statistically significant difference was observed ($p = 0.77$). There was a strong correlation between the $\delta^{15}N_{AA}$ values before and after resin treatment ($y = 0.85x - 0.31$, $R^2 = 0.96$, $p < 0.01$) (Fig. 2). To ensure the accuracy in determining $\delta^{15}N_{AA}$ values by GC–C-IRMS, the certified reference material (USGS-41a) was derived under the same conditions as the AA standard solution.

3.3. Effects of different hydrolysis conditions on $\delta^{15}N_{AA}$

The results for $\delta^{15}N_{AA}$ of 11 AAs in the feathers of little egret and night heron, sharpbelly, Algae and a biological certified reference material (Tuna) are shown in Table S1. As expected, the $\delta^{15}N_{AA}$ values of trophic AAs (e.g., Glx) were greater than those of source AAs (e.g., Phe) (Fig. 3). In general, no significant difference was observed in the $\delta^{15}N_{AA}$ values of all the samples treated with two HCl concentrations (6 M, 12 M) and four hydrolysis times (2 h, 6 h, 12 h, and 24 h) (two-way ANOVA, $p > 0.05$), except for sharpbelly between 2 h and 24 h. Specifically, the recovery of most AAs was greater at 24 h than at 2 h (Darragh et al., 1996).

In this study, the $\delta^{15}N_{AA}$ values for Tuna treated with 12 M and 6 M HCl for 24 h were not detected, except for proline, as shown in the SI.

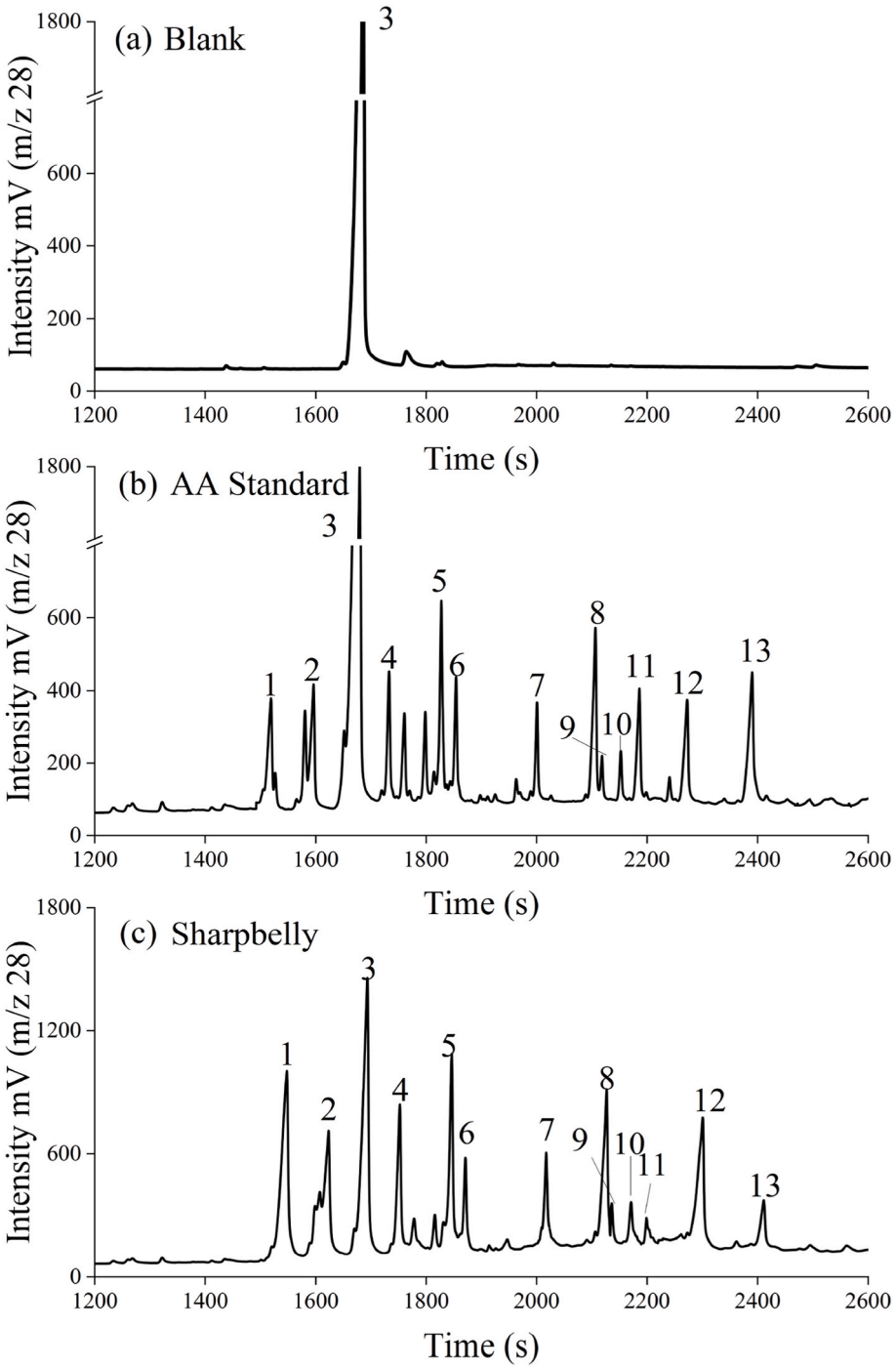

Fig. 1. GC–C-IRMS chromatograms for the analysis of AA NPIP ester derivatives. (a) The blank procedure with an internal standard after cation-exchange resin treatment. (b) AA standard solution with an internal standard. (c) Biological sample of sharpbelly with an internal standard after cation-exchange resin treatment. Only the signal at m/z 28 is presented. (1-Ala; 2-Gly; 3-AABA; 4-Val; 5-Leu; 6-Ile; 7-Pro; 8-Asx; 9-Thr; 10-Ser; 11-Met; 12-Glx; 13-Phe).

Furthermore, we doubled the sample size (20 mg) for analysis and obtained the same result, indicating that sample size was not a factor. For the detected proline, the $\delta^{15}N_{pro}$ values after treatment with 12 M and 6 M HCl for 24 h were 11.9 ± 0.8‰ and 10.2 ± 0.7‰, respectively, which was consistent with the average of 11.4 ± 2.8‰ for tuna across all treatments. This demonstrates that there was no difference between the different concentrations of HCl. However, the reason for detecting only proline was unclear, and further studies are needed in the future.

A slightly wide range of $\delta^{15}N_{Ala}$ values (ranging from 14.7‰ to 43.5‰) was observed in sharpbelly (12 M) across hydrolysis durations from 2 h to 24 h. However, no significant difference (two-way ANOVA, $p > 0.05$) was noted among the treatments, except at 2 h and 24 h ($p = 0.05$) (Fig. 3). Comparatively, the $\delta^{15}N_{Asp}$ values of sharpbelly (6 M) exhibited considerable fluctuations across different hydrolysis times when compared with those of Aglae and the reference material (Tuna), ranging from 16.3‰ to 43.3‰. Nevertheless, no clear pattern trend was discerned. The variations observed in the samples during pretreatment may be attributed to their heterogeneity. However, minimal alterations were noted across all samples for $\delta^{15}N_{Glx}$ and $\delta^{15}N_{Phe}$, regardless of varying HCl concentrations and hydrolysis times. This was particularly evident in the case of Algae (6 M) and Tuna (12 M), where Glx ranged from 10.7‰ to 12.4‰ and 21.0‰–23.5‰, and Phe ranged from 6.8‰ to

Table 1
Percentage recovery and isotopic composition of nitrogen ($\delta^{15}N$‰) in the AA standard solution.

GC-C-IRMS					
Amino Acid	Recovery	Before resin (n = 3)		After resin (n = 3)	
		$\delta^{15}N$	σ	$\delta^{15}N$	σ
Alanine (Ala)	75.9%	0.88	0.08	2.25	0.68
Glycine (Gly)	111.8%	3.67	0.25	3.11	0.64
Valine (Val)	75.1%	1.39	0.32	1.14	0.18
Leucine (Leu)	83.7%	−2.63	0.22	−2.93	0.80
Isoleucine (Ile)	78.1%	−0.06	0.01	−0.11	0.83
Proline (Pro)	75.2%	−1.13	0.53	−1.95	0.40
Aspartic acid + asparagine (Asx)	110.8%	−2.42	0.04	−2.37	1.08
Threonine (Thr)	103.3%	2.19	1.25	3.94	1.27
Serine (Ser)	110.3%	−2.35	1.08	−1.29	0.42
Methionine (Met)	102.1%	0.32	0.36	−0.25	0.15
Glutamic acid + glutamine (Glx)	106.3%	−6.51	0.11	−6.08	0.45
Phenylalanine (Phe)	107.4%	4.32	0.11	3.16	0.22

10.3‰ and 4.3‰–5.4‰, respectively, as illustrated in Fig. 3.

Interestingly, the $\delta^{15}N_{Glx}$ value of Tuna was 24.1 ± 1.1‰, which was the same as the $\delta^{15}N_{Glx}$ values observed in little egret (25.9 ± 3.4‰) and night heron (23.1 ± 3.2‰). In contrast, the $\delta^{15}N_{Phe}$ values of Tuna (6.5 ± 1.2‰) were much lower than those of little egret (12.7 ± 2.3‰) and night heron (11.5 ± 2.9‰), probably due to differences in their food and habitat. This finding verifies that $\delta^{15}N_{Phe}$ is an efficient indicator for distinguishing food sources (Zhang et al., 2021).

3.4. Effects of different hydrolysis conditions on TPs

Trophic positions (TPs) calculated according to equation (1) for sharpbelly, Algae and Tuna and equation (2) for little egret and night heron showed no differences among all samples at different HCl concentrations and hydrolysis times (Fig. 4). The TPs for the little egret treated with 12 M and 6 M HCl were 3.0 ± 0.1 and 3.0 ± 0.1 (n = 4), respectively, while for the night heron treated with 12 M and 6 M HCl, they were 2.7 ± 0.1 and 2.8 ± 0.2 (n = 4), respectively. Since both little egret and night heron primarily feed on small fish and shrimp, the estimated TPs matched the expected positions in their ecological environment. sharpbelly, which mainly feeds on phytoplankton, exhibited TPs of 2.0 ± 0.2 (12 M) and 2.2 ± 0.2 (6 M) (n = 4). The TPs of tuna treated with 12 M and 6 M HCl were 2.9 ± 0.1 (n = 3), which is consistent with the average value of 2.9 ± 0.1 reported by Hetherington et al. (2016) and significantly lower than the TP values estimated from previous studies based on BSIA (4.7 ± 0.1) (Olson et al., 2010) and (4.1 ± 0.3) (Popp et al., 2007). The TPs of the primary algae produced by the different HCl concentrations were 1.3 ± 0.1 (12 M) and 1.0 ± 0.1 (n = 4) (6 M), respectively.

It has been proven that in a single pelagic ecosystem, the TDF values of several groups decrease with increasing TPs (Hetherington et al., 2016). MacKenzie et al. (2014) reported that the $TDF_{Glx\text{-}Phe}$, calculated from the weighted mean of trophic and source AAs (5.0 ± 1.5‰) from a long-term laboratory experiment, is less than previously published values (6.4 ± 0.3‰), which is also lower than the $TDF_{Glx\text{-}Phe}$ (6.6 ± 1.7‰) from a meta-analysis of 359 marine samples (Nielsen et al., 2015). The results of the present study suggested that a $TDF_{Glx\text{-}Phe}$ concentration of 7.6‰ is not suitable for Tuna, and further experiments are needed to explore $TDF_{Glx\text{-}Phe}$ variation among different taxa.

The TPs of night heron hens at 2 h (2.8 ± 0.1), 6 h (2.8 ± 0.2), and 24 h (2.8 ± 0.1) were comparable to those at 12 h (2.7 ± 0.1). The TPs of the little egret at 6 h (3.1 ± 0.1) and 24 h (3.1 ± 0.1) were slightly greater than those at 12 h (3.0 ± 0.1) and 2 h (2.9 ± 0.1), but no significant differences were detected among the groups ($p > 0.05$). The TPs of sharpbelly plants remained consistently stable across all treatments (2 h, 6 h, 12 h, and 24 h), with values of 2.0 ± 0.1, 2.2 ± 0.2, 2.1 ± 0.2, and 2.2 ± 0.1, respectively. Similarly, the differences in the TPs in the algae at different hydrolysis times were not significant, and the values of the TPs were 1.3 ± 0.2, 1.2 ± 0.2, 1.2 ± 0.1, and 1.1 ± 0.2, respectively. The results suggest that the TPs for all samples after 2 h of hydrolysis were comparable to those achieved using the conventional 24 h hydrolysis time.

Although a significant difference was observed in the $\delta^{15}N_{AA}$ values between 2 h and 12 h in sharpbelly (12 M), as mentioned in Section 3.3,

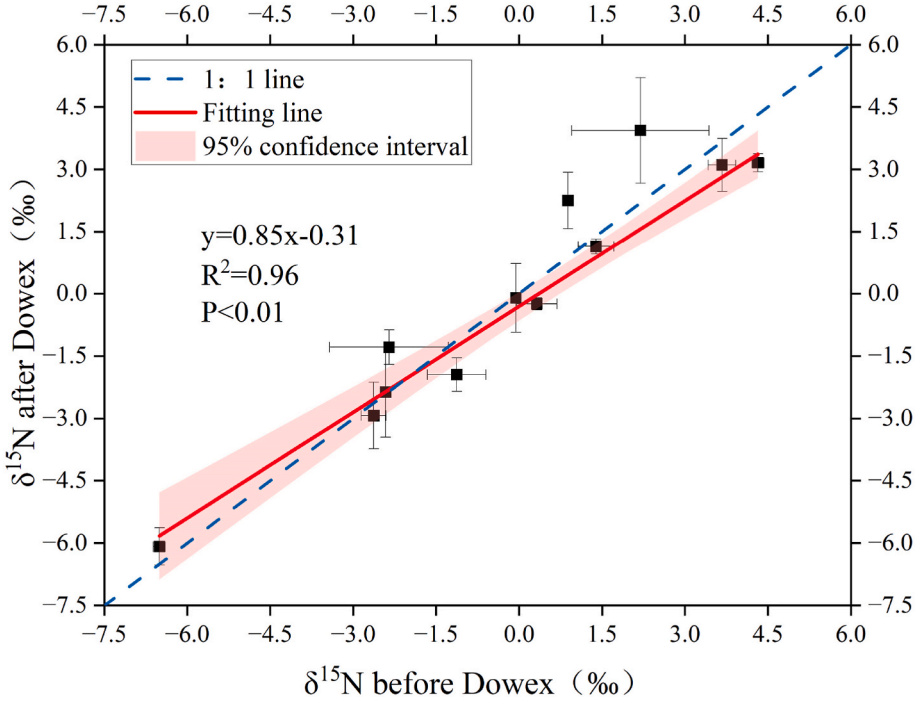

Fig. 2. Correlations between the $\delta^{15}N_{AA}$ values of a standard solution of 12 AAs determined by GC–C-IRMS before and after Dowex cation-exchange resin treatment. Error bars represent the standard deviation of three measurements.

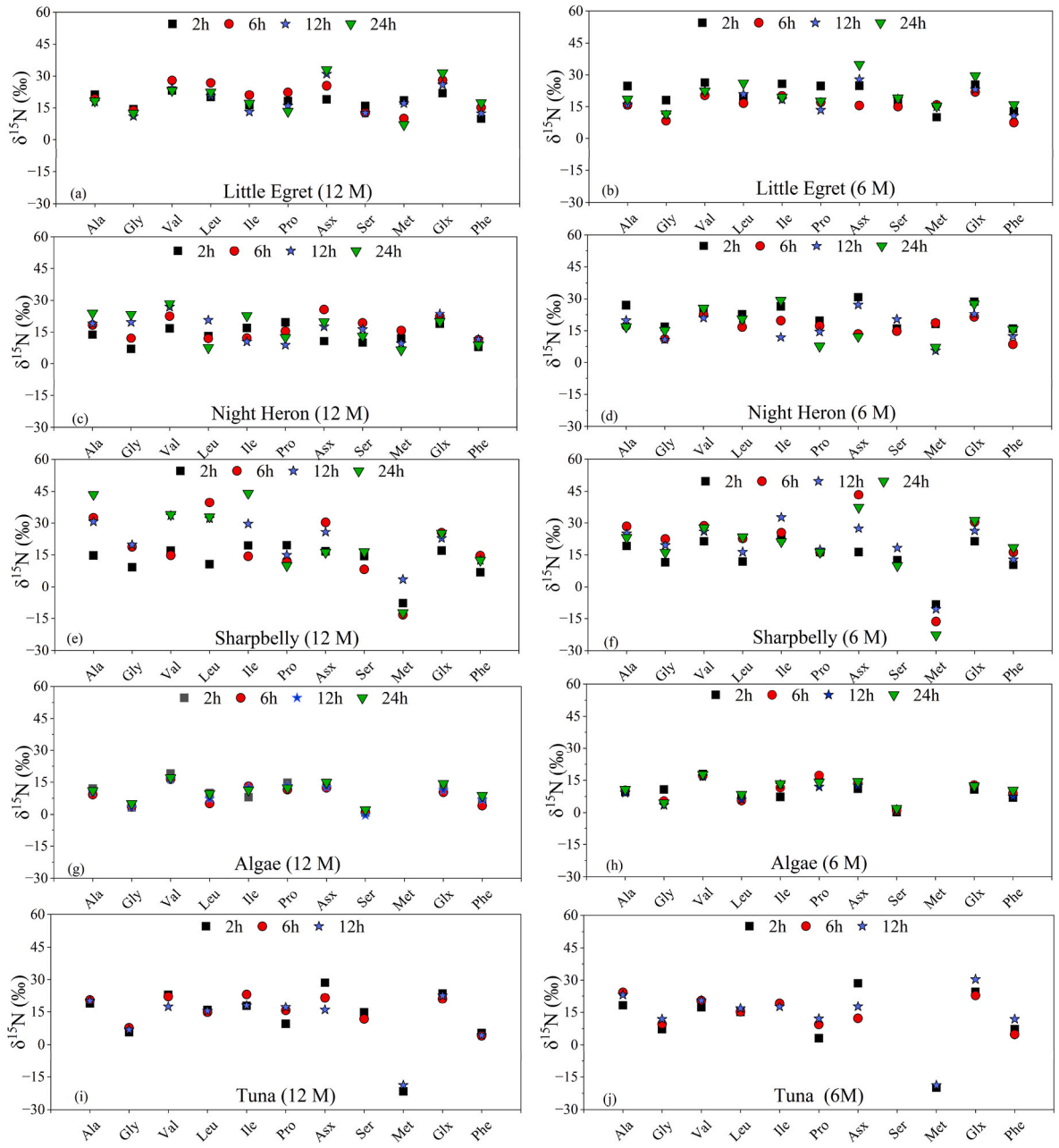

Fig. 3. $\delta^{15}N_{AA}$ values of biological samples and reference material (Tuna) treated with different HCl concentrations and hydrolysis times (the $\delta^{15}N_{AA}$ values of amino acids for Tuna at 24 h were not detected, except for proline).

no difference was detected in the TPs among the treatments ($p = 0.33$). Since our sharpbelly was a mixed sample and many studies indicate that there are differences between individuals (Bloomfield, 2011; Bradley et al., 2016), we hypothesize that the observed differences in sharpbelly might be caused by uneven sample mixing. As with the $\delta^{15}N_{AA}$ values, the TPs of Algae and the reference material (Tuna) changed very little, ranging from 1.1 to 1.3 and 2.7 to 3.0, indicating that the determination of TPs at 2 h and with 12 M or 6 M HCl is efficient and stable. Since HCl (12 M) is more volatile and easier to remove during acid hydrolysis, the use of HCl (12 M) for acid hydrolysis will shorten the pretreatment time. However, we recommend using 6 M HCl for hydrolysis treatment because it is less harmful to the human body than 12 M HCl.

Contrary to the expectation that different hydrolysis conditions would influence $\delta^{15}N_{AA}$ values and trophic positions (TPs), no significant difference ($p > 0.05$) was observed due to varying HCl concentrations and hydrolysis times. According to the analytical data, the optimal conditions for the analysis of compound-specific $\delta^{15}N_{AA}$ and the computation of trophic positions using NPIP ester were an HCl concentration of 6 M and a hydrolysis time of 2 h.

4. Conclusions

In this study, four hydrolysis periods (2 h, 6 h, 12 h, and 24 h) and two HCl concentrations (6 M, 12 M) were evaluated as hydrolysis conditions for the compound-specific isotope analysis of nitrogen in amino acids (CSIA-AA, $\delta^{15}N_{AA}$) in biota samples. No nitrogen isotope fractionation was observed when Dowex cation exchange resin was used to purify amino acids. There were no significant differences ($p > 0.05$) in the $\delta^{15}N_{AA}$ values for most of the AAs among the samples, except for a sharp difference, regardless of the HCl concentration or hydrolysis time. Trophic position (TP) values calculated with measured $\delta^{15}N_{AA}$ for all sample species also showed no significant differences ($p > 0.05$) with

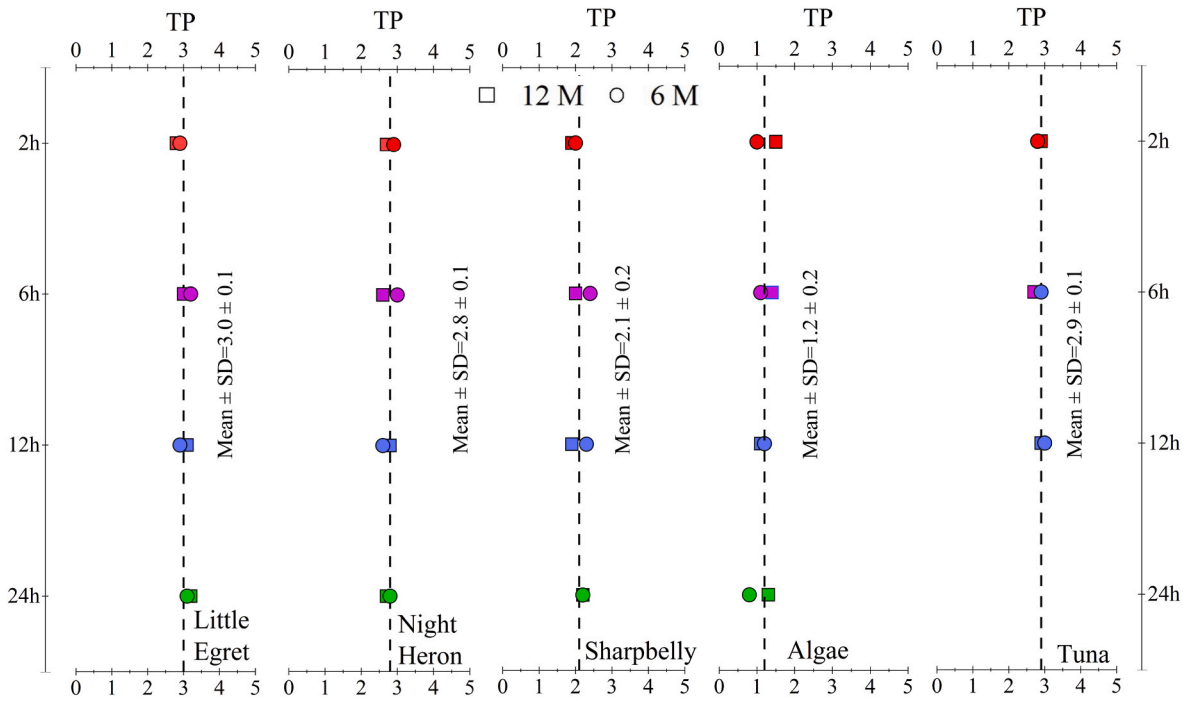

Fig. 4. TPs of biological samples and reference material (Tuna) treated with different HCl concentrations and hydrolysis times.

different HCl concentrations or hydrolysis times. Overall, contrary to our expectations, different hydrolysis conditions did not affect the $\delta^{15}N_{AA}$ or TP values of different organisms. The acid hydrolysis conditions (2 h, 6 M) allow faster, highly convenient, and efficient analysis of $\delta^{15}N_{AA}$ in biological samples, and the TPs of organisms in certain ecological food webs can be effectively calculated.

CRediT authorship contribution statement

Dawei Wang: Writing – original draft, Investigation, Data curation, Conceptualization. **Gaoen Wu:** Methodology, Investigation, Conceptualization. **Jing Tian:** Software, Methodology, Data curation. **Chan Li:** Resources. **Jiemin Liu:** Supervision, Funding acquisition. **Longchao Liang:** Methodology, Investigation, Funding acquisition. **Guangle Qiu:** Writing – review & editing, Supervision, Methodology, Funding acquisition.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2024.119223.

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