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To shred roughly or to grind? On the methods of sample preparation of the keratinous material for stable hydrogen isotope analysis

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Hydrogen stable isotope measurement in keratin samples (hair, feathers, etc.) is one of the very popular, but rather complex tasks of isotope ratio mass spectrometry. The complexity of such analyzes is associated with the rapid contamination of samples with hydrogen from atmospheric moisture. The effects of various methods of sample preparation on the measured values of δ2H is still an emerging issue in this field of study. The method of hair shredding practically does not affect the dynamics of the atmospheric moisture sorption by the samples. However, despite the same amount and isotopic composition of sorbed water, the measured δ2H values for roughly shredded and grinded hair can vary significantly and unpredictably. Equilibration the hair water content with the atmospheric moisture, even for a long time, does not completely erase the “isotope history” of the sample, which requires a review of standard sample preparation procedures.

Keywords: IRMS, deuterium, hair, mass-spectrometry, water, sorption.

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Introduction

Analysis of stable hydrogen isotopes (δ^2H) in hair and other keratin-containing tissues is applied widely. Keratin δ^2H values are used to trace the migration of birds and other animals (Chamberlain et al., 1996; Hobson and Wassenaar, 1996; Ramos et al., 2009), in anthropology (Ehleringer et al., 2008; Nardoto et al., 2006; Thompson et al., 2010), and for the forensic issues (Dunn and Carter, 2018; Meier-Augenstein and Fraser, 2008).

Nowadays, isotope ratio mass spectrometry (IRMS) is the main method for determining the isotopic composition. Despite the high accuracy of analysis and ease of handling of modern mass spectrometers, the analysis of stable hydrogen isotopes (δ^2H) in keratin materials is a rather complicated task itself. The influence of the atmospheric water on the analyzed material is the main technical difficulty when applying this method. Moisture affects keratin in two ways. Firstly, hair is a very hygroscopic material; therefore, it quickly sorbs atmospheric moisture up to 10–20% of its dry weight. Secondly, the hydrogen of keratin uncontrollably exchanges with the hydrogen of water molecules in the environment (Bowen et al., 2005; Meier-Augenstein and Kemp, 2012; Wassenaar and Hobson, 2000).

Although most of the hydrogen in complex organic compounds is directly bonded to carbon (CH– bond) and is not involved in the exchange, hydrogen atoms that are involved in functional groups, such as $–NH_2$, –COOH, –OH, –SH, easily exchange with the hydrogen of the external environment (Meier-Augenstein and Kemp, 2012). The share of such labile hydrogen varies in different organic substances from 0% for simple hydrocarbons containing only CH bonds to 20–40% of the total amount of hydrogen in complex substrates, such as collagen, cellulose, and keratin (Wassenaar and Hobson, 2000). Thus, hydrogen in keratin samples exists in three unequal states: exchangeable and non- exchangeable hydrogen of keratin molecules and hydrogen of sorbed water. For most of the practical tasks, it is important to find the δ^2H values of the non-exchangeable hydrogen, while the δ^2H values of sorbed water and the exchangeable hydrogen are a polluting factor that must always be taken into account when measuring the isotopic composition of hydrogen in keratin materials.

There are two main methods for obtaining δ^2H values of non-exchangeable hydrogen in keratin samples: the comparative two-point end-member equilibration method (Wassenaar and Hobson, 2003) and the contemporaneous two-stage equilibration method (Bowen et al., 2005).

The comparative two-point end-member equilibration method implies that the test samples are equilibrated with the atmosphere in the laboratory simultaneously with two keratin standards. During the process of equilibration, the atmospheric moisture is sorbed by the samples and hydrogen atoms are exchanged between the sorbed water and the exchangeable hydrogen in keratin. From a technical point of view, equilibration is extremely simple: samples are exposed to the laboratory atmosphere in open containers for several days. It is assumed that, upon completion of equilibration, both the studied samples and isotopic standards will have the same isotopic composition of the exchangeable hydrogen, corresponding to the isotopic composition of atmospheric moisture, and both the volume and the isotopic composition of the sorbed water will be the same. At the end of equilibration, a simultaneous (within one session) measurement of δ^2H of samples and standards is performed. The true value of the δ^2H in sample (δ^2H_{true}) is determined based on a linear regression analysis (Dunn and Carter, 2018; Wassenaar and Hobson, 2003):

$$\delta^2H_{true(sample)} = \delta^2H_{true(RM1)} + \frac{(\delta^2H_{raw(sample)} - \delta^2H_{raw(RM1)}) \times (\delta^2H_{true(RM1)} - \delta^2H_{true(RM2)})}{\delta^2H_{raw(RM1)} - \delta^2H_{raw(RM2)}},$$

where δ^2H_{true} is the true value of δ^2H of the non-exchangeable part of hydrogen, δ^2H_{raw} is the measured value of δ^2H; RM1 and RM2 are isotopic standards.

It is obvious that one does not need to know either the isotopic composition of atmospheric water or the humidity in the laboratory, which could affect the amount of sorbed moisture to use this method and follow the equation. Regard must be paid only to the parameters of the sorbed and exchangeable hydrogen, which should be the same in the samples and standard. This, in turn, implies that the measurements should be performed in a fairly short time period (i.e., within a few hours), so the atmospheric parameters will not change significantly.

The contemporaneous two-stage equilibration method (Bowen et al., 2005) implies the dividing of the test sample into two parts, each is equilibrated with water of known isotopic composition during four days. After equilibration, the samples are freeze-dried for 7 days to remove the traces of residual moisture. In order to avoid the contact of equilibrated samples with atmospheric moisture, the samples are then transferred as soon as possible (within 30 minutes after the drying has been stopped) to a special automatic sampler isolated from the environment (zero-blank or similar models).

The contemporaneous two-stage equilibration method is extremely complex and requires certain equipment to isolate samples from atmospheric moisture. Thus, for most researchers, the comparative two-point end-member equilibration method (Wassenaar and Hobson, 2003) is the only one that could be performed.

The influence of the method of sample shredding on the measurement results is one of the emerging issues of δ^2H hair analysis. It would seem that the method of sample shredding should not affect their isotopic composition. However, the measured δ^2H values of roughly shredded and grinded samples may vary significantly (Bowen et al., 2005). In our experiment, we have examined the water sorption / desorption dynamics in the human hair and hair of domestic mammals that were grinded in different ways in order to determine whether this affected the amount of sorbed water.

Another emerging issue is the rate of equilibration of the samples with the atmosphere of the laboratory. The time required for atmospheric equilibration varies significantly as reported by researches, ranging from one day (Wassenaar and Hobson, 2003) up to 35 days (Smith et al., 2009). In order to check the time necessary for equilibration, we saturated artificially the exchangeable hydrogen of the hair with deuterium, and then observed the emission dynamics.

Materials and Methods

The human hair and the hair of domestic mammals have been used as the test objects. Samples of non-dyed hair were taken from the 7-year old girl (“child”) and from the women: 70-year old (“gray”), 20-year old (“straight”), and 19-year old (“curly”). The hair of

domestic mammals was taken from a domestic goat (5-year old, female) and the Alaskan malamute breed dog (1-year old, male). Samples were marked by the shredding method and hair type (Table 1).

Approximately 1 gram of hair was taken from each test object. All samples were washed three times for 40 minutes in the 50-mL Falcon tubes in an ultrasonic bath (Derui ultrasonic cleaner DR-MS07) in the chloroform – methanol mixture (2:1 v:v) in order to remove lipids (O'Connell et al., 2001). After washing, the samples were dried in a vacuum desiccator for 48 hours. Then the samples were roughly shredded into 5–10-mm long fragments and divided into two parts. One part was grinded in a Retsch MM 200 vibratory ball mill (Laboratory of Soil Zoology and General Entomology of the A.N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences), the other part remained roughly shredded.

In order to track the sorption/desorption dynamics, the hair was divided into the two batches, no. 1 (A1, B1, A2, B2, A3, B3) and no. 2 (A4, B4, A5, B5, A6, B6). Batch no. 1 was placed in a vacuum desiccator, no. 2, in a desiccator, above the water level, for 48 hours. Then the roughly shredded and grinded samples exposed to the atmosphere after vacuum drying or under vapor saturation were weighted during two days in laboratory conditions at temperature 22 ± 1 °C and relative humidity 30 ± 5% on a Mettler Toledo WXTS3DU balance (1-μg accuracy). During the first 30 minutes after opening the desiccator, the samples were weighed with a minimum interval (2–3 minutes), then, as the measured masses reached a plateau, the time interval was increased. The experiment was repeated four times, the batches of samples subjected to drying and wetting changed each time. Therefore, the dynamics of sorption and desorption were measured twice for each batch.

The second part of the study aimed on assessing the variability of the measured δ^2H values for roughly shredded and grinded hair and the time necessary for atmospheric equilibration of the samples prior to analysis. Four samples have been taken for this study in accordance to the results of the first part of the experiment. The samples A4 and B4 were characterized by the largest difference of the water sorption/desorption rates between the roughly shredded and grinded forms, and A2 and B2, by the lowest difference. Then each sample (roughly shredded/grinded) was divided into two equal parts. The roughly shredded samples were further cut to a length of 2–3 mm. In order to assess the dynamics of equilibration the samples with the atmosphere of the laboratory, one half of the samples was equilibrated with the distilled tap water, the other, with the heavy water, for 96 hours. δ^2H of heavy water was +440‰. Due to the lack of a liquid sampler, we were not able to measure δ^2H of tap water, however, based on the GNIP data of the IAEA for Vologda (IAEA, 2020), we roughly estimated it as −110‰.

Next, the samples were dried at vacuum during four days and placed in the open tubes for equilibration with the atmospheric moisture in the laboratory at temperature 22 ± 1 °C and relative humidity of 30 ± 5%. Measurements of the isotopic composition were carried out in 4, 18, and 32 days after the equilibration has been started.

The isotopic composition was analyzed in the IRMS Laboratory of the Cherepovets State University (Russia). Samples were weighted on a Mettler Toledo WXTS3DU balance and pressed into standard silver foil capsules; the sample weight was 185 ± 20 μg. The samples were pyrolyzed at 1450 °C in a graphite pyrolytic reactor of the EA Isolink Flash IRMS elemental analyzer in a stream of pure helium. The hydrogen obtained during pyrolysis in a helium stream was transferred to a Thermo Fisher Delta V Advantage isotope mass spectrometer (IRMS) through the gas interface ConFlo IV. The measurement was carried out in continuous flow mode. The laboratory standard for hair was used as the isotope standard, it has been previously calibrated according to the standards USGS 42 (δ^2H = −72.9‰) and USGS 43 (δ^2H = −44.4‰). The isotopic composition of the non-exchangeable part of hydrogen in the laboratory standard ($\delta^2H_{true\ (RM)}$) was −89.2‰. The standards were measured at the beginning and at the end of the session, then a linear regression was calculated to find the true δ^2H values of the samples. During each session, 32 samples were measured: 4 samples with an isotopic standard at the beginning and end of the session, and 3 replicates of each studied hair sample. The δ^2H_{true} values were calculated using the formula for calibration according to the accepted standard (Dunn and Carter, 2018):

$$\delta^2H_{true(sample)} = \frac{(\delta^2H_{raw(sample)}+1)\times(\delta^2H_{true(RM)}+1)}{\delta^2H_{raw(RM)}+1} - 1.$$

Table 1. Labeling of the test samples.

	Child	Gray	Straight	Curly	Goat	Dog
Roughly shredded	A1	A2	A3	A4	A5	A6
Grinded	B1	B2	B3	B4	B5	B6

The results of the measurements are given in delta (δ) notations in accordance to the VSMOW scale:

$$\delta^2\mathrm{H} = \frac{\mathrm{R^2H}_{sample} - \mathrm{R^2H}_{RM}}{\mathrm{R^2H}_{RM}} \times 1000‰ ,$$

where R = [^{2}H]/[^{1}H].

Results

The hair actively absorb/desorb moisture during 40 minutes after opening the desiccator (Figs. 1, 2). Then, the rate of sorption/desorption decreases, reaching a plateau in 1–2 hours. Moreover, both the dynamics and the equilibrium weight practically do not differ between the roughly shredded and grinded hair. As a rule, roughly shredded hair absorb a little more water than grinded hair, but these differences are small and do not exceed 0.7% (average 0.2%) of the hair dry weight (Table 2).

The samples belonging to the groups nos. 2 and 4 were taken for the further study, they were characterized by the largest and the lowest variability of values, respectively. Half of these samples were equilibrated with normal (light) water (A2L, B2L, A4L, B4L), half, with heavy water (A2H, B2H, A4H, B4H) (Table 3, Fig. 3).

According to the IRMS analysis, for the samples of the group no. 2 equilibrated with normal water (A2L, B2L), the δ^{2}H difference between the grinded and roughly shredded hair was 13–15‰ throughout the experiment. Group no. 4 (samples A4L and B4L), on the contrary, showed an almost complete absence of differences between roughly shredded and grinded hair (1–4‰ difference). It should be noted that the opposite pattern has been observed in the experiments on the sorption/desorption of water, when the group no. 2 was characterized by the lowest variability, and the group no. 4, by the largest. The differences between the particular measurements of the same samples taken on different days did not exceed 3‰, which corresponded to the accuracy of the mass spectrometer declared by the manufacturer.

Equilibration the samples with heavy water led to different results for the sample groups nos. 2 and 4 (Fig. 3). For group no. 2, the values of δ^{2}H (A2H, B2H) were expectedly higher than the corresponding values for A2L and B2L. In this case, grinded hair showed lower values of δ^{2}H, similarly to the experiment with normal water. In the group no. 4, equilibration with heavy water had an unexpected result. Under “normal” conditions, these samples did not show differences between the roughly shredded and grinded hair, but when equilibrated with heavy water, significantly higher values of δ^{2}H were obtained for the grinded hair than for the roughly shredded hair.

During further equilibration with the atmosphere (measurements at day 18 and day 32), the δ^{2}H values for “heavy” samples were decreasing consistently. However, these values were by 7–12‰ higher than those for the corresponding “light water” samples even at day 32 of equilibration. Moreover, the val-

Table 2. The difference in sorption and desorption of water between grinded and roughly shredded hair samples. Meq – weight of the sample after reaching a plateau (stable weight), Md – dry weight of the sample, A – roughly shredded hair, B – grinded hair. Dashes (–) indicate experiment runs in which the data was lost for technical reasons.

		Meq/Md ratio								
		A1	B1	B1–A1	A2	B2	B2–A2	A3	B3	B3–A3
run 1	Sorption	1.060	1.057	–0.003	1.057	1.056	–0.001	1.058	1.056	–0.002
run 3	Sorption	1.067	1.065	–0.002	1.064	1.063	0.000	1.066	1.065	–0.001
run 2	Desorption	–	1.040	–	–	1.039	–	1.042	1.039	–0.003
run 4	Desorption	1.022	1.020	–0.002	1.020	1.020	0.000	1.021	1.020	–0.001
B–A mean				**–0.002**			**–0.001**			**–0.002**
		A4	B4	B4–A4	A5	B5	B5–A5	A6	B6	B6–A6
run 1	Desorption	1.056	1.048	–0.007	–	1.046	–	1.052	1.046	–0.006
run 3	Desorption	1.056	1.050	–0.007	1.056	1.053	–0.003	1.055	1.055	0.000
run 2	Sorption	1.054	1.055	0.001	–	1.047	–	1.051	1.045	–0.006
run 4	Sorption	1.034	1.029	–0.005	1.033	1.032	–0.001	1.033	1.034	0.001
B–A mean				**–0.004**			**–0.002**			**–0.003**

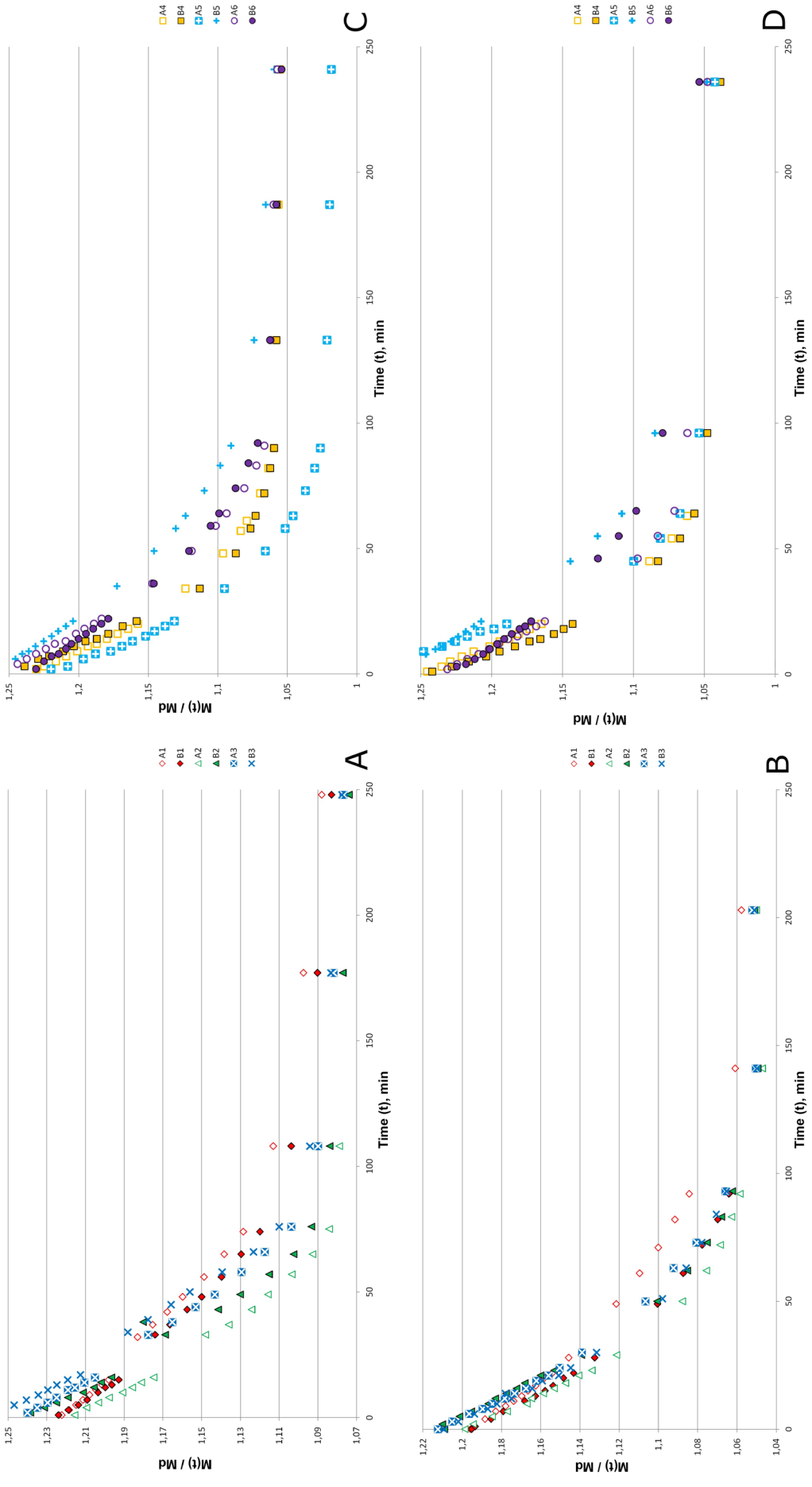

Fig. 1. Dynamics of water desorption into the atmosphere by the moistened hair samples. M(t)/Md is the ratio of the sample weight at the moment of measurement to its dry mass; **A**, **B** – samples of the groups nos. 1, 2, and 3, the first (**A**) and the second (**B**) experimental runs; **C**, **D** – samples of the groups nos. 4, 5, and 6, the first (**C**) and the second (**D**) experimental runs. For the sample labeling see Table 1.

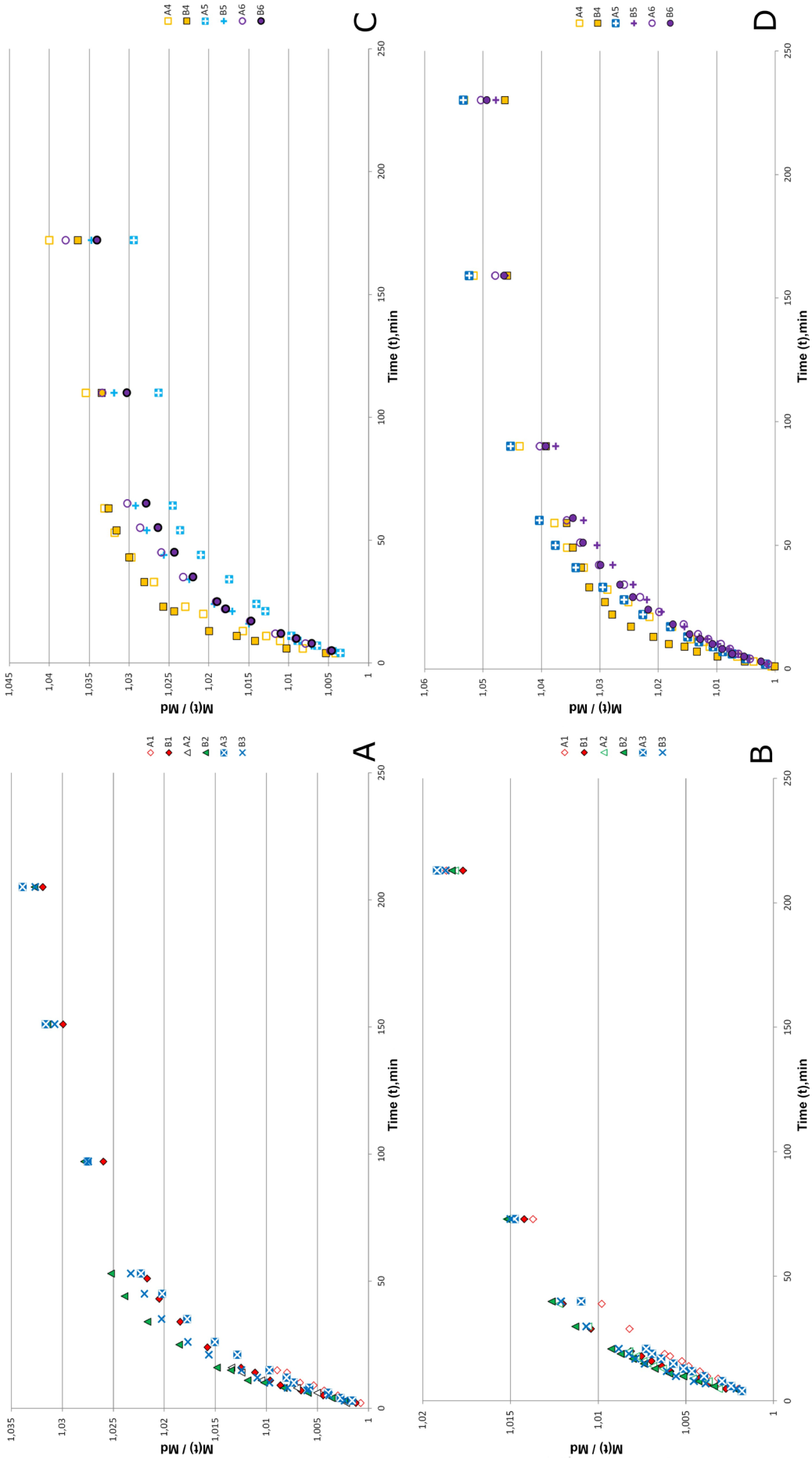

Fig. 2. Dynamics of water sorption from the atmosphere by dried hair samples. M(t)/Md is the ratio of the sample weight at the moment of measurement to its dry mass; **A**, **B** – samples of the groups nos. 1, 2, and 3, the first (**A**) and the second (**B**) experimental runs; **C**, **D** – samples of the groups nos. 4, 5, and 6, the first (**C**) and the second (**D**) experimental runs. For the sample labeling see Table 1.

Tab 3. The isotopic composition (δ2H, ‰ VSMOW) of the samples studied on the day 4, day 18, and day 32 of equilibration with the atmosphere. Mean is the average of three subsequent measurements, SD is the standard deviation of three subsequent measurements.

Sample no.		Day 4		Day 18		Day 32	
		Mean	SD	Mean	SD	Mean	SD
2	A2L	−93.7	0.8	−96.1	0.4	−94.8	1.6
	B2L	−107.2	1.2	−111.0	1.1	−108.2	0.9
	A2H	−80.9	0.9	−86.9	1.2	−87.8	0.7
	B2H	−86.5	0.1	−93.9	1.8	−95.2	0.4
4	A4L	−93.6	1.3	−94.8	1.6	−96.1	1.7
	B4L	−92.5	1.0	−93.2	0.9	−93.0	1.0
	A4H	−79.8	0.9	−86.0	1.6	−87.3	1.6
	B4H	−69.9	1.1	−78.1	0.3	−81.1	1.6

ues obtained on day 32 were only 1–3‰ lower than those on day 18. Therefore, the equilibration of these samples with the atmosphere was almost complete by day 18, but it was far from reaching the expected values.

Discussion

Experimentally, the amount of sorbed water, as well as the sorption/desorption dynamics did not depend on the method of sample shredding. During 40 minutes right after the contact with the laboratory atmosphere, both grinded and roughly shredded hair samples actively and equally sorb/desorb moisture. Rapid sorption during the very first minutes suggests that accurate measurement by the method of simultaneous two-stage equilibration (Bowen et al., 2005) without the use of any special drying devices is not possible (even at 30% humidity in the laboratory). Moreover, even pressing samples into silver foil capsules for analysis does not isolate them from contact with atmospheric moisture (original observations).

Starting this series of experiments, we expected to find either the absence of fluctuations in the isotopic composition due to the shredding method, or the regular effect of variations in the amount of sorbed water on the δ^2H measurement results. However, the obtained results contradict our original hypothesis. In our study, the proportion of sorbed water in roughly shredded and grinded samples is approximately the same, however, the results of δ^2H measurements for these samples differ in a completely unpredictable way. What is the reason? Obviously, the non-exchangeable hydrogen in keratin cannot produce such an effect. The sorbed part of hydrogen in the samples is almost the same. Thus, paradoxically, we suggest that the reasons for these variations are the exchange dynamics between the environmental water and the exchangeable hydrogen in keratin. Obviously, this dynamics should be determined primarily by the chemical structure of keratin, and not by its mechanical state. In this case, we could hardly disrupt the chemical composition of the sample just by grinding. Nevertheless, hydrogen exchange is a complex physicochemical process, and, presumably, not all of its aspects are currently understood.

The roughly shredded and grinded hair samples should be compared with caution. The generally available isotopic standards for keratin materials (such as USGS 42, USGS 43, CBS, KHS) are the powders, which means that the test samples should ideally have the same condition. However, we do not always have the opportunity to grind the samples, especially when the amount of the studied material is too small. A possible solution is to produce additional laboratory standards for the roughly shredded hair.

It was reported earlier (Bowen et al., 2005) that the exchange of hydrogen between hair keratin and atmospheric moisture was relatively fast and reached equilibrium within 3–4 days regardless of the relative humidity at which the samples were equilibrated. It has been noted earlier (Wassenaar and Hobson, 2003) that the hydrogen exchange between laboratory air moisture and the exchangeable hydrogen in keratin reached equilibrium within 24–48 hours at room temperature, at a higher temperature, it took even less time. We have found that the samples pre-equilibrated with heavy water were characterized by significantly higher δ^2H values even after four weeks of equilibration with atmospheric moisture. This means that usual approach of atmospheric equilibration does not erase the “isotopic history” of the hair even for a sufficiently large period of time, i.e. there are still traces of the isotopic composition of the water with which the hair was in contact before the equilibration started. It should be noted also that our experiment has been specially carried out in critical conditions, when the samples were equilibrated with water of a non-natural isotopic composition. Consequently, we suggest that the last issue will not be significant for practical application of this method.

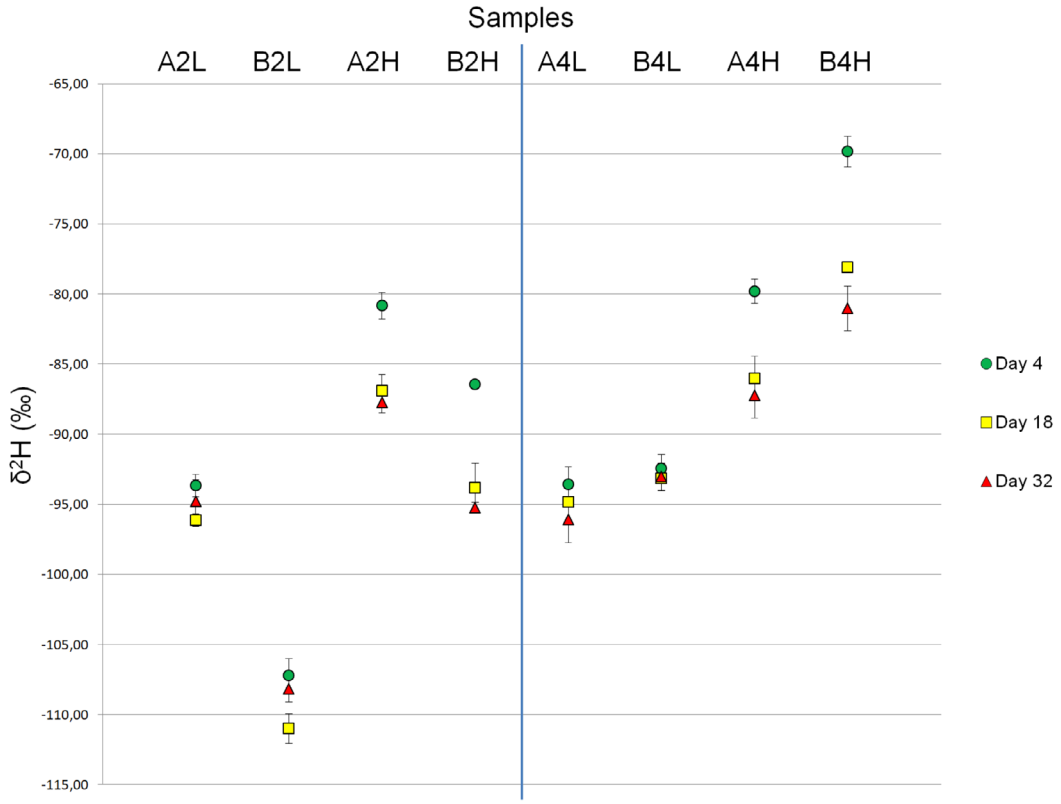

Fig. 3. Isotopic composition (δ^2H, ‰ VSMOW) of samples belonging to the groups no. 2 and no. 4 at the day 4, day 18, and day 32 of equilibration with the atmosphere. For the sample labeling see Table 1 and the description in the Results section.

Conclusions

Since completely dried hair rapidly absorbs atmospheric moisture, the contemporaneous two-stage equilibration method (Bowen et al., 2005) is not practical without maintaining zero humidity in the room or other measures to isolate the samples from the atmosphere. Thus, the comparative two-point end-member equilibration method (Wassenaar and Hobson, 2003) remains by now the only practically applicable one for most researchers. However, the sample preparation protocol for this method should be revised. Grinding of hair should be carried out by the same method as shredding of an isotope standard. Equilibration with the atmosphere should be carried out for a sufficiently long time, or it is necessary to improve the methodology for nulling the "isotopic history" of hair samples: for example, add the step of equilibration with tap water to the standard sample preparation procedure.

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