Chapter

23

Instrumental optimization of compound-specific nitrogen isotope analysis of amino acids by gas chromatography/combustion/isotope ratio mass spectrometry

Yoshito Chikaraishi*, Yoshinori Takano, Nanako O. Ogawa, and Naohiko Ohkouchi

Abstract

Compound-specific nitrogen isotope analysis of amino acids by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) has been widely used in biological, physiological, ecological, and archaeological studies. In particular, it has recently been employed as a powerful tool for estimating the trophic level of organisms and nitrogen flow in food webs. In this study, we optimize the instrumental parameters (*i.e.*, temperature of reaction furnaces, carrier gas flow rate, sample amount, derivatization, and GC column) on the GC/C/IRMS to achieve accurate and precise isotopic measurements of amino acids. Our results indicate that the nitrogen isotopic composition of amino acids can be accurately determined only at high combustion temperatures (＞925°C) and low carrier gas flow rates (0.8−1.4 mL min^{-1}); further, the GC capillary column must be packed with a polymethylsiloxane stationary phase containing nucleophilic (cyano and phenyl) groups or producing polyethylene glycol stationary phases. Furthermore, with this technique, accurate results can be obtained for the *N*-pivaloyl isopropyl (Pv/iPr) or *N*-trifluoroacetyl isopropyl (TFA/iPr) ester derivatives of amino acids. Under the conditions described above, the analytical error (1σ) in the isotope analysis is better than 0.3−0.7‰ for a minimum sample amount of 30 ngN (~2 nmolN).

Keywords: gas chromatography/combustion/isotope ratio mass spectrometry, nitrogen isotopic composition, amino acids

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INTRODUCTION

Stable isotope analysis of bulk organic materials has been conventionally employed in a number of ecological studies (Fry, 2006). In particular, the nitrogen isotopic composition of bulk organisms and their tissues has widely been used to estimate the trophic level and nitrogen flow in modern and fossil food webs (Macko et al., 1991; Hobson and Welch, 1992; Ostrom et al., 1993; Keough et al., 1996; Yoshii et al., 1999; Ogawa et al., 2001; Post, 2002). This method was recently improved with the adoption of compound-specific stable isotope analysis (CSIA) by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) (Hayes et al., 1990; Sessions, 2006; Chikaraishi and Oba, 2008). The nitrogen isotope analysis of individual amino acids gives a more precise estimate of the trophic level of organisms and the nitrogen flow in food webs than does the bulk method (McClelland and Montoya, 2002; McClelland et al., 2003; Chikaraishi et al., 2007a, 2007b, 2009, 2010). Another important advantage of the CSIA method is the small sample size (nanomolar amounts of each element) required for GC/C/IRMS; this facilitates the isotope analysis of small samples such as natural microorganisms and protein remnants in fossil bones.

Nitrogen isotope analysis of amino acids by GC/C/IRMS was first demonstrated by Merritt and Hayes (1994). They used an Ultra−2 GC column (Agilent Technologies) with *N*-acetyl *n*-propyl (Ac/*n*Pr) ester derivative for amino acid separation on the GC chromatogram. Later, some researchers used *N*-pivaloyl isopropyl (Pv/iPr) esters instead of the Ac/*n*Pr esters (Metges et al., 1996) in order to achieve better chromatographic resolution of the amino acids. Thus the Pv/iPr ester has been employed as a preferred derivative in many studies (Metges and Petzke, 1997; Simpson et al., 1997; Metges et al., 1999; McClelland and Montoya, 2002; McClelland et al., 2003; Pakhomov et al., 2004; Schmidt et al., 2004; Petzke et al., 2005; Chikaraishi et al, 2007a, 2009, 2010). This technique has also been employed for the isotope analysis of D and L enantiomers of amino acids on an optically active GC capillary column (Chirasil-Val, Alltech Associates Inc.) by *N*-trifluoroacetyl isopropyl (TFA/iPr) (Macko et al., 1997) or *N*-pentafluoropropyl isopropyl (PFP/iPr) (Veuger et al., 2005) ester derivatization. The TFA/iPr ester has been used as the preferred alternative derivative of amino acids in several studies (Fogel and Tuross, 1999, 2003; McCarthy et al., 2007; Pop et al., 2007).

The experimental conditions for GC/C/IRMS (*e.g.*, stability, sample requirement, and oxidation catalyst) for the nitrogen isotope analysis of amino acids have been provided in Merritt and Hayes (1994) and several review papers (Metges and Petzke, 1999; Meier-Augenstein, 2004; Evershed et al., 2007). Nevertheless, further optimization of the basic experimental conditions is necessary before GC/C/IRMS can be established as the standard method for the nitrogen isotope analysis of amino acids. There is limited information on the essential analytical parameters corresponding to this technique, such as temperature of the reaction furnaces and carrier gas flow rate. Further, it was noted that metal fluorides

formed when using fluorinated derivatives may cause rapid degradation of the combustion catalyst and oxidants (Hofmann et al., 2003; Metges and Petzke, 1999; Meier-Augenstein, 2004), and that care should be taken when using GC capillary column phases containing nitrogen for the isotopic exchange of amino acid nitrogen (Metges and Petzke, 1999; Meier-Augenstein, 2004). However, these issues remain to be confirmed.

In the present study, we optimize the instrumental parameters (temperature of the reaction furnaces, carrier gas flow rate, and sample amount) in the compound-specific nitrogen isotope analysis of amino acids by GC/C/IRMS. We also investigate the influence of derivatization (Pv/iPr ester versus TFA/iPr ester) of the amino acid and the type of GC capillary column (presence or absence of nitrogen and the use of a polar or nonpolar stationary phase) on the determined isotopic composition of amino acids.

Experimental procedures

Reagents and standards

All the standards, reagents, and solvents were purchased from Wako Pure Chemical Industries Ltd. Thirteen protein L-amino acid standards (alanine, aspartic acid, glutamic acid, glycine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine) and three nonprotein amino acid standards (β-alanine, γ-aminobutyric acid, and sarcosine) were used in this study. Pivaloyl chloride, isopropanol, thionyl chloride, and trifluoroacetic anhydride were used as the derivatization reagents. All the solvents were of dioxin analysis grade. We ensured that there was no substantial contamination of any of the amino acids by the reagents and solvents.

The nitrogen isotopic composition of the amino acid standards was determined by the conventional technique using a Thermo Fisher Scientific Flash EA (1112EA) coupled to a Delta^{plus}XP IRMS *via* a ConFlo III interface (*e.g.*, Ohkouchi et al., 2005; Ogawa et al., 2010). The isotopic composition is expressed as conventional δ notation against atmospheric N_2 (Air). The analytical error (1σ) in the isotopic composition measurements was better than 0.2‰ (~0.1‰ on an average).

Derivatization procedures

Pv/iPr esters of the amino acids were synthesized according to the method proposed in Chikaraishi et al. (2007a), and TFA/iPr esters were synthesized by a procedure that was a modification of the method proposed by Silfer et al. (1991). In brief, a mixture of the amino acid standards was esterified using isopropanol/thionyl chloride (4/1, v/v) at 110°C for 2 h. The residual reagents were evaporated under a gentle stream of nitrogen after the esterification. Pivaloylation or trifluoroacetylation was then performed using pivaloyl chloride/dichloromethane (1/1, v/v) or trifluoroacetic anhydride/dichloromethane (1/1, v/v), respectively, at 110°C for 2 h. After acylation, the residual reagents were again evaporated under a gentle stream of nitrogen. The obtained amino acid derivatives were dissolved in

Table 1 GC capillary columns used in this study.

Name	Stasionary phase	Length × internal diameter × film thickness	Max temperature	Nitrogen	Polarity	Optical activity	Product
Chirasil-Val	Chirasil-valine diamide polysiloxane	50 m × 0.32 mm × 0.20 μm	220°C	+	Moderate	+	Alltech Assiciates Inc.
DB−23	50% Cyanopropyl 50% methyl polysiloxane	30 m × 0.32 mm × 0.25 μm	250°C	+	High	−	Agilent Technologies
DB−35	35% Phenyl 65% methyl polysiloxane	30 m × 0.32 mm × 0.50 μm	300°C	−	Moderate	−	Agilent Technologies
DB-FFAP	Nitroterephthalic acid modified polyethylene glycol	30 m × 0.32 mm × 0.25 μm	240°C	+	High	−	Agilent Technologies
HP−1MS	Methyl polysiloxane	30 m × 0.32 mm × 1.0 μm	325°C	−	No	−	Agilent Technologies
HP-Chiral−20β	20% β-Cyclodextrin dispersed DB−35	30 m × 0.32 mm × 0.25 μm	240°C	−	Moderate	+	Agilent Technologies
HP-INNOWAX	Polyethylene glycol	30 m × 0.32 mm × 0.50 μm	260°C	−	High	−	Agilent Technologies
HP-Ultra−2	5% Phenyl 95% methyl polysiloxane	25 m × 0.32 mm × 0.50 μm	310°C	−	Low	−	Agilent Technologies

dichloromethane and stored at −20°C until further analysis.

Gas chromatography/mass spectrometry

Gas chromatography/mass spectrometry (GC/MS) analysis was performed using an Agilent Technologies 6890N GC/5973A MSD system with a Gerstel programmable temperature vaporization (PTV) injector. The amino acid derivatives were injected into the GC column using the PTV injector in solvent vent mode; the columns used for the analysis are listed in Table 1. The PTV temperature program was as follows: 50°C (initial temperature) for 0.2 min, heating from 50°G to 250°C at the rate of 600°C min^{-1}, isothermal hold at 250°C for 10 min, heating from 250°C to 350°C at the rate of 600°C min^{-1}, and isothermal hold at 350°C for 10 min. The GC oven temperature was programmed as follows: 40°C (initial temperature) for 2 min, heating from 40° to 90°C at the rate of 10°C min^{-1}, heating to 220°C at the rate of 5°C min^{-1}, and isothermal hold at 220°C for 20 min. The carrier gas (He) flow rate in the GC capillary column was controlled to 1.5 mL min^{-1} in the constant flow mode.

Gas chromatography/combustion/isotope ratio mass spectrometry*(GC/C/IRMS)*

Nitrogen isotope analysis of the amino acid derivatives was carried out by GC/C/IRMS using an Agilent Technologies 6890N GC coupled to a Thermo Fisher Scientific Delta^{plus}XP IRMS *via* a GC-C/TC III interface (Fig. 1); (Chikaraishi et al., 2007a, 2008). For GC/C/IRMS, the instrumental conditions prescribed by Merritt and Hayes (1994) were used. Accordingly, combustion was performed in a microvolume ceramic tube (length: 32 cm; internal diameter: 0.6 mm) with CuO, NiO, and Pt wires, and reduction was performed in a microvolume ceramic tube (length: 32 cm; internal diameter: 0.5 mm) with reduced Cu wires. A countercurrent dryer (Permeable membrane, Nafion™) and a liquid nitrogen

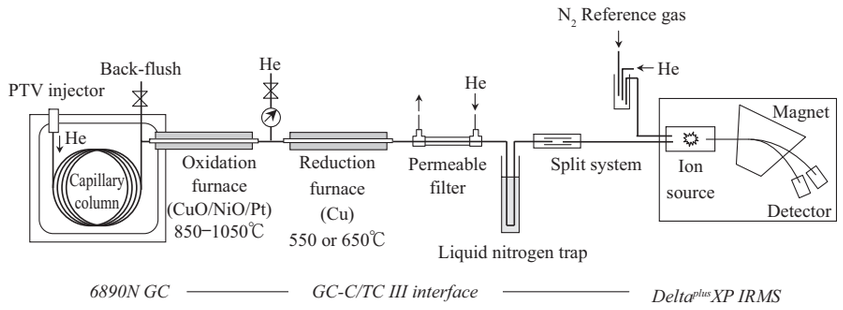


Fig. 1. Schematic of GC/C/IRMS used in the present study.

trap were installed between the reduction furnaces and the IRMS instrument for removing H_2O and CO_2 generated during amino acid combustion. All the connections were carefully tightened to prevent the GC/C/IRMS system from contamination by atmospheric N_2. The magnitude of the background signal at *m/z* 28 was reduced to 300−800 mV when the carrier gas flow rate was 1.3 mL min^{-1}. To identify the optimal conditions for the isotope analysis, the reaction furnaces were operated at various temperatures between 850°C and 1050°C during combustion and at 550°C or 650°C during reduction. The amino acid derivatives were injected into the GC column using the PTV injector in solvent vent mode; the eight columns used for the analysis are listed in Table 1. The PTV and GC oven temperature programs were the same as those used in the GC/MS analysis. The carrier gas (He) flow rate in the GC capillary column was controlled at various rates between 0.8 and 2.4 mL min^{-1} in constant flow mode.

Since accurate compound-specific isotope analysis can be carried out only after true baseline resolution of adjacent compound peaks in the GC/C/IRMS chromatogram (Chikaraishi and Oba, 2008), we used various admixtures of the amino acid standards for achieving the desired baseline resolution. For example, we analyzed the following three admixtures of the amino acid standards using an Ultra−2 column: 1) alanine, aspartic acid, glutamic acid, glycine, leucine, phenylalanine, proline, serine, tyrosine, and valine; 2) isolucine, threonine, and methionine; and 3) β-alanine, γ-aminobuthylic acid, and sarcosine. In these analyses, each amino acid was completely separated as baseline resolution on the chromatograms.

Comment for PTV injector: When using this injector, the solvent is vaporized in the injection port at a relatively low initial temperature (~50°C) and flushed through the split line. The analytes are trapped there at the initial temperature and eluted into the GC capillary column upon rapid heating (600°C min^{-1}) after a few minutes' delay. Generally, the use of a PTV injector is advantageous, particularly for the quantitative injection of high-molecular-weight organic compounds such as alkenones and sterols. However, it is less advantageous to use this injector for amino acids because they are low-molecular-weight organic compounds. Therefore, the PTV injector does not play a crucial role in the isotope

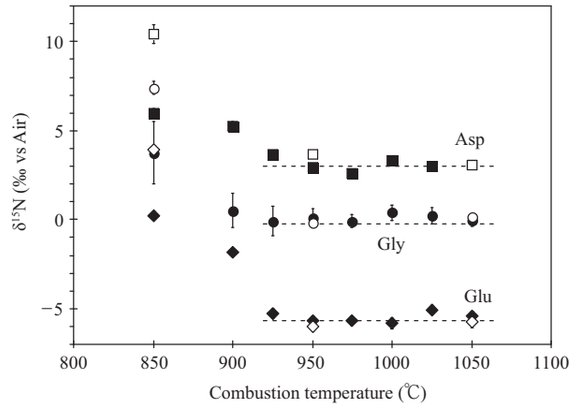


Fig. 2. Determined $\delta^{15}N$ values of Pv/iPr ester derivatives of aspartic acid (Asp), glutamic acid (Glu), and glycine (Gly) at various combustion and reduction temperatures. Dashed lines represent $\delta^{15}N$ values independently determined by EA/IRMS. Filled and open symbols indicate $\delta^{15}N$ values determined at reduction temperatures of 550°C and 650°C, respectively, and bars represent standard deviations (1σ) for triplicate analyses. GC capillary column, Ultra−2; amount of amino acids injected, 60−120 ngN. Carrier gas (He) flow rate was controlled at 1.3 mL min^{-1} in constant flow mode.

analysis of amino acids, and hence, a conventional splitless injector is generally used.

Results and discussion

Temperature of combustion and reduction furnaces

Great care should be taken when regulating the temperature of the two reaction furnaces in the GC/C/IRMS system. In general, the temperature in a combustion furnace is kept between 800°C and 1100°C to oxidize the column elutes to CO_2, H_2O, N_2, and N_xO_y; on the other hand, the temperature in a reduction furnace is kept between 550°C and 650°C for reducing N_xO_y to N_2 (Metges and Petzke, 1999; Meier-Augenstein, 2004; Evershed et al., 2007). However, the determined $\delta^{15}N$ value is questionable when the combustion is less quantitative and/or when reduction is carried out at low temperatures. In particular, precise determination of the nitrogen isotopic composition is difficult when CO is generated by incomplete combustion; this is because CO has the same mass number as N_2 (*m/z* 28). Because the natural abundance of ^{13}C (1.11%) is considerably higher than that of ^{15}N (0.36%), the $\delta^{15}N$ value can be considerably overestimated (*e.g.*, +500‰) even if there is contamination by very small quantities of CO gas.

As illustrated in Fig. 2, the $\delta^{15}N$ values measured by GC/C/IRMS for amino acids vary when the combustion temperature is in the range of 850−1050°C, although most combustion

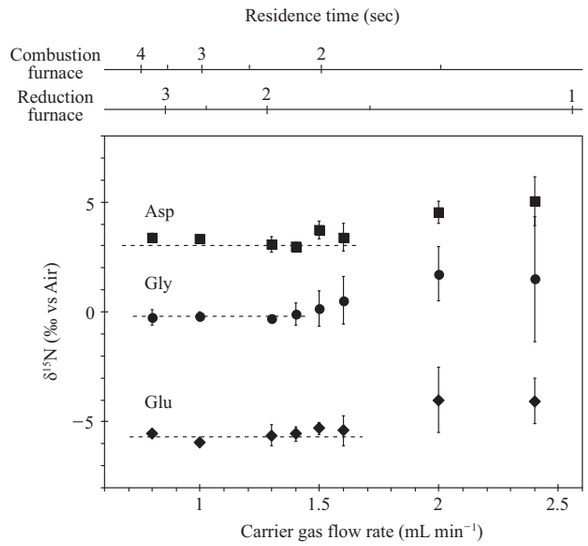


Fig. 3. Determined $\delta^{15}N$ values of Pv/iPr ester derivatives of aspartic acid (Asp), glutamic acid (Glu), and glycine (Gly) at various carrier gas flow rates. Dashed lines represent the $\delta^{15}N$ values independently determined by EA/IRMS, and bars represent the standard deviations (1σ) for triplicate analyses. Temperatures of the combustion and reduction furnaces were 1000°C and 550°C, respectively. GC capillary column, Ultra−2; amount of amino acids injected, 60−120 ngN.

processes are generally carried out in this temperature range. From the analytical errors observed in the case of EA/IRMS (0.2‰) and GC/C/IRMS (0.5‰), we state that the $\delta^{15}N$ values determined at combustion temperatures higher than 925°C are identical to those independently determined by EA/IRMS. However, the $\delta^{15}N$ values determined at temperatures below 900°C are higher (up to 6‰) than the correct values. No substantial difference is observed for the $\delta^{15}N$ values measured at reduction temperatures of 550−650°C. These results demonstrate that the $\delta^{15}N$ values for amino acids can be accurately determined at high combustion temperatures (>925°C) and that the reduction temperature does not affect the determined $\delta^{15}N$ values.

Carrier gas flow rate

The carrier gas flow rate has a direct influence on the residence time of the amino acids in the combustion and reduction furnaces, as well as on the elimination of H_2O and CO_2 in the countercurrent dryer or liquid nitrogen trap; hence, this parameter plays a crucial role in GC/C/IRMS. As shown in Fig. 3, the $\delta^{15}N$ values measured by GC/C/IRMS depend on the carrier gas flow rate. The $\delta^{15}N$ values obtained at low carrier gas flow rates (0.8−1.4 mL min^{-1}) are consistent with those determined independently by EA/IRMS, with a relatively

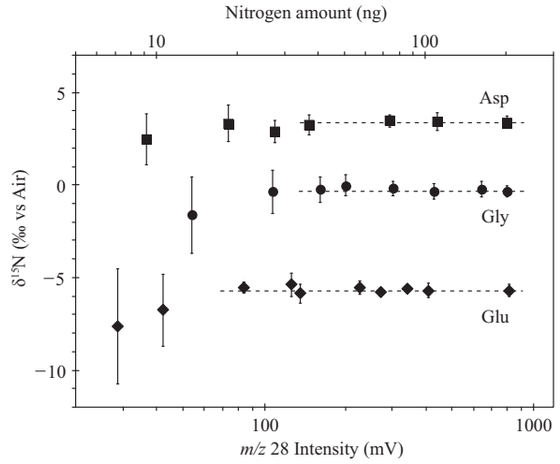


Fig. 4. Determined $\delta^{15}N$ values of Pv/iPr ester derivatives of aspartic acid (Asp), glutamic acid (Glu), and glycine (Gly) for various sample amounts. Dashed lines represent the $\delta^{15}N$ values independently determined by EA/IRMS, and bars represent the standard deviations (1σ) for triplicate analyses. Temperatures of the combustion and reduction furnaces were 1000°C and 550°C, respectively. GC capillary column used was Ultra−2. Carrier gas (He) flow rate was controlled at 1.3 mL min^{-1} in constant flow mode.

high precision (1σ < 0.5‰). This corresponds to residence times of more than 2.1 s in the combustion furnace and 1.8 s in the reduction furnace. In contrast, at high carrier gas flow rates (> 1.5 mL min^{-1}), the determined $\delta^{15}N$ values are increased by up to 2‰ with a relatively low precision (1σ ~3‰). Thus, accurate measurement of the $\delta^{15}N$ values of amino acids is possible at low carrier gas flow rates (0.8−1.4 mL min^{-1}).

Sample requirements

The amount of sample used in GC/C/IRMS is another parameter that must be considered when optimizing the accuracy and precision of the nitrogen isotope measurements. Merritt and Hayes (1994) reported that there exists a correlation between the determined $\delta^{15}N$ values and the sample amount; they found that the measurement accuracy is better than ± 0.1‰ for samples with 0.8 nmolN (~11 ngN) and better than ± 0.5‰ for samples with 0.05 nmolN (~0.7 ngN).

As depicted in Fig. 4, a similar correlation exists between the determined $\delta^{15}N$ values in this study and the sample amount. When the sample amount is between 30 ngN and 200 ngN, the determined $\delta^{15}N$ values are consistent with those determined independently by EA/IRMS, and a relatively high precision (1σ < 0.5‰) is achieved. This roughly corresponds to an *m/z* 28 intensity between 100 mV and 900 mV. In contrast, when the sample amount is less than 30 ngN, the determined $\delta^{15}N$ values differ markedly from the correct values,

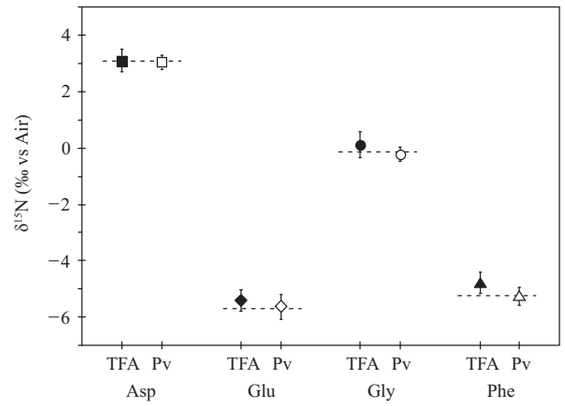


Fig. 5. Determined $\delta^{15}N$ values of two ester derivatives of aspartic acid (Asp), glutamic acid (Glu), glycine (Gly), and phenylalnine (Phe). Dashed lines represent the $\delta^{15}N$ values independently determined by EA/IRMS. Filled and open symbols indicate the $\delta^{15}N$ values of TFA/iPr and Pv/iPr ester derivatives, respectively, and bars represent the standard deviations (1σ) for triplicate analyses. Combustion and reduction temperatures were 1000°C and 550°C, respectively. GC capillary column, Ultra−2; amount of amino acid injected, 60−120 ngN. Carrier gas (He) flow rate was controlled at 1.3 mL min^{-1} in constant flow mode.

and the precision in this case is relatively low. However, the sample amount required in our study is higher than that used by Merritt and Hayes (1994), and the accuracy of our $\delta^{15}N$ values is lower. Although the exact reason for this discrepancy is not known, we speculate that one possible reason is the difference in the instruments (MAT252 versus Delta^{plus}XP IRMS) and GC/C/IRMS components (*e.g.*, on-column injector versus PTV injector) used in the two studies. In the present study, accurate measurement of the $\delta^{15}N$ values of amino acids can be carried out with sample amounts larger than 30 ngN.

Derivatization (Pv/iPr vs TFA/iPr ester)

Both Pv/iPr and TFA/iPr ester derivatives are widely used for determining the nitrogen isotopic composition of amino acids by GC/C/IRMS (Metges and Petzke, 1999; Meier-Augenstein, 2004); however, very few studies have compared the $\delta^{15}N$ values obtained when using these two ester derivatives (*e.g.*, Hofmann et al., 2003). A significant difference between these two esters is that TFA/iPr contains fluorine. As reported by Metges and Petzke (1999), fluorinated compounds may cause rapid deterioration of the combustion catalyst and oxidants. However, as shown in Fig. 5, there is no substantial difference in the $\delta^{15}N$ values determined using the two derivatives, and the $\delta^{15}N$ values are consistent with those determined independently by EA/IRMS. These results indicate that both the derivatives are suitable for the nitrogen isotope analysis of amino acids, particularly for the accurate measurement of the isotopic composition on the best condition of GC/C/IRMS

(when using a fresh combustion furnace and capillary connections, see below).

However, the repeatability and reliability of replicate isotope measurements made using the two derivatives differ significantly. When using the Pv/iPr derivative, the amplitude of peak intensity and $\delta^{15}N$ values determined during replicate isotope analyses are usually constant over 100 injections, and there is no need for reoxidation or replacement of the combustion furnace. On the other hand, when using the TFA/iPr derivative, the amplitude of peak intensity decreases gradually during analyses over 20 injections, and the determined $\delta^{15}N$ values ultimately become inaccurate. This indicates the release of fluorine in the combustion of the TFA/iPr ester; the released fluorine poisons the surface of Pt used as the combustion catalyst and/or converts the CuO and NiO oxidants to Cu_2F and Ni_2F (stable metal halides), respectively (Metges and Petzke, 1999; Hofmann et al, 2003). Moreover, a peculiar phenomenon occurs when using the TFA/iPr ester derivative; the capillary connection between the combustion and reduction furnaces becomes weak, probably implying corrosion of the capillary by HF produced in the combustion of the TFA/iPr esters.

GC capillary column

By selecting specific GC capillary columns for the analysis, we can achieve optimal baseline resolution between the peaks corresponding to the amino acids in the chromatogram. For example, Chirasil-Val, an optically active column, has previously been used for the baseline resolution of individual D and L enantiomers of amino acids during nitrogen isotope analysis (Macko et al., 1997; Veuger et al., 2005) and carbon isotope analysis (Engel et al., 1990; Docherty et al., 2001; Ostorm et al., 1994; Silfer et al., 1994; Qian et al., 1995; Uhle et al., 1997, 2007). In addition, polar phase columns such as ZB-wax and ZB-FFAP (Phenomenex Zebron) have recently been used for improving the resolution of the amino acid peaks in carbon isotope analysis (Corr et al., 2007a, 2007b). In fact, as illustrated in Fig. 6, the chromatographic resolution of the amino acids achieved with the eight types of GC capillary columns used in the present study is different. Better resolution is achieved with the highly polar DB-FFAP and nonpolar HP−1MS columns than with the less polar Ultra−2 column, which has been commonly used for amino acid isotope analysis in previous studies (McClelland and Montoya, 2002; Chikaraishi et al., 2007a).

However, when using Chirasil-Val and DB-FFAP, the determined $\delta^{15}N$ values for some of the amino acids (glutamic acid, isoleucine, threonine, and serine when using Chirasil-Val; alanine, leucine, phenylalanine, serine, and threonine when using FFAP) differ significantly (up to 4.4‰) from those independently determined by EA/IRMS (Fig. 7 and Table 2). When using the HP−1MS column, the determined $\delta^{15}N$ values for aspartic acid, leucine, serine, and valine vary slightly (1.0−1.8‰) from those independently determined by EA/IRMS. In contrast, when using the DB−23, DB−35, HP−Chiral−20β, HP−INNOWAX, and Ultra−2 columns, the determined $\delta^{15}N$ values for all the amino acids are almost consistent with those independently determined by EA/IRMS ($\Delta = 0.3-0.7$‰). In particular, the reproducibility achieved with DB−35 is the best among the reproducibilities achieved with the eight GC capillary columns used in this study.

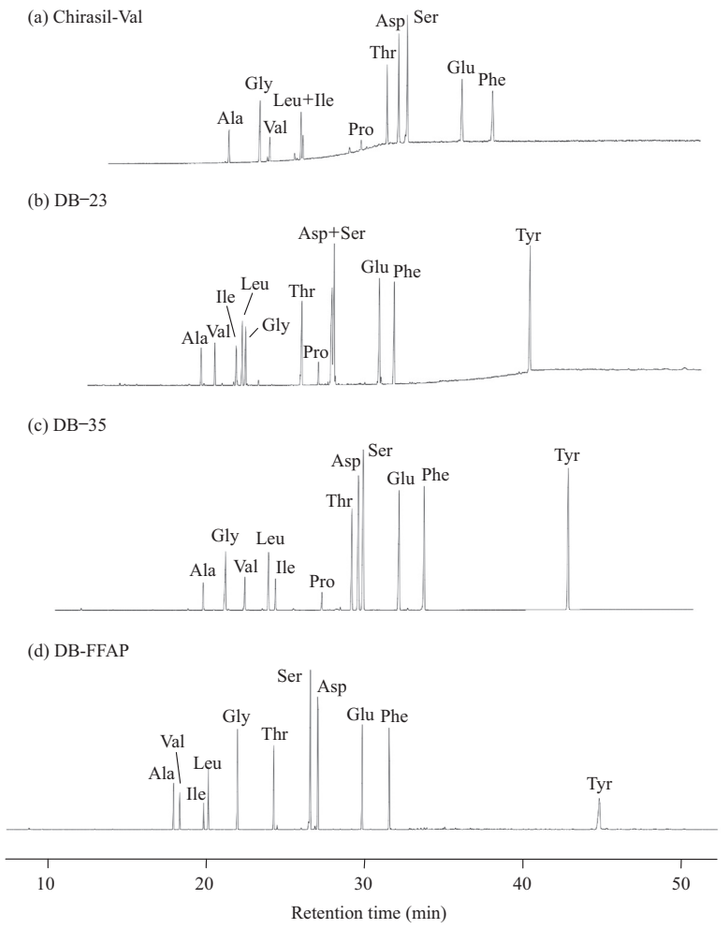
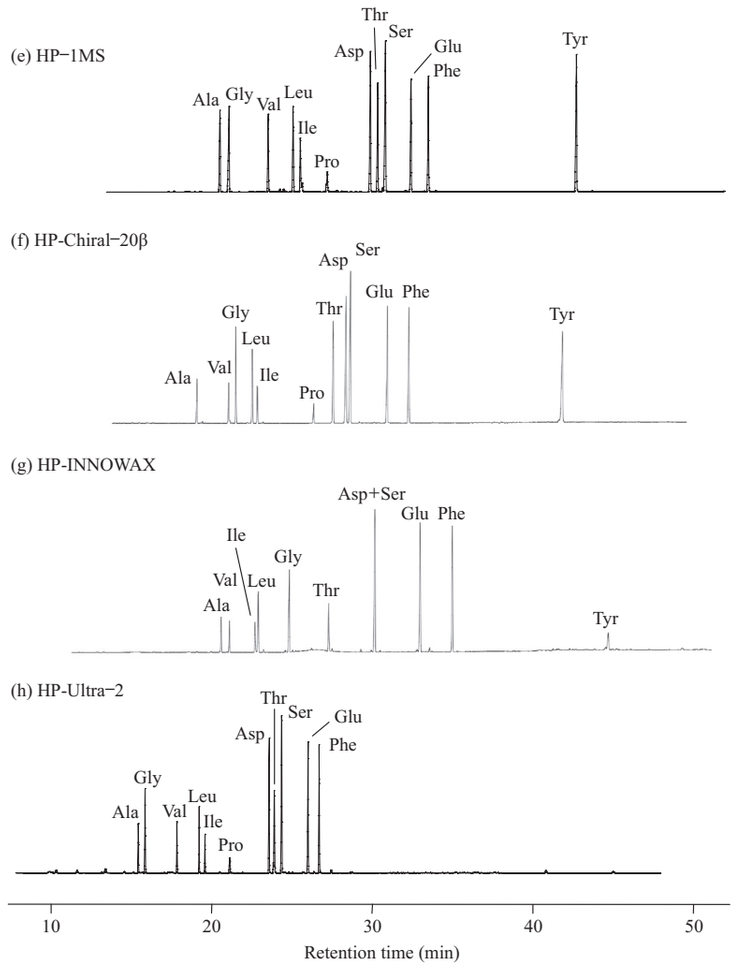


Fig. 6. Representative total ion chromatograms of amino acid standards (as Pv/iPr ester derivatives) obtained by GC/MS analysis: (a) Chirasil-Val, (b) DB−23, (c) DB−35, (d) NDB-FFAP (e) HP−1MS, (f) HP-Chiral−20β, (g) HP-INNOWAX, and (h) Ultra−2. Abbreviations are obtained from Table 2.



Chikaraishi et al., Fig. 6 (continued)

Metges and Petzke (1999) and Meier-Augenstein (2004) previously noted a caution in the isotopic exchange of nitrogen between amino acids and column phases when using the stationary phase containing nitrogen. However, in the present study, the presence or absence of nitrogen in the GC stationary phase is unrelated to the observed variation in the reproducibility. A high reproducibility is achieved with the DB−23 column but not with the Chirasil-Val and DB-FFAP columns, although the stationary phases in all these columns contain nitrogen. These results imply that the isotope exchange of nitrogen between the

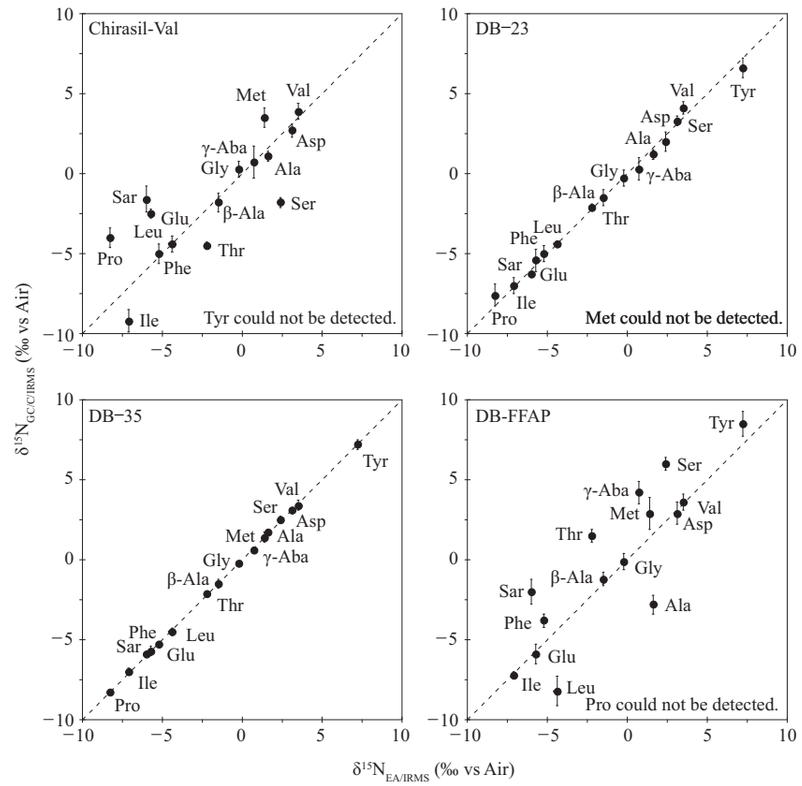
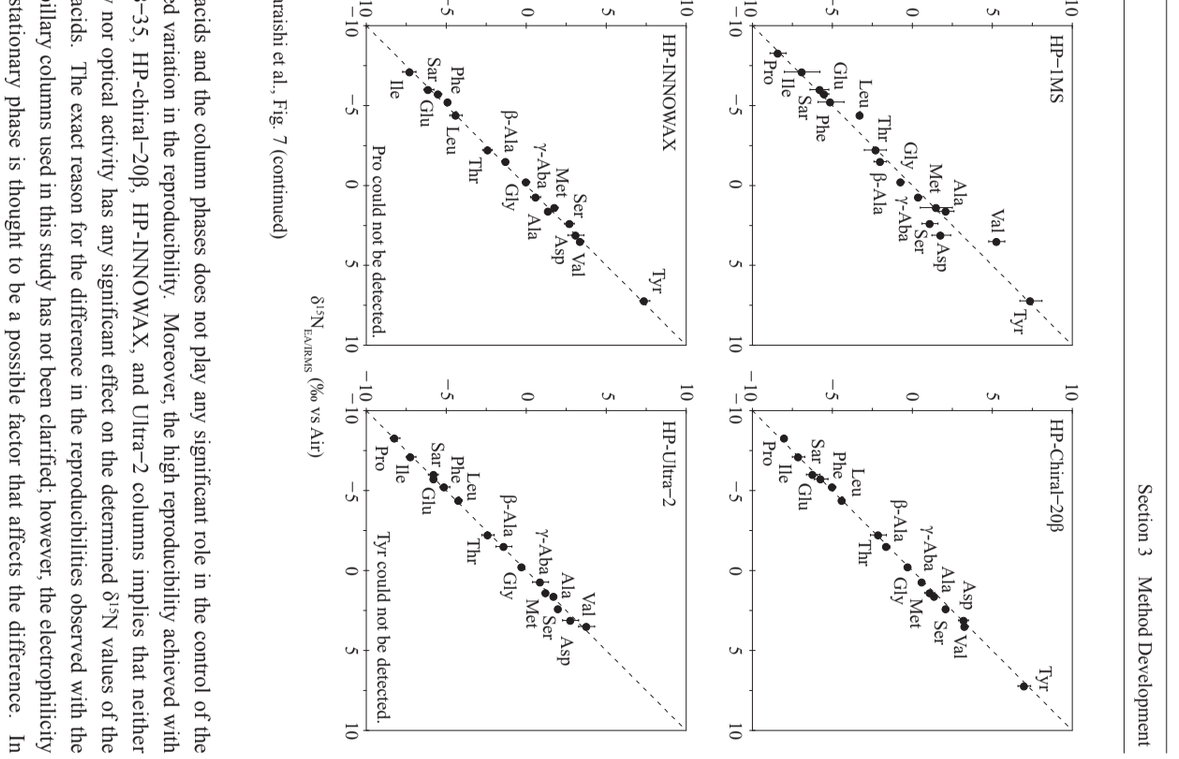


Fig. 7. Comparison of nitrogen isotopic compositions determined by GC/C/IRMS and EA/IRMS for amino acid standards (as Pv/iPr ester derivatives). Bars represent the standard deviations (1σ) for replicate analyses (N = 3−9). Combustion and reduction temperatures were 1000°C and 550°C, respectively. GC capillary column, Ultra−2; amount of amino acid injected, 60−120 ngN. Carrier gas (He) flow rate was controlled at 1.3 mL min^{-1} in constant flow mode. Abbreviations are obtained from Table 2.



Chikaraishi et al., Fig. 7 (continued)

amino acids and the column phases does not play any significant role in the control of the observed variation in the reproducibility. Moreover, the high reproducibility achieved with the DB−35, HP-chiral−20β, HP-INNOWAX, and Ultra−2 columns implies that neither polarity nor optical activity has any significant effect on the determined $\delta^{15}N$ values of the amino acids. The exact reason for the difference in the reproducibilities observed with the GC capillary columns used in this study has not been clarified; however, the electrophilicity of the stationary phase is thought to be a possible factor that affects the difference. In fact, the reproducibility of the determined $\delta^{15}N$ values is low when using Chirasil-Val, DB-FFAP, and HP−1MS, all of which contain stationary phases bearing electrophilic functionalities (carboxyl carbon or methyl carbon). In contrast, the reproducibility of the determined $\delta^{15}N$ values is very high when using the other five capillary columns, which contain polymethylsiloxane stationary phases that bear nucleophilic functionalities (cyano or phenyl groups) or polyethylene glycol stationary phases (Table 1). This is supported by

Table 2 Comparison of $\delta^{15}N$ values of amino acid standards (using Pv/*i*Pr ester derivatives) determined by GC/C/IRMS with respect to different GC capillary columns

Amino acid	Abbreviation	EA/IRMS (underivatized)		GC/C/IRMS											
				Chirasil-Val (n = 6)			DB−23 (n = 4)			DB−35 (n = 3)			DB-FFAP (n = 3)		
		$\delta^{15}N$	SD	$\delta^{15}N$	SD	Δ*	$\delta^{15}N$	SD	Δ*	$\delta^{15}N$	SD	Δ*	$\delta^{15}N$	SD	Δ*
Protein amino acids															
L-Alanine	Ala	1.6	0.1	1.1	0.3	−0.5	1.2	0.3	−0.4	1.7	0.2	0.1	−2.8	0.6	−4.4
L-Asparatic acid	Asp	3.1	0.1	2.7	0.4	−0.4	3.3	0.3	0.2	3.1	0.1	0.0	2.9	0.7	−0.2
L-Glutamic acid	Glu	−5.7	0.1	−2.5	0.3	3.2	−5.4	0.7	0.3	−5.7	0.3	0.0	−5.9	0.6	−0.2
Glycine	Gly	−0.2	0.1	0.3	0.5	0.5	−0.3	0.5	−0.1	−0.2	0.1	0.0	−0.1	0.5	0.1
L-Isoleucine	Ile	−7.1	0.1	−9.2	0.7	−2.1	−7.0	0.5	0.1	−7.0	0.2	0.1	−7.2	0.2	−0.1
L-Leucine	Leu	−4.4	0.2	−4.4	0.5	0.0	−4.4	0.2	0.0	−4.5	0.2	−0.1	−8.2	0.9	−3.8
L-Methonine	Met	1.4	0.1	3.5	0.6	2.1	n.d.	n.d.	n.d.	1.4	0.3	0.0	2.9	1.0	1.5
L-Phenylalanine	Phe	−5.2	0.0	−5.0	0.6	0.2	−5.0	0.5	0.2	−5.3	0.2	−0.1	−3.8	0.4	1.4
L-Proline	Pro	−8.3	0.1	−4.0	0.6	4.3	−7.6	0.7	0.7	−8.3	0.1	0.0	n.d.	n.d.	n.d.
L-Serine	Ser	2.4	0.0	−1.8	0.3	−4.2	2.0	0.6	−0.4	2.5	0.2	0.1	6.0	0.4	3.6
L-Threonine	Thr	−2.2	0.1	−4.5	0.2	−2.3	−2.1	0.2	0.1	−2.1	0.1	0.1	1.5	0.4	3.7
L-Tyrosine	Tyr	7.2	0.1	n.d.	n.d.	n.d.	6.6	0.6	−0.6	7.2	0.3	0.0	8.5	0.8	1.3
L-Valine	Val	3.5	0.1	3.9	0.5	0.4	4.1	0.4	0.6	3.4	0.3	−0.1	3.6	0.5	0.1
Nonprotein amino acids															
β-Alanine	β-Ala	−1.5	0.1	−1.8	0.6	−0.3	−1.5	0.5	0.0	−1.5	0.3	0.0	−1.2	0.4	0.3
γ-Aminobutyric acid	γ-Aba	0.7	0.1	0.7	1.0	0.0	0.3	0.7	−0.4	0.6	0.2	−0.1	4.2	0.7	3.5
Sarcosine	Sar	−6.0	0.1	−1.6	0.8	4.4	−6.3	0.1	−0.3	−5.9	0.0	0.1	−2.0	0.8	4.0

Δ*: Difference in $\delta^{15}N$ values determined by EA/IRMS and GC/C/IRMS

Table 2 (continued)

Amino acid	Abbreviation	EA/IRMS (underivatized)		GC/C/IRMS											
				HP−1 (n = 5)			HP-Chiral−20β (n = 3)			HP-INNOWAX (n = 3)			Ultra−2 (n = 5)		
		$\delta^{15}N$	SD	$\delta^{15}N$	SD	Δ*	$\delta^{15}N$	SD	Δ*	$\delta^{15}N$	SD	Δ*	$\delta^{15}N$	SD	Δ*
Protein amino acids															
L-Alanine	Ala	1.6	0.1	2.1	0.5	0.5	1.4	0.0	−0.2	1.4	0.2	−0.2	1.7	0.2	0.1
L-Asparatic acid	Asp	3.1	0.1	1.8	0.6	−1.3	3.2	0.3	0.1	3.1	0.5	0.0	2.8	0.5	−0.3
L-Glutamic acid	Glu	−5.7	0.1	−5.5	0.3	0.2	−5.7	0.4	0.0	−5.5	0.2	0.2	−5.8	0.2	−0.1
Glycine	Gly	−0.2	0.1	−0.7	0.2	−0.5	−0.3	0.2	−0.1	0.0	0.2	0.2	−0.3	0.2	−0.1
L-Isoleucine	Ile	−7.1	0.1	−6.9	1.1	0.2	−7.1	0.4	0.0	−7.3	0.4	−0.2	−7.2	0.3	−0.1
L-Leucine	Leu	−4.4	0.2	−3.3	0.1	1.1	−4.4	0.2	0.0	−4.4	0.4	0.0	−4.2	0.2	0.2
L-Methonine	Met	1.4	0.1	1.5	1.0	0.1	1.1	0.3	−0.3	1.8	0.2	0.4	1.2	0.2	−0.2
L-Phenylalanine	Phe	−5.2	0.0	−5.1	0.8	0.1	−5.0	0.2	0.2	−4.9	0.0	0.3	−5.1	0.3	0.1
L-Proline	Pro	−8.3	0.1	−8.4	0.5	−0.1	−8.0	0.1	0.3	n.d.	n.d.	n.d.	−8.2	0.3	0.1
L-Serine	Ser	2.4	0.0	1.1	0.5	−1.3	2.1	0.1	−0.3	2.7	0.3	0.3	2.0	0.0	−0.4
L-Threonine	Thr	−2.2	0.1	−2.3	0.7	−0.1	−2.1	0.5	0.1	−2.4	0.3	−0.2	−2.4	0.4	−0.2
L-Tyrosine	Tyr	7.2	0.1	7.4	0.7	0.2	7.0	0.4	−0.2	7.4	0.3	0.2	n.d.	n.d.	n.d.
L-Valine	Val	3.5	0.1	5.3	0.5	1.8	3.3	0.2	−0.2	3.4	0.2	−0.1	3.8	0.5	0.3
Nonprotein amino acids															
β-Alanine	β-Ala	−1.5	0.1	−2.0	0.4	−0.5	−1.6	0.2	−0.1	−1.3	0.2	0.2	−1.4	0.5	0.1
γ-Aminobutyric acid	γ-Aba	0.7	0.1	0.4	0.2	−0.3	0.6	0.2	−0.1	0.6	0.3	−0.1	0.9	0.5	0.2
Sarcosine	Sar	−6.0	0.1	−5.8	0.6	0.2	−6.2	0.4	−0.2	−6.1	0.3	−0.1	−5.8	0.3	0.2

Δ*: Difference in $\delta^{15}N$ values determined by EA/IRMS and GC/C/IRMS

our experimental results, which show that the reproducibility increases with the number of phenyl groups in the stationary phases in the DB−35, HP−1MS, and Ultra−2 columns (Table 2). Thus, Chirasil-Val and DB-FFAP are clearly unsuitable for the analysis, and the HP−1MS column is also preferably avoided when determining the nitrogen isotopic composition of amino acids. The other five GC columns−DB−23, DB−35, HP-Chiral−20β, HP-INNOWAX, and Ultra−2−can be used for the isotope analysis of amino acids.

Conclusions

In this study, we optimize several fundamental parameters (temperature of the reaction furnaces, carrier gas flow rate, and sample amount) in the compound-specific nitrogen isotope analysis of amino acids by GC/C/IRMS. The measurement accuracy is 0.3−0.7‰ or more only under the following conditions: combustion temperature, >925°C; carrier gas flow rate, 0.8−1.4 mL min^{-1}; sample amount >30 ngN (~2 nmolN). We also clarify the effect of derivatization (Pv/iPr ester versus TFA/iPr ester) and the type of GC capillary column on the isotope measurements. Although both Pv/iPr and TFA/iPr esters are widely used for the isotope analysis of amino acids, we suggest that the former is more suitable because when using the latter, rapid deterioration of the combustion furnace and capillary connection is observed. Chirasil-Val and DB-FFAP are clearly unsuitable for the analysis, and the HP−1MS column is also preferably avoided when determining the nitrogen isotopic composition of amino acids. This is probably because the stationary phases in these three columns bear electrophilic functionalities. The DB−23, DB−35, HP-Chiral−20β, HP-INNOWAX, and Ultra−2 columns are well suited for the isotope analysis of amino acids.

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