Journal of Chromatography B, 1033–1034 (2016) 382–389

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

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

Zhongyi Zhang[a,b], Jing Tian[a,b], Hongwei Xiao[c], Nengjian Zheng[a,b], Xiaofei Gao[b,d], Renguo Zhu[a,c], Huayun Xiao[a,*]

[a] *State Key Laboratory of Environmental Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences, Guiyang 550081, China*
[b] *University of Chinese Academy of Sciences, Beijing 100039, China*
[c] *Laboratory of Atmospheric Environment, Key Laboratory of Nuclear Resource and Environment (Ministry of Education), East China University of Technology, Nanchang330013, China*
[d] *Aquatic Ecohealth Group, Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China*

ARTICLE INFO

Article history:
Received 23 July 2016
Received in revised form 30 August 2016
Accepted 2 September 2016
Available online 4 September 2016

Keywords:
GC-C-IRMS
Amino acid
Trophic level
NPIP ester

ABSTRACT

The signatures of natural stable nitrogen isotopic composition ($\delta^{15}N$) of individual amino acid (AA) have been confirmed to be a potentially effective tool for elucidating nitrogen cycling and trophic position of various organisms in food webs. In the present study, a two-stage derivatisation approach of esterification followed by acylation was evaluated. The biological samples underwent acid hydrolysis and the released individual AA was derivatived into corresponding *N*-pivaloyl-isopropyl (NPIP) esters for nitrogen isotopic analysis in gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Usually, 13 individual AA derivatives were separated with fine baseline resolution based on a nonpolar gas chromatography column (DB-5 ms). The minimum sample amount required under the presented conditions is larger than 20 ngN on column in order to accurately determine the $\delta^{15}N$ values. The $\delta^{15}N$ values determined by GC-C-IRMS with a precision of better than 1‰, were within 1‰ after empirical correction compared to the corresponding measured by element analysis (EA)-IRMS. Bland-Altman plot showed highly consistency of the $\delta^{15}N$ values determined by the two measurement techniques. Cation-exchange chromatography was applied to remove interfering fraction from the extracts of plant and animal samples and without nitrogen isotope fractionation during the treatment procedure. Moreover, this approach was carried out to estimate the trophic level of various natural organisms in a natural lake environment. Results highly proved that the trophic level estimated via the presented AA method well reflected the actual food web structure in natural environments.

1. Introduction

Carbon and nitrogen are essential elemental constituents of organisms, forming a variety of biological and chemical species on Earth [1]. Accordingly, the isotopic compositions of the two elements may provide considerable process-related and source information [2]. Stable carbon and nitrogen isotopes (expressed as $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios, respectively) analysis of bulk organic materials has been conventionally employed in a number of ecological/paleoecological and environmental studies on the bases biochemical and physiological processes involved the formation and cleavage of the C—N bond. Various applications have extended from biogeochemistry to modern and fossil food web structures analysis, food and fragrance analysis, detection of doping in sports, to assessing the organic pollutants in the environment, authenticity testing of organic products, as well as assessing the dietary preferences of organisms [3–6]. However, bulk method lacks specificity, since whole tissue analysis may encompasses any molecule containing C or N into the stable isotopic ratios, each of these molecules having a different origin and metabolism [1,7,8]. Additionally, natural abundance carbon and nitrogen isotope values of bulk biological material reflect a weighted mean effect of a widely range

* Corresponding author.
E-mail address: huayunxiao@outlook.com (H. Xiao).

http://dx.doi.org/10.1016/j.jchromb.2016.09.004
1570-0232/

of biochemical and environmental processes, which are difficult to disentangle from one another [7,9]. Fortunately, compound-specific stable isotope analysis (CSIA) of the interested compounds is capable of yielding considerably valuable information from a given sample [10,11]. More particularly, CSIA of amino acid (AA) is promising since AAs are constitutes of proteins accounted for a significant portion of organismal biomass and exhibit a different degree of $\delta^{13}C$ and $\delta^{15}N$ values which may be due to a number of metabolic pathways involved, subsequent biochemical uses or the environmental factors [7,12–14]. Much of this variation has been widely exploited and proven ecologically meaningful, especially in a food web context of contemporary ecological studies of the diet or palaeodietary analysis [5,15]. Several recent studies have confirmed that the nitrogen isotopic signature of specific amino acids, especially glutamate and phenylalanine is a useful tool for estimating the trophic levels of various organisms. Recently, it has been proposed that the trophic level of various organisms in natural food webs can be precisely calculated by comparing the large and small enrichment values in glutamate (+8.0‰) and phenylalanine (+0.4‰), respectively, for each trophic level [15–17]. It is suggested that the internal difference of the isotopic fractionations between the two amino acids in individual organisms is due to the metabolic pathways. Glutamate rapidly undergoes transamination and the C—N bond is cleaved during metabolism, leading to a large enrichment in ^{15}N. In contrast, the addition of a hydroxyl group to form tyrosine is assumed to be dominant metabolic step of phenylalanine and this does not involve the cleavage of the C—N bond, leading to a small enrichment in ^{15}N [16,18].

Compared with liquid chromatography (LC)-IRMS, GC-C-IRMS is considered to be more appropriate to the measurement of individual AA $\delta^{15}N$ values. It allows determination of nitrogen isotopic composition of AA in natural abundance or slightly enriched biological samples with high precision, sensitivity and accuracy [19,20]. Recent, novel high-temperature combustion (HTC) interface linked to HPLC system for CSIA of both carbon and nitrogen is designed with better precision and trueness. The author suggested this highly efficient system may open up new possibility in CSIA-based research fields [20]. However, to our knowledge both the two commercial interfaces, LC-Isolink and LiquiFace do not allow the measurement of $\delta^{15}N$ values [21,22]. Hence, in this study the CSIA of AA $\delta^{15}N$ values is carried out on an available GC-C-IRMS system.

On the other hand, GC-C-IRMS analysis of AAs, as well as other polar compounds requires derivatization to enhance their volatility prior to GC separation until the carboxyl, amino and certain polar side-chain groups have been chemically modified with a non-polar moiety [23–25]. The goal of derivatization is to make AA more volatile and less reactive, thereby improving its chromatographic behavior. The most widely used derivatisation approaches for AA in GC/C/IRMS analysis involve silylation, esterification with subsequent trifluoroacetylation, acylation and pivaloylation [10,16,23–26]. These derivatization techniques have different advantages and disadvantages concerning individual AAs, the reaction yield and stability, their GC performance and the potentially formation of multiple reaction products. Of all the derivatisation techniques, the silylation reaction offers numerous advantages compared with others. A popular derivatization approach of silylation is to form the *t*-butyl dimethylsilyl (TBDMS) derivatives via *N*-Methyl-*N*-(*t*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA), since TBDMS groups can be attached to almost all functional groups [23,26,27]. In particular, derivatization via silylation is the only single-step reaction which can be performed quickly, simply and no additional remove of reaction reagents is needed. Besides, silylation allows the simultaneous recording of glutamate and aspartate as well as the corresponding amides, glutamine and asparagine [23,26,28]. However, the combustion of TBDMS derivatives in GC-C-IRMS can cause silicon-containing deposits in the furnace due to the formation of SiO_2. The deposition of SiO_2 would decrease the efficiency of the oxidation reactor significantly, resulting in the incomplete sample combustion, leading to a loss of sensitivity and may damage the capillary columns. Furthermore, silylation via MTBSTFA adds excessive amount of carbon atoms, at least twelve thus decreasing the precision of carbon isotope determination [16,23–26]. Besides, the presence of moisture would result in poor reaction yield since MTBSTFA is very sensitive to moisture, thus thorough water removal is necessary.

Compared with MTBSTFA, esterification followed by acylation techniques result in the converting of glutamine and asparagine into the corresponding acids, respectively, owing to the heavily acidic condition applied in the esterification step [5,24,25,29]. However, deamination also occurs under the environment adopted in the hydrolysis of proteins to release individual AAs; hence, asparagine and glutamine cannot be measured regardless of the esterification procedure. Regarding the two step reaction of esterification with subsequent trifluoroacetylation, known as *N*-trifluoroacetyl i-propyl esterification (TFA-IP esters), it is the most widely employed for GC analysis, probably due to the prominent chromatographic performance on most commonly used GC column and shorter retention times compared with the other two approaches [23,24]. However, it has been reported that fluorine in derivatives reacts with oxidation reagents (e.g. CuO, NiO) to form very stable CuF_2 and NiF_2 products and poisons the oxidation reactor catalyst, that leads to a reduction of oxidation efficiency and shorted the life of an oxidation reactors using GC-C-IRMS [10,23–25]. Moreover, fluorine in derivatives during combustion could damage the capillaries of the machines. Another derivatization approach, *N*-acetyl-*n*-propyl (NANP) esters(esterification with subsequent acylation) regards as an alternatively non-fluorinated analogue to TFA-IP esters, thus the damage caused by the combustion in term of catalyst poisons can be avoided; however, the baseline resolution of AA NANP derivatives is questionable, and at most 10 AAs can be characterized as reported [23,24]. Therefore, esterification with subsequent pivaloylation, known as *N*-pivaloyl-*iso*-propyl (NPIP) esters was developed in GC-C-IRMS analysis to enhance better GC resolution with the enlargement of the groups introduced [5]. The chromatographic baseline resolution (i.e. separation of each peak of AA derivative on the GC chromatogram) is a prerequisite for the reliability of $\delta^{13}C$ and $\delta^{15}N$ data of AA and the separation also could be different among derivatization techniques as well.

Taken together, the present study described the methodology that has been introduced to determine the individual AA $\delta^{15}N$ values from archaeological bone collagen, animal muscle tissue or plant material by GC-C-IRMS [5,15,30,31]. Plant material and freshwater fish muscle tissue were hydrolyzed into individual AAs, purified with Dowex cation-exchange resin and derivatised into NPIP esters, and then the $\delta^{15}N$ values were determined in GC-C-IRMS. Besides, the standard mixtures of known $\delta^{15}N$ values AA were also purified through cation-exchange resin and derivatised to their NPIP esters to test and verify any nitrogen isotopic fractionation associated with the effect of cation-exchange chromatography and derivatisation. Results shown that this method, involving acid hydrolysis, purification via cation-exchange chromatography and the derivatisation choice, does not cause significant nitrogen isotopic fractionation and thus can be applied to determine accurately the individual AA $\delta^{15}N$ values in muscle tissue and plant material in a natural ecosystem. Finally, a brief case study on trophic level in natural food webs was conducted to evaluate the approach applied to a real aquatic ecosystem with clear priori expectations. The trophic level was estimated by the following equations: $TL_{Glu/Phe} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 3.4)/7.6 + 1$, based on well-founded empirical principles [5,15,16,32,33].

2. Experimental

2.1. Standards and reagents

AA standard mixture was dissolved in 0.1 M HCl by the addition of 13 AAs with known $\delta^{13}C$ and $\delta^{15}N$ values, including alanine, glycine, valine, leucine, isoleucine, proline, aspartate, serine, threonine, glutamate, methionine, phenylalanine and γ-aminobutyric acid (Sigma-Aldrich, St. Louis, MO, USA). Two internal standard (norleucine and α-aminobutyric acid, Sigma-Aldrich) was added to the standard mix to enable quantification. Cation-exchange resin (Dowex 50W X8 H^+, 200–400 mesh, Sigma-Aldrich) was applied to purify the samples.

The solvents and reagents for acid hydrolysis and derivatization included ACS-grade 12.1 M HCl, HPLC-grade methanol, *n*-hexane, dichloromethane, thionyl chloride, 2-propanol, pivaloyl chloride were purchased from Aladdin (Shanghai, China) as well as anhydrous magnesium sulfate ($MgSO_4$).

2.2. Sample preparation

To obtain individual AAs, approximately 10–15 mg of well ground, homogenized samples were hydrolyzed in reactor vials with 0.5 mL of 12.1 M HCl. The vials were blown with a gentle N_2 for 1 min to remove air, then placed in an oven at 110 °C for 12 h. After hydrolysis, the samples were cooled down to room temperature. HCl was evaporated under a gentle stream of N_2. Then the hydrolysate was redissolved in 0.1 M HCl and washed with *n*-hexane/dichloromethane (3:2, v/v) three times to remove hydrophobic constituents (e.g. lipids). The solution was stored at −20 °C until required for analysis. Internal standards solution (2 mg mL^{-1} in 0.1 M HCl) was added to each sample prior to hydrolysis.

2.3. Purification of AAs with dowex cation-exchange chromatography

Cation-exchange resin (Dowex 50W X8, 200–400 mesh, H^+) was prepared to separate the AA fraction from other components such as starch, sugar, organic acid. The volume of resin demanded depends upon the sample size processed given the capacity of cation-exchange is 1.7 meq mL^{-1}. Resin was activated by washing successively (3–6 bed volumes) with 2 M HCl, ultrapure water, 2 M NaOH and finally with H_2O to pH = 7. Additionally, the resin was re-activated with 3 bed volumes of 1 M HCl to ensure that all the exchange sites were occupied by H^+ and then flushed with 3 bed volumes of H_2O before sample application. Finally, the AA fraction was eluted with 6 bed volumes of 10 wt% NH_3 aqueous solutions and completely dried in preparation for the subsequent derivatization. In addition, the potential background compounds derived from the cation-exchange resin was also examined by appropriate analysis of the blank procedures.

2.4. Preparation of amino acid derivatives (NPIP esters)

The following derivatisation procedure was according to Corr et al. [24], as shown in Fig 1. AAs were first esterified with isopropanol in 1 mL of acidified isopropanol solution at 110 °C for 2 h (addition 1 mL of thionyl chloride to 4 mL isopropanol. Since this mixing was extremely exothermic, we suggested that add thionyl chloride dropwise into isopropanol in an ice bath). The esterification was terminated by placing the reaction vials in a freezer. Dichloromethane (DCM) was added (2 × 0.25 mL) and dried up again to remove the derivative reagent completely. AA *iso*-propyl esters were then pivaloylated with 1 mL of a mixture of pivaloyl chloride and dichloromethane (1/4, v/v) at 110 °C for 2 h. Similarly, the reaction was terminated and reagents were evaporated under a gentle stream of N_2. To remove the derivative reagents completely, DCM was added (2 × 0.25 mL) and dried up again under a gentle stream of N_2. The dried NPIP derivatives of amino acids were redissolved in ~0.5 mL of *n*-hexane/dichloromethane (3/2, v/v) and 0.2 mL of distilled water and mixed vigorously. After phase separation completely, the top/organic layer containing the AA derivatives, was collected and this process was repeated three times. The total organic phase was pipetted onto a magnesium sulfate column (about 1.5 cm of $MgSO_4$ in a Pasteur pipette) to remove any residual moisture. The filtrate solvents were dried up under a gentle stream of N_2 and then diluted in dried (1% MgSO4), sonicated, DCM and stored at −20 °C until analysis.

2.5. Instrumental analysis

The abundance of each AA in the standard or the samples was determined using gas chromatography with flame ionization detection(GC-FID), calculated against the internal standard norleucine only.

A Thermo Scientific MAT253 system (EI, 70Ev. Three Faraday cup collectors for *m*/*z* 28, 29 and 30. Bremen, Germany) interfaced with a Trace GC Ultra (GC IsoLink + ConFlo IV, Thermo Scientific, Bremen, Germany) was used to determine the AA $\delta^{15}N$ values. For $\delta^{15}N$ analysis, 1.0–1.5 μL of each sample was introduced using an autosampler (Triplus, Thermo Scientific, Bremen, Germany) held at 250 °C in splitless mode. The GC oven temperature program was started at 40 °C for 2.5 min, heating at 15 °C min^{-1} to 110 °C, then at 3 °C min^{-1} to 150 °C and finally ramping at 6 °C min^{-1} to 230 °C and held for 17.5 min. Chromatography separation was accomplished based on a DB-5 ms capillary column (30 m × 0.25 mm × 0.25 μm; J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas for separation at a flow rate of 1.4 mL min^{-1}. The system was "back flushed" with helium at the beginning for about 1000 s in order to prevent solvent into the combustion reactor (and ion source), thus extending the lifetime of the catalyst reactor. The separated individual AA was online combusted into N_2, CO_2 and H_2O within a new commercial NiO tube/CuO-NiO combustion furnace reactor maintained at 1030 °C which composed of a Ni-tube with an oxidized surface that displays a greater active surface area in comparison to the conventional model. No separate reduction reactor was needed for nitrogen isotope analysis since this new commercial reactor contained an oxidation and a reduction unit (the Ni/NiO surface withdraws oxygen from nitrogen oxides thereby acting like a reduction catalyst). Water vapor was removed by a water-removing trap, consisting of a Nafion membrane. During $\delta^{15}N$ analysis, a liquid nitrogen trap was necessary which was normally added after the oxidation reactor to remove CO_2 from the oxidized and reduced analytes. The nitrogen results were expressed as $\delta^{15}N$ values (‰), using atmospheric air as the international standard for nitrogen, which has a $\delta^{15}N$ values 0‰: $\delta^{15}N = (Rsample/Rstandard-1)*1000(‰)$, where R is the $^{15}N/^{14}N$ ratio.

AA standard mixture whose $\delta^{15}N$ values were individually determined by EA-IRMS (Thermo Fisher Scientific, Bremen, Germany) was measured by GC-C-IRMS every four to six samples in order to monitor the measurement performance and to normalized the $\delta^{15}N$ values of AAs in samples. Norleucine was chosen as internal standard because it does not naturally exist and can be separated well with the adjacent peaks, and the isotopic value of norleucine was checked every sample. The analytical run was accepted when the drift in $\delta^{15}N$ values of norleucine between GC-C-IRMS and EA-IRMS was, at most ±1.0‰ for natural abundance or in slightly ^{15}N-enriched samples.

$\delta^{15}N$ values were reported relative to a laboratory reference tank of high-purity molecular nitrogen (99.998%), discharged into the IRMS at the beginning and end of each run for both standard

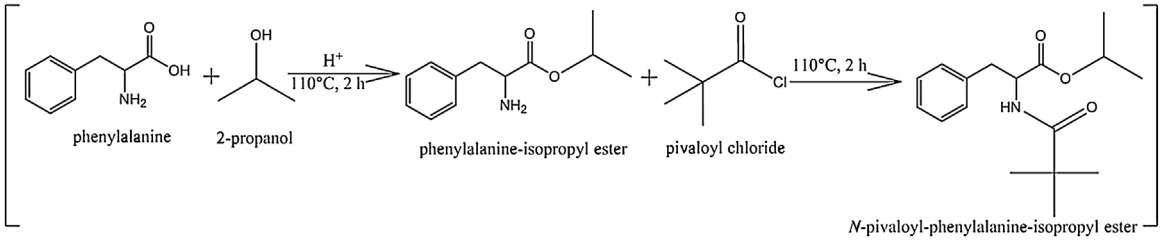

Fig. 1. The chemical derivatization pathways to produce NPIP amino acid derivatives, taking phenylalanine as an example.

mixtures and samples. All samples were measured in duplicate, and the isotope ratios were expressed in δ‰ versus atmospheric nitrogen (Air).

Data acquisition on the IRMS instrument was based on Isodata 3.0 (Thermo Fisher Scientific, Bremen, Germany). Integration time was 0.25 s. Slope sensitivity for peak start and stop definition were set to be 0.2 and 0.4 mV s^{-1}, respectively.

3. Results and discussion

3.1. Derivatisation procedure and precondition GC-C-IRMS system for δ^{15} analysis

AAs are not-volatile, polyfunctionalised compounds that must be converted into volatile derivatives prior to GC analysis until the active hydrogens on amino, carboxyl, and certain polar functional groups have been chemically modified[23,24]. Through the approach of esterification with subsequent pivaloylation, individual AA was successfully derivatived into the corresponding *N*-pivaloyl-*iso*-propyl (NPIP) ester and could be completely separated in GC-C-IRMS based on a DB-5 ms column in 62 min. The main advantage of this derivatisation method is the elimination of the need to introduce hetero atoms, especially for nitrogen isotopic analysis. Since no nitrogen atom presented in the derivatization reagents or been introduced by other solvents, corrections for nitrogen addition were not required. Furthermore, due to the enlargement of the alkyl groups introduced (pivaloyl compared with acetyl groups), improved chromatographic behavior is possible. It is important that baseline resolution is a prerequisite to obtain reliable isotopic values in GC-C-IRMS analysis [10,34].

For isotopic analysis, the surface activity of the GC capillary column and the combustion reactor is an essential in GC-C-IRMS. The deactivation of the system, especially the combustion reactor will lead to a loss of sensitivity during the analysis. AA δ^{15}N values tended to be enriched, the retention times drifted and the signal intensity decreased significantly. Thus, it is necessary that the combustion reactor furnace of the GC-IsoLink employed in this study would be reoxydized and back-flushed sufficiently every 20–25 GC-runs. Usually, the signal intensities and retention times were retrieved on the following run; nevertheless a conditioning phase with an injection of AA standard mixture was necessarily to offset the depletion in δ^{15}N value resulted from the reoxidation event. Once the GC-C-IRMS is perfectly set up (i.e. focus, tested for N_2 leaks, ref gas stability, etc.), the sample sequences are measured as described above.

3.2. Chromatographic separation in GC-C-IRMS

Peaks separation in GC is dominated by the volatility of analytes and their interaction with the stationary phase. In this study, GC separation of AA derivatives was carried out with a normally nonpolar GC column characterized by the larger temperature capability in comparison to other polar GC stationary phases. Optimal baseline resolution between the peaks corresponding to the individual amino acid in the chromatogram can be achieved based on this specific GC capillary column (Fig. 2b,c). Baseline resolution of interested peaks is regarded as primary requirement supposed that precise and accurate isotopic values are to be obtained, particularly for the complex biological samples. The length of the alkyl groups introduced by the derivative (methyl, ethyl, propyl, or isobutyl) reaction significantly affect the chromatographic behaviors of AA derivatives in GC columns, and depending on the polarity of stationary phase, some AAs may coelute [35]. By this presented approach, with a nonpolar GC column and NPIP ester derivative choice, baseline separation was achieved for 13 individual AAs (Fig. 2b,c). Ala, Gly were eluted first, as these low molecular weight, non-polar AAs do not interact strongly with the stationary phase. These are followed by the higher molecular weight, neutral AAs (e.g. Val, Leu and Ile) and then lower molecular weight polar AAs. The higher molecular weight polar and aromatic AAs elute last as these are strongly retained by stationary phases (Fig. 2c). It should be noted that asparagine and glutamine, present in most of the samples, are hydrolyzed into aspartate and glutamate, respectively. The δ^{15}N values of Asx thus represent the information of the N of aspartate and the amino N of asparagine and the δ^{15}N values of Glx represent the N of glutamate and the amino N of glutamine, similarly. Tryptophan is destroyed during acid hydrolysis and was not detected. In addition, the sulfur-containing amino acids (methionine and cysteine) are partly oxidized even under anaerobic conditions as shown in Fig. 2b.

3.3. Precision and accuracy of isotopic measurement

To evaluate the precision and simply evaluate whether the adequacy of system design for natural abundance isotope ratio measurements, 6 replicate preparations of the AA standard mixtures with δ^{15}N values determined independently by EA-IRMS were derivatized and analyzed by GC-C-IRMS in triplicate. Results clearly shown that the GC-C-IRMS system was capable of separation, oxidation and reduction for AA nitrogen isotope analysis. The AA nitrogen isotope ratios with perfect precision were achieved. The precision of GC-C-IRMS approach for any amino acid was less than 1‰ (Table 1).

In order to assess the accuracy of the obtained isotopic values, the δ^{15}N values of AA standards measured by GC-C-IRMS with the corresponding which was independently measured by EA-IRMS were compared. Fig. 3 showed the close correlation between EA-IRMS and GC-C-IRMS results and the correlation coefficient high. The difference between the δ^{15}N values in EA-IRMS and GC-C-IRMS after empirical correction was lower than ±1‰. Moreover, Bland-Altman plot was applied to assess the consistency of the δ^{15}N values of the standard amino acids obtained by EA-IRMS and GC-C-IRMS, respectively. The Bland-Altman plot indicated a bias of 0.0‰ in the GC-C-IRMS measurement with the magnitude of the δ^{15}N values within the precision of this analysis approach, suggesting that the GC-C-IRMS approach was non-fractionating for compound-specific nitrogen isotope analysis and that it maintained accurate δ^{15}N measurement as off-line method, EA-IRMS (Fig. 4).

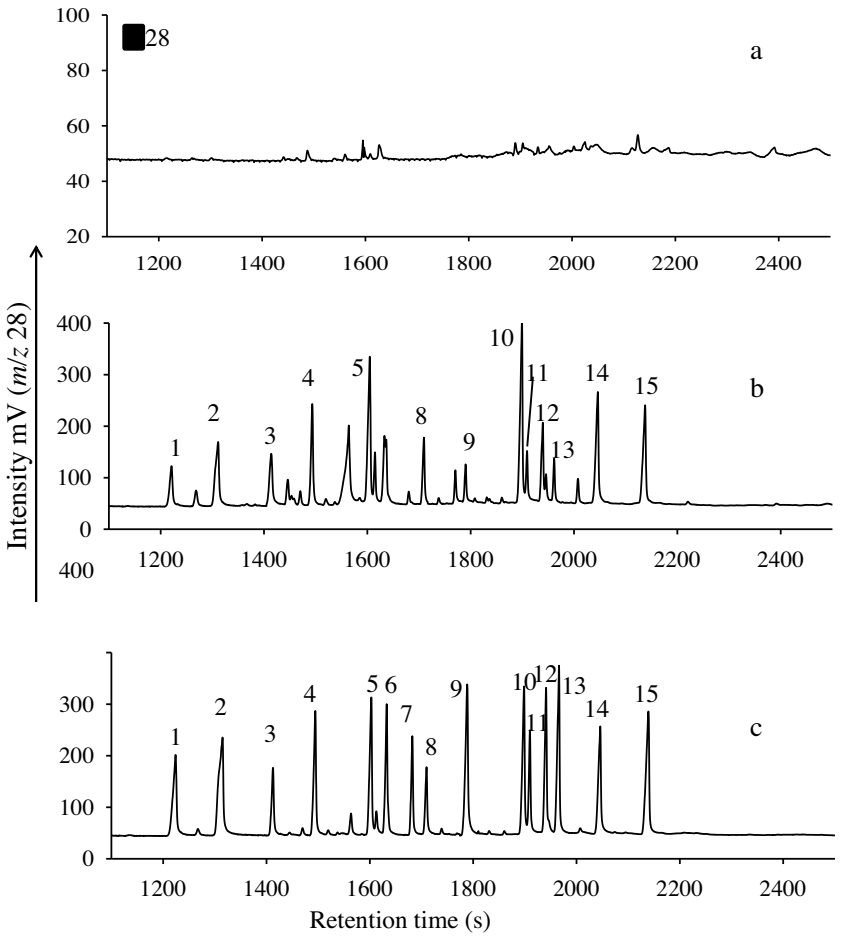

Fig. 2. GC-C-IRMS chromatogram for the analysis of amino acid NPIP ester derivatives on a DB-5 ms column. a: the blank procedure after passing through cation-exchange resin. b: muscle tissue sample of Pelteobagrus fulvidraco. c: amino acid standards mixture.
Only the signal of *m*/*z* 28 was presented. Peaks:1-Ala;2-Gly;3-I.S. (Aaba);4-Val;5-Leu;6-Ile;7-I.S. (Nle);8-GABA;9-Pro;10-Asx;11-Thr;12-Ser;13-Met;14-Glx; 15-Phe.

Table 1
Percentage recovery and the isotopic composition of N (δ^{15}N ‰) of standard amino acids.

Amino acid	GC-C-IRMS							EA-IRMS	
	After Dowex resin				Before Dowex resin				
	Recovery	σ	δ^{15}N	σ	δ^{15}N	σ	Δ*	δ^{15}N	Δ**
Alanine	102.9	1.1	−5.66	1.0	−6.29	0.8	−0.63	−6.63	0.31
Glycine	96.5	0.7	1.06	0.7	1.93	0.6	0.88	1.36	0.57
Valine	98.3	0.4	−0.08	0.4	0.01	0.7	0.09	−0.33	0.34
Leucine	95.5	1.4	1.68	0.8	2.40	0.7	0.72	2.10	0.30
Isoleucine	94.3	0.6	−2.66	0.6	−3.22	0.6	−0.56	−2.63	−0.59
Proline	101.4	0.7	3.11	0.7	3.08	0.6	−0.03	3.30	−0.22
Methionine	98.7	1.8	−1.02	0.8	−1.95	0.9	−0.94	−1.72	−0.23
Serine	100.9	0.9	2.23	0.9	1.69	0.7	−0.54	2.26	−0.57
Threonine	93.4	0.6	0.11	0.6	−0.03	0.8	−0.14	0.70	−0.73
Phenylalanine	92.1	0.2	2.54	0.2	2.64	0.5	0.10	2.28	0.36
Aspartic acid	96.4	0.8	−1.89	0.3	−2.15	0.9	−0.25	−2.32	0.17
Glutamic acid	98.4	2.5	−4.20	0.6	−3.89	0.7	0.31	−4.52	0.63

Δ*: difference in δ^{15}N values of amino acids standard mixture directly analyzed and passed through cation-exchange chromatography.
Δ**: difference in δ^{15}N values determined by EA/IRMS and GC/C/IRMS.

3.4. Sample amount requirements in CSIA for δ^{15} analysis

The absolute nitrogen amount required on column for interested compounds analysis in GC-C-IRMS is another important parameter that must be considered when optimizing the accuracy and precision of the determinations. Merritt and Hayes suggested that the determined δ^{15}N values correlated significantly with the sample amount on column [34]. Chikaraishi and Takano reported that the lower limit of accurate determination of the AA δ^{15}N values can be carried out with the amplitudes (*m*/*z* = 28) ≥100 mV, roughly equivalent to a nitrogen amount larger than 30 ngN on column [35]. Similarly, the study by Styring et al. with another derivatisation procedure (*N*-acetyl-isopropyl esters) also confirmed that highly reproducible of the δ^{15}N values were obtained within the range from 100 to 1200 mV intensity [25].

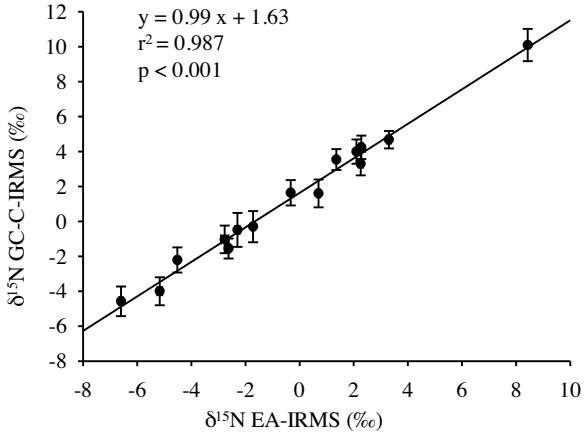

Fig. 3. The correlation between $\delta^{15}N$ values of a mixture of 15 amino acid standards determined by EA-IRMS and GC-C-IRMS. Error bars represent the SD of repeated measurements. Each point represents the average of 6 replicates for GC/C/IRMS and 3 replicates for EA/IRMS.

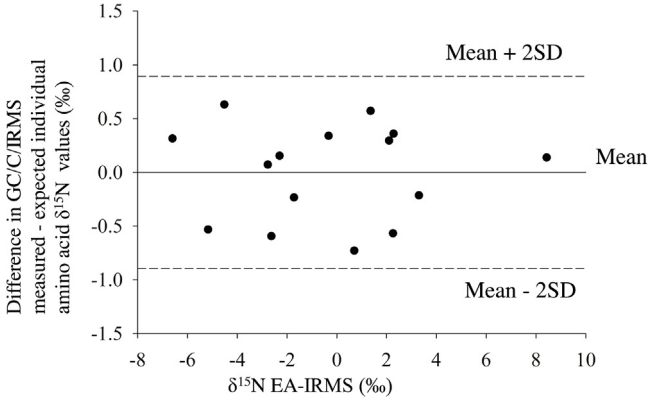

Fig. 4. Bland Altman plot comparing the expected individual amino acid $\delta^{15}N$ values (determined by EA-IRMS) with GC/C/IRMS measured $\delta^{15}N$ values. The solid line denoted the bias and the two dotted lines the 95% limits of the agreement. All GC/C/IRMS determinations are empirically corrected. The plot clearly shows the perfect agreement between the two methods.

The consistency of $\delta^{15}N$ values of standard AAs in the different range of sample amounts was also investigated in our study. Sample sizes of 0.3–4.5 nmol AA standards were derivatived and injected, and the correlations were presented in Fig. 5. Results highlight reproducibility of the $\delta^{15}N$ values when the sample amount is larger than 20 ngN on column and roughly corresponding to *m*/*z* 28 intensity of 200 mV. The difference in our results from the mentioned above may due to the difference in the IRMS instruments and the design of combustion reactors.

3.5. *Effect of dowex cation-exchange chromatography on individual AA δ^{15}values*

Sample extract pre-treatment with strong cation-exchange resin was regarded as an efficient way to purify amino acid fraction from other complex hydrophilic compounds, including sugars and organic acids, which consumed derivatization regents and also made damage to the combustion and reduction furnaces in the GC-C-IRMS system, as well as to desalt of inorganic compounds derived from calcareous and siliceous minerals [26,36,37]. In particular, for the compounds of interest which were not well-resolved from each other or from the impurities present in the sample, an artificial enrichment of the preceding peak in $\delta^{15}N$ and the depletion of the following compound during the separate integration of peaks

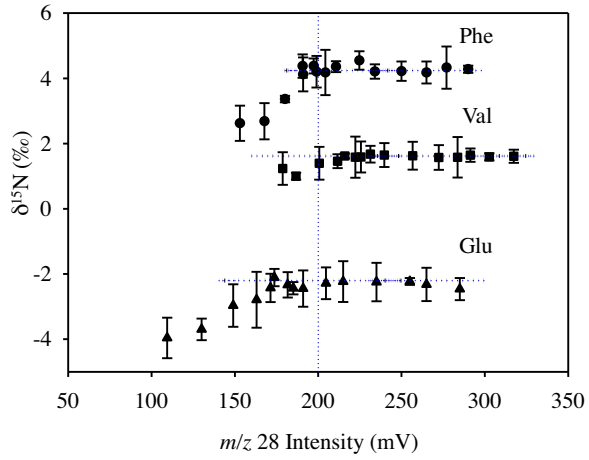

Fig. 5. The determined $\delta^{15}N$ values of NPIP ester derivatives of Glu, Val and Phe for various sample amounts by GC-C-IRMS. Dashed lines represent the $\delta^{15}N$ values of the three amino acids measured by EA-IRMS independently. Error bars represent the SD of repeated measurements.

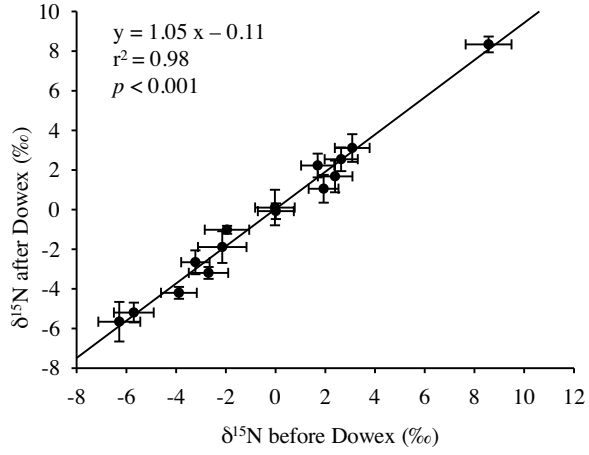

Fig. 6. The correlation between $\delta^{15}N$ values of a standard mixture of 15 amino acids determined by GC-C-IRMS before and after Dowex cation-exchange resin treatment. Error bars represent the SD of 3 repeated measurements.

areas would occur [38]. Therefore, it is necessary to eliminate these interferences in order to determine the $\delta^{15}N$ values with enough accuracy and precision.

Following passage through the cation-exchange resin, the AAs were derivatised to their NPIP esters. The recovery ratio of the AAs prior to and after cation-exchange resin was determined using GC-FID. On the basis that the yield of the two individual derivatisation reaction was 100%, average percentage recovery of the AAs after cation-exchange resin was 97.4%, calculated using the internal standard norleucine only (Table 1).

We also compared the precision and effectiveness of the pre-treatment procedure based on the $\delta^{15}N$ values obtained after cation-exchange resin, as shown in Fig. 6. Results showed the close correlation between the AA $\delta^{15}N$ values prior to and after Dowex cation-exchange resin. No statistical difference was observed between AA $\delta^{15}N$ values before and after the resin treatment, even when a 10% NH_3 aqueous solution was used to elute AA fractions from the resin.

To assess any possible background compounds derived from the Dowex cation-exchange resin, the pre-treatment procedure was applied to the analysis of the blank procedure and monitored using GC-C-IRMS. There was no significant background compound appeared in the chromatogram, as shown in Fig. 2a, thus we sug-

Table 2
Nitrogen isotopic composition of individual amino acid in freshwater organisms in the present study.

Sample	Diet devision	$\delta^{15}N$(‰)[b]									$TL_{Glu/Phe}$[a]
		Ala	Gly	Val	Leu	Pro	Ser	Met	Glu	Phe	
Spirogyra	Primary producers	0.8	1.3	7.0	5.6	4.9	−3.0	8.9	11.4	7.9	1.0
Hydrilla verticillata	Primary producers	3.6	14.1	18.0	12.6	3.7	2.9	−7.9	3.3	−1.3	1.2
Hypophthalmichthys Molitrix	Herbivores	21.7	12.3	24.7	25.2	23.2	14.4	11.9	23.9	12.2	2.1
Ctenopharyngodon idellus	Herbivores	15.4	7.6	15.9	15.2	14.5	7.0	3.8	19.7	9.8	1.9
Pelteobagrus fulvidraco	Carnivorous	31.1	13.0	32.7	30.9	29.6	17.3	15.7	32.4	14.2	2.9
Hypophthalmichthys nobilis,	Omnivores	23.1	11.2	24.8	23.7	24.8	15.1	9.3	24.2	11.2	2.3
Carassius auratus	Omnivores	22.3	9.4	26.7	24.3	22.8	11.8	10.8	23.8	9.8	2.4
Cyprinus carpio	Omnivores	14.9	5.6	15.9	15.8	17.8	6.4	2.9	20.4	7.2	2.3
Macrobranchium nipponense	Omnivores	31.8	18.5	31.4	25.7	28.4	19.3	17.7	32.9	16.5	2.7

[a] Trophic level calculated by the amino acid method using the following equation: $TL_{Glu/Phe} = (\delta^{15}N_{Glu}-\delta^{15}N_{Phe}-3.4)/7.6+1$.
[b] Mean values for the studied organisms.

gested that the pretreatment procedure with cation-exchange resin was highly effective for the elimination of certain interfering complex compounds from the extracts of biological samples.

3.6. Application of AA method to a simple case study: trophic level of freshwater organisms

A case study on the individual AA compound-specific nitrogen isotope was conducted to examine the application of this approach (hydrolysis, pre-treatment, derivatisation and GC-C-IRMS analysis of AA) for the determination of the trophic level by estimation of the $TL_{Glu/Phe}$ values of natural aquatic organisms, including aquatic plants, shrimp, fish and aquatic insects in freshwater environments. The nitrogen isotopic composition of individual AA and the resulted $TL_{Glu/Phe}$ values were summarized in Table 2.

Based on the obtained $TL_{Glu/Phe}$ values, we can effectively discriminate subsets of the communities within the freshwater ecosystem. Most food chains in freshwater start with primary producers (TL = 1, such as algae and plants), which are eaten by herbivores (TL = 2, strict plant-feeders) and omnivores (2 < TL < 3, both plant and animal feeders). The carnivores hunt herbivores and omnivores with TL > 3 (animal feeder). The represented primary procedures in this study were *Spirogyra* and *Hydrilla verticillata*, with $TL_{Glu/Phe}$ values being 1.0 ± 0.15 and 1.2 ± 0.25, respectively, as expected. The $TL_{Glu/Phe}$ values of Hypophthalmichthys Molitrix and Ctenopharyngodon idellus considered to be herbivores, range from 1.9 to 2.1. Pelteobagrus fulvidraco, known to be a ferocious carnivorous fish, registered a $TL_{Glu/Phe}$ values of 2.9 ± 0.19. Other fishes and shrimp appeared to be omnivores, including Hypophthalmichthys nobilis, Carassius auratus and Cyprinus carpio, with the $TL_{Glu/Phe}$ values ranging from 2.3 to 2.7. These obtained $TL_{Glu/Phe}$ values are considered to be corresponding to the trophic level of these omnivores and carnivores animals. Thus, we suggest that the $TL_{Glu/Phe}$ values resulted from the approach used in this study can accurately reflect the actual trophic level in natural food webs in freshwater environments, and may be able to resolve the trophic structure of complex food webs in terrestrial ecosystems.

4. Conclusion

The derivatisation of individual AA to their corresponding *N*-pivaloyl-*iso*-propyl (NPIP) esters allowed the accurate determination of $\delta^{15}N$ values of most AAs in muscle tissue and plant material. Results proved that the pretreatment with Dowex cation-exchange resin was a useful and reliable way to purify AA fraction. The treatment with Dowex cation-exchange resin had no significant effect upon the $\delta^{15}N$ values of AA standards; the differences prior to and following passage the resin were within 1‰. The amount of sample used in GC-C-IRMS was also optimized; the sample amount required in our study is not less than 20 ngN on column. It seems that the current approach is a suitable method for the measurement of individual AA $\delta^{15}N$ value of most biological samples by GC-C-IRMS.

The approach was used to estimate the trophic level by the $\delta^{15}N$ values of two AAs (glutamate and phenylalanine) from a single organism. According to the obtained $TL_{Glu/Phe}$ values, it can be effectively elucidating the trophic level of organisms in certain ecological food webs.

Conflict of interest

No conflict exists. All the authors Huayun Xiao, Zhongyi Zhang, Nengjian Zheng, Jing Tian, Hongwei Xiao, Renguo Zhu and Xiaofei Gao declare that they have no conflict of interest.

Acknowledgements

Funding: This study was kindly supported by the National Key Research and Development Program of China through grant 2016YFA0601000 (H.Y. Xiao) and National Natural Science Foundation of China through grants 41425014, 41273027 and 41173027 (H.Y. Xiao), and by the National Basic Research Program of China through grants 2013CB956703 (H.Y. Xiao).

References

[1] N. Ohkouchi, N.O. Ogawa, Y. Chikaraishi, H. Tanaka, E. Wada, Biochemical and physiological bases for the use of carbon and nitrogen isotopes in environmental and ecological studies, Prog. Earth Planet. Sci. 2 (2015).
[2] B. Fry, Stable Isotope Ecology, Springer Science & Business Media, 2007.
[3] G. Tcherkez, S. Nogues, J. Bleton, G. Cornic, F. Badeck, J. Ghashghaie, Metabolic origin of carbon isotope composition of leaf dark-respired CO2 in French bean, Plant Physiol. 131 (2003) 237–244.
[4] F. Baptist, G. Tcherkez, S. Aubert, J.Y. Pontailler, P. Choler, S. Nogues, 13C and 15N allocations of two alpine species from early and late snowmelt locations reflect their different growth strategies, J. Exp. Bot. 60 (2009) 2725–2735.
[5] Y. Chikaraishi, Y. Kashiyama, N.O. Ogawa, H. Kitazato, N. Ohkouchi, 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.
[6] F. Hyodo, S. Kusaka, D.A. Wardle, M.-C. Nilsson, Changes in stable nitrogen and carbon isotope ratios of plants and soil across a boreal forest fire chronosequence, Plant Soil 364 (2012) 315–323.
[7] Roland A. Wernera, H.-L. Schmidt, The in vivo nitrogen isotope discrimination among organic plant compounds, Phytochemistry 61 (2002) 465–484.
[8] M. Paolini, L. Ziller, K.H. Laursen, S. Husted, F. Camin, Compound-specific δ15N and δ13C analyses of amino acids for potential discrimination between organically and conventionally grown wheat, J. Agric. Food Chem. 63 (2015) 5841–5850.
[9] A.K. Styring, R.A. Fraser, A. Bogaard, R.P. Evershed, Cereal grain, rachis and pulse seed amino acid delta15N values as indicators of plant nitrogen metabolism, Phytochemistry 97 (2014) 20–29.
[10] D. Hofmann, K. Jung, H.J. Segschneider, M. Gehre, G. Schüürmann, 15N/14N analysis of amino acids with GC-C-IRMS – methodical investigations and ecotoxicological applications, Isotopes Environ. Health Stud. 31 (1995) 367–375.

[11] D. Robinson, $\delta^{15}N$ as an integrator of the nitrogen cycle, Trends Ecol. Evol. 16 (2001) 153–162.
[12] G. Wu, Amino acids: metabolism, functions, and nutrition, Amino Acids 37 (2009) 1–17.
[13] L. Handley, J.A. Raven, The use of natural abundance of nitrogen isotopes in plant physiology and ecology, Plant Cell Environ. 15 (1992) 965–985.
[14] J.M. Craine, A.J. Elmore, M.P. Aidar, M. Bustamante, T.E. Dawson, E.A. Hobbie, A. Kahmen, M.C. Mack, K.K. McLauchlan, A. Michelsen, G.B. Nardoto, L.H. Pardo, J. Penuelas, P.B. Reich, E.A. Schuur, W.D. Stock, P.H. Templer, R.A. Virginia, J.M. Welker, I.J. Wright, Global patterns of foliar nitrogen isotopes and their relationships with climate, mycorrhizal fungi, foliar nutrient concentrations, and nitrogen availability, New Phytol. 183 (2009) 980–992.
[15] Y. Chikaraishi, S.A. Steffan, N.O. Ogawa, N.F. Ishikawa, Y. Sasaki, M. Tsuchiya, N. Ohkouchi, High-resolution food webs based on nitrogen isotopic composition of amino acids, Ecol. Evol. 4 (2014) 2423–2449.
[16] Y.I. Naito, H. Bocherens, Y. Chikaraishi, D.G. Drucker, C. Wißing, M. Yoneda, N. Ohkouchi, An overview of methods used for the detection of aquatic resource consumption by humans: compound-specific delta N15 analysis of amino acids in archaeological materials, J. Archaeol. Sci.: Rep. (2015), http://dx.doi.org/10.1016/j.jasrep.2015.11.025.
[17] Y. Chikaraishi, N.O. Ogawa, H. Doi, N. Ohkouchi, 15N/14N ratios of amino acids as a tool for studying terrestrial food webs: a case study of terrestrial insects (bees, wasps, and hornets), Ecol. Res. 26 (2011) 835–844.
[18] A.K. Styring, J.C. Sealy, R.P. Evershed, Resolving the bulk δ15N values of ancient human and animal bone collagen via compound-specific nitrogen isotope analysis of constituent amino acids, Geochim. Cosmochim. Acta 74 (2010) 241–251.
[19] J.P. Godin, D. Breuille, C. Obled, I. Papet, H. Schierbeek, G. Hopfgartner, L.B. Fay, Liquid and gas chromatography coupled to isotope ratio mass spectrometry for the determination of 13C-valine isotopic ratios in complex biological samples, J. Mass Spectrom. 43 (2008) 1334–1343.
[20] E. Federherr, S. Willach, N. Roos, L. Lange, K. Molt, T.C. Schmidt, A novel high-temperature combustion interface for compound-specific stable isotope analysis of carbon and nitrogen via high-performance liquid chromatography/isotope ratio mass spectrometry, Rapid Commun. Mass Spectrom. 30 (2016) 944–952.
[21] M. Krummen, A.W. Hilkert, D. Juchelka, A. Duhr, H.J. Schluter, R. Pesch, A new concept for isotope ratio monitoring liquid chromatography/mass spectrometry, Rapid Commun. Mass Spectrom. 18 (2004) 2260–2266.
[22] T. Gilevska, M. Gehre, H.H. Richnow, Performance of the wet oxidation unit of the HPLC isotope ratio mass spectrometry system for halogenated compounds, Anal. Chem. 86 (2014) 7252–7257.
[23] 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), Isotopes Environ. Health Stud. 39 (2003) 233–244.
[24] L.T. Corr, R. Berstan, R.P. Evershed, Optimisation of derivatisation procedures for the determination of $\delta^{13}C$ values of amino acids by gas chromatography/combustion/isotope ratio mass spectrometry, Rapid Commun. Mass Spectrom. 21 (2007) 3759–3771.
[25] A.K. Styring, A. Kuhl, T.D. Knowles, R.A. Fraser, A. Bogaard, R.P. Evershed, Practical considerations in the determination of compound-specific amino acid delta15N values in animal and plant tissues by gas chromatography-combustion-isotope ratio mass spectrometry, following derivatisation to their *N*-acetylisopropyl esters, Rapid Commun. Mass Spectrom. 26 (2012) 2328–2334.
[26] G. Molero, I. Aranjuelo, P. Teixidor, J.L. Araus, S. Nogues, Measurement of 13C and 15N isotope labeling by gas chromatography/combustion/isotope ratio mass spectrometry to study amino acid fluxes in a plant-microbe symbiotic association, Rapid Commun. Mass Spectrom. 25 (2011) 599–607.
[27] R.G. Walsh, S. He, C.T. Yarnes, Compound-specific delta13C and delta15N analysis of amino acids: a rapid chloroformate-based method for ecological studies, Rapid Commun. Mass Spectrom. 28 (2014) 96–108.
[28] K.J.D. Hofmann, J. Bender, M. Gehre, G. Schüürmann, Using natural isotope variations of nitrogen in plants as an early indicator of air pollution stress, J. Mass Spectrom. 32 (1997) 855–863.
[29] M. Rubino, S. Milin, A. D'Onofrio, P. Signoret, C. Hatte, J. Balesdent, Measurement of delta13C values of soil amino acids by GC-C-IRMS using trimethylsilylation: a critical assessment, Isotopes Environ. Health Stud. 50 (2014) 516–530.
[30] S. Kruse, E.A. Pakhomov, B.P.V. Hunt, Y. Chikaraishi, N.O. Ogawa, U. Bathmann, Uncovering the trophic relationship between Themisto gaudichaudii and Salpa thompsoni in the Antarctic Polar Frontal Zone, Mar. Ecol. Prog. Ser. 529 (2015) 63–74.
[31] Y.I. Naito, Y. Chikaraishi, D.G. Drucker, N. Ohkouchi, P. Semal, C. Wißing, H. Bocherens, Ecological niche of Neanderthals from Spy cave revealed by nitrogen isotopes of individual amino acids in collagen, J. Hum. Evol. 93 (2016) 82–90.
[32] S.A. Steffan, Y. Chikaraishi, C.R. Currie, H. Horn, H.R. Gaines-Day, J.N. Pauli, J.E. Zalapa, N. Ohkouchi, Microbes are trophic analogs of animals, Proc. Natl. Acad. Sci. U. S. A. 112 (2015) 15119–15124.
[33] N. Ohkouchi, R. Tsuda, Y. Chikaraishi, K. Tanabe, A preliminary estimate of the trophic position of the deep-water ram's horn squid Spirula spirula based on the nitrogen isotopic composition of amino acids, Mar. Biol. 160 (2012) 773–779.
[34] Dawn A. Merritt, J.M. Hayes, Nitrogen isotopic analyses by isotope-ratio-monitoring gas chromatography/mass spectrometry, J. Am. Soc. Mass Spectr. 5 (1994) 387–397.
[35] Y. Chikaraishi, Y. Takano, N.O. Ogawa, N. Ohkouchi, Instrumental optimization for compound-specific nitrogen isotope analysis of amino acids by gas chromatography/combustion/isotope ratio mass spectrometry, Earth Life Isotopes 300 (2010) 365–386.
[36] Y. Takano, Y. Kashiyama, N.O. Ogawa, Y. Chikaraishi, N. Ohkouchi, 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 (2010) 2317–2323.
[37] A.J. Buczyńska, B. Geypens, R. Van Grieken, K. De Wael, Optimization of sample clean-up for the GC-C-IRMS and GC-IT-MS analyses of PAHs from air particulate matter, Microchem. J. 119 (2015) 83–92.
[38] M.P. Ricci, D.A. Merritt, K.H. Freeman, J. Hayes, Acquisition and processing of data for isotope-ratio-monitoring mass spectrometry, Org. Geochem. 21 (1994) 561–571.