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Isolation and desalting with cation-exchange chromatography for compound-specific nitrogen isotope analysis of amino acids: application to biogeochemical samples

Yoshinori Takano[1]*, Yuichiro Kashiyama[1]†, Nanako O. Ogawa[1], Yoshito Chikaraishi[1] and Naohiko Ohkouchi[1,2,3]

[1]Institute of Biogeosciences, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima, Yokosuka, Kanagawa 237-0061, Japan
[2]Department of Environmental Science and Technology, Tokyo Institute of Technology, 4259 Nagatsuda, Midori-ku, Yokohama, Kanagawa 226-8502, Japan
[3]Department of Earth and Planetary Sciences, University of Tokyo, 7-3-1 Hongo, Tokyo 113-0033, Japan

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We have established a procedure for removing interfering materials from extracts of geological and biological samples, in order to determine precise compound-specific nitrogen isotopic compositions of amino acids. We employed cation-exchange chromatography of protein and non-protein amino acids prior to derivatization for gas chromatographic separation. The average recovery of a standard amino acid solution was better than 94%, without nitrogen isotope fractionation during the cation-exchange chromatography. We applied the procedure to various environmental samples including 'hard' (calcareous, siliceous, rock and sediment samples) and 'soft' materials (aggregated microbial samples and biological soft tissue samples). We conclude that cation-exchange chromatography is a pre-treatment procedure which should be widely useful for the determination of compound-specific nitrogen isotopic compositions of amino acids. Copyright © 2010 John Wiley & Sons, Ltd.

Since the initial work of Abelson,[1] organic constituents in fossil material have been brought into focus with respect to geochemical information content. Proteins are fundamental building blocks of life and are composed of amino acids (AAs). Consequently, the preserved AAs derived from present and past biota, as well as their isotopic compositions, are potentially useful tools for understanding the dietary food web, ecology, and archaeological aspects of such biota.[2,3] However, the determination of AAs in calcareous, siliceous, microbial and sedimentary samples is troublesome, since extracts of these samples often contain a significant amount of interfering organic and inorganic substances.[e.g.,4,5] Therefore, there is a need to eliminate these interferences in order to purify AAs. Cation-exchange resins made up of sulfonic acid functional groups attached to a styrene divinyl benzene copolymer lattice are known to be useful for concentrating cationic solutes in a single-step purification procedure.[6] However, Macko *et al*. pointed out that nitrogen isotopic fractionation could occur during ion-exchange chromatography of AAs (resin: St. John Associates 9.5X).[7] Hare *et al*. reported that nitrogen isotopic fractionation of glycine ranged over 30‰ during separation using column chromatography (resin: St. John Associates).[8] Furthermore, elution of AA fractions by cation-exchange chromatography requires the use of strong solutions of ammonia. Hence, it is also necessary to determine possible nitrogen isotopic fractionation among amino moieties of AAs, desorption from cation resin and the ammonia elution procedure (see 'stationary column and nitrogen isotopic composition of amino acids'[9]).

In order to determine the nitrogen isotopic composition of AAs using gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS), carboxyl and amino groups in molecules have to be derivatized to increase the volatility. Pre-treatment of an extract has two advantages: (i) separation from complex hydrophilic compounds, including sugars and organic acids, which consume derivatization reagents during esterification/etherification; and (ii) desalting of inorganic compounds derived from calcareous and siliceous minerals, thereby preventing damage to the combustion and reduction furnaces in the GC/C/IRMS system. Here, we establish a method for the elimination of multiple interfering complex compounds from extracts of environmental samples in order to allow the precise determination of the nitrogen isotopic composition of AAs.

**Correspondence to*: Y. Takano, Institute of Biogeosciences, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima, Yokosuka, Kanagawa 237-0061, Japan.
E-mail: takano@jamstec.go.jp
† Present address: Department of Chemistry, University of Tsukuba, Tsukuba 305-8571, Japan.

Table 1. Samples analyzed after applying the described cation-exchange chromatographic procedure, including 'hard' (inorganic matrix) and 'soft' material (organic matrix)

Sample profiles			GC separation (after cation-exchange chromatography)		
Type Name	Age	Location	Baseline resolution (applicable to GC/C/IRMS)	Derivative products	Sample information
Hard materials (inorganic matrix)					
Calcareous sample					
Larger benthic foraminifera (*Calcarina gaudichaudii*)	modern	Okinawa, Japan	Yes	*N*-pivaloly *iso*-propyl esters	this study
Outer shell of decapoda (*Caridina multidentata*)	modern	Kanagawa, Japan	Yes	*N*-pivaloly *S*-(+)-2-butyl esters & *N*-pivaloly *iso*-propyl esters	[9]
Outer shell of decapoda (*Plagusia dentipes*)	modern	Kanagawa, Japan	Yes	*N*-pivaloly *S*-(+)-2-butyl esters & *N*-pivaloly *iso*-propyl esters	[9]
Archaeological human bone	middle Holocene	Hokkaido, Japan	Yes	*N*-pivaloly *iso*-propyl esters	[17]
Egg shell (*Coturnix japonica*)	modern	Culture	Yes	*N*-pivaloly *iso*-propyl esters	this study
Egg shell (*Gallus gallus*)	modern	Culture	Yes	*N*-pivaloly *iso*-propyl esters	this study
Snail shell (*Cernina fluctuata*)	modern	Cuyo, Phillipine	Yes	*N*-pivaloly *iso*-propyl esters	this study
Snail shell (*Polinices mammilla*)	modern	Cuyo, Phillipine	Yes	*N*-pivaloly *iso*-propyl esters	this study
Bivalve shell (*Crassostrea gigas*)	modern	Culture	Yes	*N*-pivaloly *iso*-propyl esters	this study
Bivalve shell (*Crassostrea gigas*)	Pleistocene	Kasumigaura, Japan	Yes	*N*-pivaloly *iso*-propyl esters	this study
Bivalve shell (*Crassostrea konbo*)	Santonian	Mikasa,Hokkaido, Japan	Yes	*N*-pivaloly *iso*-propyl esters	[16]
Siliceous sample					
Opal-A (Fossil marine diatom assemblage)	early Holocene	Lützow-Holm Bay, East Antarctica	Yes	*N*-pivaloly *iso*-propyl esters	this study
Sea sand (Quartz particles)	modern	Standard reagent	Yes	*N*-pivaloly *iso*-propyl esters	this study
Rock and sediment sample					
Serpentine rock	modern	Logatchev & Ashedze, Atlantic ocean	Yes	*N*-pivaloly *S*-(+)-2-butyl esters	[12]
Methane hydrate sediment	Pleistocene	Shimokita, Western Pacific	Yes	*N*-pivaloly *iso*-propyl esters	[11]
Soft materials (organic matrix)					
Microbial aggregated sample					
Bacteria (*Bacillus subtilis* var. *natto*)	modern	Culture	Yes	*N*-pivaloly *S*-(+)-2-butyl esters & *N*-pivaloly *iso*-propyl esters	[18]
Bacteria (*Nostoc* sp.)	modern	Culture	Yes	*N*-pivaloly *S*-(+)-2-butyl esters & *N*-pivaloly *iso*-propyl esters	[14]
Soft tissue sample					
Soy bean (*Glycine max*)	modern	Culture	Yes	*N*-pivaloly *iso*-propyl esters	this study
Sprout of Radish (*Raphanus* sp.)	modern	Culture	Yes	*N*-pivaloly *S*-(+)-2-butyl esters & *N*-pivaloly *iso*-propyl esters	this study
Outer shell of Tubeworm (*Alaysia* sp.)	modern	Sagami Bay, Japan	Yes	*N*-pivaloly *S*-(+)-2-butyl esters & *N*-pivaloly *iso*-propyl esters	[15]
Peptidoglycan of Bacteria (*Lactobacillus acidophilus*)	modern	Culture	Yes	*N*-pivaloly *S*-(+)-2-butyl esters	[18]
Psuedo-peptidoglycan of Archaea (*Methanobacterium* sp.)	modern	Culture	Yes	*N*-pivaloly *S*-(+)-2-butyl esters	[18]
Psuedo-peptidoglycan of Eukarya (*Saccharomyces cerevisiae*)	modern	Culture	Yes	*N*-pivaloly *S*-(+)-2-butyl esters	[18]

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EXPERIMENTAL

Standard and sample materials

The standards (mixtures of alanine, glycine, valine, leucine, isoleucine, proline, aspartic acid, threonine, serine, methionine, glutamic acid, phenylalanine, 2-aminobutyric acid, sarcosine, β-alanine, 3-aminoisobutyric acid, hydroxyproline and 2-aminoadipic acid) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and analytical grade cation-exchange resin (AG 50W-X8; 200-400 mesh, H^+ form) was from Bio-Rad Laboratories (Tokyo, Japan). The resin consists of sulfonated polystyrene with a cation-exchange capacity of 1.7 meq mL^{-1}, wet bead size 63–150 μm and density 0.80 g mL^{-1}. We applied the pre-treatment procedure to various geochemical samples (Table 1), including calcareous samples, siliceous samples, rock and sediment samples, and biological and microbial samples.[10–18]

Pre-treatment of AAs with cation-exchange chromatography

Samples (ca. 5–100 mg: by their organic contents) were hydrolyzed with 6 M HCl (110°C, 12 h). After cooling down, occasionally we carried out a filtering procedure to remove a precipitation by using a glass pipette column fitted with a quartz wool plug. In a liquid/liquid extraction, the hydrolysates were washed twice with two bed volumes of *n*-hexane/CH_2Cl_2 (6:5, v/v) to eliminate lipophilic compounds. After being dried with a N_2 flow, the sample was adjusted to pH 1 using 2 mL of 0.1 M HCl and the AA fraction was isolated using cation-exchange column chromatography. Figure 1 shows a schematic outline of the isolation of AAs via pre-treatment with a cation-exchange resin column (AG 50W-X8; 200–400 mesh) employed for isolation and desalting. A slurry of resin kept in deionized distilled water (hereafter, H_2O) was poured into a disposable column (Bio-Rad Laboratories) or a glass pipette column fitted with a quartz wool plug. Since the cation-exchange capacity is 1.7 meq mL^{-1}, the volume of resin used depends on the amount of sample processed. Prior to application of the sample to the column, the resin was washed successively (3 three bed volumes) with 1 M HCl, H_2O, 1 M NaOH and finally with H_2O (e.g., 2 mL AG50 resin requires 6 mL 1 M HCl as the first pre-wash step). Immediately prior to sample application, the resin was re-activated to the H^+ form using 3 bed volumes of 1 M HCl and then rinsed with 3 bed volumes of H_2O. Finally, the AA fraction was eluted with 3 bed volumes of 10 wt% NH_3 aqueous solution and dried in preparation for the subsequent derivatization. We also

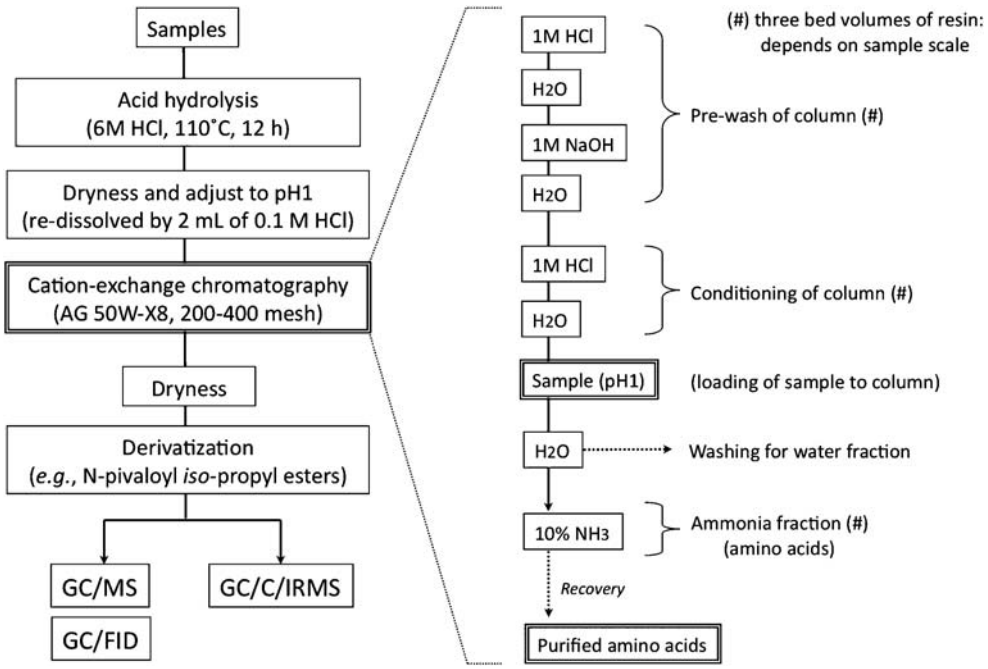

Figure 1. Schematic pathway outlining purification of amino acids via pre-treatment using cation-exchange chromatography and Bio-Rad AG 50W-X8 (200–400 mesh) resin for compound-specific and enantiomer-specific nitrogen isotope analysis (CSIA and ESIA). We can apply this procedure for molecular biology grade and biotechnology grade of Bio-Rad AG 50W-X8 (200–400 mesh) resin. However, as Evershed *et al.*[26] and their cited literature mentioned, we have to pay attention for possible disadvantages of cation-exchange chromatography to evaluate natural abundance of isotopic composition. Metges and Petzke[27] used Dowex AG 50W-X8 (Na^+ form, 200 mesh; Fluka Chemie AG, Germany) for free amino acids in soft-tissue plasma and also suggested that the isolation of amino acids by cation-exchange chromatography resulted in a small fractionation (up to 1.2‰) of nitrogen isotopic composition of glycine, lysine, and glutamic acid. The purification step with Dowex AG 50W-X8 (100–200 mesh; Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) for soil samples is summarized by Amelung and Zhang elsewhere.[28]

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RCM

examined possible background compounds derived from the cation-exchange resin (Bio-Rad AG 50W-X8; 200-400 mesh) by blank procedures (see also Fig. 4).

GC and GC/C/IRMS analysis for *N*-pivaloyl *iso*-propyl esters of AAs

The recovery ratio of the AAs prior to and following cation-exchange ion chromatography was determined using gas chromatography with flame ionization detection (GC/FID), with an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with an HP-5 column (30 m × 0.32 mm i.d., 0.52 μm film thickness; Agilent Technologies) of the AAs as their *N*-pivaloyl *iso*-propyl esters[13,19,20] and *N*-pivaloyl isobutyl esters.[10] The GC oven temperature was programmed as follows: 40°C (4 min) to 90°C at 10°C min^{-1}, then to 220°C (held 10 min) at 5°C min^{-1}. The AAs were also analyzed via GC/MS (6890N/5973MSD, Agilent Technologies) and GC/C/IRMS.[9,10,14]

The nitrogen isotopic compositions of the individual AAs were determined by GC/C/IRMS using a Delta Plus XP mass spectrometer (ThermoFinnigan, Bremen, Germany) combined with an Agilent Technologies 6890N gas chromatograph with an Ultra-2 capillary column (25 m × 0.32 mm i.d., 0.52 μm film thickness; Agilent Technologies) in combustion and reduction furnaces.[9] Combustion was performed in a microvolume ceramic tube with CuO, NiO, and Pt wires at 1000°C. Reduction was performed in a microvolume ceramic tube with a Cu wire at 550°C. The GC oven temperature was programmed as follows: initial temperature of 40°C for 4 min, ramped at 15°C min^{-1} to 130°C, ramped at 1°C min^{-1} to 160°C, and ramped at 30°C min^{-1} to 260°C, where it was maintained for 10 min. The carrier gas (He) flow rate was 1.3 mL min^{-1}. The CO_2 generated in the combustion furnace was removed with a liquid nitrogen trap.[9] As noted by Metges and Petzke,[21] fluorinated compounds may cause rapid deterioration of the combustion catalyst and oxidants in GC/C/IRMS. Since fluorine forms extremely stable

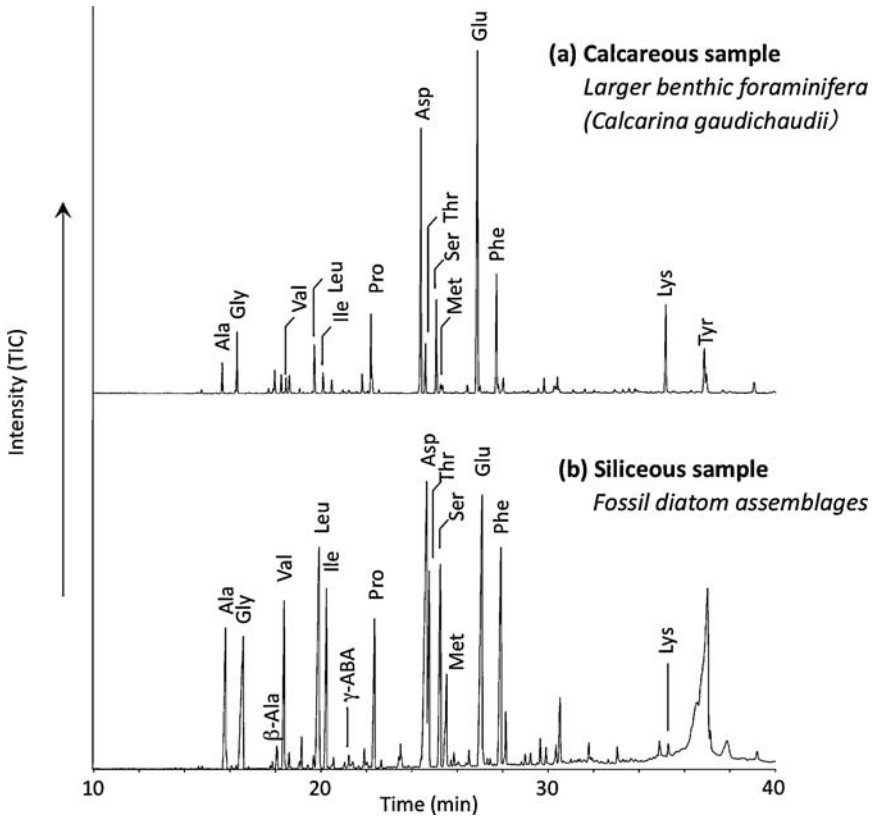

Figure 2. Representative TIC from GC/MS analysis of typical geochemical samples with interfering organic, inorganic and complex substances. (a) Calcareous sample, benthic foraminifera of *Baculogypsina sphaerulata* (modern) obtained from a sand beach, Okinawa Island, Japan. (b) Siliceous sample, sedimentary diatom (fossil) obtained from a core sample (Mw5S, 145–150 cm depth), Rundvågshetta, along the Lützow-Holm Bay area, East Antarctica. Although the two signals representing aspartic acid and threonine are not separated with baseline resolution on the HP-5 column (30 m × 0.32 mm i.d., 0.52 μm film thickness; Agilent Technologies), evaluation could be achieved using another suitable stationary phase.[9] Abbreviations, Ala, alanine; Gly, glycine; Val, valine; Leu, leucine; Ile, isoleucine; Pro, proline; Asp, aspartic acid; Thr, threonine; Ser; serine; Met, methionine; Glu, glutamic acid; Phe, phenylalanine; Lys, lysine; Tyr, tyrosine.

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fluorides with Cu (CuF_2) and Ni (NiF_2), thereby irreversibly reducing the combustion efficiency of the CuO/NiO system,[22] pivaloyl chloride was employed as the acylation reagent. The nitrogen isotopic composition of each amino acid was expressed as the per mil (‰) deviation from a standard (Air), as defined by:

$$\delta^{15}N = [(^{15}N/^{14}N)_{sample}/(^{15}N/^{14}N)_{standard} - 1] \times 1000\,(‰) \quad (1)$$

The standard deviation (1σ) of the $\delta^{15}N$ values of the AAs was better than ±0.5‰, with a minimum sample amount of 30 ng N (~2 nmol N) as injected nitrogen.[9,13]

RESULTS AND DISCUSSION

Consistency of nitrogen isotopic compositions of amino acids and its application to biogeochemical samples

Figure 2 shows representative total ion chromatograms from the GC/MS analysis of amino acid *N*-pivaloyl *iso*-propyl esters extracted from calcareous (benthic foraminifera) and siliceous samples (fossil diatom). Although these samples contained interfering materials, there was baseline resolution of protein and non-protein amino acids after the cation-exchange column (Table 1). A comparative study of AA separation and nitrogen isotopic compositions using polar and apolar stationary phases was performed by Chikaraishi *et al.*;[14] see also other AA separations by capillary columns such as Chirasil-Val (50 m × 0.32 mm i.d., 0.20 μm film thickness; Alltech Associates Inc., Deerfield, IL, USA), DB-23 (30 m × 0.32 mm i.d., 0.25 μm; Agilent Technologies), DB-35 (30 m × 0.32 mm i.d., 0.50 μm; Agilent Technologies), NDB-FFAP (30 m × 0.32 mm i.d., 0.25 μm; Agilent Technologies), HP-1MS (30 m × 0.32 mm i.d., 1.0 μm; Agilent Technologies), HP-Chiral-20β (30 m × 0.32 mm i.d., 0.25 μm; Agilent Technologies), and HP-INNOWAX (30 m × 0.32 mm i.d., 0.50 μm; Agilent Technologies).[9] We compared the precision of our procedure, based on $\delta^{15}N$ values obtained after cation-exchange chromatography (Bio-Rad AG 50W-X8; 200–400 mesh), as shown in Fig. 3. The difference in nitrogen isotopic compositions before and after the cation-exchange treatment ($\delta^{15}N_{before}$ vs. $\delta^{15}N_{after}$) was less than 0.3‰ (mean 0.02‰), even when protein and non-protein AAs samples were used (Table 2). This suggests that the precision of nitrogen isotopic composition in this procedure is related mainly to the GC/C/IRMS analysis. The recovery of AAs using the present cation-exchange procedure was 94.3 ± 11.4% ($n = 3$) on the basis that the yield of the derivatization reaction (*N*-pivaloyl *iso*-propyl esters) was 100% in both (Table 2). We also concluded that there was no isotopic fractionation of nitrogen isotopes in the protein and non-protein AAs, even when a 10% NH_3 aqueous solution was used in the final elution step.

To examine possible background compounds derived from the cation-exchange resin (Bio-Rad AG 50W-X8; 200–400 mesh), the procedure was applied to the analysis of an alanine standard and monitored using GC/MS (Fig. 4). No background compounds appeared in the chromatogram, so

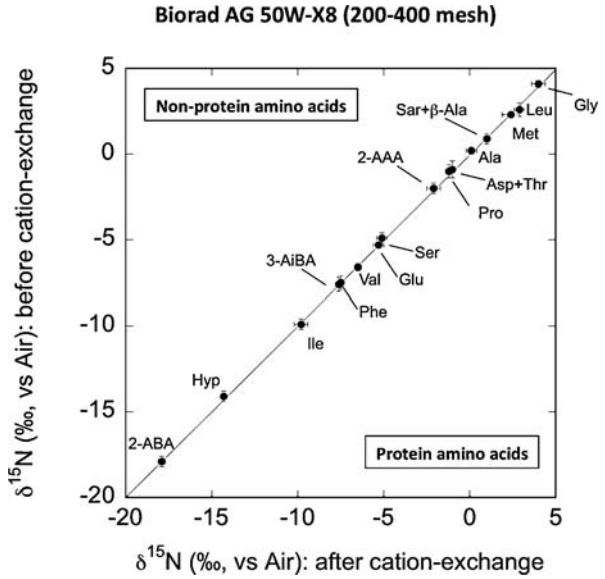

Figure 3. Verification of data consistency prior to (without) and following cation-exchange chromatography for compound-specific nitrogen isotopic composition ($\delta^{15}N$, ‰ vs. Air) using Bio-Rad AG 50W-X8 (200–400 mesh) resin. Standard AA mixtures including proteins (lower plot line) and non-proteins (upper plot line) were used. Abbreviations, Gly, glycine; Leu, leucine; Ile, isoleucine; Met, methionine; Sar; sarcosine; β-Ala, beta-alanine; Ala, alanine; Asp, aspartic acid; Thr, threonine; Pro, proline; 2-AAA, 2-aminoadipic acid; Ser; serine; Glu, glutamic acid; Val, valine; Phe, phenylalanine; 3-AiBA, 3-aminoisobutyric acid; Ile, isoleucine; Hyp, hydroxyproline; 2-ABA, 2-aminobutyric acid. We evaluate recovery (%) during this procedure by using an internal standard (e.g., α-amino adipic acid, norleucine).

the resin is resistant to elution with strong acidic and basic aqueous solutions. All AAs except glycine possess chiral center(s), and therefore D- and L-enantiomers. When optically active isobutanol, i.e. *S*-2-(+)-butanol or *R*-2-(−)-butanol, is employed in the esterification procedure, the present cation-exchange method is useful in identifying D- and L-AA enantiomers (e.g. racemic alanine; Fig. 4). Hence, chiral discrimination of D- and L-alanine does not occur during the cation-exchange chromatography. The evaluation of AA enantiomers allows the possibility of enantiomer-specific isotope analysis.[18]

This method is potentially useful for the analysis of microbial samples and other geochemical samples, including ancient fossils and geological materials,[e.g.,2,3,23,24] and therefore contributes to our general understanding of these materials through AA nitrogen isotope analysis. Since surviving and fossilized biological hard-tissue samples preserve ancient AAs as indigenous intact species,[25,26] the method is also applicable to fossil samples and therefore contributes to a comprehensive understanding of paleodietary studies. Based on a laboratory control test with the pre-treatment procedure, high recovery of AAs during cation-exchange chromatography and baseline separation during GC/C/IRMS are required for accurate and precise measurements of the isotopic compositions including ^{15}N-labeling research.

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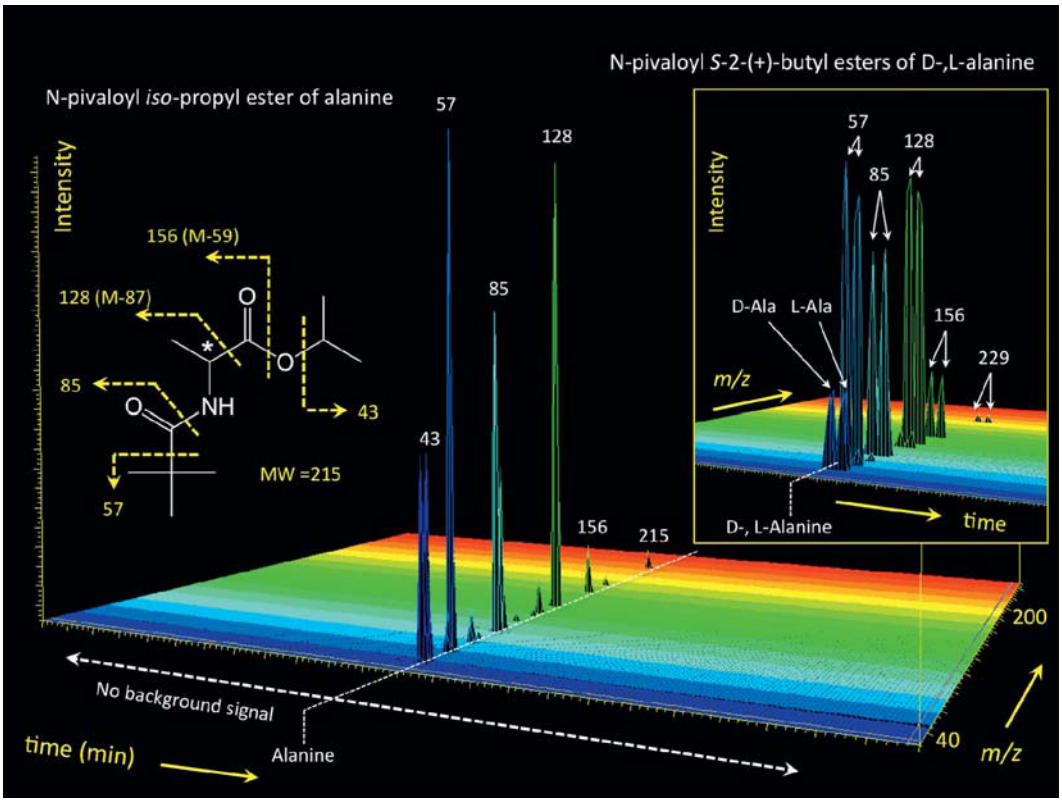

Figure 4. Examination of possible background compounds derived from cation-exchange resin (Bio-Rad AG 50W-X8; 200–400 mesh) monitored by GC/MS. The derivatives comprised *N*-pivaloyl *iso*-propyl esters and *N*-pivaloyl *S*-2-(+)-butyl esters of an alanine standard. The software employed for GC/MS was the Chemstation program for the Agilent 1100 series. We also conducted a blank procedure without any starting compounds, and no contamination was detected. To eliminate background contamination signals, precise pre-wash treatment for resin is required even when using molecular biology grade or biotechnology grade Bio-Rad AG 50W-X8 (200–400 mesh) cation-exchange resin.

Table 2. Comparison of nitrogen isotopic composition (δ^{15}N, ‰ vs. Air) for standard amino acids in mixtures before and after the cation-exchange pre-treatment procedure. Recovery of AAs from cation-exchange chromatography with Bio-Rad AG 50W-X8 resin (200–400 mesh) is also shown. The recovery average was 94.3 ± 11.4%. The error bars stand for each GC/FID multiple run analysis ($n = 3$). The initial abundance of each amino acid (without cation-exchange chromatography) was defined as 100%, and the yield of the derivatization reaction (*N*-pivaloyl *iso*-propyl esters) was also assumed as 100% in both

	Nitrogen isotopic compositions of amino acids					Recovery of amino acids	
	Before resin	1σ	After resin	1σ	Δ^{15}N		
Amino acids	δ^{15}N (‰, vs. Air)		δ^{15}N (‰, vs. Air)			Recovery (%)	1σ
Protein amino acids							
Ala	0.1	0.2	0.2	0.3	−0.1	83.9	0.6
Gly	4.0	0.2	4.1	0.4	−0.1	91.4	1.6
Val	−6.5	0.2	−6.6	0.2	0.1	100.2	1.2
Leu	2.9	0.4	2.6	0.3	0.3	95.8	0.4
Ile	−9.8	0.3	−9.9	0.4	0.1	109.2	0.6
Pro	−1.2	0.4	−1.0	0.2	−0.2	89.5	0.4
Asp	−1.0	0.5	−0.9	0.1	−0.1	104.4	0.4
Thr						102.5	0.4
Ser	−5.1	0.3	−4.9	0.3	−0.2	101.4	0.5
Met	2.4	0.0	2.3	0.5	0.1	91.4	0.3
Glu	−5.3	0.1	−5.3	0.3	0.0	100.3	0.4
Phe	−7.5	0.4	−7.5	0.2	0.0	102.3	0.4
Non-protein amino acids							
2-ABA	−17.9	0.3	−17.9	0.2	0.0	82.7	1.1
Sar + β-Ala	1.0	0.3	0.9	0.1	0.1	58.0 98.7	1.0 0.4
3-AiBa	−7.6	0.4	−7.6	0.2	0.0	90.7	0.4
Hyp	−14.3	0.3	−14.1	0.1	−0.2	87.4	0.4
2-AAA	−2.1	0.3	−2.0	0.4	−0.1	101.5	0.4
					Ave.	**94.3 ± 11.4**	

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