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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

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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

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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].