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Articles

A chloroformate based method for measuring rapidly and stably the $\delta^{15}N$ values of glutamine: implications to position-specific isotope analysis of polynitrogenous amino acids for biogeochemical studies

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Abstract

The analysis of stable nitrogen isotope ratios of amino acids ($\delta^{15}N_{AA}$) has been employed as a powerful tool to estimate the trophic position of organisms and to illustrate the trophic relationship among them in diverse ecosystems. However, applicability of this analysis is somewhat doubtful in the estimation of the trophic position of marine mammals. Unusual small values of the trophic elevation in the $\delta^{15}N_{AA}$ value have been apparently expected for marine mammals in previous studies, which may be affected by isotopic alteration during the ammonia recycling through glutamate - glutamine exchange associated with the urea-excretion. In the present study, we developed a chloroformate based method (to form isopropoxy isopropyl ester derivative) for separately measuring the $\delta^{15}N$ values of two amino groups within a single authentic glutamine molecule, as a first step accurately to evaluate the isotopic alteration during the ammonia recycling in wild animals. The isopropyl derivative of glutamine used in the present study can be preserved for longer than 47 days at −20ºC (or for up to 5 days at room temperature), unlike methyl and ethyl derivatives used in previous studies are quickly degraded within several days even at −20ºC. Moreover, together with the hydrolysis of glutamine to form glutamate, the $\delta^{15}N$ values of two amino groups within a single glutamine were measured. Thus, the chloroformate based method developed can be useful to compare the $\delta^{15}N$ values between two amino groups within a single glutamine molecule, and will provide further evaluation of the isotopic alteration on the $\delta^{15}N_{AA}$ values during the urea-excretion and related processes in marine mammals.

1. Introduction

Compound-specific isotope analysis of nitrogen within amino acids has been employed as a powerful tool to estimate the position of organisms in the trophic hierarchy of food webs (i.e., trophic position) (e.g., Chikaraishi et al., 2007; McCarthy et al., 2007; Popp et al., 2007; McMahon and McCarthy, 2016). This is based on that the elevation in the nitrogen isotopic composition of amino acids ($\delta^{15}N_{AA}$ value) at each step of trophic transfer from diet to consumer species (which is called 'trophic discrimination factor, TDF' or 'trophic enrichment factor, TEF') is universally found as relatively consistent values (in particular, approximately 7.6‰ as the subtraction of the TDF of glutamic acid minus that of phenylalanine, $TDF_{Glu-Phe}$, Chikaraishi et al., 2009) among a wide range of taxa and trophic levels of organisms (including mice, a representative mammal species) that were cultured (i.e., in controlled feeding experiments) and diet-characterized species (e.g., Chikaraishi et al., 2014; Steffan et al., 2015; Yamaguchi et al., 2017).

However, applicability of this analysis is somewhat doubtful in the estimation of the trophic position of marine mammals such that are generally difficult to carry out controlled feeding experiments. Indeed, the trophic position estimated in the $\delta^{15}N_{AA}$ value analysis is smaller than that expected in the traditional observation such as stomach content analysis, and thus apparently-small values of the TDF have been proposed for several organisms including marine mammals (e.g., seals) and birds (e.g., penguin) (Germain et al., 2013; McMahon et al., 2015). For instance, Germain et al., (2013) firstly found an apparently-small TDF (i.e., 4.3‰ as $TDF_{Glu-Phe}$) for harbor seals (*Phoca vitulina*) compared to the TDF generally found in cultured and diet-characterized species, and suggested that this ap-

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parently-small TDF may be affected by unknown, indirect isotopic alteration during the ammonia recycling through glutamate - glutamine exchange associated with the urea-excretion. In the metabolism of organisms, ammonia released from the deamination of amino acids is reacted with glutamate to produce glutamine, and forms the δ-position amino group on glutamine (the α-position amino group is derived from glutamate). On the other hand, a portion of the δ-position amino group is frequently used as the substrate of α-position amino groups to synthesize non-essential amino acids including glutamate itself (e.g., Velíšek and Cejpek, 2006). It is likely explained that the ammonia released from the deamination is depleted in ^{15}N (i.e., shows low $\delta^{15}N$ values) compared to substrate amino acids (Macko et al., 1986; Chikaraishi et al., 2007; Goto et al., 2018), and that the low $\delta^{15}N$ values is incorporated into the α-position amino groups if non-essential amino acids are synthesized in consumer species. An apparently-small TDF thus is potentially explained if the metabolic flux of ammonia recycling is activated in marine mammals and birds that have the urea- and uric acid-excretion, respectively (e.g., Germain et al., 2013).

It is simply thought that the comparison of the $\delta^{15}N$ values between α- and δ-amino groups within a single glutamine molecule will clarify the contribution of ammonia recycling to the isotopic alteration (which may cause apparently-small TDF) found in mammals and birds. To measure the $\delta^{15}N$ values of amino acids, chloroformate based methods have often been used in previous studies (e.g., Sacks and Brenna, 2005; Pan et al., 2007; Walsh et al., 2014; Yarnes and Herszage, 2017). An advantage of these methods is easy and rapid derivatization (i.e., chemical treatment to increase volatility for gas chromatographic analysis) at room temperature for within 30 minutes (e.g., Walsh et al., 2014; Ohkouchi et al., 2017), unlike other derivatization methods such as pivaloyl isopropyl esterification occurs at 100-110ºC for several hours (e.g., Metges et al., 1996; Metges and Daenzer, 2000). Moreover, the chloroformate based methods can measure the $\delta^{15}N$ values of polynitrogenous amino acids including glutamine, unlike such high reaction temperature in the other derivatization methods accelerates break down of polynitrogenous amino acids during derivatization. On the other hand, disadvantage of the chloroformate based methods is low stability derived from methyl and ethyl esters in the amino acid derivatives, which requires isotope analysis within 24 hours and therefore increases analytical errors on the measured values with time after forming derivatives (e.g., Ohkouchi et al., 2017; Yarnes and Herszage, 2017). Indeed, half life of the derivatives at −20ºC is estimated to be 3-4 days and 7-8 days for methyl and ethyl esters, respectively (Ohkouchi et al., 2017).

In the present study, we developed a new chloroformate based method that forms isopropyl esters of amino acids in the derivatization, instead of weak methyl or ethyl ester used in a previous conventional chloroformate based method (Fig. 1), and applied it for sepa-

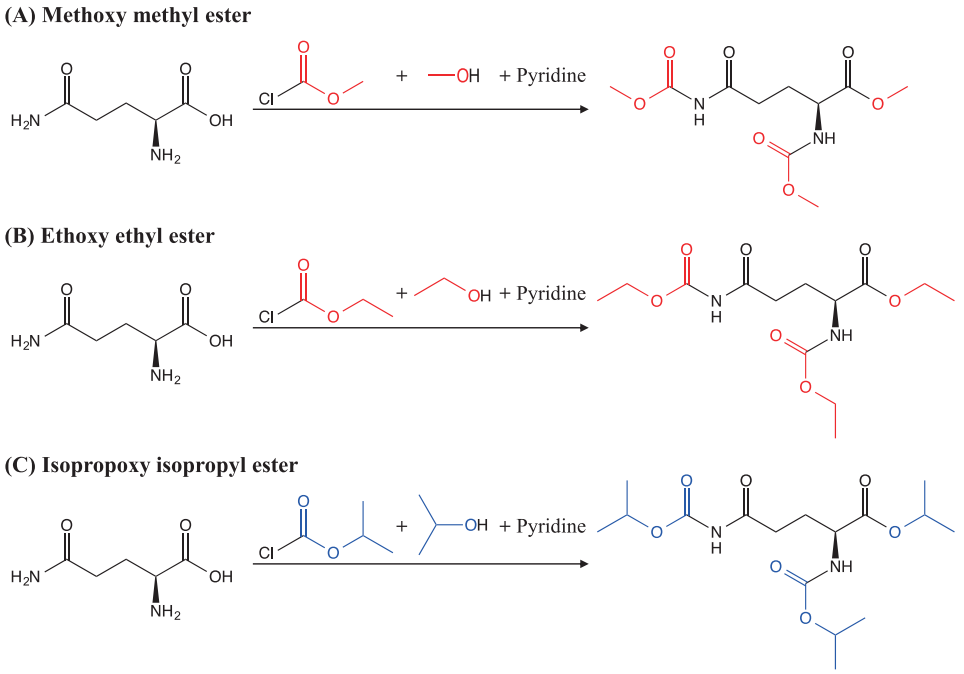

Fig. 1 Chemical reaction in chloroformate methods to produce glutamine esters: (A) Methoxy methyl ester, (B) Ethoxy ethyl ester, and (C) Isoproxy isopropyl ester.

rately-measuring the $\delta^{15}N$ values of two amino groups within a single authentic glutamine molecule. The isopropyl esters will allow us to measure the $\delta^{15}N$ values of glutamine with easy and rapid preparation and without a potential uncertainty derived from the chemically-weakness of derivatives. The separately-measuring the $\delta^{15}N$ values of two amino groups within a single glutamine will be essential, first step in further evaluation of the isotopic alteration during the ammonia recycling in wild animals.

2. Materials and Methods

Authentic glutamine

Authentic pure L-glutamine (min.99.0%, Lot. LEK1272) was purchased from Fujifilm Wako Pure Chemical Corporation. The $\delta^{15}N$ value of this glutamine is 4.2‰, which was determined by a traditional EA-IRMS (elemental analyzer - isotope ratio mass spectrometer) method. All regents and solvents used in the present study were also purchased from Fujifilm Wako Pure Chemical Corporation.

Derivatization of glutamine to isopropoxy isopropyl ester

Isopropoxy isopropyl ester of glutamine was prepared by a modified procedure according to the standard procedure for ethoxy ethyl esters in Yamaguchi et al., (2009). 1.2 mg of glutamine, 1 mL of distilled water, 500 µL of isopropanol (i.e., ethanol is used in Yamaguchi et al., 2009), and 125 µL of pyridine were added to a 10 mL vial and mixed, and 50 µL of isopropyl chloroformate (i.e., ethyl chloroformate is used in Yamaguchi et al., 2009) was added. The mixture of these materials was vortexed and reacted at room temperature for 15 minutes. After reaction, 1 mL of $NaHCO_3$ saturated solution was added and the derivative of glutamine was extracted with *n*-hexane/dichloromethane (3/2, v/v). The *n*-hexane/dichloromethane was removed under a nitrogen stream and the derivative of glutamine was redissolved in 500 µL of dichloromethane prior to injection into a gas chromatograph - isotope ratio mass spectrometer (GC-IRMS). The dichloromethane solution was partitioned to two vials, and one and the other one were stored at room temperature (ca. 21ºC) and −20ºC, respectively, until the isotope analysis.

Acid hydrolysis of glutamine to form glutamic acid

1.0 mg of glutamine was taken into a 1.0 mL reactor vial, and added along with 0.1 mL of 12N HCl. The mixture was reacted at 106ºC on a heat block for 12 hours, for hydrolyzing glutamine to form glutamic acid (Fig. 2). After hydrolysis, the HCl solution was removed under a nitrogen stream.

Derivatization of glutamic acid to pivaloyl isopropyl ester

It is known that chloroformate based derivatization is not available for the isotope analysis of nitrogen within glutamic acid, because there is an artificial, significant isotopic fractionation associated with competing reactions that involves cyclization and acylation of glutamic acid (Hušek, 1991; Ohkouchi et al., 2017). Therefore, for the isotope analysis of glutamic acid, pivaloyl isopropyl ester was prepared with a procedure in Chikaraishi et al., (2009).

Thionyl chloride/isopropanol (1/4, v/v) was added to the glutamic acid (i.e., derived from hydrolysis of glutamine) in the 1.0 mL reactor vial, and the mixture was reacted at 106ºC on a heat block for 2 hours. After esterification, the remaining thionyl chloride/isopropanol was removed under a nitrogen stream. Subsequently, pivaloyl chloride/dichloromethane (1/4, v/v) was added in the reactor vial, and the mixture was reacted at 106ºC on a heat block for 2 hours. After acylation, the remaining pivaloyl chloride/dichloromethane was removed under a nitrogen stream. The derivative of glutamic acid was redissolved in 500 µL of dichloromethane prior to injection into a GC-IRMS. The dichloromethane solution was stored at room temperature (ca. 21ºC) until the isotope analysis.

Isotope ratio analysis

Stable nitrogen isotopic composition of both derivatives was determined with a GC-IRMS using a 7890B GC (Agilent Technologies) coupled to a DeltaV IRMS

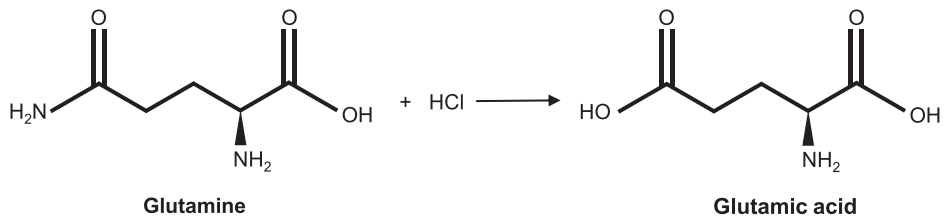

Fig. 2 Chemical reaction on the HCl hydrolysis of glutamine.

through combustion (950 °C) and reduction (550 °C) furnaces and a countercurrent dryer (NafionTM) via a GC-C/TC III interface (Thermo Fisher Scientific) (Takizawa and Chikaraishi, 2021). A 1 μL aliquot of the dichloromethane solution of derivatives was injected to GC, and derivatives were separated on an Agilent Ultra-2 capillary column (50 m length, 320 μm i.d., 0.5 μm film thickness). The carrier gas (He) flow rate was controlled using a constant flow mode at 1.4 mL min^{-1}. To assess the reproducibility of the isotope measurement, an isotopic reference mixture of amino acids (Indiana University; Shoko Science Co.) was analyzed before and after sample runs, with three pulses of reference N_2 gas discharged at the beginning and end of each run. The $\delta^{15}N$ values were expressed relative to the isotopic composition of AIR, on scales normalized to known δ values of the reference amino acids. Analytical error (1σ Standard Deviation) of the $\delta^{15}N$ values for replicate analysis was 0.4-0.7‰ in the analysis. Recovery rate (i.e., yield) that estimated by comparison with *m/z* 28 peak area of the reference amino acids were approximately 30% and 60% for isopropoxy isopropyl ester of glutamine and pivaloyl isopropyl ester of glutamic acid, respectively, for the procedures used in the present study. Analytical error of the recovery rate for replicate analysis was 15% in the analysis.

Calculation of the $\delta^{15}N$ values for α- and δ-amino groups within glutamine

The $\delta^{15}N$ values of α- and δ-amino groups ($\delta^{15}N_\alpha$ and $\delta^{15}N_\delta$, respectively) within glutamine are defined with the following equations:

$$\delta^{15}N_{Gln} = (\delta^{15}N_\alpha + \delta^{15}N_\delta) / 2 \quad (1)$$

$$\delta^{15}N_{Glu} = \delta^{15}N_\alpha \quad (2)$$

where $\delta^{15}N_{Gln}$ and $\delta^{15}N_{Glu}$ represent the measured $\delta^{15}N$ values for isopropoxy isopropyl ester of glutamine and pivaloyl isopropyl ester of glutamic acid, respectively. Based on the equations (1) and (2), $\delta^{15}N_\delta$ is given by the following equation (3):

$$\delta^{15}N_\delta = 2 \times \delta^{15}N_{Gln} - \delta^{15}N_{Glu} \quad (3)$$

3. Results and Discussion

Preservation stability of isopropoxy isopropyl ester

Isopropoxy isopropyl ester of glutamine is preserved by approximately 100% for several days after derivatization in the present study, as the GC-IRMS chromatograms clearly reveal no substantial reduction in the peak area of the glutamine derivative that stored at −20ºC (decreased by 2.5%) and at even room temperature (decreased by 9%) for 5 days (Fig. 3). Survival rates of this ester are estimated to be 84% at −20ºC and even 46% at room temperature for further 30 days (Fig. 3). These results are far different from the low stability of methyl and ethyl esters of amino acids (Ohkouchi et al., 2017), in which the methyl and ethyl esters have been frequently used in previous studies (e.g., Yarnes and Herszage, 2017).

Moreover, the $\delta^{15}N$ values of this isopropoxy isopropyl ester negligibly change at both −20ºC and room temperature within 5 days, as mean ± 1σ standard deviation of the $\delta^{15}N$ values measured are 3.9 ± 0.5‰ and 3.9 ± 0.4‰, respectively (Fig. 4). Considering 0.4-0.7‰ analytical error, we conclude that the isopropyl ester has high stability enough to measure the $\delta^{15}N$ values of glutamine by GC-IRMS analysis, which is well achieved if the the isopropyl ester was stored at −20ºC but may be obtainable even if it was stored at room temperature. We indeed confirmed that this high stability is continuously retained at −20ºC for further 42 days in the present study.

It is known that the chloroformate based methods have been easily employed as a rapid method for the isotope ratio analysis, but they have a considerable disadvantage related to chemically-weakness of methyl and ethyl esters of amino acids (e.g., Ohkouchi et al., 2017). Indeed, this disadvantage strongly requires isotope analysis within 24 hours after forming derivatives (Yarnes and Herszage, 2017). On the other hand, a new chloroformate based method (i.e., isopropyl esters of amino acids) developed in the present study allows us to measure the $\delta^{15}N$ values of glutamine with advantage on the preparation and without disadvantage on the stability. Thus, the chloroformate based method developed can be useful as a reliable method to measure the $\delta^{15}N$ values of glutamine, in particular the isopropyl ester considerably expands the lifetime of derivatives in the isotope analysis to at least 47 days after forming derivatives.

The $\delta^{15}N$ values of α- and δ-amino groups in glutamine

The mean $\delta^{15}N$ value is 3.9‰ for comparing the measured $\delta^{15}N$ values of glutamine that is stored at room temperature and −20ºC in 5 days after derivatization, and −2.7‰ for that of glutamic acid that is stored at room temperature in 3 days after derivatization. Based on these mean $\delta^{15}N$ values, the equation (3) provides that the $\delta^{15}N$ values of α- and δ-amino groups in glutamine investigated are −2.7‰ and 10.6‰, respectively. Thus the $\delta^{15}N$ values of both α- and δ-amino groups in glutamine can be determined separately in the present study, revealing to heterogeneity in the $\delta^{15}N$ value between positions, as the values of latter are significantly higher than that of former within a single glutamine molecule. To our knowledge, although this heterogeneity has not been reported in wild animal species, several data are available for authentic standards of glutamine and other polynitrogenous amino ac-

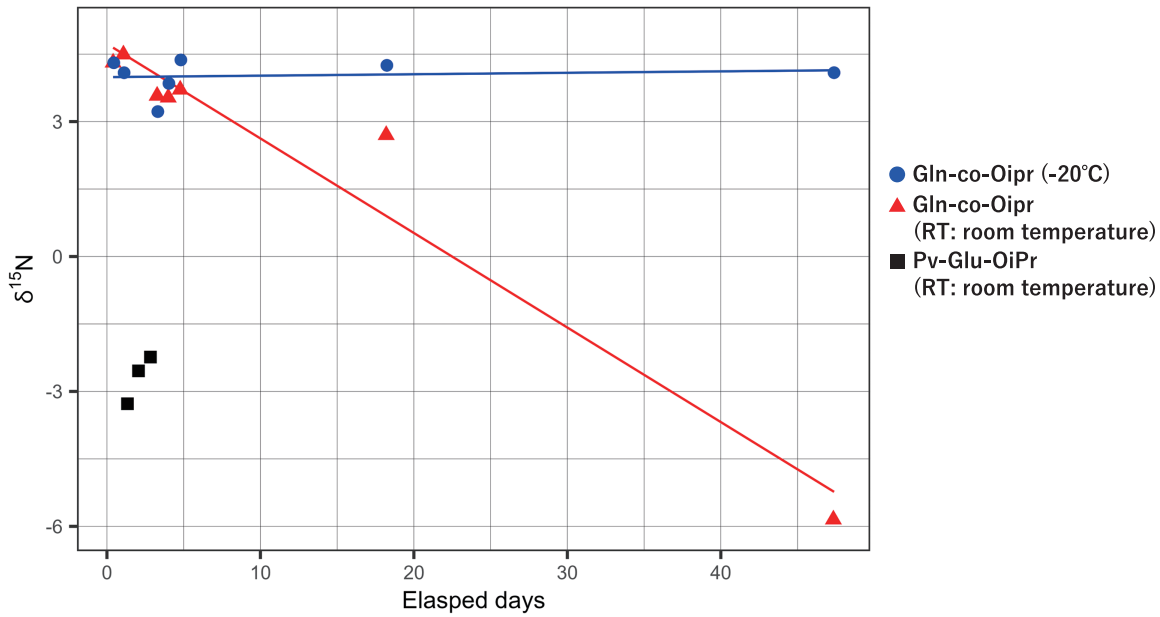

Fig. 3 Changes in the *m/z* 28 peak area (Area/nmol, vs) on GC-IRMS chromatograms with time after derivatization: preservation in −20ºC (blue circle) and room temperature (red triangle).

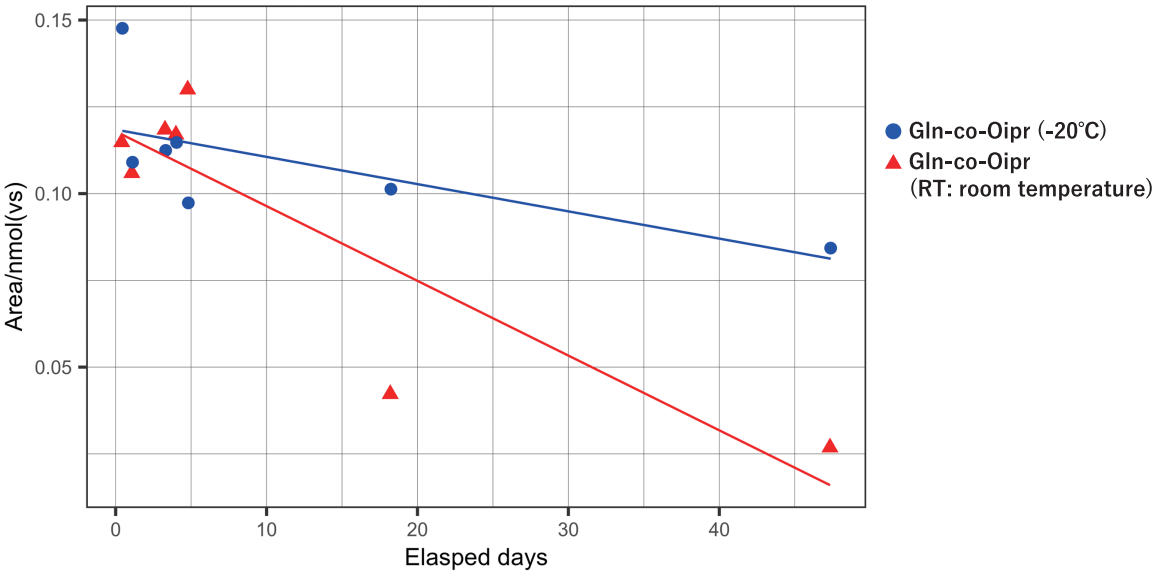

Fig. 4 Changes in the $\delta^{15}N$ value of isopropoxy isopropyl ester of glutamine with time (after derivatization): preservation in −20ºC (blue circle) and room temperature (red triangle). the $\delta^{15}N$ values of pivaloyl isopropyl ester of glutamic acids are also plotted (black square).

ids (e.g., asparagine) in a previous study that used an ethoxy-ethyl ester derivative method (Sacks and Brenna, 2005). A consistent trend for the isotopic heterogeneity between two amino groups is found in both this and previous studies. For instance, the $\delta^{15}N$ values of δ-amino group of glutamine and γ amino group of asparagine are significantly higher by 11‰ and 3‰, respectively, than that of α-amino groups within a molecule for authentic standards (Sacks and Brenna, 2005).

4. Implications

Position-specific isotope analysis of polynitrogenous amino acids

It is known that the GC-IRMS analysis requires approximately 1 hour for each run of the chromatography (Takizawa and Chikaraishi, 2020). Considering this analytical time consumption, we have to much carefully use chloroformate based methods that form methyl or ethyl ester of amino acids, because half life is shorter than one week for both esters, even if they are stored at −20ºC (Ohkouchi et al., 2017). If it is a case in the triplicate analysis of each sample within 24 hours, the number of samples measured is limited to be eight and might be possible over ten. Moreover, this number (i.e., eight/day) will be reduced depending on the temperature of sample tray on the GC-IRMS. Indeed, sample tray is placed closing to a combustion furnace (at 950ºC) of the GC-IRMS in general condition, which causes to increase temperature of sample tray more than room temperature.

On the other hand, a new chloroformate based method (i.e., isopropyl esters of amino acids) developed in the present study allows us to measure the $\delta^{15}N$ values of glutamine that is stored at −20ºC at least in 47 days or at room temperature in 5 days. These results clearly indicate that we do not have to much carefully (i.e., on the timing for analysis) use the chloroformate based method for the isotope analysis, which can minimize uncertainty in the position-specific isotope analysis for these polynitrogenous amino acids. Indeed, it is simply calculated that we will provide triplicate analysis for approximately 40 samples within 5 days either the samples are stored at at room temperature or −20ºC after forming derivatives. Moreover, this can be expanded to maximum 240 samples within one month in the latter case (i.e., stored at −20ºC).

Study for ammonia recycling with respect to the unusual TDF

In the metabolism of amino acids, ammonia is incorporated (i.e., produce amino acids), released (i.e., degrade amino acids), and recycled (i.e., transfer it between amino acids) (Velíšek and Cejpek, 2006). It is well known that, in the transamination, an ammonia released from amino acids is incorporated as the δ-position amino groups on glutamine and the γ-position of asparagine, and a portion of those is again incorporated as the α-position amino group on other amino acids including glutamate and aspartate themselves (Velíšek and Cejpek, 2006). Thus, because glutamate is a non-essential amino acid for many animal species, α-position amino group in glutamate is potentially exchangeable indirectly with the amino group in other amino acids. Therefore we have simply thought that position-specific isotope analysis of polynitrogenous amino acids, i.e., $\delta^{15}N$ analysis for each position of amino groups, will quantify the metabolic flux and associated isotopic exchange among incorporation, releasement, and recycling of the amino group. Indeed, high activity of ammonia recycling will be an explanation for better understanding a variation in the TDF among species, particularly for apparently-small TDF in specific wild animal species investigated in previous studies (e.g., Germain et al., 2013; McMahon et al., 2015). Furthermore, such understanding will promise to develop a reliable tool to estimate accurate TP of animals in natural environments.

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