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COMPARATIVE EQUILIBRATION AND ONLINE TECHNIQUE FOR DETERMINATION OF NON-EXCHANGEABLE HYDROGEN OF KERATINS FOR USE IN ANIMAL MIGRATION STUDIES

L. I. WASSENAAR[a],* and K. A. HOBSON[b]

[a]Environment Canada, National Water Research Institute, 11 Innovation Blvd., Saskatoon, SK, Canada, S7N 3H5; [b]Environment Canada, Canadian Wildlife Service, Saskatoon, SK, S7N 0X4

Stable hydrogen-isotope ratios (δD) of keratin provide a novel means for tracking geographical movements of birds and other species. Here we describe a rapid, low cost, analytical approach to facilitate online continuous-flow isotope-ratio mass spectrometry (CF-IRMS) δD analyses of keratins (120–160 samples per day) through the use of calibrated keratin working standards and "comparative equilibration" to correct for the effects of moisture on exchangeable hydrogen. It is anticipated that this analytical approach and CF-IRMS will greatly aid in providing cost effective and directly comparable δD results on keratins and feathers among various laboratories and researchers involved in animal migration studies.

Keywords: Birds migration; Deuterium; Feathers; Hydrogen exchange; Hydrogen isotopes; Natural variations; Standards

INTRODUCTION

The recent development of stable hydrogen isotope (δD) techniques to track migratory birds and mammals represents a significant advance over traditional mark-recapture methods in quantifying migratory connectivity [1, 2]. The stable hydrogen isotope tracking approach exploits the fact that predictable continent-wide patterns of deuterium abundance in rainfall occur on the continents and are transferred from plants through diet to upper trophic-level consumers such as birds and insects. Hence, stable-hydrogen isotopic analysis of metabolically inactive tissues, like feathers, can yield quantitative geographical information on natal origins of organisms [3, 4]. Since these first successful demonstrations of the utility of δD in determining breeding origins of birds and insects, this approach has rapidly gained acceptance, particularly in North America where subsequent studies have confirmed this relationship and have applied the hydrogen isotope approach to a variety of novel avian migratory questions [5–12]. Recently, interest has developed in applying this technique in Europe where numerous avian species migrate to sub Saharan Africa [13]. Numerous stable isotope

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* Corresponding author. Tel.: 306-975-5747; Fax: 306-975-5143; E-mail: len.wassenaar@ec.gc.ca

studies on migrating birds are currently underway across the US, Canada and Europe and reports in the scientific literature are expected to increase significantly in the next few years.

Despite mounting interest in using δD measurements to track large-scale terrestrial animal movement, several issues face researchers who wish to utilize this analytical tool to study animal migration. These include isotope laboratory accessibility, analytical costs, and the problem of uncontrolled hydrogen exchange in organic samples [14]. While the first two impedances are overcome by adequate project budgets, the latter is not. It is well established that δD measurements of feathers (and other organic materials) are problematical compared to $\delta^{13}C$ and $\delta^{15}N$ analyses due to the problem of uncontrolled hydrogen isotopic exchange between "exchangeable" organic hydrogen in the feathers with isotopically variable ambient moisture in the laboratory environment. If left uncorrected, δD measurements of the total hydrogen in an identical feather will yield different results at analytical laboratories located at different geographic locations, as well as over time due to geographical and seasonal changes in the hydrogen isotopic composition of ambient moisture [14]. The net result, unfortunately, is incomparable concerning δD results for feathers and other organic materials both among labs and studies. As stable hydrogen isotope research in avian studies increases and data begin to accumulate, it is imperative that keratin δD results among labs and studies be made comparable in order to link study results and to generalize our findings.

Previously, we described in detail an offline steam equilibration method for feathers and organics that provided δD measurements that were not influenced by uncontrolled hydrogen isotope exchange with ambient moisture in the laboratory. This approach also yielded isotopic values for the non-exchangeable hydrogen (δD_n) of feathers [14]. This offline method, however, required intensive preparations and dual-inlet hydrogen isotope analyses, and hence suffered from low sample throughput ($\sim$60 samples per week) and high cost due to labor costs and preparative equipment required. While this approach was a step forward in making δD results comparable among avian studies, its adoption today requires an adaptation to accommodate the most recent developments in hydrogen isotope analysis of organic compounds by online continuous-flow isotope-ratio mass spectrometry (CF-IRMS, [15]).

Here we describe a modification of our previous method of [14] to facilitate high-throughput CF-IRMS keratin deuterium analyses (120–160 samples per day), and a "comparative equilibration" approach through the use of calibrated keratin working standards. It is anticipated that this analytical approach and the use of CF-IRMS will greatly aid in providing researchers with cost effective and directly comparable δD results on feathers and other keratin-based samples among various laboratories and studies.

MATERIALS AND METHODS

Keratin Standards

We prepared three in-house keratin standards (chicken feather (CFS), cow hoof (CHS), and bowhead whale baleen (BWB-II)). The CFS was obtained from a single batch of chicken feathers from a poultry processing operation located in Wynyard, Saskatchewan, Canada. Approximately 2 kg of feathers were obtained. The CHS (approximately 500 g) was obtained by cutting hoof from a single cow carcass at an abattoir in Saskatoon, Canada. The BWB-II was a powdered whale baleen obtained from Don Schell at the University of Alaska (Fairbanks). Each keratin type was obtained from a single geographic location, and represented both terrestrial and marine end members in anticipation of obtaining a wide range in δD_n values.

All of these keratin standards were solvent cleaned (2:1 chloroform:methanol solution), dried, cryogenically ground, and homogenized using a mill in large enough quantities (>0.5 kg) sufficient to last several years in our laboratory The in-house keratin standards were then analyzed 6 times each using the offline equilibration dual-inlet method described by Wassenaar and Hobson (2000) and yielded the following results for δD_n: CFS $= -138 \pm 5$‰ (VSMOW), CHS $= -187 \pm 2$‰ (VSMOW), and BWB-II $= -108 \pm 4$‰ (VSMOW). The δD_n values reported for our in-house keratin standards include an assumed equilibrium isotope fractionation factor of 80‰ between the steam (130 °C) and the exchangeable hydrogen in keratin (see details in 14). All of these keratin materials had similar chemical and exchangeable hydrogen ($15 \pm 3\%$) properties as feathers.

A Comparative Equilibration Approach

Previous studies showed that hydrogen exchange between lab air moisture and exchangeable hydrogen in keratins is fast, and will reach equilibrium within 24–48 hours at room temperatures [5, 16], and in less time at higher temperature [14]. Because the previous offline steam equilibration approach was both costly and laborious and was required to be applied to all samples, an alternative solution was to air equilibrate pre-calibrated keratin working standards (see above) and unknown feather samples with lab air moisture. The co-equilibrated keratin standards and unknown feathers were then analysed in a single analysis session. The benefit of comparative equilibration is that it eliminated the need for offline steam equilibration of samples and the associated costly equipment, thereby significantly reducing analytical cost and processing time by not having to process samples offline. Further, this approach follows the recommended Principle of Identical Treatment (PIT) for stable isotope analyses, whereby samples and working standards are not only identical in their chemical composition, but also go through exactly the same preparation and analysis steps [17].

The target weight of feather (and in-house keratin standard) required for routine online hydrogen isotope analysis by CF-IRMS in our laboratory was 350 ± 10 μg. Feather samples were cut (not powdered) from the same position on each feather and weighed using a microbalance (Sartorius M2P) and transferred into 4.0 mm × 3.2 mm isotope grade silver capsules (Elemental Microanalysis, Part #D2000). Capsules containing the keratin standard powder or feather were folded into tiny balls. All weights were recorded and the samples were stored in 96 position culture trays (Elisa plates), loosely covered with the lid. Samples and keratin standards were then allowed to "air equilibrate" on the shelf with ambient lab air moisture at room temperature for >96 hours prior to stable hydrogen isotope analysis. The hydrogen exchange process could be sped up by holding the samples and working standards in an oven at higher temperatures since [14] showed that complete exchange with moisture occurs in less than 30 minutes at temperatures above 100 °C. For practical purposes, however, a 96-hour equilibration at room temperature was deemed sufficient and easier, and could be easily implemented as a standard laboratory analysis procedure. Following 96-hour comparative equilibration, all samples and standards were immediately loaded into the auto sampler carousel of the CF-IRMS and analysed for δD, as described below.

Online Pyrolysis and CF-IRMS Measurements

Stable hydrogen isotope measurements on feathers and in-house keratin standards were performed on H_2 derived from high-temperature flash pyrolysis and by CF-IRMS. Pure H_2 was used as the sample analysis gas and the isotopic reference gas. A Eurovector 3000™ high temperature elemental analyzer (EA) with auto sampler was used to automatically pyrolyse feather samples to a single pulse of H_2 gas (and N_2 and CO gas). The pyrolysis

column consisted of a standard ceramic tube with an inner glassy carbon tube, which was filled from the base to the heated zone with glassy carbon chips [see setup details in Ref. 15]. The pyrolysis column was held at 1270 °C and the GC column at 100 °C. The GC column used to resolve the sample H_2 from N_2 and CO was a 1 meter 5 Å packed molecular sieve column. The column flow rate was set to 100 ml He/min and the sample purge rate to 50 ml He/min. The resolved H_2 sample pulse was then introduced to the isotope ratio mass spectrometer (Micromass Isoprime[TM] with electrostatic analyser) via an open split capillary.

The δD of sample hydrogen was calculated by measurement of HD isotopes at a m/z ratio of 3/2 (after standard H^{3+} corrections) and comparison to 2 reference pulses (10 nA and 5 nA) of research grade (99.999%) H_2 gas having a δD of about $-71‰$ (VSMOW). The IRMS source was empirically tuned and tested for optimum source linearity for H_2 at a trap current of 400 μA (0.5‰ per nA or V). The two 30-second reference H_2 pulses were automatically introduced to the source using the dual-inlet reference and sample bellows. Samples were automatically dropped into the Eurovector EA pyrolysis furnace 15 seconds after initiation of the analysis run, with the sample H_2 pulse appearing in the IRMS at around 65 seconds. Single feather and keratin analysis time, after allowing for complete elution of N_2 and CO from the GC column, was 165 seconds.

All feather and keratin δD_n results are reported in units of per mil (‰) relative to the VSMOW-SLAP standard scale. Repeated analyses of hydrogen isotope inter-comparison material IAEA-CH-7 ($-100‰$ VSMOW), routinely included as a check, yielded an external repeatability of better than $\pm 1.5‰$ for δD_n.

RESULTS AND DISCUSSION

A typical example δD analysis of keratin standards and unknown migrant bird feathers are shown in Table I. The uncorrected hydrogen isotope values of the keratin standards from each CF-IRMS auto run (~2.5 hours for 50 samples) were subjected to a least squares regression to derive a correction formula to be applied to all the keratin standards and feather samples within that auto run (Fig. 1). A typical auto run included our in-house keratin standards at the beginning, 2 keratin standards after every 10–12 unknowns, and 2 or 3 keratin standards at the end of the auto run.

A further check on the quality of online δD analysis was to continuously monitor and record sample H_2 peak height and H_2 sample peak retention times, as shown in Table I. Peak height outliers sometimes occurred due to human sample weighing errors. Because of slight IRMS source non-linearity any samples weighed to 350 ± 10 μg that yielded peak heights greater or less than 1 nA (Tab. I) of the mean were discarded and repeated. In Table I this would correspond to a peak height of 9.5 nA, and so any samples with a peak height less than 8.5 nA and greater than 10.5 nA would be discarded. This criterion can be determined experimentally for each specific CF-IRMS instrument's source linearity. Alternately, because sample peak height correlates strongly with peak area, this parameter could also be routinely monitored as a quantitative and comparative measure of yield.

Of course, sample peak height is also a function of the H content of the sample, and so any minor variations in H content due to sample matrix or feather type were accommodated for by either adjusting the weight of the keratin standards or the weight of the samples to ensure an equivalent yield of H_2 gas by pyrolysis. To avoid unneeded complexity, we do not recommend mixing of different sample and standard types within a single auto run, as the PIT also recommends [16].

After ~180 samples, accumulation of Ag from the sample capsules will begin to induce column plugging. The symptom of column plugging is a shift in sample peak retention

TABLE I Selected δD Results From an Auto Run of $350 \pm 10\,\mu$g Feather Samples and All Keratin Standards (Boldface). Data Evaluation Includes Examination of Sample Retention Time (RT) and Peak Height. Final Recalculation of δD_n (VSMOW) is Based on a Least Squares Regression of the Known Keratin Standards as in Fig. 1. Sample 39 is an Outlier*, Likely Due to Weighing Error, and So Was Discarded in the Correction Due to Our Peak Height Criteria.

Sample number	Sample name	Peak retention time (Sec)	Peak height (nA)	δD (uncorrected)	δD_n (VSMOW)
1	CFS	64.6	9.9	-58.1	-141.0
2	CFS	64.7	10.3	-59.4	-142.6
3	CHS	64.7	9.8	-92.8	-184.7
4	CHS	64.6	10.3	-95.4	-188.0
5	BWB	64.6	10.0	-30.1	-105.7
6	BWB	64.5	10.2	-29.9	-105.5
7	f1	64.7	9.3	-21.7	-95.1
8	f2	64.8	8.9	-7.1	-76.7
9	f3	64.6	9.5	-35.6	-112.7
20	g2	64.7	9.8	-13.4	-84.7
21	g3	64.7	9.5	-17.9	-90.4
22	g4	64.9	10.1	-22.5	-96.2
23	CFS	64.7	9.9	-58.6	-141.6
24	CHS	65.0	10.0	-91.5	-183.1
25	BWB	64.7	9.5	-30.7	-106.5
26	g5	64.7	9.4	-21.6	-95.0
27	g6	64.7	9.6	-15.9	-87.8
35	h2	65.2	9.2	-19.7	-92.6
36	h3	65.1	9.5	-19.6	-92.5
37	h4	65.0	8.5	-16.1	-88.1
38	CFS	65.1	9.8	-57.9	-140.8
39	CHS*	65.1	6.7	-89.2	*
40	BWB	65.3	9.3	-31.5	-107.5

Note: Mean CFS $= -58.5$, Stdev $= 0.7$, n $= 4$; Mean CHS $= -93.2$, Stdev $= 2.0$, n $= 3$; Mean BWB $= -30.6$, Stdev $= 0.7$, n $= 4$.

time and concomitant drop in sample peak height (Tab. I), as well as variable results. Prior to reaching this point, removal and cleaning of the pyrolysis furnace column is required. The use of larger Ag capsules (e.g. 4×6 mm) resulted in earlier constriction of the columns, after $\sim$90 samples. Rejuvenation of the pyrolysis column simply required cooling and removal of the ceramic and glassy carbon tube, removal of the silver plug (it rolls out) and the glassy carbon chips from the carbon tube, sieving and cleaning of the chips to remove ash, and reassembly of the column as per manufacturer specifications. Our experience has shown that a single ceramic and glassy carbon tube combination will last for years, but we replaced our glassy carbon chips after about 2000 samples, based on visual inspection.

Frequently, $150\,\mu$g samples of IAEA CH-7 polyethylene intercomparison material (having no exchangeable H) and a value of -100‰ (VSMOW) are included in several sample auto runs each week to monitor instrument performance and also to observe the apparent effect of changes in ambient lab moisture on our keratin standards. Over a 3-month period in the spring of 2002 we observed a maximum variation of 25‰ in a single keratin working standard relative to CH-7 simply due to effects of seasonal changes in the isotopic composition of lab air moisture on exchangeable H.

In 2002, the cost of online keratin δD analyses ranges from \$20–35 USD per sample. As more laboratories make this type of δD analysis available the costs will likely decrease. Considering the cost of dual inlet organic δD analyses ($>$\$100) or, more importantly, the huge cost and efforts involved in largely ineffective conventional mark-recapture programs, it is clear that stable hydrogen isotope analyses heralds an effective and highly affordable means to begin to unravel migratory connectivity issues for many migrating species.
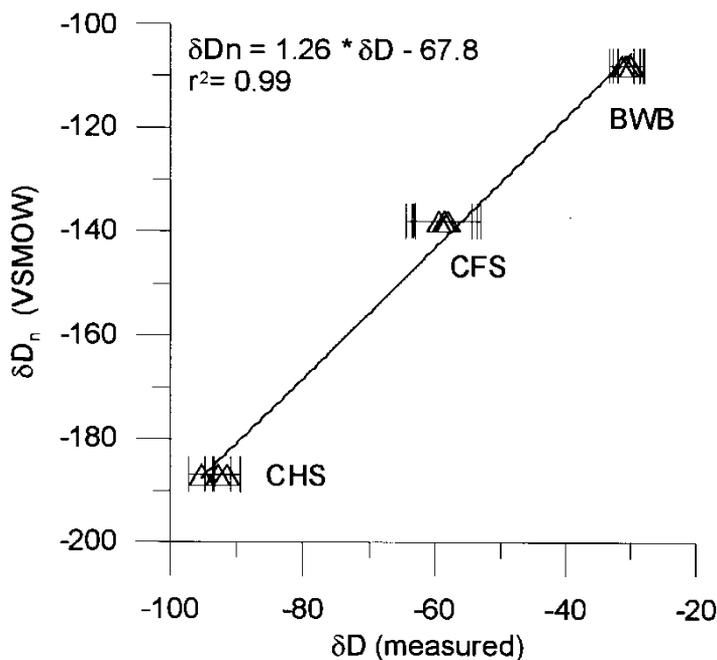
FIGURE 1   Least squares regression of δD measured versus δD$_n$ (with offline error bars) of keratin working standards (CFS, CHS, BWB) and correction equation applied to samples in a single auto run.

SUMMARY

Here we describe how online keratin δD analyses by CF-IRMS technology and the introduction of keratin standards and comparative equilibration can be used to provide cost effective and rapid δD analyses for large-scale animal migration research programs. We strongly recommend that researchers utilizing organic δD analyses adopt this protocol in order to provide findings that are comparable among laboratories. The development and distribution of feather and keratin working standards that have a wide range of δD$_n$ can be done on an individual basis, or preferably, developed and distributed as a future collaborative effort among avian researchers and stable isotope laboratories that provide feather δD analyses.

Acknowledgements

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