

## New organic reference materials for hydrogen, carbon, and nitrogen stable isotope-ratio measurements: caffeines, n-alkanes, fatty acid methyl esters, glycines, L-valines, polyethylenes, and oils

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New organic reference materials for hydrogen, carbon, and nitrogen stable isotope-ratio measurements: caffeines, *n*-alkanes, fatty acid methyl esters, glycines, L-valines, polyethylenes, and oils

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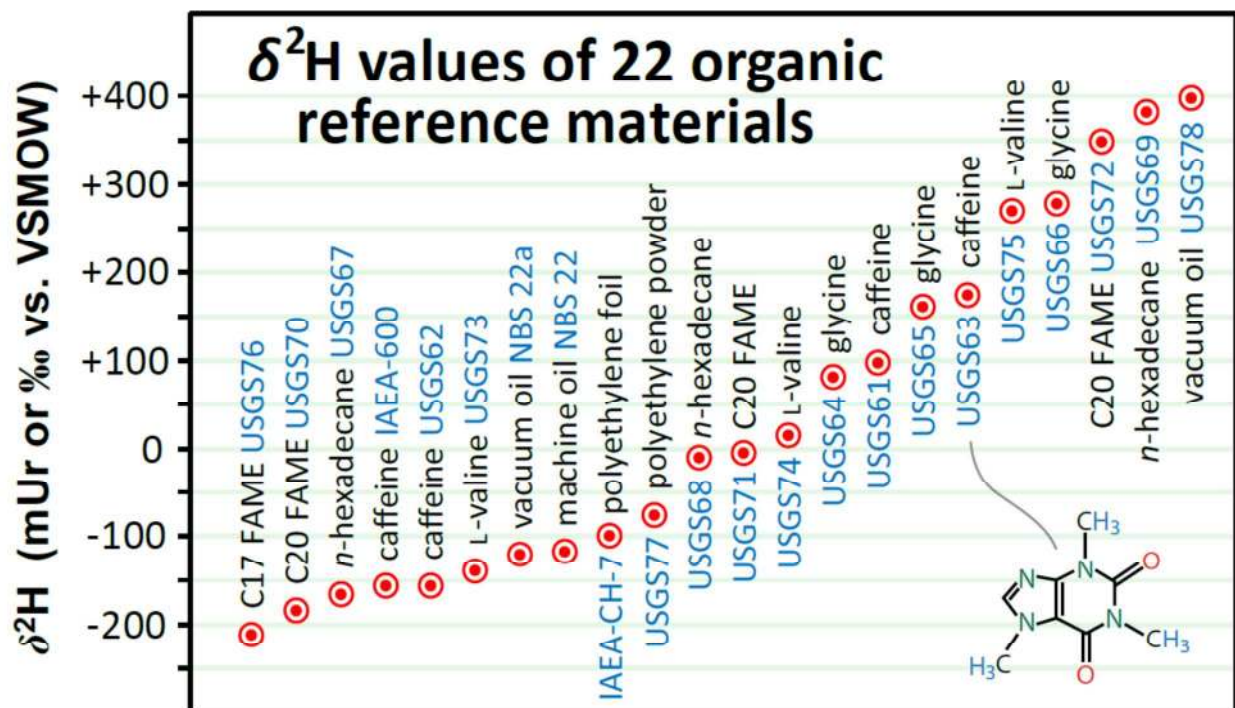
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**Key words:** amino acid, alkane, caffeine, calibration, carbon isotope, fatty acid methyl ester, glycine, hydrogen isotope, L-valine, nitrogen isotope, isotope standard, reference material, scale normalization, stable isotope ratio

## Abstract

An international project developed, quality-tested, and determined isotope- $\delta$  values of 19 new organic reference materials (RMs) for hydrogen, carbon, and nitrogen stable isotope-ratio measurements, in addition to analyzing pre-existing RMs NBS 22 (oil), IAEA-CH-7 (polyethylene foil), and IAEA-600 (caffeine). These new RMs enable users to normalize measurements of samples to isotope- $\delta$  scales. The RMs span a range of  $\delta^2\text{H}_{\text{VSMOW-SLAP}}$  values from  $-210.8$  to  $+397.0$  mUr or ‰, for  $\delta^{13}\text{C}_{\text{VPDB-LSVEC}}$  from  $-40.81$  to  $+0.49$  mUr, and for  $\delta^{15}\text{N}_{\text{Air}}$  from  $-5.21$  to  $+61.53$  mUr. Many of the new RMs are amenable to gas and liquid chromatography. The RMs include triads of isotopically contrasting caffeines,  $\text{C}_{16}$  *n*-alkanes, *n*- $\text{C}_{20}$ -fatty acid methyl esters (FAMEs), glycines, and L-valines, together with polyethylene powder and string, one *n*- $\text{C}_{17}$ -FAME, a vacuum oil (NBS 22a) to replace NBS 22 oil, and a  $^2\text{H}$ -enriched vacuum oil. Eleven laboratories from 7 countries used multiple analytical approaches and instrumentation for 2-point isotopic normalization against international primary measurement standards. The use of reference waters in silver tubes allowed direct normalization of  $\delta^2\text{H}$  values of organic materials against isotopic reference waters following the principle of identical treatment. Bayesian statistical analysis yielded the mean values reported here. New RMs are numbered from USGS61 through USGS78, in addition to NBS 22a. Due to exchangeable hydrogen, amino acid RMs currently are recommended only for carbon- and nitrogen-isotope measurements. Some amino acids contain  $^{13}\text{C}$  and carbon-bound organic  $^2\text{H}$ -enrichments at different molecular sites to provide RMs for potential site-specific isotopic analysis in future studies.

## Abstract graphic:



## Introduction

Semi-automated, economical, and fast stable isotope-ratio analysis (SIRA) in continuous flow mode with on-line elemental analysis (EA), gas chromatography (GC), or liquid chromatography (LC) interfaced with isotope-ratio mass spectrometry (IRMS) has become a routine and invaluable tool in science and industry, including applications in ecology, (paleo)environmental research, fossil fuel and biofuel research, food authentication, forensics, medical diagnostics, paleoceanography, archaeology, and other fields.<sup>1,2,3,4</sup> Accurate determinations of relative stable isotope ratios of hydrogen ( $\delta^2\text{H}$ ), carbon ( $\delta^{13}\text{C}$ ), and nitrogen ( $\delta^{15}\text{N}$ ) require 2-point normalization<sup>5</sup> using at least two isotopic reference materials (RMs, also called standards) with contrasting isotopic compositions to (i) anchor the isotopic scale and (ii) compensate for differences in responses of instruments, which commonly compress isotope- $\delta$  scales.<sup>6,7,8,9</sup> The development of suitable organic stable isotope RMs has not kept pace with the rapid development of analytical continuous flow IRMS methods, forcing many practitioners to resort to a single RM and accept the increased analytical uncertainties that accompany ignorance of the relevant isotope- $\delta$  scale compression factor. In order to minimize matrix effects, a standard should be chemically as similar as possible to the unknown sample, and therefore organic RMs should be used to normalize  $\delta$  values of organic samples. The fundamental “principle of identical treatment of sample and RM” in the analytical process<sup>10</sup> does not permit the use of pulses of “standard gases” of  $\text{CO}_2$ ,  $\text{H}_2$ , or  $\text{N}_2$  as primary anchors of isotope- $\delta$  scales because such gas

pulses have not passed through the same GC, EA, combustion of high-temperature-conversion (HTC), etc. interfaces before being admitted into the mass-spectrometer. The proper role of “standard gas pulses” is that of a mediator between the introduction of RMs within the same sequence, to correct for drift of the IRMS over time, whether during the course of a long GC analysis or between sequential EA analyses. Instead, the RMs (local or international) are interspersed among unknowns, and only through them can the measurements be normalized to isotope- $\delta$  scales. These newly developed organic RMs will enable the stable isotope community to adhere more closely to established guidelines and recommended practices when measuring stable isotope ratios of organic samples.

The development of new organic RMs was prompted mainly by the lack of appropriate RMs at a time when novel on-line IRMS methods were implemented by numerous disciplines. The lack of GC-amenable organic RMs has been particularly problematic.<sup>11,12</sup> The calibration of in-house standards by individual laboratories was typically hampered by the inability to comparatively measure organic hydrogen and the hydrogen in VSMOW and SLAP while adhering to the principle of identical treatment of standard and sample. Our development of new RMs is based on direct isotopic comparisons between organic hydrogen and standard waters sealed in silver tubes.<sup>13</sup>

Chemical choices of new RMs were guided by the need for chemical stability, low volatility and toxicity, homogeneity, and ease of handling. In particular, it is preferred that RMs for hydrogen stable isotopes should not be hygroscopic and should contain no exchangeable hydrogen, even though this is not always possible for the wide variety of matrices sought in this project.

We comply with IUPAC guidelines for the International System of Units (SI) when expressing  $\delta$  values<sup>14</sup> and we use the unit mUr instead of ‰ when expressing  $\delta^2\text{H}$ ,  $\delta^{13}\text{C}$ , and  $\delta^{15}\text{N}$  values<sup>1,15</sup> because the use of the ‰ symbol has been deprecated.<sup>16</sup> The term urey (symbol Ur) is suited for diverse isotope scales of all elements. A  $\delta$  value in the traditional form of  $-15\text{‰}$  can be expressed as  $-15\text{ mUr}$ . The  $\delta^2\text{H}$  values are normalized to a scale in which VSMOW = 0 mUr and SLAP =  $-428\text{ mUr}$ <sup>6</sup>; the  $\delta^{13}\text{C}$  values are normalized to a scale in which NBS 19 is  $+1.95\text{ mUr}$  and LSVEC is  $-46.6\text{ mUr}$ <sup>17</sup>; the  $\delta^{15}\text{N}$  values are normalized to a scale in which atmospheric nitrogen = 0 mUr and USGS32 =  $+180\text{ mUr}$ .<sup>18,8</sup>

## Materials and Methods

### Raw materials and preparation of new organic RMs

*n*-Hexadecanes (USGS67, USGS68, USGS69): Two batches of 125 and 175 mL from Fluka Analytical (CAS # 544-76-3) with GC-based purities of at least 99.9 % and two isotopically enriched *n*-hexadecanes from Aldrich ( $\text{C}_{16}^2\text{H}_{33}$ ,  $^2\text{H}$  fraction = 98 %; *n*-hexadecane-1,2- $^{13}\text{C}_2$ ,  $^{13}\text{C}$  fraction = 99 %) were used in the preparation of 3 *n*-hexadecanes with different  $\delta^2\text{H}$  and  $\delta^{13}\text{C}$  values (Table 2). Each stirred, homogeneous liquid was subdivided into glass containers and flame-sealed under argon. Users will receive aliquots of 50  $\mu\text{L}$  sealed in glass tubes as RMs for  $\delta^2\text{H}$  and  $\delta^{13}\text{C}$  normalization.

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3 *n-Heptadecanoic acid methyl ester (USGS76)*: Custom-synthesized C<sub>17</sub> fatty acid methyl ester  
4 (C<sub>18</sub>H<sub>36</sub>O<sub>2</sub>, CAS # 1731-92-6) was obtained from Sigma-Aldrich with a purity of ≥99 %. The  
5 supply of 200 g was melted, subdivided as a homogeneous liquid into glass ampoules, and  
6 flame-sealed under argon. Users will receive an aliquot of this RM of 50 µL sealed in a glass  
7 tube for δ<sup>2</sup>H and δ<sup>13</sup>C normalization.  
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10 *Icosanoic acid methyl esters (USGS70, USGS71, USGS72)*: Also known as methyl eicosanoate  
11 or arachidic acid methyl ester (C<sub>21</sub>H<sub>42</sub>O<sub>2</sub>, CAS # 1120-28-1), a 2-kg batch was custom-  
12 synthesized by Spectrum Chemical Mfg. Corp. (New Brunswick, New Jersey) with a purity of  
13 99.9 %. A fraction of 800 g of the original material termed icosanoic acid methyl ester (USGS  
14 70) was melted to a homogeneous liquid and dripped through a glass capillary into liquid  
15 nitrogen, where it shock-solidified to ~2-mg waxy beads with uniform isotopic composition. The  
16 remaining original ester was subdivided into two aliquots for adding medium and high <sup>2</sup>H and  
17 <sup>13</sup>C enrichments at the methyl group *via* transesterification with methan-<sup>2</sup>H-ol (i.e. <sup>2</sup>H<sup>1</sup>H<sub>2</sub>COH,  
18 CAS no. 4206-31-9; <sup>2</sup>H fraction = 98 %, Aldrich) and methanol-<sup>13</sup>C (<sup>13</sup>C fraction = 99 %,  
19 Aldrich). Each transesterified product was distilled under vacuum and only the center fractions  
20 were used to ultimately produce waxy beads as described above. Multi-gram amounts of beads  
21 of each type were flame-sealed into glass containers under argon. The three different icosanoic  
22 acid methyl esters have increasing δ<sup>2</sup>H and δ<sup>13</sup>C values in the order from USGS70, USGS71, to  
23 USGS72 (Table 2). Users will receive aliquots of 100 mg of beads in glass vials as RMs for δ<sup>2</sup>H  
24 and δ<sup>13</sup>C normalization.  
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30 *Glycines (USGS64, USGS65, USGS66)*: Ten kilograms of glycine were purchased from Acros  
31 Organics (C<sub>2</sub>H<sub>5</sub>NO<sub>2</sub>, CAS # 56-40-6, purity ≥99 %). Three kilograms were dissolved in ultrapure  
32 water and the homogeneous solution dripped through a glass capillary into liquid nitrogen where  
33 drops were flash frozen. Freeze-drying these pellets then produced fine-grained glycine  
34 (USGS64) with uniform isotopic composition. The two glycines enriched in <sup>2</sup>H, <sup>13</sup>C, and <sup>15</sup>N  
35 (USGS65 and USGS66) were prepared by small additions of isotopically spiked glycines to the  
36 unspiked original glycine from Acros Organics. The following spikes were purchased from  
37 ICON: (i) glycine-2,2-d<sub>2</sub>, <sup>2</sup>H fraction = 98 %; (ii) glycine-1-<sup>13</sup>C, <sup>13</sup>C fraction = 99 %; (iii) glycine-2-  
38 <sup>13</sup>C, <sup>13</sup>C fraction = 99 %; (iv) glycine-<sup>15</sup>N, <sup>15</sup>N fraction = 99 %. The homogeneous solutions of  
39 glycines USGS65 and USGS66 in ultrapure water were dripped into liquid nitrogen and freeze-  
40 dried as described above. Aliquots of glycines were flame-sealed under vacuum into multiple  
41 round-bottom Pyrex<sup>®</sup> flasks. <sup>1</sup>H NMR analyses at JAMSTEC indicated that >99.99 % of total  
42 hydrogen in all three RMs is in glycine. Bulk δ<sup>2</sup>H, δ<sup>13</sup>C, and δ<sup>15</sup>N values increase in the order  
43 from USGS64, USGS65, to USGS66 (Table 2). Glycines USGS65 and USGS66 feature  
44 contrasting <sup>13</sup>C-enrichments at molecular sites 1 and 2, namely in an atom ratio 1:2 in USGS65  
45 and 2:1 in USGS66 for future use in site-specific carbon isotopic measurements. Users will  
46 receive aliquots of 0.5 g in glass vials as RMs for δ<sup>13</sup>C and δ<sup>15</sup>N normalization. Note that the  
47 preliminary bulk δ<sup>2</sup>H values are based on partially exchangeable hydrogen in glycine that may  
48 not be conservative upon handling in a moist atmosphere. The use of these RMs for  
49 normalization of δ<sup>2</sup>H measurements will have to await the development of analytical methods  
50 that can reliably account for the influence of exchangeable hydrogen in individual amino acids.  
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3 *L-Valines (USGS73, USGS74, USGS75)*: Four kilograms of L-valine ( $C_5H_{11}NO_2$ , CAS #72-18-4)  
4 were donated by the Amino GmbH (Frellstedt, Germany). The L-valine was mixed in an  
5 industrial-strength blender without adding any isotopic spikes, sieved through a 40-mesh-metal  
6 sieve, and was labeled USGS73. The two  $^2H$ ,  $^{13}C$ , and  $^{15}N$ -enriched L-valines USGS74 and  
7 USGS75 were prepared from small additions of isotopically spiked L-valines to amounts of 2  
8 and 1.6 kg of unspiked L-valine from Alfa-Aesar, respectively. The following spikes were used:  
9 (i) L-valine-3-d1,  $^2H$  fraction = 98 %, Cambridge Isotope Laboratories, Inc.; (ii) L-valine-2-d1,  $^2H$   
10 fraction = 99 %, CDN Isotopes; (iii) L-valine-2- $^{13}C$ ,  $^{13}C$  fraction  $\geq$  99 %, Isotech Laboratories,  
11 Inc.; (iv) L-valine-1- $^{13}C$ ,  $^{13}C$  fraction = 99 %, Cambridge Isotope Laboratories, Inc.; (v) L-valine-  
12  $^{15}N$ ,  $^{15}N$  fraction = 98 %, Aldrich. Unlike glycine, the lower solubility of L-valine made it  
13 impractical to dissolve kilogram amounts in ultrapure water. Instead, for USGS74 and USGS75,  
14 only the small amounts of isotopic spikes were jointly dissolved in ultrapure water and added to  
15 powdered, water-wet regular L-valine. Each paste was stirred for 5 hours and subsequently  
16 extruded as strings into liquid nitrogen. The frozen strings were freeze-dried, mixed in an  
17 industrial-strength blender, sieved through a 40-mesh-metal sieve, and crushed in a stainless-  
18 steel, refrigerated shatter box. Dry powder was finally tumbled for 12 weeks with stainless steel  
19 balls under nitrogen in rotating metal cans until the medium-enriched L-valine USGS74 was  
20 demonstrated by analyses at the USGS in Reston, Virginia to be sufficiently homogeneous at  
21 the 0.1 mg level. In spite of tumbling for 6 months, the highly enriched L-valine USGS75 only  
22 reached acceptable homogeneity at the 0.2 mg level. In order to approach bulk isotopic mean  
23 values, 3 g of the tumbled L-valine #3 were dissolved in ultrapure water, dripped into liquid  
24 nitrogen, freeze-dried, and aliquots were sent to participating laboratories. The purity of L-  
25 valines was evaluated at JAMSTEC using  $^1H$  NMR. The purity is 99.999 % for USGS73 from the  
26 Amino GmbH, whereas 99.54 % for USGS74 and 99.50 % for USGS75 may reflect a  
27 combination of the different Alfa Aesar raw material batches, the added isotopic spikes, and the  
28 extended homogenization efforts. At 99.5 % purity, the magnitude of contamination remains  
29 limited. Bulk  $\delta^2H$ ,  $\delta^{13}C$  and  $\delta^{15}N$  values increase in the order from USGS73, USGS74, to  
30 USGS75 (Table 2). L-valines USGS74 and USGS75 feature contrasting  $^2H$ - and  $^{13}C$ -  
31 enrichments at different molecular sites (i.e. carbon atoms in positions 1, 2, and 3). Future  
32 methods for site-specific carbon and hydrogen isotopic characterization may find value in the  
33 USGS74  $^2H$ -spike ratio 2:1 for H-molecular positions 3 and 2, as well as  $^{13}C$ -spike ratio 2:1 for  
34 C-molecular positions 2 and 1. In contrast, USGS75 has a  $^2H$ -spike ratio 1:2 for H-molecular  
35 positions 3 and 2, and a  $^{13}C$ -spike ratio 1:2 for C-molecular positions 2 and 1. Aliquots of L-  
36 valines were flame-sealed under vacuum into round-bottom Pyrex<sup>®</sup> flasks. Users will receive  
37 aliquots of 0.5 g in glass vials as RMs for  $\delta^{13}C$  and  $\delta^{15}N$  normalization. As with the glycines, the  
38 preliminary  $\delta^2H$  values are based on the total hydrogen, which includes partially exchangeable  
39 hydrogen that may not be conservative upon handling in a moist atmosphere. The use of these  
40 RMs for normalization of  $\delta^2H$  measurements will have to await the development of analytical  
41 methods that can reliably account for the influence of exchangeable hydrogen in individual  
42 amino acids. Tumbled L-valine USGS75 has limited homogeneity below a sample size of 0.2  
43 mg.  
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55 *Caffeines (USGS61, USGS62, USGS63)*: Two kilograms of caffeine ( $C_8H_{10}N_4O_2$ , CAS # 58-08-  
56 2) from Alfa Aesar with a purity of 99.5 % (chromatographic purity >99.9 %) were mixed in an  
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3 industrial-strength blender, sieved through a 20-mesh-metal sieve, and labeled caffeine  
4 USGS61. However, this caffeine was only moderately depleted in  $^2\text{H}$ . It was not possible to find  
5 caffeine with a combined strong depletion in  $^2\text{H}$  and  $^{13}\text{C}$  because industrial decaffeination mixes  
6 coffees from different areas and yields isotopic ranges far smaller than those observed in  
7 natural caffeines.<sup>19,20</sup> Instead, these three caffeines form a sequence USGS61 – USGS62 –  
8 USGS63 where only the abundances of  $^{13}\text{C}$  and  $^{15}\text{N}$  are increasing, whereas the sequence for  
9 increasing  $^2\text{H}$  abundance is USGS62 – USGS61 – USGS63. Isotopically fractionated caffeines  
10 for spiking included (i) caffeine-d3 (1-methyl-d3),  $^2\text{H}$  fraction = 99.8 %, ICON Isotopes, (ii)  
11 caffeine 3-methyl- $^{13}\text{C}$ ,  $^{13}\text{C}$  fraction = 99 %, Cambridge Isotope Laboratories, Inc.; (iii) caffeine  
12 1,3- $^{15}\text{N}_2$ ,  $^{15}\text{N}$  fraction = 99 %, Cambridge Isotope Laboratories, Inc. Caffeine USGS62 contains  
13 1.8 kg of  $^2\text{H}$ -depleted caffeine from the Coffein Compagnie (Bremen, Germany) with added  $^{13}\text{C}$   
14 and  $^{15}\text{N}$ -spiked caffeines. Caffeine USGS63 contains 900 g of caffeine from Alfa Aesar with  
15 added  $^2\text{H}$ ,  $^{13}\text{C}$ , and  $^{15}\text{N}$ -spiked caffeines. Similar to the preparative routes described for L-valines  
16 above, for caffeines USGS62 and USGS63 only the small amounts of isotopic spikes were  
17 jointly dissolved in ultrapure water and added to powdered, water-wet regular caffeine. Each  
18 paste was stirred for 5 hours and subsequently extruded as strings into liquid nitrogen. The  
19 frozen strings were freeze-dried, mixed in an industrial-strength blender, sieved through a 20-  
20 mesh-metal sieve, and crushed in a stainless-steel, refrigerated shatter box. Homogeneity was  
21 demonstrated to be satisfactory at the 0.1-mg level by the USGS in Reston, Virginia. All  
22 caffeines were tested to confirm the absence of crystal water, by heating pre-weighed aliquots  
23 in vacuum to 80 °C for at least 3 hr and re-weighing after cooling. No significant weight loss was  
24 detected. Aliquots of caffeines were flame-sealed under vacuum into multiple round-bottom  
25 Pyrex<sup>®</sup> flasks. Users will receive aliquots of 0.5 g in glass vials as RMs for  $\delta^2\text{H}$ ,  $\delta^{13}\text{C}$ , and  $\delta^{15}\text{N}$   
26 normalization. Note that caffeine can have a strong physiological effect and express toxicity.  
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34 *Vacuum oils (NBS 22a, USGS78)*: We purchased 19 L of Fisher Scientific Maxima C Plus  
35 Vacuum Pump Oil representing a triple-distilled hydrocarbon fraction that had been  
36 hydrogenated to reduce the abundance of aromatic components with exchangeable hydrogen  
37 and to increase chemical stability. The vapor pressure at 25 °C of 0.133 mPa guarantees the  
38 absence of volatile components. A volume of 4.3 L (i.e. 3.35 kg) of vacuum oil was enriched in  
39  $^2\text{H}$  by heating it to 60 °C and dissolving 250 mg of perdeuterated *n*-tetracosane ( $\text{C}_{24}\text{H}_{50}$ ,  $^2\text{H}$   
40 fraction = 99.1 %, MSD Isotopes) to yield a homogeneous solution. Fourteen liters of the  
41 original vacuum oil NBS 22a and 4.3 L of the  $^2\text{H}$ -enriched vacuum oil USGS78 were flame-  
42 sealed under vacuum into multiple round-bottom Pyrex<sup>®</sup> flasks for long-term storage. Users will  
43 receive 1-mL aliquots sealed in glass containers as RMs for  $\delta^2\text{H}$  and  $\delta^{13}\text{C}$  normalization.  
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47 *Polyethylene powder (USGS77) and extruded string PE77*: Custom-synthesized low density  
48 polyethylene powder with a grain size of ~0.1 mm was purchased from Alfa Aesar (10 kg,  
49  $(\text{CH}_2\text{CH}_2)_n$ , CAS # 9002-88-4). Multiple round-bottom Pyrex<sup>®</sup> flasks were filled and flame-sealed  
50 under vacuum. Users will receive aliquots of 1 g in glass vials as RMs for  $\delta^2\text{H}$  and  $\delta^{13}\text{C}$   
51 normalization. Two kilograms of the polyethylene powder were extruded to form a string with a  
52 diameter of ~1 mm in collaboration with the Australian Nuclear Science and Technology  
53 Organisation (ANSTO). Isotopic measurements at the USGS and Indiana University indicated  
54 that USGS77 powder and the string PE77 have indistinguishable isotopic compositions. Some  
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laboratories may prefer string PE77 over powder if electrostatic dispersion of powder is problematic. String PE77 is available exclusively from ANSTO.<sup>21</sup>

**Table 1:** Stable isotope approaches and equipment of participating laboratories.

[n.a. = not applicable.]

Laboratory	$\delta^2\text{H}$ measurements	$\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ measurements
California Institute of Technology, Pasadena, California, USA	On-line, Thermo Scientific Delta <sup>plus</sup> XP, Thermo Trace GC-Ultra gas chromatograph connected <i>via</i> Thermo GC/C pyrolysis interface, operated at 1440 °C. <sup>22</sup>	n.a.
ETH Zürich, Switzerland	On-line, Costech Zero-Blank autosampler, HEKAtech HTO with reversed flow, SiC outer tube and glassy carbon inner tube with glassy carbon chips without graphite crucible; reactor 1450 °C, GC 40 °C, He flow ~90 mL min <sup>-1</sup> ; trap filled with charcoal, Ascarite <sup>®</sup> and Mg(ClO <sub>4</sub> ) <sub>2</sub> between reactor tube and GC column; connected <i>via</i> Thermo ConFlo III interface to Thermo Scientific Delta <sup>plus</sup> XP.	n.a.
Helmholtz Centre Leipzig, Germany	On-line, Euro 3000 Elemental Analyzer (EuroVector, Italy), oven filled with chromium, one-oven system, 1050 °C, He flow ~100 mL min <sup>-1</sup> <i>via</i> ConFlo IV connected to Thermo Scientific MAT 253 <sup>™</sup> . <sup>23,24</sup>	On-line, Euro 3000, two-oven system, <i>via</i> ConFlo IV connected to Thermo Scientific MAT 253 <sup>™</sup> , peak jump from N <sub>2</sub> to CO <sub>2</sub> during each run; combustion at 1020 °C, reduction at 600 °C, GC 60 °C, He flow 100 mL min <sup>-1</sup> .
Helmholtz Centre Munich, Germany	On-line, thermal decomposition by pyrolysis (1480 °C/SiC-tube filled with glassy carbon and ~1 g nickel-coated graphite), He flow 90 mL min <sup>-1</sup> . Separation column at 90 °C. Thermo ConFlo II Interface, Thermo Scientific MAT 253 <sup>™</sup> .	On-line, combustion by EA (EuroVector) <i>via</i> ConFlo II Interface, Thermo Scientific MAT 253 <sup>™</sup> ; oxidation 1000 °C, reduction 600 °C. Separation column at 85 °C. He flow 70 mL min <sup>-1</sup> . Measurements of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ <i>via</i> peak jumping.
Indiana University, Bloomington, USA	Off-line combustion in evacuated and sealed SiO <sub>2</sub> ampoules with CuO, Cu, Ag at 800 °C, reduction of H <sub>2</sub> O to H <sub>2</sub> with uranium at 800 °C on vacuum line, purification and collection of analyte gases H <sub>2</sub> , CO <sub>2</sub> , and N <sub>2</sub> on vacuum line, manual dual-inlet measurements with Thermo Scientific Delta XP IRMS.	
JAMSTEC, Japan	n.a.	On-line, Thermo Scientific Delta <sup>plus</sup> XP IRMS coupled to Flash EA1112 <i>via</i> ConFlo III; oxidation 1050 °C, reduction 750 °C.
Max-Planck-Institute, Jena, Germany	On-line, Thermo Scientific Delta <sup>plus</sup> XL, Hekatech HTO with reversed flow connected <i>via</i> Thermo ConFlo III interface. SiC outer tube and glassy carbon inner tube filled with glassy carbon chips without graphite	On-line, Thermo Scientific Delta <sup>plus</sup> , CE 1100 EA connected <i>via</i> ConFlo III interface. Oxidation 1050 °C, reduction 620 °C, GC 40 °C. He flow 90 mL min <sup>-1</sup> . <sup>25,10,26</sup>

	crucible. Reactor at 1450 °C, GC 90 °C, helium flow 60 mL min <sup>-1</sup> . Later, the upper glassy carbon chip layer was mixed with chromium chips. <sup>27</sup>	
James Hutton Institute, Dundee, United Kingdom	On-line, Thermo Scientific Delta <sup>plus</sup> XP coupled to TC/EA <i>via</i> ConFlo III; TC/EA reactor: ceramic tube with inner glassy carbon tube filled with sieved glassy carbon chips and graphite crucible; TC/EA 1425 °C; GC 85 °C; He flow 90 mL min <sup>-1</sup> .	On-line, Thermo Scientific Delta V <sup>TM</sup> Advantage coupled to Flash 1112 EA <i>via</i> ConFlo IV; combustion at 980–1000 °C; reduction 640 °C; GC 42 °C; He flow 100 mL min <sup>-1</sup> .
Université du Québec, Canada	n.a.	On-line, continuous flow, no peak jumping, <i>via</i> either (i) Isoprime 100, Elementar Vario MicroCube, or (ii) Isoprime Vision and Elementar Vario PyroCube.
University of Groningen, the Netherlands	On-line, GVI Isoprime. <i>Method #1</i> : glassy carbon tube with glassy carbon chips, 1450 °C. <i>Method #2</i> : quartz tube filled with Patinal Cr powder at 1030 °C; HekaTech HTC with zero-blank autosampler, coupled to GVI Isoprime <i>via</i> open split "ref box"; GC at 90 °C, He flow 90 mL min <sup>-1</sup> , top-feed arrangement.	On-line, Elementar Isotope Cube EA in CN arrangement with zero-blank autosampler, connected with open split reference box to Isoprime 100; combustion at 980 °C, reduction at 650 °C, He flow 240 mL min <sup>-1</sup> .
U.S. Geological Survey, Reston, Virginia, USA	On-line, Thermo Scientific Delta <sup>plus</sup> XP, Thermo Scientific TC/EA connected <i>via</i> Thermo ConFlo IV interface. <i>Method #1</i> : Glassy carbon tube filled with glassy carbon chips without graphite crucible. Reactor 1450 °C, GC 90 °C, He flow 120 mL min <sup>-1</sup> . <i>Method #2</i> : Removed glassy carbon tube. Filled ceramic tube from bottom to top with glassy carbon chips, quartz wool and 3 cm chromium powder. Reactor 1150 °C, GC 90 °C, He flow 120 mL min <sup>-1</sup> .	On-line, Thermo Scientific Delta V <sup>TM</sup> Plus, Costech EA connected <i>via</i> ConFlo II interface; oxidation 1020 °C, reduction 650 °C, GC 60 °C, He flow 110 mL min <sup>-1</sup> .

### On-line and off-line isotopic analyses

Eleven participating laboratories used a variety of analytical approaches for measuring  $\delta^2\text{H}$ ,  $\delta^{13}\text{C}$ , and  $\delta^{15}\text{N}$  values (Table 1). The isotopic homogeneity of new RMs was tested and confirmed at the 0.1 mg level, except for L-valine #3 where homogeneity required 0.2 mg- aliquots. Ten laboratories used on-line methodologies based on a variety of elemental analyzer (EA) and gas chromatographic (GC) interfaces, as well as mass spectrometers from different manufacturers. All laboratories used combinations of primary and secondary international measurement standards VSMOW2, SLAP2, NBS 19, NBS 22, LSVEC, USGS32, USGS40,

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3 USGS41, IAEA-N-1, IAEA-N-2, IAEA-CH-7, and IAEA-600 for two-point calibrations  
4 (normalization) of isotope  $\delta$ -scales anchored by VSMOW, SLAP, VPDB, LSVEC, atmospheric  
5 nitrogen, and USGS32. An additional, new  $^2\text{H}$ -enriched reference water IAEA-604 with  $\delta^2\text{H} =$   
6  $+799.9$  mUr was used for normalization of RMs having positive  $\delta^2\text{H}$  values.<sup>28</sup> Waters and oils  
7 were crimp-sealed into segments of silver tubing for use in on-line EA-interfaces.<sup>13</sup> The use of  
8 reference waters in silver tubes made it possible to directly measure  $\delta^2\text{H}$  values of organic  
9 materials against known  $\delta^2\text{H}$  values of reference waters<sup>29</sup> following the principle of identical  
10 treatment.<sup>10</sup>  
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14 For on-line measurements of  $\delta^2\text{H}$  values in nitrogen-containing organic compounds with an EA  
15 interface, the conventional high-temperature conversion in a glassy carbon environment (also  
16 termed TC/EA) has recently been found to fractionate hydrogen isotopes due to the generation  
17 of HCN as a pyrolytic byproduct.<sup>23,27</sup> The use of chromium for high-temperature conversion of  
18 organic hydrogen to gaseous  $\text{H}_2$  eliminated the bias both for EA<sup>23</sup> and GC interface  
19 applications<sup>24</sup> and resulted in on-line  $\delta^2\text{H}$  values that were comparable with off-line  
20 measurements at Indiana University. The traditional off-line combustion at Indiana University of  
21 milligram amounts of organic material with copper oxide (CuO) at 800 °C in evacuated and  
22 sealed “quartz” ampoules overnight offers sufficient time at high temperature to yield  
23 quantitatively  $\text{H}_2\text{O}$ ,  $\text{CO}_2$ , and  $\text{N}_2$  after cooling to 300 °C. Immediately after combustion, after  
24 cooling to 200 °C, individual ampoules with break-seals were transferred to the inlet system of a  
25 glass vacuum line. Cryogenic separation of combustion products in a vacuum line yielded pure  
26  $\text{N}_2$  (collected with a Toepler pump and flame-sealed in a glass tube) and  $\text{CO}_2$  (collected and  
27 sealed in a glass tube at the temperature of liquid nitrogen). A subsequent step of reducing  $\text{H}_2\text{O}$   
28 to  $\text{H}_2$  in the presence of uranium metal at 800 °C is a classical method for preparation of  $\text{H}_2$  for  
29 stable isotope mass spectrometry<sup>30</sup> and was applied uniformly to reference waters and for  
30 waters from combustion of organics.<sup>31</sup> We found no difference in isotopic composition between  
31 reference waters that were either “pre-combusted” with CuO in quartz glass or directly admitted  
32 to hot uranium in the vacuum line.  
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39 Complete oxidative combustion of organic materials was used by all laboratories to generate  
40  $\text{CO}_2$  and  $\text{N}_2$  analyte gases in on-line or off-line modes for measurements of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$   
41 values. At Indiana University,  $\text{CO}_2$  reference gases were prepared from NBS 19 and LSVEC  
42 carbonates with anhydrous phosphoric acid at 25 °C, followed by additional “combustion” of the  
43  $\text{CO}_2$  with CuO at 800 °C in evacuated and sealed “quartz” ampoules overnight to match the  
44 oxygen stable isotopic composition of  $\text{CO}_2$  from combusted organic materials.  
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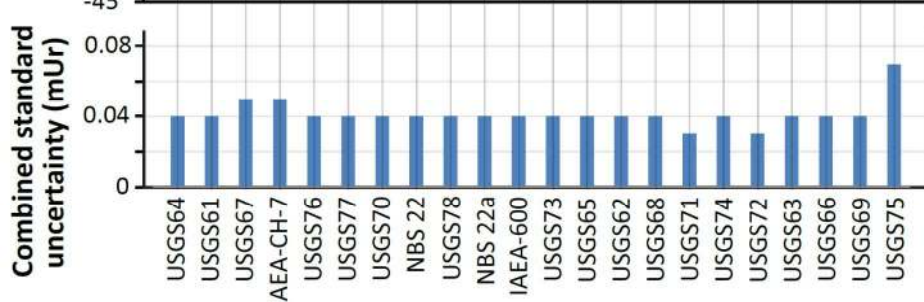
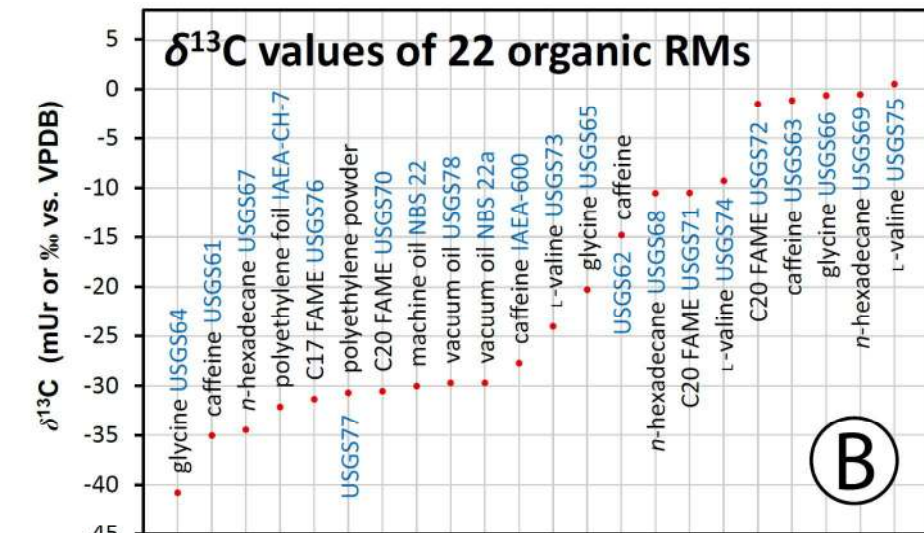
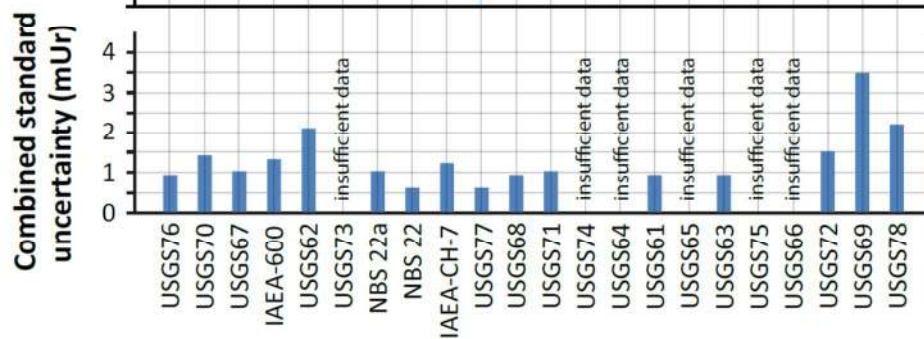
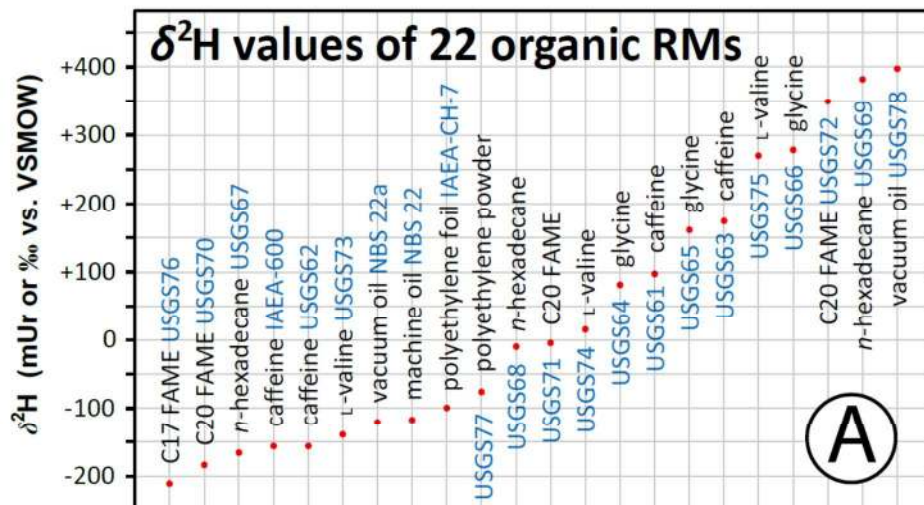
## 47 Results

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49 Only 2-point-normalized data were accepted. Additional data, including averages and standard  
50 deviations, and associated graphs are provided in the Supporting Information. All 2-point-  
51 normalized  $\delta^2\text{H}$  values were accepted except for those that had been measured on-line with  
52 high-temperature-conversion methodologies (e.g., conventional TC/EA) for nitrogen-containing  
53 compounds in the absence of chromium to scavenge nitrogen. All  $\delta^{13}\text{C}$  values were accepted  
54 except for the measurements of four materials from a single laboratory that had used peak  
55 jumping when measuring  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values in the same runs. We eliminated these four sets  
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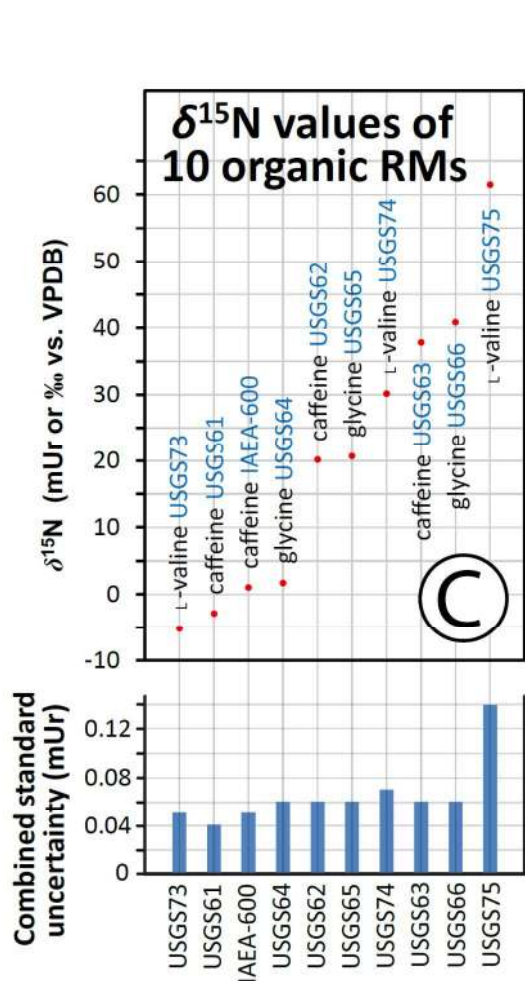
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3 of  $\delta^{13}\text{C}$  values as outliers (see Supporting Information). We also eliminated the  $\delta^{15}\text{N}$  values of  
4 one material from a single laboratory due to outlier status. All accepted data were used for  
5 Bayesian Random Effects statistical analysis to yield mean values with standard uncertainties  
6 and expanded uncertainties (~95 % uncertainty bounds; see description of statistical details in  
7 Supporting Information).<sup>32</sup> There was one exception to the statistical treatment; because the  
8  $\delta^{13}\text{C}$  values of the regular vacuum oil NBS 22a and the  $^2\text{H}$ -enriched vacuum oil USGS78 are  
9 known to be essentially identical, the results for these two materials were harmonized as  
10 described in section 2 of the Supporting Information.  
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14 A statistical overview of mean  $\delta$  values from a Bayesian Random Effects model approach with  
15 combined standard uncertainty is presented in Table 2. Multiple graphs in the Microsoft Excel<sup>®</sup>  
16 file (see Supporting Information) document the typically excellent agreement of data among  
17 participating laboratories and testify to the group's analytical agreement as a consequence of  
18 shared primary and secondary RMs, strict adherence to 2-point normalization, and high levels of  
19 analytical proficiency. Combined standard uncertainties take into account not only measurement  
20 uncertainties from Bayesian statistical analysis of our group's combined results, but additionally  
21 include published uncertainties of  $\delta$  values from RMs that were used for normalization, for  
22 example VSMOW2 and SLAP2.<sup>8</sup> A primary reason that uncertainties are larger is that most  
23 laboratories used materials for normalization other than anchor materials, and many of these  
24 materials had significant uncertainty associated with them. These are shown in Tables S3, S4,  
25 and S5 of the Supporting Information Excel<sup>®</sup> file. The inclusion of these sources of uncertainty  
26 enlarged our confidence intervals in comparison to earlier ring-tests.<sup>33,17</sup> Some primary  
27 international RMs like NBS 19 and VSMOW define anchors and fixed points along isotope- $\delta$   
28 scales and by definition have zero uncertainty.<sup>7</sup> There is evidence that USGS41 L-glutamic acid  
29 is hygroscopic, likely due to the presence of pyroglutamic acid<sup>34</sup>, and its measured  $\delta^{13}\text{C}$  value  
30 can be too low; therefore, the uncertainty of USGS41 used for normalization has been  
31 increased from the value of 0.05 mUr<sup>8</sup> to 0.25 mUr. There is evidence that LSVEC can increase  
32 in  $\delta^{13}\text{C}$  value, perhaps by reaction with atmospheric carbon dioxide<sup>34</sup>, and the uncertainty of  
33 LSVEC used for normalization has been increased from 0 mUr<sup>17</sup> to 0.15 mUr.  
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40 The Bayesian Random Effects mean  $\delta$  values of organic RMs are presented for  $\delta^2\text{H}_{\text{VSMOW-SLAP}}$ ,  
41  $\delta^{13}\text{C}_{\text{VPDB-LSVEC}}$ , and  $\delta^{15}\text{N}_{\text{Air}}$  (Figure 1A-C) in ascending order with assigned combined standard  
42 uncertainties (mUr) and range from -210.8 to +397.0 mUr for  $\delta^2\text{H}_{\text{VSMOW-SLAP}}$ , from -40.81 to  
43 +0.49 mUr for  $\delta^{13}\text{C}_{\text{VPDB-LSVEC}}$ , and from -5.21 to +61.53 mUr for  $\delta^{15}\text{N}_{\text{Air}}$ . A modified, alternative  
44 assessment scheme for the calculation of combined standard uncertainties for these new RMs  
45 is discussed in component 6 of the Supporting Information.  
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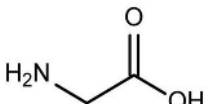




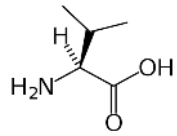
**Figure 1:** (A) Bayesian Random Effects mean  $\delta^2\text{H}_{\text{VSMOW-SLAP}}$  values 22 organic RMs in ascending order with assigned combined standard uncertainties (mUr). The  $\delta^2\text{H}_{\text{VSMOW-SLAP}}$  values of L-valines USGS74 and USGS75 are rough estimates without assigned uncertainties due to limited data. The  $\delta^2\text{H}_{\text{VSMOW-SLAP}}$  values of amino acids have not been corrected for exchangeable hydrogen. Assigned combined uncertainties of amino acids do not reflect isotopic interference by geographically and seasonably variable air humidity and isotopic composition. (B) Bayesian Random Effects mean  $\delta^{13}\text{C}_{\text{VPDB-LSVEC}}$  values of 22 organic RMs in ascending order with assigned combined uncertainties (mUr). (C) Bayesian Random Effects mean  $\delta^{15}\text{N}_{\text{Air}}$  values of 10 organic RMs in ascending order with assigned combined standard uncertainties (mUr).

**Table 2:** Alphabetical listing of new and pre-existing (\*) organic reference materials with Bayesian Random Effects mean values with associated combined standard uncertainties.

[The unit mUr is synonymous with deprecated ‰; n.a., not applicable; FAME, fatty acid methyl ester. The  $\delta^2\text{H}_{\text{VSMOW-SLAP}}$  values are normalized to a scale in which  $\delta^2\text{H}$  of SLAP =  $-428$  mUr; the  $\delta^{13}\text{C}$  values are normalized to a scale in which NBS 19 is  $+1.95$  mUr and LSVEC is  $-46.6$  mUr; the  $\delta^{15}\text{N}$  values are normalized to a scale in which the  $\delta^{15}\text{N}$  of atmospheric nitrogen =  $0$  mUr and that of USGS32 =  $+180$  mUr.]

Reference ID	Chemical name	Structure or composition of material	Ring-test reference values with combined standard uncertainties (mUr or ‰)		
			$\delta^2\text{H}_{\text{VSMOW-SLAP}}$	$\delta^{13}\text{C}_{\text{VPDB-LSVEC}}$	$\delta^{15}\text{N}_{\text{Air}}$
USGS61	caffeine		$+96.9 \pm 0.9$	$-35.05 \pm 0.04$	$-2.87 \pm 0.04$
USGS62	caffeine		$-156.1 \pm 2.1$	$-14.79 \pm 0.04$	$+20.17 \pm 0.06$
USGS63	caffeine		$+174.5 \pm 0.9$	$-1.17 \pm 0.04$	$+37.83 \pm 0.06$
IAEA-600*	caffeine		$-156.1 \pm 1.3$	$-27.73 \pm 0.04$	$+1.02 \pm 0.05$
USGS64	glycine		<i>no values indicated due to the presence of exchangeable hydrogen</i>	$-40.81 \pm 0.04$	$+1.76 \pm 0.06$
USGS65	glycine		$-20.29 \pm 0.04$	$+20.68 \pm 0.06$	
USGS66	glycine		$-0.67 \pm 0.04$	$+40.83 \pm 0.06$	
USGS67	<i>n</i> -hexadecane	$\text{C}_{16}\text{H}_{34}$	$-166.2 \pm 1.0$	$-34.50 \pm 0.05$	n.a.
USGS68	<i>n</i> -hexadecane		$-10.2 \pm 0.9$	$-10.55 \pm 0.04$	n.a.
USGS69	<i>n</i> -hexadecane		$+381.4 \pm 3.5$	$-0.57 \pm 0.04$	n.a.
USGS70	icosanoic acid methyl ester (C20 FAME)	$\text{C}_{20}\text{H}_{39}\text{OOCH}_3$	$-183.9 \pm 1.4$	$-30.53 \pm 0.04$	n.a.
USGS71	icosanoic acid methyl ester (C20 FAME)		$-4.9 \pm 1.0$	$-10.50 \pm 0.03$	n.a.
USGS72	icosanoic acid methyl ester (C20 FAME)		$+348.3 \pm 1.5$	$-1.54 \pm 0.03$	n.a.

continued

USGS73	L-valine		<i>no values indicated due to the presence of exchangeable hydrogen</i>	-24.03 ± 0.04	-5.21 ± 0.05
USGS74	L-valine			-9.30 ± 0.04	+30.19 ± 0.07
USGS75	L-valine			+0.49 ± 0.07	+61.53 ± 0.14
USGS76	methylheptadecanoate (C17 FAME)	C <sub>17</sub> H <sub>33</sub> OOCH <sub>3</sub>	-210.8 ± 0.9	-31.36 ± 0.04	n.a.
IAEA-CH-7*	polyethylene foil	(C <sub>2</sub> H <sub>4</sub> ) <sub>n</sub>	-99.2 ± 1.2	-32.14 ± 0.05	n.a.
USGS77	polyethylene powder (also extruded string)	(C <sub>2</sub> H <sub>4</sub> ) <sub>n</sub>	-75.9 ± 0.6	-30.71 ± 0.04	n.a.
NBS 22*	oil	n.a.	-117.2 ± 0.6	-30.02 ± 0.04	n.a.
NBS 22a	vacuum oil, regular	n.a.	-120.4 ± 1.0	-29.72 ± 0.04	n.a.
USGS78	vacuum oil, <sup>2</sup> H-enriched	n.a.	+397.0 ± 2.2	-29.72 ± 0.04	n.a.

## Discussion

The principle of identical treatment of sample and RM<sup>10</sup> was followed by each participating laboratory according to their own interpretation. Some laboratories employed identical sample preparation and analysis conditions (e.g., the same microbalances, tin or silver capsules from the same batch, concurrent analyses of RMs and quality control samples within the same EA or GC batch run with unknowns), while other laboratories additionally adhered to the notion that RMs, quality control samples, and unknowns should reflect a chemical matrix match with similar chemical compositions. For example, some laboratories used the carbonates NBS 19 and LSVEC for two-point  $\delta^{13}\text{C}$  scale normalization of on-line EA measurements, whereas others used organic RMs such as USGS40, USGS41, and IAEA-CH-7. The remarkable agreement of data from multiple, independent approaches testifies to the practicality and suitability of careful laboratory practices, both on-line and off-line.

In addition to 19 new organic RMs, our ring-test re-calibrated 3 older organic RMs NBS 22 (oil), IAEA-CH-7 (polyethylene foil), and IAEA-600 (caffeine) and arrived at new  $\delta$  values (with tightened confidence intervals and lower combined standard uncertainties) that supersede earlier recommended values.<sup>35,36,8</sup> The quality of the results is evidenced by the  $\delta^{13}\text{C}_{\text{VPDB-LSVEC}}$  value of NBS 22 of  $-30.02 \pm 0.04$  mUr (Table 2) compared to the earlier value of  $-30.03 \pm 0.05$  mUr.<sup>17</sup>

The hydrogen exchange of organic hydrogen in functional groups can currently not be controlled satisfactorily in monomeric amino acids to warrant the recommendation of bulk hydrogen  $\delta^2\text{H}$  values for glycine and L-valine RMs. We refrain from listing any bulk  $\delta^2\text{H}$  values of amino acids in Table 2 for RM purposes, but the few preliminary bulk  $\delta^2\text{H}$  values on non-equilibrated amino acids graphed on a coarse scale (Figure 1A) demonstrate that the  $^2\text{H}$ -spiking of USGS65, USGS66, USGS74, and USGS75 in molecular positions bound to carbon atoms was successful. This bodes well for refined and reproducible hydrogen-isotopic characterization and use as RMs by future analytical techniques capable of measuring only isotopically non-exchangeable hydrogen in monomeric amino acids. We note that the much higher abundance of exchangeable hydrogen in monomeric amino acids poses more difficult challenges than for analytically less demanding polypeptides, such as collagen and keratin, where the formation of peptide bonds has eliminated much of the original exchangeable hydrogen from amino acid building blocks.

Some glycine and L-valine RMs have been isotopically spiked with  $^2\text{H}$  (bonded to carbon) and  $^{13}\text{C}$  at specific molecular positions resulting in distinct, contrasting abundances of molecular isotopologues. Future analytical techniques capable of discerning the intra-molecular isotopic distribution at natural  $^2\text{H}$  and  $^{13}\text{C}$  abundances (e.g., high-resolution NMR)<sup>37</sup> may take advantage of this additional, built-in quality of these new RMs. We note that problems in an earlier laboratory inter-comparison of hydrogen isotope abundances measured *via* IRMS and Site-specific Natural Isotope Fractionation measured by Nuclear Magnetic Resonance (SNIF-NMR) have been likely caused by incomplete hydrogen yields during high-temperature conversion without the use of chromium.<sup>38</sup>

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3 Logistic and administrative constraints precluded the development of RMs with characteristics  
4 that may complicate international distribution, such as hydrocarbon gases, mixtures of  
5 compounds in a common organic solvent, and highly toxic substances.  
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8 Organic RMs are sensitive to UV radiation and heat. Although none of the new RMs expresses  
9 significant volatility (except for hexadecane and FAMES under vacuum), and only amino acids  
10 have a small propensity for slow oxidation, it is strongly recommended that refrigerated, dark,  
11 and dry storage be used for all organic RMs. Non-polymeric RMs are subject to microbial  
12 degradation in the presence of moisture. Glass containers should be warmed to room  
13 temperature prior to opening in order to avoid condensation of atmospheric water.  
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## 16 17 18 **Conclusions**

19  
20 Nineteen new organic reference materials for hydrogen, carbon, and nitrogen stable isotope-  
21 ratio measurements have been developed and analyzed using primary and secondary  
22 hydrogen, carbon, and nitrogen isotopic reference materials by an international group of  
23 laboratories, and will be distributed jointly by the USGS<sup>39</sup>, IAEA<sup>40</sup>, and Indiana University<sup>41</sup>.  
24 Polyethylene string PE77 is available from ANSTO.<sup>21</sup> Revised  $\delta$  values are being recommended  
25 for pre-existing reference materials NBS 22 (oil), IAEA-CH-7 (polyethylene foil), and IAEA-600  
26 (caffeine). The use of reference waters VSMOW2, SLAP, and IAEA-604 in silver tubes made it  
27 possible to directly calibrate  $\delta^2\text{H}$  values of organic materials against known  $\delta^2\text{H}$  values of  
28 reference waters following the principle of identical treatment. The available organic reference  
29 materials include various compound classes to facilitate adherence to the principle of identical  
30 treatment of organic sample and reference material. It is recommended that efforts be invested  
31 in the future to check for heterogeneity of prepared aliquots for routine distribution. It may also  
32 be appropriate to revisit the scheme for reference material uncertainty evaluation in light of  
33 emerging new conventions and statistical approaches (details provided in component 6 of the  
34 Supplementary Information).  
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40 Recommended  $\delta$  values of reference materials are subject to occasional revision, which may  
41 not result in prompt updates on Web sites of distributing agencies. Each user of isotopic  
42 reference materials is encouraged to consult the Web site of the *Commission on Isotopic*  
43 *Abundances and Atomic Weights*<sup>42</sup> (CIAAW) as a dedicated and up-to-date source of  
44 information about current  $\delta$  values of isotopic reference materials.  
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47 Future analytical techniques capable of (i) distinguishing between molecular isotopologues with  
48 site-specific  $^2\text{H}$  and  $^{13}\text{C}$  enrichments and (ii) measuring only isotopically non-exchangeable  
49 hydrogen in amino acids will take advantage of the fact that some of the new glycine and L-  
50 valine reference materials have been spiked at distinct molecular positions with  $^2\text{H}$  (bonded to  
51 carbon) and  $^{13}\text{C}$ . Refrigerated, dark, and dry storage is strongly recommended for organic RMs.  
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## 55 56 **Acknowledgements**

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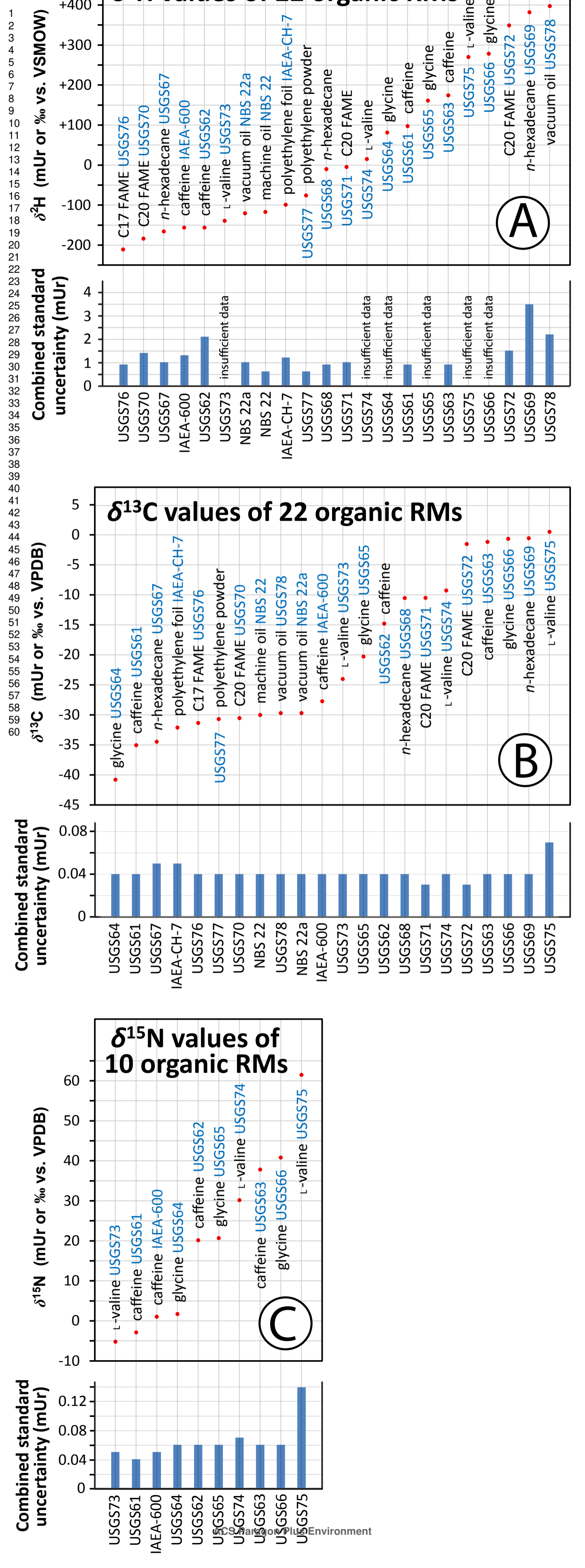
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