Stable-carbon and hydrogen isotope ratios reveal breeding origins of Red-winged Blackbirds

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Improved Method for Determining the Stable-Hydrogen Isotopic Composition (δD) of Complex Organic Materials of Environmental Interest

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Despite new and increasing applications in environmental studies, determinations of the stable-hydrogen isotope (δD) composition of complex organic substrates are hampered by laborious preparation techniques and uncontrolled isotopic exchange between labile hydrogen in the sample and ambient water vapor. To date, there has been little agreement in the way laboratories prepare, measure, and correct for uncontrolled hydrogen isotopic exchange in complex organic samples, resulting in incomparable δD results. Previously, nitration and preparative equilibration procedures aimed at controlling exchangeable hydrogen were designed for specific sample types (e.g. cellulose, chitin) but required extensive processing of individual samples. Here we describe a static, high-temperature preparative equilibration technique that provides increased sample throughput and δD measurements that are not compromised by uncontrolled isotopic exchange. By adopting this approach, it will be possible to compare complex organic δD measurements among laboratories. The current inconsistency in organic δD measurements among laboratories seriously hampers comparison of results among studies. As pyrolitic continuous-flow δD analyses become available, the preparative equilibration method described can be adapted to suit the smaller size requirements.

Introduction

In the past 2 decades, numerous studies have demonstrated the power of using measurements of naturally occurring stable isotopes of several elements in order to investigate ecological and environmental questions (*1*−*3*). In particular, the stable-carbon and -nitrogen isotope ratios of animal tissues have been used extensively to evaluate dietary sources and establish animal trophic relationships (*4*−*6*). To date, however, despite tremendous potential applications, relatively few studies have utilized stable-hydrogen isotope (δD) measurements to gain information on the ecology of organisms in diverse aquatic and terrestrial food webs (*7*−*11*). Because of the extensive variation in δD in nature between marine and terrestrial biomes, across continents as a result of climatic and meteorological processes (*12*), and from equilibrium and kinetic isotope effects (*13, 14*), stable hydrogen isotope measurements have tremendous potential application in ecosystem research. For example, δD measurements have been used successfully to quantify breeding origins of migratory songbirds and insects that migrate across naturally occurring hydrogen isotopic gradients in North America (*11, 15*). The quantitative information gained in such isotope studies can be used to improve wildlife management decisions and has recently focused attention on detrimental effects of genomics on nontarget species (*16*). Measurements of δD can also be used to evaluate biological isotopic fractionation (*8*), decipher trophic relationships (*7*), and aid in paleoclimatic reconstructions using fossil materials (*9, 17*).

Analytical Considerations. Stable-hydrogen isotope analyses of complex organic materials are significantly more complicated compared to the conventional measurements for δ^{13}C and δ^{15}N, and therefore relatively few studies have used δD measurements to address ecological questions. Despite recent advances in δD measurements by online-pyrolysis to improve sample throughput (*18*), δD analyses of biological and complex organic materials are generally hampered by laborious offline sample preparation techniques and the problem of uncontrolled hydrogen isotopic exchange between "labile" organic hydrogen in the sample matrix and ambient water vapor (*13, 19*−*22*). Unless the exchangeable proportion of hydrogen is accounted for and corrected, δD results from complex organic materials remain incomparable among laboratories. To date, however, there has been little agreement or uniformity in the way independent laboratories account and correct for hydrogen isotopic exchange in complex organic samples.

Although most of the hydrogen in biological tissues and complex organic matter is bound to carbon (C−H) and is not exchangeable with ambient moisture at low temperatures (*13, 19, 22*), a significant fraction of the total organic hydrogen, mainly in the form of −COOH, $-NH_2$, and −SH functional groups involving N−H and O−H bonds, readily exchanges hydrogen atoms with ambient water or vapor (*20*). For proteins, the proportion of such exchangeable hydrogen may be on the order of 20% or more of the total hydrogen (*13, 23*). Stable-hydrogen isotopic measurements of complex organic samples, on the other hand, can only be made on the total hydrogen (δD_t) content of a sample.

During the process of hydrogen exchange between organic materials and ambient water, there exists a temperature-dependent equilibrium isotopic fractionation (ϵ_{x-w}, in ‰) between the proportion of exchangeable organic hydrogen (f_e) in the sample and ambient moisture or equilibration water (δD_w). The laboratory measured isotope value for the total hydrogen (δD_t) in a sample can be expressed as follows (*13, 20, 22*)

$$\delta D_t = f_e \delta D_{ex} + (1 - f_e)\delta D_n \quad (1)$$

where δD_n is the isotopic composition of the carbon-bound nonexchangeable hydrogen and δD_{ex} is the exchangeable hydrogen. The fraction of exchangeable hydrogen (f_e) varies among organic matrices from 0.0 for simple hydrocarbons containing only C−H bonds (e.g. methane, lipids) up to 0.2−0.4 (e.g. 20−40% of the total H) for more complex substrates such as collagen, cellulose, and keratin (*13, 19*−*21, 24*). The value for the isotopic fractionation, ϵ_{x-w} (‰), between exchangeable hydrogen in the sample and ambient moisture

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can be defined as follows (*20*):

$$\epsilon_{x-w}(‰) \equiv 1000((\delta D_{ex} + 1000)/(\delta D_w + 1000) - 1) \quad (2)$$

A complication is that the isotopic value of the exchangeable hydrogens, δD_{ex}, cannot be measured directly, and the hydrogen isotope fractionation, ϵ_{x-w}, in eq 2 between water vapor and exchangeable organic hydrogen remains unclear for most complex organic samples. Values for ϵ_{x-w} have been derived for specific substrates such as cellulose and chitin where exchangeable hydrogens were eliminated using chemical preparative treatments such as nitration, and the results contrasted with equilibrated samples (*17, 19, 21*). Unfortunately, complete removal of exchangeable hydrogens by chemical replacement or nitration techniques is possible for only a few specific organic materials. δD analyses of most complex natural organic materials must therefore rely on preparative sample equilibration techniques to quantify hydrogen-isotope exchangeability by controlling the isotopic exchange parameters (*22*). Moreover, due to the temperature dependence of ϵ_{x-w}, it is crucial that such isotopic exchange preparations be conducted using precisely controlled isothermal (±0.1 °C) conditions (*19, 20*). Recently, (*20*) reported values of ϵ_{x-w} at various equilibration temperatures (25−150 °C) for organic compounds that ranged between −46‰ to +110‰. Values of +60‰ to +100‰ were typical of complex organics at higher equilibration temperatures. However, Schimmelmann et al. (*20*) recommend the use of a provisional ϵ_{x-w} value of +80‰ for complex natural organic substrates at higher temperature equilibrations, with a sensitivity analysis over a ϵ_{x-w} range of +60‰ to +100‰. Currently, such a range of ϵ_{x-w} values are believed to reflect a reasonable range in natural complex organic substrates, but this assumption requires careful consideration for each specific organic substrate analyzed. Nevertheless, simply disregarding the effects of hydrogen isotopic exchange with ambient moisture (e.g. samples stored on the shelf) will produce variable δD_t results that depend to a certain degree on the season of analysis and geographic location of the analytical laboratory (*12*). Such unpredictable analytical uncertainty and incomparable results among laboratories is unacceptable for environmental or ecological isotopic research.

Despite these challenges in applying δD measurements to complex organic samples, several approaches can be used to obtain significant environmental information from these materials. However, a preparative equilibration technique (or nitration if applicable) to correct for the exchangeable hydrogen is required. For comparative environmental studies using samples of identical or similar chemical composition and hydrogen exchangeability (e.g. fixed f_e and ϵ_{x-w} in eqs 1 and 2), δD measurements can provide important environmental information because *differences* among δD_t values of equilibrated samples ultimately reflect differences in the isotopic composition of the nonexchangeable hydrogen in the sample. Such an approach was used successfully to determine breeding origins of migrant songbirds and insects through the measurement of equilibrated δD_t values of feathers and insect wing membranes (*11, 15*). Also, based on a clearly stated provisional ϵ_{x-w} for mixed organic substrate types, but where proportions of exchangeable hydrogen differ among matrices, equilibrated sample δD_t values may be normalized according to the proportion of exchangeable hydrogen for each organic sample type. In such cases, the δD_t results also reflect differences in the nonexchangeable hydrogen portion of samples (*10*).

Previously published hydrogen isotopic preparative equilibration techniques for complex organics include high-temperature dynamic-flow steam equilibration (105−156 °C over 25 h) (*19, 20*) as well as static equilibration with water at temperatures of 0 °C to 92 °C over 20 h to 20 days (*22, 25, 26*). In all cases, rather extensive time periods were required to process individual samples. One alternate approach uses no direct preparative equilibration of samples (in actual fact a dynamic ambient moisture equilibration at room temperature) and correction of results by comparison to a chemically similar standard material stored and measured along with all samples (e.g. ref *13*). Although seemingly less complicated, an impractical aspect to this approach is that standard materials are required for all organic substrates of interest, sufficient standard material must be stored and analyzed concurrently with samples, and constantly changing isotopic exchange corrections due to varying room humidity δD and T must be maintained. With a rapidly increasing interest in the application of organic hydrogen isotope measurements to environmental investigations our goal was to significantly improve sample throughput over previous methods, to demonstrate an easily maintained and inexpensive preparative sample equilibration device, and to provide a reliable and reproducible sample equilibration and analysis procedure. The static, high temperature, preparative equilibration technique described here enables rapid and reproducible δD_n analyses for organic materials of ecological interest using dual-inlet isotope-ratio mass spectrometry. As online pyrolitic D/H stable isotopic analyses become commercially available in the future, this equilibration technique can be readily adapted to accommodate the smaller online sample size requirements.

Materials and Methods

Sample Processing. Thousands of environmental samples of ecological interest have been tested successfully for hydrogen exchangeability and δD_n measurements in our laboratory. Here we present selected results from a wide-ranging variety of materials of ecological interest including animal keratin (feathers, insect wing membranes), body tissues (lipid, liver, muscle, blood, yolk, albumen), plant matter (homogenized animal feed, cotton, plant leaves), oil, and humic acids (Na^+ form).

Because lipids have a wide range of δD values and are typically much more depleted in deuterium than other tissue types (*27, 28*), and since lipid content in tissues can vary among samples, all lipids and surface oils were removed from samples using multiple rinses in a solution of 2:1 chloroform:methanol (*29*). Samples, depending on their handling properties, were either dried in a fume hood (e.g. keratin), freeze-dried (e.g. animal tissues), or oven-dried (e.g. plant material) (80 °C) to remove residual water and solvent. For δD analysis of extracted lipids, samples were first sonicated for 1 h in solvent, and dissolved lipids were then filtered through a glass fiber (GFC) filter. The solvent solution was then evaporated at room temperature in a fume hood for approximately 48 h, and the remaining lipid sample was frozen prior to analysis.

The amount of hydrogen required for routine dual-inlet isotope-ratio analysis is generally on the order of 200−300 μg H. The mass of organic substrate required for preparative processing therefore depends on the total hydrogen content of the sample. The total weight-percent hydrogen for each sample type was directly assayed by commercial hydrogen elemental analysis or determined empirically using a range of weights and measuring hydrogen yield at the mass spectrometer. For example, most animal keratins (e.g. feathers, nails, hair) contain approximately 4−5 wt % hydrogen, and so about 7−8 mg of cleaned, dry sample material was required. All samples were solvent cleaned, dried, and stored in glass scintillation vials and subsequently handled using tweezers or spatulas to avoid contamination.

Samples were weighed into boats made from 2 cm lengths of 6 mm o.d. Vycor tubes sealed at one end or into 8 × 5 mm elemental analyzer tin cups (Elemental Microanalysis).

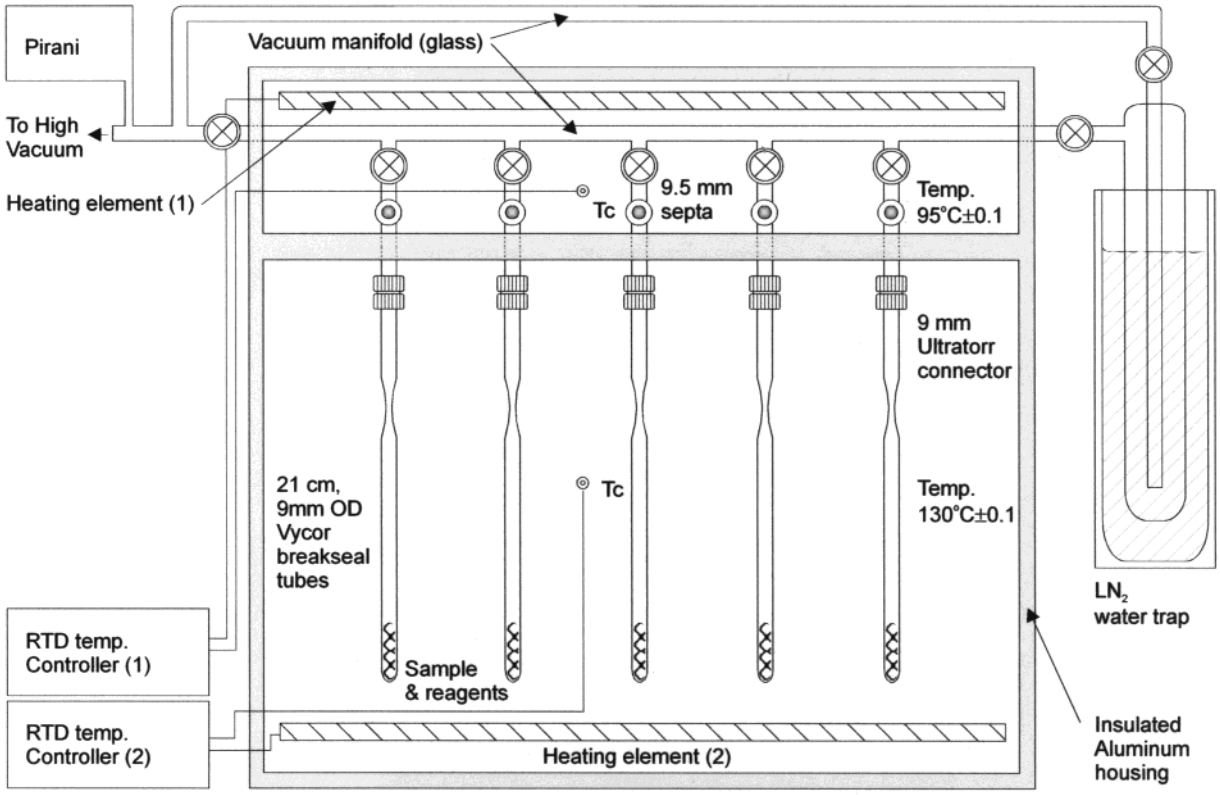

FIGURE 1. Schematic diagram of the sample hydrogen equilibration manifold: Tc, thermocouple and LN_2, liquid nitrogen water trap.

Problems with static charge generally prevented direct loading of powdered samples into large sample breakseal combustion tubes. Vycor boats or tin cups containing the sample were introduced gently into 21 cm × 9 mm o.d. Vycor breakseal tubes over 1 g of cupric oxide and 2 cm of pure silver wire. The breakseal tubes were then "necked down" using a flame torch, placed on the equilibration manifold, evacuated, and equilibrated, as described below.

Static Equilibration System. Preparative equilibration of organic samples with steam of known hydrogen isotopic composition was done using a simple static equilibration manifold shown in Figure 1. This equilibration device consisted of a custom-built Pyrex vacuum manifold to which 10 or more sample ports were attached (five shown). Each sample port consisted of a high vacuum valve, septum port, and 9 mm UtraTorr fitting, to which the loaded sample breakseal tubes described above were attached. The entire manifold was enclosed in a reflective and insulated aluminum housing. The front panels were attached using hinges to allow easy access for attaching and sealing samples and cleaning the valves. The upper (i.e. the section containing the connection manifold) and lower sections of the apparatus were separated by an insulated divider and were individually heated by standard strip heating elements controlled by 2 Barnant Model 621-8620 RTD digital temperature controllers and thermocouples. The upper portion was held at 95 ± 0.1 °C, the maximum allowable temperature for the high vacuum valves, whereas the lower section containing only the sample vessels was kept at 130 ± 0.1 °C.

After breakseal tubes containing samples were attached to the manifold, the front panel was closed, and each sample valve carefully opened in turn to fully evacuate the samples. Samples were allowed to degas for ~30 min or more (or until 1×10^{-4} Torr) as the system reached 130 ± 0.1 °C in order to drive off all adsorbed moisture. Once thermal stability was achieved and all adsorbed moisture was removed the valves were closed. Samples were then equilibrated individually with 300 μL of water of known isotopic composition injected through the individual sample septum. The δD isotopic composition of injection waters used were −135, −73, +115, +312, and +525 ‰ relative to the Vienna Standard Mean Ocean Water (VSMOW) standard. This injection H_2O immediately volatilized to steam and equilibrated with the sample at 130 ± 0.1 °C. The use of equilibration temperatures below 100 °C is not recommended because we found water droplets inside the combustion tube and manifold apparatus. Conversely, the use of equilibration temperatures above 150 °C may lead to thermal degradation of samples, and therefore isothermal equilibration temperatures between 110 and 140 °C are recommended (*19*).

We also conducted sample equilibration experiments at 25 °C using 7.5 mg keratin samples in the presence of 10 mL of water enclosed for 48 h in sealed Vacutainers (data not shown) and found that repeated measurements on the same sample yielded poor reproducibility (e.g. ±15‰). Such poor reproducibility was also found by others using a similar approach (*25*). This suggested that hydrogen isotopic exchange was either incomplete over a 48-h period of equilibration or was otherwise complicated by the presence of water in two phases.

The amount of water (steam) required can be precisely calculated and optimized for specific isotopic exchangeability and sample mass; however, we determined that 300 μL of H_2O was generally sufficient, since the ratio of steam hydrogen to exchangeable hydrogen was usually >95:1 for most sample types we have encountered. For the equilibration system described here, injection of >500 μL of H_2O resulted in condensation of water droplets in the sample vessels, leading to excessive water removal times and potential fractionation effects between water vapor and droplets. Following sample equilibration, valves were carefully opened in sequence, and all samples were evacuated for 1 h or more (or until $<1 \times 10^{-4}$ Torr) at 130 ± 0.1 °C to completely remove all residual water vapor. The water removed was collected in a large

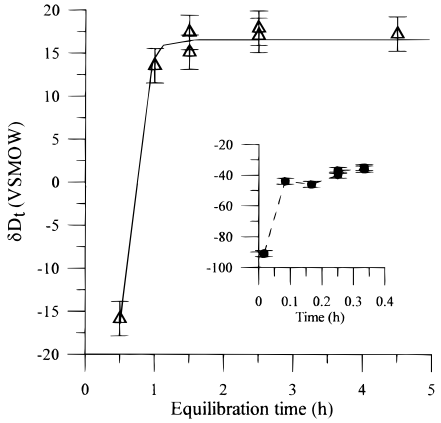

FIGURE 2. Rates of hydrogen isotopic equilibration between exchangeable hydrogens in butterfly wing membranes (keratin) and water vapor at 130 ± 0.1 °C over time frames of 30 min to 5 h. Inset graph denotes the early part of equilibration from 0 to 25 min for Quail feather keratin. For both samples equilibrium was reached between 1 and 2 h.

cryogenic trap located before the vacuum pumps. This preparative equilibration procedure effectively replaced all available exchangeable hydrogen with hydrogen of fixed isotopic composition.

The Vycor sample breakseal tubes containing fully equilibrated and evacuated organic samples were then individually flame sealed under vacuum. Samples were combusted at 850 °C in a muffle furnace for 2 h, followed by slow cooling. The waters produced from sample combustion were quantitatively separated from CO_2 and N_2 on a vacuum preparation line. These waters were then cryogenically transferred to 6 mm Pyrex break seal vessels containing 120 mg of zinc alloy (Biogeochemical Laboratories, Indiana University). The 6 mm Pyrex vessels were sealed and placed in a heating block and reacted at 510 °C for 35 min to reduce all H_2O to H_2 gas. Yields of CO_2, N_2 (on the cryogenic preparation line), and H_2 (on the mass spectrometer) were recorded for each sample as a check to ensure quantitative sample yield. Invariably, poor δD results could be traced back to sample preparation errors or low gas yield. Stable hydrogen isotope measurements on sample H_2 gas were performed using dual inlet isotope-ratio mass spectrometry (Micromass Optima) at the National Water Research Institute, Saskatoon. Results are expressed in the typical delta notation, in units of per mil (‰), and normalized on the VSMOW-SLAP standard scale. Repeated analyses of hydrogen isotope intercomparison material IAEA-CH-7 (−100‰), routinely included as a check, yielded a long-term external repeatability of better than ±2‰ for δD.

Results and Discussion

Rate of Equilibration. Using the static equilibration technique described above, the rate of hydrogen equilibration at 130 ± 0.1 °C was determined for various organic sample types over time intervals from minutes to days. Experimental samples for the determination of rates were equilibrated with +525‰ injection waters in order to gain a significant change in the measured sample δD_t. A representative set of time series experimental results for two homogenized keratin samples (feathers and insect wing membrane) are shown in Figure 2. Measurements of δD_t versus time showed that under the experimental conditions, full equilibration was reached after about 1.5 h. Similar results were obtained for numerous other organic matrices (e.g. blood, muscle, plants), and therefore a 2-h equilibration time was deemed sufficient for most sample types. A comparison of 2- and 20-h sample

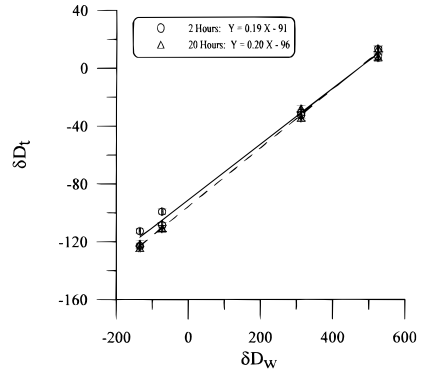

FIGURE 3. Comparison of monarch butterfly wing membrane (keratin) equilibrations using a wide isotopic range of vapors for 2 and 20 h at 130 ± 0.1 °C.

equilibration with a range of equilibration waters showed little difference in δD values and confirmed that a 2-h equilibration was satisfactory (Figure 3). This improvement in preparative equilibration time alone represented a significant advance in sample processing compared to the 20-h to 20-day equilibration times used previously (e.g. refs *19, 25, 26*) and does not require maintaining and analyzing concurrent reference materials for each sample type (e.g. ref *13*). Nevertheless, equilibration rates should be determined for the organic sample type of interest under the experimental temperature and equilibration conditions used.

Measurement Repeatability. Repeated analyses of intercomparison reference materials (IAEA-CH-7) were always included within sample groups to ensure quality control. Results from IAEA-CH-7 yielded values of −100 ± 2‰ (n = 127). Replicated 2-h equilibration of single samples with injection steam of −135‰ yielded the following selected examples of δD_t reproducibility (normalized to VSMOW): powdered and homogenized common milkweed (*A. syriaca*) −60 ± 2‰ (n = 7) and commercial poultry starter food (homogenized) −49 ± 2‰ (n = 3). Nonhomogenized sections of a single butterfly (*D. plexippus*) wing membrane equilibrated with injection water of +525‰ yielded a δD_t of +2 ± 2‰ (n = 6). In another experiment, five individual tail feathers (nonhomogenized) taken from a single Japanese quail (*Coturnix coturnix japonica*) (see ref *10*) were equilibrated with injection water of +525‰ and yielded a mean δD_t of −4 ± 6‰ (n = 5). In the latter two cases, no attempts were made to homogenize the samples and so are a reflection of hydrogen isotopic variability among like tissues within an individual organism.

Quantification of Hydrogen Exchangeability. To determine the proportion of hydrogen exchangeability, f_e, a series of controlled isothermal isotopic equilibrations with steam having a wide range of δD values was performed on each specific organic sample type. A plot of δD_w versus δD_t yielded a series of straight line relationships (Figure 4), the slopes being a function of f_e and the equilibrium fractionation factor ϵ_{x-w} (*19, 22*). Several approaches have been used previously to determine hydrogen exchangeability on organic substrates. Several authors describe the slope of these lines solely as a function of hydrogen exchangeability by implicitly or explicitly assuming a constant ϵ_{x-w} (*13, 25*), which is a valid approach provided all samples are chemically identical. However, a more rigorous approach requires accurate knowledge of ϵ_{x-w}, determined experimentally through nitration techniques and elimination of exchangeable hydrogen forms, although as indicated earlier, this process is not feasible for most complex organic substrates. Instead, a simple two end-member isotopic equilibration procedure developed by Schimmelmann et al. (*20*) was used to calculate the proportion of hydrogen exchangeability (f_e) for tissues

TABLE 1. δD_n Determinations (±SD) of Test Samples Using Measured Total Hydrogen Values after Paired Equilibrations with a Range of Equilibration Water Vapors and Assumed ϵ_{x-w} Values of +60‰ to +100‰ (*20*) and Eq 4[a]

	measured parameters (VSMOW)					δD_n (VSMOW)			% H exchangeability (of total H)		
sample	δD_{ta}	δD_{tb}	δD_{wa}	δD_{wb}		$\epsilon = 60$‰	$\epsilon = 80$‰	$\epsilon = 100$‰	$f_e, \epsilon = 60$ ‰	$f_e, \epsilon = 80$ ‰	$f_e, \epsilon = 100$ ‰
whole blood	−76	+46	−135	+525		−75	−78	−82	0.17	0.17	0.17
(freeze-dried)	+8	+46	+312	+525		−69	−73	−76	0.17	0.17	0.16
	−76	+8	−135	+312		−74	−78	−82	0.18	0.17	0.17
					mean:	−73 ± 3	−76 ± 3	−80 ± 3	17.3 ± 0.01	17.0 ± 0.01	16.6 ± 0.01
muscle	−80	+58	−135	+525		−79	−83	−87	0.20	0.19	0.19
(lipid free)	+15	+58	+312	+525		−75	−79	−83	0.19	0.19	0.19
	−80	+15	−135	+312		−79	−83	−87	0.20	0.20	0.19
					mean:	−78 ± 2	−82 ± 2	−86 ± 2	19.6 ± 0.01	19.3 ± 0.01	0.19 ± 0.01
liver	−130	+6	−135	+525		−142	−146	−149	0.19	0.19	0.19
(lipid free)	−40	+6	+312	+525		−149	−153	−156	0.20	0.20	0.20
	−130	−40	−135	+312		−142	−145	−149	0.19	0.19	0.18
					mean:	−145 ± 4	−149 ± 4	−152 ± 4	19.3 ± 0.01	19.3 ± 0.01	19.0 ± 0.01
feathers	−135	+19	−135	+525		−149	−154	−158	0.22	0.22	0.21
(quail)	−73	+19	+115	+525		−141	−145	−149	0.21	0.21	0.20
	−134	−73	−135	+115		−149	−154	−158	0.23	0.23	0.22
					mean:	−147 ± 5	−151 ± 5	−155 ± 5	22.0 ± 0.01	22.0 ± 0.01	21.0 ± 0.01
turkey feed	−75	+73	−135	+525		−73	−78	−82	0.21	0.21	0.20
	+27	+73	+312	+525		−68	−73	−77	0.21	0.20	0.20
	−75	+27	−135	+312		−73	−78	−82	0.21	0.21	0.21
					mean:	−71 ± 3	−76 ± 3	−80 ± 3	21.0 ± 0.01	20.6 ± 0.01	20.3 ± 0.01
cotton	−37	+126	−135	+525		−23	−28	−34	0.23	0.23	0.22
(medical)	+73	+126	+312	+525		−24	−30	−35	0.23	0.23	0.23
	−37	+73	−135	+312		−23	−29	−34	0.23	0.23	0.22
					mean:	−24 ± 2	−29 ± 2	−34 ± 2	23.0 ± 0.01	23.0 ± 0.01	21.3 ± 0.01
humic acid	−128	−67	−135	+312		−135	−137	−139	0.13	0.13	0.12
(Aldrich Co.)	−92	−116	+115	−73		−130	−132	−134	0.12	0.12	0.12
					mean:	−132 ± 4	−134 ± 4	−137 ± 4	12.5 ± 0.01	12.5 ± 0.01	12.0 ± 0.01
olive oil	−133	−136	−135	+525		−133	−133	−133	0.00	0.00	0.00
(100% virgin)	−138	−135	+115	+525		−139	−140	−140	0.00	0.00	0.00
	−135	−136	−73	+525		−135	−135	−135	0.00	0.00	0.00
					mean:	−136 ± 4	−136 ± 4	−136 ± 4	0.0 ± 0	0.0 ± 0	0.0 ± 0
butterfly	−123	+13	−135	+525		−133	−136	−140	0.19	0.19	0.19
(wing keratin)	−31	+13	+312	+525		−133	−137	−141	0.19	0.19	0.19
	−123	−31	−135	+312		−133	−136	−140	0.19	0.19	0.19
						−133 ± 2	−137 ± 2	−140 ± 2	19.0 ± 0.01	19 ± 0.01	19 ± 0.01

[a] Comparison of hydrogen exchangeability (f_e) determinations of selected individual complex organic sample matrices using eq 3 (±SD). All test sample-water vapor equilibrations were conducted 130 ± 0.1 °C. Subscripts in measured parameters columns are defined in eq 3.

(T) based on tissue δD_t values equilibrated with one or more trials of two waters (W) having widely separated δD values

$$f_e = (\delta_{TA} - \delta_{TB})/((\delta_{WA} - \delta_{WB})(1 + \epsilon_{x-w}/1000)) \quad (3)$$

where the A and B subscripts refer to widely separated equilibration waters. Equation 3 requires knowledge or a provisional estimate of ϵ_{x-w}. We used two or more pairs of static equilibration experiments for each organic sample type to determine an average substrate f_e (e.g. −135‰ vs +525‰, +312‰ vs +525‰, −135‰ vs +312‰), allowing a provisional ϵ_{x-w} to range between +60 and +100‰ (Table 1). The results of a series of static equilibrations for various organic sample matrices are shown in Figure 4. It is readily apparent that the slopes of the regressions are indeed primarily a function of hydrogen exchangeability. For example, as anticipated, olive oil having no exchangeable hydrogen showed no response to equilibration with a wide isotopic range of waters. Ancient humic acid showed smaller hydrogen isotope exchange effects than modern materials. Modern organic and tissue samples, on the other hand, all had a significant response to isotopically varied equilibration waters. For comparison purposes, we provided the results of a series of determinations of hydrogen exchangeability derived using eq 3 and a range of provisional ϵ_{x-w} for various sample substrates (Table 1). Overall, hydrogen exchangeability determined this way was only slightly less than using the slopes of the regression as a proxy for % exchangeable hydrogen (Figure 4 and Table 1). For example, humic acid, feather, cotton, and poultry starter feed were all about <1−2% lower in estimated exchangeable hydrogen when the provisional range of ϵ_{x-w} was taken into consideration. For this reason, we recommend using an average of the values for δD_n derived from multiple two-endpoint estimates and a sensitivity analysis over a range of values expected for ϵ_{x-w}.

For routine processing of samples of identical composition we suggest the preparative equilibration procedure outlined in Figure 5. Thus, to determine δD_n values for specific organic substrates the following information is required: δD_w of the equilibration water, the δD_t of the equilibrated sample (all δD results normalized on VSMOW/SLAP scale), the experimentally determined value for f_e for that substrate (eq 3), and measured or assumed provisional value for ϵ_{x-w} (‰) for the sample type. The δD_n value of similar unknown samples are then calculated using

$$\delta D_n = (\delta D_t - (f_e * \alpha * \delta D_w))/1 - f_e \quad (4)$$

where $\alpha = \ln^{-1}(\epsilon_{x-w}/1000)$. Determinations of δD_n for a variety of organic sample substrates are presented in Table 1 using a range of provisional ϵ_{x-w} values, our experimentally measured δD_t, δD_w, f_e, and eq 4. For each provisional ϵ_{x-w} the results of 2−3 repeated equilibration pairs generally yielded δD_n precisions better than ±4‰ for δD_t (Table 1). These precisions are acceptable for ecological and stable isotope studies. A sensitivity analysis using provisional ϵ_{x-w} values

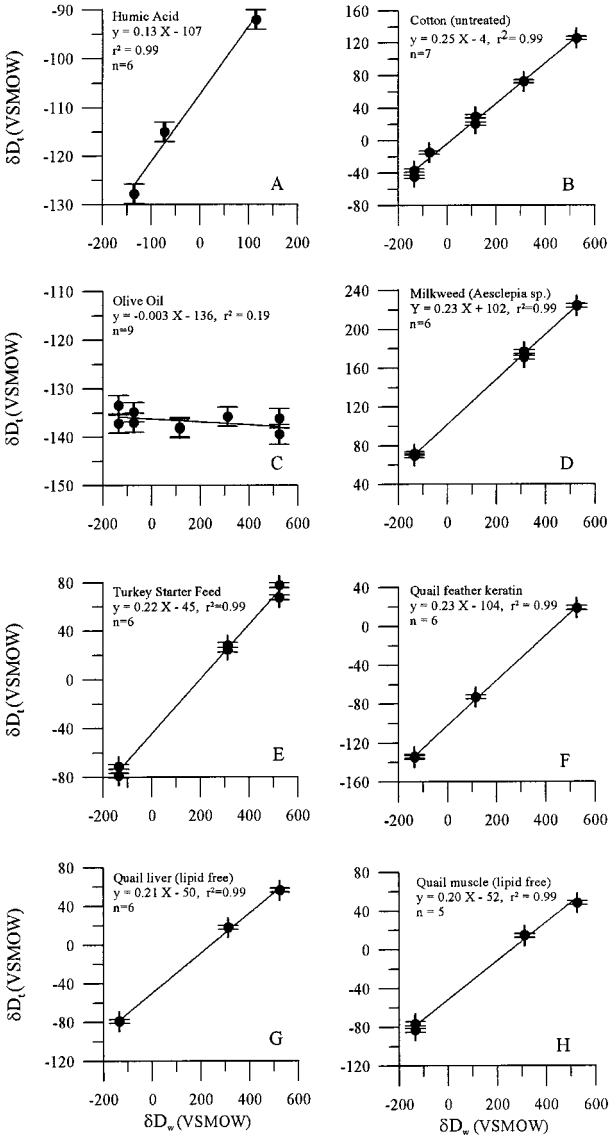

FIGURE 4. A series of 2-h equilibrations (130 ± 0.1 °C) of selected complex environmental organic sample matrices and the resulting least squares regression of δD_w versus δD_t. Note the scales on the *Y*-axis are not identical.

ranging from 60‰−100‰ (*20*) showed a spread of only 5−10‰ for δD_n (Table 1), demonstrating that a large range in uncertainty for ϵ_{x-w} values do not significantly affect δD_n. For analyses of chemically identical substrates, ϵ_{x-w} may be considered to be constant; therefore in actual practice the δD_n precisions anticipated using this approach are better than ±4‰.

Toward a Consistent Approach for δD Measurements in Organics. We have described a reliable and improved method for preparative equilibration of complex organic materials to be used in ecological and environmental studies involving δD measurements. This method provides δD_n measurements that are not influenced by uncontrolled hydrogen isotope exchange with ambient moisture in the laboratory. If other laboratories adopt such a rigorous approach, it would be possible to compare organic δD_n measurements among laboratories. Currently, the lack of consistency severely hampers direct comparison of organic δD_n results among studies and laboratories. A solution to this situation is becoming urgent as interest in δD_n measurements of complex organic materials becomes increasingly widespread in environmental and ecological studies.

Organic Hydrogen-Isotope Sample Equilibration Procedure

1. Using clean, dry organic sample Determine wt. % H
2. Experimentally determine equilibration time on representative sample (e.g. Figure 2)
3. Determine / assign ϵ_{x-w} for each substrate type
4. Experimentally determine % exchangeable hydrogen (f_e) (Equation 3)
5. Equilibrate samples with δD_w (Figure 1)
6. Measure Sample δD_t
7. Determine sample δD_n using measured δD_t, δD_w, f_e, and provisional or determined ϵ_{x-w} (Equation 4)

FIGURE 5. Recommended organic δD sample equilibration and processing procedure. For each sample type (e.g. keratin, chitin, cellulose) steps 1−4 are required initially. Once completed, routine sample processing involves only repeating Steps 5−7.

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