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B-HIVE: Beeswax hydrogen isotopes as validation of environment. Part I: Bulk honey and honeycomb stable isotope analysis

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ABSTRACT

Stable isotope analysis is an established method for detecting honey adulteration. We extend its application to include honey and honeycomb region-of-origin assignment using hydrogen (δ^2H) and oxygen ($\delta^{18}O$) isotopes. We observed that liquid honey δ^2H and $\delta^{18}O$ values had the potential to change because of water absorption and H atom exchange between sugars and water vapour. This suggested that liquid honey has limited use for geo-location, because specimens will record analysis location humidity. Paired liquid honey and honeycomb δ^2H values were significantly correlated; therefore, we propose using wax δ^2H values for region-of-origin assignment. We observed significant correlations between beeswax δ^2H values and both mean annual precipitation and tap water δ^2H values predicted for hive locations, suggesting that geographical variation in water δ^2H values are recorded by beeswax δ^2H values. This survey demonstrates the promise of stable isotopes for region-of-origin assignment, using honeycomb wax δ^2H values, which complements carbon isotope analysis to detect adulteration.

1. Introduction

The joint Codex Alimentarius Commission (www.codexalimentarius.net) of the Food and Agricultural Organization (FAO) and World Health Organization (WHO) defines honey as "the natural sweet substance produced by honey bees from the nectar of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honeycomb to ripen and mature." However, honey can be easily adulterated with additives or mislabelled as to source or origin (Anklam, 1998), misleading consumers who believe they are purchasing pure honey from a particular region while generating large profits for unscrupulous sellers. The practices of adulteration and misidentification of honey in the domestic American food market have recently prompted a grassroots movement for the implementation of national legislation to standardise its labelling. For example, a standard of identity for honey was formally adopted in July, 2009, by the state legislature of Florida (Rule 5K-4.027; www.flrules.org/gateway/ruleno.asp?id=5K-4.027) and several other states in the US may soon follow suit.

Carbon stable isotope ($\delta^{13}C$) analysis is an established and reliable method for detecting adulteration of honey with cheaper sweeteners, such as corn syrup and cane sugar (White, 1992; White, Winters, Martin, & Rossmann, 1998). On the other hand, methods for determining the geographical region-of-origin of honey have historically been limited to analysis of its organoleptic (e.g., aroma, colour, and taste), physicochemical (e.g., density, pH, and water content), and microscopic (e.g., melissopalynology) properties (Anklam, 1998). There has been a recent flurry of activity to develop chemical analysis methods to provenance honey. For example, trace element analysis has been shown to relate to honey region-of-origin (Bogdanov, Haldimann, Luginbühl, & Gallmann, 2007; Kropf et al., 2010; Tuzen, Silici, Mendil, & Soylak, 2007). A study in the European Union has demonstrated that it may be possible to analyse individual components of honey [e.g., protein (Schellenberg et al., 2010)] for the stable isotope ratios of the elements hydrogen and oxygen in order to determine region-of-origin. However, there has been no concerted effort to analyse the hydrogen (δ^2H) and oxygen ($\delta^{18}O$) stable isotope ratios of the two main substances produced by honeybees – honey and beeswax – for investigations into region-of-origin.

The hydrogen and oxygen stable isotope analysis of honey and honeycomb may prove useful for geographic region-of-origin assignment, because biological materials record the isotope ratios of local environmental water (West, Bowen, Cerling, & Ehleringer,

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2006). Hydrogen and oxygen ratios of water vary across landscapes, creating a predictable pattern in meteoric water hydrogen and oxygen isotope ratios, which can be represented graphically in an isotope landscape, or isoscape (Bowen, Ehleringer, Chesson, Stange, & Cerling, 2007; Bowen & Revenaugh, 2003; West et al., 2006). Plant tissues record the hydrogen and oxygen isotopic composition of water available to the plant during growth (Schmidt, Werner, & Eisenreich, 2003; Schmidt, Werner, & Rossmann, 2001; Sternberg, 1988) and the measured stable isotope ratios of plant-derived food products are useful for determining the original production location (Rummel, Hoelzl, Horn, Rossmann, & Schlicht, 2010; West, Ehleringer, & Cerling, 2007). While not of zip code precision, stable isotope analyses can provide information about bands or zones consistent with region-of-origin. Similarly, animals record the stable isotope ratio of consumed water in their tissues (Boner & Förstel, 2004; Heaton, Kelly, Hoogewerff, & Woolfe, 2008) and the stable isotope analysis of meats can be used to answer questions of food origin (Franke et al., 2008).

For this investigation of honey and honeycomb region-of-origin, we assumed that the hydrogen and oxygen isotope ratios of plant nectar collected by bees are linked to environmental water, because plants use local water to produce nectar. We further assumed that the isotopic composition of locally collected nectar is then recorded in the products made by honeybees, either directly (honey) or indirectly (beeswax) from the nectar. Thus, we hypothesised that the stable isotope analysis of bee products could be used to investigate and validate the region-of-origin of honey and honeycomb. To test this hypothesis, we collected honey and honeycomb throughout the USA and produced within a single year. We first demonstrate and discuss the potential and challenges of analysing both liquid honey and beeswax for hydrogen and oxygen stable isotope ratios. Next, we explore the relationship between region-of-origin (as represented by local water isotope ratios) and the isotopic composition of wax. Finally, we discuss limitations and future applications of the stable isotope analysis of bee products.

2. Materials and methods

2.1. Sample acquisition

Samples of comb and chunk honey (i.e., liquid honey surrounding a piece of honeycomb) were purchased in January–February, 2009, from US honey producers/packers. Producers/packers were identified using the National Honey Board's Honey Locator website (www.honeylocator.com). Upon receipt, honey and honeycomb were transferred from original packaging to pre-baked 1-quart glass canning jars. Jar mouths were covered with baked aluminium foil prior to the lid being screwed into place. During transfer, liquid honey and a cap of wax covering a single honeycomb cell were collected from each sample and placed in separate pre-baked 1-dram glass vials with Teflon-lined caps. Because the interior wax of honeycomb cells is often left for the bees after honey harvest, we chose to collect and analyse wax caps, which are removed during honey harvest and must be replaced by the bee during next season's honey production. Thus, the cap wax should be more closely associated with the honey than the interior wax.

2.2. Sample processing

Honey sub-samples were stored in 1-dram vials prior to being weighed. The 1-dram vials, containing cap wax sub-samples, were filled with deionised water and sonicated for 10 min before the water was decanted. The process was repeated twice (30 min total sonication time) to remove residual honey. Cleaned wax sub-samples were dried under a stream of purified air, and then stored prior to being weighed.

Honey and wax sub-samples were weighed (150 μg ± 10%) into pre-baked Ag capsules for hydrogen and oxygen stable isotope ratio analysis. Honey sub-samples were weighed by dipping the end of a clean paper clip into the honey and smearing honey into the bottom of the capsule until the desired weight was reached. Wax sub-samples were weighed by slicing small pieces of cap wax from the collected sample, using a clean razor blade; pieces were dropped into the capsule, using clean stainless steel forceps, until the desired weight was reached. Weighed honey and cap wax samples were stored under vacuum for a minimum of 5 days prior to analysis.

2.3. Sample analysis

Samples were analysed, in duplicate, in a Thermo-Finnigan stable isotope ratio mass spectrometer (MAT Delta Plus XL; Bremen, Germany) with a high temperature conversion elemental analyzer (TC/EA) attached. Samples were introduced to the pyrolysis column via a zero-blank autosampler (Costech Analytical, Valencia, CA, USA). Samples were converted to H_2 and CO_2 gases in the pyrolysis column, which was held at 1400 °C; gases were separated using a 1 m, 0.25 in (outer diameter) molecular sieve 5 Å gas chromatography column (Costech Analytical).

Honey samples were analysed alongside a cellulose reference material. Wax samples were analysed alongside a set of *normal*-alkane reference materials (for H data normalisation) and a set of benzoic acid reference materials (for O data normalisation). The stable isotope ratios of honey and wax samples are reported in "delta" (δ) notation, expressed in units of ‰, calculated as

$$\delta = (R_A/R_{STD} - 1) \times 1000$$

where R_A and R_{STD} are the ratios of rare to common isotopes (e.g., $^2H/^1H$) in the sample and an international standard, respectively. The international standard for both hydrogen and oxygen is Vienna Standard Mean Ocean Water. The analytical precision for honey analysis was 2‰ for H and 0.2‰ for O. Analytical precisions for cap wax analysis were 2‰ and 0.6‰ for H and O, respectively.

2.4. Statistical analysis

Statistical analysis was completed using Prism® 5.0c (GraphPad Software, La Jolla, CA) for Mac OS X. The δ^2H and $\delta^{18}O$ values of analysed honey samples, as well as the measured δ^2H values of paired honey and wax sub-samples, were compared using Deming regression (total least squares regression, TLS), an errors-in-variables model. Ordinary least squares (OLS) regression was used to compare the measured δ^2H values of wax to predicted mean annual precipitation and predicted tap water. Regression lines were fitted to data only when the slope of the line was significantly different from 0 at the $\alpha = 0.05$ level. Mean annual precipitation δ^2H values were predicted using a raster dataset downloaded from www.waterisotopes.org (Bowen & Revenaugh, 2003). The data layer was imported into ArcGIS™ 9.2 (ESRI®, Redlands, CA) then intersected with a point shape (vector) file containing latitude, longitude and elevation data for sample locations, in order to predict precipitation for each sample. Tap water hydrogen isotope ratios were predicted in the same manner, using a previously generated tap water data layer for the USA [see (Bowen et al., 2007)].

3. Results and discussion

3.1. Purchase locations spanned the maximum range of expected precipitation isotope ratios

Thirty-eight samples of comb and chunk honey were purchased from 33 producers/packers that operated online stores in 19 of the 50 states of the USA. The distribution of seller locations is shown in Fig. 1. We attempted to contact each producer/packer for more information regarding the location of honey and honeycomb production and verified the actual hive locations for 32 of the 38 samples. Hives were located in 14 of the 19 states that were home to sellers (Fig. 1). Two hives were not located in the same state as the producer/packer, but were located in a nearby state instead. For example, a store in Maryland sold honey produced in Virginia while a store based in Indiana sold honey from Kentucky.

While the samples originated in only 16 states, the locations spanned the maximum range of mean annual precipitation δ^2H values predicted for the continental USA [see Fig. 1; (Bowen & Revenaugh, 2003)]. The continental USA exhibits a large range in hydrogen and oxygen isotope ratios of water, measured as both precipitation and tap water (Bowen et al., 2007). The water isotope ranges observed in the US represent a large amount of the natural water isotope variation seen across the globe (Bowen & Revenaugh, 2003). Thus, the general patterns, between honey, honeycomb, and environmental water, observed in this dataset are likely valid for samples collected globally.

3.2. Liquid honey isotope ratios can change over time

Several honeys were analysed for hydrogen and oxygen isotope ratios, multiple times, over the course of 1 month. We observed that the mean δ^2H values (and, to a lesser degree, the $\delta^{18}O$ values) of pairs of capsules analysed across the course of the month were not similar: the greater the time between analysis attempts, the larger the difference between the measured stable isotope ratios of each analysis. For example, the mean measured δ^2H value of one honey sub-sample ranged from −29‰ to −59‰ over a span of 6 weeks, a change of 30‰. We hypothesised that honey was (a) absorbing water from the atmosphere, (b) exchanging water with the atmosphere, or (c) both as the sub-samples sat on the laboratory bench at ambient temperature.

To test this hypothesis, we purchased a jar of store brand filtered clover honey from a local supermarket. The jar was opened and immediately emptied into 14 polyethylene 2-oz. Nalgene® bottles (each ~½ full). The bottles were promptly tightly capped and stored upside down. The bottles were then uncapped and placed, one at a time, over 19 days, into a glass airtight chamber with a reservoir of isotopically heavy water (+350‰ for H and +16.5‰ for O) at the bottom. After 19 days, all bottles of honey were removed and weighed as described above. A sample of honey from the original purchased jar was also weighed to represent $t = 0$ (i.e., no exposure to the isotopically heavy water vapour). Weighed samples were not kept under vacuum for 5 days but analysed immediately.

Hydrogen (panel A) and oxygen (panel B) isotope ratio data for the exchange experiment are shown in Fig. 2. The original honey δ^2H value was −84‰. After 1 day in the high relative humidity environment, the honey δ^2H value was −33‰; by the end of experiment ($t = 19$), the δ^2H value was +22‰ (Fig. 2A). The change in $\delta^{18}O$ values was not as large, increasing from 23.9‰ ($t = 0$) to 26.5‰ after 19 days in the chamber (Fig. 2B). We also observed that the sample bottle with the longest residence time in the chamber gained ~2 g of mass during its 19-day exposure to the high relative humidity environment.

A two-phase exponential decay model could be fitted to the measured hydrogen stable isotope ratio data ($r^2 = 0.97$; model fit not shown) but a two-phase decay model could not be fitted to

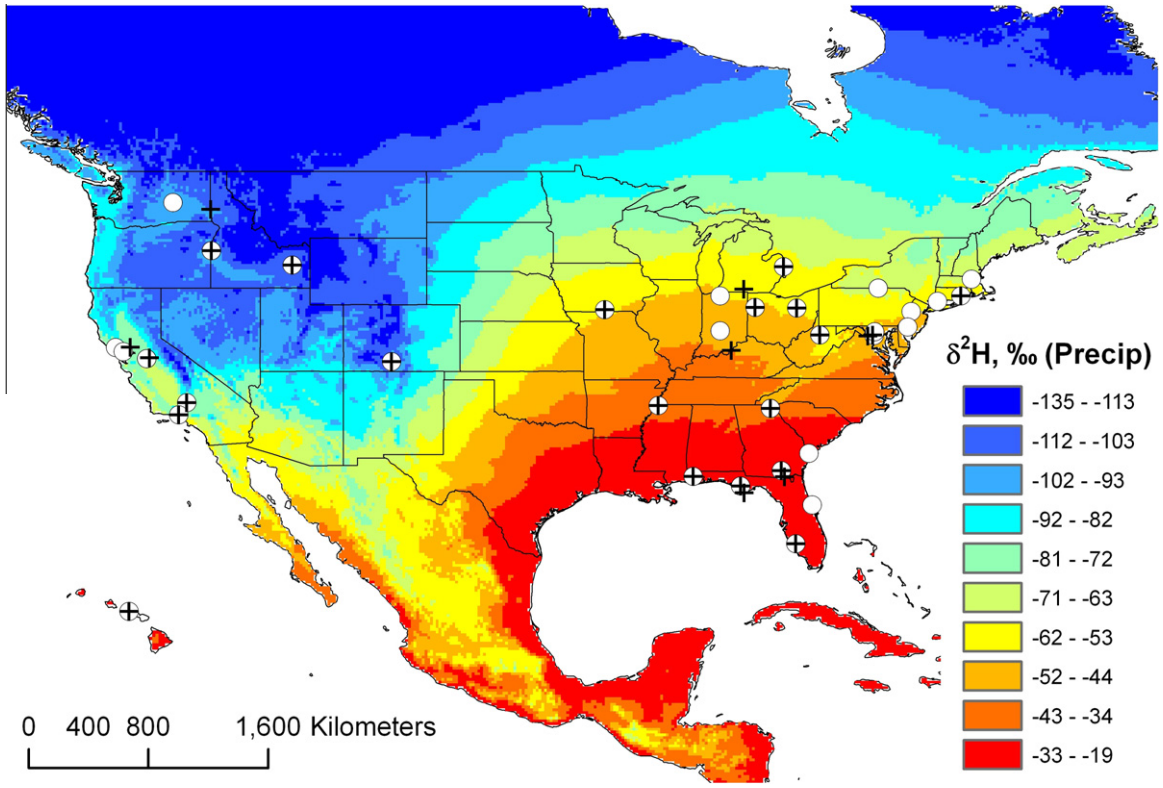

Fig. 1. The locations of producers/packers (circles) that supplied the honey samples collected in this survey, shown on a predicted mean annual precipitation isoscape for the USA. Hive locations are also shown (crosses) for samples that could be verified by contacting the seller.

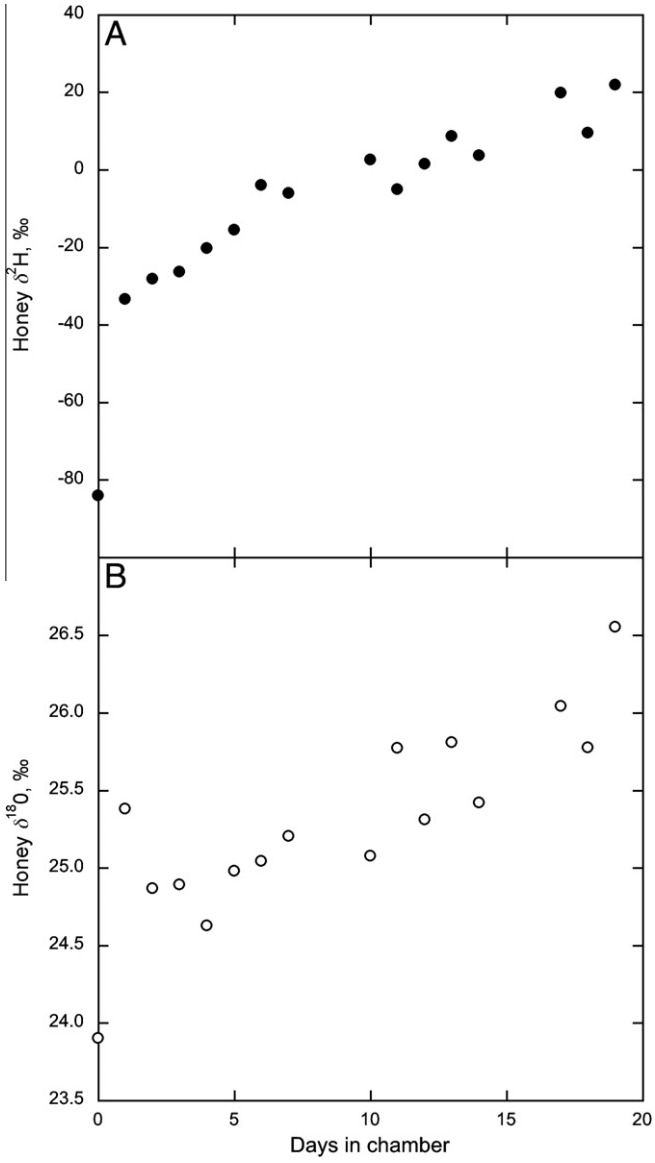

Fig. 2. The measured δ^2H (panel A) and δ^{18}O (panel B) values of liquid honey sub-samples exposed to isotopically heavy water vapour for varying amounts of time.

the measured δ^{18}O values. This suggests that the effects of exposure to isotopically heavy water vapour were not equivalent for hydrogen and oxygen. We assume this is because liquid honey incorporated the heavy δ^2H value of the ambient water vapour in the chamber via two mechanisms: (1) water absorption and/or (2) exchange of H atoms between sugar (within the honey) and the surrounding water vapour. The oxygen stable isotope ratios of the liquid honey were likely affected by water absorption only and thus the relative effect of surrounding water vapour on measured δ^{18}O values between honey samples at $t = 0$ and $t = 19$ was smaller than that for measured hydrogen isotope ratios.

Despite the possibility of water absorption from and exchange with the local analytical (laboratory) environment, the measured δ^2H and δ^{18}O values of honey sub-samples from each of the 38 purchased samples were correlated ($r^2 = 0.64$; Fig. 3); the TLS regression line fitted to the data was described by the equation

$$\delta^2H = 6.3^*\delta^{18}O - 250‰$$

We hypothesise this is because most purchased honeys were sub-sampled and transferred to limited head-space vials, and then analysed soon after receipt. The combination of rapid processing

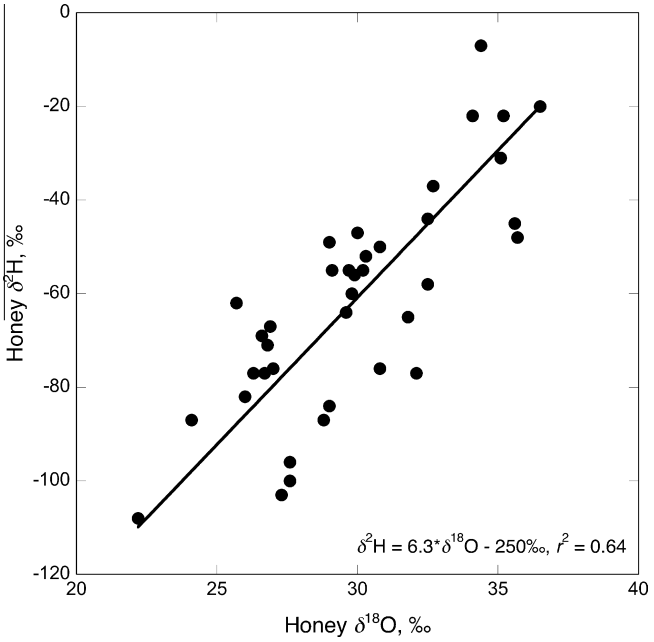

Fig. 3. Bi-plot of the measured δ^2H and δ^{18}O values of liquid honey samples collected in this survey. Hydrogen and oxygen isotope ratios were correlated and the equation for the Deming regression line (in solid black) is given.

and controlled storage conditions likely prevented much absorption from and exchange with local ambient water vapour prior to analysis. We also note that the analysis location (Salt Lake City, UT) is characterised by a naturally low humidity environment. Still, some of the scatter about the regression line, especially on the *y*-axis (measured δ^2H values), in Fig. 3 could be due to the impact of local atmospheric conditions at the time of analysis.

3.3. Wax can be used as a proxy for honey

Each of the purchased samples also contained wax in the form of honeycomb. Waxes, in general, do not absorb water and contain little to no exchangeable H atoms. The measured hydrogen isotope ratios of honey (see previous section) were correlated with the measured δ^2H values of cap wax collected from the same sample ($r^2 = 0.90$; Fig. 4). A Deming regression line fitted to the paired data was described by the equation

$$\delta^2H_{honey} = 0.9^*\delta^2H_{wax} + 170‰$$

Worker bees produce honeycomb both to house larvae (brood comb) and to store honey and pollen (Dadant&Sons, 1975). To produce beeswax, bees must consume six to eights times more honey by mass; the bees then convert the sugars from the honey into wax. Thus it is not surprising that the hydrogen isotope ratios of honey and beeswax are linked because one is used as the raw material for the other. Because honeycomb is much easier to sample and weigh, and because wax hydrogen isotope ratios are not likely to change when exposed to ambient water vapour in the laboratory, we propose that the measured δ^2H values of wax and not honey be used for investigations into the influence of local environmental conditions on bee products.

3.4. Wax isotopes are significantly correlated with local water

Due to our greater confidence in original source for samples with verified hive location information, we used only those sample locations ($n = 33$) to predict mean annual precipitation and tap

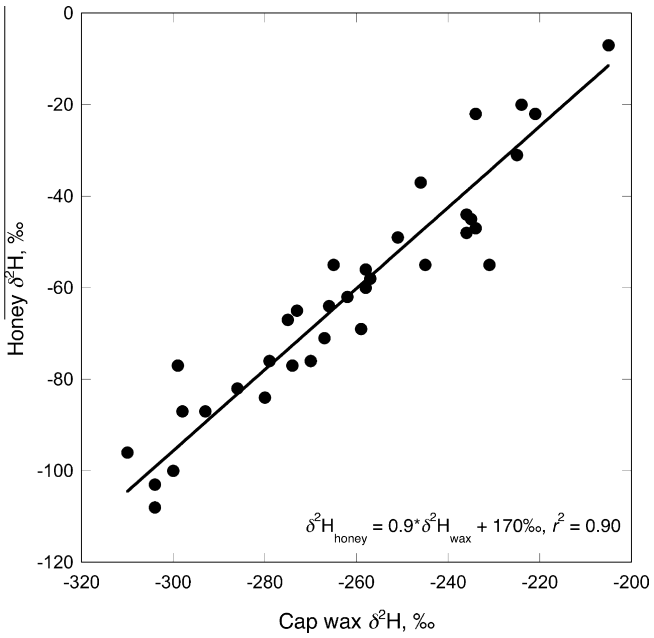

Fig. 4. Bi-plot of the measured δ^2H values of honey and a wax cap collected from the same purchased sample. The hydrogen isotope ratios were correlated and the equation for the Deming regression line (in solid black) is given.

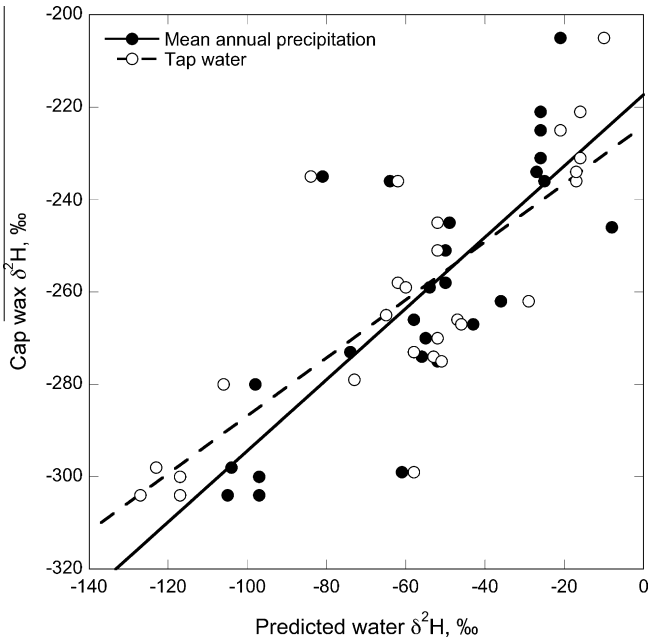

Fig. 5. Bi-plot of the measured δ^2H values of cap wax *versus* the δ^2H values of mean annual precipitation (closed circles) and tap water (open circles) predicted from the verified hive locations. Wax δ^2H values were correlated with both precipitation ($r^2 = 0.61$) and tap water ($r^2 = 0.63$). The OLS regression line describing the relationship between wax and water had the form of $y = 0.8x - 217‰$ for mean annual precipitation (solid line) and $y = 0.6x - 224‰$ for tap water (dashed line).

water δ^2H values. The hydrogen isotope ratio analysis of three wax samples did not meet quality assurance specifications. Additionally, despite verifying the hive location by contacting the seller, the measured δ^2H value of one wax sample purchased from Georgia, but (supposedly) produced in Florida, was an outlier in the dataset. This is likely because large producer/packer operations buy honey for resale from smaller producers and often do not themselves know the exact location of the hives that produced the honey. Thus the sample count for analysed wax caps was 29.

The measured hydrogen isotope ratios of cap wax and predicted mean annual precipitation at verified hives were correlated ($r^2 = 0.61$; Fig. 5); the equation describing the ordinary least squares regression line fitted to the data was

$$\delta^2H_{wax} = 0.8^*\delta^2H_{MAP} - 217‰$$

Predicted tap water δ^2H values were also correlated with measured wax cap δ^2H values ($r^2 = 0.63$; Fig. 5) and the equation of the OLS line fitted to the data was

$$\delta^2H_{wax} = 0.6^*\delta^2H_{tap} - 224‰$$

The lines fitted to the wax *versus* predicted mean annual precipitation or predicted tap water hydrogen isotope ratios were not different. The amount of variation in measured wax cap δ^2H values, explained by water, were approximately equal for predicted precipitation (61%) and predicted tap water (63%) but relatively modest for both water datasets. This could be due to the inclusion of hive locations in our predictions that were not accurate, despite our attempts to verify the locale of honey and wax production. It could also be due to our analysis of bulk cap wax samples, which are composed of a variety of compounds that may differentially record environmental isotope ratios. The extraction and stable isotope analysis of a specific lipid fraction from the cap wax samples may generate stronger correlations between wax and predicted water δ^2H values.

Despite the modest correlations, predicted mean annual precipitation and tap water δ^2H values are both independent and equally valid measures of the local water available to a nectar-producing plant. Most plants visited by bees, when gathering nectar, are likely either (i) accessing groundwater, which is an amalgamation of regional precipitation; or (ii) managed crops, which are irrigated using locally available tap water. Furthermore, the measured δ^2H values of mean annual precipitation and tap water generally agree in most regions of the USA (Bowen et al., 2007). As we hypothesised, nectar-producing plants record the isotopes of local environmental water and that record is preserved in the beeswax, albeit with some transformation due to fractionation processes as nectar is transformed to honey and honey is then used to make wax.

4. Conclusions and implications

From this survey of honey and honeycomb throughout the USA, we have shown that (1) the hydrogen and oxygen stable isotope analysis of liquid honey is difficult because of water absorption and exchange by the honey and local water vapour; (2) cap wax δ^2H values are a good proxy for honey hydrogen isotope ratios and (3) the hydrogen isotope ratios of wax are correlated equally well with two independent predictions of local water isotope ratios. This investigation has also highlighted future work that should be done to develop the use of stable isotope analysis for honey provenancing.

First, for the ease of statistical analysis we grouped all honey and honeycomb samples, regardless of varietal type. The specific environmental conditions favoured by different nectar-producing plants (for example, sage or tupelo trees) could affect the relationship between environmental water and wax δ^2H values among different types of honeys. Second, we had the luxury of analysing cap wax collected from pieces of honeycomb present in the purchased samples. In filtered liquid honey samples, there is no honeycomb to collect and analyse, thereby hindering a possible origin assignment by using the stable isotope analysis of wax. Third and finally, there are many components within a single jar of honey that could be collected and analysed, including (but not limited to) honey, pollen, protein and free amino acids, sugars, wax, and potentially

foreign debris. For this work we have focused on only two of those components, honey and honeycomb wax. The collection and stable isotope analysis of other components may yield yet more information useful for geographic origin assignment, as demonstrated recently with protein precipitated from honey (Schellenberg et al., 2010).

Nevertheless, this initial survey of chunk and comb honeys, purchased from across the USA, demonstrates the potentially powerful application of stable isotope analysis for investigating the origin of honey via wax. Using the measured δ^2H value of cap wax, we have demonstrated that the hydrogen isotope ratio of beeswax is related to the hive's local environmental water. Based on this, through future work, it may be possible to predict the hydrogen isotope ratio of precipitation or tap water local to the hive where a wax sample was produced, generating predictive regions-of-origin for a sample of interest. At a time when both industry and consumers are demanding increased regulation and verification of specialty food items, such as honey, the use of stable isotope analysis to validate the environmental conditions of beeswax source is extremely promising.

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