Nitrogen Isotopic Analyses by Isotope-Ratio-Monitoring Gas Chromatography/Mass Spectrometry

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Amino acids containing natural-abundance levels of ^{15}N were derivatized and analyzed isotopically using a technique in which individual compounds are separated by gas chromatography, combusted on-line, and the product stream sent directly to an isotope-ratio mass spectrometer. For samples of N_2 gas, standard deviations of ratio measurement were better than 0.1‰ (Units for δ are parts per thousand or per million (‰).) for samples larger than 400 pmol and better than 0.5‰ for samples larger than 25 pmol (0.1‰ ^{15}N is equivalent to 0.00004 atom % ^{15}N). Results duplicated those of conventional, batchwise analyses to within 0.05‰. For combustion of organic compounds yielding CO_2/N_2 ratios between 14 and 28, in particular for N-acetyl *n*-propyl derivatives of amino acids, δ values were within 0.25‰ of results obtained using conventional techniques and standard deviations were better than 0.35‰. Pooled data for measurements of all amino acids produced an accuracy and precision of 0.04 and 0.23‰, respectively, when 2 nmol of each amino acid was injected on column and 20% of the stream of combustion products was delivered to the mass spectrometer. (*J Am Soc Mass Spectrom* 1994, 5, 387–397)

Measurements of natural variations in the abundances of stable isotopes have been used widely in biochemistry, archaeology, geochemistry, and ecology to determine sources of materials and processes governing their synthesis and degradation. Results of these investigations are expressed quantitatively in terms of the differential parameter, δ ,

$$\delta = [(R_{\text{sample}}/R_{\text{reference}}) - 1] 10^3 \quad (1)$$

where R is the isotope ratio (e.g., $^{15}\text{N}/^{14}\text{N}$) and the reference point is some widely available and isotopically homogenous material (atmospheric N_2 in the case of N). Units for δ are parts per thousand or per million (‰). Measurements of $\delta^{15}\text{N}$ have provided information about the synthesis and metabolism of amino acids [1–6], the diets of ancient humans and other animals [7–9], transformations that occur during burial, diagenesis, and catagenesis of biological matter [10–13], and biogeochemical, environmental, and ecological processes [14–21].

Although diverse spectroscopic techniques have been used to measure nitrogen isotopic abundances, highly precise and accurate measurements ($\pm 0.5\text{‰}$) in small quantities of naturally occurring materials depend mainly on isotope-ratio mass spectrometry. Typi-

cally, potentially interfering elements are removed before analyses of nitrogen isotopic abundances in natural materials by converting organic nitrogen to N_2 after Kjeldahl digestion [22] or combustion [20, 23–26], although different techniques, such as inductively-coupled or microwave-induced plasmas [27, 28], have been used to transform organic materials to suitable forms for mass spectrometric measurements.

Automated, continuous-flow systems have been developed for nitrogen-isotopic analyses of bulk materials [24–26]. These provide excellent efficiency but usually require micromole quantities of nitrogen and are not appropriate for the examination of individual compounds in mixtures. Matthews and Hayes [29] first reported a technique involving on-line separation of individual compounds for nitrogen isotopic measurements. Amino acids with low enrichments of ^{15}N were derivatized and separated by gas chromatography, and gas chromatographic effluents were combusted and sent to the mass spectrometer continuously in a helium carrier stream. This technique, referred to as isotope-ratio-monitoring gas chromatography-mass spectrometry (irmGCMS), has since been applied successfully to measure subtle variations in carbon isotope ratios in natural materials.

The first reports of successful irmGCMS measurements of natural-abundance levels of nitrogen isotopes in amino acids and other natural materials appeared in 1991 [30]. These analyses demonstrated that accurate

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and precise measurements were obtained for samples of N_2 containing 6–1000 pmol and for N-acetyl *n*-propyl derivatives of glycine and alanine yielding 200 pmol N_2 . Triplicate analyses of alanine and glycine yielded standard deviations of better than 0.3‰ and were accurate to within $\pm 0.35\%$. Subsequently, a second laboratory has reported the application of isotope-ratio-monitoring techniques to organic materials with relatively low C/N ratios [31].

Here we document presently attainable levels of performance and consider optimization of the postcolumn combustion system. We demonstrate the stability of the instrument, recommend methods for introduction of isotopic standards, and demonstrate that accurate and precise measurements can be obtained for N_2 introduced to the ion source in a carrier stream. We demonstrate that accurate and precise measurements were obtained for N_2 samples containing 30–1000 pmol N_2 with accuracy and precision similar to those obtained in conventional measurements. We also demonstrate that for materials with relatively low levels of nitrogen ($14 < CO_2/N_2 < 28$), in particular individual derivatized amino acids, accurate and highly precise nitrogen isotope ratios can be obtained quickly and conveniently.

Experimental

Instrumentation

The irmGCMS system employed for nitrogen isotopic measurements is shown in Figure 1. This system is similar to that used in carbon isotopic analyses [32] except that a CO_2 trap has been placed between the water-separator and the open split. In a typical analytical sequence: (1) individual compounds were separated chromatographically, (2) effluents were combusted continuously to CO_2 , N_2 , H_2O , etc., (3) water of combustion was removed as the effluent stream passed through a microvolume drying unit, (4) CO_2 was removed from the carrier stream, and (5) a portion of the effluent stream was delivered continuously to the ion source through a capillary leak. The sequence

Figure 1. Schematic of the combustion interface and related inlets.

was modified for analyses of N_2 gas, which was injected onto a chromatographic column and sent directly to the mass spectrometer via the open split and capillary leak. In separate trials, a reduction reactor was placed between the combustion reactor and the water separator and/or components of the system were altered or removed in order to investigate specific effects.

Gas chromatography. The system utilized a model 3400 (Varian, Sugarland, TX) gas chromatograph. Samples were injected using an on-column injector for liquid or solid samples in solution (Hewlett Packard, Avondale, PA) or a 1- μ L internal-volume injection valve for N_2 samples (Valco Instruments, Houston, TX). For gas analyses, porous-layer open-tubular (PLOT) columns were employed. PLOT columns contained either bonded molecular sieve particles (Chrompack, Raritan, NJ: Molsieve 5Å, 25 m $\times$ 0.32 mm $\times$ 10 μ m particle size, operated at room temperature) or a porous polymeric adsorbent (Chrompack: PoraPLOT Q, styrene-divinylbenzene, 25 m $\times$ 0.32 mm $\times$ 10 μ m, operated at room temperature). An Ultra-2 column was employed for separation of amino acids and other organic materials (Hewlett Packard: 5% phenyl, 95% methyl polysiloxane, 50 m $\times$ 0.32 mm $\times$ 0.52 μ m film thickness), and the temperature of the column was programmed to increase from 45 $^{\circ}C$ to 280 $^{\circ}C$ at a rate of 3 $^{\circ}C/min$. In all analyses, columns were operated with a carrier stream of 1 mL He/min.

Combustion of organic material. The effluent stream entered a unit consisting of a combustion reactor and water separator [32]. The former was a nonporous alumina tube with an inner diameter of 0.5 mm containing copper or nickel wire oxidized to nickel oxide or cupric oxide. Reactors containing CuO were operated at 850 $^{\circ}C$ whereas NiO reactors were operated at 1100 $^{\circ}C$. Water was removed as the effluent stream passed through a semipermeable nafion membrane tube. A reactor for reduction of nitrogen oxides and scavenging of O_2 from the effluent stream was constructed by inserting three 0.1-mm diameter, 20-cm length copper wires into a nonporous alumina tube (30.5-cm length, 3-mm o.d., 0.5-mm i.d.). In some experimental trials, this reactor was connected between the combustion reactor and water separator using transfer capillaries and 1/8-in. to 1/32-in. reducing unions with graphite or graphitized-vespel ferrules (SGE, Austin, TX). Void volumes were minimized by inserting the capillaries approximately 1 cm into the ends of the reactor tube. The temperature of the unit was maintained at 500 $^{\circ}C$ using a reactor furnace described previously [32].

Removal of CO_2 . Carbon dioxide, which would otherwise contribute to ion currents at mass 28, 29, and 30 via its CO^+ fragment, was removed from the efflu-

ent stream by adsorption, chemical conversion, or cryogenic trapping. In most experiments CO₂ was trapped cryogenically by inserting a coiled length of the transfer capillary (0.32-mm i.d., 1.0- to 1.5-m length) downstream from the water separator and upstream from the open split into a dewar filled with liquid N₂. Otherwise, CO₂ was removed from the effluent stream by chemical trapping by placing the Molsieve PLOT column in series between the water separator and open split.

Mass spectrometer. Nitrogen entered the ion source via the capillary leak connected to the open split. A mass spectrometer with a high-sensitivity source operating at an accelerating potential of 10 kV was employed (MAT 252; Finnigan MAT, Bremen, Germany). Ions were generated by electron impact (70 eV), and the source and analyzer regions of the instrument were differentially pumped. Ion currents were measured continuously for *m/z* 28, 29, and 30 using triple Faraday cups connected to high-speed amplifiers. Electronic offsets were also applied to the collectors by adding small, constant currents to the summing point for the electrometers [33].

Flow Pathways

Several operational modes were available by selection among gas pathways through the interface. Pathways of gas streams were controlled by the three computer-controlled, air-actuated valves shown in Figure 1 [33, 35].

During normal operation, the inlet capillary transmitted 0.2 mL/min from the effluent stream to the ion source. The remainder (0.8 mL/min) exited through a vent attached to the open split. During backflush operation, vent A (upstream from the reactor) was opened while vent B (attached to the open split) was closed. Consequently, He flowed in both directions from the open split: to the ion source and to vent A. The system was backflushed during the first 10 min of most analyses to prevent solvent from entering the interface. Finally, metal oxides in combustion reactors were re-

generated by admitting a stream of O₂ (1-5 mL/min) into the carrier stream upstream from the reactor.

Experimental Procedures

Samples. In analyses of N₂, samples were prepared over a range of 40 nmol to 5 pmol N₂ by filling an exponential dilution flask (EDF) with N₂ and diluting it slowly with He. The approach was similar to that used previously in analyses of CO₂, CH₄, and propane [32, 33].

An aqueous solution of nine amino acids was prepared for use as a test sample (Table 1). N-acetyl *n*-propyl derivatives were prepared using a modified version of a procedure outlined in a review by Engel and Hare [34]. Specifically, a 100- μ L aliquot of the amino acid solution was transferred to a 5-mL vial with a Teflon-lined cap and treated as follows: (1) water was evaporated at 110 °C under a nitrogen stream, (2) carboxyl groups were esterified using 300 μ L 3N HCl/*n*-propanol (~2000 $\times$ excess, 15 min at 110 °C), (3) reagents were evaporated at 110 °C under a nitrogen stream, (4) amino groups were acetylated using 1 mL of a 1:1 mixture of acetyl chloride in dichloromethane (~3000 $\times$ excess, 30 min at 90 °C), (5) reagents were evaporated at 90 °C under a nitrogen stream, and (6) products were dissolved in 125 μ L ethyl acetate. For each 1- μ L injection containing 1.3-2.6 nmol of these amino acids (Table 1), 130-260 pmol N₂ per compound were delivered to the ion source (80% of the effluent stream exited through the open split). Additionally, single-component solutions of nitrobenzene, nitropropane, and N-acetyl *n*-propyl derivatives of alanine, glycine, and leucine were prepared. Nitrogen isotopic compositions of these compounds and of nonderivatized amino acids were determined independently by conventional isotope-ratio mass spectrometry [20, 23].

Introduction of isotopic standards. Isotopic standards were introduced by admitting pure N₂ from the variable-volume inlet [35]. The isotopic composition of the N₂ standard was calibrated by conventional isotope-

Table 1. Composition of the amino acid mixture

Compound	Designation	Concentration (nmol/ μ L)	Derivative (CO ₂ /N ₂)	pmol N ₂ to Ion Source ^a
Alanine	A	2.2	16	220
Glycine	G	2.6	14	260
Leucine	L	1.6	22	160
Norleucine	NI	1.6	22	160
Serine	S	1.8	20	180
Proline	P	2.0	20	200
Aspartic acid	D	1.5	24	150
Hydroxyproline	H _p	1.7	24	170
Phenylalanine	F	1.3	28	130

^a injection size = 1 μ L. 20% of the effluent stream was analyzed.

ratio mass spectrometry by comparison with N_2 in air ($\delta^{15}N \equiv 0\text{‰}$) and by comparison with N_2 prepared from ammonium sulfate standards NBS-N1 ($0.43 \pm 0.03\text{‰}$) and NBS-N2 ($20.40 \pm 0.07\text{‰}$) (Collister, J. W. Personal communication, Indiana University, 1991).

Calculations and Presentation of Results

Data collection and processing. The mass spectrometer was controlled by a Compaq 386/20 MHz computer, and data acquisition was under software control [36]. Ion currents at m/z 28, 29, and 30 were integrated continuously and digitized at 0.25-s intervals. The resulting data files were processed to define chromatographic peaks. Differences in chromatographic retention times for isotopic species of N were corrected, if necessary, by fitting a parabolic function to each of the three collector signals and shifting the integration intervals for m/z 28 and 30 to align with that of m/z 29. Next, for all peaks, total ion currents for m/z 28, 29, and 30 were integrated, background signals were subtracted, and the ratio of the sample-derived ion current at m/z 29 to that at 28 (^{29}R) was calculated for each peak.

Calculation of $\delta^{15}N$. To calibrate the isotopic compositions of samples versus isotopic standards, the operator identified peaks corresponding to standards and entered the δ value for each standard into an algorithm which calculated isotope ratios. To correct for proportional mass-discrimination effects, we distinguish between measured and true ratios by denoting the latter by R^* . We can then write

$$(R_{\text{sample}}/R_{\text{standard}})_{\text{measured}} = R^*_{\text{sample}}/R^*_{\text{standard}} \quad (2)$$

The values on the right side of the equation are independent of mass discrimination, and R^*_{sample} can be calculated from measured δ values if R^*_{standard} is known. For each standard introduced during the analysis, the true ratio can be calculated from its known δ value:

$$^{15}R^*_{\text{standard}} = (10^{-3\delta} + 1) ^{15}R^*_{\text{air}} \quad (3)$$

where $^{15}R^*_{\text{air}}$ is equal to 0.003676 ± 0.000008 [37]. The true ion current ratio ($^{29}R^*$) is then given by

$$^{29}R^*_{\text{standard}} = 2^{15}R^*_{\text{standard}} \quad (4)$$

Typically, standards are introduced periodically to allow detection of and correction for drifts in instrument performance. As described previously for carbon isotope measurements [36], given the ion-current ratio observed for the first standard, the ratio expected for the second is calculated and compared to the observed ratio. Subsequent standards can be either later injections of the same material or compounds with different (but accurately known) isotopic compositions. Any

difference between observed and expected ratios represents drift and provides a means for estimating instrument characteristics at intervening points where samples appear. Specifically, for any two sequentially observed standard peaks, i and $i + 1$, we can write

$$^{29}R^p_{i+1} = ^{29}R^*_{i+1} (^{29}R_i / ^{29}R^*_i) \quad (5)$$

where $^{29}R^p_{i+1}$ is the ion-current ratio predicted for the second standard peak and $^{29}R_i$ is the measured ratio for the first standard. To correct for differences in observed versus predicted ratios, the ion-current ratio that would be observed if the i th standard were reintroduced at any time between the two standard peaks can be estimated by linear interpolation:

$$^{29}R_{iu} = [(t_u - t_i)/(t_{i+1} - t_i)] (^{29}R_{i+1} - ^{29}R^p_{i+1}) + ^{29}R_i \quad (6)$$

where t_i and t_{i+1} are the actual times of appearance for standards and $^{29}R_{iu}$ is a proxy for the ion-current ratio which would be observed for a standard with $^{29}R^* = ^{29}R^*_i$ appearing at time t_u . The true ratio for an unknown peak appearing at t_u is then given by

$$^{29}R^*_u = ^{29}R^*_i (^{29}R_u / ^{29}R_{iu}) \quad (7)$$

and its δ value is given by

$$\delta = 10^3 [(^{29}R^*_u / ^{29}R^*_{\text{air}}) - 1] \quad (8)$$

Results are also reported in terms of Δ values in order to express the accuracy of isotopic measurements:

$$\Delta = \delta_m - \delta_e \quad (9)$$

where the subscripts m and e refer to measured and expected values of δ . To summarize the quality (combining both accuracy and precision) of a set of n measurements of δ values, we report root-mean-square Δ (rms Δ):

$$\text{rms}\Delta = (\Sigma \Delta^2 / n)^{0.5} \quad (10)$$

Results and Discussion

Analysis of Nitrogen (N_2) Samples

Appearance of data. To provide a basis for consideration of signal processing and instrument performance it is useful to examine the nature and appearance of the recorded signals. As shown in Figure 2a, signals responded rapidly when the switching valves were manipulated to mix N_2 admitted from the variable-volume reservoir with He flowing through the inlet capillary shown in Figure 1. All signals quickly returned to baseline values after the switching valves

Figure 2. Representative ion-current and ratio traces produced during introduction of N_2 (a) from the variable volume inlet ($0.3 \text{ nmol N}_2/\text{s}$) or (b) via the carrier gas stream ($0.34 \text{ nmol N}_2/\text{s}$). The same sample was used to generate both traces. The small, secondary peak in (b) results from inadvertent introduction of air because of imperfect seals in a gas-sampling valve.

were returned to their original positions. Because the rate of flow of N_2 from the variable-volume reservoirs was constant, recordings of ion currents approximated square waves. Additionally, since the isotopic composition of the N_2 did not vary with time, ^{29}R was constant over these regions. For cases in which samples were admitted to the mass spectrometer after passing through a chromatographic column (Figure 2b), ion-current traces corresponding to introduction of sample N_2 have gaussian peak profiles. The same nitrogen sample was used to generate traces shown in Figure 2.

As with irmGCMS analyses of CO_2 , because the ratio of total ion currents was plotted as a function of time, the appearance of the recording was dependent not only on the signals produced by the sample but also on the magnitudes of background signals. In the present case, background signals derived almost entirely from electronic-offset currents applied to the summing point of the electrometer for each collector [33], and resulting values of ^{29}R were greater than those characteristic of the sample. As a result, ^{29}R decreased when the sample was admitted. Because separation of nitrogen isotopic species was minimal, interpretation was not complicated by consideration of chromatographic isotope effects.

Instrument performance. Stability. In conventional, dual-inlet procedures, each integration of sample ion currents is immediately preceded and/or followed by an equivalent observation of ion currents derived from an isotopic standard. In isotope-ratio-monitoring procedures, observations of samples and standards may be separated by a considerable interval. In order to examine limitations imposed by instabilities in components and operating conditions, experiments were performed in which ion-current ratios from a single gas sample were observed repeatedly. The first observation in the series was arbitrarily taken as the standard, and all subsequent observations were treated as unknown samples. Any sample versus standard differences which then appeared indicated the occurrence of drifts that, in real measurements, would degrade the accuracy of computed isotope ratios unless multiple standards were distributed throughout the run in order to allow recognition of and correction for such drifts.

Results from three sets of observations for N_2 introduced from the variable-volume inlet are summarized in Figure 3. In the first two experiments (Figure 3a and b), gas samples were repeatedly introduced in the absence of carrier gas. The background pressure in the ion source was $0.01 \mu\text{torr}$ and the indicated pressure during sample introduction was $0.1 \mu\text{torr}$. The sample introduction rate was approximately $0.2 \text{ nmol N}_2/\text{s}$. The resulting ion current (mass 28) was 8 nA . Integration of ion currents for 20 s was thus expected to produce—if the system displayed perfect stability and performance at the shot-noise limit (see discussion below)—a population of δ values with a mean of zero and a standard deviation of 0.024% .

To examine the stability of mass-discrimination characteristics using these data, δ values were first referred to a single standard defined at the beginning of the series. As shown in Figure 3a, δ values, expressed as Δ , drifted at a rate of approximately $-0.030\%/ \text{min}$. Effects of this drift in mass discrimination could be neutralized by definition of additional standards within the series. In a second series of observations (Figure 3b), δ values did not drift significantly during the first 25 min but drifted at a rate of approximately $0.010\%/ \text{min}$ during the remainder of the analysis. The two runs indicated (Figure 3a and b) are typical and were separated in time by twelve hours. As shown in Figure 3c, drift rates were similar when a stream of carrier gas (0.2 mL He/min , indicated source pressure of $5 \mu\text{torr}$) was admitted to the ion source.

Drift characteristics are summarized in Table 2. The entries tabulated under "representative maximum drift rates" refer to the slopes of the single-standard δ trends in Figure 3a and c. The practical effects of these instabilities depend on the nature of the drifts. For example, if drifts were perfectly linear, their effects could be neutralized by use of only two standards, one at the beginning of an analytical run and one at the end. As demonstrated in Figure 3, however, more

Figure 3. Plots of the drift in $\delta^{15}\text{N}$ values versus time for repeated introduction of identical samples via the variable-volume inlet. The sample introduction rate was approximately $0.2 \text{ nmol N}_2/\text{s}$ ($\delta^{15}\text{N} = -7.4\text{‰}$ versus AIR). The three lines in each graph represent Δ values deriving from three different standardization schemes. In one case, each ion-current ratio in a series of 17 regularly spaced observations was compared only to the first observation in the series. Computed values of δ then drifted as a result of uncompensated instabilities, the trend being indicated by the most widely divergent line in each graph. In a second case, the first and seventeenth observations were taken as standards and δ values were computed using an interpolative procedure (eqs 5-8). Values of Δ were thus tethered to zero at the beginning and end of the series and effects of drift greatly reduced. Finally, two additional, intermediate points within each series were designated as standards. The maximum interval between standards was then 1000 s and values of Δ ranged only been -0.06 and 0.05‰ . Positions of standards are indicated by the black dots "anchoring" Δ to the zero line. The series in graphs (a) and (b) were both recorded in the absence of carrier gas. They differ only because of random variations in drift characteristics and together provide a view of typical performance. The series in graph (c) was recorded in the presence of carrier gas, which was never observed to have any significant effect on drift characteristics.

frequent standardization is often advantageous. Entries in Table 2 indicate how frequently standards must be introduced if specified levels of accuracy are to be obtained, whereas data in Table 3 indicate accuracy and precision obtained when standards were introduced at intervals of 57, 30, and 20 min. These data indicate that systematic drifts in instrument response can be adequately corrected if standards are introduced at 20-30 min intervals; more frequent standardization did not improve performance.

Sample requirements. Samples of N_2 , ranging from 17-1200 pmol were prepared using an exponential-dilution system. Samples were injected onto a Poraplot Q column and introduced directly from the column into the mass spectrometer through a capillary leak exiting the open split. Resulting values of δ are shown in Figure 4, and lines designating multiples of theoretical levels of precision are indicated (discussed below). Results in Figure 4a demonstrate absence of systematic errors between 75-1000 pmol N_2 , although δ values trend toward more positive values for smaller samples at a rate of nearly $0.008\text{‰}/\text{pmol N}_2$, probably due to introduction of atmospheric contamination during sample dilution (in these experiments effects of drift were neutralized by admission of standards from the variable-volume inlet at 20-min intervals). These problems were corrected for analyses shown in Figure 4b and c.

Precision and accuracy of measurements. When standards are introduced frequently enough to neutralize effects of drift, the performance of this technique will be limited by instrument sensitivity and sample size. A theoretical maximum level of performance can be established by consideration of shot noise and its effects on δ . The standard deviation for the precision-controlling ratio for nitrogen isotopic measurements, $R = {}^{29}\text{i}/{}^{28}\text{i}$, will be given by

$$\sigma_R^2 = (\partial R / \partial {}^{29}\text{i})^2 \sigma_{29}^2 + (\partial R / \partial {}^{28}\text{i})^2 \sigma_{28}^2 \quad (11)$$

where ${}^{28}\text{i}$ and ${}^{29}\text{i}$ are the mass-28 and mass-29 ion currents and σ_{29} and σ_{28} are the standard deviations of the ion currents. The following expression for the maximum attainable (i.e., shot-noise limited) performance can be derived by modifying equations presented previously in derivations for carbon isotopic measurements [33]:

$$\sigma_\delta^2 = 2 \times 10^6 (\sigma_R/R)^2 = 2 \times 10^3 (1 + R)^2 / \text{EmN}_A R \quad (12)$$

Table 2. Drift rate versus frequency of standardization

He Flow (mL/min)	Length of runs (h)	Number of Observations	Drift, Representative Maximum (‰/min)	σ_{δ} (snl) ^a	σ_{δ} (obsd)	Maximum Time between Standards (min)	
				(‰)	(‰)	$\sigma_{\text{obs}} \leq 3\sigma_{\text{snl}}$	$\sigma_{\text{obs}} \leq 0.1\text{‰}$
0	1.0	32	±0.030	0.024	0.032	20	60
0.2	1.0	16	±0.010	0.024	0.027	30	60

^a snl = Shot-noise limit defined by eq 14.

Table 3. Precision of isotopic measurements versus frequency of standardization

He Flow (mL/min)	Number of Observations	Interval Between Standards (min)	mean Δ^a (‰)	σ_δ , obs ^b (‰)
0	16	57	-0.19	0.083
0	15	30	-0.021	0.045
0	14	20	0.004	0.035
0.2	16	57	-0.12	0.068
0.2	15	30	-0.017	0.024
0.2	14	20	-0.010	0.033

^aThe $\delta^{15}\text{N}$ value measured for this sample was $-7.42 \pm 0.06\text{‰}$, $n = 6$.

^bThe shot-noise limited precision for these samples was $\sigma_\delta = 0.024\text{‰}$.

Figure 4. Values of $\delta^{15}\text{N}$ for N_2 samples of varying size generated using an exponential dilution flask. (a) Samples transmitted directly from column effluent to the ion source by way of an open split; (b) after insertion of water-removal unit (tubular Nafion membrane) in sample pathway; (c) after addition of a CuO combustion reactor (850°C) to the sample pathway. Error envelopes were calculated using eq 14: (a) $\pm 5\sigma$, $42\text{-V}\cdot\text{s}$ standard; (b) $\pm 7\sigma$, $46.5\text{-V}\cdot\text{s}$ standard; (c) $\pm 10\sigma$, $42\text{-V}\cdot\text{s}$ standard.

where σ_δ is the standard deviation characteristic of the distribution of observations of δ , R is the ion-current ratio characteristic of the gas being analyzed (approximately 0.0073 for $^{29}/^{28}$ in the mass spectrum of N_2), E is the number of molecular ions received at the collector per molecule of gas introduced to the ion source, m is the number of moles of sample gas, and N_A is Avogadro's number. If we substitute $^{28}\text{A}/R_I q_e$ for the product EmN_A in the above equation, where

^{28}A is the "area," or integrated signal ($\text{V}\cdot\text{sec}$) and $q_e = 1.6 \times 10^{-19}$ coulombs, $R_I = 3 \times 10^8$ ohms, and $R = 0.0073$, a simplified expression for the shot-noise limited precision as a function of the integrated signal is obtained:

$$\sigma_\delta^2 = 0.01353 / ^{28}\text{A} \quad (13)$$

This expression is applicable when values of ^{29}R for standard and sample gases have been determined with equal precision. A more general expression can be written for instances in which integrated ion currents for the sample and standard are not equal:

$$\sigma_\delta^2 = 0.00676 (1 / ^{28}\text{A}_{\text{sample}} + 1 / ^{28}\text{A}_{\text{standard}}) \quad (14)$$

The approach of the system to these theoretical-maximum levels of performance is examined in Figure 4. In Figure 4a points represent values of Δ (i.e., the deviation from the correct result) observed when N_2 -He mixtures prepared by exponential dilution were introduced to the He stream just upstream from the open split. If the shot noise treated in eqs 11-14 were the only significant source of error, 99% of the Δ values should fall within $\pm 2.5\sigma_\delta$ of zero. In fact, in order to enclose nearly all the points in the 70- to 1000-pmol range, it has been necessary to place the error envelope at $\pm 5\sigma$, thus indicating that actual performance falls short of the theoretical maximum by roughly a factor of two. Systematic errors that are evident near the end of the dilution run, for samples containing less than 70 pmol N_2 , were caused by leakage of atmospheric N_2 into the sample-preparation system (the δ value for the test sample was -7.4‰ versus AIR; deviations associated with the air leak are thus positive) and do not indicate some low-sample failure of the mass spectrometer system. Apart from this artifact, average values of δ do not differ significantly from the expected result (i.e., values of Δ are scattered around zero), demonstrating the accuracy of the procedures for measurement and calibration.

In order to determine their effect, if any, on accuracy and precision, interface components were added sequentially to the flow pathway and the test analyses repeated. Figure 4b indicates the scatter observed when the water separator was added and Figure 4c indicates

the scatter observed when sample gases also flowed through a CuO combustion reactor heated to 850 °C. Error envelopes in parts b and c have been placed at ± 7 and ± 10 σ , indicating levels of performance approximately three- and fourfold short of theoretical-maximum levels.

The loss in performance when the CuO reactor was inserted into the flow pathway was not caused by CO₂ contamination released from the reactor since the liquid nitrogen trap collected CO₂ before it entered the ion source. The possibility that CO released from the reactor is responsible for loss of performance can be dismissed because data did not trend strongly to more positive values at smaller sample sizes. A more plausible explanation may be variable formation of nitrogen oxides in the reactor. Support for this is found in observations made using NiO reactors. The partial pressure of O₂ over NiO is very low [32], but when O₂ was added upstream from the reactor so that the mole fraction of O₂ was 0.005 (equal to that expected from equilibration with CuO at 850 °C) and the reactor was maintained at 1050 °C, formation of nitrogen oxides was so efficient that <1% of the expected signal was observed at masses 28–30. When the O₂ stream was removed, N₂ was transmitted quantitatively and accurate δ values were obtained. In the case of CuO reactors, O₂ was always available in the gas phase and formation of nitrogen oxides was possible, albeit at much lower levels that were a result of the lower temperature (850 °C, max) and likely catalytic destruction of NO_x that were a result of the presence in the system of traces of Cu metal. On balance, these considerations suggest but do not prove that the results summarized in Figure 4c, in which precision was degraded but transmission of N₂ was essentially quantitative, are indicative of the formation of traces of isotopically fractionated nitrogen oxides. However, when a "reduction reactor" (Cu at 500 °C) was placed in the gas stream downstream from the combustion reactor, no improvement in performance could be demonstrated.

Analysis of Nitrogen in Organic Materials

Removal of CO₂ from the effluent stream. Since CO⁺ fragments are generated from CO₂ in the ion source and produce ion currents at m/z 28–31 which interfere with measurement of ion currents for N₂ (m/z 28–30), CO₂ produced during the combustion of organic material must be removed from the effluent stream. In early experiments, CO₂ was removed from the effluent by sorption in a microbore chromatographic column containing molecular sieve (Molsieve 5A PLOT column) or in packed tubes containing molecular sieve or ascarite. High efficiencies of removal of CO₂ could not be obtained without degrading peak shape. In contrast, cryogenic trapping proved efficient and simple. It was necessary only to increase the length of the transfer capillary connecting the water separator and the open

split so that a portion could be immersed in liquid nitrogen. Although at least four temperature-programmed chromatographic analyses of the amino acid mixture could be completed before solid CO₂ accumulated and restricted flow, the capillary was warmed briefly after each chromatographic run.

Comparison of performance using CuO or NiO combustion reactors. Cupric oxide reactors. Postcolumn reactors in which CuO served as O₂ donor and oxidation catalyst were used in previous irmGCMS analyses of nitrogen in organic materials [30, 31]. Here, their performance was documented by analyzing derivatives of alanine and glycine individually. Approximately 2 nmol of each derivatized amino acid was injected onto the chromatographic column. Amounts of N₂ reaching the mass spectrometer were thus 200 pmol per compound. Results are summarized in Table 4 and document several points: (1) results were accurate within 0.35‰, thus neither the isotope-ratio-monitoring procedure nor the derivatization led to systematic errors, (2) standard deviations frequently differed by less than a factor of two from those obtained in conventional measurements and were within a factor of 10 of the theoretical-maximum precision (0.029‰ for 200 pmol N₂), (3) accurate and precise results were obtained using nearly 5000 $\times$ less sample than required for conventional measurements, and (4) a Cu reduction reactor was not required to achieve acceptable performance for these analyses, presumably because traces

Table 4. Results of analyses using cupric oxide reactors

Compound	$\delta^{15}\text{N}$, ‰	
	irmGCMS	Conventional Analysis
Alanine ^a	-1.57	-1.63
	-1.59	-1.48
	-1.48	-1.74
	-1.98	
Mean	-1.66	-1.62
Standard deviation	0.22	0.13
Glycine ^a	-16.64	-16.10
	-16.20	-16.20
	-16.18	
	-16.34	-16.15
Mean	-16.34	-16.15
Standard deviation	0.26	0.06
Nitrobenzene ^b	-20.04	-19.90
	-19.60	
	-20.17	
	-19.28	
Mean	-19.77	-19.90
Standard deviation	0.41	

^aCuO combustion reactor only.

^bCuO reactor followed by Cu reduction reactor.

of elemental copper were present in the reactor at all times. The leucine derivative was also analyzed, but results were erratic, apparently because samples of this carbon-rich amino acid exceeded the oxidation capacity of the reactor. When nitrobenzene was analyzed, N_2 ion currents were barely visible above baseline when combustion reactors were used alone but were sufficient when a reduction reactor was inserted (Table 4). Use of a reduction reactor was generally required—and uniformly successful [31]—when analytes contained N—O bonds.

Nickel oxide reactors. Nickel oxide reactors were preferred in carbon isotopic analyses because of their higher oxidation capacity and insignificant loss of O_2 from thermal dissociation of metal oxide [32]. When operated at 1100 °C without addition of gas-phase O_2 , NiO reactors displayed several advantages versus CuO reactors: (1) larger capacity for oxidation of organic material, (2) better retention of oxidizing power during successive analyses, (3) operability at temperatures greater than 1100 °C during continuous operation without loss of oxidation capacity, (4) no requirement for a separate reduction reactor, and (5) if a reduction reactor is nevertheless employed, the near-zero bleed of O_2 from the NiO extends the life of the reduction unit.

To evaluate the performance of NiO reactors, the isotopic compositions of derivatized amino acids and of nitropropane were measured. A representative chromatogram is shown in Figure 5. It was necessary to introduce greater than 2 nmol of each amino acid to generate at least 200 pmol N_2 per component. For most compounds, these quantities of organic material overloaded the Ultra-2 chromatographic column (liquid phase thickness, 0.52 μ m). Results allowing comparison of conventional and irmGCMS analyses (with and without a Cu reactor for reduction of traces of NO_x) are summarized in Table 5. The precision of the irmGCMS analyses is only slightly poorer than that of the conventional measurements, which required much larger samples (quantities of material adequate to generate $> 5 \mu$ mol N_2) and were much slower. There is

Figure 5. Ion-current and ratio traces for a chromatographic analysis of a mixture containing N-acetyl *n*-propyl amino acids. Isotopic standards (*) and individual amino acids are identified (refer to codes in Table 1). Standards were introduced automatically from the variable-volume inlet at the rate of 0.3 nmol N_2 /s for 20-s periods. Injection times for standards were selected so that standards bracketed peaks of interest and were introduced during baseline regions of the chromatogram.

no evidence for inaccuracy, although, because of difficulty in separating isotopic contributions from individual sample components, coelution degrades accuracy and precision. In contrast with carbon isotopic analyses, in which separation of isotopic species often leads to systematic errors when compounds coelute [36], the accuracy of nitrogen isotopic measurements for slightly overlapped peaks was not significantly degraded. However, the precision of analyses decreased as demonstrated by results obtained for the closely eluting pairs alanine and glycine, serine and

Table 5. Comparison of data obtained for individual amino acids (nickel oxide and reduction reactors)

Compound	Conventional isotope-ratio Mass Spectrometry			irmGCMS (NiO Reactor Only)			irmGCMS (NiO and Cu Reactors)		
	$\delta^{15}N$ (‰)	σ_δ (‰)	n	$\delta^{15}N$ (‰)	σ_δ (‰)	n	$\delta^{15}N$ (‰)	σ_δ (‰)	n
Alanine	-1.62	0.13	3	-1.55	0.14	4	-1.75	—	2
Glycine	-16.15	0.07	2	-16.11	0.32	4	-16.17	—	2
Leucine	0.91	0.14	3	0.95	0.06	4	0.93	0.11	4
Norleucine	0.43	0.10	4	0.69	0.36	4	0.42	0.19	4
Serine	-8.84	0.06	3	-8.88	0.31	4	-8.88	0.19	4
Proline	-7.73	0.24	3	-7.88	0.25	4	-7.61	0.43	4
Aspartic acid	-1.31	0.08	3	-1.19	0.14	4	-1.55	0.15	4
Hydroxyproline	8.19	0.20	3	8.00	0.04	3	8.37	0.25	4
Phenylalanine	-3.06	0.08	4	-2.95	0.07	4	-3.02	0.26	4
Nitropropane	—	—	—	11.56	0.32	4	11.53	0.37	4

proline, and hydroxyproline and phenylalanine (Table 5). Through use of the NiO reactors, accurate and precise data were obtained for the leucine derivative and for other derivatives with high CO_2/N_2 ratios. The absence of a requirement for a reduction reactor is demonstrated by comparison of results for nitropropane and derivatized amino acids in which the combustion reactor was used alone or in combination with the reduction reactor.

Sample quantities used here were controlled by: (1) requirements of the mass spectrometer, (2) splitting of the carrier-gas stream, (3) sample capacities of chromatographic columns, and (4) the combustion capacity of NiO reactors. The acceptable range of sample sizes could be extended downward by modifying the split ratio so that a greater portion of the effluent stream entered the ion source or upward by increasing the sample capacity of the chromatographic column (e.g., by utilizing larger-bore or thicker-film columns). In practice, the latter option would be preferred because the precision of irmMS measurements and the sensitivity of the mass spectrometer can decrease dramatically if, due to admission of more carrier gas, the pressure in the ion source approaches 1 μtorr (indicated pressure, corresponding to an actual pressure of $\sim 8 \mu\text{torr}$ He, taking into account gauge sensitivity) [33]. The use of thicker-film columns will, however, cause higher rates of column bleed and thus lead to more rapid depletion of the oxygen reservoirs of combustion reactors.

Results from these experiments also demonstrate that procedures for definition and subtraction of background ion currents, initially developed for use in carbon-isotopic analyses, performed adequately in nitrogen irmGCMS experiments. For example, as shown in Figure 5, the background ion current increased dramatically because small leaks in interface fittings inside the chromatographic oven were temperature dependent and caused amounts of air in the carrier gas stream to increase steadily. Errors associated with the background correction will propagate, causing random errors that will further limit attainable levels of precision. This has been quantitatively treated for the case of carbon-isotopic analyses [33]. An exactly parallel derivation for the nitrogen-isotopic case leads to the expression

$$\sigma_{\delta}^2 = 0.00676 \left\{ \left[(A_{\Sigma} - a_b) / (A_{\Sigma} - wa_b/t_b)^2 \right]_{\text{sample}} + \left[(A_{\Sigma} - a_b) / (A_{\Sigma} - wa_b/t_b)^2 \right]_{\text{standard}} \right\} \quad (15)$$

where A_{Σ} and a_b are the integrated total and background signals ($\text{V} \cdot \text{sec}$) for mass 28, with a_b having been observed over a background-observation time t_b (typically only 1 s or less) and A_{Σ} having been integrated over the full chromatographic peak width w (s). For the analyses performed under the conditions pertinent to Figure 5, values of σ_{δ} based on eq 14 (i.e., without taking into account propagation of back-

ground-correction errors) are 0.03‰. Use of eq 15 leads to $\sigma_{\delta} = 0.06‰$. The latter value is independent of background levels. Peaks with higher backgrounds happened to be larger because increased column temperature was associated with decreased carrier-gas flow rate and thus with a more favorable split ratio. The resulting gain in precision offset the loss associated with higher background levels. As shown in Table 5, the standard deviations observed for quadruplicate analyses range from 0.04 to 0.43‰ and were thus less than $10 \times$ larger than the σ values predicted by eq 15 (see discussion of results summarized in Figure 4). Moreover, as shown in Figure 6, attained levels of precision and accuracy were independent of background levels despite background ion currents approaching 5.5 nA at m/z 28.

Conclusions

Although challenges inherent in nitrogen irmGCMS measurements are similar to those encountered in the analyses of carbon [32, 33, 35, 36], there are additional factors that complicate nitrogen isotopic measurements. These include [31] the relatively low natural abundance of ^{15}N , the commonly low abundance of N relative to C, the possibility of forming nitrogen oxides and the difficulty achieving quantitative conversion of organic material to N_2 , the pervasiveness of atmospheric N_2 and consequent problems with contamination, and the relatively low ionization efficiency of N_2 . This article indicates both that these factors can be overcome and that their impact is not negligible, leading to sample requirements that are high and levels of

Figure 6. Accuracy and precision of amino acid analyses as a function of background ion currents at m/z 28. Electronic offset on the mass-28 signal accounts for 0.3 nA, the remainder is due to small leaks in fittings in the combustion interface. The initial background ion current was 1.7 nA, but the first amino acid derivative did not elute until background ion currents had risen to the levels indicated (see Figure 5). Points shown here are representative of data in Table 5 (circles, NiO reactor only; triangles, NiO and Cu reactors).

precision that are low in comparison to performance obtained in carbon isotopic analyses.

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