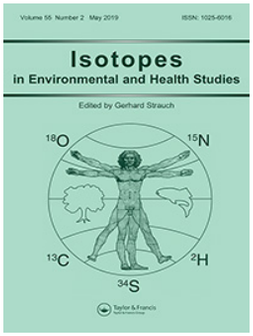

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A guide for proper utilisation of stable isotope reference materials*

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ABSTRACT

Many scientific publications about stable isotope ratios suffer from flawed practices regarding calibration and normalisation of raw δ values in conjunction with prescribed δ values of reference materials. Violations of the identical treatment principle with regards to samples and standards (i.e. reference materials) and lack of adherence to SI-mandated and IUPAC-recommended nomenclature exacerbate the widespread problem of lackadaisical analytical practice and reporting. Science is supposed to strive for exactness, whereas ambiguity and jargon confound interdisciplinary communication. This contribution aims to expose typical misconceptions and avoidable errors and offers guidance toward the reproducible generation of isotope data, isotopic scale normalisation, and proper data reporting. We offer a comprehensive overview of sources of light stable isotope reference materials to best match sample matrices encountered by stable isotope practitioners with chemically similar reference materials.

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1. Introduction

The exponential growth of global scientific output fosters compartmentalisation into sub-disciplines with specialised jargons and cryptic conventions. Practitioners of stable isotope analyses are spread over many poorly connected scientific fields from archaeology over ecology and forensics to geochemistry to name but a few [1–5]. Fruitful interdisciplinary communication mandates that scientific results should be measured reproducibly and reported unambiguously based on internationally accepted scientific units and nomenclature [6,7].

This contribution aims to provide a guide towards generating and reporting stable isotope data with quality assurance and transparency. Adherence to such principles will facilitate publication of results as well as interdisciplinary understanding.

It is probably fair to say neither reviewers nor editors of scientific journals would accept and publish manuscripts reporting quantitative data based on mass spectrometric (MS) analysis if such data were not supported by a multi-point calibration. So, why should manuscripts reporting isotope abundance data based on isotope ratio mass spectrometric

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(IRMS) analysis be treated any differently? The answer, of course, is they should not. While failure to calibrate an analytical method to compensate for method and/or instrument inherent differences between measured and accepted (known to be true) values will present problems in any subject of natural or life sciences, this is of particular concern in subject areas impacting on public health and safety including consumer protection such as food authenticity (or food forensics), authenticity of pharmaceutical drugs or forensic science. Lack of traceability to internationally recognised scale anchors and lack of inter-laboratory comparability of such non-calibrated data undermines not just confidence in the data but confidence in the conclusions drawn. This would be particularly regrettable if such data were to be presented and refuted in court [8]. Yet, many an example of articles reporting such data can be found in journals dedicated to food authenticity or forensic science to this day.

This article, therefore, aims to dispel any misconceptions that may still exist concerning isotope abundance calibration or isotopic scale normalisation of measured stable isotope abundance data by explaining the concept of reference materials (RMs) in this context and by illustrating how to use them and how not to.

2. Why multi-point isotopic calibration is a must

Having referred to multi-point calibration being a prerequisite for quantitative MS analysis, let us briefly explore the similarity or analogous principles between quantitative compound MS analysis and compound isotope abundance analysis by IRMS. In quantitative MS analysis calibration aims to determine the relation between compound amount or concentration and detector response. Even though the detector response of MS instruments is directly proportional to compound amount, performance variability of the different instrument components results in uncertainty levels of >10 % relative standard deviation (RSD), too poor for reliable quantification. Repeatedly carried out calibration series can, therefore, yield widely differing calibration curves even though individually each calibration curve can be all but perfectly linear (Figure 1(a)). To overcome this problem quantitative MS analysis relies on the use of internal standards (IS). Calibration curves are based on the analysis of varying concentrations of the target compound(s) to which always the same amount of internal standard(s) has been added. Calibration curves are constructed by plotting abundance ratios of quantifying ion of compound over quantifying ion of IS (Figure 1(b)). This approach is associated with uncertainty levels of <1 % RSD. As a consequence of dividing compound quantifying ion abundance by IS quantifying ion abundance, any factors ultimately affecting detector response will cancel each other out because these factors will affect the measurement of quantifying ions of target compound and IS in the same way.

The situation we encounter when analysing compounds for the abundance of the heavier isotope of a given light element by IRMS is similar and yet somewhat different. One could say calibration in quantitative MS and IRMS are two different sides of the same coin. Isotopic abundance analysis also aims to determine a quantitative answer, namely the true amount of a given isotope present in a compound or material. However, for a number of reasons the analytical answer is not an absolute amount but a ratio of the heavier isotope abundance over the lighter isotope abundance of the

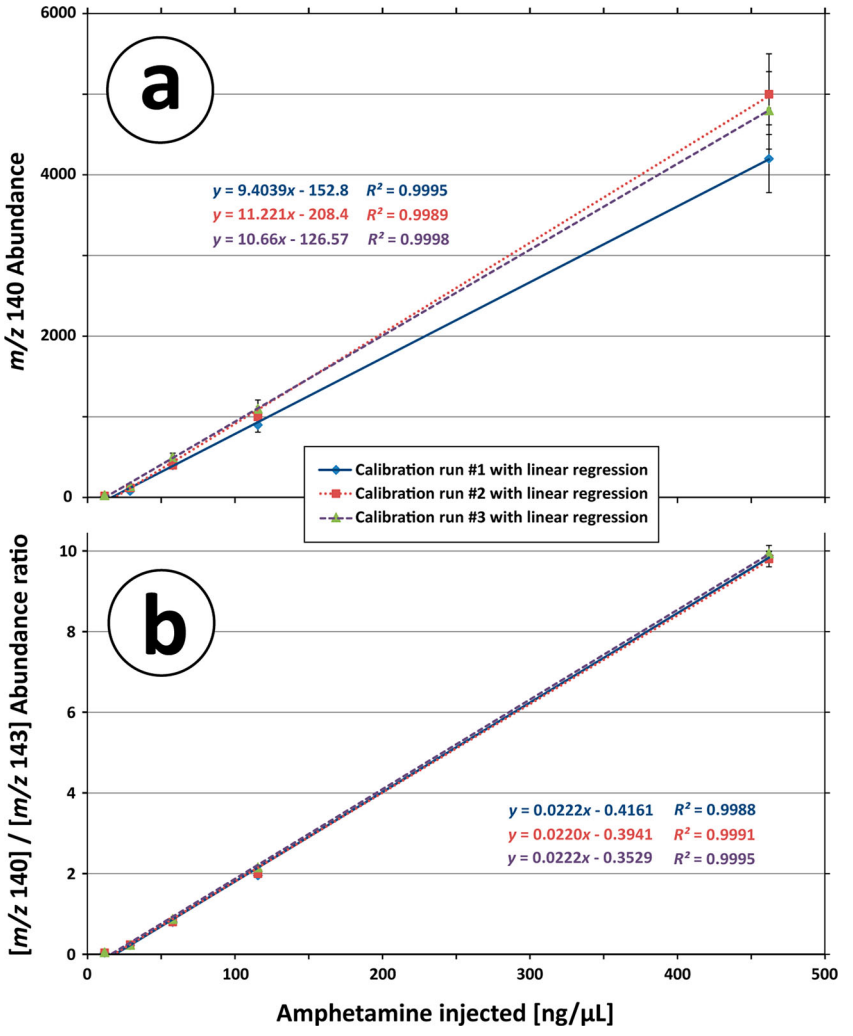

Figure 1. Comparison of variability and thus the precision of quantitative mass spectrometric analysis based (a) on absolute *m/z* abundance measurement and (b) on *m/z* abundance ratio determination.

sample (S) relative to the known isotope abundance ratio of a standard (STD).

$$\delta^h E_{S/STD} = \frac{R_S - R_{STD}}{R_{STD}} = \frac{R_S}{R_{STD}} - 1. \quad (1)$$

The result of this ratio-of-ratios calculation is the delta value (δ) of the heavier isotope *h* of a given element E (Equation (1)). Numerical results of this equation are typically quite small, e.g. −0.02996, which is why for reasons of convenience they may be expressed as ‰ values where −0.02996 is written as -29.96×10^{-3} or −29.96 ‰ since 10^{-3} = ‰ analogue to 10^{-2} = %. Therefore, like the quantitative MS analysis of compound abundance IRMS analysis of stable isotope abundance yields a quantitative answer yet with one subtle difference. Unlike quantitative MS analysis, the result of IRMS analysis can be either a negative or a positive number because measurement results are expressed

relative to an internationally agreed scale zero point. A negative number, therefore, indicates the abundance of the heavier isotope *h* in a given compound is less than that of the primary RM defining the scale zero point. Conversely, a positive number indicates the abundance of the heavier isotope *h* in a given compound is higher than that of the primary RM defining the scale zero point.

It is important to note, neither % nor ‰ are units or unit modifiers in the SI (SI = Système International, International System) but purely a mathematical convention for expressing small numbers. Should one wish to avoid the ‰ notation (and thus the ‰ symbol) being confused with an SI unit, particularly in graphs and tables, one can present relative abundance data as $10^3 \times \delta$ values [9,10]. In line with the established practice to attribute SI supplementary units to values derived from ratios where units cancel each other (e.g. the Reynolds number [Re]), the term 'urey' (symbol Ur) has been proposed so that δ values traditionally written as e.g. −29.96 ‰ can be written as −29.96 mUr [6].

While in theory any compound could be used as standard as long as its isotopic abundance with regards to ^{h}E were known or defined, it is obvious that δ values thus obtained could not be compared between different laboratories unless all laboratories would use subsamples of the same standard material. However, would one standard suffice to quantify isotope abundance of a given element in a given compound or material? The answer is no of course. We know ^{13}C abundance in chemically identical compounds like sugars can differ widely depending by which photosynthetic pathway they were produced [11–13]. In other words, in analogy to the need in quantitative MS analysis for concentration or abundance calibration based on several but at least two standard preparations containing different amounts of the target compound, there is a similar need in quantitative isotope abundance IRMS analysis for several but at least two standards of preferably identical chemical composition but of different isotope abundance (i.e. isotopic specific quantity). For this reason alone δ values obtained by comparative measurement to a single cylinder gas are neither calibrated nor scale-normalised. It could, of course, be argued multi-point calibration should not be necessary considering we are measuring relative isotope abundance values. However, such an argument fails to take into account a phenomenon particular to IRMS analysis called scale compression [14,15]. Scale compression is essentially the sum of all mass discriminatory effects associated with sample gas transfer to the IRMS instrument, sample gas admission into the ion source of the IRMS instrument and possibly processes inside the ion source itself. The measurable consequence of scale compression is most noticeable and thus most detrimental to accuracy in ^{2}H abundance analysis as illustrated by the examples presented in Table 1. In that instance, the difference between SLAP2's measured δ^2H value and its scale-normalised and thus isotopically calibrated δ^2H_{VSMOW} value was 31.8 ‰ (Vienna Standard Mean Ocean Water (VSMOW) and Standard Light Antarctic Precipitation (SLAP)). Similarly, the difference between the measured δ^2H value of NBS 22 and its scale-calibrated

Table 1. Difference between measured δ^2H values and δ^2H_{VSMOW} values calibrated by two end-point scale normalisation.

Compound name	$10^3 \times \delta^2H_{measured}$	$10^3 \times \delta^2H_{VSMOW}$	$10^3 \times \Delta^2$H
VSMOW2	−0.36	0.00	0.36
NBS 22; $10^3 \times \delta^2H_{accepted} = -117.2$	−108.54	−117.00	8.46
SLAP2	−395.68	−427.50	31.82

δ^2H_{VSMOW} value of −117.0 ‰ (accepted δ^2H_{VSMOW} value of −117.2 ‰ [16]) was 8.5 ‰, representing a relative difference of 7.25 % between measured and accepted ^2H abundance value. This example alone illustrates the importance of using RMs when carrying out stable isotope abundance measurements. Equally, this example illustrates the importance of reporting which international RMs have been used as scale anchors for the respective stable isotope scales and which accepted δ values have been employed [7]. There are numerous instances where well-established international RMs like NBS 22 or IAEA-CH-7 have been repeatedly isotopically re-evaluated over time.

3. Why internationally accepted reference materials are important

The inescapable conclusions from the foregoing are these: Internationally accepted RMs are required that satisfy two needs.

(I) Ensuring relative isotope abundance measurements can be calibrated to internationally agreed methodology, so calibrated δ values can be compared between laboratories on a like-for-like basis.
(II) Ensuring relative isotope abundance measurements can be corrected for any scale-distorting effects, so calibrated δ values are anchored to internationally agreed isotope abundance scales as defined for a particular element.

In addition to delivering highly comparable data, RMs meeting these two conditions will also put in place a good traceability system. There are other practical but equally important reasons why internationally accepted RMs are needed in addition to the primary, scale-defining RMs [7] such as VSMOW (now VSMOW2) and SLAP (now SLAP2) which scale anchor the ^2H as well as the ^{18}O δ scale [6,10,17,18]. For one, for historical reasons the primary scale-defining RMs are inorganic compounds whose chemical elemental composition does not match that of organic compounds and materials. For another, limited stocks of primary RMs place restrictions on how much and at which intervals they can be supplied to any one laboratory. To overcome these constraints, internationally accepted and distributed secondary RMs have been made available 'to bridge the materials and chemistry gap' [7]. Secondary RMs are traceable to the scale-defining primary RMs but unlike the defined δ values of primary RMs, specified δ values of secondary RMs are associated with an uncertainty envelope (Tables 2 and 3). A list of distributors of RMs is given in Table 4.

However, even secondary RMs (Tables 2 and 3) are not available in limitless supply, which means it is not practically feasible to use them directly as Equation (1) implies. They are therefore more efficiently used to scale-normalise and thus calibrate 'raw' δ values that have been measured against a working gas. It must be emphasised that measured δ values calculated by instrument software on the basis of a working gas peak are neither calibrated nor scale-normalised, even if the isotopic composition of the working gas is known. As illustrated by the example shown in Table 1, scale normalisation requires at least two RMs acting as scale anchors. Furthermore, in continuous-flow IRMS (CF-IRMS) instruments working gas pulses are introduced in a gas stream separate to the sample gas stream and are not subject to the same physical and chemical processes as the samples. For these two reasons the use of working gas pulses does not meet with the identical treatment principle [7,19,20]. Last but not least, even if the isotopic

Table 2. Select list of reference materials for δ^2H, δ^{13}C and/or δ^{18}O analysis that can serve as scale anchors (SCAN). Some reference materials listed include a matching quality control (QC).

Reference material ID	Compound name	$10^3 \times \delta^2H_{VSMOW}$	$10^3 \times \delta^{13}C_{VPDB}$	$10^3 \times \delta^{18}O_{VSMOW}$
VSMOW2[a]	Water (SCAN #1)	0.0 ± 0.3		0.00 ± 0.02
GISP2[a]	Water (QCl)	−258.3 ± 0.3		−33.43 ± 0.02
SLAP2[a]	Water (SCAN #2)	−427.5 ± 0.3		−55.50 ± 0.02
USGS48[b]	Water (SCAN #1)	−2.0 ± 0.4		−2.22 ± 0.01
USGS47[b]	Water (QCl)	−150.2 ± 0.5		−19.80 ± 0.02
USGS49[b]	Water (SCAN #2)	−394.7 ± 0.4		−50.55 ± 0.04
USGS67[b,c]	Hexadecane (SCAN #1)	−166.2 ± 1.0	−34.50 ± 0.05	
USGS68[b,c]	Hexadecane (QC)	−10.2 ± 0.9	−10.55 ± 0.04	
USGS69[b,c]	Hexadecane (SCAN #3)	+381.4 ± 3.5	−0.57 ± 0.04	
USGS70[b,c]	Icosanoic acid methyl ester (C20:0 FAME) (SCAN #1)	−183.9 ± 1.4	−30.53 ± 0.04	
USGS71[b,c]	Icosanoic acid methyl ester (C20:0 FAME) (QC)	−4.9 ± 1.0	−10.50 ± 0.03	
USGS72[b,c]	Icosanoic acid methyl ester (C20:0 FAME) (SCAN #2)	+348.3 ± 1.5	−1.54 ± 0.03	

[a]Reference material IDs and their δ values taken from: http://www.ciaaw.org/reference-materials.htm
[b]Reference material IDs and their δ values taken from: https://isotopes.usgs.gov/lab/referencematerials.html
[c]See also reference [16].

composition of a permanent gas in a full cylinder is known, this still does not turn the cylinder gas into a 'reference gas'. A gas cylinder constitutes a finite and thus limited reservoir of a compound comprising N_0 molecules of a relative isotopic abundance value δ_0 at time t_0 when the cylinder is full. Continued withdrawal of gas will, therefore, be associated with a change in isotopic composition according to

$$\delta = (1 + \delta_0) \times (N/N_0)^{\alpha-1} - 1. \quad (2)$$

Here α is the isotopic fractionation factor, N/N_0 is the remaining fraction of the original reservoir and δ_0 is the isotope abundance value of the original reservoir. The phenomenon of changing isotopic composition of a cylinder gas as the gas cylinder contains less and less gas is particularly noticeable with CO_2 once the cylinder pressure starts to drop.

Table 3. Select list of reference materials for δ^{13}C, δ^{15}N and/or δ^{18}O analysis that can serve as scale anchors (SCAN). Some reference materials listed include a matching quality control (QC).[a]

Reference material ID	Compound name	$10^3 \times \delta^{13}C_{VPDB}$	$10^3 \times \delta^{15}N_{Air}$	$10^3 \times \delta^{18}O_{VSMOW}$
USGS40[a]	L-glutamic acid (SCAN #1)	−26.39 ± 0.04	−4.52 ± 0.06	
USGS41a[a]	L-glutamic acid (SCAN #2)	+36.55 ± 0.08	+47.55 ± 0.15	
USGS64[b,c]	Glycine (SCAN #1)	−40.81 ± 0.04	+1.76 ± 0.06	
USGS65[b,c]	Glycine (QC)	−20.29 ± 0.04	+20.68 ± 0.06	
USGS66[b,c]	Glycine (SCAN #2)	−0.67 ± 0.04	+40.83 ± 0.06	
IAEA-601[a]	Benzoic acid (^{18}O SCAN #1)	−28.81 ± 0.04		+23.14 ± 0.19
IAEA-602[a]	Benzoic acid (^{18}O SCAN #2)	−28.85 ± 0.04		+71.28 ± 0.36
USGS34[a]	KNO_3 (^{15}N or ^{18}O SCAN #1)		−1.8 ± 0.1	−27.78 ± 0.37
USGS32[a]	KNO_3 (^{15}N SCAN #2; ^{18}O QC)		+180.0 ± 1.0[d]	+25.4 ± 0.20
IAEA-NO-3[a]	KNO_3 (^{18}O QC)		+4.7 ± 0.42[d]	+25.32 ± 0.29
USGS35[a]	$NaNO_3$ (^{15}N QC; ^{18}O SCAN #2)		+2.7 ± 0.1	+56.81 ± 0.31

[a]Reference material IDs and their δ values taken from: http://www.ciaaw.org/reference-materials.htm.
[b]Reference material IDs and their δ values taken from: https://isotopes.usgs.gov/lab/referencematerials.html.
[c]See also reference [16].
[d]Information for $\delta^{15}N_{Air}$ values of USGS32 and IAEA-NO-3 taken from: https://nucleus.iaea.org/rpst/referenceproducts/referencematerials/Stable_Isotopes/15N14N/index.htm.

Table 4. Distributors of light stable isotope reference materials (RMs) in alphabetical order.

Distributor	Web portal or contact	Comments	RM categories
AirLiquide (international)	http://isotope.airliquide-expertisecenter.com/	Gases like CO_2 and SO_2 from cylinders can only serve as monitoring gases in on-line applications	Air, trace gases in air, CO, CO_2, SO_2, NO_x, SF_6, hydrocarbons
ANSTO (Australia)	http://www.ansto.gov.au/ResearchHub/OurInfrastructure/ACNS/CurrentResearch/ScientificHighlights/NDF-PE77/index.htm; tde@ansto.gov.au	NDF-PE77 is isotopically indistinguishable from USGS77 powder	Polyethylene line NDF-PE77
Elemental Microanalysis (United Kingdom)	http://www.elementalmicroanalysis.com/product_list.php?top = IRMS%20supplement&category = 204&sub = Certified	Website offers insufficient documentation of isotopic characterisation (13 September 2018)	2 waters, 3 organic RMs
ERM®, European Reference Materials (Belgium)	https://crm.jrc.ec.europa.eu/c/By-application-field/Stable-isotopes/40476/	ERM® and IRMM RMs are identical and available from various vendors	Inorganic RMs
IAEA, International Atomic Energy Agency (Austria)	https://nucleus.iaea.org/rpst/referenceproducts/referencematerials/Stable_Isotopes/index.htm	Isotope data and inventory not up-to-date on website as of 13 September 2018	Predominantly inorganic RMs, many waters
Indiana University, Department of Earth and Atmospheric Sciences (USA)	https://arndt.schimmelmann.us/welcome.html	e.g. Schimmelmann et al., 2016, *Analytical Chemistry* **88**, 4294–4302 http://dx.doi.org/10.1021/acs.analchem.5b04392	Organic RMs (gases, liquids, solids, GC-IRMS mixtures)
IRMM, Institute for Reference Materials and Measurements (Belgium)	https://crm.jrc.ec.europa.eu/c/By-application-field/Stable-isotopes/40476/	RMs from ERM® and IRMM are identical and available from various vendors	Inorganic RMs
Isometric Instruments (Canada)	http://www.isometricinstruments.com/gasstandards.html	Website offers insufficient documentation of isotopic characterisation (13 September 2018)	Methane in air
NIST, National Institute of Standards and Technology (USA)	https://www-s.nist.gov/srmors/detail.cfm?searchstring = isotope	Isotope data and inventory not up-to-date on website as of 13 September 2018	Predominantly inorganic RMs
NMI, National Measurement Institute (Australia)	chemref@measurement.gov.au; available only for WADA-accredited forensic laboratories	Tobias and Brenna, 2018, *Drug Testing and Analysis* **10** (4), 781–785 https://doi.org/10.1002/dta.2309	Steroids for carbon stable isotope ratios only
OZTECH (USA)	isotopems@gmail.com http://www.si-science.co.jp/product/data/oztech.pdf	Insufficient documentation of isotopic characterisation (13 September 2018)	Pure gases CO_2, N_2, H_2
Sercon Limited, Crewe (UK)	https://serconlimited.com/sercon_systems/standards/	Insufficient documentation of isotopic characterisation on RMs proprietary to Sercon (i.e. RMs with the prefix SC) (13 September 2018)	Inorganic and organic RMs; waters; flours and soils
SHOKO Science (Japan)	https://www.si-science.co.jp/global/en/index.html	RMs partially co-developed with JAMSTEC and Indiana University	Amino acids and waters
USGS, United States Geological Survey, Reston, Virginia (USA)	https://isotopes.usgs.gov/lab/referencematerials.html	Up-to-date isotope data on website; waters available in crimp-sealed silver capillary segments Also available: LIMS for stable isotope laboratories: https://isotopes.usgs.gov/research/topics/lims.html	Inorganic and organic RMs, including collagen and keratin
USGS, United States Geological Survey, Denver, Colorado (USA)	https://energy.usgs.gov/GeochemistryGeophysics/GeochemistryLaboratories/GasStandards.aspx	Dai et al., 2012, *Chemical Geology* **310–311**, 49–55. https://doi.org/10.1016/j.chemgeo.2012.03.008	Natural gas RMs of different geological origins

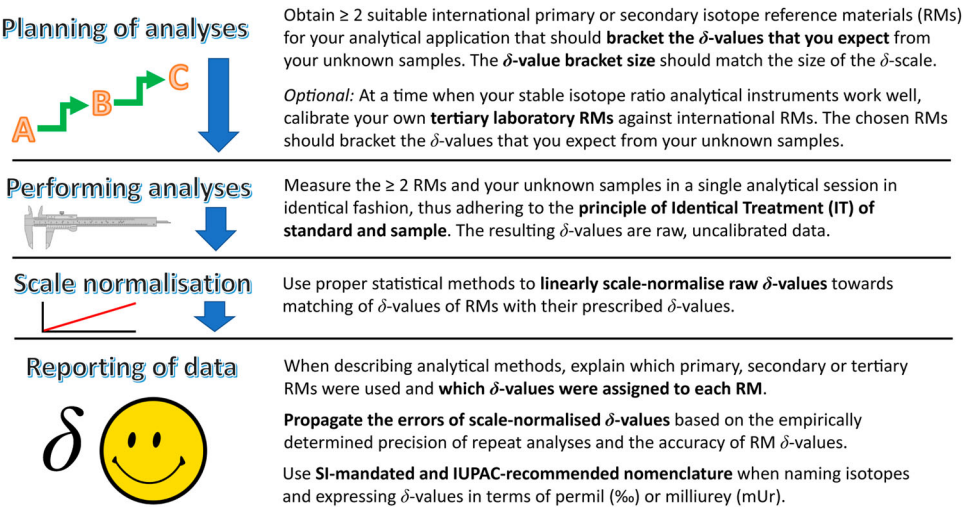

Figure 2. Flowchart for isotope abundance analysis observing the identical treatment principle for both samples and RMs serving as scale anchors.

Cylinder gases, therefore, do not meet one of the key prerequisites for a RM, namely for its isotopic composition to remain constant over time.

What does work however while meeting all requirements discussed above is using RMs and working gases in combination (Figure 2; Table 5). This means both samples and RMs serving as scale anchors are analysed under identical conditions and their measured δ values are expressed relative to a working gas. Measured δ values of RMs 1 and 2 (RM1 and RM2) are subsequently compared to their accepted δ values on the relevant isotope reference scale. A linear regression line is fitted through the data points ($RM1_{measured}$, $RM1_{accepted}$) and ($RM2_{measured}$, $RM2_{accepted}$) to yield a scale normalisation

Table 5. Generic batch sequence run sheet favouring high sample throughput under stable experimental conditions using ^{13}C abundance analysis as example.

Sample ID[a]	$10^3 \times \delta^{13}C$			$10^3 \times \delta^{13}C_{VPDB}$
	Measured	Corrected	Mean ± S.D.	Accepted
BLANK				
RM1 (USGS40)				−26.39
RM1 (USGS40)				−26.39
RM1 (USGS40)				−26.39
RM1 (USGS40)				−26.39
Samples k to l				
AQC (IAEA-CH-6)				−10.45
AQC (IAEA-CH-6)				−10.45
AQC (IAEA-CH-6)				−10.45
AQC (IAEA-CH-6)				−10.45
Samples m to n				
RM2 (USGS41a)				+36.55
RM2 (USGS41a)				+36.55
RM2 (USGS41a)				+36.55
RM2 (USGS41a)				+36.55
BLANK				

[a]RM: International Reference Material/s (here USGS40 and USGS41a) used to scale anchor measured $\delta^{13}C$ values on the VPDB scale; AQC: acquisition quality control sample/s (here IAEA-CH-6) used to control quality of scale normalisation.

equation of the form

$$\delta^h E_{\text{scale}} = s \times \delta^h E_{\text{measured}} + b, \tag{3}$$

where $s = (\text{RM2}_{\text{accepted}} - \text{RM1}_{\text{accepted}})/(\text{RM2}_{\text{measured}} - \text{RM1}_{\text{measured}})$
and $b = \text{RM1}_{\text{accepted}} - (s \times \text{RM1}_{\text{measured}}) = \text{RM2}_{\text{accepted}} - (s \times \text{RM2}_{\text{measured}})$.

Given what has been said thus far, it should be obvious that in principle the δ value of the working gas does not have to be known and could be set arbitrarily to any value. However, there are practical reasons why it is useful for the approximate δ value of the working gas to be known. This is easily achieved by measuring pulses of the working gas against a single RM. The most important practical reason for doing so is for raw δ values (i.e. based on working gas δ values) to serve as decision enabling acceptance criteria. More often than not, sample material is in limited supply so a decision as to whether a batch run analysis can proceed or should be aborted has to be made early on, e.g. based on measured δ values of RM1 and thus before any samples (Table 5). In fact, standard operating procedures for accredited analytical processes require defined acceptance criteria on which basis a decision is made if an analytical batch sequence has to be stopped or is permitted to proceed. In this context, a brief mention is owed to two laboratory information management systems (LIMS) that offer users easy scale normalisation of results of isotopic abundance measurements (e.g. LIMS for Lasers and LIMS for Light Stable Isotopes; see entry for USGS Reston in Table 4). However, it must be emphasised that the use of appropriately chosen RMs as scale anchors is a prerequisite for the software applications to be deployed successfully.

4. Why and which secondary reference materials are appropriate scale anchors

As discussed above, in terms of sample turn-around and efficient use of RMs, working gases offer a convenient way to generate raw δ values indicative of a sample's actual isotope abundance. Cylinder gases, however, well their isotopic composition may be known, are not equivalent to the scale-defining primary RMs that anchor a particular δ^hE value reference scale. Most scale-defining primary RMs are however inorganic compounds or materials that are either not directly amenable or directly comparable to stable isotope analysis of organic compounds and materials by continuous-flow IRMS. For this reason, numerous secondary (often organic) RMs have been developed (Tables 2 and 3). Like primary RMs, secondary RMs are compounds or materials which are administered and distributed by internationally recognised organisations (Table 4). Their scale-normalised δ values are based on statistically valid results submitted by stable isotope laboratories that had participated in international inter-laboratory exercises organised by the IAEA or other organisations. The purpose of internationally distributed RMs is to anchor δ^hE scales and to enable comparable measurement results in stable isotope laboratories no matter their location in the world. The majority of these secondary international RMs are listed on the website of the International Union of Pure and Applied Chemistry's (IUPAC) Commission on Isotopic Abundances and Atomic Weights (CIAAW) at www.ciaaw.org. The importance of international isotopic RMs to produce robust, traceable and internationally comparable

results has been emphasised by IUPAC guidelines that have been widely published [6,7,10].

- If a secondary international measurement standard defines the size of a δ^hE scale, such as SLAP2 water in case of the VSMOW/SLAP scale for δ^2H and δ^{18}O measurements, δ^hE values should be normalised using both standards. The authors should state this clearly in their articles and reports.
- Authors are also encouraged to analyse with their samples and report applied δ^hE values of further internationally distributed RMs as appropriate for the measurement method concerned. Secondary internationally distributed isotopic RMs that are of a nature similar to those of the unknowns being measured (amino acid, cellulose, fatty acid, nitrate, sulphate, etc.) should be analysed. This has been called the identical treatment principle [19] and minimises systematic errors by subjecting sample unknowns and RMs to exactly the same chemical and other manipulation steps, including the transfer pathway to the ion source of the IRMS. In this manner, measurement results can be adjusted in the future as analytical methods improve and consensus values of internationally distributed isotopic RMs are amended.

Therefore it follows from the above that preferably any two secondary RMs whose δ^hE values extend closely to the size of the relevant δ^hE scale can serve as appropriate scale anchors. Even more preferably, two such secondary RMs should be a good matrix match with regards to chemical nature and relative elemental composition of the samples. For example, RMs USGS40 and USGS41a are both glutamic acid but of different ^{13}C and different ^{15}N abundances (Table 3). As amino acid, the elemental C/N ratio of glutamic acid resembles the C/N ratio typically associated with that of peptides and proteins. Similarly, for relative ^{2}H or ^{13}C abundance analysis of hydrocarbons or long chain fatty acids, RM pairs USGS67/USGS69 or USGS70/USGS72, respectively, are ideally matched and can thus serve as appropriate scale anchors (Table 2). For bulk ^{2}H abundance analysis of organic compounds, in particular, solids by TC/EA-IRMS (Thermal Conversion/Elemental Analyser-IRMS), the need for well matched RMs was a major concern up until recently. Thanks to the efforts by Haiping Qi and Tyler Coplen (USGS Reston, VA, USA) scale anchoring primary RMs VSMOW and SLAP2 have become available sealed in cold-welded silver tubes. This makes them thus amenable to be loaded on autosamplers for solids and to be analysed side-by-side with solid samples for both bulk ^{2}H and bulk ^{18}O abundance analysis by TC/EA-IRMS [21]. Recognising the resource constraints put on the daily use of VSMOW2 and SLAP2, secondary reference waters have been developed, also available sealed in silver tubes from USGS Reston, whose δ^2H_{VSMOW} and $\delta^{18}O_{VSMOW}$ values cover the range of the VSMOW/SLAP scale (Tables 2–4).

5. Why appropriate choice of reference materials is crucial

The importance of which pair of RMs is chosen to act as end-points, i.e. scale anchors, cannot be stressed enough. The narrower the δ value range or bracket covered by one's choice of RMs is, the less accurate the resulting normalisation will be of measured δ values outside that bracket (Figure 3). In the example shown in Figure 3 the δ^2H_{VSMOW} values of RMs IAEA-CH-7 and IA-R002 bracket merely a δ^2H value range of 10.9 ‰, i.e. nowhere near

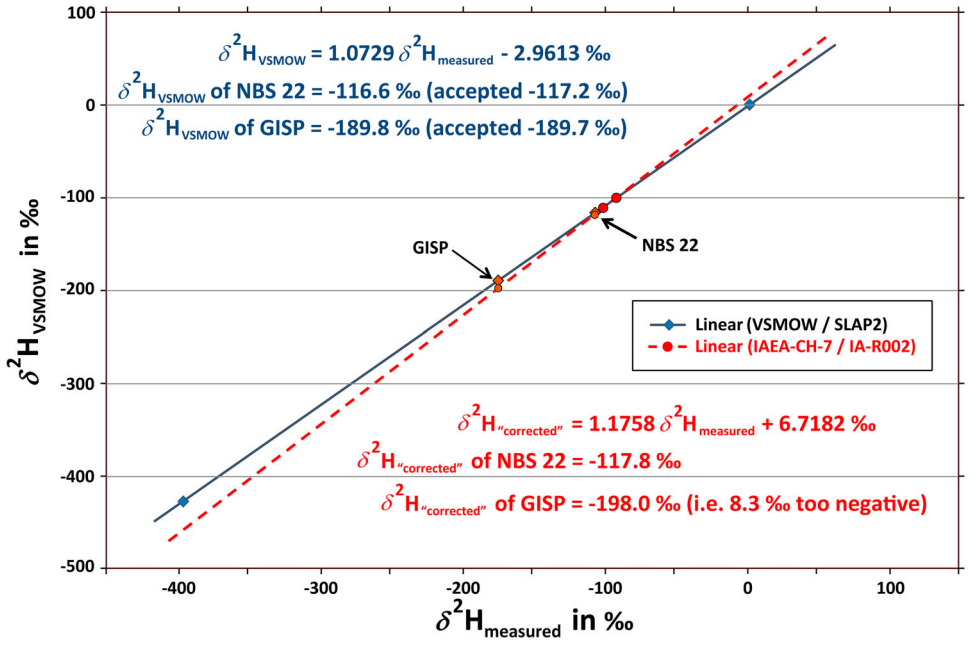

Figure 3. Impact of RM choice as end-points for δ^2H scale normalisation on correction equation and corrected δ^2H values.

the δ^2H value range of 427.5 ‰ covered by VSMOW2 and SLAP2. As a consequence, the linear correction equation based on IAEA-CH-7 and IR-R002 is significantly different from the scale normalisation equation obtained from contemporaneously analysed VSMOW and SLAP2 yielding as it does a 'calibrated' δ^2H value of −198.0 ‰ for GISP that compared to its accepted δ^2H_{VSMOW} value of −189.7 ‰ is 8.3 ‰ too negative.

An analogous impact of too narrow a δ^{13}C value range is illustrated by the example shown in Figure 4(a). Here the δ^{13}C range of 1.38 ‰ covered by RMs IAEA-600 and USGS40 chosen as end-points has a significant impact on the accuracy of 'corrected' δ^{13}C values. The difference of 0.283 ‰ may seem small between the 'not-to-scale-calibrated' δ^{13}C value of −10.733 ‰ for IAEA-CH-6 and its scale-normalised δ^{13}C value of −10.45 ‰. However, based on the acceptance criterion for modern IRMS instruments of 0.06 ‰ for repeatability of δ^{13}C measurements, the difference of 0.283 ‰ of (accepted minus corrected) is 4.72 times higher than this and thus statistically significant.

Considering how close proximity of two end-points results in pronounced differences for slope and off-set between the corresponding linear regression equation and that obtained from using appropriate scale anchors, one can understand why so-called single-point calibrations cannot be and are not fit for purpose (Figure 4(b)). Obviously, a single point is not sufficient to define a linear equation given the slope s of a line is given by the ratio of $(y2 - y1)/(x2 - x1)$. A so-called single-point 'calibration' does most certainly not meet the aforementioned IUPAC requirement of normalising measured δ values to the size of the relevant δ scale. Furthermore, by using merely one RM as a single point of comparison to correct measured δ values one either assumes a slope equal to 1 ($y = x + b$) or an off-set of zero ($y = a \times x$). In case of the former, the off-set 'b' would be determined by

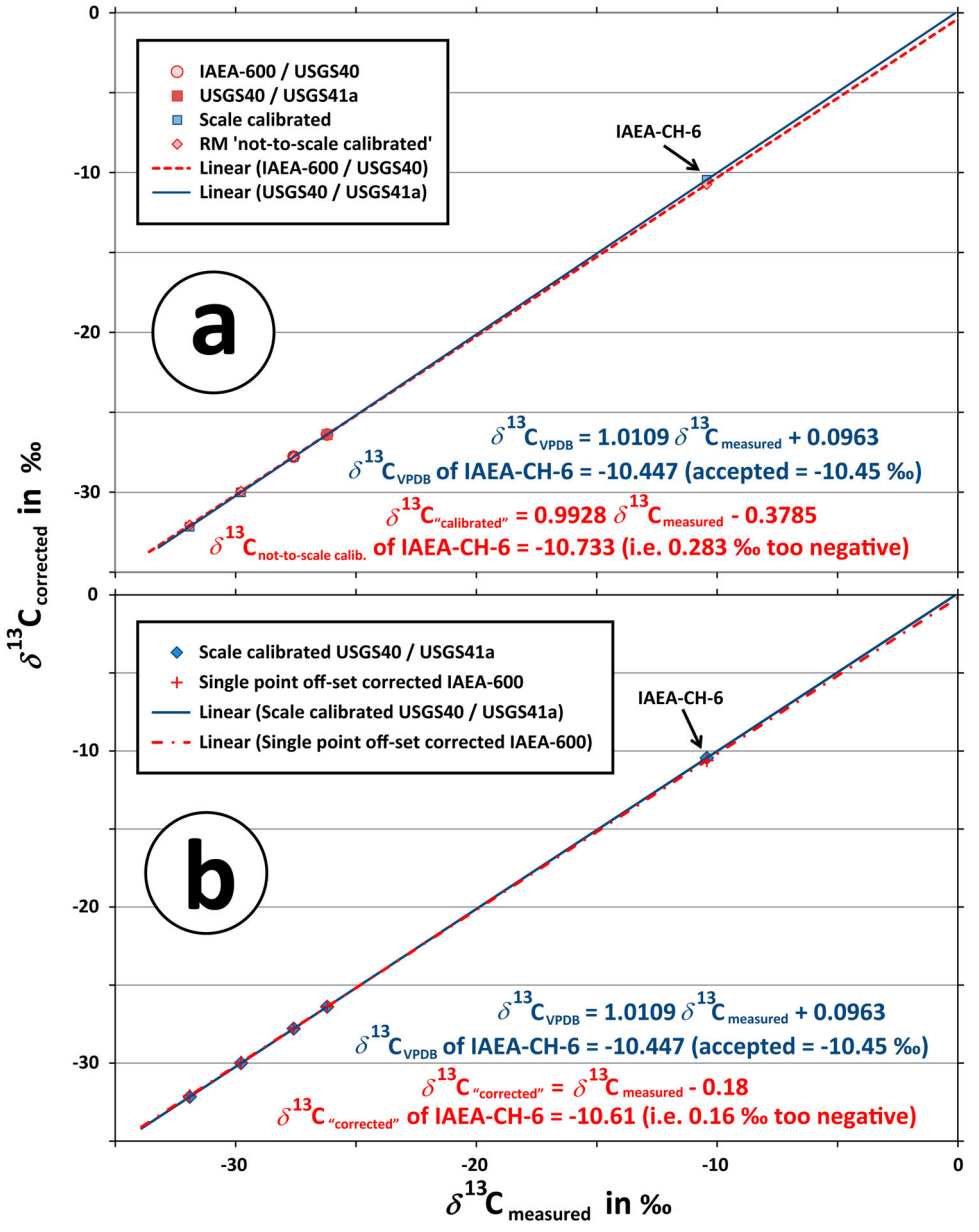

Figure 4. Comparison of impact of RM choice (a) as end-points or (b) as single-point for $\delta^{13}C$ scale normalisation on correction equation and corrected $\delta^{13}C$ values.

($RM_{accepted} - RM_{measured}$). In case of the latter the correction factor '*a*' would be determined by ($RM_{accepted}/RM_{measured}$). However, either approach does not yield accurate, let alone scale-normalised δ values as Figure 4(b) illustrates for the scenario $y = x + b$. While the difference of 0.16 ‰ between single-point corrected and scale-normalised $\delta^{13}C$ value of IAEA-CH-6 seems small, it is still 2.67 times higher than the acceptance criterion of 0.06 ‰ for repeatability of $\delta^{13}C$ measurements.

The examples discussed above illustrate quite clearly the importance of ensuring the two RMs chosen as scale anchors are indeed end-points whose δ values extend to if not match the size of the relevant δ scale. Furthermore, in addition to meeting with this condition, one should also take care when selecting RMs with regards to their physical properties and chemical elemental composition.

Modern on-line interfaces perform chemical conversion into a mixture of simple gases and subsequent chromatographic separation of this gas mixture into individual gas peaks. The combination of these different on-line processes in conjunction with transporting permanent gases of low molecular weight in a carrier gas stream may be subject to potential mass discriminatory effects. Further factors affecting measurement accuracy of δ values are memory effects but also peak detection and peak integration by proprietary instrument software. Adherence to the identical treatment principle of sample and standard can minimise any analytical bias only when samples and RMs are of comparable chemical and elemental compositions, which is termed matrix matching. Attention is drawn to instances when sample matrix effects during combustion or thermolysis do not result in quantitative compound conversion into the desired analyte gas. Non-quantitative sample conversion is subject to mass discriminatory effects. Methods that would result in incomplete sample conversion are therefore unsuitable for the particular application because the analyte gas has to represent the isotopic composition of the sample. Measuring δ^2H values of nitrogen-rich organic compounds is such a case. Yield of H_2 is not quantitative if such compounds are converted in a high temperature reactor comprising an outer ceramic tube and an inner glassy carbon tube filled with glassy carbon chips because of side-reactions producing HCN [22,23]. In such instances, sample conversion methods have to be adapted to achieve quantitative sample conversion [23]. Matrix-match RMs of known true isotopic composition should still be used as scale anchors of course.

For example, stable isotope analysis of ammonia salts or nitrogen-bearing organic compounds for their ^{15}N abundance is one example where the appropriate choice of RMs in terms of matrix matching is extremely important. In ammonium salts or organic amines or amides, nitrogen is present in states of oxidation ranging from –IV to –I. However, RMs USGS32 and USGS34 are both nitrates where nitrogen is present in its highest state of oxidation, namely + V. While in ammonium salts and nitrogen-bearing organic compounds nitrogen will be oxidised to states of oxidation of 0 (N_2) or possibly + II (NO), nitrogen in nitrates cannot be oxidised any further but has to be reduced to + IV (NO_2) or + II (NO). Therefore, organic RMs such as L-valine or caffeine should be chosen as scale anchors to ensure identical treatment during compound conversion of nitrogen-bearing organic compounds such as amino acids or hetero-aromatic compounds, respectively.

Conversely, when analysing nitrate samples for their ^{15}N abundance, it is equally important to use nitrate RMs as scale anchors. This is not only important from a matrix matching point of view but also from a sample conversion point of view. The conversion of nitrates into ultimately N_2 gas relies on thermolysis of $[NO_3]^-$ into NO, NO_2 and O_2, i.e. a reaction that generates oxygen rather than combustion, which consumes and thus requires oxygen. This means the conversion of nitrates in an EA has to be carried out without the otherwise customary pulse of oxygen [24].

Last but not least, attention is drawn to the perhaps confusing situation concerning scale normalisation of $\delta^{18}O$ values. For historic reasons, there are three δ scales in use; $\delta^{18}O_{VSMOW}$, $\delta^{18}O_{VPDB}$ and $\delta^{18}O_{Air\text{-}O2}$. While reports of $\delta^{18}O_{Air\text{-}O2}$ values are few and far between, reporting $\delta^{18}O$ values of carbonates as $\delta^{18}O_{VPDB}$ values is still quite common even though the CIAAW's list of ^{18}O RMs now states $\delta^{18}O_{VSMOW}$ values throughout, including carbonate RMs. It is crucially important to stay with one $\delta^{18}O$ reference scale when reporting and dealing with $\delta^{18}O$ values, and especially when performing calculations involving $\delta^{18}O$ values. This may seem obvious, yet a glance at published articles reveals it is not [25,26]. Subtracting $\delta^{18}O_{VSMOW}$ values from $\delta^{18}O_{VPDB}$ values makes about as much sense as subtracting kJ from kcal or ounces from grams. For meaningful scale normalisation of measured $\delta^{18}O$ values to either the VSMOW or the VPDB scale, accepted $\delta^{18}O$ values of the two RMs chosen as scale anchors must be expressed on the same reference scale. Should instrumental or experimental set-up make it necessary for measurement results of two different sets of samples to be expressed on either of the two reference scales, this should be clearly stated in reports and publications. Furthermore, prior to any calculation involving both data sets, $\delta^{18}O_{VSMOW}$ values have to be converted into $\delta^{18}O_{VPDB}$ values or *vice versa*. The conversion equations originally given by Friedman and O'Neil [27] were:

$$\delta^{18}O_{VSMOW} = 1.03086 \times \delta^{18}O_{VPDB} + 30.86, \tag{4}$$

$$\delta^{18}O_{VPDB} = 0.97006 \times \delta^{18}O_{VSMOW} - 29.94. \tag{5}$$

Please note, in Equations (4) and (5) scale reference points SMOW and PDB in [27] have been amended to read VSMOW and VPDB in line with latest IUPAC guidelines [10].

However, with continuously advancing technology, new studies and resulting new insights, relations such as expressed in Equations (3) and (4) are continuously being refined. Readers are therefore advised to visit e.g. the CIAAW website to check for the latest information [28]. With regards to the relation between $\delta^{18}O_{VSMOW}$ and $\delta^{18}O_{VPDB}$ values the CIAAW web page for oxygen RMs currently states this equation [7,29]:

$$\delta^{18}O_{VPDB} = 0.97001 \times \delta^{18}O_{VSMOW} - 29.99. \tag{6}$$

6. Conclusions

- Most published methods sections describing stable isotopic analytical approaches and resulting δ values reflect shortcomings or even blatant misconceptions with regard to the proper use of RMs, calibration, isotopic scale normalisation, and adherence to SI-rules and recommended IUPAC isotopic nomenclature.
- This paper offers detailed guidance about the mandatory use of isotopic RMs for two end-point scale normalisation and calibrations against common isotopic scales.
- Practical examples of small data sets with various underlying scale normalisation and calibration strategies demonstrate the severity of isotopic artefacts resulting from inappropriately chosen 'end'-points, inadmissible single-point calibration and violations of the principle of identical treatment of sample and standard.

- We offer a list of prominent suppliers of light stable isotope RMs to best match sample matrices encountered by stable isotope practitioners with chemically similar RMs to adhere to the identical treatment principle of sample and standard (Table 4).
- Proper use of RMs and reporting of δ values fosters interdisciplinary communication while minimising ambiguity and reducing the jargon of scientific sub-disciplines.

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