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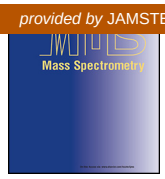

Isolation of underivatized amino acids by ion-pair high performance liquid chromatography for precise measurement of nitrogen isotopic composition of amino acids: Development of comprehensive LC × GC/C/IRMS method

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ABSTRACT

Nitrogen isotopic composition of amino acids has been widely applied to biochemical, ecological, archeological, and biogeochemical studies in an attempt to trace nitrogen source and transformation processes. For accurate isotope analysis of individual amino acids, we validated a preparative method involving the isolation of underivatized amino acids by ion-pair chromatographic separation and confirmed the consistency of nitrogen isotope composition. Ion-pair reversed-phase liquid chromatography coupled with electrospray ionization mass spectrometry (LC/ESI-MS) and gas chromatography/combustion coupled with isotope ratio mass spectrometry (GC/C/IRMS) were conducted for the purpose of separation of underivatized amino acids and nitrogen isotopic analysis, respectively. Firstly, we confirmed the resolution of proteinogenic and non-proteinogenic amino acids by the preparative ion-pair LC separation. Diagnostic product ions determined by mass spectrometry can support the rapid identification of individual amino acids in screening analyses. Secondly, we observed no dependency on nitrogen isotopic composition for the injection amount of underivatized amino acids and even for different chemical formula including neutral, acidic, sulfur-containing, heterocyclic, and aromatic species. The recovery during the LC was 91.7 ± 4.3% (n = 3). The present method and strategy of LC coupled with GC/C/IRMS (i.e., comprehensive LC × GC/C/IRMS) are useful for the high precision determination of the nitrogen isotopic composition of amino acids, in conjunction with an appropriate pre-treatment of cation-exchange chromatographic procedures.

1. Introduction

Ion-pair chromatography, first developed by Schill and coworkers [1,2], is a useful separation technique for organic molecules having cationic, anionic, and zwitterionic characteristics [3–5]. Appropriate ion-pair reagents used with reversed-phase stationary columns in LC have been utilized for the separation of amino organic compounds [6–8]). To optimize the separation of target amino compounds, the ion pairing effects of perfluoroalkyl carboxylic acids including trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA), heptafluorobutyric acid (HFBA), nonafluoropentanoic acid (NFPA), tridecafluoroheptanoic acid (TDFHA), and pentadecafluorooctanoic acid (PDFOA) were verified on a number of reversed-phase columns [5,7,8]. Subsequently, several online detectors including evaporative light scattering detector (ELSD), corona charged aerosol detector (Corona CAD) and electrospray ionization mass spectrometry (ESI-MS) were also comparably validated [8–12].

Focusing on nitrogen isotopic composition, although Tripp et al. reported ion-pair separation of amino acids for isotopic measurements and an isotopically-dispersed validation by elemental analyzer/isotope ratio mass spectrometry (EA/IRMS), resulting the nitrogen isotopic difference (*i.e.*, $\Delta^{15}N$, the difference between $\delta^{15}N_{before}$ and $\delta^{15}N_{after}$ LC separation) ranged from −9.0‰ (vs. Air; valine) to +9.6‰ (vs. Air; proline) (mean, −0.1 ± 4.6‰, n = 13; [13]). Subsequently, Broek et al. also investigated an ion-pair LC procedure using the SiELC Primesep A column with elemental analyzer/isotope ratio mass spectrometry (EA/IRMS) for amino acids (nitrogen isotopic difference, $\Delta^{15}N < 2.33$‰, n = 14) and an application to biological samples by gas

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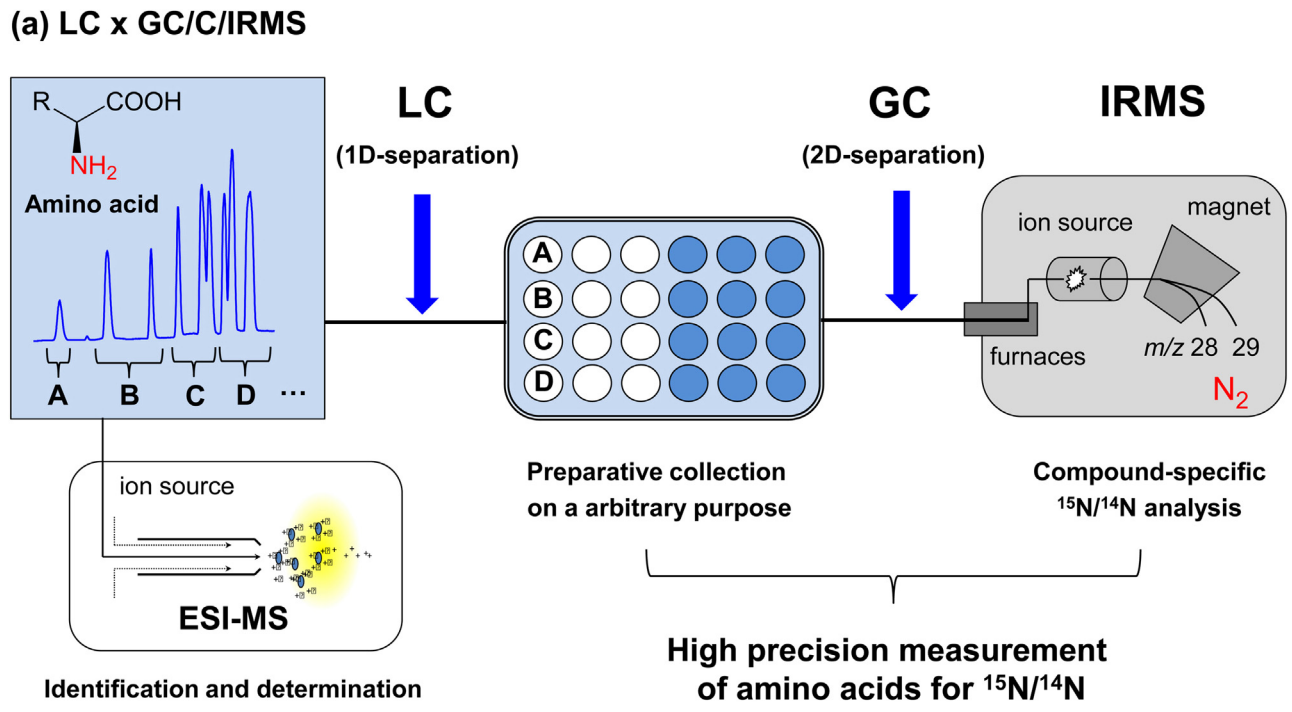
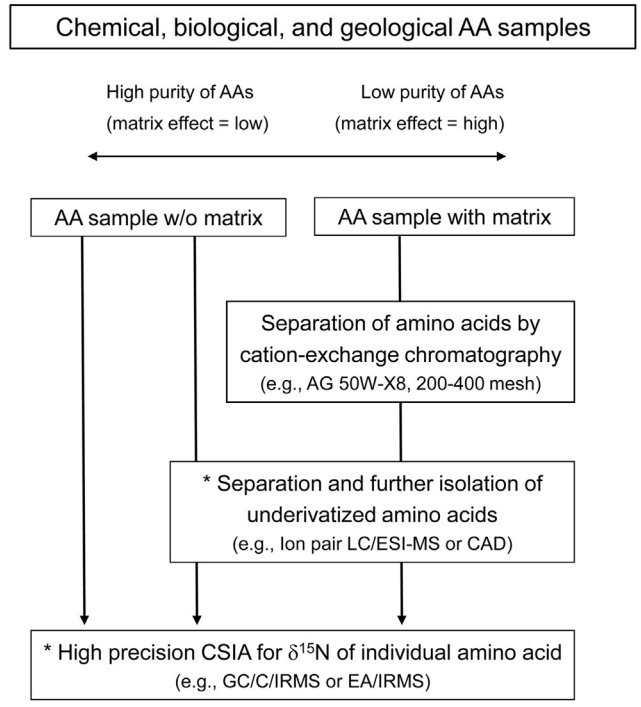




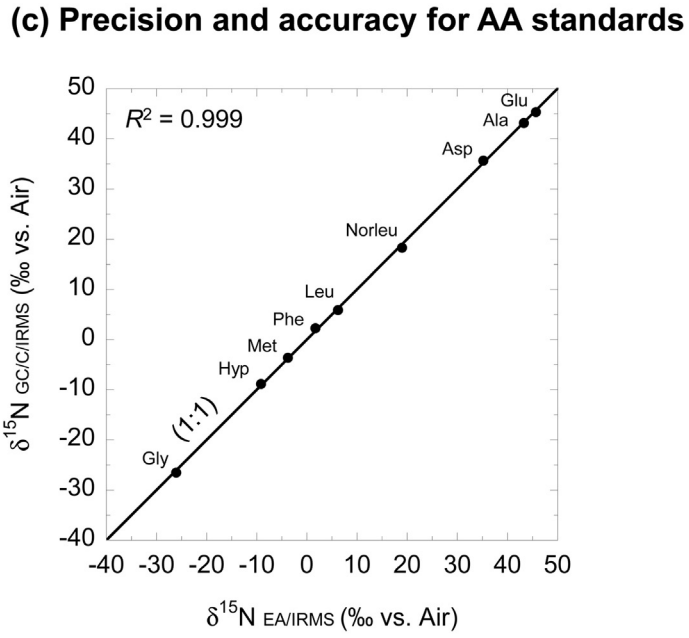


Fig. 1. (a) The concept of comprehensive LC × GC/C/IRMS (liquid chromatography × gas chromatography/combustion/isotope ratio mass spectrometry) for precise measurement of nitrogen isotopic composition of amino acids. (b) Experimental workflow for high-precision CSIA for $\delta^{15}N$ values of amino acids investigated in chemical, biological, and geological samples, with or without (w/o) a matrix effect. The asterisk (*) represents this study. Fractionation and purification of amino acids by cation-exchange chromatography were previously validated using appropriate resins (e.g., AG50W-X8 200–400 mesh [21]; Dowex AG50W-X8 200 mesh (e.g., [39,40])). (c) Precision and accuracy of working standard for GC/C/IRMS by the reference mixtures of 9 amino acids (alanine, glycine, leucine, isoleucine, aspartic acid, methionine, glutamic acid, phenylalanine, and hydroxyproline: each amino acid in 1 sigma range) with known $\delta^{15}N$ values for EA/IRMS (elementary analyzer combined with an isotope ratio mass spectrometry). The summary data is shown in Supplementary Information.

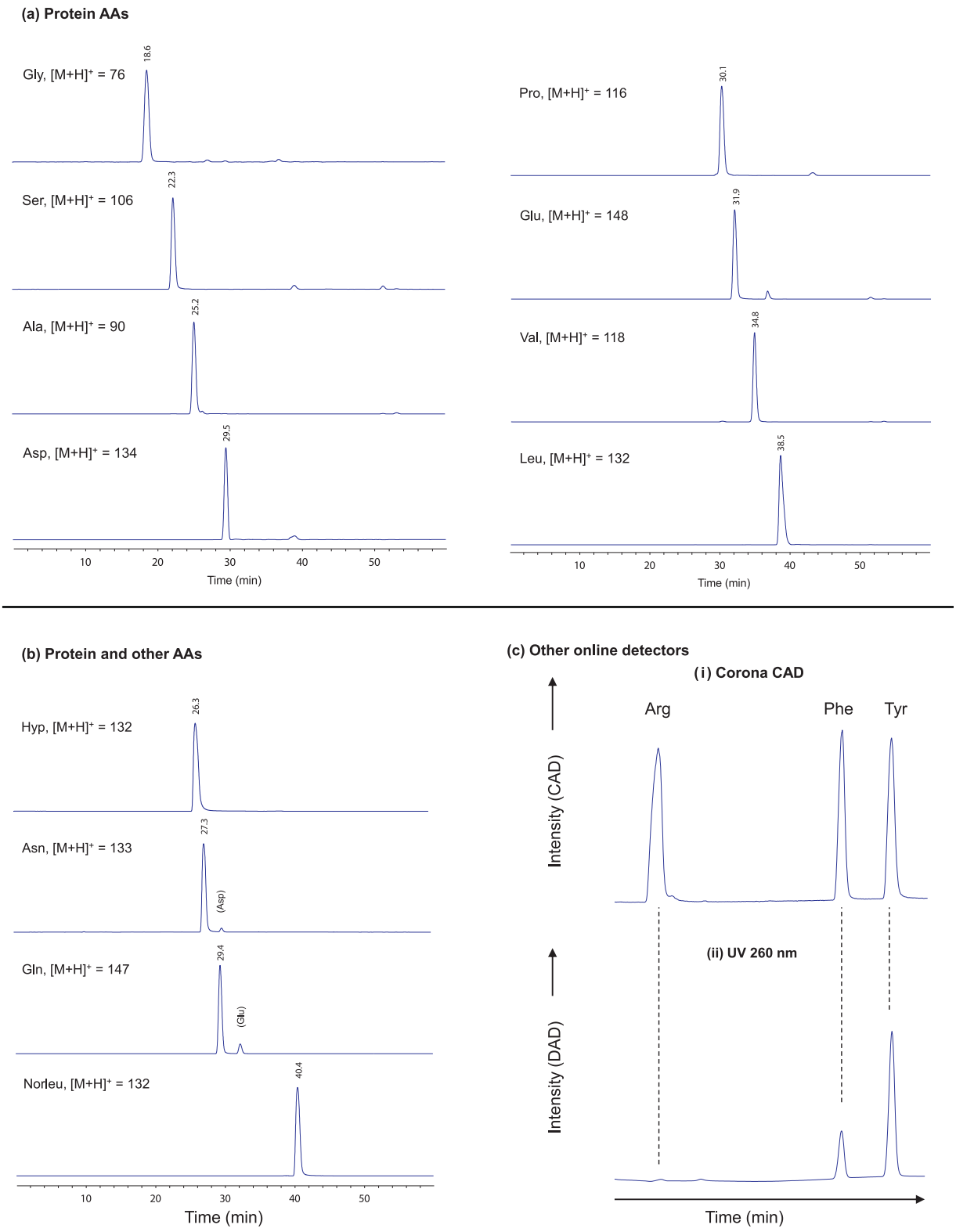


Fig. 2. Representative extracted ion chromatogram (EIC) for LC/ESI–MS analysis of underivatized amino acids. (a) Protein type amino acids. *Abbreviations*: Gly, glycine; Ser, serine; Ala, alanine; Asp, aspartic acid; Pro, proline; Glu, glutamic acid; Val, valine; Leu, leucine. (b) Protein and other amino acids. Small amounts of Asn and Gln were co-injected with Asp and Glu, respectively. Norleu was used as an internal standard for GC/C/IRMS analysis. *Abbreviations*: Hyp, hydroxyproline; Asn, asparagine; Gln, glutamine; Norleu, norleucine. Asn and Gln will convert to Asp and Glu, respectively, after hydrolysis. (c) Comparison of responses determined by other online detectors for corona CAD and photodiode array detector (DAD) for a UV absorbance of 260 nm. The summary data are also shown in Table 1 and Supplementary Information for other non-protein amino acids.

Table 1
Summary of the ion-pair reversed-phase LC for underivatized amino acids showing elution order, chemical formula, molecular weight, parent ion, and fragment (m/z) by electrospray ionization mass spectrometry. In the right-hand column, 'P' and 'NP' represent protein amino acid and non-protein amino acid, respectively. #1, hydroxyproline is one of the important amino acids in collagen protein. #2, #3, Asparagine (Asn) and glutamine (Gln) will convert to aspartic acid (Asp) and glutamic acid (Glu), respectively, after hydrolysis. #4, The chromatographic co-elution of leucine and isoleucine may occur on this ion-pair LC separation. However, if the eluent and gradient program was modified with same ion-pair reagent, leucine and isoleucine were separated as shown in Supplementary Information. Please see other non-protein type amino acids shown in Fig. 3 and Supplementary Information.

Elution order	Abbreviation	Formula	Molecular weight	Retention time (min)	Parent ions $[M+H]^+$	Product ions (m/z)	Remarks
Glycine	Gly	$C_2H_5NO_2$	75	18.6	76	–	P
Serine	Ser	$C_3H_7NO_3$	105	22.3	106	88	P
Alanine	Ala	$C_3H_7NO_2$	89	25.2	90	–	P
Hydroxyproline	Hyp	$C_5H_9NO_3$	131	26.3	132	–	NP (#1)
Threonine	Thr	$C_4H_9NO_3$	119	27.0	120	102, 74	P
Asparagine	Asn	$C_4H_8N_2O_3$	132	27.3	133	116, 87	P (#2)
Glutamine	Gln	$C_5H_{10}N_2O_3$	146	29.3	147	130, 101	P (#3)
Aspartic acid	Asp	$C_4H_7NO_4$	133	29.5	134	116, 88	P
Proline	Pro	$C_5H_9NO_2$	115	30.1	116	–	P
Glutamic acid	Glu	$C_5H_9NO_4$	147	31.9	148	130, 102	P
Valine	Val	$C_5H_{11}NO_2$	117	34.8	118	72	P
Lysine	Lys	$C_6H_{14}N_2O_2$	146	36.6	147	130	P
Leucine	Leu	$C_6H_{13}NO_2$	131	38.5	132	86	P (#4)
Isoleucine	Ile	$C_6H_{13}NO_2$	131	38.5	132	86	P (#4)
Methionine	Met	$C_5H_{11}NO_2S$	149	39.0	150	133, 104	P
Histidine	His	$C_6H_9N_3O_2$	155	40.1	156	110	P
Arginine	Arg	$C_6H_{14}N_4O_2$	174	43.1	175	–	P
Phenylalanine	Phe	$C_9H_{11}NO_2$	165	51.3	166	120	P
Tyrosine	Tyr	$C_9H_{11}NO_3$	181	53.2	182	165, 136	P

chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) [14]. Here, we report the development and validation of a method for underivatized amino acid separation by the ion-pair LC procedure. Then, we focused on the verification of nitrogen isotopic composition by separation, injection volume, the ion pairing interaction, and overall elution procedures using a reversed-phase column. We established a preparative isolation method for the high-precision measurement of the nitrogen isotopic composition of proteinogenic (protein type) and non-proteinogenic (non-protein type) amino acids.

2. Experimental

2.1. Standard and analytical materials

Standard amino acids purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Shanghai Hanhong Chemical Co. (Shanghai, China), Ltd., and Sigma–Aldrich Co. LLC. (Tokyo, Japan) were used: glycine, L-serine, sarcosine, L-alanine, L-hydroxyproline, β-alanine, L-threonine, L-asparagine, N-ethylglycine, L-glutamine, β-aminoisobutyric acid, L-aspartic acid, α-aminoisobutyric acid, γ-aminobutyric acid, L-proline, D,L-α-aminobutyric acid, L-glutamic acid, L-isovaline, L-valine, α-aminoadipic acid, L-norvaline, L-lysine, L-leucine, L-isoleucine, L-methionine, L-histidine, L-norleucine, L-arginine, L-phenylalanine, and L-tyrosine. Each standard amino acid was dissolved in distilled water (pH 1, adjusted by 0.1 M HCl). Nonafluoropentanoic acid (NFPA; molecular weight: 264.05) purchased from Tokyo Chemical Industry Co., Ltd. was used as a volatile ion pairing reagent and a surface active agent (surfactant) for the LC separation of underivatized amino acids. HPLC grade acetonitrile was purchased from Wako Pure Chemical Industries, Ltd. and used as a mobile phase.

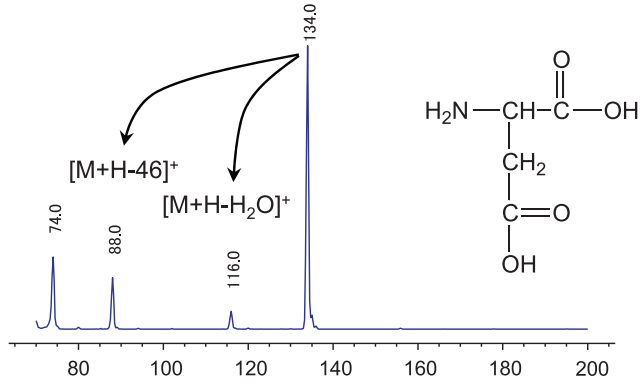
2.2. Separation and detection for underivatized amino acids

For the separation of underivatized amino acids, we used an ion-pair liquid chromatograph (Agilent Technologies Inc., 1100 series; Tokyo, Japan) coupled with either an electrospray ionization mass spectrometer (Agilent Technologies Inc., 1100 series; Tokyo, Japan) or a corona charged aerosol detector (Corona CAD; Dionex K.K./Thermo Fisher Scientific Inc.; Kanagawa, Japan) and a diode array detector (DAD; Agilent Technologies Inc.; Tokyo, Japan). We used a reversed-phase Hypercarb column (4.6 mm × 150 mm, particle size 5 μm; stationary phase, porous graphitic carbon, Thermo Fisher Scientific Inc.; Kanagawa, Japan) and a guard column (4.6 mm × 10 mm, particle size 5 μm; stationary phase as above) with a Cool pocket column cooler (Thermo Fisher Scientific Inc., Kanagawa, Japan). The Hypercarb column is useful over the entire pH range (0–14).

The mobile phase consisted of two solvents. Solvent A was 20 mM NFPA in distilled water, while solvent B was acetonitrile. For conditioning of the ion-pair column, we used overnight elution of solvent A at a flow rate of 0.1 mL min^{-1}. The eluent program for an analysis run employed a linear gradient from 0 min (A: 100%, B: 0%) to 60 min (A: 40%, B: 60%), with a flushing time of 60–70 min using 60% B and the equivalent time of 30 min. The flow rate and column temperature were held constant at 0.2 mL min^{-1} and 10 °C, respectively.

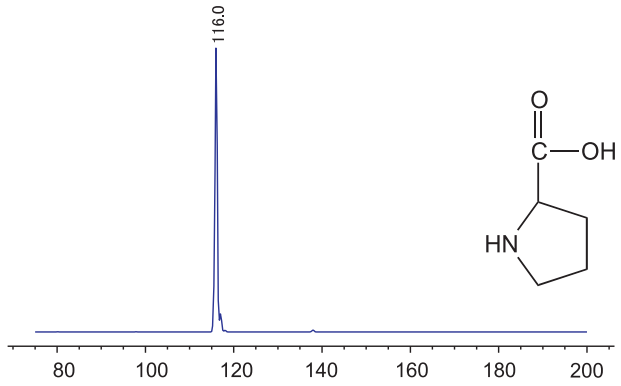
Table 2
Recovery of representative amino acids between before ion-pair LC and after LC. The recovery average during the LC was 91.7 ± 4.3% (n = 3) for the injection of 20 nmol by the GC/NPD multiple run analysis. The initial abundance of each amino acid (without ion-pair LC) was defined as 100%, and the yield of the derivatization reaction (N-pivaloyl *iso*-propyl esters) was also assumed as 100% in both.

Amino acids	Recovery % (Ave. n = 3)	1σ
Neutral		
Glycine	90.8	2.1
Alanine	91.4	4.4
Valine	87.8	3.1
Leucine	92.2	6.2
Isoleucine	90.7	3.1
Acidic		
Aspartic acid	93.8	1.5
Glutamic acid	92.8	1.2
Sulfur containing		
Methionine	85.5	1.1
Heterocyclic		
Proline	89.1	1.9
Hydroxyproline	102.5	1.9
Aromatic		
Phenylalanine	92.3	1.4
Ave.	**91.7 ± 4.3**	



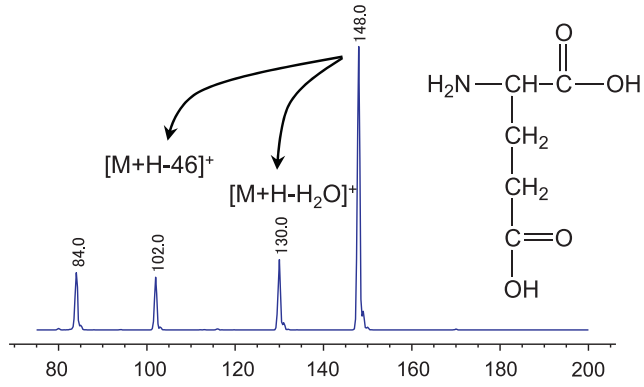
Asp, $[M+H]^+$ = 134

Chemical Formula: $C_4H_7NO_4$
Exact Mass: 133.04
Molecular Weight: 133.10



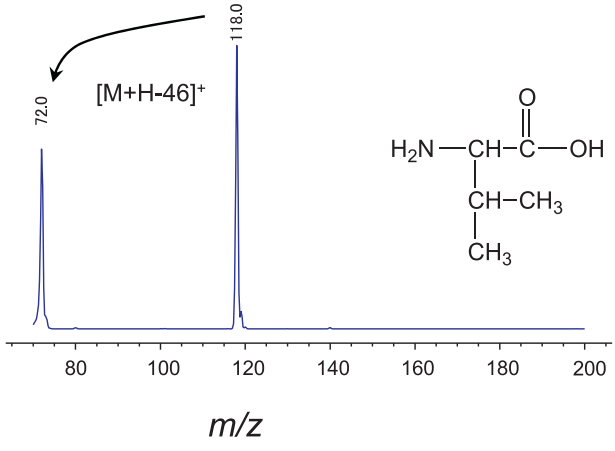
Pro, $[M+H]^+$ = 116

Chemical Formula: $C_5H_9NO_2$
Exact Mass: 115.06
Molecular Weight: 115.13



Glu, $[M+H]^+$ = 148

Chemical Formula: $C_5H_9NO_4$
Exact Mass: 147.05
Molecular Weight: 147.13



Val, $[M+H]^+$ = 118

Chemical Formula: $C_5H_{11}NO_2$
Exact Mass: 117.08
Molecular Weight: 117.15

Fig. 3. Observed product ions by the ESI–MS for the identification of representative underivatized amino acids. The chemical formula, exact mass, molecular weight, and theoretical values of their corresponding masses (m/z) are shown. The data summary is also shown in Table 1 and Supplementary Information for other non-protein amino acids.

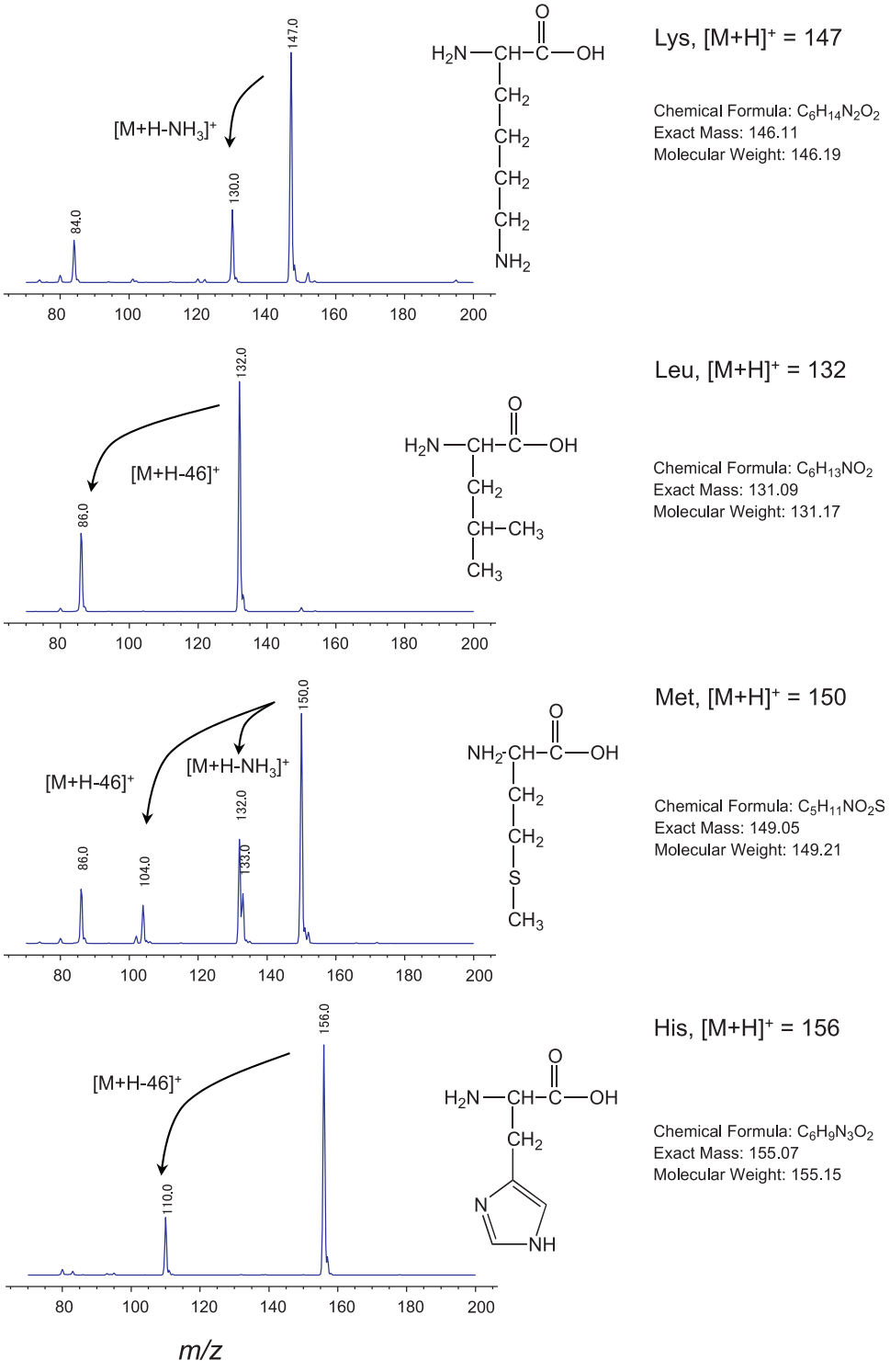


Fig. 3. (*Continued*)

Conditions for the electrospray ionization–mass spectrometry (ESI–MS) were as follows: nebulizer pressure 50 psi, drying gas (N_2) temperature 200 °C, drying gas flow (N_2) 10 L min^{-1}, and capillary voltage 3000 V. A positive ion mode was used with a *m*/*z* range of 70–400 for further extracted ion chromatogram (EIC). The theoretical ion products of underivatized amino acids for $[M+H]^+$ are summarized in Table 1. For corona CAD condition, the nitrogen gas pressure was constantly 35 ± 0.1 psi unit with the corona voltage (<3400 V).

After a preparative collection of underivatized amino acids by the ion-pair LC and subsequent dry-up by nitrogen flow, we conducted the derivatization procedure for *N*-pivaloyl *iso*-propyl esters of amino acids [15–18] (cf. the relationship between GC stationary columns and nitrogen isotopic composition of amino acids; [15]). As it is important to keep combustion efficiency of the CuO/NiO system on GC/C/IRMS [19,20], we carefully eliminate the ion-pair reagents (i.e., fluorinated compounds) by liquid/liquid extraction (i.e., water/organic phase separation) in the

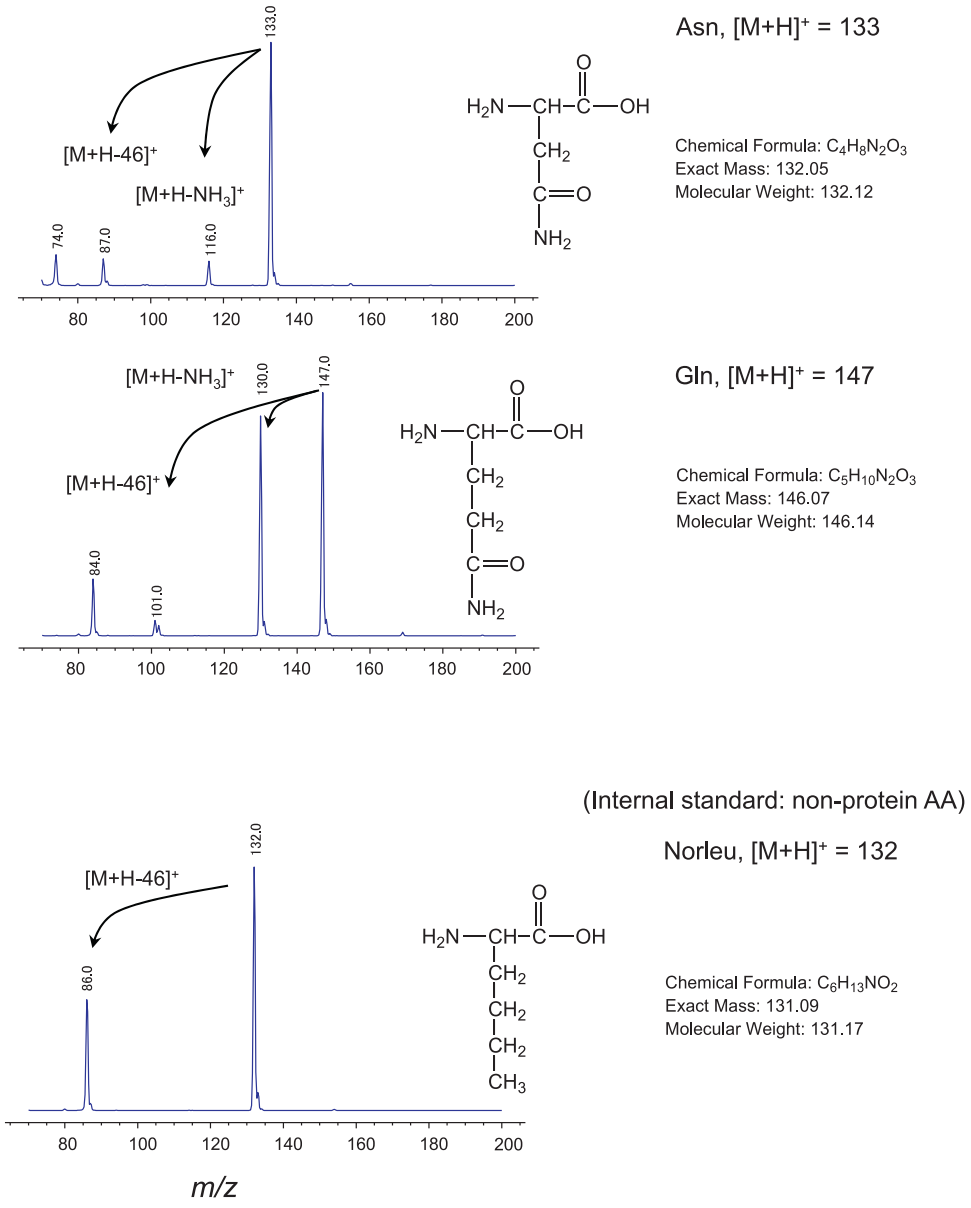


Fig. 3. (*Continued*)

derivatization process prior to GC/C/IRMS analysis [15]. The recovery of representative amino acids between before ion-pair LC and after LC was determined by gas chromatography (GC) using a 6890N GC instrument connected to the flame ionization detector (FID) and nitrogen phosphorus detector (NPD) (Agilent Technologies Inc., Tokyo, Japan). The separation was performed by VF-35ms capillary column (30 m × 0.52 mm; film thickness, 0.50 μm; Agilent Technologies Inc., Tokyo, Japan).

2.3. GC/C/IRMS analysis for N-pivaloyl iso-propyl esters of amino acids

The nitrogen isotopic composition of the individual amino acids (derivatized as *N*-pivaloyl *iso*-propyl esters) was determined using GC/C/IRMS (Thermo Finnigan Delta Plus XP; Thermo Fisher Scientific Inc., Kanagawa, Japan) combined with an Agilent 6890 N GC system (Agilent Technologies Inc., Tokyo, Japan). For the GC separation, we used an Ultra-2 capillary column (25 m × 0.32 mm i.d., 0.52 μm film thickness; stationary phase, 5% phenyl 95% methyl polysiloxane; Agilent Technologies Inc.; Tokyo, Japan) [15,18]. The GC oven temperature was programmed as follows: heating from 40 to 110 °C at a rate of 15 °C min^{-1} after 3 min at the initial temperature, heating from 110 to 150 °C at a rate of 3 °C min^{-1}, 150° to 220 °C at a rate of 6 °C min^{-1}, and then holding isothermally at 220 °C for 17.3 min. The combustion furnace was performed in a micro-volume ceramic tube with CuO, NiO, and Pt wires at 950 °C. The reduction furnace was performed in a micro-volume ceramic tube with a Cu wire at 550 °C. The nitrogen isotopic composition is expressed as the per mil (‰) deviation from the standard (vs. Air), as conventionally defined by the following equation: $\delta^{15}N = [(^{15}N/^{14}N)_{sample}/(^{15}N/^{14}N)_{standard} - 1] \times 1000$. The standard deviation (1σ) of the analytical precision was estimated to be within ±0.4‰ based on the repeated injection of laboratory working standards [15].

3. Results and discussion

3.1. Separation and identification of underivatized amino acids by ion-pair LC

Fig. 1a and b represents the scheme of comprehensive LC × GC/C/IRMS and a workflow diagram for high-precision

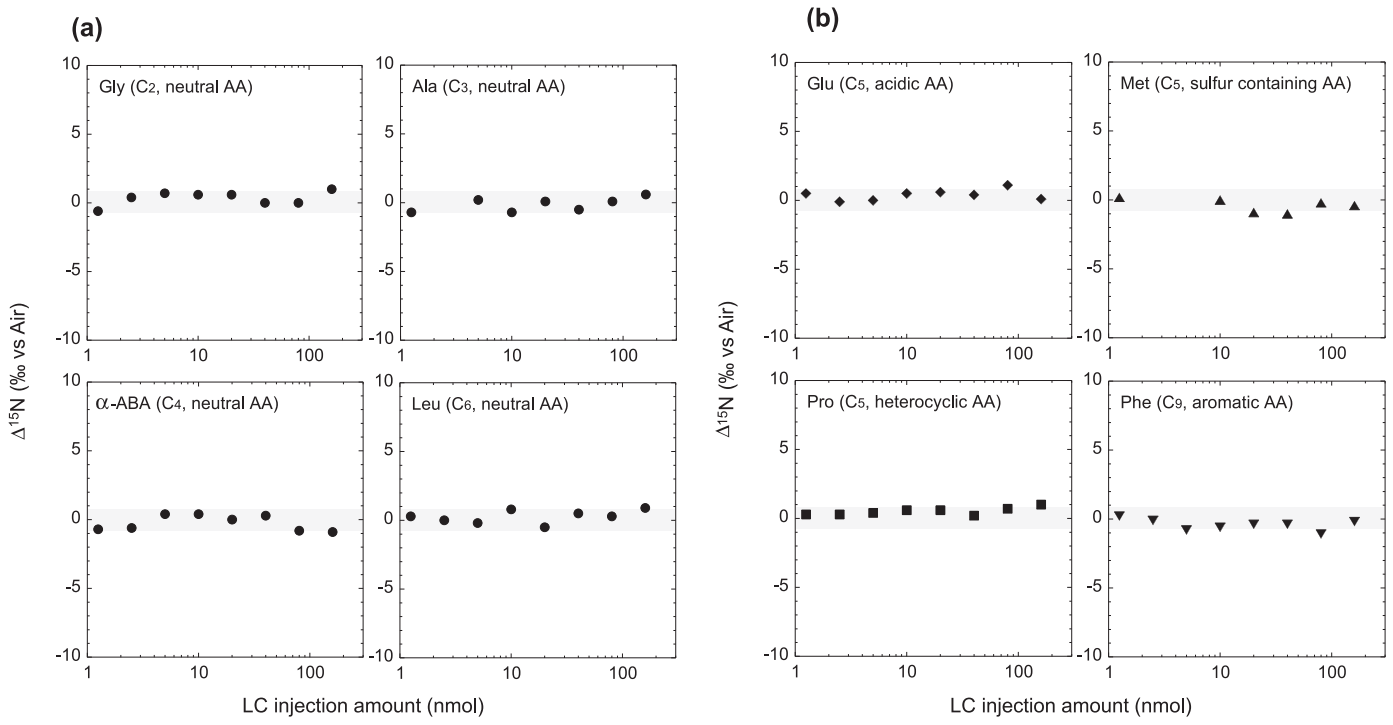


Fig. 4. Relationship between LC injection amount (nmol) for underivatized amino acids and $\Delta^{15}N$ (representing the difference between $\delta^{15}N_{before}$ and $\delta^{15}N_{after}$ LC separation). Results are shown for representative alkyl (e.g., C_2–C_6; Gly, Ala, α-ABA, Leu), acidic (e.g., Glu), sulfur containing (e.g., Met), heterocyclic (e.g., Pro), and aromatic (e.g., Phe) amino acids. The gray layer (zero, normalized as $\Delta^{15}N$) represents the 2σ range (>95% of the mean values) for the precision of the GC/C/IRMS in this verification. The analytical scale of the GC/C/IRMS analysis was approximately 30 ng (ca. 2 nmol N as injected quantity of nitrogen) [15]. The raw data are also shown in Table 3.

measurement of the nitrogen isotopic composition of individual amino acids. To assess the reproducibility of the isotope measurement and obtain the amino acid isotopic composition, reference mixtures of 9 amino acids (alanine, glycine, leucine, isoleucine, aspartic acid, methionine, glutamic acid, phenylalanine, and hydroxyproline; Fig. 1c) with known $\delta^{15}N$ values (ranging from −26.1‰ to +45.7‰, Indiana University, USA and SI science Co., Tokyo Japan) were analyzed after every four to six samples runs.

We previously reported the nitrogen isotopic consistency [21] and practical applications using a cation-exchange resin (Bio-Rad Laboratories AG 50W-X8; 200–400 mesh; Tokyo, Japan) to eliminate possible matrix effects and to purify the amino acid fraction [22–24]. The workflow can be adapted according to the amount of impurities present in a sample. Representative extracted ion chromatograms (positive ion mode, $[M+H]^+$; Fig. 2) of underivatized amino acids (adjusted to pH 1) are shown for the LC/ESI-MS and other detectors (corona CAD and DAD using a UV absorbance at 260 nm). The response of the corona CAD is independent of the chemical structure and constant among amino acids (e.g., [10,11]). Within the protein amino acids, only phenylalanine and tyrosine have sufficient UV absorbance, i.e., DAD is not helpful for other amino acid detection. We confirmed that corona CAD detection is

Table 3
Comparison of the nitrogen isotopic composition of underivatized amino acids ($\delta^{15}N$ ‰ vs. Air) before and after LC injection to validate $\Delta^{15}N$ (the difference between $\delta^{15}N_{before}$ and $\delta^{15}N_{after}$ LC separation).

Amino acid	Cn	Nitrogen isotopic composition of amino acids											
		Before LC injection	After LC injection (nmol)								Average	$\Delta^{15}N$	1σ
		–	1.25	2.5	5	10	20	40	80	160			
		$\delta^{15}N$ (‰ vs. Air)	$\delta^{15}N$ (‰ vs. Air)										
Protein type													
Gly	2	**4.2**	4.9	3.9	3.6	3.6	3.6	4.3	4.2	3.2	**3.9**	**0.3**	**0.5**
Ala	3	**−1.7**	−1.0		−2.0	−1.1	−1.8	−1.2	−1.9	−2.3	**−1.6**	**−0.1**	**0.5**
Ser	3	**−4.8**	−5.2			−3.6	−4.2	−3.4	−3.4	−4.1	**−4.0**	**−0.8**	**0.7**
Asp+Thr	4	**−3.7**	−3.1	−3.1	−3.4	−3.4	−3.4	−2.9	−2.1	−1.7	**−2.9**	**−0.8**	**0.6**
Val	5	**3.5**					3.9	3.4	3.0	3.8	**3.5**	**0.0**	**0.4**
Glu	5	**−4.9**	−5.4	−4.8	−4.9	−5.4	−5.5	−5.3	−6.0	−5.0	**−5.3**	**0.4**	**0.4**
Pro	5	**−0.4**	−0.8	−0.7	−0.8	−1.0	−1.0	−0.6	−1.2	−1.4	**−0.9**	**0.5**	**0.3**
Met	5	**8.0**	7.8			8.0	9.0	9.0	8.2	8.4	**8.4**	**−0.4**	**0.5**
Leu	6	**6.0**	5.8	6.1	6.3	5.3	6.6	5.6	5.8	5.2	**5.8**	**0.2**	**0.5**
Ile	6	**−3.2**	−3.0	−3.8	−3.7	−2.9	−4.0	−3.2	−3.9	−2.3	**−3.4**	**0.2**	**0.6**
Phe	9	**−3.8**	−4.0	−3.7	−3.1	−3.2	−3.5	−3.5	−2.8	−3.6	**−3.4**	**−0.4**	**0.4**
Non-protein type													
α-ABA	4	**−7.4**	−6.7	−6.9	−7.9	−7.9	−7.5	−7.8	−6.6	−6.5	**−7.2**	**−0.2**	**0.6**
Hyp	5	**−8.4**	−7.3	−8.1	−7.8	−7.6	−8.8	−8.1	−8.3	−8.6	**−8.1**	**−0.3**	**0.5**
Norleu	6	**18.0**				17.9	18.5	18.0	18.0	18.5	**18.2**	**−0.2**	**0.3**

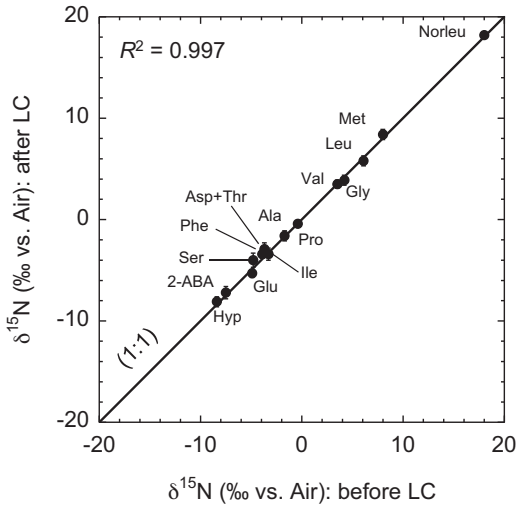


Fig. 5. Experimental verification of data consistency for compound-specific nitrogen isotope composition ($\delta^{15}N$, ‰ vs. Air). This is shown for before LC (*x*-axis) and after LC (*y*-axis) using ion-pair reversed-phase liquid chromatography (Hypercarb column, 4.6 mm × 150 mm, 5 μm; Thermo Fisher Scientific Inc.) and the analytical conditions (determined in the Method section). For practical analysis scale for GC/C/IRMS, the experimental plots (>1.25 nmol) were used. The correlation coefficient was $R^2 = 0.994$. All the raw data are also listed in Table 3.

useful for detecting and fraction collection of underivatized amino acids for further compound-specific analysis.

ESI–MS spectra are useful for the rapid identification, screening, and diagnosis of underivatized amino acids using their retention times and corresponding product ion(s) (Fig. 3 and Supplementary Information). In the ESI-MS analysis of neutral alkyl amino acids (e.g., Val, Norval, Isoval, Leu and Norleu), abundant protonated molecule and their product ions [M+H − 46] which corresponds to neutral formic acid loss (—HCOOH) were observed. Ammonia product for [M+H − 17] was also observed in basic amino acids (e.g., Lys, Asn and Gln). Furthermore, loss of water [M+H − 18] was observed for some acidic amino acids including Ser, Thr, Asp and Glu. Tracing the retention time and specific ion transitions, each underivatized amino acid was identifiable on the ion-pair LC/ESI-MS. A co-injection of threonine ($[M+H]^+ = 120$) and phenylalanine ($[M+H]^+ = 166$) for $[M+H-46]^+$ (= 120) showed the same ion (*m*/*z* 120) at differing retention times. Table 1 summarizes the elution order, retention times, and fragment ions of ESI–MS for protein and non-protein amino acids.

3.2. Nitrogen isotopic compositions of amino acids before and after LC separation

The difference of nitrogen isotopic composition of amino acids before and after the ion-pair LC, where $\Delta^{15}N$ represents the difference between $\delta^{15}N_{before}$ and $\delta^{15}N_{after}$ LC separation are shown in Fig. 4. The injection volume of underivatized amino acids ranged from 1 to 100 nmol on the ion-pair LC. The recovery average during the LC was 91.7 ± 4.3% ($n = 3$) for the injection of representative amino acids (Table 2). Fig. 4a represents the consistency of nitrogen isotopic compositions for alkyl amino acids including Gly (C_2), Ala (C_3), α-ABA (C_4), and Leu (C_6). We also confirmed this consistency for acidic (Glu, C_5), sulfur-containing (Met, C_5), heterocyclic (Pro, C_5), and aromatic amino acids (Phe, C_9) (Fig. 4b). Consequently, the nitrogen isotopic composition of these underivatized amino acids is independent of injection volume onto the ion-pair LC column. Hare et al. (1991) reported a large isotopic variability ($\delta^{15}N > 30‰$) within a chromatographic peak of glycine during LC separation with a resin (St. John Associates, Adelphi, MD, USA) [25]. Therefore, to precisely determine the nitrogen isotopic composition, baseline resolution of amino acids and careful isolation of the entire peak should be required prior to further GC analysis (Table 3).

The comparison of nitrogen isotopic compositions of underivatized amino acids between before and after the ion-pair LC separation (>1.25 nmol) indicated good correlation with the mean of difference ($\Delta^{15}N$) within −0.1% ($R^2 = 0.997$ in 14 amino acids; Fig. 5). Given this close correlation, the nitrogen isotope compositions for the amino acids are independent of the chemical structure of the investigated amino acids. The present ion-pair LC and offline IRMS method can compensate a ^{15}N assessment of minor amino acids (e.g., Met, Pro) and the resolution of some amino acids (e.g., Asp, almost co-elution with Thr on an Ultra-2 capillary column; [15]) by GC.

4. Implication and perspectives

This analytical procedure using LC × GC/C/IRMS is applicable to the high-precision analysis of amino acids obtained from microbial, ecological, and biogeochemical materials [26–29], if high-resolution fingerprinting of amino acids is necessary. The method is also applicable to fossil materials preserved in hard tissues, as commonly examined in archeological and paleo-dietary research [30–33]. Amino acids contain chiral center for their D- and L-enantiomers with some exceptions (e.g., glycine in protein type and α-aminoisobutyric acid in non-protein type). The preparative isolation procedure demonstrated here is thus useful for the accurate evaluation of D- and L-enantiomers when chiral separation by further GC or LC analysis is employed. In this manner, accurate evaluation of D- and L-enantiomers of amino acids originated from pristine abiotic processes [34–38] could be possible for opening up the possibility of high-precision enantiomer-specific isotope analysis (ESIA). The present results contribute to the refinement of nitrogen isotope analysis in determining the biotic or abiotic origin of amino acids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijms.2014.11.012.

References

[1] S. Eksborg, G. Schill, Ion pair partition chromatography of organic ammonium compounds, Anal. Chem. 45 (1973) 2092–2100.
[2] S. Eksborg, P.O. Lagerström, R. Modin, G. Schill, Ion-pair chromatography of organic compounds, J. Chromatogr. 83 (1973) 99–110.
[3] R. Gloor, E.L. Johnson, Practical aspects of reverse phase ion pair chromatography, J. Chromatogr. Sci. 15 (1977) 413–423.
[4] J.H. Knox, R.A. Hartwick, Mechanism of ion-pair liquid chromatography of amines, neutrals, zwitterions and acids using anionic hetaerons, J. Chromatogr. 204 (1981) 3–21.
[5] T. Cecchi, Ion pairing chromatography, Critical Rev. Anal. Chem. 38 (2008) 161–213.
[6] K. Petritis, P. Chaimbault, C. Elfakir, M. Dreux, Ion-pair reversed-phase liquid chromatography for determination of polar underivatized amino acids using perfluorinated carboxylic acids as ion pairing agent, J. Chromatogr. A 833 (1999) 147–155.

[7] P. Chaimbault, K. Petritis, C. Elfakir, M. Dreux, Ion-pair chromatography on a porous graphitic carbon stationary phase for the analysis of twenty underivatized protein amino acids, J. Chromatogr. A 870 (2000) 245–254.
[8] M. Armstrong, K. Jonscher, N.A. Reisdorph, Analysis of 25 underivatized amino acids in human plasma using ion pairing reversed phase liquid chromatography/time of flight mass spectrometry, Rapid Commun. Mass Spectrom. 21 (2007) 2717–2726.
[9] K. Petritis, P. Chaimbault, C. Elfakir, M. Dreux, Parameter optimization for the analysis of underivatized protein amino acids by liquid chromatography and ionspray tandem mass spectrometry, J. Chromatogr. A 896 (2000) 253–263.
[10] J. Qu, Y. Wang, G. Luo, Z. Wu, C. Yang, Validated quantitation of underivatized amino acids in human blood samples by volatile ion-pair reversed-phase liquid chromatography coupled to isotope dilution tandem mass spectrometry, Anal. Chem. 74 (2002) 2034–2040.
[11] R.W. Dixon, D.S. Peterson, Development and testing of a detection method for liquid chromatography based on aerosol charging, Anal. Chem. 74 (2002) 2930–2937.
[12] D.L. Liu, L.W. Beegle, I. Kanik, Analysis of underivatized amino acids in geological samples using ion-pairing liquid chromatography and electrospray tandem mass spectrometry, Astrobiology 8 (2008) 229–241.
[13] J. Tripp, J. McCullagh, R. Hedges, Preparative separation of underivatized amino acids for compound-specific stable isotope analysis and radiocarbon dating of hydrolyzed bone collagen, J. Sep. Sci. 29 (2006) 41–48.
[14] T.A. Broek, B.D. Walker, D.H. Andreasen, M.D. McCarthy, High-precision measurement of phenylalanine $\delta^{15}N$ values for environmental samples: a new approach coupling high-pressure liquid chromatography purification and elemental analyzer isotope ratio mass spectrometry, Rapid Commun. Mass Spectrom. 27 (2013) 1–11.
[15] Y. Chikaraishi, Y. Takano, N.O. Ogawa, N. Ohkouchi, Instrumental optimization for compound-specific nitrogen isotope analysis of amino acids by gas chromatography/combustion/isotope ratio mass spectrometry, in: N. Ohkouch, I. Tayasu, K. Koba (Eds.), Earth, Life, and Isotopes, Kyoto Univ. Press, Kyoto, 2010, pp. 367–386.
[16] C.C. Metges, K.J. Petzke, U. Hennig, Gas chromatography combustion isotope ratio mass spectrometric comparison of N-acetyl- and N-pivaloyl amino acid esters to measure ^{15}N isotopic abundances in physiological samples: a pilot study on amino acid synthesis in the upper gastro-intestinal tract of minipigs, J. Mass Spectr. 31 (1996) 367–376.
[17] L.T. Corr, R. Berstan, R.P. Evershed, Optimisation of derivatisation procedures for the determination of delta C-13 values of amino acids by gas chromatography/combustion/isotope ratio mass spectrometry, Rapid Commun. Mass Spectrom. 21 (2007) 3759–3771.
[18] Y. Chikaraishi, N.O. Ogawa, Y. Kashiyama, Y. Takano, H. Suga, A. Tomitani, H. Miyashita, H. Kitazato, N. Ohkouchi, Determination of aquatic food-web structure based on compound-specific nitrogen isotopic composition of amino acids, Limnol. Oceanogr. Methods 7 (2009) 740–750.
[19] C.C. Metges, J.K. Petzke, The use of GC-C-IRMS for the analysis of stable isotope enrichment in nitrogenous compounds, in: A.E. El-khoury (Ed.), Methods for Investigation of Amino Acid and Protein Metabolism, CRC Press LCC, Boca Raton, FL, 1999, pp. 121–132.
[20] W. Meier-Augenstein, Handbook of Stable Isotope Analytical Techniques, vol. I, Elsevier, Amsterdam, 2004 (Chapter 8).
[21] Y. Takano, Y. Kashiyama, N.O. Ogawa, Y. Chikaraishi, N. Ohkouchi, Isolation and desalting with cation-exchange chromatography for compound-specific nitrogen isotope analysis of amino acids, Rapid Commun. Mass Spectrom. 24 (2010) 2317–2323.
[22] Y. Takano, Y. Chikaraishi, N.O. Ogawa, H. Kitazato, N. Ohkouchi, Compound-specific nitrogen isotope analysis of D-L-alanine and valine: application of diastereomer separation to $\delta^{15}N$ and microbial peptidoglycan studies, Anal. Chem. 81 (2009) 394–399.
[23] Y.I. Naito, Y. Chikaraishi, N. Ohkouchi, M. Yoneda, Evaluation of carnivory in inland Jomon hunter–gatherers based on nitrogen isotopic compositions of individual amino acids in bone collagen, J. Archaeol. Sci. 40 (2013) 2913–2923.
[24] N. Ohkouchi, R. Tsuda, Y. Chikaraishi, K. Tanabe, A preliminary estimate of the trophic position of the deep-water ram's horn squid *Spirula spirula* based on the nitrogen isotopic composition of amino acids, Mar. Biol. 160 (2013) 773–779.
[25] P. Hare, M. Fogel, T. Stafford, A. Mitchell, T. Hoering, The isotopic composition of carbon and nitrogen in individual amino acids isolated from modern and fossil proteins, J. Archaeol. Sci. 18 (1991) 277–292.
[26] H.M. Engel, S.A. Macko, Organic Geochemistry: Principles and Applications, Plenum Press, New York, 1993.
[27] J.M. McClelland, J.P. Montoya, Trophic relationships and the nitrogen isotopic composition of amino acids in plankton, Ecology 83 (2002) 2173–2180.
[28] R. Michener, K. Lajtha, Stable Isotopes in Ecology and Environmental Science, Blackwell Publishing, Oxford, 2007.
[29] N. Ohkouchi, Y. Takano, Organic nitrogen: sources, fates, and chemistry, in: B. Birrer, P. Falkowski, K. Freeman (Eds.), Treatise on Geochemistry, vol. 12: Organic Geochemistry, Elsevier, 2014, pp. 251–289.
[30] A.G. Goodfriend, M.J. Collins, M.L. Fogel, S.A. Macko, J.F. Wehmiller, Perspectives in Amino acid and Protein Geochemistry, Oxford Univ. Press, New York, 2000.
[31] M. Collins, C. Nielsen-Marsh, J. Hiller, C. Smith, J. Roberts, R. Prigodich, T. Wess, J. Csapo, A. Millard, G. Turner-Walker, The survival of organic matter in bone: a review, Archaeometry 44 (2002) 383–394.
[32] R.P. Evershed, I.D. Bull, L.T. Corr, M.Z. Crossman, B.E. van Dongen, C.J. Evans, S. Jim, H.R. Mottram, A.J. Mukherjee, R.D. Pancost, Compound-specific stable isotope analysis in ecology and paleoecology, in: R. Michener, K. Lajtha (Eds.), Stable Isotopes in Ecology and Environmental Science, Blackwell Publishing, Oxford, 2007, pp. 480–540.
[33] O.A. Sherwood, M.F. Lehmann, C.J. Schubert, D.B. Scott, M.D. McCarthy, Nutrient regime shift in the western North Atlantic indicated by compound-specific $\delta^{15}N$ of deep-sea gorgonian corals, Proc. Natl. Acad. Sci. 108 (2011) 1011–1015.
[34] J. Cronin, S. Chang, Organic matter in meteorites: molecular and isotopic analyses of the Murchison meteorite, in: J.M. Greenberg, C.X. Mendoza-Gómez, V. Pirronello (Eds.), The Chemistry of Life's Origins, Kluwer Academic Publishers, Dordrecht, 1993, pp. 209–258.
[35] U. Meierhenrich, Amino Acids and the Asymmetry of Life, Springer, Berlin, 2008.
[36] D.P. Glavin, M.P. Callahan, J.P. Dworkin, J.E. Elsila, The effects of parent body processes on amino acids in carbonaceous chondrites, Meteor. Planet. Sci. 45 (2011) 1948–1972.
[37] C. Meinert, P. de Marcellus, L. Le Sergeant d'Hendecourt, L. Nahon, N.C. Jones, S.V. Hoffmann, J.H. Bredehöft, U.J. Meierhenrich, Photochirogenesis: photochemical models on the absolute asymmetric formation of amino acids in interstellar space, Phys. Life Rev. 8 (2011) 307–330.
[38] Y. Takano, Y. Chikaraishi, N. Ohkouchi, Enantiomer-specific isotope analysis (ESIA) of D- and L-alanine: nitrogen isotopic hetero- and homogeneity by microbial and chemical processes, in: N. Ohkouchi, I. Tayasu, K. Koba (Eds.), Earth, Life, and Isotopes, Kyoto Univ. Press, Kyoto, 2010, pp. 387–402.
[39] C.C. Metges, K.J. Petzke, Measurement of N-15/N-14 isotopic composition in individual plasma free amino acids of human adults at natural abundance by gas chromatography combustion isotope ratio mass spectrometry, Anal. Biochem. 247 (1997) 158–164.
[40] A.K. Styring, A. Kuhl, T.D.J. Knowles, R.A. Fraser, A. Bogaard, R.P. Evershed, Practical considerations in the determination of compound-specific amino acid $\delta^{15}N$ values in animal and plant tissues by gas chromatography-combustion–isotope ratio mass spectrometry, following derivatisation to their N-acetyl isopropyl esters, Rapid Commun. Mass Spectrom. 26 (2012) 2328–2334.