

Nicotine, acetanilide and urea multi-level ^2H -, ^{13}C - and ^{15}N -abundance reference materials for continuous-flow isotope ratio mass spectrometry[†]

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Accurate determinations of stable isotope ratios require a calibration using at least two reference materials with different isotopic compositions to anchor the isotopic scale and compensate for differences in machine slope. Ideally, the δ values of these reference materials should bracket the isotopic range of samples with unknown δ values. While the practice of analyzing two isotopically distinct reference materials is common for water (VSMOW-SLAP) and carbonates (NBS 19 and L-SVEC), the lack of widely available organic reference materials with distinct isotopic composition has hindered the practice when analyzing organic materials by elemental analysis/isotope ratio mass spectrometry (EA-IRMS). At present only L-glutamic acids USGS40 and USGS41 satisfy these requirements for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, with the limitation that L-glutamic acid is not suitable for analysis by gas chromatography (GC). We describe the development and quality testing of (i) four nicotine laboratory reference materials for on-line (i.e. continuous flow) hydrogen reductive gas chromatography-isotope ratio mass-spectrometry (GC-IRMS), (ii) five nictines for oxidative C, N gas chromatography-combustion-isotope ratio mass-spectrometry (GC-C-IRMS, or GC-IRMS), and (iii) also three acetanilide and three urea reference materials for on-line oxidative EA-IRMS for C and N. Isotopic off-line calibration against international stable isotope measurement standards at Indiana University adhered to the 'principle of identical treatment'. The new reference materials cover the following isotopic ranges: $\delta^2\text{H}_{\text{nicotine}} -162$ to -45% , $\delta^{13}\text{C}_{\text{nicotine}} -30.05$ to $+7.72\%$, $\delta^{15}\text{N}_{\text{nicotine}} -6.03$ to $+33.62\%$; $\delta^{15}\text{N}_{\text{acetanilide}} +1.18$ to $+40.57\%$; $\delta^{13}\text{C}_{\text{urea}} -34.13$ to $+11.71\%$, $\delta^{15}\text{N}_{\text{urea}} +0.26$ to $+40.61\%$ (recommended δ values refer to calibration with NBS 19, L-SVEC, IAEA-N-1, and IAEA-N-2). Nictines fill a gap as the first organic nitrogen stable isotope reference materials for GC-IRMS that are available with different $\delta^{15}\text{N}$ values. Comparative $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ on-line EA-IRMS data from 14 volunteering laboratories document the usefulness and reliability of acetanilides and ureas as EA-IRMS reference materials. Published in 2009 by John Wiley & Sons, Ltd.

Semi-automated stable isotope ratios analysis (SIRA) in continuous flow by on-line elemental analysis (EA) or gas chromatography (GC) interfaced with isotope ratio mass-spectrometry (IRMS) has become a routine and invaluable approach to characterize and trace organic compounds. It is commonly used in studies of ecology, environmental sciences, food science, forensics, geochemistry, and medical sciences. Rapid advances in instrumentation have reduced sample size requirements and increased sample throughput, for example in the GC-IRMS of hydrogen,¹ carbon^{1,2} and nitrogen,²⁻⁴ and in the EA-IRMS of hydrogen,⁵ carbon⁶ and

nitrogen.⁶ Although continuous-flow techniques involve the isotopic monitoring of reference gas from a gas cylinder, the pulses of reference gas are typically injected directly into, or in parallel to, He streams carrying analytes from the gas chromatograph or elemental analyzer peripheral to the IRMS instrument. Thus these gas pulses have not passed through the same analytical train of interfaces and are not affected by the same isotope fractionation as the analyte gas from combusted organic samples. Ideal measurements and calibration of isotopic data rely on the 'principle of identical treatment' of unknowns and isotopic reference materials⁷ and thus require organic stable isotope reference materials rather than an external 'calibration gas'. The need for normalization of measured isotopic compositions to isotope reference scales⁸ and to compensate for differences in δ -scaling in mass spectrometers has been recognized for hydrogen⁹ and more

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recently for carbon¹⁰ and has resulted in the recommended use of pairs of isotopically different reference materials of the same or similar chemical compositions.

Unfortunately, modern continuous-flow GC-IRMS and EA-IRMS analytical methods are restricted to organic reference materials that are chemically similar to the unknown analytes, in contrast to traditional off-line SIRA methods that were able to reliably convert both organic samples and inorganic international measurement standards such as VSMOW and SLAP waters, carbonates NBS 19 and L-SVEC, and ammonium sulfates IAEA-N-1 and IAEA-N-2 into H₂, CO₂ and N₂ analyte gases for direct comparative measurement and calibration.^{11,12} The development of suitable organic stable isotope reference materials or standards¹³ for continuous-flow methods has not kept pace with existing and emerging needs of analysts. For example, the International Atomic Energy Agency (IAEA¹⁴) and the National Institute of Standards and Technology (NIST¹⁵) do not offer any $\delta^{15}\text{N}$ organic reference materials for GC-IRMS,¹⁶ although the analytical GC-IRMS method has been in use for over 15 years.^{17,18} (IAEA 600 caffeine lists an approximate $\delta^{15}\text{N}$ value of unspecified origin and reliability; a single compound is insufficient for proper control of the isotopic instrument scale.)

For GC-IRMS, we describe the development of five nicotine reference materials with different $\delta^2\text{H}$, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. Nicotine is of significant toxicological interest and its stable isotope ratios may serve as forensic indicators for the origin of tobacco.¹⁹ For EA-IRMS, Qi *et al.*²⁰ developed a pair of L-glutamic acid (C₅H₉NO₄, R(C,N)=5) reference materials with different $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. Analysts benefit from having a choice of organic reference materials with different C-N molar ratios. Here we describe the development of three acetanilides (R(C,N)=8) with different $\delta^{15}\text{N}$ values, and of three ureas (R(C,N)=0.5) with different $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. Urea and acetanilide are suitable for laboratory reference materials because they are chemically stable, have low toxicity, and can be powdered to ensure isotopic homogeneity and to facilitate weighing into EA capsules. However, because it does not suffer from static problems, L-glutamic acid is easier to weigh on a microbalance than acetanilide.

EXPERIMENTAL

Materials

Nicotines with multiple levels of ²H-, ¹³C-, and ¹⁵N-abundance

We used four isotopically different nicotine starting materials. Two separate batches of nicotine were purchased from Sigma Aldrich Co. (Fluka, St. Louis, MO, USA), $\geq 99\%$ by GC assay, and by Sigma Aldrich Laborchemikalien GmbH (Riedel-de Haën, Seelze, Germany), 99.8% by GC assay. Nicotine (R, S) with $\sim 90\%$ ¹³C at the methyl position was synthesized as described by Mesnard *et al.*²¹ Doubly ¹⁵N-labelled ($\sim 73\%$ ¹⁵N) nicotine (S) was obtained by extraction of *Nicotiana tabacum* leaves grown with K¹⁵NO₃ and (¹⁵NH₄)₂SO₄ fertilizers (Eurisotop, Saint-Aubin, France) on sterilized sand. After adding 1 M H₂SO₄ to leaves, cellular material was disintegrated with an Ultra-Turrax[®] blender (IKA[®] Werke GmbH & Co. KG, Staufen, Germany) at 13 500 rpm for 1 min. The pH was raised to >12 with 1 M

NaOH, the suspension was filtered, and the filtrate was passed through a preconditioned (washed with 20 mL methanol and 20 mL water adjusted with NaOH to pH > 12) solid-phase extraction cartridge (5 g Discovery C18, Supelco Inc., Bellefonte, PA, USA) fitted to a Visiprep vacuum manifold (Supelco Inc.). The cartridge was rinsed with 5 mL water (pH > 10) and dried with a flow of N₂ gas for 1 h, followed by extraction of compounds with methanol, evaporation of methanol at 33°C under reduced pressure, and removal of traces of water by co-evaporation with toluene. Labeled nictines were separated from nornicotine and other tobacco alkaloids by preparative reversed-phase high-performance liquid chromatography (HPLC).²² The structures of the nictines were confirmed by one-dimensional ¹H NMR and one-dimensional ¹H-decoupled ¹³C or ¹⁵N NMR. The purities were estimated at 90% by weight for ¹³C- and ¹⁵N-labelled nictines by high-resolution electro-spray mass spectrometry (HR-ESMS).

High purity of reference compounds for GC-IRMS is required for off-line isotopic calibration because isotope ratios are determined for bulk sub-samples without prior gas chromatography. Although sufficient purity of commercially available nicotine starting materials was suggested by GC and GC-MS, these analytical techniques are unable to detect non-volatile contaminants. In addition, the commercially available nictines used as starting materials ranged in color from pale yellow to amber, indicating the presence of trace amounts of oxidized compounds; for example, nicotine-N-1'-oxide, nicotinic acid, cotinine and nicotyrine.^{23,24} All four nicotine starting materials were therefore vacuum-sublimated at room temperature to produce colorless nicotine samples devoid of non-volatile contaminants.

Before mixing of nictines, we measured preliminary $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the two purified, commercially available nictines (off-line technique described below). Milligram amounts of the purified, highly ¹³C- or ¹⁵N-enriched nictines were mixed with larger amounts of one of the purified commercial nictines (#1) to provide two intermediate working mixtures with R(¹³C,¹²C) and R(¹⁵N,¹⁴N) ratios that were intentionally too high to be useful in reference materials but could be measured via off-line MS with rough accuracy. Based on the approximate $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of these mixtures, we performed isotopic mass-balance calculations and mixed appropriate volumes to produce three nicotine mixtures, #2, #3, #4, with elevated $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values within the desired ranges for reference materials. Together with the two commercial nictines, #1 and #5, we thus arrived at a total of five prospective nicotine reference materials that were sealed under nitrogen or argon in glass ampoules and glass capillaries and are being stored aphotically in a freezer to preserve their chemical and isotopic integrity. Multiple aliquots of each nicotine were taken from freshly opened capillaries as needed for detailed GC-MS and isotopic analyses. The purities of the final nictines were evaluated by GC-MS using a Varian 4000 ion trap GC/MS/MS system (Varian, Palo Alto, CA, USA). The purities of nictines #1, #4, and #5 were measured, respectively, as 99.68, 99.67, and 99.90% from their GC-MS chromatographic peak areas. These tests prove that all the nicotine end-members and their resulting mixtures are sufficiently pure to serve as reference materials.

Acetanilides with multiple levels of ^{15}N -abundance and ureas with multiple levels of ^{13}C - and ^{15}N -abundance

Acetanilide #1 and urea #1 with natural stable isotopic abundances and purities of $\geq 99\%$ and $\geq 99.5\%$ by weight were purchased from Merck KGaA (Darmstadt, Germany) and Sigma Aldrich Co. (Fluka, St. Louis, MO, USA), respectively. Acetanilide with $\geq 98\%$ ^{15}N -enrichment and two batches of urea with 99% ^{13}C -enrichment and 5% ^{15}N -enrichment were purchased from Cambridge Isotope Laboratories Inc. (CIL, Andover, MA, USA). Compounds from CIL have purities of $\geq 98\%$ by weight. In a similar fashion as described above for nicotine, we first determined approximate $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of acetanilide #1 and urea #1, followed by mass-balance calculations and mixing of appropriate amounts of the various substances to produce acetanilides #2 and #3 with medium and high $\delta^{15}\text{N}$ values, and ureas #2 and #3 with medium and high $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, respectively.

Each solid prospective reference material required homogenization. Acetanilide #1 with natural isotopic abundances was powdered in an industrial-strength blender (Waring Laboratory Science, Torrington, CT, USA). The effectiveness of powdering was limited because frictional heat in the blender eventually caused clumping. Acetanilides #2 and #3 were individually melted in pre-annealed Pyrex[®] beakers with aluminum foil covers by placing each beaker into the chamber of a GC oven, raising the temperature to 2°C above the melting point of acetanilide ($113\text{--}114^\circ\text{C}$), and waiting for the shortest time possible until each melt was homogenous. Molten acetanilide was poured onto pre-annealed, heavy-duty aluminum foil placed on a cold baking tray, thus causing instantaneous crystallization in the form of a thin crust that easily peeled off the foil and was subsequently powdered in the industrial-strength blender. Due to the limitation of powdering, the resulting acetanilides were homogenous enough to warrant the filling of gram amounts into pre-annealed glass vials for permanent storage in a freezer, but further grinding in an agate mortar with a pestle was performed routinely prior to analyses to ensure homogeneity at the sub-milligram level.

The high solubility of urea in water offered a convenient method for homogenization, by first dissolving each urea (#1, #2, #3) in the least amount of double distilled water. A peristaltic pump was used to drip each solution slowly into a large cup of pre-annealed, heavy-duty aluminum foil that had been placed into a large Dewar flask and filled with liquid nitrogen. Drops froze instantaneously and sank into liquid nitrogen. Rapid freezing caused the fast growth of small urea crystals. Each batch of frozen drops in its aluminum cup was placed into the vacuum chamber of a freeze-dryer. The final dry urea products still bear the shape of frozen drops that can be easily powdered or re-dissolved in water.

Methods

Off-line measurements of H, C, N stable isotope ratios at Indiana University

Although the accuracy of on-line analyses has improved, data are still susceptible to analytical uncertainty due to different C-N molar ratios, sample weights, combustion efficiency, peak shape and peak resolution. In contrast, traditional off-line

methods circumvent such problems by quantitative combustion in sealed 'quartz' (i.e. vitreous SiO_2) ampoules, cryogenically preparing pure analyte gases, and equalizing the pressures of analyte gas and standard gas using bellows in the mass spectrometer, thus achieving high accuracy. Our off-line determinations of isotope ratios utilized traditional procedures¹² and followed the 'principle of identical treatment' in the preparation of analyte gases and the manual dual-inlet isotopic measurement of $R(^2\text{H}, ^1\text{H})$ and $R(^{15}\text{N}, ^{14}\text{N})$ ratios of acetanilides, ureas, nicotines, and international measurement standards VSMOW, SLAP, IAEA-N-1 and IAEA-N-2. The off-line determination of $R(^{13}\text{C}, ^{12}\text{C})$ ratios was based on (i) CO_2 from combusted aliquots of organic materials and (ii) calibration relative to CO_2 from phosphoric acid-digested international carbonate measurement standards NBS 19 and L-SVEC.²⁵ In brief, multiple 1.5 to 3 mg aliquots of pure nicotines #1 to #5 or of NBS 22 oil were sealed in the central sections of freshly prepared quartz capillaries with Z-shaped bends²⁶ to avoid partial evaporation and isotope fractionation during evacuation (Fig. 1). In addition to nicotine, each sealed quartz capillary also contained $<0.1\text{ mm}^3$ of air at the ends, which represents $<0.3\%$ of the total hydrogen and nitrogen in a capillary containing 2 mg of nicotine. The isotopic influence of this small contamination can be ignored because in the area of Indiana where the measurements were made atmospheric water $\delta^2\text{H}$ (the local Bloomington precipitation annual mean $\delta^2\text{H}$ is $\sim -43\text{‰}$) and $\delta^{15}\text{N}$ (atmospheric N_2 has a $\delta^{15}\text{N}$ value of $\sim 0\text{‰}$) are not far from the respective organic δ values. Sealed

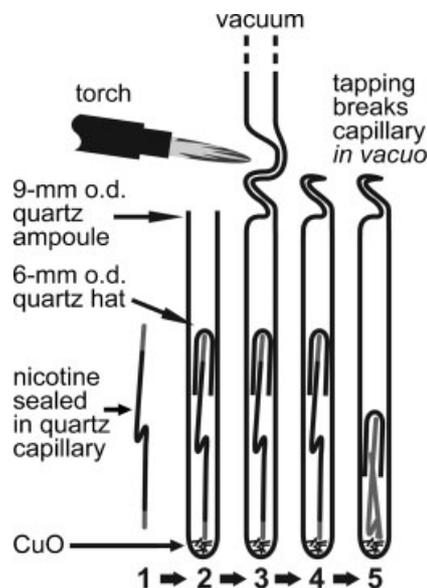


Figure 1. Between 1.5 to 3 mg of nicotine is sealed in the center of a Z-shaped quartz capillary (step 1). A 9-mm o.d. quartz ampoule with a length of $\sim 10\text{ cm}$ receives $\sim 80\text{ mg}$ copper(II) oxide wire, in addition to a sealed capillary and a 6-mm o.d. quartz 'hat' riding on the capillary (step 2). Using a glass blowing torch, a 9-mm o.d. quartz extension is attached to the ampoule and a break-seal is fashioned (step 3). The extended quartz ampoule is connected to a vacuum line, the ampoule is heated with a heat gun to desorb water, and the torch is used to separate the ampoule from the extension (step 4). The evacuated ampoule is tapped onto a hard surface causing the capillary to break under vacuum and release the nicotine (step 5).

capillaries were rinsed with dichloromethane, dried, and individually placed into 9-mm o.d. quartz combustion ampoules (~10 cm long) as outlined in Fig. 1. With the exception of the nicotine-filled capillaries, all components had been pre-heated to 500°C overnight to burn off organic contaminants.

Differences in $\delta^{13}\text{C}$ were observed for certain compounds when analyzed by on-line and off-line techniques. Carbon blanks associated with all the reagents used in off-line combustions were quantified by using pure O_2 as the oxidant or in the presence of varying amounts of copper(II) oxide (CuO) and/or granular copper with a surface layer of CuO. As a result, subsequent off-line measurements used only ~200% of the stoichiometrically needed amounts of CuO wire. Off-line combustions compared $\delta^{13}\text{C}$ values of urea powders with those from aqueous solutions of urea samples that had been sealed into quartz capillaries as described for nicotine.

Aliquots of finely ground acetanilides weighing ~2 mg, or of reference materials (USGS40, USGS41, IAEA-CH-7), were loaded directly into quartz ampoules and 100 mg amounts of ureas were dissolved in double distilled water to produce stock solutions. Volumetric aliquots containing the equivalent of ~1.5 mg urea were pipetted into quartz combustion ampoules that were placed into a freeze-drier. After the addition of ~200% of the stoichiometric amount of CuO wire needed for complete combustion to each ampoule,²⁷ break-seals were formed and the ampoules were evacuated at room temperature and sealed off.

IAEA-N-1 and IAEA-N-2 ammonium sulfates were loaded and processed in quartz ampoules in the same fashion as urea and acetanilide. VSMOW and SLAP were not loaded into quartz ampoules because their 'combustion' is chemically unnecessary. This shortcut is justified because heating of water in quartz ampoules introduces no significant isotopic shift.²⁸

All ampoules were combusted overnight at 800°C converting organic substances and IAEA nitrogen stable isotope standards into H_2O , CO_2 , and N_2 . The ampoules were kept in the oven at ~200°C until they were individually retrieved and immediately processed on a vacuum line, in order to reduce the possibility of developing hydrous copper carbonates and isotope fractionation during storage at room temperature.²⁹ The break-seal of an ampoule was broken in vacuum by a magnetically operated iron bar. Cryogenic separation of N_2 from CO_2 and H_2O occurred at the temperature of liquid nitrogen. N_2 was collected with a Toepler pump and sealed into 9-mm o.d. Pyrex[®] glass tubes. CO_2 was separated from H_2O at the temperature of dry ice, frozen into 6-mm o.d. Pyrex[®] glass tubes at the temperature of liquid nitrogen and sealed off with a torch. H_2O was reduced to H_2 by passage through 800°C uranium metal turnings, followed by collection with a Toepler pump and sealing of H_2 into 9-mm o.d. Pyrex[®] glass tubes. VSMOW and SLAP waters were first sealed into Pyrex[®] glass capillaries with Z-bends, followed by introduction into the vacuum line, crushing of capillaries *in vacuo* with an iron bar, and processing of H_2O with uranium as described above.

All isotope ratios are presented in customary δ notation on isotopic scales defined by pairs of international measurement standards. Measurement of δ values occurred in manual dual-inlet mode using a Delta Plus XP isotope ratio mass

spectrometer (Thermo Finnigan, Bremen, Germany) at Indiana University. The $\delta^2\text{H}$ values are reported relative to VSMOW $\equiv 0\text{‰}$ and normalized to SLAP $\equiv -428\text{‰}$ according to IUPAC guidelines.⁹ The $\delta^{13}\text{C}$ values were calibrated relative to the VPDB isotopic scale using international measurement standards NBS 19 $\equiv +1.95\text{‰}$ and L-SVEC $\equiv -46.6\text{‰}$.¹⁰ The reliability of NBS 19 for partially defining the $\delta^{13}\text{C}_{\text{VPDB}}$ scale was recently confirmed by Brand *et al.*³⁰ The $\delta^{15}\text{N}$ values were calibrated relative to air nitrogen $\equiv 0\text{‰}$ using international measurement standards IAEA-N-1 $\equiv +0.43\text{‰}$ and IAEA-N-2 $\equiv +20.41\text{‰}$ ammonium sulfates.³¹ At natural isotopic abundance levels, the $\delta^2\text{H}$, $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$ values carry an analytical precision of $\pm 2\text{‰}$, $\pm 0.05\text{‰}$, and $\pm 0.07\text{‰}$, respectively (i.e., $\pm 1\sigma$). The analytical uncertainty increases for δ values that result from linear extrapolation of isotopic scales beyond the δ values of utilized international measurement standards.

In addition to gases from the above-named standards, we also measured CO_2 and N_2 gases that had been prepared off-line from NBS 20, NBS 22, IAEA-CH-7, USGS40 and USGS41. Measurements of all reference materials with defined isotopic compositions should ideally yield linear calibrations across the covered wide ranges of 84.23‰ for $\delta^{13}\text{C}$ and 52.09‰ for $\delta^{15}\text{N}$. The linearity of the Delta Plus XP mass spectrometer at Indiana University fulfilled this expectation within the stated isotopic precisions of the reference materials and the precisions of the measurements, albeit with one notable exception. USGS41 is characterized by extremely positive $\delta^{13}\text{C} = +37.63 \pm 0.10\text{‰}$ and $\delta^{15}\text{N} = +47.57 \pm 0.22\text{‰}$ values.²⁰ Our manual dual-inlet measurements of CO_2 and N_2 gases from USGS41 yielded $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values that are 0.21‰ and 0.16‰ below their previously reported^{10,20} values on extrapolated isotopic scales that are linearly defined by NBS 19/L-SVEC and IAEA-N-1/IAEA-N-2, respectively. The measured 0.16‰ offset is not significant for $\delta^{15}\text{N}$ because it is within the stated uncertainty of $\pm 0.22\text{‰}$ for the assigned $\delta^{15}\text{N}_{\text{USGS41}}$ value. In contrast, the lack of linearity for $\delta^{13}\text{C}$ is significant because the measured offset of 0.21‰ is larger than the stated uncertainty of $\pm 0.10\text{‰}$ for $\delta^{13}\text{C}_{\text{USGS41}}$, and $\delta^{13}\text{C}$ values are measured more precisely at $\pm 0.05\text{‰}$. Forcing a linear $\delta^{13}\text{C}$ regression across imperfectly aligned reference materials including USGS41 would violate scale-defining international measurement standards NBS 19 and L-SVEC. Our off-line $\delta^{13}\text{C}$ calibration is therefore based exclusively on CO_2 gases from NBS 19 and L-SVEC that were measured in manual dual-inlet mode. In contrast, the on-line measurements used USGS40 and USGS41 for calibration (see next section). Fortunately, the resulting $\delta^{13}\text{C}$ discrepancy between off-line and on-line calibrations is limited because the $\delta^{13}\text{C} = +11.71\text{‰}$ value of our most ^{13}C -enriched new reference material urea 3 is much closer to that of NBS 19 (+1.95‰) than to that of USGS41 (+37.63‰).

Continuous-flow EA-IRMS comparisons of acetanilides and ureas

Fourteen laboratories participated in an effort to use on-line EA-IRMS methods for C- and N-isotopic comparison of the six acetanilides and ureas relative to L-glutamic acid reference materials USGS40 and USGS41. Participating laboratories and their analytical equipment are listed in Table 1. (An

insufficient number of qualified labs were available for H, C, N isotopic GC-IRMS comparison of nictines.) Isotopic anchoring and two-point isotopic scale normalization, and thus the calibrations of on-line δ values of acetanilides and ureas, relied exclusively on USGS40 and USGS41. We adopted the assigned values $\delta^{13}\text{C}_{\text{USGS40}} \equiv -26.39 \pm 0.09\%$, $\delta^{13}\text{C}_{\text{USGS41}} \equiv +37.63 \pm 0.10\%$, $\delta^{15}\text{N}_{\text{USGS40}} \equiv -4.52 \pm 0.12\%$, and $\delta^{15}\text{N}_{\text{USGS41}} \equiv +47.57 \pm 0.22\%$.¹⁰ In other words, each laboratory's data matrix was uniformly adjusted to match the prescribed δ values for USGS40 and USGS41. The calibration of on-line data ignored data from any other reference materials and international measurement standards in order to maintain mutual compatibility of the on-line data sets.

RESULTS AND DISCUSSION

Rationale for reference materials with different ^2H -, ^{13}C -, and ^{15}N -abundances

All determinations of $\delta^2\text{H}$, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values require careful standardization and calibration for several reasons. First, the use of pairs or triplets of reference materials with

different $\delta^2\text{H}$, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values ensures proper anchoring to isotopic scales and post-acquisition adjustment of all data to standardize the scaling of the instrument via linear regression. Second, the achievable accuracy is improved when the isotopic composition of the unknown sample is similar to that of a reference material and the errors from extrapolating along isotopic scales are minimized. The availability of multiple reference materials with different δ values increases the odds for a good match between sample and reference material. Proper calibration of isotopic data sets obtained with an EA carousel autosampler or a GC autoinjector should also employ a correction for sample size, by combusting the same material in different amounts and compensating for any weight-related trend in measured isotopic values. Finally, multiple aliquots of the same material in identical quantities should be periodically measured within a sequence to correct for instrument drift over time.

The dynamic measurement of δ values in transient pulses of analyte gases in a flow of helium carrier gas requires the complete chromatographic separation of the analyte from all other combustion products and contaminant gases. The

Table 1. Participating laboratories in alphabetical order, their analytical equipment, and analysts

Laboratory	Equipment	Analysts
Austrian Research Centers GmbH ARC, Biogenetics-Natural Resources, Seibersdorf, Austria	Carlo Erba EA with Finnigan MAT 251; Vario EL EA with Thermo Delta Plus	Micha Horacek
Indiana University, Department of Geological Sciences, Bloomington, IN, USA	Costech EA with Thermo Delta Plus XP	Andrea Albertino, Peter E. Sauer
NERC Life Sciences Mass Spectrometry Facility, Scottish Universities Environmental Research Centre (NERC LSMSFC-SUERC), East Kilbride, UK	Costech ECS 4010 EA + autosampler with Thermo Delta XP Plus	Jason Newton
Scottish Crop Research Institute SCRI, Invergowrie, Dundee, UK	ANCA-GSL with SerCon 20-20, Costech ZB Flash EA + autosampler with Thermo Delta V Plus	Wolfram Meier-Augenstein
Smithsonian Institution, Museum Conservation Institute, Suitland, MD, USA	Costech ECS 4010 EA with Thermo Delta V Advantage	Gregory A. Henkes
TÜV Rheinland Agroislab GmbH, Jülich, Germany	EA Carlo Erba NA 1500 Isochrome Micromass/Elementar	Hilmar Förstel, M. Boner, C. Erven
University of Bradford, Division of Archaeological, Geographical and Environmental Sciences, Bradford, UK	Flash EA 1112 elemental analyzer with Thermo Delta Plus XL	Julia Lee-Thorp, Andrew Gladhill
University of California Santa Barbara, Marine Science Institute, Santa Barbara, CA, USA	Costech ECS 4010 EA with Thermo Delta Plus Advantage	Georges L. Paradis, Robert Petty
University of Michigan, Department of Geological Sciences, Ann Arbor, MI, USA	Costech ECS 4010 EA with Thermo Delta V Plus	Lora L. Wingate
University of St Andrews, School of Geography & Geosciences, St. Andrews, Fife, UK	Costech EA zero blank autosampler with Thermo Delta Plus XL, via Conflo III	Michael Bird, Chris Wurster
University of Washington, Department of Earth and Space Sciences, Seattle, WA, USA	Costech ECS 4010 EA with Finnigan MAT 253, via Conflo III	Andrew Schauer
U.S. Department of Agriculture, ARS, National Soil Tilth Laboratory, Ames, IA, USA	NA1500 CN analyzer with Thermo Delta V Advantage	David DenHaan
U.S. Geological Survey, Reston, VA, USA	Carlo Erba NA2500 EA with Thermo Delta V Plus via Conflo II	Ty Coplen, Haiping Qi
Woods Hole Oceanographic Institution, Dept. of Marine Chemistry and Geochemistry, Woods Hole, MA, USA	at WHOI: Carlo Erba EA 1107 with Delta Plus via Conflo II; at Brown University: Costech EA with Thermo Delta V Plus via Conflo III	Carl G. Johnson

demands are relatively straightforward in hydrogen reductive continuous-flow IRMS methods where the generated H₂ gas can be easily separated from CO and N₂ and where it faces few problems with interfering isobaric ions due to its low mass/charge (*m/z*) ratio of 2 or 3 (²H⁺, (²H¹H)⁺), except when much N₂ is present.³² The situation is more complicated for the continuous-flow determination of δ¹³C and δ¹⁵N. Reproducible oxidation of organic materials by EA methods demands complete flash combustion, but the chemistry of the analytes may lengthen the combustion time, for example, in the case of polycondensed aromatic molecules (i.e. clusters of aromatic rings). Generated gas pulses of CO₂ and N₂ need to be fully separated by GC before they are admitted sequentially into the ion source because the presence of any oxygen and carbon in the ion source during the measurement of δ¹⁵N values would generate isobaric (¹³C¹⁶O)⁺ and (¹²C¹⁷O)⁺ with *m/z* 29, mimicking the presence of excess (¹⁵N¹⁴N)⁺. A suite of different organic nitrogen stable isotope reference materials with similar δ¹⁵N values and different C-N molar ratios, hence different CO₂/N₂ peak ratios, is invaluable for diagnosing and quantifying EA-IRMS analytical artifacts.

Nicotines for GC-IRMS

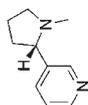
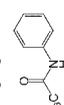
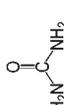
Nicotine is a suitable reference material not only for δ¹³C and δ¹⁵N, but also for δ²H, because the hydrogen atoms in nicotine are exclusively bonded to carbon (Table 2), and because they have high proton acidity *pK_a* values and therefore have limited isotopic exchangeability in the presence of H₂O.³³ In contrast, acetanilide and urea are not suitable as stable isotope reference materials for hydrogen because they both contain isotopically exchangeable hydrogen atoms linked to nitrogen that continuously re-equilibrate with H₂O, including atmospheric moisture. ²H-enriched nicotine could not be obtained for spiking, but fortunately the two commercially available nicotines #1 and #5 differ naturally in δ²H by ~117 ‰ (Table 2), presumably because they were derived from tobacco crops grown in separate geographic areas with contrasting meteoric water δ²H values.¹⁹

Our off-line δ¹⁵N values of nicotines could not be complemented by independent and reliable δ¹⁵N values from continuous-flow GC-C-IRMS because there are no suitable organic nitrogen stable isotope reference materials that can pass through a GC column.¹⁶ In fact, this lack of GC-C-IRMS reference materials had motivated us to develop our nicotines. Heretofore, any determinations of organic δ¹⁵N values via GC-C-IRMS would ultimately have to rely on the use of nitrogen calibration gas peaks, which violates the principle of identical treatment.

Acetanilides and ureas for EA-IRMS

The compilation of δ¹³C and δ¹⁵N on-line EA-IRMS data of three acetanilides and three ureas in comparison with off-line generated data (Figs. 2 and 3) shows that results from 14 different laboratories exhibit significant scatter (1σ up to 0.40‰ in δ¹³C and up to 0.36‰ in δ¹⁵N), despite all laboratories using the same USGS40 and USGS41 reference materials for normalization. The observed variance tends to be larger for δ¹⁵N than for δ¹³C. A prominent source of δ¹⁵N EA-IRMS uncertainty is caused by tailing of large CO₂

Table 2. Off-line stable isotope results for five nicotines, three acetanilides and three ureas (mean value, standard deviation, number of measurements, range). All analyses were performed at Indiana University using international measurement standards VSMOW, SLAP, NBS 19, L-SVEC, IAEA-N-1 and IAEA-N-2 for calibration. n.d. = not determined

Formula	Compound #	δ ² H vs VSMOW (‰)	δ ¹³ C vs VPDB (‰)	δ ¹⁵ N vs N ₂ of Air (‰)
C ₁₀ H ₁₄ N ₂ 	Nicotine #1	-44.8 ± 1.7 (n = 4) [-42.4 to -46.2]	-29.98 ± 0.01 (n = 5) [-29.97 to -30.00]	-5.82 ± 0.05 (n = 4) [-5.75 to -5.88]
	Nicotine #2	n.d.	+7.72 ± 0.02 (n = 7) [+7.68 to +7.75]	-5.94 ± 0.15 (n = 7) [-5.72 to -6.18]
	Nicotine #3	-90.0 ± 2.8 (n = 4) [-87.3 to -92.5]	-30.05 ± 0.02 (n = 7) [-30.03 to -30.07]	+33.62 ± 0.18 (n = 7) [+33.40 to +33.83]
	Nicotine #4	-105.3 ± 2.1 (n = 4) [-103.4 to -107.2]	-2.06 ± 0.02 (n = 5) [-2.04 to -2.08]	+15.49 ± 0.13 (n = 7) [+15.31 to +15.68]
	Nicotine #5	-162.0 ± 1.4 (n = 7) [-160.8 to -164.6]	-29.63 ± 0.01 (n = 5) [-29.61 to -29.65]	-6.03 ± 0.04 (n = 5) [-5.97 to -6.08]
CAS #54-11-5 	Acetanilide #1	n.d.	-29.53 ± 0.01 (n = 6) [-29.51 to -29.54]	+1.18 ± 0.02 (n = 4) [+1.16 to +1.21]
	Acetanilide #2	n.d.	-29.50 ± 0.02 (n = 4) [-29.48 to -29.53]	+19.56 ± 0.03 (n = 7) [+19.53 to +19.60]
	Acetanilide #3	n.d.	-29.50 ± 0.02 (n = 4) [-29.49 to -29.52]	+40.57 ± 0.06 (n = 6) [+40.52 to +40.66]
CAS #103-84-4 	Urea #1	n.d.	-34.13 ± 0.03 (n = 6) [-34.17 to -34.09]	+0.26 ± 0.03 (n = 7) [+0.20 to +0.28]
	Urea #2	n.d.	-8.02 ± 0.05 (n = 5) [-7.96 to -8.08]	+20.17 ± 0.06 (n = 6) [+20.09 to +20.25]
	Urea #3	n.d.	+11.71 ± 0.03 (n = 6) [+11.69 to +11.76]	+40.61 ± 0.02 (n = 7) [+40.58 to +40.63]

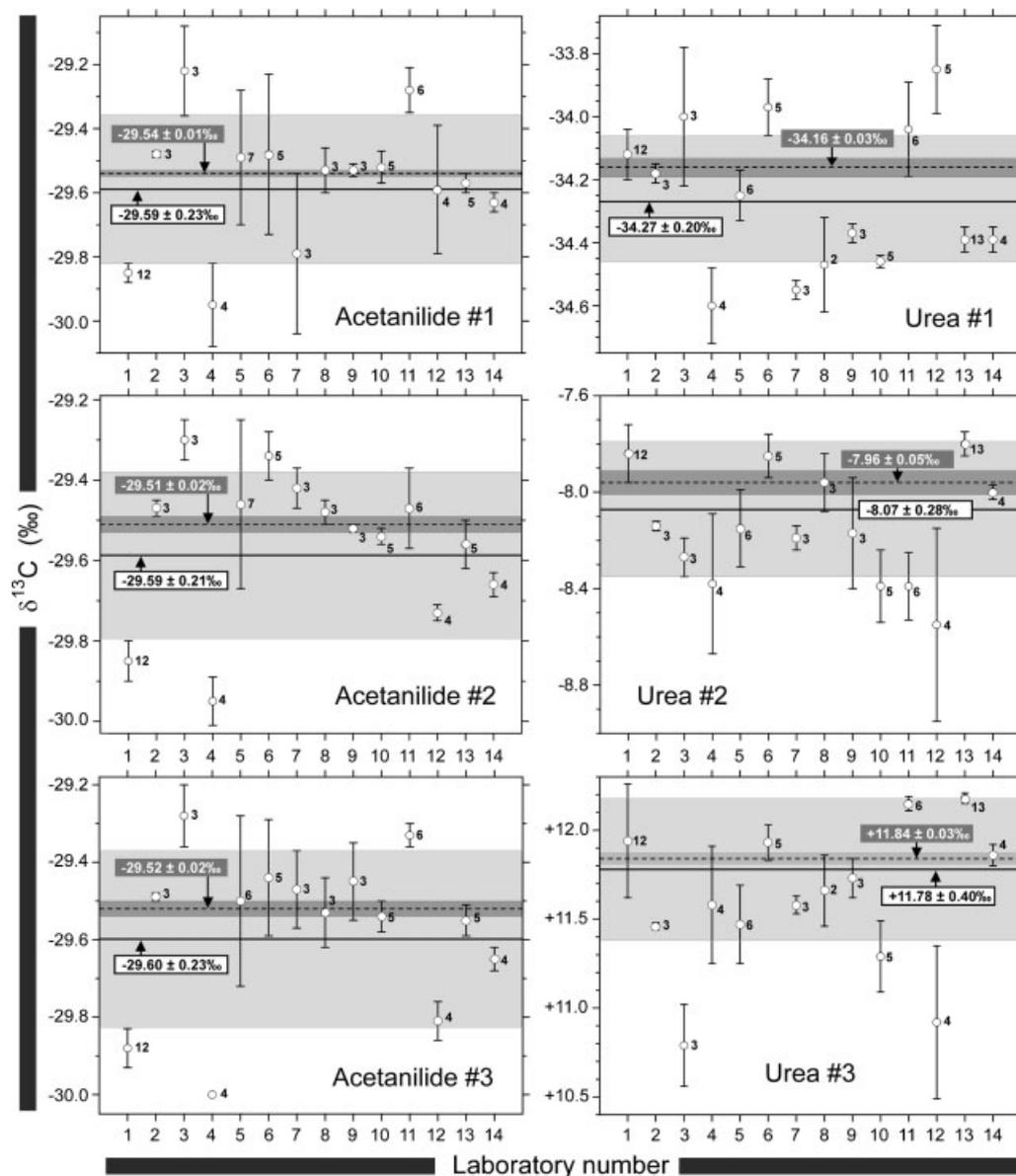


Figure 2. Fourteen laboratories used oxidative EA-IRMS in a $\delta^{13}\text{C}$ comparison of three acetanilides and three ureas. Lab numbers were randomly assigned and do not follow the alphabetical listing in Table 1. Individual data points representing reported means for each compound are accompanied by error bars indicating the standard deviation, and by the number of underlying measurements. All δ values in this figure are based exclusively on normalization relative to USGS40 and USGS41 and are thus not identical with the recommended δ values in Table 2 where normalization is based on NBS 19, L-SVEC, IAEA-N-1, and IAEA-N-2. Horizontal solid lines and white boxes with black text indicate overall average values of all reported EA-IRMS mean values, whereby each laboratory's average δ values are weighted proportionally to (i) the number of underlying analyses and (ii) the inverse of the standard deviation of the results. Light gray shading represents the standard deviation. Dashed lines and grey boxes with white text indicate the off-line determined $\delta^{13}\text{C}$ mean values after calibration against USGS40 and USGS41, with dark gray shading representing their standard deviation. Note that the latter calibration was chosen in this figure to maintain direct comparability between off-line and on-line $\delta^{13}\text{C}$ data. The USGS40/USGS41 calibration deviates slightly from the NBS 19/L-SVEC calibration of off-line $\delta^{13}\text{C}$ data that is used elsewhere in this study. Note that $\delta^{13}\text{C}$ -scales differ among panels.

peaks into the nitrogen peak of the subsequent sample.⁷ CO_2 and N_2 peak sizes need to result in ion currents that are within acceptable dynamic ranges of the mass spectrometer. The C-N molar ratio of organic compounds is of critical importance because many organic compounds have high C-

N molar ratios making it necessary to load an excess of C in order to supply sufficient N. Extremely low and high C-N molar ratios make it impractical to use peak jumping and sequential determination of both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values within a single continuous-flow IRMS run, but instead require

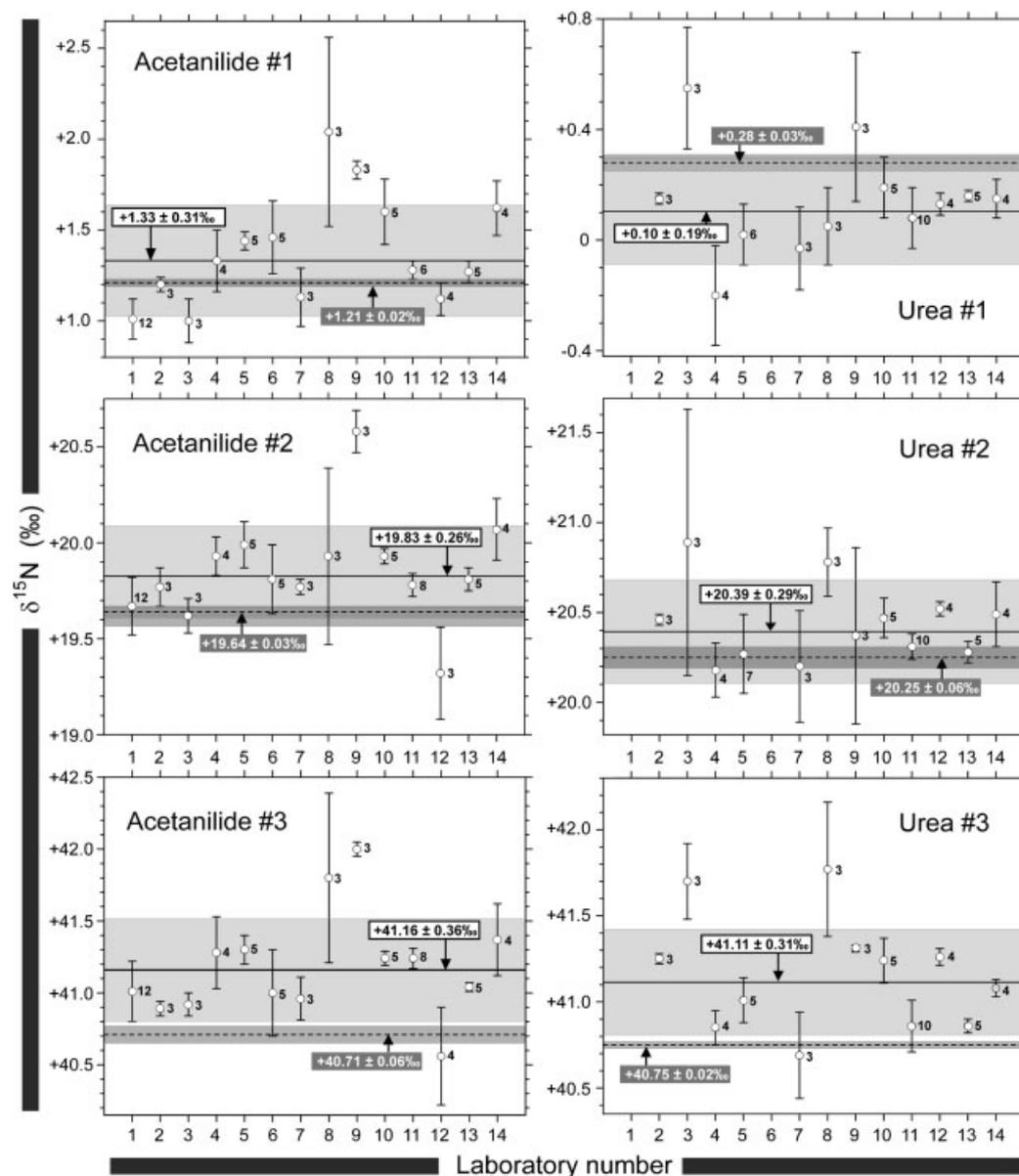


Figure 3. Fourteen laboratories used oxidative EA-IRMS in a $\delta^{15}\text{N}$ comparison of three acetanilides and 12 laboratories reported oxidative EA-IRMS $\delta^{15}\text{N}$ results for three ureas. All δ values in this figure are based exclusively on normalization relative to USGS40 and USGS41 and are thus not identical with the recommended δ values in Table 2 where normalization is based on NBS 19, L-SVEC, IAEA-N-1, and IAEA-N-2. See additional explanations in the caption of Fig. 2, except that Fig. 3 concerns $\delta^{15}\text{N}$.

separate combustions with different sample sizes for determinations of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. Valuable practical insight with regard to the use of acetanilide and other reference materials for calibration and quality control of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in EA-IRMS was submitted to the ISOGEOCHEM list on 4 November 2008.³⁴ We caution that urea crystals are known to adsorb smaller molecules, for example, straight-chain *n*-alkanes.³⁵ Our combustion experiments with urea stock solutions suggest that CO_2 and/or methane from glass-blowing flames can be trapped in urea crystals and shift bulk $\delta^{13}\text{C}$ values.

Reassuringly, the more accurately determined off-line $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the acetanilides and ureas are positioned in Figs. 2 and 3 within one standard deviation ($\pm 1\sigma$) of the means of the respective EA-IRMS data (for this graphic comparison the off-line data were also normalized

with USGS40 and USGS41, like the EA-IRMS data). In other words, the dashed line (i.e. the off-line mean value) is always positioned within the band of light grey shading ($\pm 1\sigma$ of on-line determinations; Figs. 2 and 3). The only exceptions are the most ^{15}N -enriched acetanilide #3 and urea #3 where less positive off-line $\delta^{15}\text{N}$ values fall below 1σ of EA-IRMS $\delta^{15}\text{N}$ values. Trapped air in folded EA capsules would be expected to have the opposite effect. The magnitude of the discrepancy depends on the statistical approach used in Figs. 2 and 3, where each laboratory's average δ values are weighted proportionally to (i) the number of underlying analyses and (ii) the inverse of the standard deviation of the results. Data from a laboratory performing many analyses with high reproducibility weigh more than data from a laboratory providing few results with large standard deviation.

We wish to avoid over-interpreting discrepancies between the laboratories who volunteered for this study (in some cases with just three replicate analyses of each compound). However, even for acetanilide #3 and urea #3, the average off-line $\delta^{15}\text{N}$ matches the on-line means $\delta^{15}\text{N}$ well within the 2σ (95%) uncertainties, if not the 1σ uncertainties. The differences in results reported from the various laboratories illustrate the value of measuring multiple common reference materials with distinct δ values to enhance the inter-comparability of results from different laboratories.

CONCLUSIONS

- A set of five pure nictines with various $\delta^2\text{H}$, $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$ values is useful for versatile calibration and as scaling laboratory reference materials in reductive and oxidative GC-IRMS. Nictines fill a long-standing gap with regard to $\delta^{15}\text{N}$ organic reference materials that can be passed through a GC column.
- Three acetanilides (C-N molar ratio $R(\text{C},\text{N})=8$) with different $\delta^{15}\text{N}$ values and three ureas ($R(\text{C},\text{N})=0.5$) with different $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were developed as oxidative EA-IRMS laboratory reference materials. These C-N molar ratios complement the two L-glutamic acids ($R(\text{C},\text{N})=5$) USGS40 and USGS41. Acetanilide and urea contain some exchangeable hydrogen and therefore are inappropriate as reference materials for $\delta^2\text{H}$.
- Fourteen laboratories participated in a direct EA-IRMS comparison of our acetanilides and ureas with L-glutamic acids USGS40 and USGS41. Almost all mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values from EA-IRMS analyses of the acetanilides and ureas agree within one standard deviation ($\pm 1\sigma$) with more accurately determined off-line $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. Only the most ^{15}N -enriched acetanilide #3 and urea #3 expressed $\delta^{15}\text{N}$ disagreements between off-line and on-line data of more than 1σ of EA-IRMS $\delta^{15}\text{N}$ values.
- Manual dual-inlet measurements of CO_2 and N_2 with Indiana University's Delta Plus XP mass spectrometer suggest slight non-linearities of extrapolated carbon isotopic NBS 19/L-SVEC and nitrogen isotopic IAEA-N-1/IAEA-N-2 scales when attempting to incorporate the recommended $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for USGS40 and USGS41. Although the discrepancies are within the stated isotopic uncertainties for USGS40 and USGS41, we observed that normalization with carbonates NBS 19 and L-SVEC (for $\delta^{13}\text{C}$) or ammonium sulfates IAEA-N-1 and IAEA-N-2 (for $\delta^{15}\text{N}$) does not yield identical results compared with normalization with L-glutamic acids USGS40 and USGS41. The isotopic differences are negligible within the typical isotopic ranges of natural samples, but become relevant when δ values are unnaturally positive, similar to USGS41.

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