Chapter

*22*

# An improved method for precise determination of carbon isotopic composition of amino acids

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**Abstract**

Compound-specific carbon isotope analysis of amino acids by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) has been employed as a powerful tool in various fields such as biology, physiology, ecology, archaeology, and cosmology studies. However, since amino acids have low volatility and high polarity, they must be derivatized for GC elution. Problems related to derivatization (*i.e.*, significant uncertainty resulting from the incorporation of a large number of carbons, isotopic fractionation during derivatization, and incorporation of heteroatoms in the derivative groups) remain matters of concern. In the present study, we report an improved method, which is a combination of one-step ethyl ester derivatization and GC/C/IRMS analysis employing a GC capillary column coated with a polar stationary phase. With this methods, a high analyte-to-derivative carbon ratio is obtained, and fractionation of the carbon isotopes during derivatization and incorporation of heteroatoms in the derivatives can be avoided. Using this method, the carbon isotopic composition of alanine, valine, leucine, proline, and aspartic acid can be determined with a standard deviation (1σ) of 0.2−0.7‰.

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

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## INTRODUCTION

Stable carbon isotopic composition ($\delta^{13}C$) of amino acids has been widely employed as a powerful tool in various studies, including biological, physiological, ecological, archaeological, and cosmological studies for tracing the source and fate of amino acids and for understanding the process of formation and degradation of amino acids (Macko et al. 1987; Engel et al. 1990; Johnson et al. 1993; Corr et al. 2005; O'Brien et al. 2005; Petzke et al. 2005; McCullagh et al. 2006; Scott et al. 2006; O'Donnel et al. 2007; Uhle et al. 2007). In these studies, compound-specific stable isotope analysis (CSIA) by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) (Hayes et al., 1990; Sessions, 2006) has been frequently adopted for the isotope analysis of amino acids. This is because GC/C/IRMS has high selectivity and sensitivity, which in turn is because of the excellent chromatographic resolution of each amino acid achieved with this method.

However, because of the low volatility and high polarity of amino acids, derivatization is necessary for GC elution. Various derivatives, including acetyl/methyl (Ac/Me) (Corr et al., 2007a, 2007b), acetyl/*n*-propyl (Ac/*n*Pr) (Merritt and Hayes 1994), ethoxycarbonyl/ethyl (EtOC/Et) (Montigon et al. 2001), heptafluorobutyl/isopropyl (HFB/iPr) (Balagopal et al. 1996), pentafluoropropyl/isopropyl (PFP/iPr) (Veuger et al. 2005), pivaloyl/isopropyl (Pv/iPr) (Metges and Daenzer 2000), *tert*-butyl dimethyl silyl (*t*-BDMS) (Shinebarger et al 2002; Hofmann et al., 2003), and trifluoroacetyl/isopropyl (TFA/iPr) (Silfer et al., 1991) esters, are used for the carbon isotope analysis of amino acids by GC/C/IRMS. Unfortunately, because of these derivatizations, several significant problems are encountered during isotope analysis, and hence, the accuracy and precision of the obtained results are always low (Rieley 1994; Docherty et al. 2001; Shinebarger et al., 2002).

First, the incorporation of a large number of carbons in the derivative groups brings about a significant uncertainty in the obtained isotopic composition of the desired amino acids. Generally, the isotopic composition of amino acids ($\delta^{13}C_{AA}$) is determined by using the isotope mass balance calculation from the isotopic composition of the derivatized amino acids ($\delta^{13}C_{DAA}$) and carbons incorporated during the derivatization ($\delta^{13}C_D$), as shown in equation (1):

$$\delta^{13}C_{AA} = (n_{DAA} \times \delta^{13}C_{DAA} - n_D \times \delta^{13}C_D)/n_{AA}, \quad (1)$$

where $n$ is the number of carbon atoms, and the subscripts $AA$, $D$, and $DAA$ indicate amino acids, carbon atoms incorporated during derivatization, and the derivatized amino acids, respectively. In this correction, the error ($\sigma$) in the $\delta^{13}C_{DAA}$ and $\delta^{13}C_D$ values propagates into that in the $\delta^{13}C_{AA}$ values, as shown in equation (2):

$$\sigma_{AA}^2 = \sigma_{DAA}^2 \times (n_{DAA}/n_{AA})^2 + \sigma_D^2 \times (n_D/n_{AA})^2 \quad (2).$$

Therefore, the potential uncertainty in the estimated isotopic composition of amino acids ($\sigma_{AA}$) increases with a decrease in the analyte-to-derivative carbon ratio. For example,



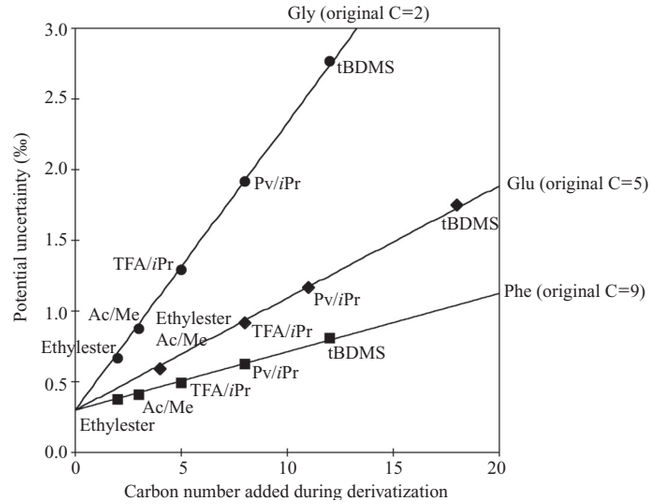


**Fig. 1.** Potential uncertainty in carbon isotopic compositions of amino acids with respect to various types of derivatizations. The uncertainty is calculated by using eq. (2) assuming a standard deviation of 0.3‰ for both the isotopic composition of the incorporated carbon atoms during derivatization and the derivatized amino acids.

TFA/iPr and *t*BDMS derivatizations involve the addition of a minimum of five and twelve derivative carbon atoms, respectively. When the errors in the $\delta^{13}C_{DAA}$ and $\delta^{13}C_D$ values are both 0.3‰, the potential uncertainty in the $\delta^{13}C_{AA}$ values become 0.5‰ for phenylalanine, 0.9‰ for glutamic acids, and 1.9‰ for glycine during TFA/iPr derivatization, and become 0.8‰ for phenylalanine, 1.8‰ for glutamic acids, and 2.8‰ for glycine during *t*BDMS derivatization (Fig. 1).

Second, significant fractionation of carbon isotopes occurs during acylation in Ac/Me, Ac/*n*Pr, EtOC/Et, HFB, PFP/iPr, Pv/iPr, and TFA/iPr derivatizations (Rieley 1994). These derivatizations involve esterification of the carboxylic acid group with acidified alcohol and acylation of the amine group with an acid anhydrate or acid halide. As shown in Fig. 2, the esterification and acylation result in the formation of carbon-oxygen and carbon-nitrogen bonds, respectively; thus, isotope fractionation of the carbonyl carbon may occur in both these reactions. However, since esterification usually proceeds in the presence of excess alcohol and the carbonyl carbon reacts quantitatively, fractionation of carbon isotopes does not occur in this step. In contrast, since acylation involves the use of excess acid and the carbonyl carbon does not react quantitatively, fractionation of carbon isotopes occurs in the derivatization reagent but not in the amino acids of interest. The degree of fractionation depends on the kinetic isotope effect in the reaction and the molar ratio between the amino acid and the derivatization reagent. Silfer et al. (1991) observed such isotopic fractionations

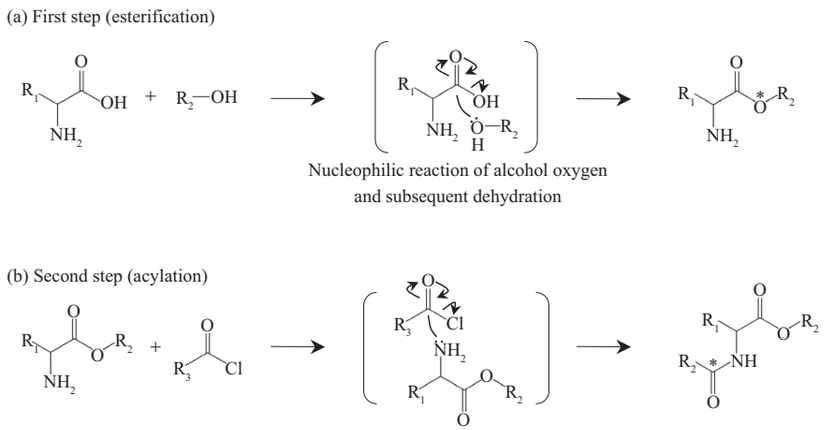


**Fig. 2.** Reaction mechanism of esterification and acylation in amino acid derivatization. Asterisk indicates atoms where significant isotopic fractionation occurs.

during TFA acylation and reported that the resulting error in the estimated carbon isotopic composition of the derivatives ranges −3.3 to −1.0‰ (the error depends on the type of amino acid). Metges and Daenzer (2000) also observed isotopic fractionations during Pv acylation and reported that the resulting errors in the estimated carbon isotopic composition are in the range −4.0 to +2.1‰.

Third, several GC/C/IRMS components may be contaminated by heteroatoms such as silicon (Si) and fluorine (F) present in the derivative groups. For example, the combustion of Si-containing derivatives (*e.g.*, *t*-BDMS) may result in the formation of Si deposits and silicon carbide at the combustion interface used in GC/C/IRMS (Prévost et al. 2001; Shinebarger et al. 2002). Similarly the combustion of F-containing derivatives (*e.g.*, HFB/iPr, PFP/iPr, and TFA/iPr) may result in the formation of stable metal halides such as $Cu_2F$ and $Ni_2F$ at the combustion interface (Hofmann et al., 2003; Metges and Petzke, 1999; Meier-Augenstein, 2004). The formation of these deposits reduces the combustion efficiency and accuracy of the determined isotopic compositions (Chikaraishi et al., 2010).

Although various derivatizations have been utilized for the carbon isotope analysis of amino acids by GC/C/IRMS in previous studies, the problems occurring during derivatization remain issues of concern. Recently, Corr et al. (2007a, 2007b) reported a method based on the combination of Ac/Me derivatization and the correction for the kinetic isotope effect during the derivatization for minimizing the error in the obtained isotopic composition of amino acids. They succeeded in reducing errors in the carbon isotope analysis of amino acids to 0.6‰ for phenylalanine, leucine, and isoleucine, and 1.1‰ for serine and glycine. In the present study, we report an alternative method for the



carbon isotope analysis of amino acids; this method is a combination of one-step ethyl ester derivatization and GC/C/IRMS analysis employing a GC capillary column coated with a polar stationary phase.

## Experimental procedures

### *Reagents and standards*

All the standards, reagents, and solvents were purchased from Wako Pure Chemical Industries Ltd. Ten protein L-amino acid standards, namely, alanine, aspartic acid, glutamic acid, glycine, leucine, phenylalanine, proline, serine, tyrosine, and valine, were used in this study. Ethanol, pivaloyl chloride, isopropanol, and thionyl chloride were used as the derivatization reagents. All the solvents were of dioxin analysis grade.

The carbon isotopic composition of the amino acid standards was determined by the conventional technique using Thermo Fisher Scientific Flash EA (1112EA) coupled to a Delta$^{plus}$XP IRMS *via* a ConFlo III interface (Ohkouchi et al., 2005; Ogawa et al., 2010). The isotopic composition was expressed by the conventional δ notation against an international standard PeeDee Belemnite (PDB). The analytical error (1σ) in the isotopic measurement was less than 0.3‰ (~ 0.1‰ on an average).

Five admixtures of amino acid standards (A to E) were prepared in the present study (Table 1). Standard B was prepared as the decinormal solution of standard A, and standard C was adjusted abundance to similar in total but different in the amino acid compositions from the standard A. Admixtures A, B, and C were used for Pv/iPr derivatization, and D and E were used for ethyl ester derivatization.

### *Derivatization procedure*

*Pivaloyl/isopropyl (Pv/iPr) esters*

Pv/iPr esters of the amino acids were prepared according to the procedure reported by Chikaraishi et al. (2007). In brief, standards A–C were esterified using 1.0 mL of isopropanol/thionyl chloride (4/1, v/v) at 110°C for 2 h. After the esterification, the residual reagents were evaporated under a gentle stream of nitrogen. Pivaloylation was then performed at 110°C for 2 h using 1.0 mL of pivaloyl chloride/dichloromethane (1/1, v/v). After acylation, the residual reagents were again evaporated under a gentle stream of nitrogen. The amino acid derivatives thus obtained were dissolved in 100 mL of dichloromethane and stored at −20°C until further analysis.

*Ethyl esters*

Ethyl esters of the amino acids were prepared according to the improved procedure reported by Chikaraishi et al. (2007). Standards D and E were esterified at 110°C for 2 h using 1.0 mL of ethanol/thionyl chloride (4/1, v/v), and the obtained ester derivatives were stored at −20°C until further analysis. Before the isotope analysis, the residual reagents were evaporated under a gentle stream of nitrogen, and the ethyl esters of the amino acids

**Table 1** Abundance (mg) of each amiono acid in five admixtures of standards used in the present study

| Amino acid | STD-A | STD-B | STD-C | STD-D | STD-E |
|---|---|---|---|---|---|
| Alanine | 2.3 | 0.2 | 2.3 | - | 2.8 |
| Aspartic acid | 3.3 | 0.3 | 0.4 | 3.1 | 5.4 |
| Glycine | 3.2 | 0.3 | 8.5 | 4.0 | – |
| Glutamic acid | 2.4 | 0.2 | 6.9 | 3.0 | – |
| Leucine | 2.4 | 0.2 | 2.4 | – | 4.2 |
| Phenylalanine | 2.8 | 0.3 | 2.8 | – | – |
| Proline | – | – | – | – | 2.7 |
| Serine | 3.6 | 0.4 | 2.6 | – | – |
| Tyrosine | 3.0 | 0.3 | 0.4 | – | – |
| Valine | 3.8 | 0.4 | 0.5 | – | 2.5 |

were redissolved in 100 mL of ethyl acetate. The ethyl acetate solution was stored at −20°C until injection into the GC/C/IRMS system.

The esterified amino acids are stable in acidic solvents but not in neutral solvents (including ethyl acetate), in which they form diketopiperazine condensates. Therefore, in the present study, the acidic reagent (ethanol/thionyl chloride) was evaporated just before the isotope analysis, and the ethyl acetate solution was carefully stored at −20°C until injection for GC/C/IRMS. During this process, diketopiperazine condensates were not detected in the ethyl acetate solution, and ethyl esters of the amino acids were satisfactorily stable during the isotope analysis.

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

Carbon 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* GC-C/TC III interface (Chikaraishi et al., 2008, 2010). Combustion was performed in a microvolume ceramic tube with CuO, NiO, and Pt wires at 1000°C, and reduction was performed in a microvolume ceramic tube with reduced Cu wires at 550°C. A countercurrent dryer (Permeable membrane, Nafion™) was installed between the reduction furnaces and the IRMS instrument to eliminate the $H_2O$ generated during the combustion. 1.0 µl of the amino acid derivatives was injected by using a Gerstel programmable temperature vaporization (PTV) injector using the following temperature program: 0.2 min at the initial temperature (50°C for Pv/iPr esters and 75°C for ethyl esters), heating from the initial temperature to 250°C at the rate of 600°C min$^{-1}$, isothermal hold at 250°C for 10 min, heating to 350°C at the rate of 600°C min$^{-1}$, and isothermal hold at 350°C for 10 min. The carrier gas (He) flow rate in the GC capillary column was controlled at 1.4 mL min$^{-1}$ in the constant flow mode. The amino acid derivatives were analyzed on the following two GC capillary columns and oven temperature programs:

1) HP-Ultra−2 column: 5% phenyl 95% methyl polysiloxane (low polarity) stationary phases; 25 m length × 0.32 mm i.d. × 0.52 µm film thickness, Agilent Technologies. Oven temperature program: 4 min at the initial temperature (60°C), heating from 60°C to 300°C at the rate of 10°C min$^{-1}$, and isothermal hold at 300° C for 10 min (for ethyl esters) or 2 min at the initial temperature (40°C), heating from 40°C 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 (for Pv/iPr esters)
2) HP-INNOWAX column: polyethylene glycol (high polarity) stationary phase, 30 m length × 0.32 mm i.d. × 0.50 µm film thickness; Agilent Technologies. Oven temperature program: 4 min at the initial temperature (60°C), heating from 60°C to 260°C at the rate of 10°C min$^{-1}$, and isothermal hold at 260°C for 20 min.



## Results and discussion

***Isotopic fractionation in carbon isotopes in the Pv/iPr derivatives***

To evaluate whether or not the fractionation in the carbon isotopes during acylation is significant, we first determined the carbon isotopic composition of the Pv/iPr derivatives standards A, B, and C, which have different abundances or amino acids compositions, under identical derivatization conditions. As described above, the degree of fractionation depends on the kinetic isotope effect in the reaction and the molar ratio between the amino acid and the derivatization reagent (Rieley 1994). If isotopic fractionation is insignificant, the determined isotopic composition of the Pv/iPr derivatives should be the same for standards A, B, and C. However, as shown in Fig. 3, the determined isotopic composition of the Pv/iPr derivatives differs considerably among the standards, and the magnitude of the difference depends on the type of amino acid. For example, in the case of glutamic acid, a relatively large difference of 1.2−1.5‰ is observed between the isotopic compositions of the standards, whereas a very small difference is observed for serine. These results are consistent with those reported in the previous studies, i.e., significant fractionation occurs in the carbon isotopes during acylation (Silfer et al. 1991; Metges and Daenzer 2000; Docherty et al. 2001; Corr et al. 2007a, 2007b). Further, our results clearly indicate that control or correction of the fractionation process would be difficult because the degree of fractionation depends on the kinetic isotope effect, which is specific to a given amino acid, the molar ratio between the amino acid and the derivatization reagent, and the amino acid composition.

***Isotope analysis by ethyl ester derivatization***

The first step in the derivatization process, *i.e.*, esterification, helps avoid the problems involved in traditional derivatization methods. This is because a high analyte-to-derivative carbon ratio can be achieved, and fractionation of carbon isotopes does not occur during derivatization; further, heteroatoms are not incorporated in the derivatives. In fact, the formation of ethyl ester derivatives of amino acids involves only the addition of two derivative carbons in the case of neutral amino acids (*e.g.*, glycine and phenylalanine) and the addition of four derivative carbons in the case of acidic amino acids (*e.g.*, aspartic acid). Therefore, the resulting error in the obtained isotopic composition of amino acids is expected

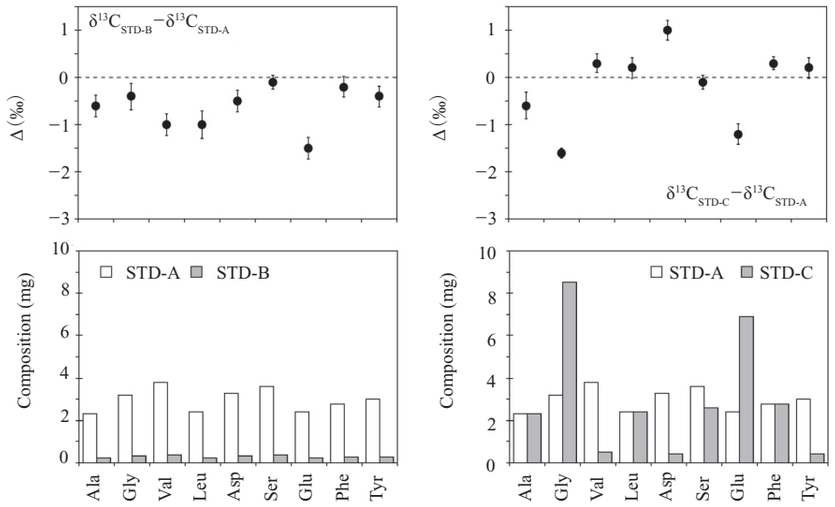


**Fig. 3.** Molecular composition of amino acids in the standards A−C and the difference in the carbon isotopic composition of amino acids as Pv/*i*Pr derivatives, determined by GC/C/IRMS, between these standards.

to be small, i.e., in the range of 0.4‰ (for phenylalanine) to 0.7‰ (for glycine and aspartic acid) (Fig. 1). The disadvantage of this method is poor resolution of the peak due to each amino acid in the GC chromatogram; this is because of the high polarity and low volatility of the ester derivatives. As shown in Fig. 4, a significantly poor resolution of the peaks due to the ethyl esters of the amino acids is observed in the GC/C/IRMS chromatogram when an HP-Ultra−2 GC capillary column (a low-polarity column generally used for amino acid isotope analysis in previous studies) is employed. The isotopic composition of the ethyl esters cannot be determined using such low-resolution chromatograms.

However, this disadvantage can be overcome by employing a GC capillary column coated with a polar stationary phase such as methyl polysiloxane containing a cyanopropyl group (*e.g.*, DB−23, Agilent Technologies) or polyethylene glycol (*e.g.*, HP-INNOWAX and FFAP, Agilent Technologies). In fact, the chromatographic resolution of the ethyl esters of the amino acids is dramatically improved when HP-INNOWAX (Fig. 4) is used, and therefore the carbon isotopic composition of the amino acids can be accurately determined with a standard deviation (1σ) of 0.2−0.7‰ (Table. 2). This analytical error is the lowest reported value for the carbon isotope analysis of amino acids.

In the present study, we demonstrate the isotope analysis of only five amino acids (alanine, valine, leucine, proline, and aspartic acid) using a polar column (HP-INNOWAX). We have not discussed which type of GC column is most suited for the isotope analysis. Because of the low volatility of the ethyl esters of the amino acids, a GC column that can be operated



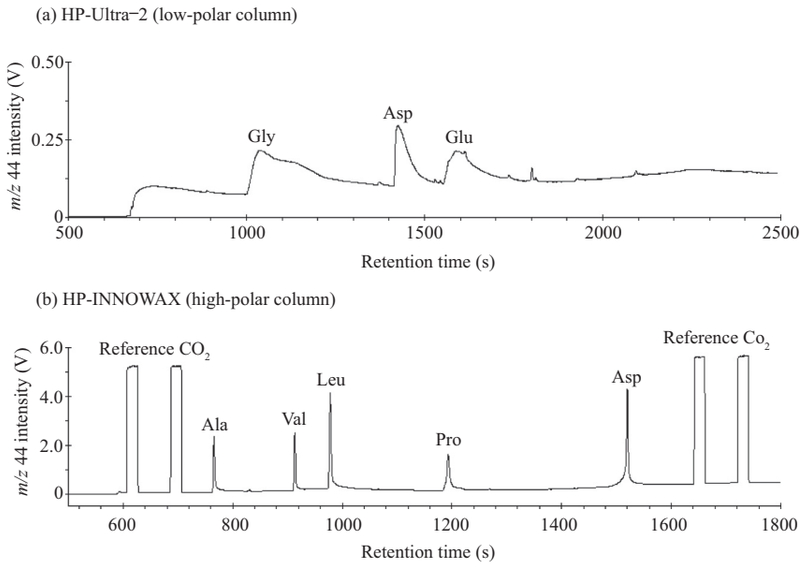


**Fig. 4.** *m/z* 44 chromatograms on GC/C/IRMS analysis for ethyl ester derivatives of amino acids: (a) standard D on the HP-Ultra−2 and (b) standard E on the HP-INNOWAX GC capillary columns.

**Table 2** Comparison of the carbon isotopic composition of amino acids ($\delta^{13}C$, vs PDB) determined by EA/IRMS and GC/C/IRMS

| Amino acid | EA/IRMS | | GC/C/IRMS (N=3)* | | | | $\Delta_{GC\text{-}EA}$ |
|---|---|---|---|---|---|---|---|
| | $\delta^{13}C$ | 1σ | $\delta^{13}C_{AAEt}$ | $1\sigma_{AAEt}$ | $\delta^{13}C_{AA}$ | $1\sigma_{AA}$ | |
| Alanine | −21.9 | 0.1 | −26.5 | 0.3 | −22.0 | 0.5 | −0.1 |
| Aspartic acid | −21.7 | 0.0 | −26.3 | 0.3 | −21.7 | 0.7 | 0.0 |
| Leucine | −13.6 | 0.1 | −19.3 | 0.1 | −13.5 | 0.2 | 0.1 |
| Proline | −15.9 | 0.3 | −21.0 | 0.2 | −16.0 | 0.3 | −0.1 |
| Valine | −23.6 | 0.1 | −26.3 | 0.4 | −23.5 | 0.6 | 0.1 |

*: Carbon isotopic compositon of amino acids ($\delta^{13}C_{AA}$) is corrected by isotopic mass balance calculation between that of amino acid ethyl ester ($\delta^{13}C_{AAEt}$) and of incroporated carbon derived from ethanol during derivatization (−29.0‰). The standard deviation of amino aicds ($1\sigma_{AA}$) is shown as the combination error between that of amino acid ethylester ($1\sigma_{AAEt}$) and of incroporated carbon derived from ethanol during derivatization (0.3‰).

at high temperatures (>300°C) is necessary for the isotope analysis of other amino acids such as glutamic acid and phenylalanine. In the case of HP-INNOWAX, elution of aspartic acid occurs at the maximum column temperature (260°C) (Fig. 4b), implying that at this temperature, glutamic acid may also be eluted from this column, but phenylalanine, lysine, and tyrosine are not. Moderately polar columns such as DB−17ms (maximum temperature:

320°C; Agilent Technologies) and the recently developed, highly inactive GC column HP−5 ultra inert (maximum temperature: 325°C, Agilent Technologies) could be suitable for the carbon isotope analysis. In addition to the selection of a suitable GC column, further optimization of the preparation (*e.g.*, purification and recovery) of amino acid ethyl esters and the instrumental parameters (*e.g.*, temperature of the reaction furnace, carrier gas flow rate, sample amount) related to GC/C/IRMS is necessary before our method can be extensively established as a routine method for the carbon isotope analysis for protein and nonprotein amino acids.

Nevertheless, we conclude that a combination of one-step ethyl ester derivatization of amino acids and GC/C/IRMS analysis using a GC capillary column with a polar stationary phase is well suited for the isotope analysis of amino acids. With further optimization, this method will enable the precise determination of the carbon isotopic composition of amino acids in natural samples.

## Conclusions

In the present study, we report a new method based on the combination of one-step ethyl ester derivatization and GC/C/IRMS analysis using a GC capillary column with a polar stationary phase. With this method, a high analyte-to-derivative carbon ratio can be achieved, fractionation of carbon isotopes during derivatization and incorporation of heteroatoms in the derivatives can be avoided. We demonstrate that by our method, the carbon isotopic composition of alanine, valine, leucine, proline, and aspartic acid can be accurately determined with a standard deviation (1σ) of 0.2−0.7‰, which is the lowest analytical error observed till date. The present method is important for the carbon isotope analysis of amino acids, although further optimization of the method is required before using it for the determination of carbon isotopic composition of amino acids in natural samples.

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## References

Balagopal P, Charles G, Ebenstein DB, Nadeau DA, Sceekumaran Nair K (1996) Mass spectrometric methods for determination of [$^{13}$C]Leucine enrichment in human muscle protein. Anal Biochem 239: 77−85

Chikaraishi Y, Kashiyama Y, Ogawa NO, Kitazato H, Ohkouchi N (2007) Biosynthetic and metabolic controls of nitrogen isotopic composition of amino acids in marine macroalgae and gastropods: Implications for aquatic food web studies. Mar Ecol-Prog Ser 342: 85−90

Chikaraishi Y, Kashiyama Y, Ogawa NO, Kitazato H, Satoh M, Nomoto S, Ohkouchi N (2008) A compound-specific isotope method for measuring the stable nitrogen isotopic composition of tetrapyrroles. Org. Geochem. 39: 510−520

Chikaraishi Y, Takano Y, Ogawa NO, Ohkouchi N (2010) Instrumental optimization for compound-specific nitrogen isotope analysis of amino acids by gas chromatography/combustion/isotope ratio mass spectrometry. In: Earth, Life, and Isotopes (Ohkouchi N, Tayasu I, Koba K, Eds), pp. 367−386, Kyoto: Kyoto University Press (in this volume)

Corr LT, Sealy JC, Horton MC, Evershed RP (2005) A novel marine dietary indicator utilising compound-specific bone collagen amino acid $\delta^{13}C$ values of ancient humans. J Archaeol Sc 32: 321−330

Corr LT, Berstan R, Evershed RP (2007a) 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: 3759−3771

Corr LT, Berstan R, Evershed RP (2007b) Development of N-acetyl methyl ester derivatives for the determination of $\delta^{13}C$ values of amino acids using gas chromatography-combustion-isotope ratio mass spectrometry. Anal Chem 79: 9082−9090

Docherty G, Jones V, Evershed RP (2001) Practical and theoretical considerations in the as chromatography/combustion/isotope ratio mass spectrometry $\delta^{13}C$ analysis of small polyfunctional compounds. Rapid Commun Mass Spectrom 15: 730−738

Engel MH, Macko SA, Silfer JA (1990) Carbon isotope composition of individual amino acids in the Murchison meteorite. Nature 348: 47−49

Hayes JM, Freeman KH, Popp BN, Hoham CH (1990) Compound-specific isotopic analyses: A novel tool reconstruction of ancient biogeochemical processes. Org Geochem 16: 115−1128

Hofmann D, Gehre M, Jung K (2003) Sample preparation techniques for the determination of natural $^{15}N/^{14}N$ variations in amino acids by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Isotopes Environ Health Stud 39: 233−244

Johnson BJ, Fogel ML, Miller GH (1993) Paleoecological reconstructions in southern Egypt based on the stable carbon and nitrogen isotopes in the organic fraction and stable carbon isotopes in individual amino acids of fossil ostrich eggshell. Chem Geol 107: 493−497

Macko SA, Fogel, ML, Hare PE, Hoering TC (1987) Isotopic fractionation of nitrogen and carbon in the synthesis of amino acids by microorganisms. Chem Geol 65: 79−92

McCullagh JSO, Juchelka D, Hedges REM (2006) Analysis of amino acid 13C abundance from human and faunal bone collagen using liquid chromatography/isotope ratio mass spectrometry. Rapid Commun Mass Spectrom 20: 2761−2768

Meier-Augenstein W (2004) GC and IRMS technology for $^{13}C$ and $^{15}N$ analysis on organic compounds and related gases. In de Groot PA (ed) Handbook of Stable Isotope Analytical Techniques, Volume-I, Elsevier, pp. 153−181

Merritt DA, Hayes JM (1994) Nitrogen isotopic analyses by isotope-ratio-monitoring gas chromatography/mass spectrometry. J Am Soc Mass Spectrom 5: 87−397

Metges CC, Petzke KJ (1999) The use of GC-C-IRMS for the Analysis of Stable Isotope Enrichment in

Nitrogenous Compounds. In El-Khoury AE (ed) Methods for Investigation of Amino acid and Protein Metabolism, CRC Press, pp. 121−133

Metges CC, Daenzer M (2000) $^{13}$C gas chromatography-combustion isotope ratio mass spectrometry analysis of *N*-pivaloyl amino acid esters of tissue and plasma samples. Anal Biochem 278: 156−164

Montigon F, Boza JJ, Fay B (2001) Determination of $^{13}$C- and $^{15}$N-enrichment of glutamine by gas chromatography/mass spectrometry and gas chromatography/combustion/isotope ratio mass spectrometry after N(O, S)-ethoxycarbonyl ethyl ester derivatisation. Rapid Commun Mass Spectrom 15: 116−123

O'Brien DM, Boggs CL, Fogel ML (2005) The amino acids used in reproduction by butterflies: A comparative study of dietary sources using compound-specific stable isotope analysis. Physiol Biochem Zool 78: 819−827

O'Donnel TH, Macko SA, Wehmiller JF (2007) Stable carbon isotope composition of amino acids in modern and fossil *Mercenaria*. Org Geochem 38: 485−498

Ogawa NO, Nagata T, Kitazato H, Ohkouchi N (2010) Ultra sensitive elemental analyzer/isotope ratio mass spectrometer for stable nitrogen and carbon isotope analyses. In: Earth, Life, and Isotopes (Ohkouchi N, Tayasu I, Koba K, Eds), pp. 339−353, Kyoto: Kyoto University Press (in this volume)

Ohkouchi N, Nakajima Y, Okada H, Ogawa NO, Suga H, Oguri K, Kitazato H (2005) Biogeochemical processes in a meromictic Lake Kaiike: Implications from carbon and nitrogen isotopic compositions of photosynthetic pigments. Environ Microbiol 7: 1009−1016

Petzke KJ, Boeing H, Metges CC (2005) Choice of dietary protein of vegetarians and omnivores is reflected in their hair protein $^{13}$C and $^{15}$N abundance. Rapid Commun Mass Spectrom 19: 1392−1400

Prévost S, Nicol T, Monteau F, André F, Le Bizec B (2001) Gas chromatography/combustion/isotope ratio mass spectrometry to control the misuse of androhens in breeding animals: New derivatization method applied to testosterone metabolites and precursors in urine samples. Rapid Commun Mass Spectrom 15: 2509−2514

Rieley G (1994) Derivatization of organic compounds prior to gas chromatographic-combustion-isotope ratio mass spectrometric analysis: identification of isotope fractionation processes. Analyst 199: 915−919

Scott JH, O'Brien DM, Emerson D, Sun H, McDonald GD, Salgado A, Fogel ML (2006) An examination of the carbon isotope effects associated with amino acid biosynthesis. Astrobiology 6: 867−880

Sessions AL (2006) Isotope-ratio detection for gas chromatography. J Separation Sci 29: 1946−1961

Shinebarger SR, Haisch M, Matthews DE (2002) Retention of carbon and alteration of expected $^{13}$C-tracer enrichments by silylated derivatives using continuous-flow combustion-isotope ratio mass spectrometry. Anal Chem 74: 6244−6251

Silfer JA, Engel MH, Macko SA, Jumeau EJ (1991) Stable carbon isotope analysis of amino acid enantiomers by conventional isotope ratio mass spectrometry and combined gas chromatography/isotope ratio mass spectrometry. Anal Chem 63: 370−374.

Uhle ME, Sikes EL, Nodder SD, Pilditch CA (2007) Sources and diagenetic status of organic matter in the Hauraki Gulf, New Zealand: Evidence from the carbon isotopic composition of D- and L-amino acids. Org Geochem 38: 440−457

Veuger B, Middelburg JJ, Boschker HTS, Houtekamer M (2005) Analysis of $^{15}$N incorporation into D-alanine: A new method for tracing nitrogen uptake by bacteria. Limnol Oceanogr: Meth 3: 230−240