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$^{15}N/^{14}N$ Position-Specific Isotopic Analyses of Polynitrogenous Amino Acids

Gavin L. Sacks and J. Thomas Brenna*

Division of Nutritional Sciences, Cornell University, Savage Hall, Ithaca, New York 14853

$^{15}N/^{14}N$ isotope ratios are widely used to study processes and systems involving amino acids. Nitrogen isotope fractionation in biological processes occurs primarily at sites of bond-breaking and formation; the finest discrimination for "isotopic fingerprinting" and studies of isotopic fluxes is thus obtained at the position-specific level. While there are numerous reports of natural intramolecular carbon isotope variability, there are no literature reports of $^{15}N/^{14}N$ position-specific isotopic analysis (N-PSIA) of biologically relevant molecules. We report a methodology for high-precision N-PSIA of four polynitrogenous α-amino acids (asparagine, glutamine, lysine, histidine) and the first survey of natural intramolecular $^{15}N/^{14}N$ in these biomolecules. Selective liberation of N-atoms from multiple commercial standards of each parent amino acid was achieved by an appropriate enzymatic reaction or by acid hydrolysis. $^{15}N/^{14}N$ measurements were performed on *N*-ethoxycarbonyl ethyl ester derivatives of the parent amino acids and their analogues by gas chromatography combustion isotope ratio mass spectrometry, and the average precision for replicate injections was found to be SD($\delta^{15}N$) = 0.3‰. Position-specific $\delta^{15}N$ values of the parent amino acid were directly observed or indirectly calculated using mass balance. The average precision obtained for directly measured positions was SD($\delta^{15}N$) = 0.2−0.4‰. The average precision for indirectly obtained positions was SD($\delta^{15}N$) = 0.6−1.3‰ as a result of propagation of errors. Enrichment in the side chain-N with respect to the peptide-N was observed in nearly all of the amino acid sources, most notably in asparagine (average $\Delta\delta_{side-peptide} = +11$‰), which may be indicative of its method of production. In some cases, it was possible to distinguish commercial sources by N-PSIA that could not be distinguished at the compound-specific level.

Stable isotope analysis by gas chromatography combustion-isotope ratio mass spectrometry (GCC−IRMS) is a powerful technique for providing clues to the processes and sources that formed a sample. Typical applications include the authentication of flavors and fragrances and the assignment of place of origin for foodstuffs,[1,2] sourcing of environmental pollutants,[3] and reconstruction of contemporary and historical dietary behaviors.[4,5] These applications are based heavily on empirical data and, as such, treat the isotopic information as a "fingerprint". However, variations in isotope ratios are clearly a result of rational (bio)chemical processes. Isotopic fractionation in biological systems is primarily a result of kinetic isotope effects associated with bond-breaking and formation; therefore, nonstatistical isotope distributions are expressed, and most clearly observed, at the position-specific level.[6] Recent reviews[7−9] have called for a robust study of nonstatistical isotopic distributions within an organism, and the authors of the reviews stress the importance of developing routine methods for high-precision position-specific isotope analysis (PSIA) to facilitate this goal.

While examples of carbon PSIA (C-PSIA) using IRMS are becoming more commonplace,[7] intramolecular $^{15}N/^{14}N$ isotope measurements are nearly nonexistent. It has been known for some time that the α-amino fraction of amino acids is depleted in ^{15}N with respect to the amide fraction,[10] implying that the peptide-N of glutamine should be depleted with respect to its amide-N. However, this intramolecular variation has not been measured directly. In fact, aside from a 1982 extended abstract by Medina et al.,[11] there are no literature reports of N-PSIA on biologically relevant molecules. The lack of data may have been partly due to the relative scarcity of small molecules that contain more than one nitrogen atom, but also because of the lack of facile methods for rapid, on-line N-PSIA measurements. Recently, several groups have begun to study isotopomers of N_2O, a technique that shows considerable promise in understanding the cycling of this important atmospheric pollutant.[12,13] However, these measurements take advantage of the unique attributes of N_2O in the IRMS source, specifically the dissociation of N_2O^+ to NO^+, and do not provide a framework for N-PSIA studies of other organic molecules. A

* To whom correspondence should be addressed. E-mail: jtb4@cornell.edu. Voice: (607)255-9182. Fax: (607)255-1033.

(1) Lichtfouse, E. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1337−1344.
(2) Asche, S.; Michaud, A. L.; Brenna, J. T. *Curr. Org. Chem.* **2003**, *7*, 1527−1543.
(3) Schmidt, T. C.; Zwank, L.; Elsner, M.; Berg, M.; Meckenstock, R. U.; Haderlein, S. B. *Anal. Bioanal. Chem.* **2004**, *378*, 283−300.
(4) Mottram, H. R.; Dudd, S. N.; Lawrence, G. J.; Stott, A. W.; Evershed, R. P. *J. Chromatogr., A* **1999**, *833*, 209−221.
(5) Meier-Augenstein, W. *Anal. Chim. Acta* **2002**, *465*, 63−79.
(6) Hayes, J. M. In *Stable isotope geochemistry*; Valley, J. W., Cole, D. R., Eds.; Mineralogical Society of America: Washington, DC, 2001; pp 225−277.
(7) Brenna, J. T. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1252−1262.
(8) Werner, R. A.; Schmidt, H. L. *Phytochemistry* **2002**, *61*, 465−484.
(9) Schmidt, H. L. *Naturwissenschaften* **2003**, *90*, 537−552.
(10) Gaebler, O. H.; Vitti, T. G.; Choitz, H. C.; Vukmirov. R *Can. J. Biochem. Physiol.* **1963**, *41*, 1089-&.
(11) Medina, R.; Olleros, T.; Schmidt, H. L. *4th International Conference on Stable Isotopes*; Elsevier Scientific Publishing Co.: New York, 1982; pp 77−82.
(12) Toyoda, S.; Yoshida, N. *Anal. Chem.* **1999**, *71*, 4711−4718.
(13) Rockmann, T.; Brenninkmeijer, C. A. M.; Wollenhaupt, M.; Crowley, J. N.; Crutzen, P. J. *Geophys. Res. Lett.* **2000**, *27*, 1399−1402.

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more widely applicable option is to use on-line pyrolysis (Py) coupled to a GCC−IRMS system.[14] This technique has been successfully used for C-PSIA measurements of alkanes,[15] amino acids,[16] and short-chain organic acids,[17,18] and a modification of this system was used to measure site-specific $^{18}O/^{16}O$ isotope ratios in vanillin.[19] The primary advantage of the Py-GCC−IRMS system for C-PSIA is that it permits fragmentation and isolation of sites that are not readily accessible by off-line chemical methods. However, Py-GCC−IRMS systems are not commercially available at this time. Furthermore, pyrolytic fragmentation is nonquantitative, which leads to isotopic fractionation and accompanying difficulties in measuring absolute isotope ratios.[16]

In the case of the polynitrogenous amino acids, the nitrogen sites are often discrete moieties (such as amine or amide groups) that can be isolated by chemical means with relative ease. Off-line fragmentation and isolation is therefore well-suited for N-PSIA. In this paper, we report a rapid method for N-PSIA of four of the six polynitrogenous proteinogenic amino acids, asparagine (Asn), glutamine (Gln), lysine (Lys), and histidine (His), and evaluate the analytical figures of merit for the overall procedure. We then apply the procedure to a series of commercially obtained amino acids to demonstrate the existence of natural variability of intramolecular $\delta^{15}N$ in biomolecules.

EXPERIMENTAL SECTION

Amino Acid Standards. Four or five standards of each amino acid (L-Asn, L-Gln, L-Lys, L-His) and one standard each of their respective analogues (L-Asp, L-Glu, δ-aminovaleric acid (DAVA), urocanic acid) were purchased from commercial vendors and used without further preparation. The vendors used were Acros (Geel, Belgium; "Acr"), Aldrich (St. Louis, MO; "Ald"), Avocado Organics (Heysham, England; "Avo"), J. T. Baker (Phillipsburg, NJ; "Bak"), Fluka (Buchs, Switzerland; "Flu"), and Sigma (St. Louis, MO; "Sig"). The sources for each amino acid were as follows. Asn: Acr, Ald, Avo, Flu. Asp: Sig. Gln: Acr, Ald, Avo, Bak, Flu. Glu: Sig. Lys: Ald, Avo, Flu, Sig. DAVA: Ald; His: Acr, Ald, Avo, Sig. Urocanic acid: Sig.

Acid Hydrolysis: Asn and Gln. The amino acids were prepared as 10 mg/mL solutions in water. A 100-μL aliquot of each standard solution was added along with 100 μL of 2 N HCl to a 2-mL reaction vial. The mixture was capped with a Teflon seal, vortexed, and incubated at 80 °C for 24 h.

Enzymatic Reactions: Asn, Gln, Lys, and His. Asparaginase (EC 3.5.1.1), glutaminase (EC 3.5.1.2), and lysine oxidase (EC 1.4.3.14) were purchased as lyophilized powders from Sigma-Aldrich and prepared as a 5 unit/mL solution in an appropriate buffer (50 mM asparaginase, pH 8.6 Tris; glutaminase, pH 4.9 sodium acetate; lysine oxidase, pH 8.0 Tris), where 1 unit will liberate 1 μmol of NH_3 from the substrate amino acid in 1 min at 37 °C in the specified buffer. Histidase (EC 4.3.1.3) was purchased from MP Biomedicals (Aurora, OH) and prepared as a 5000 unit/mL suspension in a pH 9.0 Tris buffer, where 1 unit will deaminate 1 μg of His/min at 25 °C.

The amino acid standards were prepared as 10 mg/mL solutions in water. A 100-μL aliquot of standard solution, 80 μL of the appropriate buffer, and 20 μL of the enzyme solution were added to a 2-mL reaction vial. The mixture was capped with a Teflon seal, vortexed, and incubated at 37 °C for 24 h. Asparaginase, glutaminase, and histidase generate the amino acid analogues shown in Figure 1 directly. Lysine oxidase converts lysine to 2-oxo-6-aminohexanoic acid, which spontaneously decarboxylates in the absence of catalase to form DAVA.[20]

***N*-Ethoxycarbonyl Ethyl Ester (ECEE) Derivatization of Amino Acids.** ECEE derivatives were prepared by a procedure adapted from Husek[21] and from Montigon et al..[22] A 200-μL aliquot of the substrate (either the analogue reaction mixture or a 5 mg/mL solution of the standard in an appropriate buffer), 100 μL of EtOH, and 25 μL of pyridine were added to a 2-mL reaction vial. For derivatization of DAVA, the reaction was acidified with 50 μL of 2 N HCl. Ethyl chloroformate (ECF, 10 μL) was added. The mixture was capped, vortexed, and allowed to sit for 5 min. A 300-μL aliquot of saturated $NaHCO_3$ and 1 mL of CH_2Cl_2 were added, and the aqueous layer was removed. Anhydrous $CaCl_2$ was added, and the dried organic layer was transferred to another vial. The CH_2Cl_2 was removed under a nitrogen stream, and the product was redissolved in 50 μL of CH_2Cl_2 before introduction to the GCC−IRMS. Identification of the derivatives was performed by a Varian Saturn 2000 ion trap (Walnut Creek, CA) operated in positive ion electron impact mode. The identities of the α-amino acid ECEE derivatives were confirmed by comparing characteristic ion peaks to data collected by Huang and colleagues.[23] ECEE derivatization of DAVA and urocanic acid has not been previously reported. Under acidic conditions, ECEE derivatization of DAVA formed a cyclic product (2-piperidone), and identification was aided by the Wiley mass spectral library. The urocanic acid−ECEE derivative showed characteristic ion peaks at $m/z = 238$, 193, 166, 121, 94, and 66; these peaks correlate with the fragmentation pattern observed in His-ECEE following loss of the α−amino-*N*-ethoxycarbonyl group.[23]

GCC−IRMS of Amino Acid Derivatives. The system we used is similar to that described by Merritt and Hayes.[24] Briefly, the sample was injected, splitless, via an autosampler (Varian, Inc. 8200; Walnut Creek, CA) into a GC (Varian 3400CX). Components were separated on a 15 m × 0.32 mm × 3.0 μm VB-1 capillary column (cross-linked dimethylpolysiloxane; VICI; Houston, TX). The initial GC oven temperature was 80 °C, held for 5 min, ramped to 260 °C at 15 °C/min, and held for 5 min. The head pressure was set at 10 psi, resulting in a flow rate of 2 mL/min at a GC temperature of 80 °C. An electronically controlled Valco rotary valve was used to divert the solvent and to provide an auxiliary

(14) Corso, T. N.; Brenna, J. T. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 1049−1053.
(15) Corso, T. N.; Brenna, J. T. *Anal. Chim. Acta* **1999**, *397*, 217−224.
(16) Sacks, G. L.; Brenna, J. T. *Anal. Chem.* **2003**, *75*, 5495−5503.
(17) Dias, R. F.; Freeman, K. H.; Franks, S. G. *Org. Geochem.* **2002**, *33*, 161−168.
(18) Yamada, K.; Tanaka, M.; Nakagawa, F.; Yoshida, N. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 1059−1064.
(19) Dennis, M. J.; Wilson, P.; Kelly, S.; Parker, I. *J. Anal. Appl. Pyrolysis* **1998**, *47*, 95−103.
(20) Kusakabe, H.; Kodama, K.; Kuninaka, A.; Yoshino, H.; Misono, H.; Soda, K. *J. Biol. Chem.* **1980**, *255*, 976−981.
(21) Husek, P. *J. Chromatogr.* **1991**, *552*, 289−299.
(22) Montigon, F.; Boza, J. J.; Fay, L. B. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 116−123.
(23) Huang, Z. H.; Wang, J.; Gage, D. A.; Watson, J. T.; Sweeley, C. C.; Husek, P. *J. Chromatogr.* **1993**, *635*, 271−281.
(24) Merritt, D. A.; Hayes, J. M. *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 387−397.

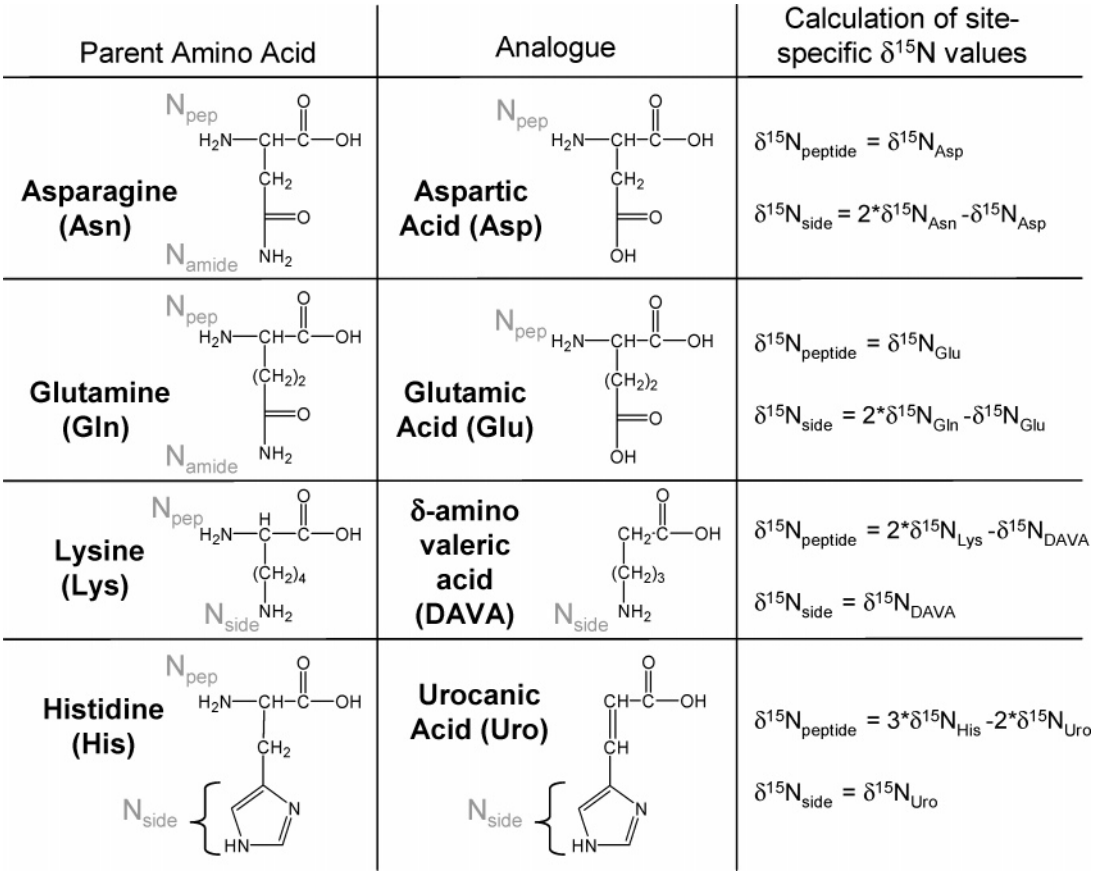


Figure 1. Structures of α-amino acids and their analogues. The conversion of Lys to DAVA involves a subsequent decarboxylation following enzymatic deamination. Position-specific δ^{15}N values of the original α-amino acids were calculated either directly or indirectly by mass balance, using the equations in the rightmost column.

flow of O_2 for recharging the furnace, in place of a back-flush system.[25]

The carrier stream exited the rotary valve by fused-silica transfer line and was coupled within the GC oven to the combustion furnace using Valco vespel ferrules and zero dead volume connectors. The combustion furnace consisted of a 30 cm × 0.5 mm ceramic tube packed with oxidized Cu and resistively heated to 950 °C by a Fibercraft furnace (Thermcraft; Winston-Salem, NC). The combustion products were directed to a similarly constructed reduction furnace, packed with Cu metal, and held at 550 °C, to convert N_xO_y to N_2. CO_2 and H_2O were removed by an LN_2 cold trap, and a portion of the effluent stream was admitted via an open split to an APP2003 IRMS (GV Instruments; Manchester, England). High-purity N_2 was calibrated against air and used as a reference.

Consecutive runs were automated by macros written in the native APP2003 scripting language. Data acquisition was performed by the APP2003, and the raw data were exported to SAXICAB,[26] a home-written LabVIEW[27] software package, for calculation of δ^{15}N values. The background in the area around the peaks was low and nearly flat, and the individual summation method for background correction described by Ricci et al.[28] was found to give satisfactory results.

Elemental Analyzer (EA)-IRMS of Amino Acid Derivatives. The system consisted of an NC2500 Carlo Erba EA interfaced to a Finnigan MAT Delta Plus IRMS (Bremen, Germany) through a ConFlo II open split. In the EA, the combustion conditions were 1000 °C over Cr_2O_3 and CuO, and the reduction conditions were 650 °C over copper filings. Samples (~1 mg) were loaded into tin cups (Costech; Valencia, CA) before introduction into the EA by autosampler. High-purity He (120 mL/min) was used as a carrier gas, and the N_2 reference gas was of highest purity.

Reporting Isotope Ratios. The accepted method for reporting isotope measurements is to compare the isotope ratio of the analyte to the isotope ratio of a standard. For nitrogen studies, analytes are indirectly calibrated against N_2 in air and expressed in delta notation:

$$\delta^{15}N_{air} = (R_{spl} - R_{air})/R_{air} \times 1000 \qquad R_x = [^{15}N_x/^{14}N_x]$$

where R_{spl} is the isotope ratio of the sample and $R_{air} = 0.003\,678\,2$.

RESULTS AND DISCUSSION

The structures of the investigated amino acids (Asn, Gln, Lys, His) and their analogues are shown in Figure 1. The conversion

(25) Goodman, K. J. *Anal. Chem.* **1998**, *70*, 833−837.
(26) Sacks, G. L.; Brenna, J. T.; Sepp, J. T. *49th ASMS Conference on Mass Spectrometry*, Chicago, IL, 2001.
(27) National Instruments. *Labview 6i*; Austin, TX, 2000
(28) Ricci, M. P.; Merritt, D. A.; Freeman, K. H.; Hayes, J. M. *Org. Geochem.* **1994**, *21*, 561−571.

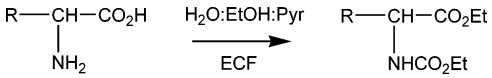


Figure 2. General reaction scheme for the preparation of ECEE derivatives of amino acids.

of Gln, Asn, and His to their corresponding analogues is straightforward. The enzymatic deamination of Lys generates 2-oxo-6-aminohexanoic acid, which spontaneously decarboxylates to form DAVA.[20] The amino acids were made amenable to GC analysis by reaction with ECF in a H_2O/EtOH/pyridine solvent system to form ECEE derivatives. The reaction proceeds by the general reaction scheme shown in Figure 2, with simultaneous derivatization of the N-containing moieties and the carbonyl group.[21,23] ECEE derivatization for GCC−IRMS $^{13}C/^{12}C$ measurements of Gln has been reported previously[22] and provides several advantages over more common derivatization schemes. Most sample preparation techniques for $^{15}N/^{14}N$ measurements by GCC−IRMS rely on esterification followed by either trifluoroacetylation, acetylation, or pivaloylation.[29] Unfortunately, these methods are inappropriate for the analysis of amides (Asn, Gln) due to the acidic conditions of the esterification step. Tert-butylmethylsilation (t-BDMS) derivatization is also routinely used for GCC−IRMS, and the procedure is amenable to amides. However, t-BDMS derivatives are unstable at room temperature, add a large carbon load, and can be damaging to the combustion furnace.[22,30] In contrast, ECEE derivatives are stable for at least 1 week at room temperature, introduce no heteroatoms aside from oxygen, give excellent chromatography, and are compatible with all of the proteinogenic amino acids except for arginine.[21]

Reproducibility of Analogue Preparation and ECEE Derivatization. Asn and Gln analogues (Asp and Glu, respectively) were prepared by both enzymatic reaction and under acidic conditions; Lys and His analogues (DAVA and urocanic acid, respectively) were prepared only by enzymatic reaction. In these procedures, no trace of the starting amino acid was detectable after 24 h. Representative chromatograms of the conversion of Asn to Asp by asparaginase are shown in Figure 3, and similar results were achieved with the other procedures. Meier-Augenstein has cautioned that the nonquantitative nature of the chloroformate derivatization is a potential hazard for GCC−IRMS[31] and could result in severe and irreproducible fractionation. Other groups have reported recoveries ranging between 75 and 100% for ECF derivatization of amino acids,[32,33] and we achieved similar efficiencies in our work (data not shown). Incomplete reactions in derivatization schemes that cause reproducible isotopic fractionation are well known and, if properly taken into account, do not seriously degrade the quality of isotope ratio measurement.[34] To assess the reproducibility of the fractionation, the ECEE derivatization was run in duplicate, "prep 1" and "prep 2", for multiple commercial sources of each amino acid and its analogue. $\delta^{15}N$ values for the two runs were measured by GCC−IRMS with an average precision of SD($\delta^{15}N$) = 0.3‰ for three or four replicate injections. The difference between prep 1 and prep 2, Δ_{prep}, was calculated as

$$\Delta_{prep} = \delta^{15}N(\text{prep 1}) - \delta^{15}N(\text{prep 2})$$

The average value and standard deviation of Δ_{prep} are reported in Table 1 for each compound. Two criteria were defined for determining whether the analogue preparation and derivatization procedures behaved acceptably. First, if the average value of Δ_{prep} was smaller than SD[Δ_{prep}], then the difference between the runs was considered to be within experimental error. In all cases, the average value of Δ_{prep} was small (<0.3‰), and met this criterion. Second, the run-to-run precision, SD[Δ_{prep}], should be similar to (or better than) the precision typically associated with $\delta^{15}N$ measurements by GCC−IRMS, SD($\delta^{15}N$) = 0.5‰.[3] Measurements of the parent amino acids yielded SD[Δ_{prep}] of less than 0.3‰. However, some of the analogue preparation procedures resulted in unacceptably high values of SD[Δ_{prep}]. For example, enzymatic hydrolysis of Gln to Glu yielded poor results (SD[Δ_{prep}] = 0.98‰) compared to acid hydrolysis (SD[Δ_{prep}] = 0.35‰). Husek[23] reported that ECF derivatization of Glu forms two products: the expected ECEE−Glu derivative and a dehydrated ECEE−pyro-

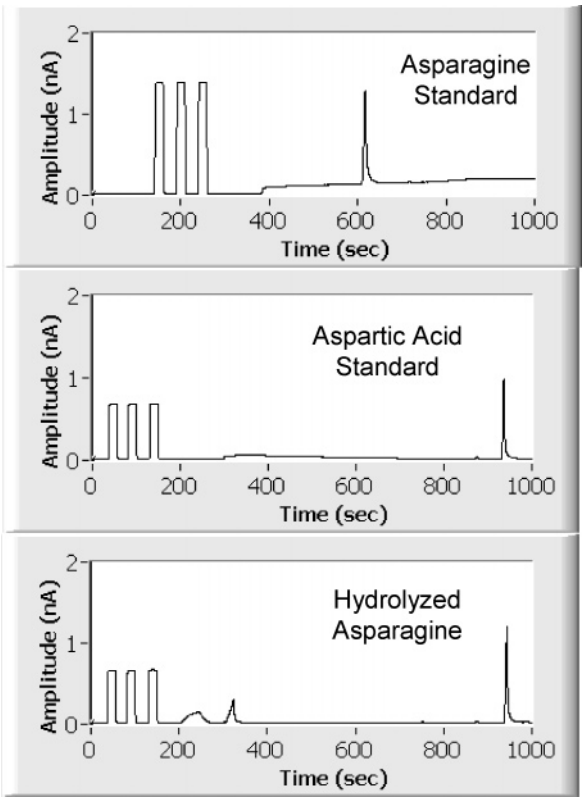


Figure 3. GCC−IRMS chromatograms of ECEE derivatives of Asn standard, Asp standard, and deaminated Asn, showing complete conversion of Asn to Asp by asparaginase. Three N_2 standard pulses were introduced at the beginning of each run. The additional peaks in the deaminated bottom run are from contaminants in the asparaginase and are unrelated to the analyte.

(29) Hofmann, D.; Gehre, M.; Jung, K. *Isot. Environ. Health Stud.* **2003**, *39*, 233−244.
(30) Shinebarger, S. R.; Haisch, M.; Matthews, D. E. *Anal. Chem.* **2002**, *74*, 6244−6251.
(31) Meier-Augenstein, W. *Curr. Opin. Clin. Nutr. Metab. Care* **1999**, *2*, 465−470.
(32) Nozal, M. J.; Bernal, J. L.; Toribio, M. L.; Diego, J. C.; Ruiz, A. *J. Chromatogr., A* **2004**, *1047*, 137−146.
(33) Silva, B. M.; Casal, S.; Andrade, P. B.; Seabra, R. M.; Oliveira, M. B.; Ferreira, M. A. *Anal. Sci.* **2003**, *19*, 1285−1290.
(34) Docherty, G.; Jones, V.; Evershed, R. P. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 730−738.

Table 1. Reproducibility of Isotope Ratio Analysis for ECEE Derivatization of Parent Amino Acids and Their Corresponding Analogues[a]

amino acid	source	parent			analogue (‰)	
		prep1	prep2	Δ_{prep}[b]	enzymatic	acid hydrolysis
Asn	Acr	0.74	0.43	0.31		
	Ald	−1.88	−1.99	0.11		
	Avo	0.16	0.26	−0.10		
	Flu	−3.25	−3.14	−0.11		
	av Δ_{prep}[c] (mean±SD, ‰)			0.05 ± 0.20	−0.02 ± 0.26	−0.34 ± 2.11
Gln	av Δ_{prep} (‰)			−0.13 ± 0.14	0.15 ± 0.98	−0.26 ± 0.35
Lys	av Δ_{prep} (‰)			−0.01 ± 0.11	−0.07 ± 0.30	n/a[d]
His	av Δ_{prep} (‰)			−0.05 ± 0.26	−0.42 ± 0.65	n/a

[a] Reactions were done in duplicate, prep 1 and prep 2, and $\delta^{15}N$ was measured for each by GCC−IRMS (mean precision, SD($\delta^{15}N$) = 0.3‰, for three or four replicate injections). Complete data for Asn (Parent) are reported and summary data for other amino acids presented for brevity. [b] $\Delta_{prep} = \delta^{15}N_{Prep\ 1} - \delta^{15}N_{Prep\ 2}$. [c] Av Δ_{prep}, mean ± SD: calculated as the mean of Δ_{prep} for Asn; SD calculated as the pooled error for the four Δ_{prep} values. Other av Δ_{prep} defined equivalently for the other amino acids. [d] n/a, not attempted.

Table 2. Comparison of $\delta^{15}N$ Values of ECEE Derivatives (GCC−IRMS, $n = 7$) and Underivatized Standards (EA-IRMS, $n = 2$)[a]

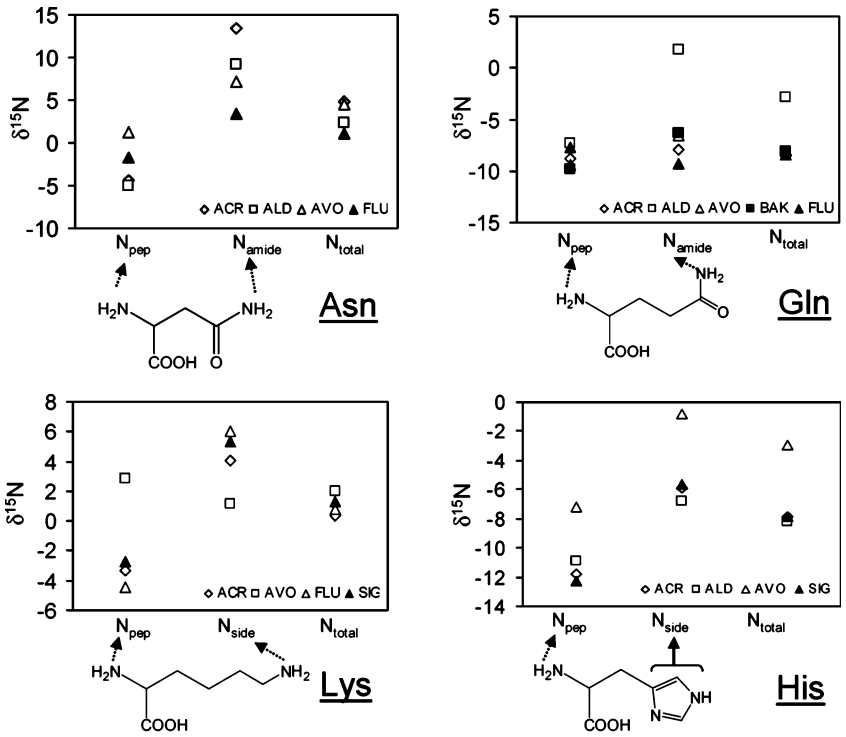
amino acid	vendor	GCC−IRMS	EA-IRMS	α (‰)	amino acid	vendor	GCC−IRMS	EA-IRMS	α (‰)
Asn	Acr	0.59	4.17	−4.04 ± 0.44	Lys	Ald	−7.10	0.88	−7.45 ± 0.40
	Ald	−1.93	1.99			Avo	−5.46	1.54	
	Avo	0.21	4.22			Flu	−6.67	0.58	
	Flu	−3.20	1.43			Sig	−6.15	1.33	
Asp	Sig	−10.09	−8.56	−1.53	DAVA	Sig	−6.02	−5.93	−0.09
Gln	Acr	−9.34	−8.33	−0.98 ± 0.40	His	Acr	−9.96	−7.76	−2.09 ± 0.19
	Ald	−3.77	−2.13			Ald	−10.24	−8.84	
	Avo	−8.97	−8.38			Avo	−5.05	−2.91	
	Bak	−9.05	−8.16			Sig	−9.96	−7.76	
	Flu	−9.42	−8.65						
Glu	Sig	−3.17	−4.59	+1.43	urocanic acid	Sig	−7.16	−8.25	+1.09

[a] Fractionation factors (α) are reported in the rightmost column where $\alpha = \delta^{15}N$ (GC) − $\delta^{15}N$ (EA). For compounds where samples from multiple vendors were run, the mean ± SD α value is calculated.

glutamic acid derivative. Under alkaline conditions, the two products are formed in roughly equal amounts, but under acidic conditions (pH ∼1), we saw nearly exclusive formation of the ECEE−pyroglutamic acid. The opposite effect was seen for the conversion of Asn to Asp, which showed far better reproducibility with the enzymatic reaction (SD[Δ_{prep}] = 0.26‰) than with acid hydrolysis (SD[Δ_{prep}] = 2.11‰). It is not clear why ECEE−Asp derivatization yields poor reproducibility under acidic conditions. ECEE derivatives of DAVA and urocanic acid have not been previously reported. We observed that at pH 8.0, DAVA forms both ECEE−DAVA predicted from the mechanism in Figure 2 and 2-piperidone via dehydration. In our work, it was necessary to acidify the enzymatic reaction mixture following deamination in order to preferentially form the dehydrated product, from which good run-to-run precision was achieved (SD[Δ_{prep}] = 0.30‰). ECEE−urocanic acid, formed through enzymatic deamination of His, had a reproducibility (SD[Δ_{prep}] = 0.65‰) close to the precision typically associated with $^{15}N/^{14}N$ GCC−IRMS. In summary, in our hands, reproducible fractionation during analogue formation and ECEE derivatization can be achieved by acid hydrolysis of Gln and by enzymatic treatment of Asn, Lys, and His.

Fractionation during Analogue Preparation and Derivatization. Because the nitrogen sites are part of the bond-breaking and bond-forming steps during the ECF derivatization, two steps that might be subject to isotopic fractionation are the derivatization of the parent amino acid or its analogue. A third, less probable source of fractionation is during the analogue preparation because the nitrogen sites in the analogue are not part of the bond-breaking step and thus are at low risk for fractionation;[35] consequentially, we disregard this step as a potential source of fractionation. To assess the extent of fractionation, $\delta^{15}N$ of the underivatized amino acids and their analogues were measured by EA-IRMS and compared to the values obtained by GCC−IRMS. The fractionation factors (α) were calculated for each substrate as $\alpha = \delta^{15}N_{GC} - \delta^{15}N_{EA}$ and are reported in Table 2. For the parent amino acids, multiple commercial sources were used, and an average fractionation factor is reported. α was in the range of ±2‰ for most substrates, although it was significantly larger for Asn and Lys. However, the reproducibility of the fractionation was very good for the parent amino acids [SD(α) ≤ 0.4‰], even in the case of the sizable fractionation of Lys ($\alpha_{Lys} = -7.45 \pm 0.40$‰). The magnitude of the α's cannot be completely explained by the fractionation during the ECEE derivatization, which was <0.2‰ for the parent amino acids (Table 1). It is possible that N-containing impurities present in the bulk samples skewed the EA

(35) Rieley, G. *Analyst* **1994**, *119*, 915−919.



Amino Acid	Average Precision, SD(δ15N), for moiety or total molecule, n=3 or 4 replicates. Units are in per mil (‰)		
	Peptide	Amide or Side	Total
Asn	0.2	0.6	0.3
Gln	0.4	0.7	0.3
Lys	0.8	0.3	0.4
His	1.3	0.3	0.4

Figure 4. Position-specific δ^{15}N values of multiple sources of polynitrogenous amino acids, showing wide variation at the intramolecular level. The average precision for each site, and for the total molecule, is in the bottom table.

analyses but are excluded from the GC analyses because of the separation.

Calculation of Position-Specific δ^{15}N Values and Error Analysis. Based on the reliable procedures established in the reproducibility experiments, data from the enzymatic reactions of Asn, Lys, and His, and from the acid hydrolysis of Gln, were used for calculation of PSIA δ^{15}N values. Data from the acid hydrolysis of Asn and enzymatic hydrolysis of Gln were of unacceptably poor reproducibility for these duplicate reactions and were not further explored.

The δ^{15}N measurements from each set of duplicate runs were pooled, and the mean and standard deviation were recalculated for each amino acid and analogue. The average δ^{15}N values were corrected using the experimentally measured fractionation factors. The position-specific isotope ratios were then calculated for each amino acid, either directly from the δ^{15}N value of its analogue or indirectly by subtraction of the isotopic contribution of the analogue from the parent amino acid. The equations used to calculate these values are listed in the rightmost column of Figure 1. For Asn, Gln, and Lys, the δ^{15}N of the side chain represents a single N, while for His, it represents a composite of the two nonequivalent imidazole nitrogen sites. The average precision achieved for the directly measured positions (peptide-N of Asn and Gln, side chain-N of Lys and His) was SD(δ^{15}N) = 0.2−0.4‰. The precision achieved for the indirectly measured (i.e., calculated) position (side chain-N of Asn and Gln, peptide-N of Lys and His) was greater, averaging 0.6−1.3‰, due to propagation of errors.

The average and fractionation-corrected position-specific and compound-specific ("total-N") isotope ratios for each polynitrogenous amino acid are presented in Figure 4. The position-specific δ^{15}N show a range at least double that shown by total δ^{15}N. In 11 of the 13 amino acids, the peptide-N is depleted with respect to the side chain-N, and in the two exceptions, the differences are relatively small.

More specific interpretation of the variation of position-specific δ^{15}N is hindered because production methods for these samples are not known. We can, however, speculate based on published methods of industrial amino acid production. The side chain-N of Asn is enriched with respect to the peptide-N in all four sources (average $\Delta\delta_{side-pep} = +11‰$), and in Gln in four of the five sources (average $\Delta\delta_{side-pep} = +3‰$). This is consistent with Gaebler's observation that the bulk amide-N fraction is enriched with respect to the bulk peptide-N fraction in plants[10] and with more recent reports that show enrichment in heteroaromatic compounds that receive their N from Gln[36]. Glu acts as a general donor of peptide-N to the other amino acids by transamination, which favors ^{15}N enrichment in the remaining Glu and depletion in Asp; this may explain Asn's prominent depletion in the peptide-N compared

to the side chain amide-N.[37] However, it should be pointed out that industrial production of Gln, Lys, and Asp is typically accomplished by microbial fermentation,[38,39] while Asn is often produced from Asp by a secondary chemical synthesis step. Furthermore, the peptide-N of the four Asn sources (−5 to +1‰) is in a range appropriate for microbial production, while the side chain amide-N shows much more variability and is quite enriched (+3 to +13‰). Therefore, the relative enrichment of the side chain-N of Asn may well be a reflection of the isotopic nature of the feedstock used in its production.

All four sources of His show enrichment at the imidazole side chain compared to the peptide-N (average $\Delta\delta_{side-amino} = +9‰$). Three of the four sources of Lys (Acr, Flu, Sig) show enrichment at the side chain-N (average $\delta^{15}N = +4‰$) with respect to the peptide-N (average $\delta^{15}N = -3.5‰$). The other source (Avo) does not show a significant difference between the peptide- and side chain-N positions. Assuming that all four lysine samples were produced through microbial fermentation, this suggests that Avo was produced under different physiological conditions or by a different organism than the other three Lys sources.

The $^{15}N/^{14}N$ position-specific measurements provide a tool for distinguishing between sources that cannot be separated by GCC−IRMS at the compound-specific level. For example, by CSIA, Gln from Acr was indistinguishable from Gln from Flu at the 95% confidence limit. Using PSIA, the peptide-N of Acr Gln ($\delta^{15}N = -8.85 \pm 0.41‰$) is distinguishable from the same position on Flu ($-7.66 \pm 0.58‰$). Similarly, Asn from Acr and Avo are distinguishable at peptide-N ($\delta^{15}N = -4.31$ and $+1.18$, respectively) although they are nearly identical at the CSIA level.

(36) Zalkin, H.; Smith, J. L. In *Advances in Enzymology*; Purich, D. L., Ed.; John Wiley & Sons Inc.: New York, 1998; Vol. 72, pp 87−144.
(37) Macko, S. A.; Fogel, M. L.; Hare, P. E.; Hoering, T. C. *Chem. Geol.* **1987**, *65*, 79−92.
(38) Rowlands, R. T. *Enzyme Microb. Technol.* **1984**, *6*, 3−10.
(39) Kusumoto, I. *J. Nutr.* **2001**, *131*, 2552S−2555S.

CONCLUSIONS

High-precision $\delta^{15}N$ measurements of low-microgram quantities of polynitrogenous amino acids were accomplished by selective off-line removal of an N-containing moiety, followed by derivatization with ethyl chloroformate. This represents the first literature report of intramolecular $^{15}N/^{14}N$ measurements of biologically relevant molecules and also provides a general framework for how other N-PSIA studies of polynitrogenous compounds may proceed. For example, the peptide-N of tryptophan may be liberated by commercially available tryptophanase and that of ornithine by ornithine oxo acid transaminase. The ability to observe isotopic variation at the position-specific level provides detailed information for refining models of biosynthetic pathways and their resulting isotopic fluxes, as well as improved specificity for "isotopic fingerprinting" studies. In this work, we observed a general pattern of enrichment in the side chain-N with respect to the peptide-N. In some cases, we could rationalize this pattern in terms of biosynthetic fractionation (Gln) or as an artifact of the synthetic process (Asn). Exceptions to this trend (Gln from Flu, Lys from Avo) may indicate different biological sources or different production techniques. To facilitate our interpretation of PSIA data, we intend to study polynitrogenous amino acids from organisms grown under controlled conditions in future work.

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