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Analytical Methods

B-HIVE: Beeswax hydrogen isotopes as validation of environment, part II. Compound-specific hydrogen isotope analysis

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ABSTRACT

Hydrogen isotope analysis has proven a useful tool to understand region-of-origin of food items and biologic materials. Here its application is extended to compound-specific hydrogen isotope analysis of authentic *Apis mellifera* beeswax and *normal*-alkanes in order to elicit geographic information. We found *A. mellifera* produced a characteristic distribution of hydrocarbons that was present in both lipid extracts from beeswax and liquid honey. We observed significant correlations between hydrogen (δ^2H) isotope values of beeswax-extracted *n*-alkanes and both mean annual precipitation and tap water δ^2H values. These data suggest the geographical variation in water δ^2H values was reflected in beeswax *n*-alkane δ^2H values while isotopic variation was likely related to local environment as potentially influenced by plant varietal. Further, extraction of *n*-alkanes from liquid honey is shown to be a viable method to expand compound-specific isotope analysis to liquid honeys.

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1. Introduction

The market for pure honey produced from monospecific and/or region-specific plant varietals is growing (Alissandrakis, Tarantilis, Harizanis, & Pollissiou, 2007; Cotte et al., 2007) and as the demand for these boutique honeys has grown, so too have incidences of honey adulteration with non-honey additives and of honey mislabelling concerning its botanical and geographical origin (Anklam, 1998). As an example, New Zealand's honey exports increased by 74% from 2007 to 2009 with an estimated value of approximately US$70 million (New Zealand Trade, 2010). In particular, roughly US$50 million of the total New Zealand exports derived from Manuka honey (Rogers, Somerton, Rogers, & Cox, 2010). On the global market, Manuka honey commands high prices and is highly sought after due to its antibacterial and neutraceutical properties (Cooper, Molan, & Harding, 1999; Molan, 1992; Rogers et al., 2010). These properties and the associated economic incentives increase the risk of fraudulent botanical and geographical labelling of Manuka honey and adulteration with less-expensive honeys or sweeteners (Padovan, De Jong, Rodrigues, & Marchini, 2003). To combat the loss of confidence by international buyers due to mislabelling, New Zealand's Horticulture Export Authority (HEA) and Ministry of Agriculture are preparing to include Manuka honey under the HEA Act 1987 in order to standardise, regulate, and certify Manuka honey exports (Manuka Honey Steering Group – New Zealand Horticulture Export Authority, 2010).

Two of the most common methods to establish a honey's specific region-of-origin are (1) examination of plant-derived components (e.g., pollen) extracted from honey (Diez, Andres, & Terrab, 2004), and (2) investigation of chemical markers (e.g., volatile compounds and trace elements) within honey (Aliferis, Tarantilis, Harizanis, & Alissandrakis, 2010; Baroni et al., 2006; Bogdanov, Haldimann, Luginbuhl, & Gallmann, 2007). These methods focus on the environmental or floral characteristics imparted to the honey and are well developed for discrete regions (Kropf et al., 2010; Soria, Gonzalez, de Lorenzo, Martinez-Castro, & Sanz, 2004) and honey varietals (Castro-Vazquez, Diaz-Maroto, Gonzalez-Vinas, & Perez-Coello, 2009; Lusic, Koprivnjak, Curic, Sabatini, & Conte, 2007; Tananaki, Thrasyvoulou, Giraudel, & Montury, 2007). In addition, several works have recently focused on stable isotope analysis of honeybee-derived components present in honey (e.g., protein and beeswax), rather than the floral- or environmental-derived components, to determine region-of-origin (Chesson, Tipple, Erkkila, Cerling, & Ehleringer, 2011; Schellenberg et al., 2010).

Techniques that focus on honeybee-derived components within honey-like wax-hold great promise to determine a honey's region-of-origin, given the global distribution of the European honey bee, *Apis mellifera* L., which produces most commercially available honey.

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Young *A. mellifera* bees manufacture wax from specialised glands in order to construct honeycomb to store honey, pollen, and eggs/larvae/pupae (Winston, 1987). The biochemical pathway for production of beeswax compounds (primarily made of the elements carbon, hydrogen, and oxygen) are well established (Hepburn, 1986; Nelson, 1993) with plant nectar (via honey) and pollen as the primary molecular inputs (Seeley, 1995). Hydrogen (δ^2H) and oxygen (δ^{18}O) stable isotope values of meteoric waters vary predictably as a function of geography (Bowen & Revenaugh, 2003b; Craig, 1961), where δ^2H and δ^{18}O values generally decrease from low-latitude, low elevation coastal regions toward continental interior, high-latitude, and mountainous regions (Bowen, Ehleringer, Chesson, Stange, & Cerling, 2007; Bowen & Revenaugh, 2003a; West, Bowen, Cerling, & Ehleringer, 2006). Because organisms incorporate the isotopic signal from their local waters, the δ^2H and δ^{18}O values of organic materials are useful in identifying the region-of-origin of organic materials such as food (Chesson, Valenzuela, O'Grady, Cerling, & Ehleringer, 2010) and other plant/animal products (Chamberlain et al., 1997; Hobson, Atwell, & Wassenaar, 1999; Kreuzer-Martin, Lott, Dorigan, & Ehleringer, 2003; Sharp, Atudorei, Panarello, Fernandez, & Douthitt, 2008; Wassenaar & Hobson, 1998).

Given that all molecular contributions to beeswax reflect meteoric water and that the hydrogen and oxygen ratios of water vary across landscapes in a predictable pattern (Bowen & Revenaugh, 2003a; Bowen et al., 2007; West et al., 2006), it has been shown that the hydrogen and oxygen stable isotope analysis of honeycomb wax and protein is useful for geographic origin assignment (Chesson et al., 2011; Schellenberg et al., 2010). Chesson et al. (2011) established the groundwork to compare meteoric water and beeswax hydrogen (δ^2H) and oxygen (δ^{18}O) isotope ratios. In their study, they found liquid honey and bulk solid beeswax δ^2H values were strongly correlated and that beeswax δ^2H values were in turn strongly correlated with both mean annual precipitation δ^2H values and locally-available tap water δ^2H values for a given hive location. While solid beeswax is often found in raw or unfiltered honey, visible beeswax is lacking in highly processed and/or filtered honey, limiting the applicability of bulk beeswax δ^2H analysis. However, the primary compounds found in *A. mellifera* beeswax [monoesters (35–45%), complex esters (15–27%), long chain hydrocarbons (12–16%), and fatty acids (12–14%) (Tulloch, 1980)] are often found in liquid honey (Bonaga, Giumanini, & Gliozzi, 1986; Tan, Holland, Wilkins, & Molan, 1988; Tan, Wilkins, Holland, & McGhie, 1989; Tan, Wilkins, Holland, & McGhie, 1990; Terrab, Vega-Perez, Diez, & Heredia, 2002). Many studies have investigated volatile honey compounds (Aliferis et al., 2010; Baroni et al., 2006; Castro-Vazquez et al., 2009; Dahler & Gulacar, 2008; Graddon, Morrison, & Smith, 1979; Jerkovic, Tuberoso, Marijanovic, Jelic, & Kasum, 2009; Soria, Martinez-Castro, & Sanz, 2008; Soria, Sanz, & Martinez-Castro, 2009; Soria et al., 2004; Tananaki et al., 2007) and hydrocarbon compositions of beeswax (Aichholz & Lorbeer, 1999; Aichholz & Lorbeer, 2000; Hepburn et al., 1991; Namdar, Neumann, Sladezki, Haddad, & Weiner, 2007; Tulloch, 1972; Tulloch, 1980; Tulloch & Hoffman, 1972) and honey (Bonaga et al., 1986; Tan et al., 1988; Tan et al., 1989; Tan et al., 1990; Terrab et al., 2002), but to our knowledge no study has examined the stable isotope ratios of these compounds. Potentially, these molecular components and their isotope ratios may yield information regarding a honey's geographical, climatic, and/or botanical origin, especially in instances where solid beeswax is not present.

Here we present the molecular distributions and stable hydrogen isotope compositions of *n*-alkanes extracted from *A. mellifera* beeswax and honeys in order to investigate its reliability to validate the region-of-origin of honey and honeycomb. We hypothesized that geographic information (i.e., the isotopic signature of local water) is recorded in the compound-specific hydrogen isotope ratios of beeswax hydrocarbons, similar to the information recorded by bulk beeswax. To test this hypothesis, we collected and analysed honey and honeycomb throughout the United States produced during the 2008 growing season. We first discuss the distinctive beeswax *n*-alkane distribution and then explore the relationship between region-of-origin (as represented by local water isotope ratios) and the hydrogen isotopic composition of *n*-alkanes extracted from bulk beeswax. Finally, we discuss the role of honey varietals in determining the hydrogen isotope ratio of *n*-alkanes and the applicability of extracting honeybee-derived *n*-alkanes from liquid honey for isotope analysis.

2. Materials and methods

2.1. Sample acquisition and preparation

Thirty-eight comb and chunk honey samples from eight floral varietals were ordered January–February 2009 from US honey producers/packers operating Internet stores. Online stores were identified using the National Honey Board's Honey Locator website (www.honeylocator.com). We contacted each producer/packer for more information regarding the location of honey and honeycomb production but could only verify the actual hive locations for 31 of the 38 samples. Hives were located in 14 of the 19 states that were home to the producer/packer (Fig. 1). Two hives were not located in the same state as the producer/packer, but were located in a nearby state instead.

Upon receipt, honeycombs were transferred from original packaging to pre-baked 1-quart glass canning jars. A cap of wax covering a single honeycomb cell was collected from each sample and placed in separate pre-baked 4-ml glass vials with Teflon-lined caps. Cap wax was sampled exclusively, as the interior honeycomb cells within the hive frame are often reused by the beekeeper. Thus, the restricted use of cap wax ensures the associated honey and waxes reflect the same growing season and geographic location. The 4-ml vials containing cap and interior wax sub-samples were filled with deionised water and sonicated for 10 min. The supernatant was decanted and 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 then stored tightly capped in baked 4-ml vials in the laboratory until weighed.

An additional series of 53 liquid honey samples were purchased from national chain and local grocers throughout North America in February 2008. These purchased liquid honeys were categorised as either filtered or raw samples based on label information. These honeys were stored in the original packaging, unopened, with the hermetic seal preserved.

2.2. Extraction, separation, and analytical method

For total lipid extraction from raw honeycomb, 5–10 mg of the wax cap (roughly ¼ of a cap) was sub-sampled into a baked 4-ml glass vial. Lipids were extracted from wax cap by sonication with a 2:1 (v:v) mixture of dichloromethane:methanol for 15 min. Total lipid extracts (TLEs) were concentrated under a stream of purified air using a 40 °C Pierce evaporator. The concentrated TLEs were then separated by column chromatography using 1 g deactivated silica gel (70–230 mesh) in a baked 3.5″ Pasteur pipette, and eluted with a solvent sequence of hexane, dichloromethane, and methanol. The aliphatic hydrocarbons were collected with the hexane fraction. Other solvent fractions were archived.

For liquid–liquid extraction of dispersed hydrocarbons, 10 g of liquid honey was sub-sampled into a baked 40-ml glass vial with a Teflon-lined cap. Hydrocarbons were extracted three times with OmniSolv® hexanes (20 ml) with a vortexer. Hydrocarbons were concentrated under a stream of purified air using a 40 °C Pierce

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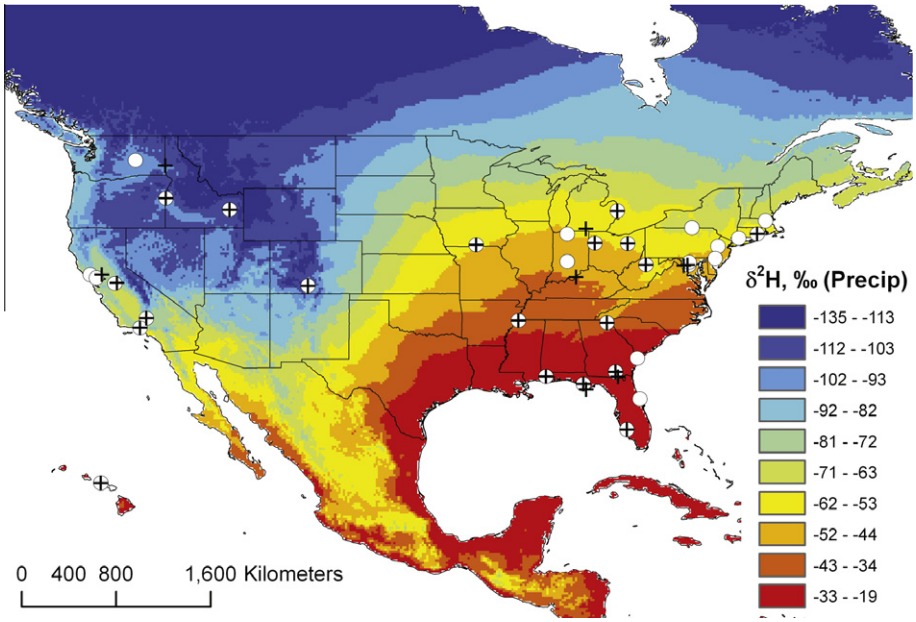

Fig. 1. The locations of producers/packers (circles; $n = 38$) that supplied the honey samples collected in this survey, shown on a estimated mean annual precipitation isotopic landscape (isoscape) for the USA. Hive locations are also shown (crosses) for samples that could be verified by contacting the seller.

evaporator. The concentrated hydrocarbon fractions were then passed through 1 g deactivated silica gel (70–230 mesh) in an baked 3.5″ Pasteur pipette with hexane to remove any non-aliphatic compounds.

Concentrations for both honeycomb and honey-extracted hydrocarbon fractions were determined using a Thermo Trace 2000 gas chromatograph (GC) fitted with a split–splitless injector coupled to a Thermo MS structural mass spectrometer. Hydrocarbons were separated on a fused silica, DB-5 phase column [30 m × 0.25 mm I.D., 0.25 μm film thickness (Supelco, Inc.)] with helium as the carrier gas at a flow of 1.5 ml/min. The GC oven temperature program utilised was 60–320 °C at 10 °C/min with an isothermal for 30 min. *Normal*-alkanes and other hydrocarbons were identified through comparison of elution times with known *n*-alkane standards and mass spectra.

Individual *n*-alkane peak areas were calculated using Xcaliber software version 1.5. Changes in average chain length (ACL) were calculated using the following relationship:

$$\mathrm{ACL} = \frac{(A_{25}(25)) + (A_{27}(27)) + (A_{29}(29)) + (A_{31}(31))}{(A_{25} + A_{27} + A_{29} + A_{31})},$$

where A_{xx} corresponds to the area of the individual *n*-alkane peak from the chromatograph trace.

2.3. Compound-specific isotope analysis (CSIA) of hydrogen

Isotope analyses were performed using a Thermo Trace 2000 GC coupled to a Finnigan MAT Delta V Plus (Bremen, Germany) isotope ratio mass spectrometer interfaced with a high temperature conversion system (Thermo Scientific). Hydrocarbons were separated on a fused silica, DB-5 phase column [30 m × 0.25 mm I.D., 0.25 μm film thickness (Supelco, Inc.)] with helium as the carrier gas at a flow of 1.5 ml/min. The GC oven temperature program utilised was 102–350 °C at 6 °C/min with an isothermal for 8 min. Hydrogen isotope analysis required conversion of eluting hydrocarbons to H_2 and CO at 1400 °C and delivery to the mass spectrometer via an open split. The H_3^+ factor was determined daily prior to standard calibration and sample analysis. Peaks were quantified using Isodat 2.5 and an individual baseline for hydrogen measurements.

Using delta notation, stable isotope ratios (δ) are calculated as:

$$\delta = [(R_{\mathrm{samp}}/R_{\mathrm{std}}) - 1],$$

where R represents the $^2H/^1H$ abundance ratio, and R_{samp} and R_{std} are the ratios in the sample and standard, respectively. δ^2H values are expressed relative to the standard Vienna Standard Mean Ocean Water (VSMOW). Individual *n*-alkane isotope ratios were corrected to in-house *n*-alkane reference materials [*n*-C_{18} (−69.9‰), *n*-C_{20} (−53.9‰), *n*-C_{22} (−38.6‰), *n*-C_{24} (−36.0‰), *n*-C_{28} (−250.1‰), and *n*-C_{32} (−236.4‰)] analysed daily at several concentrations. Accuracy in isotope determinations for reference *n*-alkanes was ±3‰ for δ^2H measurements.

The difference between the hydrogen isotope ratios of *n*-alkanes and bulk wax is described as

$$\Delta^2H_{\mathrm{alkane-wax}} = \delta^2h_{\mathrm{alkane}} - \delta^2H_{\mathrm{wax}}.$$

The apparent fractionation ($\varepsilon_{\mathrm{water-alkane}}$) between *n*-alkanes and the isotopic composition of environmental waters can be described as

$$\varepsilon_{\mathrm{water-alkane}} = [(R_{\mathrm{alkane}}/R_{\mathrm{water}}) - 1],$$

where R represents the $^2H/^1H$ abundance ratio, and R_{alkane} and R_{water} are the ratios in the *n*-alkane and environmental water, respectively.

2.4. Statistical analysis

Statistical analysis was completed using Prism® 5.0c (GraphPad Software, La Jolla, CA) for Mac OS X. The measured δ^2H values of paired *n*-alkane and bulk wax cap 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 *n*-alkanes to estimated mean annual precipitation and estimated tap water. Regression lines were fitted to data only when the slope of the line was significantly different from 0 at the $\alpha = 0.01$ level. The slopes and the *y*-intercept of the regression lines for the data sets were

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compared with a line of slope 1 and intercept 1 using one-way analysis of variance (ANOVA) and a Tukey's post hoc test to identify differences at $\alpha = 0.01$. Mean annual precipitation δ^2H values were estimated using a raster dataset downloaded from www.wateriso topes.org (Bowen & Revenaugh, 2003a). The data layer was imported into ArcGIS™ 9.2 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 estimated in the same manner, using a previously generated tap water data layer for the United States (see Bowen et al., 2007).

3. Results and discussion

3.1. A. mellifera beeswax n-alkane distributions are distinctive

A. mellifera produced a characteristic homologue of saturated odd and even hydrocarbons from n-C_{21} to n-C_{35}, with n-C_{25}, n-C_{27}, n-C_{29}, and n-C_{31} as the dominant n-alkanes in beeswax (Fig. 2). In addition, *A. mellifera* produced n-olefins from n-C_{29} to n-C_{33}, with n-C_{31} and n-C_{33} as the dominant n-alkenes. These findings are consistent with previous studies (Namdar et al., 2007; Tulloch, 1980). Comparisons of beeswax hydrocarbons have shown each honeybee species to have a distinct distribution (Tulloch, 1980). We found all samples analysed had the distinctive *A. mellifera* hydrocarbon pattern (Fig. 2) with a strong odd-over-even predominance and an average chain length (ACL) of 28.0 ± 0.2 (1σ).

We also found ACL was not related to mean annual temperature (MAT) and/or mean annual precipitation (MAP; $r^2 = 0.00$ and 0.04, respectively). Analysis of plant leaf waxes have shown significant relationships between climatic parameters (MAT, MAP, and aridity) and hydrocarbon distributions (Hughen, Eglinton, Xu, & Makou, 2004; Sachse, Radke, & Gleixner, 2006). While leaf waxes and beeswax are produced through similar biosynthetic pathways, plant waxes are exposed to the external environment and beeswax is produced under specific hive temperature and humidity conditions (Winston, 1987) likely limiting or masking the climatic signal recorded in the hydrocarbon distribution. The lack of climatic-forced variation and presence of consistent hydrocarbon distribution and ACL for all *A. mellifera* beeswax is potentially useful given the global distribution of the European honeybee. We predict *A. mellifera* beeswax produced worldwide should record a similar hydrocarbon distribution, no matter the environmental conditions. In addition, this characteristic pattern and ACL may be useful to distinguish *A. mellifera* hydrocarbons from potential environmental (i.e., leaf-waxes, atmospheric deposition, etc.) and commercial (i.e., plasticizers, etc.) contaminants.

3.2. Hydrocarbon hydrogen isotopes are significantly correlated with, but offset from, bulk wax δ^2H values

Each of the honey samples purchased from online stores in 2009 contained visible wax in the form of raw honeycomb. Beeswax does not absorb water, contains little to no exchangeable H atoms, and bulk beeswax δ^2H values have been shown to reflect meteoric waters δ^2H values (Chesson et al., 2011). As beeswax is made up of many individual compounds (Tulloch, 1980), we predicted that these individual components' δ^2H values would be related to bulk beeswax and meteoric waters δ^2H values as well. The measured hydrogen isotope values of bulk beeswax (δ^2H_{wax}) were linearly correlated with the hydrogen isotope ratio of n-alkanes (δ^2H_{alkane}) collected from the same sample ($r^2 = 0.94$, $p < 0.0001$; Fig. 3). For ease of presentation and discussion we present the mean-weighted average of n-C_{23} to n-C_{31} individual δ^2H values as $\delta^2H_{alkanes}$. A total least squares regression line fitted to the paired data was described by the equation

$$\delta^2H_{alkane} = 1.1^{*}\delta^2H_{wax} + 49‰ \; (F_{1,27} = 435.4,\; p < 0.0001).$$

The slope was not different than 1 at an α of 0.01. The one-to-one relationship and high correspondence between wax cap n-alkane and beeswax δ^2H values suggests an identical H-source for both materials. *Normal*-alkanes are formed from extension of palmitic and stearic acid with acetate via the fatty acid synthesis pathway (Morgan, 2004). Similarly, the fatty acid pathway forms the other major molecular components of beeswax, mono- and diesters.

The $\Delta^2H_{alkane-wax}$ between paired n-alkanes and bulk wax indicate n-alkanes are more enriched in ^{2}H relative to the beeswax by 20 ± 7‰ (1σ). The ^{2}H enrichment in n-alkanes is not unexpected given that n-alkanes comprise ~15% of the total molecular composition of beeswax (Tulloch, 1980). These data suggest the other molecular components of beeswax (e.g., esters and fatty acids) were more depleted in ^{2}H relative to bulk beeswax and n-alkanes hydrogen isotope values.

3.3. Normal-alkane isotope ratios are significantly correlated to local water isotopes

Due to increased confidence for samples with verified hive location information, we used only those sample locations to predict

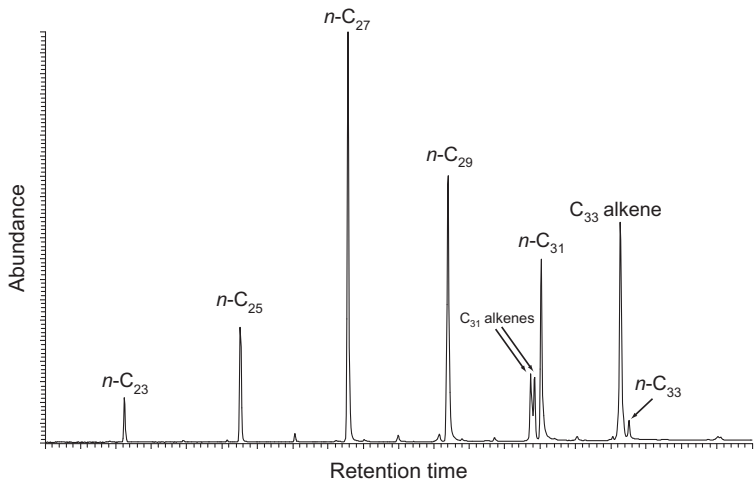

Fig. 2. Representative beeswax hydrocarbons total ion current GC–MS trace.

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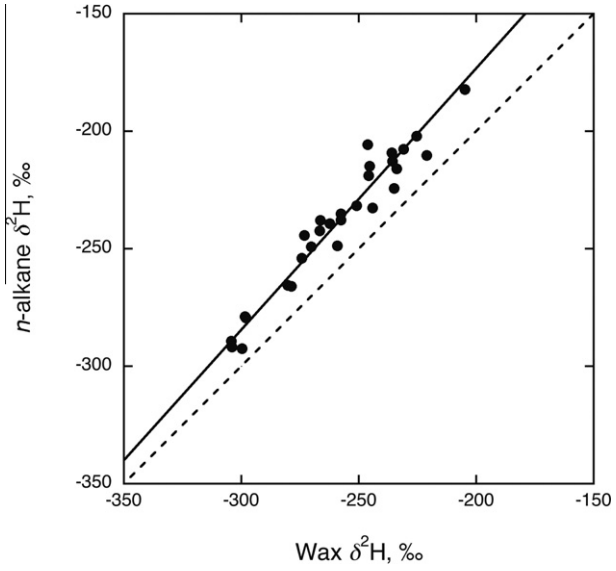

Fig. 3. Cross plot of the measured δ^2H values of bulk cap wax and *n*-alkanes collected from the same sample. The hydrogen isotope ratios were correlated and the equation for the total least square regression line (in solid black) is $\delta^2H_{alkane} = 1.1 * \delta^2H_{wax} + 49‰$.

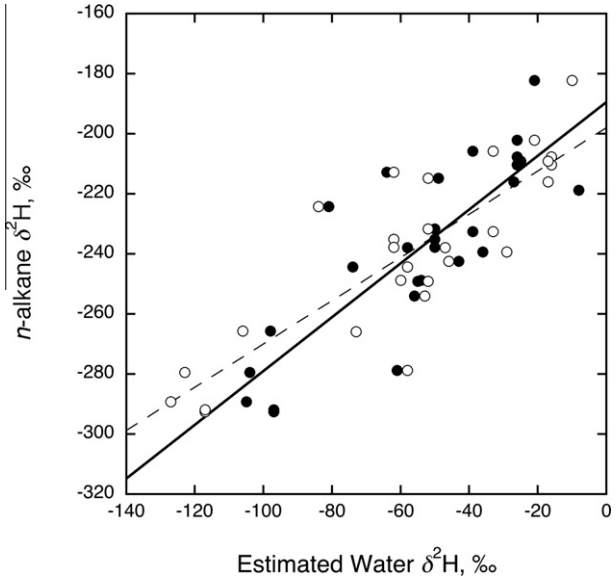

Fig. 4. Cross plot of the measured δ^2H values of *n*-alkanes *versus* the δ^2H values of mean annual precipitation (closed circles) and tap water (open circles) estimated from the verified hive locations. *Normal*-alkane δ^2H values were correlated with both precipitation ($r^2 = 0.82$) and tap water ($r^2 = 0.84$). The ordinary least square regression line describing the relationship between *n*-alkanes and water had the form of $\delta^2H_{alkane} = 0.9 * \delta^2H_{ppt}$-189‰ for mean annual precipitation (solid line) and $\delta^2H_{alkane} = 0.7 * \delta^2H_{tap}$-198‰ for tap water (dashed line).

mean annual precipitation and tap water δ^2H values from GIS layers. Sample hive location for three samples ordered online could not be confirmed, thus they were excluded from predictions. Additionally, despite verifying the hive location by contacting the seller, the measured δ^2H_{alkane} value of one sample supposedly produced in the Florida panhandle was a clear outlier in the dataset, leaving 29 of the original 33 *n*-alkanes fractions for this analysis.

The measured δ^2H values of cap wax *n*-alkanes and mean annual precipitation (δ^2H_{ppt}) estimated from verified hive locations were linearly correlated ($r^2 = 0.82$, $p < 0.0001$; Fig. 4) and can be described by the ordinary least squares (OLS) regression line equation

$$\delta^2H_{alkane} = 0.9^*\delta^2H_{ppt} - 189‰ \ (F_{1,27} = 56.89, p < 0.0001).$$

Tap water δ^2H values estimated for verified hive locations were also correlated linearly with δ^2H_{alkane} ($r^2 = 0.84$, $p < 0.0001$; Fig. 4) and can be describe by the OLS regression line

$$\delta^2H_{alkane} = 0.7^*\delta^2H_{tap} - 198‰ \ (F_{1,26} = 63.77, p < 0.0001).$$

The slopes for δ^2H values of tap water and precipitation *versus* cap wax *n*-alkanes were not different than 1 at $\alpha = 0.01$. In addition, the lines fitted to the wax *versus* estimated mean annual precipitation or estimated tap water hydrogen isotope ratios were not different ($p > 0.01$) from one another and can be described by a pooled slope and intercept of

$$\delta^2H_{alkane} = 0.8^*\delta^2H_{water} - 195‰.$$

The amount of variation in measured wax cap *n*-alkane δ^2H values explained by water isotope values was approximately equal for mean annual precipitation (82%) or tap water (84%). Thus, we conclude that both estimated mean annual precipitation and tap water δ^2H values were 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. As we hypothesized, nectar-producing plants reflect the estimated isotopes of local environmental water and that record is preserved in the molecular components of beeswax.

3.4. The apparent isotope fractionation between hydrocarbons and water varies with honey varietal

For biological processes, the apparent fractionation (ε value should be relatively constant between reactants and products (Hayes, 2002; Sachse et al., 2006). Honeybees use the fatty acid elongation biosynthetic pathway to produce hydrocarbons used in beeswax. We therefore predicted relatively fixed ε values between the source H pool (reactants) and resulting hydrocarbons (products). However, we observed $\varepsilon_{PPT-alkane}$ values ranging from −156‰ to −232‰ with a mean of −195‰ (Fig. 5). In addition, $\varepsilon_{PPT-alkane}$ varied as a function of honey varietal (Fig. 5), even though there were no observable differences in hydrocarbon distribution between individual varietals.

As an example, we observe beeswax *n*-alkanes from sage honeys have the most positive $\varepsilon_{PPT-alkane}$ values. Sage honeys originate from southern California and occur in chaparral environments where summers are hot and dry. These climatic conditions have been shown to evaporatively enrich environmental waters (Feakins & Sessions, 2010) and thus may be reflected in higher beeswax *n*-alkane δ^2H values, and thus higher e values. These observations suggest $\varepsilon_{PPT-alkane}$ can change due to an external, non-biologic factor, such as local environmental conditions. *A. mellifera* primarily use nectar and the resulting honey as their energy and H-source. The hydrogen isotope ratio of plant materials (i.e., cellulose, lipids, etc.) and stem waters, as well as the e values between these materials, can be altered by the local environment (Hou, D'Andrea, & Huang, 2008; Pedentchouk, Sumner, Tipple, & Pagani, 2008; Roden & Ehleringer, 2000). While our knowledge of the isotopic variation of plant nectar is nascent, a logical assumption is that floral nectar is subject to similar environmental factors as other plant materials. Thus, we suggest that while this work establishing ε values between *n*-alkanes and local water (estimated precipitation) is an important first step in order to create a predicative model of beeswax hydrocarbon δ^2H values, a more appropriate comparison may be between nectar and hydrocarbon δ^2H values. In future applications, we predict by comparing hydrocarbon δ^2H values to the true source

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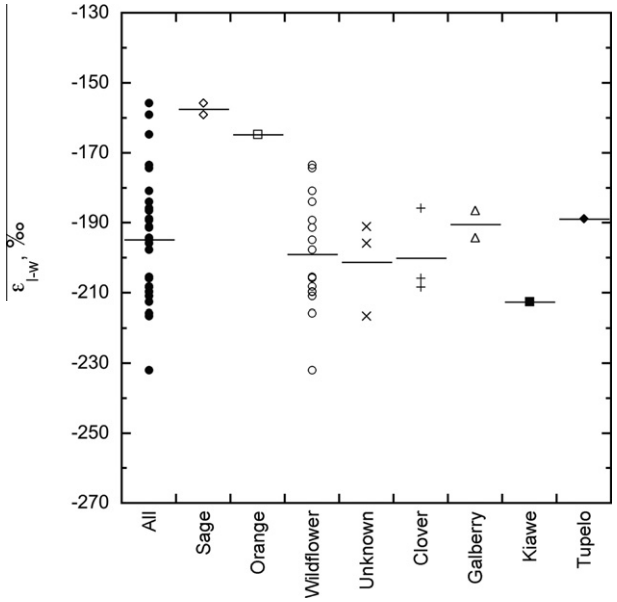

Fig. 5. Dot plot of apparent fractionations (ε) between mean annual precipitation and *n*-alkane δ^2H values. Apparent fractionations are grouped by honey varietals. Lines represent mean values for each varietal.

material (i.e., nectar) rather than water (i.e., precipitation), the variability currently observed in calculated ε values will be reduced.

3.5. Distinctive beeswax normal-alkanes can be extracted from liquid honey for isotope analysis

Even when visible beeswax or honeycomb were not available, extraction of dispersed hydrocarbons from 52 liquid honeys purchased from regional and national chain grocery stores throughout the United States, northern Mexico, and southern Canada in 2008 yielded hydrocarbon distributions distinctive to *A. mellifera*. We found hydrocarbon extracted from liquid honeys to have identical distribution to and ratio of *n*-alkanes and *n*-olefins as those extracted from solid beeswax. In addition, we found hydrocarbon extracted from liquid honeys to have an average chain length (ACL) of 27.1 ± 0.3, similar to beeswax. Thus, we ascribe these compounds as derivatives of the original honeycomb, not contamination from processing or waxes from plants visited by bees. Long-chain hydrocarbons attributed to beeswax have been previously reported in liquid honey (Bonaga et al., 1986; Tan et al., 1988; Tan et al., 1989; Tan et al., 1990; Terrab et al., 2002) from unifloral honey samples. To our knowledge, this is the first report of beeswax-derived hydrocarbons in mass-produced, highly processed liquid honeys. These data suggest that although beeswax is carefully separated from commercial liquid honeys, beeswax unavoidably contaminates liquid honey even with extensive filtration and heating applied in order to make mass-produced honeys shelf-stable.

Hydrocarbon distributions confirmed the presence of beeswax contamination, but in and of themselves yielded no geographic information due to the global dispersion of *A. mellifera* and lack of climatic/environmental variability (Section 3.1). On the other hand, hydrogen isotope values between bulk beeswax and hydrocarbons were strongly correlated (Fig. 3) and both were significantly correlated with estimated water δ^2H values (Fig. 4).

Given the additional laboratory and instrument time required for compound-specific isotope analysis (CSIA), there is little advantage to perform CSIA on the hydrocarbon fraction when bulk beeswax is visibly present and easily extractable. However, one advantage of CSIA is that only trace amounts of material are needed to generate isotope values and to quantify the presence of distinctive compounds.

Table 1
Hydrocarbon concentrations of three different honey types collected via liquid–liquid extraction.

Sample Type	*n*	Hydrocarbon Conc. (μg)/(g)	σ	Maximum (μg)/(g)	Minimum (μg)/(g)
Filtered	29	69	57	268	16
Raw	23	164	360	1764	19
Chunk	9	1362	1849	5802	32

From an extraction of highly processed and commercially available liquid honeys, we find hydrocarbons in concentrations sufficient to perform CSIA (Table 1). Given that the δ^2H value of hydrocarbons extracted from beeswax recorded the geographic variation in the δ^2H value of precipitation and tap water (Fig. 4), we predicted beeswax derivatives extracted from liquid honey should also exhibit this relationship. Degree of processing relates to concentration of hydrocarbons, where the least amount of processing (i.e., raw honey) yields the highest concentrations of hydrocarbons. Importantly, all honey types yielded hydrocarbon concentration adequate for CSIA (~200 ng; Table 1). As the liquid honey samples purchased from grocery stores in 2008 were not authentic materials from known hive locations, they should not be included in the development of a honey region-of-origin model, but they did provide a potential future application to determine region-of-origin of liquid honeys via CSIA of extracted hydrocarbons. CSIA of extracted hydrocarbon carbon isotope values may also relate to liquid honey carbon isotope values similar to the established relationship between liquid honey and protein (White & Winters, 1989; White, Winters, Martin, & Rossmann, 1998). Compound-specific and other recently developed GC-based techniques (Ruiz-Matute, Soria, Martinez-Castro, & Sanz, 2007) are likely the imminent methods needed to combat the future advancements in honey adulteration (e.g., the deliberate addition of proteins and/or sugar with similar carbon isotope values).

4. Conclusions

Here we examined the relationships between *A. mellifera* wax compounds' hydrogen isotope ratios and their region-of-origin. We have shown that *A. mellifera* produced a characteristic hydrocarbon distribution that was maintained regardless of honey varietal and environmental conditions. In this first study to present compound-specific hydrogen isotope analysis of beeswax-derived hydrocarbons, we found hydrocarbon δ^2H values were significantly correlated with estimated water δ^2H values for hive location. In addition, we observed potentially much of the isotopic difference between hydrocarbon and water δ^2H values is determined by environmental conditions. This work has also highlighted directions of future research in order to develop the use of stable isotope analysis for honey provenance-determination. Finally, this survey of honeys from the USA demonstrated the potential applications of compound-specific isotope analysis for investigation of the origin of honey, especially processed liquid honey containing no visible wax.

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