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Evaluating uncertainty in the calculation of non-exchangeable hydrogen fractions within organic materials

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We calculated the fraction of exchangeable hydrogen atoms in proteinaceous materials commonly analyzed for stable isotopic composition related to the region-of-origin of an animal. These included several types of α- and β-keratin, and muscle tissue. We find that the fraction of H atoms in keratin available for exchange at a biologically relevant temperature (25°C) averaged 9% across a range of ground organic materials, but was as high as ~17% in cut hair; muscle tissue has ~12% exchangeable H atoms. Under most analysis conditions, the difference in exchangeable fractions due to physical sample processing has a minimal effect on the calculated δ^2H values of the non-exchangeable H atoms within a keratin-containing tissue (<2‰). However, extreme mismatches between sample and reference material types could affect δ^2H values. Copyright © 2009 John Wiley & Sons, Ltd.

Recent studies on the geographical region-of-origin of animals have included analyses of beef,[1,2] bird feathers,[3,4] butterfly wings,[5] human hair,[6,7] human fingernails,[8] lamb meat,[9] microbial spores,[10] and wood rat hair.[11] The ability to regionally geo-locate an animal using the stable isotopic composition of its tissues is possible because the stable isotopes of hydrogen (δ^2H) and oxygen (δ^{18}O) in the global water cycle vary predictably throughout the world, decreasing from low-latitude, low-elevation coastal regions to inland, high-latitude, and mountainous regions.[12,13] The isotopic composition of animal tissues is strongly correlated with that of drinking water[11,14] and thus an examination of δ^2H and δ^{18}O values for a collected material can describe broad patterns related to the geographic origin of the animal, with applications in ecology,[14] food provenancing,[15] and forensics.[8]

The pre-analysis treatment of organic samples, such as animal tissues, is especially important when measuring δ^2H values. A fraction of the H atoms within a sample may exchange with atmospheric water vapor post-collection,[16] leading to potentially erroneous results unless controlled as part of the measurement and/or analysis process. To control H exchange, some materials can be easily derivatized to a form without labile H atoms (e.g., cellulose nitration[16]). Other compounds do not easily lend themselves to a nitration process (e.g., hair or feathers), such that the substitution between exchangeable H atoms in the sample and the atmosphere cannot be prevented through chemical means.[17]

One experimental approach to calculate the fraction of exchangeable H atoms in a material used high-temperature steam to equilibrate unknowns with water vapors of varying isotopic composition. The difference between the total δ^2H values of an unknown equilibrated with two distinct water vapors could then be used to calculate the contribution of exchangeable H atoms.[17,18] Steam equilibration required each unknown to undergo intensive preparation prior to analysis and also exposed the sample to a temperature extreme that greatly exceeded biologically relevant temperatures. In response, Wassenaar and Hobson developed a Principle of Identical Treatment (PIT),[19] a comparative equilibration technique. Using PIT, calibrated reference materials and unknowns are both simultaneously equilibrated with ambient water vapor under identical conditions prior to isotopic analysis. Post-analysis, the measured total δ^2H values for the reference material(s) are compared with the defined non-exchangeable δ^2H value, and the calculated difference is used to remove the effect of exchangeable H atoms on the δ^2H value of the unknowns.[18,20,21]

In the comparative equilibration technique, the difference between the exchangeable H fraction in reference and unknown materials is assumed to be negligible. However, when Bowen *et al*.[20] calculated the fraction of labile H atoms in keratin from horse hair, they determined that ~15% of H atoms in cut hair were exchangeable, but that this value was reduced to ~10% in ground hair. In addition, details on the actual non-exchangeable fraction of H atoms within other keratin-containing tissues are often unknown, but are assumed to be similar to horse hair as measured by Bowen

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et al.[20] Analyzing different animal tissues using comparative equilibration thus raises an important question of how similar unknown keratin and reference materials must be for PIT to apply?

In this study, we determined values for the exchangeable H fractions in different materials commonly collected during ecological and forensics studies, including α- and β-keratin and muscle tissue. We also explored potential uncertainties in determination of δ^2H values of a sample if the exchangeable H pool size in reference materials is different from that of unknowns. Specifically, we addressed the following questions:

1. Are the fractions of H atoms available for exchange at biologically relevant temperatures in different types of keratin similar to each other?
2. Is there a difference in the fraction of exchangeable H atoms in ground and cut hair? If so, can the contribution of exchangeable H atoms in ground unknowns be corrected using cut reference materials?

EXPERIMENTAL

Design

We calculated the fraction of H atoms available for exchange with ambient water vapor in wool, human hair, horse hair, a commercially produced powdered keratin, cow horn, and springbok horn. We also evaluated a composite muscle tissue, a hamburger (beef) sample. The materials were exposed to water vapors of known ^{2}H isotopic composition, then dried prior to analysis for total δ^2H values, as detailed previously.[20] Briefly, samples were weighed (150 μg ± 10%) in Ag capsules and loaded into plastic 96-well trays. The trays were loosely covered, then placed in sealed chambers and exposed to vapor in equilibrium with either light (δ^2H = −123‰) or heavy (δ^2H = +350‰) water for a minimum of 72 h at ambient laboratory temperature, as per Bowen *et al*.[20] who measured maximum equilibration within 3–4 days. After equilibration, the trays were transferred as quickly as possible (<60 s) to plastic desiccators and held under vacuum for a minimum of 5 days. The trays were isolated – one tray per desiccator, one desiccator per vacuum pump – to prevent back-equilibration between H atoms in materials from different treatments. The dried samples were quickly transferred (<10 min) from evacuated desiccators to the autosampler of a high-temperature conversion elemental analyzer (TC/EA), which was subsequently evacuated and purged with He, preventing any back-equilibration between exchangeable H atoms in the samples and ambient water vapor. The total time for which an equilibrated sample was exposed to ambient atmosphere during transfer events was less than 15 min. Bowen *et al*.[20] previously observed back-equilibration between samples and ambient water vapor over a time-scale of hours. While back-equilibration could have occurred in this experimental design, exposure to ambient vapor was of limited duration and probably contributed only slightly to the overall uncertainty in measurements and calculations. Unlike the previous work of Bowen *et al*.[20] using an in-house cellulose reference material, the total δ^2H values of materials were determined using a polyethylene foil standard (IAEA-CH-7, δ^2H = −100.3‰). The standards were carefully weighed (75 μg ± 10%) to closely match the peak areas of the samples.

We also analyzed ten human hair samples (prepared as ground samples) and corrected these data with reference materials that included both ground and cut hair material. These samples were chosen from a set of hair collected across the 48 contiguous states (excluding Alaska and Hawaii) of the USA.[7] Human hair samples were equilibrated for 48 h under ambient laboratory conditions alongside both ground and cut laboratory reference materials, Florida Horse tailhair (FH) and Utah Horse tailhair (UH), for which the fraction of exchangeable H atoms had been previously determined.[20] After equilibration, unknowns and reference materials were weighed and dried prior to measurement of δ^2H values. Data were corrected twice, once using ground reference materials (i.e., 'like-with-like') and once using cut reference materials, to calculate the isotopic composition of the non-exchangeable H atoms in the unknowns.

Material preparation

For determination of exchangeable H fractions, samples were obtained from a variety of locations. Wool was collected from sheep in the Orkney Islands, UK. Human hair was collected at a hairdressing salon in Salt Lake City, UT, USA; all other human hair samples were collected as detailed in Ehleringer *et al*.[7] Horse tailhair was collected from a horse from Powell, WY, USA. Commercial powdered keratin was procured from Voight Global Distribution (Lawrence, KS, USA). Cow and springbok horns were obtained from The Bone Room (Berkely, CA, USA) (no provenance information is known). The hamburger sample was purchased from a fast food restaurant in Salt Lake City.

Wool was washed with laundry detergent (Woolite®) and hot water to remove lanolin. Once clean, the wool – and all other hair samples – were washed in a 2:1 chloroform/methanol solvent mixture. Solvent-cleaned hair samples were ground to a homogeneous powder (hereafter, 'ground') using a mixer mill (Retsch, Newton, PA, USA) and stored in glass vials. Aliquots of the solvent-washed wool, human hair collected from Salt Lake City, and horse hair were set aside before grinding and analyzed whole (hereafter, 'cut'). Horn surfaces were cleaned using 2:1 chloroform/methanol. Small pieces of each horn were ground to powder in batches. The hamburger sample was delipified and ground as detailed in Chesson *et al*.[22] No cleanup or processing was done to the commercial powdered keratin.

Stable isotope analysis

Total δ^2H values were measured using a ThermoFinnigan-MAT Delta Plus XL isotope ratio mass spectrometer (Bremen, Germany) operated in continuous flow mode, with a zero-blank autsosampler (Costech Analytical, Valencia, CA, USA) and TC/EA (ThermoFinnigan) attached. High-temperature pyrolysis at 1400°C ensured complete reduction of organic H and conversion of O within the samples to gaseous H_2 and CO, respectively. The resultant gases were separated on a 1-m, 0.25″ (o.d.) molecular sieve 5 Å gas chromatography (GC) column (Costech Analytical) held at 95°C. The GC column flow rate was 100 mL He/min. The H_3^+

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factor was determined prior to the first batch analysis. There was no effect of peak area on the measured δ^2H values of IAEA-CH-7 throughout subsequent batch analyses. All analyses were conducted at the Stable Isotope Ratio Facility for Environmental Research (SIRFER, http://sirfer.net) at the University of Utah. The analytical precision for total δ^2H measurements, based on analysis of an unequilibrated powdered keratin reference material, was ±4‰ (1σ, $n = 16$).

The stable isotopic composition is reported in 'delta' (δ notation:

$$\delta = (R_{sample}/R_{standard} - 1) \times 1000 \quad (1)$$

where R_{sample} and $R_{standard}$ are the molar ratios of rare (heavy) to abundant (light) isotopes (e.g., $^2H/^1H$) in the sample and standard, respectively. All δ-values are expressed in per mil (‰) units relative to the international standard V-SMOW. Isotopic fractionation (α) is defined as the difference between two phases (A and B) in equilibrium:

$$\alpha_{AB} = (1000 + \delta_A)/(1000 + \delta_B) \quad (2)$$

and the isotopic enrichment (ε) between phases, in per mil units, is:

$$\varepsilon_{AB} = (\alpha_{AB} - 1) \times 1000 \quad (3)$$

Because the standard δ notation for reporting stable isotope abundances is non-linear, calculations based on δ values can lead to errors. Hence we used molar ratios for the calculations, although data are ultimately reported in per mil units.

In a complex organic material, H atoms comprise a mixture of exchangeable and non-exchangeable pools, so that the total isotopic composition of H atoms within a sample (R_{total}) can be described as:

$$R_{total} = R_{ex}(f_{ex}) + R_{nex}(1 - f_{ex}) \quad (4)$$

where R_{ex} is the isotopic composition of exchangeable H atoms within the sample, f_{ex} is fraction of H atoms available for exchange, and R_{nex} is the isotopic composition of non-exchangeable H atoms within the material. The value of R_{ex} will vary, depending on the isotopic composition of the atmospheric water vapor available for exchange (R_w) and the fractionation (α_{ex}) between H atoms in vapor and the material, such that:

$$\alpha_{ex} = R_{ex}/R_w \quad (5)$$

We assume that both f_{ex} and R_{nex} are constant for a material under investigation, regardless of treatment method. We also explicitly assume that $\alpha_{ex} = 1$, as per Bowen *et al*.[20] We note this assumption is counter to most work on exchangeable H in complex biological materials, which (1) assume α is constant throughout an experiment, without explicitly defining a value;[5,17,23,24] (2) use a value for α calculated from experimental results;[25–27] or (3) use an assumed value for α, ranging from 1.060 to 1.100.[4,18,19,28–31] See Table 2 for more details. We calculate the fraction of H atoms within a material available for exchange using the total H isotopic composition of the material equilibrated with two isotopically distinct waters according to the equation:

$$f_{ex} = \frac{R_{total1} - R_{total2}}{R_{w1} - R_{w2}} \quad (6)$$

Differences in H isotopic composition between repeated analyses of a sample ($\Delta\delta^2$H) are calculated via subtraction:

$$\Delta\delta^2H = \delta^2H_{t1} - \delta^2H_{t2} \quad (7)$$

where δ^2H_{t1} and δ^2H_{t2} denote the H isotopic composition of a sample analyzed two separate times. Note that data are not converted into ratios for difference calculations.

RESULTS AND DISCUSSION

Calculated f_{ex} and 1σ values at ambient temperatures for the nine types of keratinous material and one muscle tissue investigated in this study exhibited only a modest range and are presented in Table 1. Experimentally determined f_{ex} values for ground keratin samples were 0.084 to 0.095, a range of ~1%. Both cut keratin samples – cut human hair and cut horse hair – had a larger fraction of exchangeable H atoms (0.159 and 0.173, respectively) than ground counterparts (0.094 and 0.092, respectively), similar to the findings of Bowen *et al*.[20] The single muscle tissue investigated, ground beef, had a similar, but slightly larger fraction of H atoms available for exchange than ground keratin samples. The f_{ex} value for beef muscle (0.127) was less than f_{ex} values published for other muscle and organ tissues (~0.20)[4,18] that had been pre-treated with high temperatures.

The f_{ex} values that we calculated appear to be similar across the range of keratin tissues collected from different biological organisms (Table 1). The major pattern that appears is a difference among studies associated with the pre-treatment temperatures (Table 2). Our study was conducted at ambient temperature, so as to be relevant for the exchange processes that will take place as a tissue is transported from one region to another. Our equilibration treatment was at ~25°C, similar to studies on American redstart feathers[3] and human hair.[6] Both ambient temperature studies calculated fractions of exchangeable H atoms similar in size to the values calculated in this work: ~13% and ~9% for feather[3] and hair,[6] respectively. Elevated temperatures during sample-water vapor equilibration are likely to expose H atoms for exchange that are normally shielded within the material. Thus, it is not surprising that the f_{ex} values observed in the study (Table 1) are lower than values previously published for other types of keratin,

Table 1. Calculated fractions of exchangeable H atoms (f_{ex}) for nine different types of keratinous material and a single muscle tissue. Data are means with ±1 standard deviations (σ) also given

Material	Physical processing	n	f_{ex}
wool	ground	16	0.087 ± 0.005
	cut	15	0.064 ± 0.010
human hair	ground	24	0.094 ± 0.006
	cut	16	0.159 ± 0.010
horse hair	ground	16	0.092 ± 0.005
	cut	8	0.173 ± 0.005
commercial keratin	ground	16	0.084 ± 0.010
cow horn	ground	16	0.091 ± 0.010
springbok horn	ground	16	0.095 ± 0.008
hamburger meat	ground	16	0.127 ± 0.016

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Table 2. Literature observations for calculated fractions of exchangeable H atoms (f_{ex}) within a range of biological materials, with details on experimental equilibration conditions. The fractionation factor (α) between H atoms in water and sample is also provided

Material	Tissue	Source	Temp. (°C)	α	*n*	f_{ex}	Ref.
cellulose	amylose	unknown	92	1.300§	4	0.30	26
cellulose	cellulose	*Pinus longeava*	92	1.230§	9	0.22	26
cellulose	cellulose	*Pinus radiata*	92	1.240§	16	0.22	26
cellulose	cellulose	*Pinus sylvestris*	105	1.082§	3	0.24	27
cellulose	cellulose	unknown	114	1.080§	8	0.27	17
cellulose	cellulose	non-aboreal	114	nd	6	0.27	24
cellulose	cellulose*	*Pinus contorta*	0	1.213§	7	0.26	25
cellulose	cellulose*	*Lagarostrobos franklini*	0	1.243§	6	0.26	25
cellulose	cotton	medical	114	nd	17	0.16	17
cellulose	cotton	medical	130	1.080†	3	0.23	18
cellulose	cotton*	medical	0	1.254§	6	0.27	25
cellulose	holocellulose	*Pinus tabulaeformis*	130	nd	8	0.22	24
cellulose	whole wood	*Pinus tabulaeformis*	130	nd	8	0.17	24
chemical	humic acid	marine sediment	114	nd	7	0.19	17
chemical	humic acid	Aldrich Co.	130	1.080†	3	0.13	18
chitin	carapace	lobster	114	nd	2	0.16	17
chitin	exoskeleton	arthropods	130	nd	13	0.15	24
collagen	bone	bison	24	nd	5	0.21	23
collagen	bone	muskox	24	nd	6	0.21	23
collagen	bone	seal	24	nd	7	0.22	23
collagen	bone	bison	135	1.080†	4	0.20	31
collagen	gelatin	unflavored	24	nd	6	0.23	23
collagen	tendon	bovine	110	nd	1	0.17	24
keratin	baleen	whale	130	1.080†	6	0.15	19
keratin	feather	American redstart	25	nd	3	0.13	3
keratin	feather	quail	130	1.060–1.100†	3	0.19	4
keratin	feather	quail	130	1.080†	3	0.22	18
keratin	feather	chicken	130	1.080†	6	0.15	19
keratin	hair	human (beard)	25	nd	3	0.09	6
keratin	hair	horse (cut)	25	1.000†	16	0.16	20
keratin	hair	horse (ground)	25	1.000†	16	0.09	20
keratin	hair	bat	130	1.080†	?	0.17	29
keratin	hoof	cow	130	1.080†	6	0.15	19
keratin	wing	butterfly	130	nd	27	0.20	5
keratin	wing	butterfly	130	1.080†	3	0.19	18
kerogen	kerogen	Miocene	114	nd	2	0.06	17
kerogen	kerogen	Recent	114	nd	6	0.11	17
kerogen	protokerogen	types I-III & IIS	115	1.080†	2	0.12	28
lipid	fat	abdominal	130	1.060–1.100†	3	0.00	4
lipid	oil	olive	130	1.080†	3	0.00	18
protein	muscle**	pectoral	130	1.060–1.100†	3	0.20	4
protein	muscle**	unknown	130	1.080†	3	0.19	18
protein	organ**	liver	130	1.060–1.100†	3	0.19	4
protein	organ**	liver	130	1.080†	3	0.19	18
shell	shell	freshwater bivalve	150	1.080†	3	0.36	30

* NaOH pre-treated to increase exchangeability; ** delipified. nd = not defined (assumed constant).
§ calculated.
† assumed.

including butterfly wings,[5,18] cow hoof,[19] feathers,[3,4,18,19] hair,[6,20,29] and whale baleen[19] that were pre-treated with much higher temperatures (Table 2). The thermal stress to keratin equilibrated with steam may physically alter the primary and/or secondary structure of the material, modifying the number of sites available for exchange, as shown in the physical changes to the cuticle of human hair after cycles of wetting and drying.[32]

Alternatively, our use of an ambient temperature combined with what could be perceived as a short equilibration period may have prevented some intramolecular portions of material from reaching complete equilibration with water vapor. Wassenaar and Hobson[18] observed a variation among different types of keratin equilibrated at 25°C for 48 h of 15‰. Chamberlain *et al.*[3] also reported similar variation among feathers equilibrated at 25°C for 4 and 8 days, although the variability appeared to decrease after 8 days. In contrast, Bowen *et al.*[20] found that vapor-keratin exchange was complete within 3–4 days under conditions nearly identical to the ones used in this study. However, we cannot eliminate the possibility of incomplete equilibration, causing us to underestimate the fraction of exchangeable H atoms in our samples and leading to lower calculated f_{ex} values than previously published.

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Table 3. The δ^2H_{nex} values for ground hair unknowns analyzed alongside reference materials physically processed in a similar (ground, column B) and different (cut, column C) manner. Unknowns had been previously analyzed; original data are given in column A. The difference between original and new data (A–B) is given in column D. The difference between the data correction methods from this work (B–C) is shown in column E

	Original		This work			Difference	
	δ^2H_{nex} (‰)		δ^2H_{nex} (‰)			$\Delta\ \delta^2H_{nex}$ (‰)	
State	A: ground	Date	B: ground	C: cut	date	D: A-B	E: B-C
ID	−125	09/04	−124	−124	08/08	−1.2	0.2
MT	−120	09/04	−120	−120	08/08	0.0	0.4
ND	−115	09/04	−115	−116	08/08	0.4	0.7
CO	−110	09/04	−106	−108	08/08	−3.2	1.2
WY	−105	09/04	−105	−106	08/08	−0.6	1.3
NM	−99	06/04	−97	−99	08/08	−2.1	1.8
IL	−95	09/04	−93	−95	08/08	−1.7	1.9
IN	−90	09/04	−90	−92	08/08	−0.8	2.2
AR	−85	01/05	−86	−89	08/08	1.1	2.3
LA	−81	06/04	−81	−83	08/08	−0.5	2.7
Average						−0.9	1.5

We also assume that $\alpha_{ex} = 1$ and the isotopic composition of exchangeable H atoms in a completely equilibrated sample does not differ from the isotopic composition of the water vapor with which it equilibrated. If our assumption was incorrect, the calculated values for f_{ex} would also be incorrect. However, using theoretical α_{ex} values of 1.06, 1.08, and 1.10 would negligibly change the calculated exchangeable H value by less than 0.01.

Correcting sample materials using cut versus ground reference materials

In Table 3, we present the δ^2H_{nex} values and original analysis dates for ten ground human hair samples collected across the USA that were analyzed as part of a study investigating relationships between geography and the stable isotopic composition of hair.[7] The reference materials for the measurements in 2004–2005 were ground hair (FH and UH). Four years later, these same ten samples were again analyzed, but this time using either ground or cut FH and UH reference materials. There is a strong agreement in the measurements across years and irrespective of cut vs. ground reference materials. The δ^2H_{nex} values for individual hair samples agree well with the original analyses, varying on average by <1‰ between dates (column D, Table 3). We interpret this as an independent assessment that the preparation and analysis protocols using PIT result in highly reproducible data, regardless of time of year, ambient weather or atmospheric conditions, isotope ratio mass spectrometer specification changes, and/or laboratory personnel turnover.

In this study, the ground human hair samples were analyzed at the same time as ground and cut hair laboratory reference materials, which differed in known f_{ex} with values of 0.09 and 0.16 for ground and cut, respectively.[20] Data for human hair samples were corrected using both ground and cut reference materials; the corrected δ^2H_{nex} values are shown in Table 3. On average, the difference in correction methods was 1.5‰, below the accepted overall analytical precision of ±2‰. However, there were systematic differences between the calculated δ^2H_{nex} values using ground and cut reference materials.

As the δ^2H_{nex} value calculated using ground reference materials increased, the disparity between the two correction methods also increased. In other words, the difference between the two correction methods was greatest when the δ^2H_{nex} values were heavier. This systematic difference is perhaps unusual and associated with our analysis location (Salt Lake City, UT, with low atmospheric water vapor stable isotope values), because the relationship is dependent solely on the isotopic composition of the exchangeable H atom pool in the samples. Due to our location inland and at high elevation, the local precipitation (and thus, the local water vapor) is depleted in ^2H compared with many other locations in the contiguous USA.[12] Thus, when a sample is equilibrated in Salt Lake City, the exchangeable H atoms have a relatively depleted signal. For hair samples collected in a region similar to Utah (i.e., human hair from Idaho), the difference between the isotopic composition of the exchangeable and non-exchangeable pools of H atoms in the samples is small. For hair samples from a region with higher δ^2H values (i.e., human hair from Louisiana), the disparity between the isotopic compositions of the two H pools appears to be greater.

The maximum difference in exchangeable H atom fraction among keratin materials investigated here is 11%, ranging from cut wool at 6% to cut horsehair at 17% (Table 1). The difference between the actual δ^2H_{nex} value of a sample with ~6% exchangeable H atoms (e.g., cut wool) and the δ^2H_{nex} value calculated for that sample by using a reference material with ~17% exchangeable H atoms (e.g. horsehair) can approach and exceed an overall measurement precision value of ±2‰. Thus, it is important to recognize that f_{ex} values must be determined for the keratin material of interest so that any mismatch between sample and reference material f_{ex} values is minimized.

This discussion highlights several cautionary points when applying the comparative equilibration technique. Identical treatment can be overlooked at three levels:

1. The equilibration temperature (i.e., steam vs. ambient temperature) used to calculate f_{ex} could result in a mismatch between the calculated and actual size of the exchangeable H atom pool.
2. The physical processing of material could physically alter the exchangeable H atom pool size (e.g., ground vs. cut hair).
3. The type of material used for data correction and the types of material analyzed could have inherently different-sized pools of exchangeable H atoms (e.g., hair vs. cellulose[17] or shell[30]).

For the ground materials investigated in this work (keratin and muscle tissue), the variation among calculated f_{ex} values is minimal (<5%). Among ground keratin only, the

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difference is almost nonexistent (<1%). Thus, we feel that a ground keratin reference material (or set of ground keratin reference materials) could be used for data correction of all types of ground keratin samples. In most cases, a ground keratin reference material could also be used for data correction of cut keratin samples. However, depending on the isotopic composition of ambient water vapor, the error generated by over- or underestimating f_{ex} could result in meaningful differences among calculated δ^2H_{nex} values when using PIT. Thus, we believe that the processing of reference material and unknowns must be uniform at three levels: (1) calibration and calculation of f_{ex}; (2) physical processing; and (3) classification of materials by type. If the treatment is mismatched at any level, it must only be done with a full understanding of the potential uncertainty introduced by that mismatch.

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