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Calibration of Isotope Ratio Mass Spectrometry Working Standard for $^2H/^1H$ Ratio Analysis

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A method is described for the calibration of an isotope ratio mass spectrometry working standard for $^2H/^1H$ analysis using highly purified (>99.99% enriched) deuterium oxide (^2H_2O). Serial dilution of such a compound can be used to construct a standard curve for calibration purposes using the relationship

$$APE/100 = \frac{\delta D(\mathrm{SA})_{\mathrm{WR}} - \delta D(\mathrm{BKG})_{\mathrm{WR}}}{1000} \times r_{\mathrm{WR}}\,.$$

where *APE* is the atom percent excess. It was found that the determined r_{WR} had a precision of ±1%. Most of the variation came from sample handling, which includes the serial dilution of the weighed deuterium oxide, the reduction of water to hydrogen gas and the introduction of the hydrogen gas into the mass spectrometer. The use of this calibration procedure allows the direct determination of the isotope ratio r_{SA}, and APE from a standard curve over a wide range of enrichment. Unlike the reference standards V-SMOW and SLAP, the highly purified deuterium oxide is non-exhaustible and readily available. The highly purified deuterium oxide should be used in place of secondary standards to calibrate working references for $^2H/^1H$ analysis in biological studies. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: isotope ratio mass spectrometry; calibration; working standard; $^2H/^1H$ ratio

INTRODUCTION

Isotope ratio mass spectrometry (IRMS) is increasingly used for the assay of ^{18}O, ^2H and ^{13}C enrichment in metabolic studies with stable isotope tracers.[1–3] Traditionally, enrichment determined by IRMS is expressed as delta (δ) or del. This is necessitated by the fact that isotope ratios of the sample (r_{SA}) and the working standard or working reference (these terms are used interchangeably in this paper) (r_{WR}) are the primary measurements in IRMS. (A double-inlet instrument measures enrichment as incremental isotope ratio, which is different in concept from the isotope ratio ($^2H/^1H$) of the single inlet instrument; δ is the incremental isotope ratio multiplied by 1000 out of convenience as a means to express small differences in highly precise isotope ratios.) Delta (δ), a measure of incremental enrichment, is calculated using these ratios and the equation

$$\delta\ (\text{per mil}) = \frac{r_{\mathrm{SA}} - r_{\mathrm{WR}}}{r_{\mathrm{WR}}} \times 1000‰ \qquad (1)$$

For the purpose of standardized reporting, r_{WR} is first determined or ‘calibrated’ such that the results can be converted to delta against Standard Mean Ocean Water (SMOW). However, the common units of measure in tracer dilution studies are the tracer/tracee ratio ($R = r_{\mathrm{SA}} - r_{\mathrm{BKG}}$) and atom percent excess (*APE*). These are related to δ by the following equations:[4]

$$r_{\mathrm{SA}} = \left(\frac{\delta}{1000} + 1\right) \times r_{\mathrm{WR}} \qquad (2)$$

$$APE = \frac{\delta r_{\mathrm{WR}}}{(\delta r_{\mathrm{WR}} + 1000)} \times 100 \qquad (3)$$

It can be seen from Eqns (2) and (3) that the accurate determination of r_{WR} is critical for the accurate determination of enrichment of stable isotope tracers. The propagation of error in the estimation of r_{WR} potentially results in significant under- or over-estimation of the true enrichment.

Customarily, the isotope ratio of the working reference (WR) is calibrated against one or more reference standards (available from NIST, Gaithersburg, MD, USA) such as Vienna Standard Mean Ocean Water (V-SMOW) or Standard Light Antarctic Precipitation (SLAP). More often, the working reference is calibrated against secondary standards. The accuracy of such a calibration procedure depends on the accuracy of the reference standards and the precision of the IRMS analysis. If the accuracy of one of the reference standards is in doubt, the traditional method of calibrating the working reference cannot be used to determine which secondary standard has the correct value.

Recent advances in separation technology have produced highly purified (>99.99% enriched) labeled compounds such as deuterium oxide (^2H_2O) and sodium [^{13}C]carbonate. Serial dilution of such a compound can

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be used to construct a standard curve for calibration purposes. In this paper, we examine the effects of an error in the calibration on the determined values of δ and r_{SA}. A method of calibrating the working reference using samples prepared with ^2H_2O of known enrichment is described. An error analysis of the method is also presented.

EXPERIMENTAL

We prepared three identical series of standard solutions of ^2H_2O with known molarity by diluting a highly enriched deuterium oxide (D = 99.9%, Cambridge Isotope, DLM-4 Lot PSO GG-253). Deuterium oxide contained in 1 ml was first weighed and distilled water (^1H_2O) was added to a total volume of 10 ml. The $^2H/^1H$ ratio of the calibration sample is given by

$$\frac{D}{H} = \frac{aA/M_A + (1-b)B/M_B}{bB/M_B + (1-a)A/M_A}$$

where a, A and M_A are the fractional water content, its mass and molecular mass of heavy water respectively,[5] and b, B and M_B are the fractional water content, its mass and molecular mass of light water. *APE* is defined as the difference between $^2H/^1H$ of the standard solution and $^2H/^1H$ of the distilled water (since the mass of deuterium oxide is very small compared with light water in these standard solutions, $APE = (aA/M_A)(bB/M_B)$; in our case, a and b are $>99.9\%$). Serial dilution of the deuterium oxide with distilled water gave solutions with *APE* ranging from 0.0050 to 0.0400%. The deuterium enrichment of each solution was analyzed as the corresponding amount of hydrogen gas produced on reduction by zinc, and expressed as δ against a working standard (δD_{WR}) using a Finnegan Delta-S IRMS system. Briefly, the procedure for determining δD_{WR} is as follows. The reference gas (Oztech Trading, Dallas, TX, USA) was introduced into both the reference and sample chambers to a final pressure between 40 and 60 mbar for determination of the H3 factor (the fraction of H^{3+} ion formed at the given pressure of hydrogen gas). Then the sample chamber was evacuated and the hydrogen generated from the water sample was introduced. The gas pressure in both chambers was again equalized. The reference and the sample gases were introduced alternately into the ionizing chamber, and several δD_{WR} values were determined for each sample. The working reference was purchased from Oztech Trading and had a calibrated $\delta D_{SMOW} = -95.68$. From Eqns (1)–(3), it can be derived that

$$r_{SA} - r_{BKG} = \frac{\delta D(SA)_{WR} - \delta D(BKG)_{WR}}{1000} \times r_{WR} \qquad (4)$$

Since r_{BKG} is $\ll 1$, $(r_{SA} - r_{BKG}) \times 100$ is essentially *APE*. Therefore,

$$APE/100 = \frac{\delta D(SA)_{WR} - \delta D(BKG)_{WR}}{1000} \times r_{WR} \qquad (5)$$

and r_{WR} can be determined from the slope of the linear plot of $APE/100$ against $[\delta D(SA)_{WR} - \delta D(BKG)_{WR}]/1000$.

RESULTS

To examine the effect of an error in calibrating r_{WR} on the estimation of enrichment as *APE*, δD_{SMOW} and r_{SA}, we first calculated the theoretical δD_{WR} values of seven hypothetical hydrogen samples with $^2H/^1H$ ratios ranging from 0.000 12 to 0.000 45 and two reference gases as if the samples were analyzed by IRMS against a working standard with $r_{WR} = 0.000\,165$ (columns 2 and 3 in Table 1). The corresponding correct values of δ versus V-SMOW, APE and δD_{SMOW} were calculated from r_{WR} and δD_{WR} (columns 4 and 5 in Table 1). The calculations were then repeated using a new r_{WR} with a 3% error introduced (by setting r_{WR} equal to 0.000 17 instead of the 'correct' value). This is the same as assigning a δD_{SMOW} of 89.74 to the working standard instead

Table 1. Effects of an error in $^2H/^1H$ ratio of the working standard on the estimation of isotope enrichment *APE*, δD_{SMOW} or r_{SA}

		Calculated using 'correct' r_{WR}			Calculated using 'incorrect' r_{WR}				
Sample No.	r_{SA}	δD_{WR} [b]	*APE* [a]	δD_{SMOW} [b]	δD_{SMOW} [c]	Error (%) [d]	*APE* [c]	r_{SA} [c]	Error (%)
1	0.00012	−272.73	−0.0045	−230.77	−207.46	10.10	−0.0046	0.0001236	3.03
2	0.00014	−151.52	−0.0025	−102.56	−75.37	26.52	−0.0026	0.0001442	3.03
3	0.00016	−30.30	−0.0005	25.64	56.72	121.21	−0.00052	0.0001648	3.03
4	0.00017	30.30	0.0005	89.74	122.77	36.81	0.00052	0.0001752	3.03
5	0.00018	90.91	0.0015	153.85	188.81	22.73	0.00154	0.0001855	3.03
6	0.000225	363.64	0.0060	442.31	486.01	9.88	0.0062	0.0002318	3.03
7	0.00045	1727.27	0.0285	1884.62	1972.03	4.64	0.0294	0.0004636	3.03
SMOW	0.000156	−54.55	−0.0009	0	30.30303	— [e]	−0.00093	0.0001607	3.03
WRSTD	0.000165	0	0	57.69	89.74	55.5	0	0.00017	3.03

[a] *APE* is atom percent in excess of WR. It was calculated using Eqn (3).

[b] δD_{SMOW} and δD_{WR} are calculated from r_{SA} using r^5_{SMOW} of 0.000 156 and r_{WR} of 0.000 165.

[c] When there is an error in the calibration of the working standard, δD_{WR} will remain unchanged. However, δD_{SMOW}, *APE* and r_{SA} calculated from δD_{WR} using the wrong r_{WR} will be in error. In this example, δD_{SMOW}, *APE* and r_{SA} were calculated assuming r_{WR} to be 0.000 17 instead of its true value (0.000 165).

[d] Error (%) was calculated from the absolute difference between δD_{SMOW}, *APE* and r_{SA} and their respective correct values using the correct values as 100%.

[e] Indeterminate, value divided by zero.

of the correct value of 57.69. This error is reflected in the calculation of δD_{SMOW}, APE and r_{SA} as shown in columns 6, 8 and 9 in Table 1. When there is an error in the calibration of the working standard, δD_{WR} will remain unchanged. However, δD_{SMOW}, APE and r_{SA} calculated from δD_{WR} using the wrong r_{WR} will be in error. As predicted by Eqn (2), the APE and r_{SA} are consistently off by 3.03%. However, the error in the estimation of δD_{SMOW} ranges from 4.6% to 121%, inversely related to the magnitude of δD_{SMOW} as suggested by Eqn (1).

The results in Table 1 illustrate that if the accuracy of one of the reference standards is in doubt, the traditional method of calibrating the working standard cannot discriminate the correct from the incorrect reference standards. When an incorrect δD_{SMOW} is assigned to the reference working standard, the analysis of the correct reference standards gives incorrect values of $\delta D(\mathrm{SA})_{\mathrm{SMOW}}$, as illustrated by the deviation of $\delta D(\mathrm{SA})_{\mathrm{SMOW}}$ from the true values in the last two rows of Table 1. Such a disagreement can be frustrating to resolve.

The δD_{WR} values of the standard solutions prepared from serial dilution of deuterium oxide are given in Table 2. The percentage variation of APE attributed to weighing was about 0.12%, whereas that in δD_{WR} ranged from 1.2 to 2.9%. Regression of APE/100 against $[\delta D(\mathrm{SA})_{\mathrm{WR}} - \delta D(\mathrm{BKG})_{\mathrm{WR}}]/1000$ yielded a straight line with a slope of $(158 \pm 1.4) \times 10^{-6}$ r_{WR} was determined from the slope by regression analysis to be $0.000\,158 \pm 0.000\,001\,4$. Therefore, the working reference has a δD_{SMOW} of 12.8, which is substantially different from the manufacturer's calibration.

The procedure for preparing the standard curve involves several independent steps: weighing of the deuterium oxide, serial dilutions using volumetric pipettes and volumetric flasks, zinc reduction in sealed vacuum tubes, introduction of the gaseous samples and mass spectrometric analysis. For these independent processes, the variance of the entire procedure is equal to the sum of variances of each individual process. The overall standard deviation of r_{WR} is about 1% of its determined value. The mass of 1 ml of deuterium oxide was determined to be 1.1117 ± 0.0015 g, with a coefficient of variation of 0.14%. The standard deviation for repeated IRMS analyses of the same sample was of the order of 0.5‰, which is <0.1%. We also investigated the variation due to zinc reduction and sample handling by performing IRMS analyses on several samples from the same solution, and found the standard deviation to be of the order of 1%. Therefore, the major part of the variance of the standard curve procedure comes from the sample preparation of serially diluted solutions and IRMS analysis. Since the purity of the deuterium oxide was previously determined by NMR to be 99.9%, the manufacturer's calibration must be erroneous since its falls outside of the expected range of error.

Table 2. Observed δD_{WR} of the standard solutions prepared from serial dilution of deuterium oxide

APE	SD[a]	δD_{WR}	SD[b]
0.04002	4.92×10^{-5}	2515.04	74.51
0.02001	2.46×10^{-5}	1255.18	18.95
0.01001	1.23×10^{-5}	621.19	7.75
0.0005003	6.15×10^{-6}	304.07	4.85

[a] *APE* and its associated standard deviation (SD) were determined from the average mass of the three deuterium oxided samples used ($n = 3$).

[b] δD_{WR} and its standard deviation (SD) were determined from duplicate analyses of each of the dilutions from three serial dilutions ($n = 6$).

DISCUSSION

The accuracy of enrichment determination by IRMS depends heavily on the calibration of the working standard. An error in the calibration of the working standard results in an error in δD_{SMOW} which is inversely related to the enrichment, whereas the same error results in a constant error in the isotope ratio r_{SA}. Therefore, discrepancies in reported δD_{SMOW} are common in experimental studies using low deuterium enrichment.[6] Such errors can be avoided by running the sample analyses along with SMOW and SLAP. When two known reference standards are used, the enrichment of the sample can be normalized as follows:

$$\delta D(\mathrm{SA})_{\mathrm{SMOW}} = \frac{\delta D(\mathrm{SA})_{\mathrm{WR}} - \delta D(\mathrm{SMOW})_{\mathrm{WR}}}{\delta D(\mathrm{SLAP})_{\mathrm{WR}} - \delta D(\mathrm{SMOW})_{\mathrm{WR}}} \times \delta D(\mathrm{SLAP})_{\mathrm{SMOW}} \quad (6)$$

The deltas against the working standard (WR) of the sample $[\delta D(\mathrm{SA})_{\mathrm{WR}}]$, SMOW $[\delta D(\mathrm{SMOW})_{\mathrm{WR}}]$ and SLAP $[\delta D(\mathrm{SLAP})_{\mathrm{WR}}]$ are first determined. The ratio of the differences in deltas is then multiplied by a constant factor (delta of SLAP against SMOW) to obtain the adjusted or normalized delta versus SMOW of the sample.[5,7] This method of determining $\delta D(\mathrm{SA})_{\mathrm{SMOW}}$ is independent of the knowledge of $\delta D(\mathrm{WR})_{\mathrm{SMOW}}$ or r_{WR}. There is no need to calibrate the working reference. However, the error in each of these measurements is propagated into the calculation of the final enrichment. The variance of such a measurement is the sum of variances of four separate determinations as shown in Eqn (6), and is therefore larger than that of a single measurement [the propagation of error can be seen by obtaining the differential equation of Eqn (6) for $\delta D(\mathrm{SA})_{\mathrm{SMOW}}$; the error in $\delta D(\mathrm{SA})_{\mathrm{SMOW}}$ is the sum of terms containing any errors in $\delta D(\mathrm{SA})_{\mathrm{WR}}$, $\delta D(\mathrm{SMOW})_{\mathrm{WR}}$ and $\delta D(\mathrm{SLAP})_{\mathrm{WR}}$]. Since primary standards are non-replenishable resources and can be exhausted, secondary standards are often used in practice. When secondary standards are used in place of SLAP and SMOW, the error in calibrating the working reference is dependent on the error in the calibration of the secondary standards and the calibration of the working standard. In many instances, the true error is unknown. Another approach in tracer studies which can avoid the need for calibration of the working reference is to report tracer incorporation in enrichment relative to the enrichment of the tracer dose as determined by the same IRMS using the same working reference.[8] This approach does not permit easy comparison of results from different laboratories.

In addition to the above two approaches, the method presented in this paper of calibrating isotope ratio reference gas for the determination of $^2H/^1H$ ratio using 99.9% enriched deuterium oxide should be a useful alternative. The method is similar to the approach used by de Wit *et al.*[5] in their determination of the $^2H/^1H$ ratio of V-SMOW and SLAP using known mixtures of pure ^1H_2O and ^2H_2O. This method has the advantage over the traditional calibration that a full range of enrichment is used, and the range of linear response by the instrument can be assessed. For biologists who are not interested in the use of δD_{SMOW}, this method allows the direct determination of r_{SA} and APE from the standard curve. The method can also be adapted for calibration of CO_2 gas for $^{13}C/^{12}C$ and $^{18}O/^{16}O$ analyses. Unlike V-SMOW and SLAP, the highly purified deuterium oxide is non-exhaustible and readily available. The highly purified deuterium oxide should be used in place of secondary standards to calibrate working references for $^2H/^1H$ analysis in biological studies.

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