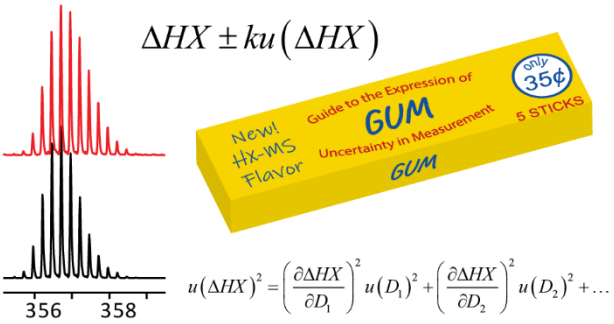
Recommendations for the propagation of uncertainty in hydrogen exchange-mass spectrometric (HX-MS) measurements

David D. Weis

Invited contribution to Michael Gross Special Focus Issue

Department of Chemistry
The University of Kansas
1567 Irving Hill Rd.
Lawrence, KS 66045 USA
dweis@ku.edu
+1-785-864-1377
ORCID ID: 0000-0003-3032-1211



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Abstract

Hydrogen exchange-mass spectrometry (HX-MS) is used widely to characterize higher-order protein structure and to locate changes in protein structure and dynamics that accompany, for example, ligand binding and protein-protein interactions. Quantitative differences in the amount of hydrogen exchange between two states (*i.e.,* differential HX) are taken as evidence of significant differences in higher-order structure or dynamics. The quantitative measures range from simple mass differences at one HX labeling time to differences averaged across an HX time course with correction for deuterium recovery. This work applies the principles of uncertainty propagation to differential HX measurements to facilitate the identification of significant differences. Furthermore, it is shown that pooled estimates of experimental uncertainty result in a lower false positive rate than estimates of uncertainty based on individual standard deviations.

INTRODUCTION

With improvements in automation of HX-MS measurements over the past decade, there has been a substantial increase in the use of differential HX to quantitatively identify changes in HX between two protein states. The improved precision has led many to consider issues related to the limits of detection. Accurate propagation of experimental uncertainty is essential for a rigorous consideration of the decision "when is a difference real?"[1, 2]

In the first part of this work, the principles of uncertainty propagation [3, 4] are applied to all of the different ways in which differential HX can be quantified. These different ways include taking differences at individual HX labeling times and approaches where differences across all HX labeling times are accumulated (*e.g.*, sum of differences, arithmetic mean of differences). In addition, cases with and without back-exchange correction are also treated. It is assumed in this treatment that the measurement uncertainties, u, in the centroid masses of the peptides are random and uncorrelated. The second part of this work is concerned with whether individual uncertainties or a pooled estimate of uncertainty is more accurate. Example of differential HX-MS calculations and their associated uncertainties are provided in the final part of this work.

A few definitions are needed to initially establish the derivations. Let the labels *A* and *B* denote two samples that are the subject of differential HX-MS measurements. The peptide of interest has N_{H} exchangeable amides hydrogen atoms. *m* denotes the measured centroid mass of a peptide. D_t denotes the amount of deuteration measured in a peptide at HX exchange time *t*

$$D_t = m_t - m_0 \tag{1}$$

where the subscript zero denotes the mass of the undeuterated peptide. Typically the peptide mass is measured in *n* replicates,[5] so the following treatment assumes that the arithmetic mean of the replicates is used as the measured quantity represented by m_t. The standard uncertainty in the centroid mass, represented as $u(m)$, may be estimated based on the standard deviation of the mean, $s(m)$:

$$u(m) = \frac{s(m)}{\sqrt{n}} = \sqrt{\frac{\sum_{i=1}^{n}(m_i - \bar{m})^2}{(n-1)n}} \quad (2)$$

where the index i increments over replicate measurements of the peptide mass and the overbar notation denotes the arithmetic mean of replicate measurements. Table 1 summarizes this work by setting forth various expressions of differential HX and their associated combined uncertainties in a standardized notation. Derivations of these quantities follow. (In the treatment that follows, the notations $s(x)$ and $u(x)$ denote, respectively, the standard deviation in the mean of x and the standard uncertainty in the mean of x, and thus, $s(x)^2$ and $u(x)^2$ should be understood as the squares of those quantities.)

Following modern recommendations on the use and reporting of measurement uncertainties,[3, 4] traditional confidence intervals (*e.g.,* based upon a *t* statistic) are replaced by an expanded uncertainty that may be obtained by taking the combined uncertainty in differential HX measurements, u, multiplied by a coverage factor, *k*:

$$U = ku \quad (3)$$

In many areas of chemical analysis the coverage factor is taken as $k = 2$, however the choice of a coverage factor to test significance in a particular context will depend on the number of measurements. In differential HX-MS measurements, the null hypothesis is an HX difference of zero, thus an HX difference would be deemed significant when its absolute value exceeds ku. In other words, when zero is not within the confidence interval $\pm U$. Null differential HX-MS measurements (i.e., the sample compared to itself) may be useful in defining a suitable value for the coverage factor since, by definition, all measured HX differences arise from random errors in the measurements. A thorough consideration of the selection of an appropriate coverage factor, however, is outside the scope of the present work. The coverage factor used should be reported when the uncertainty is reported in published work.[6]

PROPAGATION OF UNCERTAINTY IN DIFFERENTIAL HX-MS

Case 1: Individual HX differences

The fundamental unit of differential HX-MS measurement of a peptide at labeling time *t* is

$$\Delta D_t = D_{A_t} - D_{B_t} = \left(\bar{m}_{A_t} - m_0\right) - \left(\bar{m}_{B_t} - m_0\right) = \bar{m}_{A_t} - \bar{m}_{B_t} \quad (4)$$

Note that inclusion of the mass of the undeuterated peptide, m_0 is unnecessary since it cancels out. Furthermore, inclusion of independent measurements of m_0 in *A* and *B* unnecessarily introduces additional uncertainty into ΔD_t. Hence it is recommended that differential HX should be calculated without reference to the mass of the undeuterated molecule.

Uncorrelated random uncertainty in (4) propagates as

$$u\left(\Delta D_t\right)=\sqrt{u\left(m_{A_t}\right)^2+u\left(m_{B_t}\right)^2} \tag{5}$$

where $u\left(m_{A_t}\right)$ and $u\left(m_{B_t}\right)$ denote the standard uncertainties as defined in (2). (Throughout this work, parentheses in equations are used only to denote either order of operation or to label a variable, parentheses are not used as a shorthand for multiplication.)

Fractional HX is represented here as *HX*. The simplest example is representing the amount of HX as a fraction of the number of exchanging amides in the peptide, N_{H}:

$$\Delta HX_t=\frac{\Delta D_t}{N_{\mathrm{H}} m_\delta} \tag{6}$$

Here, the mass m_δ (1.0063 Da), the difference between the atomic masses of deuterium and protium, serves simply to render ΔHX as a truly dimensionless quantity. With current HX-MS technology, the uncertainty in m_δ (approximately ±0.0003 Da) will be negligible compared to measurement uncertainty in centroid mass (approximately ±0.1 Da) and can be safely dispensed with by taking $m_\delta \approx 1$ Da. Since the number of exchangeable amides in a peptide is an exact number, it has no uncertainty associated with it. Thus the combined uncertainty is simply scaled by the mass of the exchangeable amide hydrogens:

$$u\left(\Delta HX_t\right)=\frac{u\left(\Delta D_t\right)}{N_{\mathrm{H}} m_\delta} \tag{7}$$

Similarly, changing to a percentage scale would require multiplication of both ΔHX_t and $u\left(\Delta HX_t\right)$ by 100%.

Case 2: Accumulated HX difference

In some cases, the accumulated HX difference, summed across all HX labeling times, $t \in \mathrm{T}$ where T denotes the set of labeling times. The number of distinct HX labeling times, $n_{\mathrm{T}}=\left|\mathrm{T}\right|$ (*i.e.,* the cardinality of set T).

$$\Delta D_\Sigma=\sum_{t\in \mathrm{T}} \Delta D_t \tag{8}$$

Here the subscript Σ is added to denote accumulation across HX labeling times. (The summation in (8) is formed from the mean values of replicates.) Following the method for propagation of random uncertainty in sums and differences yields

$$u\left(\Delta D_{\Sigma}\right)=\sqrt{\sum_{t\in \mathrm{T}}\left(u\left(m_{A_t}\right)^2+u\left(m_{B_t}\right)^2\right)} \tag{9}$$

In some cases, the arithmetic mean of ΔD_{Σ} is reported [7]

$$\Delta\overline{D}=\frac{\Delta D_{\Sigma}}{n_{\mathrm{T}}}=\frac{\sum_{t\in \mathrm{T}}\Delta D_t}{n_{\mathrm{T}}} \tag{10}$$

This quantity can be described either as the 'mean difference' or the 'difference of the means'.

Since the number of HX labeling times, n_{T}, is an exact quantity, the uncertainty in (9) is simply scaled by n_{T}:

$$u\left(\Delta\overline{D}\right)=\frac{u\left(\Delta D_{\Sigma}\right)}{n_{\mathrm{T}}}=\frac{\sqrt{\sum_{t\in \mathrm{T}}\left(u\left(m_{A_t}\right)^2+u\left(m_{B_t}\right)^2\right)}}{n_{\mathrm{T}}} \tag{11}$$

Accumulated HX differences can also be expressed as a fraction of the of exchangeable amides:

$$\Delta\overline{HX}=\frac{\Delta D_{\Sigma}}{n_{\mathrm{T}}Nm_{\delta}}=\frac{\sum_{t\in \mathrm{T}}\Delta D_t}{n_{\mathrm{T}}Nm_{\delta}} \tag{12}$$

which again just scales the propagated uncertainty in (9)

$$u\left(\Delta\overline{HX}\right)=\frac{u\left(\Delta D_{\Sigma}\right)}{n_{\mathrm{T}}N_{\mathrm{H}}m_{\delta}}=\frac{\sqrt{\sum_{t\in \mathrm{T}}\left(u\left(m_{A_t}\right)^2+u\left(m_{B_t}\right)^2\right)}}{n_{\mathrm{T}}N_{\mathrm{H}}m_{\delta}} \tag{13}$$

Case 3: Individual HX difference with measured deuterium recovery correction

Introduction of an experimental determination of the deuterium recovery (*i.e.,* after correction for back-exchange) [8] through the measurement of a deuteration control introduces an additional source of uncertainty. Here, it is assumed that the mass of a deuterated form of the peptide (e.g., a deuteration control) has been determined, m_{∞}, such that

$$D_{\infty}=m_{\infty}-m_0 \tag{14}$$

with an associated standard uncertainty of $u\left(D_{\infty}\right)$. (To avoid additional sources of error, it is recommended that m_0 be based on the theoretical average mass of the peptide as in (4), however,

if there is a systematic bias in centroid mass determination,[9, 10, 11] it may be necessary to account for such bias.) D_∞ can then be used to adjust the HX difference in various ways such as fractional HX difference:

$$\Delta HX_t^* = \frac{\Delta D_t}{D_\infty} \tag{15}$$

where the asterisk is appended to denote correction for measured deuterium recovery. Propagation of random uncertainty in this case (see derivation in the Appendix) results in

$$\begin{aligned} u\left(\Delta HX_t^*\right) &= \frac{1}{D_\infty}\sqrt{u\left(m_{A_t}\right)^2 + u\left(m_{B_t}\right)^2 + \left(u\left(D_\infty\right)\frac{\Delta D_t}{D_\infty}\right)^2} \\ &= \frac{1}{D_\infty}\sqrt{u\left(m_{A_t}\right)^2 + u\left(m_{B_t}\right)^2 + \left(u\left(D_\infty\right)\Delta HX_t^*\right)^2} \end{aligned} \tag{16}$$

Equations (15) and (16) can be expressed as percentages by simply multiplying both quantities by 100%.

In some cases, it is preferred to apply a back-exchange correction in order to obtain mass units, or equivalently, number of exchanged amides:

$$\Delta D_t^* = \frac{\Delta D_t}{D_\infty} N_{\mathrm{H}} m_\delta \tag{17}$$

Since this merely scales the uncertainty by $N_{\mathrm{H}} m_{\mathrm{H}}$, the uncertainty becomes

$$u\left(\Delta D_t^*\right) = \frac{N_{\mathrm{H}} m_\delta}{D_\infty}\sqrt{u\left(m_{A_t}\right)^2 + u\left(m_{B_t}\right)^2 + \left(u\left(D_\infty\right)\Delta D_t^*\right)^2} \tag{18}$$

Case 4: Accumulated HX difference with measured deuterium recovery correction

This case simply uses the principles established under cases 2 and 3. The simplest instance is summed differences corrected for back-exchange:

$$\Delta D_\Sigma^* = \frac{\Delta D_\Sigma}{D_\infty} N_{\mathrm{H}} m_\delta = \frac{\sum_{t\in \mathrm{T}} \Delta D_t}{D_\infty} N_{\mathrm{H}} m_\delta \tag{19}$$

In this case, the propagated uncertainty is a simple extension of (18)

$$u\left(\Delta D_\Sigma^*\right) = \frac{N_{\mathrm{H}} m_\delta}{D_\infty}\sqrt{\sum_{t\in \mathrm{T}}\left(u\left(m_{A_t}\right)^2 + u\left(m_{B_t}\right)^2\right) + \left(u\left(D_\infty\right)\Delta D_\Sigma^*\right)^2} \tag{20}$$

The mean deuteration difference can also be corrected for back-exchange:

$$\Delta\bar{D}^{*} = \left(\frac{\sum_{t\in \mathrm{T}} \Delta D_t}{n_{\mathrm{T}}} \right) \frac{N_{\mathrm{H}} m_{\delta}}{D_{\infty}} \tag{21}$$

It is apparent that the propagated uncertainty is merely (20) scaled by n_{T}.

$$u\left(\Delta\bar{D}^{*}\right) = \frac{N_{\mathrm{H}} m_{\delta}}{n_{\mathrm{T}} D_{\infty}} \sqrt{\sum_{t\in \mathrm{T}} \left(u\left(m_{A_t}\right)^2 + u\left(m_{B_t}\right)^2 \right) + \left(u\left(D_{\infty}\right) \Delta\bar{D}^{*} \right)^2} \tag{22}$$

Finally, the mean fractional HX can also be corrected for back-exchange:

$$\Delta\overline{HX}^{*} = \frac{\sum_{t\in \mathrm{T}} \Delta D_t}{n_{\mathrm{T}} D_{\infty}} \tag{23}$$

For which the propagated uncertainty is

$$u\left(\Delta\overline{HX}^{*}\right) = \frac{1}{n_{\mathrm{T}} D_{\infty}} \sqrt{\sum_{t\in \mathrm{T}} \left(u\left(m_{A_t}\right)^2 + u\left(m_{B_t}\right)^2 \right) + \left(u\left(D_{\infty}\right) \Delta\overline{HX}^{*} \right)^2} \tag{24}$$

Expression of $\Delta\overline{HX}^{*}$ as a percentage simply requires multiplying (23) and (24) by 100%.

ESTIMATION OF UNCERTAINTY IN HX-MS MEASUREMENTS.

Having established how uncertainty propagates in all of the common scenarios for expression of differential HX-MS measurements, we can now turn to approaches that can be taken to estimate uncertainty in centroid measurements. Replicated measurements provide two benefits: (i) the mean value of the measurements is more accurate and (ii) the variation in the replicated measurements provides an estimate of the uncertainty. What is atypical, from an analytical chemistry perspective, about HX-MS data is that the data set is usually composed of hundreds to thousands of replicated measurements, each one with a small number of replicates, typically three. Uncertainty can be estimated from the standard deviation of the measurand based on the small number of replicate measurements as described by equation (2). However, estimates of standard deviation based on triplicate measurements are notoriously unreliable. Figure 1 illustrates this point by showing the distribution of standard deviations obtained from 2000 triplicate measurements, simulated using a random data following a gaussian distribution.

Previously, my colleague and I have argued that pooling the standard deviations of all peptide centroids is a more accurate estimate of the uncertainty in centroid determination:[2, 12, 13]

$$s_{\mathrm{p}} = \sqrt{\frac{\sum_{j}\left(n_j - 1\right)s_j^2}{\sum_{j}\left(n_j - 1\right)}} \tag{25}$$

where the index *j* runs over all samples, all peptides, and all labeling times and the subscript p has been added to denote that the standard deviation is based upon a pooled estimate. The pooled standard deviation is depicted by the red vertical line in Figure 1, and is very close to the true value of the uncertainty (1.009 vs. 1). The standard pooled uncertainty is then simply

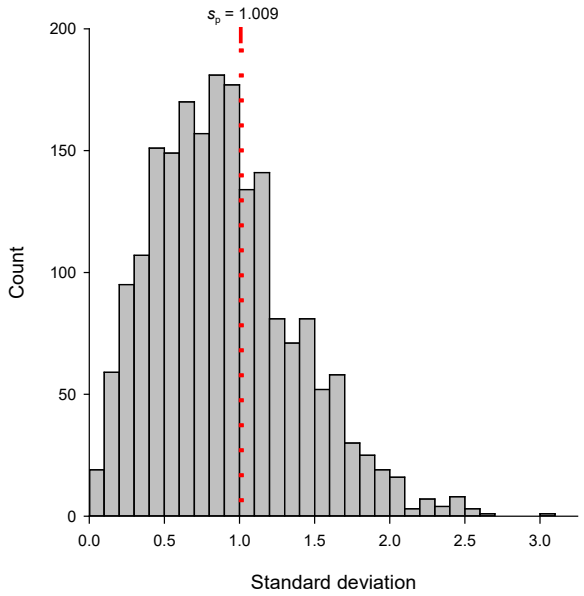

Figure 1. Standard deviations from 2000 sets of triplicate random numbers drawn from a randomized gaussian distribution with a population mean of 0 and a population standard deviation of 1. The red reference line denotes the pooled standard deviation (s_{p}). Numbers were generated using Gaussian Random Number Generator hosted on random.org.

$$u_{\mathrm{p},j} = \frac{s_{\mathrm{p}}}{\sqrt{n_j}} \tag{26}$$

Replacement of the individual uncertainties in each replicated centroid determination with a global estimated uncertainty simplifies the combined uncertainty determination, especially if the same number of replicates was used in all centroid measurements. This is advantageous because calculation of the combined uncertainty equations, particularly in cases 3 and 4, is cumbersome.

Tables 2 and 3 recast the combined uncertainties in Table 1, replacing individual standard deviations with a pooled estimate of uncertainty in cases with and without identical numbers of replicates in all centroid determinations. Table 3 represents the simplest case, but only applies for equal numbers of replicates across the entire data set.

Beyond the convenience, estimating uncertainty by pooling standard deviations is more accurate. Equation (3) defines a significance limit or confidence interval: HX differences larger than the propagated uncertainty are treated as significantly different. In other words, such differences are too large to be explained by the uncertainty in the measurements if the two samples were identical. If the samples are in fact identical, an observed difference that exceeds the limits is a false positive: a difference has been detected where no true difference exists. Figure 2 illustrates false positives (red symbols) that arise when uncertainty is estimated from individual standard deviations. Two sets of triplicate measurements were drawn from a random number generator 1000 times. The random numbers had a gaussian distribution with a population mean of zero and a standard deviation of 1. (The distribution of standard deviations was shown in Figure 1.) The result, with $k = 4$ for the coverage factor in equation (3), is a false positive rate of 1.7%. In contrast, Figure 3 shows that use of the pooled standard deviation for the uncertainty estimate results in zero false positives. The results obtained with different values of the coverage factor are shown in Figure 4. As might be expected, more permissive uncertainty estimates (*i.e.*, smaller coverage factors) do result in larger false positive rates, but the false positive rate with pooled uncertainty estimate is always lower than that obtained with individual standard deviations. These false positives arise because in some cases, the triplicate data happen to cluster together, resulting in a small standard deviation. Indeed, this is evident in Figure 1. While figure 4 suggests that a coverage factor of $k = 4$ will be sufficient to essentially eliminate false positives from differential HX data based on triplicated centroid measurements, a suitable coverage factor will depend on the size of the differential HX-MS data set and its variability.

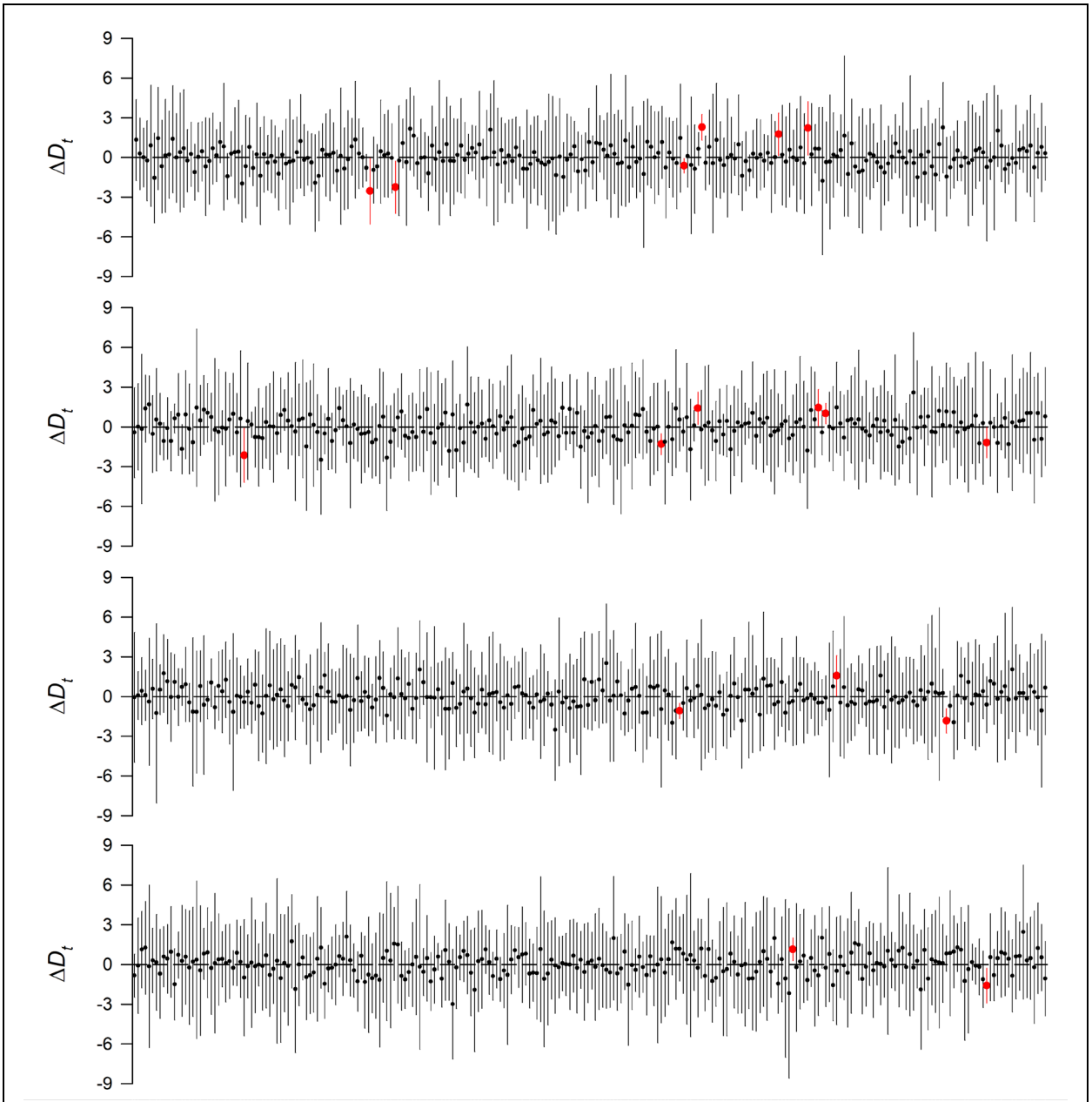

Figure 2. Differences between 1000 pairs of triplicate random numbers drawn from a randomized gaussian distribution with a population mean of 0 and a population standard deviation of 1. For clarity, the differences are spread across four panels. The bars represent the expanded uncertainty based on individual standard deviations (shown in Figure 1) with a coverage factor of $k = 4$. The red symbols denote false positives as defined in the text. Numbers were generated using Gaussian Random Number Generator at random.org.

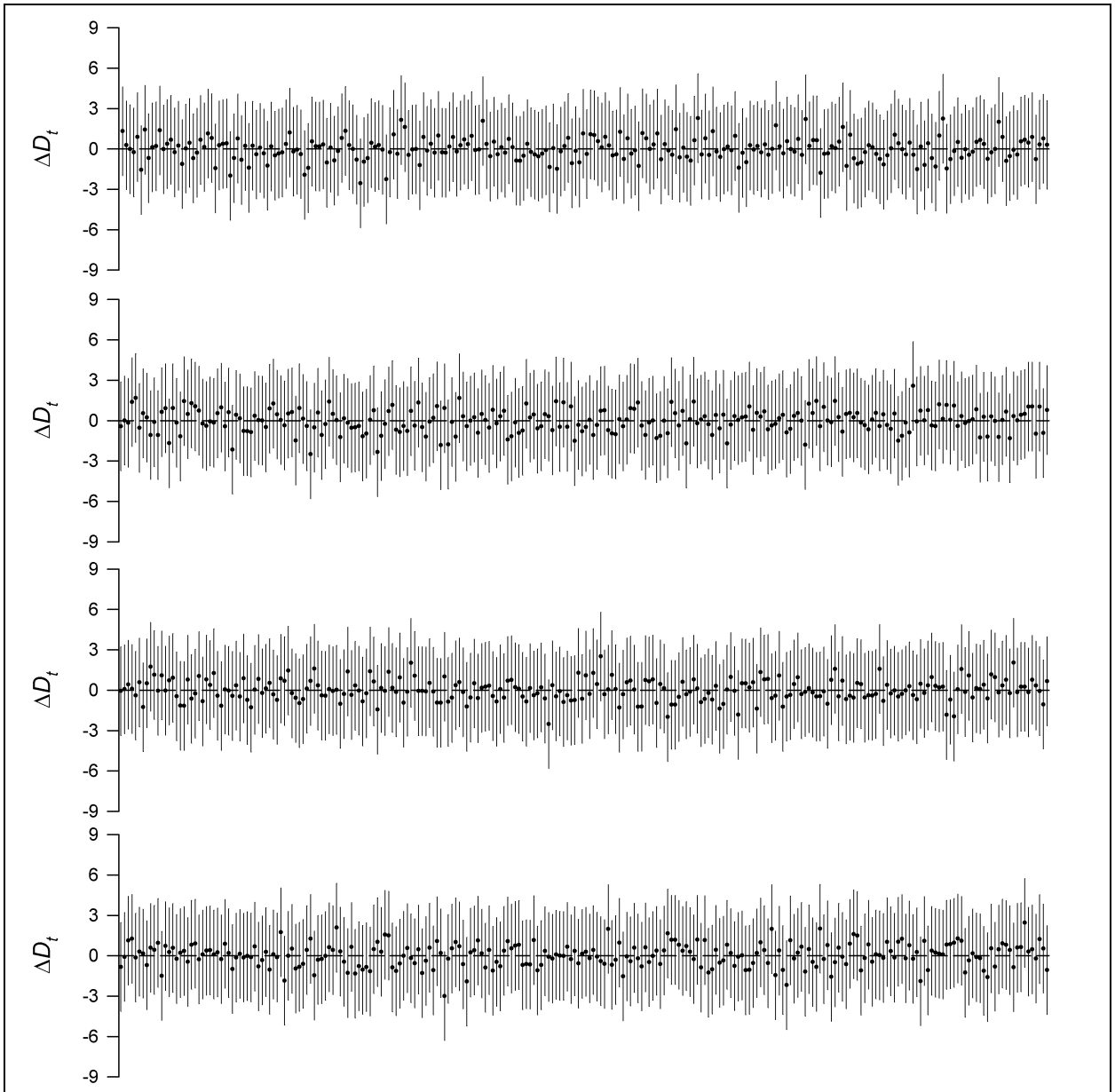

Figure 3. Differences between 1000 pairs of triplicate random numbers drawn from a randomized gaussian distribution, as described in Figure 2. For clarity, the differences are spread across four panels. The bars represent the expanded uncertainty based on pooled standard deviations (shown in Figure 1) with a coverage factor of $k = 4$. There are no false positives. Numbers were generated using Gaussian Random Number Generator at random.org.

ILLUSTRATION OF DIFFERENTIAL HX-MS UNCERTAINTY CALCULATIONS

Figure 5 represents typical data from a differential HX-MS measurements from two samples in which the peptide of interest exhibits a small differences in HX. The peptide contains 7 exchangeable amide hydrogens ($N_{\mathrm{H}} = 7$), measured at 9 HX times ($n_{\mathrm{T}} = 9$) and has an undeuterated average mass of 1000 Da ($m_0 = 1000$ Da). All measurements were obtained in triplicate ($n = 3$). The raw data for this simulation are provided in the Supporting Information. Table 3 provides the mean deuteration values (D_{A_i} and D_{B_i}), their associated standard deviations, all expressions of individual HX differences and their corresponding combined uncertainties. Table 4 provides the accumulated HX differences. All results were tested for significance , using a coverage factor $k = 4$, according to

$$\begin{aligned} H_0 &: |\Delta x| \le ku(\Delta x) \\ H_1 &: |\Delta x| > ku(\Delta x) \end{aligned} \tag{27}$$

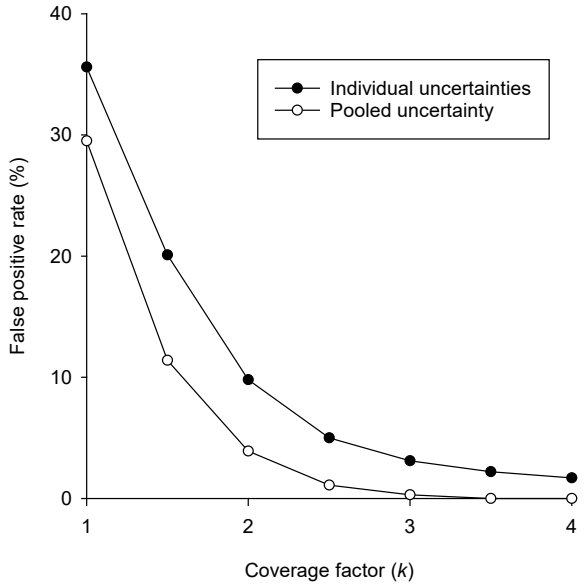

Figure 4. Effect of coverage factor, k, on the false positive rates for the data shown in Figures 2 and 3.

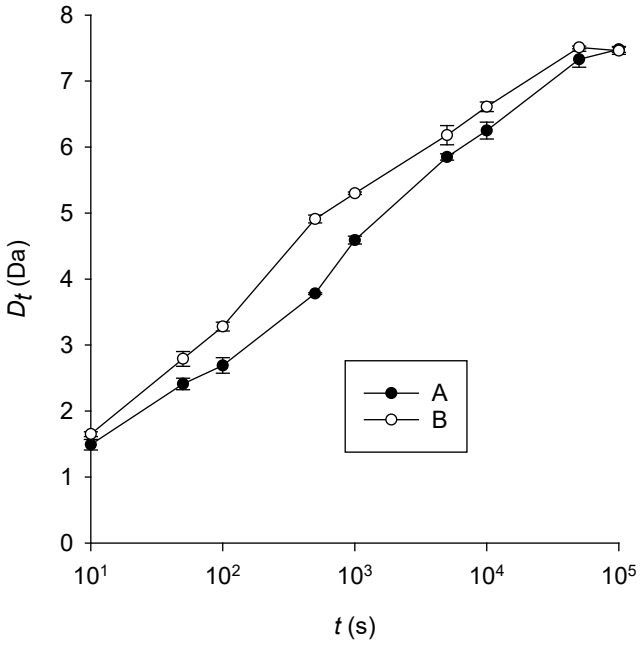

Figure 5. Simulated HX uptake plot to illustrate the calculation of HX differences and their uncertainties. The error bars denote the standard deviations. See text for details.

where Δx denotes an HX difference. Here, H_1 indicates a significant difference (*i.e.,* rejection of the null hypothesis).

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APPENDIX

Propagation of uncertainty in fractional differential HX with uncertainty in numerator and denominator. The simplest form of fractional differential HX with uncertainty in the numerator and denominator is

$$\Delta HX_t^* = \frac{\Delta D_t}{D_\infty} \tag{28}$$

For simplicity, let this equation be represented as

$$f = \frac{x-y}{z} \tag{29}$$

having associated random uncertainties $u(x)$, $u(y)$, and $u(z)$. The combined random uncertainty[4] may be found using

$$u(f)^2 = u(x)^2\left(\frac{\partial f}{\partial x}\right)^2 + u(y)^2\left(\frac{\partial f}{\partial y}\right)^2 + u(z)^2\left(\frac{\partial f}{\partial z}\right)^2 \tag{30}$$

which yields

$$u(f)^2 = \left(\frac{u(x)}{z}\right)^2 + \left(\frac{u(y)}{z}\right)^2 + \left(\frac{-(x-y)u(z)}{z^2}\right)^2 = \frac{1}{z^2}\left[u(x)^2 + u(y)^2 + \left(\frac{(x-y)u(z)}{z}\right)^2\right] \tag{31}$$

Substitution of (29) into (31) and taking the positive square root gives

$$u(f) = \frac{1}{z}\sqrt{u(x)^2 + u(y)^2 + (fu(z))^2} \tag{32}$$

Hence, by comparison of (28) and (29), equation (32) becomes

$$u\left(\Delta HX_t^*\right) = \frac{1}{D_\infty}\sqrt{u\left(m_{A_t}\right)^2 + u\left(m_{B_t}\right)^2 + \left(u\left(D_\infty\right)\Delta HX_t^*\right)^2} \tag{33}$$

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Table 1. Summary of combined uncertainties for different expressions of differential HX.		
deuteration difference at a single HX time	$\Delta D_t = \overline{m}_{A_t} - \overline{m}_{B_t}$	$u\left(\Delta D_t\right) = \sqrt{\frac{s\left(m_{A_t}\right)^2}{n\left(m_{A_t}\right)} + \frac{s\left(m_{B_t}\right)^2}{n\left(m_{B_t}\right)}}$
fractional difference at a single HX time based on maximum theoretical exchange	$\Delta HX_t = \frac{\Delta D_t}{N_{\mathrm{H}} m_\delta}$	$u\left(\Delta HX_t\right) = \frac{u\left(\Delta D_t\right)^2}{N_{\mathrm{H}} m_\delta}$
deuteration difference summed over all HX times	$\Delta D_\Sigma = \sum_{t \in \mathrm{T}} \Delta D_t$	$u\left(\Delta D_\Sigma\right) = \sqrt{\sum_{t \in \mathrm{T}} u\left(\Delta D_t\right)^2}$
arithmetic mean deuteration difference across all HX times	$\Delta \overline{D} = \frac{\Delta D_\Sigma}{n_{\mathrm{T}}} = \frac{\sum_{t \in \mathrm{T}} \Delta D_t}{n_{\mathrm{T}}}$	$u\left(\Delta \overline{D}\right) = \frac{1}{n_{\mathrm{T}}} \sqrt{\sum_{t \in \mathrm{T}} u\left(\Delta D_t\right)}$
arithmetic mean fractional difference across all HX times based on maximum theoretical exchange	$\Delta \overline{HX} = \frac{\Delta D_\Sigma}{n_{\mathrm{T}} N m_\delta} = \frac{\sum_{t \in \mathrm{T}} \Delta D_t}{n_{\mathrm{T}} N m_\delta}$	$u\left(\Delta \overline{HX}\right) = \frac{1}{n_{\mathrm{T}} N_{\mathrm{H}} m_\delta} \sqrt{\sum_{t \in \mathrm{T}} u\left(\Delta D_t\right)^2}$
deuteration difference at a single HX time corrected for back-exchange	$\Delta D_t^* = \frac{\Delta D_t}{D_\infty} N_{\mathrm{H}} m_\delta$	$u\left(\Delta D_t^*\right) = \frac{N_{\mathrm{H}} m_\delta}{D_\infty} \sqrt{\frac{s\left(m_{A_t}\right)^2}{n\left(m_{A_t}\right)} + \frac{s\left(m_{B_t}\right)^2}{n\left(m_{B_t}\right)} + \frac{s\left(D_\infty\right)^2}{n\left(D_\infty\right)} \Delta D_t^{*2}}$
fractional difference at a single HX time corrected for back-exchange	$\Delta HX_t^* = \frac{\Delta D_t}{D_\infty}$	$u\left(\Delta HX_t^*\right) = \frac{1}{D_\infty} \sqrt{\frac{s\left(m_{A_t}\right)^2}{n\left(m_{A_t}\right)} + \frac{s\left(m_{B_t}\right)^2}{n\left(m_{B_t}\right)} + \frac{s\left(D_\infty\right)^2}{n\left(D_\infty\right)} \Delta HX_t^{*2}}$
deuteration difference summed over all HX times corrected for back-exchange	$\Delta D_\Sigma^* = \frac{\Delta D_\Sigma}{D_\infty} N_{\mathrm{H}} m_\delta = \frac{\sum_{t \in \mathrm{T}} \Delta D_t}{D_\infty} N_{\mathrm{H}} m_\delta$	$u\left(\Delta D_\Sigma^*\right) = \frac{N_{\mathrm{H}} m_\delta}{D_\infty} \sqrt{\sum_{t \in \mathrm{T}} \left(\frac{s\left(m_{A_t}\right)^2}{n\left(m_{A_t}\right)} + \frac{s\left(m_{B_t}\right)^2}{n\left(m_{B_t}\right)} \right) + \frac{s\left(D_\infty\right)^2}{n\left(D_\infty\right)} \Delta D_\Sigma^{*2}}$
arithmetic mean deuteration difference across all HX times corrected for back-exchange	$\Delta \overline{D}^* = \left(\frac{\sum_{t \in \mathrm{T}} \Delta D_t}{n_{\mathrm{T}}} \right) \frac{N_{\mathrm{H}} m_\delta}{D_\infty}$	$u\left(\Delta \overline{D}^*\right) = \frac{N_{\mathrm{H}} m_\delta}{n_{\mathrm{T}} D_\infty} \sqrt{\sum_{t \in \mathrm{T}} \left(\frac{s\left(m_{A_t}\right)^2}{n\left(m_{A_t}\right)} + \frac{s\left(m_{B_t}\right)^2}{n\left(m_{B_t}\right)} \right) + \frac{s\left(D_\infty\right)^2}{n\left(D_\infty\right)} \Delta \overline{D}^{*2}}$

arithmetic mean fractional difference across all HX times corrected for back-exchange	$\Delta\overline{HX}^{*} = \frac{\sum_{t\in\mathrm{T}} \Delta D_t}{n_\mathrm{T} D_\infty}$	$u\left(\Delta\overline{HX}^{*}\right) = \frac{1}{n_\mathrm{T} D_\infty}\sqrt{\sum_{t\in\mathrm{T}}\left(\frac{s\left(m_{A_t}\right)^2}{n\left(m_{A_t}\right)} + \frac{s\left(m_{B_t}\right)^2}{n\left(m_{B_t}\right)}\right) + \frac{s\left(D_\infty\right)^2}{n\left(D_\infty\right)}\Delta\overline{HX}^{*2}}$

$\overline{m}$ denotes the arithmetic mean of replicate measurements of the centroid mass of a peptide, A and B denote the two samples in a differential HX measurement, t denotes an HX labeling time $\left(t \in \mathrm{T}\right)$ where T is the set of labeling times, n_T is the number of distinct HX labeling times (*i.e.,* the cardinality of T). The notation $u\left(x\right)^2 = \frac{s\left(x\right)^2}{n\left(x\right)}$ denotes the square of the standard uncertainty in x as defined by (2) where $n\left(x\right)$ is the number of replicate measurements of x and $s\left(x\right)$ is the standard deviation. Importantly, in this parenthetical notation $n\left(x\right) \neq nx$. N_H denotes the number of exchangeable amide hydrogens in the peptide, $m_\delta \approx 1$ Da is the difference between the mass of deuterium and protium, and D_∞ is measured mass increase of a deuteration control as defined by (14) .

Table 2. Summary of combined uncertainties for different expressions of differential HX using pooled standard deviation.

With unequal replicates	With equal replicates, n, is all measurements
$u\left(\Delta D_t\right) = s_p\sqrt{\frac{1}{n\left(m_{A_t}\right)}+\frac{1}{n\left(m_{B_t}\right)}}$	$u\left(\Delta D_t\right) = s_p\sqrt{\frac{2}{n}}$
$u\left(\Delta HX_t\right) = \frac{s_p}{N_{\mathrm{H}}m_\delta}\sqrt{\frac{1}{n\left(m_{A_t}\right)}+\frac{1}{n\left(m_{B_t}\right)}}$	$u\left(\Delta HX_t\right) = \frac{s_p}{N_{\mathrm{H}}m_\delta}\sqrt{\frac{2}{n}}$
$u\left(\Delta D_\Sigma\right) = s_p\sqrt{\sum_{t\in\mathrm{T}}\frac{1}{n\left(m_{A_t}\right)}+\frac{1}{n\left(m_{B_t}\right)}}$	$u\left(\Delta D_\Sigma\right) = s_p\sqrt{\frac{2n_{\mathrm{T}}}{n}}$
$u\left(\Delta\overline{D}\right) = \frac{s_p}{n_{\mathrm{T}}}\sqrt{\sum_{t\in\mathrm{T}}\frac{1}{n\left(m_{A_t}\right)}+\frac{1}{n\left(m_{B_t}\right)}}$	$u\left(\Delta\overline{D}\right) = s_p\sqrt{\frac{2}{n_{\mathrm{T}}n}}$
$u\left(\Delta\overline{HX}\right) = \frac{s_p}{n_{\mathrm{T}}N_{\mathrm{H}}m_\delta}\sqrt{\frac{1}{n\left(m_{A_t}\right)}+\frac{1}{n\left(m_{B_t}\right)}}$	$u\left(\Delta\overline{HX}\right) = \frac{s_p}{N_{\mathrm{H}}m_\delta}\sqrt{\frac{2}{n_{\mathrm{T}}n}}$
$u\left(\Delta D_t^*\right) = \frac{s_pN_{\mathrm{H}}m_\delta}{D_\infty}\sqrt{\frac{1}{n\left(m_{A_t}\right)}+\frac{1}{n\left(m_{B_t}\right)}+\frac{\Delta D_t^{*2}}{n\left(D_\infty\right)}}$	$u\left(\Delta D_t^*\right) = \frac{s_pN_{\mathrm{H}}m_\delta}{D_\infty}\sqrt{\frac{2+\Delta D_t^{*2}}{n}}$
$u\left(\Delta HX_t^*\right) = \frac{s_p}{D_\infty}\sqrt{\frac{1}{n\left(m_{A_t}\right)}+\frac{1}{n\left(m_{B_t}\right)}+\frac{\Delta HX_t^{*2}}{n\left(D_\infty\right)}}$	$u\left(\Delta HX_t^*\right) = \frac{s_p}{D_\infty}\sqrt{\frac{2+\Delta HX_t^{*2}}{n}}$
$u\left(\Delta D_\Sigma^*\right) = \frac{s_pN_{\mathrm{H}}m_\delta}{D_\infty}\sqrt{\sum_{t\in\mathrm{T}}\left(\frac{1}{n\left(m_{A_t}\right)}+\frac{1}{n\left(m_{B_t}\right)}\right)+\frac{\Delta D_\Sigma^{*2}}{n\left(D_\infty\right)}}$	$u\left(\Delta D_\Sigma^*\right) = \frac{s_pN_{\mathrm{H}}m_\delta}{D_\infty}\sqrt{\frac{2n_{\mathrm{T}}+\Delta D_\Sigma^{*2}}{n}}$
$u\left(\Delta\overline{D}^*\right) = \frac{s_pN_{\mathrm{H}}m_\delta}{n_{\mathrm{T}}D_\infty}\sqrt{\sum_{t\in\mathrm{T}}\left(\frac{1}{n\left(m_{A_t}\right)}+\frac{1}{n\left(m_{B_t}\right)}\right)+\frac{\Delta\overline{D}^{*2}}{n\left(D_\infty\right)}}$	$u\left(\Delta\overline{D}^*\right) = \frac{s_pN_{\mathrm{H}}m_\delta}{n_{\mathrm{T}}D_\infty}\sqrt{\frac{2n_{\mathrm{T}}+\Delta\overline{D}^{*2}}{n}}$
$u\left(\Delta\overline{HX}^*\right) = \frac{s_p}{n_{\mathrm{T}}D_\infty}\sqrt{\sum_{t\in\mathrm{T}}\left(\frac{1}{n\left(m_{A_t}\right)}+\frac{1}{n\left(m_{B_t}\right)}\right)+\frac{\Delta\overline{HX}^{*2}}{n\left(D_\infty\right)}}$	$u\left(\Delta\overline{HX}^*\right) = \frac{s_p}{n_{\mathrm{T}}D_\infty}\sqrt{\frac{2n_{\mathrm{T}}+\Delta\overline{HX}^{*2}}{n}}$

Table 3. Differential HX, combined uncertainty, and significance testing for individual HX times.

t (s)	D_{A_t} (Da)	$s\left(D_{A_t}\right)$ (Da)	D_{B_t} (Da)	$s\left(D_{B_t}\right)$ (Da)	ΔD_t (Da)	$u\left(\Delta D_t\right)$ (Da)	significant[a]	ΔHX_t	$u\left(\Delta HX_t\right)$	significant[a]
10	1.49	0.080	1.65	0.036	-0.16	0.051	FALSE	-0.0163	0.0050	FALSE
50	2.41	0.087	2.78	0.112	-0.37	0.082	TRUE	-0.0368	0.0081	TRUE
100	2.68	0.118	3.26	0.066	-0.58	0.078	TRUE	-0.0579	0.0078	TRUE
500	3.73	0.013	4.81	0.060	-1.08	0.036	TRUE	-0.1069	0.0035	TRUE
1000	4.49	0.060	5.11	0.019	-0.61	0.037	TRUE	-0.0610	0.0036	TRUE
5000	5.41	0.049	5.40	0.146	0.01	0.089	FALSE	0.0005	0.0088	FALSE
10000	5.47	0.127	5.40	0.073	0.07	0.085	FALSE	0.0073	0.0084	FALSE
50000	7.33	0.121	7.51	0.020	-0.17	0.071	FALSE	-0.0174	0.0070	FALSE
100000	7.48	0.045	7.46	0.056	0.02	0.041	FALSE	0.0017	0.0041	FALSE
∞[b]	7.57	0.044	7.57	0.044						

t (s)	ΔD_t^* (Da)	$u\left(\Delta D_t^*\right)$ (Da)	significant[a]	ΔHX_t^*	$u\left(\Delta HX_t^*\right)$	significant[a]
10	-0.218	0.068	FALSE	-0.0217	0.0067	FALSE
50	-0.493	0.110	TRUE	-0.0490	0.0108	TRUE
100	-0.774	0.107	TRUE	-0.0770	0.0103	TRUE
500	-1.430	0.068	TRUE	-0.1422	0.0047	TRUE
1000	-0.817	0.056	TRUE	-0.0812	0.0048	TRUE
5000	0.007	0.118	FALSE	0.0007	0.0117	FALSE
10000	0.098	0.113	FALSE	0.0097	0.0112	FALSE
50000	-0.232	0.095	FALSE	-0.0231	0.0094	FALSE
100000	0.023	0.055	FALSE	0.0023	0.0055	FALSE

[a]Signficance testing with a coverage factor $k = 4$ according to equation (27). [b]Denotes a measured deuteration control.

Table 4. Differential HX, combined uncertainty, and significance testing for accumulated differences.

Quantity	Value	Uncertainty	significant[a]
ΔD_{Σ}	-3.8088 Da	0.1993 Da	TRUE
$\Delta \bar{D}$	-0.4232 Da	0.0221 Da	TRUE
$\Delta \overline{HX}$	-0.0519	0.0022	TRUE
ΔD_{Σ}^{*}	-5.0638 Da	0.2538 Da	TRUE
$\Delta \bar{D}^{*}$	-0.5626 Da	0.0293 Da	TRUE
$\Delta \overline{HX}^{*}$	-0.0559	0.0029	TRUE

[a]Signficance testing with a coverage factor $k = 4$ according to equation (27)