Measurement precision and accuracy of high artificial enrichment ^{15}N and ^{13}C tracer samples

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Abstract:	<p>RATIONALE: Oversaturation of the Faraday cup amplifiers of isotope ratio mass spectrometers when using tracers that are highly enriched in heavier isotopes (up to 99.9%) remains a major bottleneck to obtaining high-precision measurements. The memory effect plays a key role in reducing tracer sample measurement precision and accuracy. Several sample preparation approaches are known to reduce memory effects and to improve tracer sample measurement precision. However, the potential benefits when using very high enrichment tracer samples ($> +1000$ mUr) have not been tested.</p> <p>METHODS: In this study, we test how specific sample positioning for measurements and frequent use of natural isotope abundance reference materials within the sequence affects the precision and accuracy of isotopic ratio analyses when using a Flash elemental analyser coupled to a Delta^{plus}XP isotope ratio mass spectrometer for very high enrichment (> 22000 mUr) ^{15}N tracer sample measurements. Furthermore, we investigate if tracer sample dilution with natural isotope abundance materials reduce memory effect and increase measurement precision and accuracy when measurements of high enrichment ^{15}N and ^{13}C biomass tracer samples are conducted.</p> <p>RESULTS: Frequent use of natural isotope abundance materials and specific positioning increased ^{15}N tracer sample precision, but it had a negative effect on the precision of quality control substances. ^{15}N and ^{13}C tracer sample dilution improved measurement precision by a maximum of ± 0.9 mUr; however, a strong linear relationship between the original and the calculated δ values was found. Highly enriched ^{15}N tracer samples caused a maximum memory effect of 0.11%. High levels of ^{15}N abundance within the samples affected measurement accuracy by an average of 6.7%.</p> <p>CONCLUSIONS: We conclude that highly enriched tracer samples do not require dilution before analysis. Tracer sample precision can be</p>

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	improved using a specific measurement order of anticipated isotope abundance and by the frequent use of natural abundance reference materials.

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Measurement precision and accuracy of high artificial enrichment ^{15}N and ^{13}C tracer samples

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Abstract

RATIONALE: Oversaturation of the Faraday cup amplifiers of isotope ratio mass spectrometers when using tracers that are highly enriched in heavier isotopes (up to 99.9%) remains a major bottleneck to obtaining high-precision measurements. The memory effect plays a key role in reducing tracer sample measurement precision and accuracy. Several sample preparation approaches are known to reduce memory effects and to improve tracer sample measurement precision. However, the potential benefits when using very high enrichment tracer samples ($> +1000$ mUr) have not been tested.

METHODS: In this study, we test how specific sample positioning for measurements and frequent use of natural isotope abundance reference materials within the sequence affects the precision and accuracy of isotopic ratio analyses when using a Flash elemental analyser coupled to a Delta^{plus}XP isotope ratio mass spectrometer for very high enrichment (>22000 mUr) ^{15}N tracer sample measurements. Furthermore, we investigate if tracer sample dilution with natural isotope abundance materials reduce memory effect and increase measurement precision and accuracy when measurements of high enrichment ^{15}N and ^{13}C biomass tracer samples are conducted.

RESULTS: Frequent use of natural isotope abundance materials and specific positioning increased ^{15}N tracer sample precision, but it had a negative effect on the precision of quality control substances. ^{15}N and ^{13}C tracer sample dilution improved measurement precision by a maximum of ± 0.9 mUr; however, a strong linear relationship between the original and the calculated δ values was found. Highly enriched ^{15}N tracer samples caused a maximum memory effect of 0.11%. High levels of ^{15}N abundance within the samples affected measurement accuracy by an average of 6.7%.

CONCLUSIONS: We conclude that highly enriched tracer samples do not require dilution before analysis. Tracer sample precision can be improved using a specific measurement order of anticipated isotope abundance and by the frequent use of natural abundance reference materials.

Keywords: stable isotopes, nitrogen, carbon, tracer sample dilution, reference material

Introduction

Stable isotopes are being used in an increasing number of fields including biology, paleoclimatology, environmental sciences, geology, oceanography, atmospheric sciences, medicine, food and drug authentication and forensic applications.¹ An increasing number of labelling studies require high accuracy and precision of isotopic ratio analyses.²⁻⁴ The use of isotope ratio mass spectrometer to measure samples with high artificial enrichment in the heavier isotopes may lead to Faraday cup amplifier saturation and distinct inter-sample memory effects (i.e., isotope composition of the previous sample(s) influencing the measurement of the isotope composition of the following sample(s)). Memory effects may be caused by either (i) sample preparation and handling before production of the measuring gases, (ii) conversion of analyte molecules to sample measuring gases (i.e., oxidation to CO₂ or oxidation/reduction to N₂), and/or (iii) the chromatographic separation process of the measuring gases.⁵

There are several options for how to avoid or minimise the biasing influence of memory effects. First, samples with a natural isotope abundance and samples with an artificially high amount of (heavier) isotopes should be separated (separate measurement sequences). Second, biasing influences of a memory effect can be minimised by analysing several sample aliquots in series, discarding the measurement results of the initial aliquots.^{5,6} Third, a mathematical correction of the memory effect⁷ may be applied, provided the memory effect is constant over the measurement sequence. The impact of the memory effect can further be minimised by running samples in ascending or descending order of isotope enrichment.⁸ However, this approach requires samples to be pre-measured in advance, which increases instrumental time and the cost of the study.

To the best of our knowledge, the relevance of memory effects, measurement accuracy, and precision to tracer data quality was previously not evaluated at very high ¹³C and ¹⁵N enrichment levels (> +1000 mUr). The typical precision of the measurement of samples with natural isotope abundance vary between ±0.06–0.3 mUr for δ¹³C and ±0.3–0.5 mUr for δ¹⁵N.⁹⁻¹² Increased dosing of the “tracer” (material with increased artificial enrichment in heavier isotopes) (e.g. *x*^E(*E*) of 0.8–1%) may decrease precision and accuracy of the corresponding measurement^{11,13,14}; however, actual numbers are commonly not reported.

This lack of scientific publications reporting precision and the accuracy of the measurement of samples spiked with a high enrichment in heavier isotopes is in stark contrast to the active discussion in the Isogeochem mailing list¹⁵, triggered by the need to understand the significance of errors in tracer data and to ensure precision control procedures of isotopic ratio analyses. However, an absence of quality control procedures in laboratories, as well as a lack of international reference materials with (high) enrichment in heavier isotopes, might contribute to an unwillingness to report these numbers. Moreover, approaches including tracer sample preparation and precision and accuracy assessments typically rely on internal laboratory procedures that are not standardized among laboratories.

A proper referencing scheme should include 'Identical Treatment' of samples and reference materials⁷, with the possibility of calibrating quality control substances with international reference materials with adequate enrichment in heavier isotopes (material with similar δ -value or atom fraction of the heavier isotope). However, the international reference materials with the highest enrichments for ^{15}N and ^{13}C available at the International Atomic Energy Agency (IAEA) are IAEA-311 ($\delta^{15}\text{N}$ of $+4693(\pm 110)$ mUr measured in 29 laboratories) and IAEA-309B ($\delta^{13}\text{C}$ of $+535.3(\pm 5)$ mUr measured in 12 laboratories).¹⁶ This corresponds to a 2.1% atom fraction for nitrogen ($x(^{15}\text{N})$) and 1.7% atom fraction for carbon ($x(^{13}\text{C})$), enabling quality control of the measurement up to this level of enrichment in heavier isotopes. However, these reference materials carry high uncertainty.¹⁷ The lack of highly enriched reference materials in the range of $+10000$ – 20000 mUr discourages the use of highly enriched tracer samples and reduces the flexibility of labelling studies by limiting the amounts of heavy isotopes that can be used in the experiments. Furthermore, the cost of highly enriched reference materials would significantly affect the budget of the study, thus, it might not be suitable for all laboratories. To the best of our knowledge, there are no guidelines for quality control procedures concerning measurements of highly enriched tracer samples.

The aim of this study was to evaluate precision and accuracy of the measurement of samples with high enrichment in ^{15}N and ^{13}C (tracer samples with δ -values of $> +1000$ mUr) and to find a fast and reliable approach to reduce memory effects. In this study, we compared four approaches with different pre-treatments of tracer samples. First, we tested whether specific biomass and ammonium nitrate salt sample positioning (samples with similar enrichment in ^{15}N grouped together) could have an effect on measurement precision. Two identical sets of biomass samples were packed into tin capsules. The first set was measured in a random ^{15}N

enrichment order, while the second set was additionally interspersed by reference urea samples with natural isotope abundance and measured in ascending ^{15}N enrichment order. Furthermore, to evaluate measurement accuracy and to compare the precision of isotopic ratio analyses, we created our own reference material that is cheap and easily accessible. In brief, a series of diluted ^{15}N ammonium nitrate reference samples (ranging between $\delta^{15}\text{N} +66\text{--}23000$ mUr) were prepared and measured in an ascending ^{15}N enrichment order. Lastly, we diluted ^{13}C and ^{15}N biomass samples with the natural abundance reference material to significantly reduce their isotopic signatures and to test whether that would minimise memory effects and improve precision and accuracy of isotopic ratio analyses.

Material and methods

Biomass sample preparation

Two sets of *Ceiba pentandra* (Kapok Silk cotton tree) biomass samples from two different labelling experiments in 2015 were used in this study. A first set of ^{15}N tracer samples originated from a labelling experiment carried out in growth chambers in Zurich, Switzerland (Experiment 1), while a second set of ^{13}C and ^{15}N samples originated from a dual labelling experiment performed in Tel Aviv, Israel (Experiment 2). For the first experiment, *C. pentandra* saplings were grown in 5 L pots (17.9×22.7 cm, Soparco AG, Chaingy, France) filled with 0.7–1.2 mm sand (Carlo Bernasconi AG, Zurich, Switzerland) in climate chambers, with a diel cycle consisting of 15-h day (28°C , 55% relative humidity (RH) and 8-h night (22°C , 65% RH) and a 2×0.5 h transition time (dusk and dawn). Saplings were fertilized six times per week with 0.5 L of Hoagland's nutrient solution.¹⁸ Two-month-old saplings were pulse-labelled with 160 mg/L of 99.1% ($x(^{15}\text{N})$) $^{15}\text{NH}_4^{15}\text{NO}_3$ (Sigma-Aldrich Co. LLC., Buchs, Switzerland) solution for 2 h. After 2 h labelling, we used tap water to clean the label from the roots and sand and to stop the labelling process. After pulse-labelling, saplings were continuously sampled for 3 days and harvested at 72 h.

The second labelling experiment (^{13}C and ^{15}N dual-labelling) was performed in a large-scale aeroponic system at the "Sarah Racine Root Research Laboratory" (Tel Aviv University, Tel Aviv, Israel).¹⁹ The top parts of the canopy of nine-month old *C. pentandra* saplings were pulse-labelled for 2 h with 99.9% ($x(^{13}\text{C})$) $^{13}\text{CO}_2$ (Cambridge Isotope Laboratories, Inc., Andover, MA, USA) using airtight chambers. At the same time, the root systems were enclosed in custom-made water tight chambers and pulse-labelled for 90 minutes with 160mg/L of

99.1% ($x(^{15}\text{N})$) ammonium nitrate ($^{15}\text{NH}_4^{15}\text{NO}_3$). After labelling, tree roots were rinsed with tap water, and trees were continuously sampled for 72 h. After the harvest, biomass samples from both experiments were treated in the same way. Leaf, stem and root biomass samples were washed and microwaved for 30 seconds, then oven dried at 60°C for 3 days.

To determine carbon and nitrogen isotope composition, biomass samples were ground with a ball mill (Retsch GmbH, Haan, Germany) and 3–4 mg were weighed into tin capsules (5×9mm, Sántis Analytical AG, Teufen, Switzerland). The quality control substances caffeine (Fluka Chemie AG, Buchs, Switzerland, $\delta^{13}\text{C}$ –43.11 mUr, $\delta^{15}\text{N}$ –5.79 mUr), and tyrosine (Fluka Chemie AG, Buchs, Switzerland, $\delta^{13}\text{C}$ –24.0 mUr, $\delta^{15}\text{N}$ +5.16 mUr) were placed in the measurement sequence together with samples of high enrichment in ^{13}C and ^{15}N according to the suggestion of Werner and Brand.⁷ The samples were analysed using a Flash elemental analyser (1112 Series, Thermo Italy, former CE Instruments, Rhodano, Italy) coupled to a Delta^{plus}XP isotope ratio mass spectrometer (Finnigan MAT, Bremen, Germany) via a 6-port valve²⁰ and a ConFlo III interface (Finnigan MAT, Bremen, Germany).²¹ The Flash elemental analyser was additionally equipped with a home-made Nafion-trap backed by a conventional $\text{Mg}(\text{ClO}_4)_2$ trap followed by a 4-port valve²² between the reduction tube and the GC column to avoid frequent exchange of $\text{Mg}(\text{ClO}_4)_2$ and to allow for easy maintenance of the oxidation and the reduction tubes without closing the isolation valve of the mass spectrometer. Post-run off-line calculations, such as blank and offset corrections for assigning the final δ -values on the V-PDB and AIR- N_2 scales, were performed according to Werner and Brand.⁷ Calibration of the internal laboratory standard acetanilide (Merck KGaA, Darmstadt, Germany, $\delta^{13}\text{C}$ –27.21 mUr, $\delta^{15}\text{N}$ +0.77 mUr) was periodically done by comparison with international reference materials provided by the IAEA (Vienna, Austria).

Sample preparation approaches

All samples were measured separately for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. For this study, we used four tracer sample preparation approaches. For the first approach (App1), nine aliquots of pre-measured highly enriched ^{15}N biomass samples (3 leaf, 5 trunk and 1 root sample) with different δ -values were packed into 5×9 mm tin capsules (3.5–4 mg of biomass each). Six replicates were prepared for each sample and they were measured in a random enrichment order together with quality control substances (Figure 1A). For the second approach (App2), we used aliquots of the same nine biomass samples as in App1. Six replicates were prepared for each sample and the samples were placed in the measurement series in selective order (with increasing

enrichment in heavier isotope). One urea sample (natural isotope abundance) was placed after each tracer sample to evaluate memory effects (Figure 1B).

To test measurement accuracy of ^{15}N tracer samples and to evaluate whether ammonium nitrate can be used as commercially available reference material, for the third approach (App3), we prepared a series of diluted ^{15}N tracer ammonium nitrate samples at 13 different enrichment levels. In brief, two stock solutions were prepared. The ^{15}N stock solution was prepared by mixing 3 mg of 99.1% ($x(^{15}\text{N})$) ammonium nitrate ($^{15}\text{NH}_4^{15}\text{NO}_3$, Sigma-Aldrich Co. LLC., Buchs, Switzerland) with 30 mL of deionized water. The ^{14}N stock solution was prepared by mixing 2100 mg of natural abundance ammonium nitrate (0.37% ($x(^{15}\text{N})$), Riedel-de Haën, Seelze, Germany) with 300 mL of deionized water. To create 13 different enrichment levels, both stock solutions were mixed in accordance with Table 1. Liquid samples were pipetted into 5×9 mm tin capsules and dried in a highly sensitive drying oven at 60°C until a dry weight of 0.3 mg per sample was reached. Six replicates were prepared for each enrichment level. Diluted ammonium nitrate samples were placed in selective measurement order (with ascending enrichment in heavier isotope determined by expected $\delta^{15}\text{N}$ values) and measured together with quality control substances in five measurement sequences (Figure 1C).

For the fourth approach (App4), 0.5–0.7 mg of enriched ^{15}N and ^{13}C plant biomass (ranging from +17 to +6267 mUr) were diluted with 4–6 mg of the natural isotope abundance biomass (from the same species with known isotopic signature and with comparable carbon and nitrogen contents as the original samples). Diluted samples were packed into 5×9 mm tin capsules and analysed for carbon and/or nitrogen isotope composition in random order. After the measurement, $\phi^{13}\text{C}$ and $\phi^{15}\text{N}$ were calculated using Equation 5 (given below in the Calculations section). During dilution, heavier isotope levels were reduced 3–50 times, which was expected to improve measurement precision and reduce memory effects. As a control for App4, all tracer samples used in App4 were measured undiluted and in random order. The original measured values of the tracer samples were used.

Urea sample preparation

Natural isotope abundance urea (Fluka Chemie AG, Buchs, Switzerland) was selected for App2 since it contains both N and C. To avoid problems with sample heterogeneity, 550 mg of urea was dissolved in 50 mL of double deionized water (DDW). The solution was then pipetted into 5×9 mm tin capsules and dried at 60°C in a drying cabinet. Twenty μL of the solution resulted

in ~0.21 mg of urea per capsule. A liquid urea solution is especially convenient to use when a large number of capsules is needed.

Memory effect evaluation

The memory effect created by the highly enriched ^{15}N tracer samples in App2 was evaluated using natural isotopic abundance urea samples as reference materials (described above). In App2, one urea tin capsule was placed after each tracer sample and measured for $\delta^{15}\text{N}$. The memory effect (in %) was calculated as follows:

$$\text{Memory effect} = \frac{\delta^{15}\text{N } U_{\text{sample}} - \delta^{15}\text{N } U_{\text{ref}}}{\delta^{15}\text{N }_{\text{sample before urea}}} \cdot 100 \quad (1)$$

where U_{sample} is the urea measured after each tracer sample and U_{ref} is the urea reference value of $\delta^{15}\text{N} + 0.19 \text{ mUr}$.

Calculations

Carbon and nitrogen isotopic signature δ -values were calculated using the isotope ratio equation as follows:

$$\delta = \frac{R_{\text{sample}}}{R_{\text{std}}} - 1 \quad (2)$$

where R_{sample} equals $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$ of the sample and R_{std} $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$ of the used international standard.²³

Corso and Brenna²⁴ proposed the use of the ϕ notation when working with high enrichment tracer samples since the quantity δ is not proportional to the atom fraction. The notation ϕ (in mUr) was calculated as shown below:

$$\phi = \frac{F_{\text{sample}}}{F_{\text{std}}} - 1 \quad (3)$$

where F_{sample} equals $R_{\text{sample}}/(1+R_{\text{sample}})$ and F_{std} equals $R_{\text{std}}/(1+R_{\text{std}})$.²⁴ The notation ϕ has the advantage of being nearly identical to the δ -values at natural enrichment levels and it has increasing linearly up to 100%.²⁵

The following isotope balance equations were used to determine expected ϕ notations (ϕ_{sample}) after mixing two ϕ components:

$$\phi_{\text{mix}} = \phi_{\text{sample}} \times \text{pool}_1 + \phi_{\text{dilution agent}} \times \text{pool}_2 \quad (4)$$

$$\varphi_{sample} = \frac{\varphi_{mix} - (\varphi_{dilution\ agent} \times pool_2)}{pool_1} \quad (5)$$

where φ_{mix} is the φ of the mixed samples and $pool_1$ and $pool_2$ represent the mass fractions of materials used in the mixture φ_{mix} , with $pool_1 + pool_2$ equal to 1.²⁶

The ^{15}N ammonium nitrate tracer used in App3 had 99.1% ($x(^{15}\text{N})$). However, the atom fraction on the tracer package was rounded up to the nearest tenth, which lead to inevitable inaccuracy in the calculated $\varphi^{15}\text{N}$ since the ^{15}N ammonium nitrate tracer could have been anything between 99.05% and 99.14% ($x(^{15}\text{N})$). To evaluate diluted sample accuracy, we evaluated how the expected $\varphi^{15}\text{N}$ would have changed if the ^{15}N tracer ranged between 99.05% and 99.14% ($x(^{15}\text{N})$). The uncertainty (given in $\pm\text{mUr}$) was calculated as follows:

$$\varphi_{99.1\% \text{ } ^{15}\text{N accuracy}} = \frac{\varphi_{99.14\% \text{ } ^{15}\text{N}} - \varphi_{99.05\% \text{ } ^{15}\text{N}}}{2} \quad (6)$$

where $\varphi_{99.14\% \text{ } ^{15}\text{N}}$ represents the calculated φ of the selected sample if the ^{15}N tracer is 99.14% ($x(^{15}\text{N})$), and $\varphi_{99.05\% \text{ } ^{15}\text{N}}$ represents the calculated φ if the ^{15}N tracer is 99.05% ($x(^{15}\text{N})$). The accuracy of the measurement was evaluated as the difference between the measured $\varphi^{15}\text{N}$ and the expected $\varphi^{15}\text{N}$ (App3), which was calculated using Equation 5 after mixing ^{15}N and ^{14}N stock solutions.

Statistical analysis

R statistical software²⁷ (version 3.3.1) was used for data processing. We used a general linear regression model `lm`. A linear model with a log-transformed target variable y (standard deviation `mUr`) was used to fit the regression line with the sample standard deviation data ($y=10^{a \cdot x} \cdot 10^t$). The statistical package `regr0` was used when a linear model with log-transformed target variable y (urea ^{15}N `mUr`) was employed to fit the regression line to the data ($y=10^{a \cdot x} \cdot 10^t - 1$). Additionally, we used an exponential model of $y=a \cdot \exp(-kx)$ to fit the App3 coefficient of variation, where the variable “ a ” represents the maximum value of the ^{15}N coefficient of variation and “ k ” is a rate constant of ^{15}N coefficient of variation decrease.

Results and discussion

Measurement precision

To ensure sufficient labelling of the entire plant biomass, pulse-labelling techniques require short applications of highly enriched tracers. For this reason, in our experiments, the

application of a tracer often resulted in highly enriched tracer samples (Figure 2). These highly enriched tracer samples contributed greatly to the finding to our studies; however, the two highest enrichment international reference materials (IAEA-31 and IAEA-305B) are costly and insufficient to cover a wide sample distribution. Therefore, the performances of App1, App2, App3, and App4 were compared using the measurement precision of quality control substances caffeine and tyrosine (Table 2).

Tracer samples measured for App1 and App2 ranged from $\delta^{15}\text{N}$ +542 to +18553 mUr (Figure 3). Our target was to reach similar values for diluted ^{15}N ammonium nitrate samples for App 3, where samples ranged between $\delta^{15}\text{N}$ +63 and +22777 mUr. The amplitude of m/z 29 traces for nitrogen and m/z 45 traces for carbon (resistor – $3.E+10$ Ohm) of samples used in App1 and App2 reached 39 V out of a maximum 50 V (maximal and saturating ion current – $2.E-09$ Ampere), while samples used in App3 reached 47 V. This indicates that the Faraday cup amplifiers of our isotope ratio mass spectrometer were not saturated with heavier isotopes (results reported below). For App1, App2, and App3, tracer sample measurement precision was evaluated using the variation of $\delta^{15}\text{N}$ between six replicates measured for each sample (Figure 3). Tracer samples measured in accordance with App2 and App3 showed lower $\delta^{15}\text{N}$ variation than samples measured in App1. However, standard deviations (SD) increased with higher $\delta^{15}\text{N}$ values in all three approaches (Figure 4). The SD of App2 and App3 ranged from ± 1.4 to ± 154.4 mUr, while in App1, it reached a maximum of ± 1169 mUr. This suggests that tracer sample measurement precision can be improved by using a specific sample order during the measurement and by using non-enriched reference substances interspersed between tracer samples. Moreover, the use of an ascending enrichment order and urea samples in App2 improved the coefficient of variation (CV%), which was 0.21–1.1% compared to ± 3.3 –12.8% in App1. We found a higher CV for low enrichment tracer samples in App1 and App3. This was most probably not due to lower biomass sample homogeneity in App1 since dissolved ammonium nitrate samples demonstrated even greater variation at low $\delta^{15}\text{N}$ values in App3; however, the difference in the chemical nature of inorganic and organic samples will certainly contribute to an unknown extent. A higher CV with low enrichment samples serves as a good illustration of how even a small memory effect and fluctuating measurement precision can have a large influence when working with low enrichment or natural abundance samples, particularly in ecological studies.

App2 resulted in lower quality control substance precision than App1. This might have been triggered by the larger magnitude of the memory effect generated by the specific tracer sample positioning used in App2. We suggest that the random order in App1 distributed the impact of the memory effect more evenly to all measured quality control substances. As expected, App3 resulted in the lowest measurement precision of quality control substances, most likely due to the highest sample $\delta^{15}\text{N}$ levels.

In this study, the memory effect was evaluated using reference urea samples from App2. Isotopic compositions of urea samples became more positive with higher ^{15}N tracer sample enrichments (Figure 5A). A maximum increase of +16.83 mUr was recorded (from the reference urea value of $\delta^{15}\text{N} = +0.19$ mUr). An average memory effect of +0.36 mUr was seen at isotopic abundance levels of $\delta^{15}\text{N} +700$ to +7000 mUr and +8 mUr at +12000 to +18000 mUr. The memory effect, evaluated as proportional increase in the isotopic composition compared to the reference urea δ -value, ranged between -0.03% and +0.11%. The highest memory effect of 0.11% was created by a sample with a $\delta^{15}\text{N}$ value of +12027 mUr. We hypothesize that our observed memory effect is too small to significantly influence the quality of the tracer data, as 0.11% only represents a memory effect of +13.1 mUr. In many labelling studies, isotopic composition variations among replicates or even within a replicate exceed 1400 mUr (Figure 7). For this reason, we argue that high measurement precision can be considered irrelevant at very high tracer levels.

Biomass sample dilution (App4) resulted in the highest quality control substance precision of ± 0.10 – 0.11 mUr for ^{15}N and ± 0.03 – 0.09 mUr for ^{13}C (Table 2), which is most likely a result of a reduced memory effect. For both isotopes, the precision was slightly lower than the long-term precision of natural abundance quality control substances (± 0.12 – 0.17 mUr for ^{13}C and ± 0.15 mUr for ^{15}N) and much lower than the precision of quality control substances in control measurements of App4 (Table 2). However, the long-term precision was evaluated using more than 8 years of data, and it was expected to reflect larger variations. Although App4 resulted in the highest precision, its daily application would result in extended study time. Additionally, unaccounted inaccuracies involved in sample dilution could play a role in final sample precision.

Previously, only a few studies evaluated measurement precision and accuracy of enriched tracer samples.^{29–31} The measurement precision of gas chromatography/combustion/isotope ratio mass spectrometry (GC/C-IRMS) when using ^{15}N enriched glutamic acids at +1000 mUr

resulted in a variation of ± 91.83 mUr.²⁹ Measurements of ^{13}C enriched tracer fatty acids at an enrichment of $+925$ – 957 mUr yielded an isotopic ratio analysis precision of ± 0.17 – 1.56 mUr SD when using IRMS with 3–5 sample replicates.³⁰ Measurement of cholesterol samples of lower ^{13}C enrichment resulted in an accuracy of ± 0.18 to ± 0.8 mUr when measuring cholesterol samples with a ^{13}C enrichment ranging from $+61$ to $+73.6$ mUr.³¹ Additionally, a memory effect of 1.5% at $+61$ mUr ^{13}C enrichment levels was reported.³¹ However, measurement precision and memory effects at higher ^{13}C and ^{15}N enrichment levels were not reported. When dealing with the precision of isotopic ratio analyses, it is important to keep in mind that in the majority of labelling studies, the differences associated with method selection (e.g. labelling time, tracer concentration, etc.) might largely outweigh the error generated by the isotope ratio mass spectrometer.³²

Measurement accuracy

The highest enrichment diluted ^{15}N ammonium nitrate sample (App3) resulted in an instrumentally measured $\phi^{15}\text{N}$ of $+20881$ mUr (mean value) (Figure 6A, Table 3). We found a strong relationship between the expected (i.e., calculated before dilution) and original measured $\phi^{15}\text{N}$ values ($R^2=0.9998$, $p<0.001$). However, measured values were less positive than expected values (Table 3). Neither diluted nor pure samples in App3 and App4 caused Faraday cup amplifier saturation. Insufficient enrichment information supplied by the ^{15}N ammonium nitrate tracer manufacturer could have resulted in a difference of $\pm 0.05\%$ (mean value) in the calculated $\phi^{15}\text{N}$ data; however, we suggest that this potential error is minimal and a mathematical correction is therefore unnecessary. On the other hand, instrumentally measured data could be mathematically corrected using calculated $\phi^{15}\text{N}$ values if more accurate data are required. In this study, the differences between calculated and instrumentally measured $\phi^{15}\text{N}$ ranged between $+2.6$ mUr and $+1994$ mUr, which represents a difference of 3.7 to 9.6%. Unfortunately, ammonium nitrate is not best suited as reference material for organic samples, as ideally, reference materials should have similar chemical properties as the experimental samples. For the correction of scale contraction, the best approach would be to relate to an isotope difference of two reference materials.

Biomass samples used in App4 ranged between $\phi^{13}\text{C}$ of $+47$ and $+4303$ mUr and between $\phi^{15}\text{N}$ of $+17$ and $+6104$ mUr (Figure 5B, C). Instrumentally measured and calculated $\phi^{15}\text{N}$ and $\phi^{13}\text{C}$ values showed strong relationships ($R^2=0.994$ and 0.945 , $p<0.001$). However, 80% of measured $\phi^{15}\text{N}$ values were less positive than calculated values after dilution. This is in line

with the results of App3. In contrast, the ^{13}C biomass sample dilution showed an opposite trend, with 70% of measured $\phi^{13}\text{C}$ values being more positive than calculated $\phi^{13}\text{C}$ values. These differences indicate that samples with high ^{13}C enrichment could be corrected to less positive values. However, App4 should only be used for measurement precision comparison, as instrumentally measured $\delta^{13}\text{C}$ were used to calculate $\phi^{13}\text{C}$ after sample dilution.

Considering the tracer data accuracy recorded in this study, we argue, that in labelling studies with high sample enrichments, the effects of the applied treatments should be clearly visible even without accuracy correction of tracer data.

Conclusions

Our objectives were to evaluate whether preparation approaches for high ^{15}N and ^{13}C enrichment samples could improve measurement precision and to find a straightforward way to reduce memory effects. A comparison of four approaches indicated that the frequent use of natural abundance reference materials and specific sample measurement order (App2) is the most practical way to increase measurement precision of tracer samples. Even though this approach had a negative impact on the precision of our laboratory quality control substances, it successfully reduced variability between very high ^{15}N enrichment sample replicates. The ^{15}N samples with up to +23000 mUr showed relatively high variation in δ -values. However, this observed variation had little impact when highly positive $\delta^{15}\text{N}$ data were considered. In addition, we conclude that frequent use of natural abundance reference materials (like urea) can be used to monitor and reduce the impact of memory effects.

The currently available highly enriched international reference materials carry high variation in δ -values and are too imprecise to be used for mathematical correction. Furthermore, based on the 'Identical Treatment' principle, they are also not best suited as reference materials for measurement of organic samples. However, in this study, we demonstrated an improved precision of tracer sample isotopic ratio analyses without the use of highly enriched international reference materials. High enrichment ^{15}N samples created a maximum memory effect of 0.11%, which we consider to be too small to impact findings in ecological studies. However, for even lower memory effects, we recommend measuring high enrichment tracer samples separately from low enrichment tracer samples.

Tracer sample dilution prior to measurements (App4) yielded the best quality control substance precision. When ^{13}C and/or ^{15}N enrichment of the samples leads to an oversaturation of the

Faraday cup amplifiers in the isotope ratio mass spectrometer, either the level of ^{13}C and/or ^{15}N enrichment of the samples or the measuring device need to be adapted. This could be done by either "diluting" highly enriched tracer material with known amounts of material with natural isotope abundance or by changing e.g. resistors of the Faraday cups used.

In this study, we also evaluated the accuracy of the $^{15}\text{N}/^{14}\text{N}$ ratio analyses with enrichment of up to +23000 mUr. Our results indicated that measured values could be mathematically corrected by +6.7% (mean value). However, only the level of data quality required by individual studies could determine if this mathematical correction is really necessary, especially when dealing with very high enrichment samples. Common variations introduced by the labelling procedure and natural variability between the plant replicates (and even within replicate) exceed the size of the inaccuracy of isotopic ratio analyses. Nonetheless, the accuracy of the measurement could be improved by having reference materials with similar chemical properties and similar ^{13}C and/or ^{15}N enrichment in comparison to experimental samples. In practice, the best approach would be to relate to an isotope difference of two reference materials and use this difference to correct scale contraction issues. No matter which approach is employed, the long-term assessment of the memory effect and measurement precision and accuracy should be employed in every laboratory working with tracer samples.

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Table 1. Sample preparation method for Approach 3 (App3). Quantities of ^{15}N and ^{14}N ammonium nitrate stock solutions were mixed together for ^{15}N ammonium nitrate tracer dilution. Samples were measured in the ascending $\delta^{15}\text{N}$ order determined by the expected $\delta^{15}\text{N}$ values calculated after the mixture of two stock solutions.

Sample Nr.	^{15}N stock solution (μL)	^{14}N stock solution (μL)	Expected $\delta^{15}\text{N}$ values (mUr)
1	100	5714.3	69
2	100	1857.1	209
3	200	1142.9	677
4	200	600.0	1289
5	300	685.7	1691
6	400	628.6	2460
7	900	720.0	4830
8	1400	740.0	7308
9	2000	657.1	11754
10	2200	597.1	14227
11	2600	557.1	18019
12	3000	514.3	22520
13	3400	528.6	24831

Table 2. The precision of isotope ratio analyses using an isotope ratio mass spectrometer with various enrichment tracer and natural abundance ^{15}N and ^{13}C samples. Results are shown as 1 standard deviation of quality control substances. For Approach 1 (App1), biomass samples were measured in a random ^{15}N enrichment order, while for Approach 2 (App2), the same biomass samples were measured in ascending ^{15}N enrichment order with additional reference urea samples interspersed after each tracer sample. A series of ^{15}N ammonium nitrate diluted tracer samples were prepared for Approach 3 (App3) and measured in ascending ^{15}N enrichment order. For Approach 4 (App4), various enrichments of ^{15}N and ^{13}C biomass tracer samples were diluted with a natural abundance biomass material and measured in random enrichment order, while control of App 4 was the same biomass samples measured undiluted. Long-term precision was evaluated using more than 8 years of various enrichment tracer and natural abundance measurements data.

Quality control substances	App1	App2	App3	App4	Control of App4	Long-term (>8 years)	
						Natural abundance samples	Tracer samples
¹⁵ N (mUr)							
Caffeine	0.87	2.97	10.29	0.10	0.99	0.15	1.26
Tyrosine	2.59	3.12	6.86	0.11	0.48	0.15	4.06
¹³ C (mUr)							
Caffeine				0.09	0.26	0.17	0.30
Tyrosine				0.03	0.05	0.12	0.24

Table 3. The difference between measured and calculated $\phi^{15}\text{N}$ values derived from ^{15}N ammonium nitrate tracer dilution (App3), including estimation of potential inaccuracy resulting from insufficient 99.1 ($x(^{15}\text{N})$) tracer package labelling supplied by the $^{15}\text{NH}_4^{15}\text{NO}_3$ manufacturer. Shown are mean values for 6 sample replicates.

Sample Nr.	$\phi^{15}\text{N}$ measured (mUr)	$\phi^{15}\text{N}$ calculated (mUr)	Difference (calculated –measured)		$^{15}\text{NH}_4^{15}\text{NO}_3$ tracer accuracy 99.14% - 99.05%	
			(mUr)	(%)	($\pm$ mUr)	($\pm$ %)
1	66.2	68.7	2.6	3.7	0.03	0.04
2	200.1	208.5	8.4	4.0	0.09	0.05
3	629.9	673.3	43.4	6.5	0.31	0.05
4	1260.9	1278.3	17.4	1.4	0.58	0.05
5	1559.0	1674.9	115.9	6.9	0.76	0.05
6	2312.4	2428.7	116.3	4.8	1.11	0.05
7	4268.3	4728.2	459.9	9.7	2.15	0.05
8	6539.9	7091.7	551.7	7.8	3.23	0.05
9	10283.3	11227.6	944.3	8.4	5.12	0.05
10	12248.8	13472.9	1224.1	9.1	6.14	0.05
11	15526.6	16840.7	1314.2	7.8	7.67	0.05
12	18733.0	20726.7	1993.8	9.6	9.43	0.05
13	20881.2	22676.2	1794.9	7.9	10.34	0.05
			Mean		Mean	
			6.7		0.05

Figure 1. Tin capsule measurement order used to compare the precision of isotopic ratio analyses using an isotope ratio mass spectrometer with different sample preparation approaches. Biomass samples were measured in random ^{15}N enrichment order for Approach 1 (A), biomass and urea samples were measured in ascending ^{15}N enrichment order for Approach 2 (B), and diluted ^{15}N ammonium nitrate tracer samples were measured in ascending enrichment order for Approach 3. Acetanilide, tyrosine, and caffeine capsules were measured in nearly identical order with all three approaches.

205x183mm (600 x 600 DPI)

Figure 2. Comparison of ^{15}N tracer samples measured in our laboratory and available reference materials. A set of ^{15}N biomass tracer samples (A) and acetanilide, tyrosine, and caffeine materials measured together with tracer samples (B). No approaches to reduce the memory effect were used. Two horizontal dash lines represent international reference materials IAEA-305B and IAEA-311 (not used in this study).

206x142mm (600 x 600 DPI)

Figure 3. $\delta^{15}\text{N}$ variation between sample replicates measured using three sample preparation approaches. Biomass samples measured in random ^{15}N enrichment order using Approach 1 (A), biomass samples measured in ascending ^{15}N enrichment order using Approach 2 (B), and diluted ^{15}N ammonium nitrate tracer samples measured in ascending enrichment order for Approach 3 (C). Six replicates of each sample are shown.

206x111mm (600 x 600 DPI)

Figure 4. Comparison of data variation between three sample preparation approaches. Shown are standard deviations (in mUr) and coefficients of variation (in %) for Approach 1 (A, C), Approach 2 (B, D), and Approach 3 (B, D). Six replicates of each sample are displayed.

238x142mm (600 x 600 DPI)

Figure 5. Size of the memory effect derived from reference urea samples used in Approach 2. Shown in mUr (A) and in percentage (B). The first horizontal dashed line indicates reference urea value of $\delta^{15}\text{N} + 0.19$ mUr (A); the second - memory effect of 0% (B).

152x152mm (600 x 600 DPI)

Figure 6. Relationship between calculated tracer sample enrichment (after dilution) and measured values. Expressed as ϕ for Approach 3 (A) using ^{15}N ammonium nitrate samples ($n=78$) and for Approach 4 using ^{15}N biomass samples (B) ($n=131$) and ^{13}C biomass samples (C) ($n=158$). Equations 4 and 5 were used to calculate ϕ after sample dilution.

117x196mm (600 x 600 DPI)

Figure 7. An example of enrichment variation in samples obtained during one pulse-labelling experiment. $\delta^{15}\text{N}$ variation among non-woody white roots of six tree replicates (A), trees continuously sampled over a 72 h experiment. The $\delta^{13}\text{C}$ variation within the labelled top canopy (70 cm) of one experimental tree harvested at 72 h after the labelling (B).

190x103mm (600 x 600 DPI)