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Hydrogen and oxygen stable isotope analysis of pollen collected from honey

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Abstract
Analyses of stable isotope ratios of pollen present within complex matrices may be useful in sample provenance or climate change applications. We developed a method for separating pollen from honey and preparing pollen for hydrogen and oxygen stable isotope ratio analysis. The method combined several treatments: ethanol dilution to separate pollen; hexane washing to remove wax; and acetolysis to digest all but the external layer of pollen granules. There were large isotopic effects associated with treatments but effects were consistent between replicate applications of the method. We used the method to separate pollen from 45 honey samples from across the United States. Pollen δ^2H and $\delta^{18}O$ isotope values were significantly positively correlated ($P < 0.05$), but there was little variation in δ^2H values explained by $\delta^{18}O$ values ($r^2 = 0.17$). Pollen δ^2H values were significantly correlated with δ^2H values of precipitation predicted for hive locations ($P < 0.05$, $r^2 = 0.24$), but there was no significant correlation between pollen and precipitation $\delta^{18}O$ values ($P = 0.34$, $r^2 = 0.03$). These results suggest hydrogen and oxygen stable isotope analysis of pollen may not be as useful as analysis of liquid honey or beeswax in investigating provenance.

Keywords: *stable isotope ratio, geo-location, region-of-origin, isotope ratio mass spectrometry (IRMS), honeybee*

The separation of pollen granules from a complex sample matrix, such as honey or soil, is an oft-employed method in the fields of botany, ecology and geology to identify plant species. The identification of pollen granules to genus or species can be used to investigate historic patterns in land use and species prevalence (Gaillard et al., 1994; Tinner et al., 1996) as well as changes in climate (Van der Knaap & Van Leeuwen, 1995; Peyron et al., 2000; Rudaya & Li, 2012). At modern timescales, the identification of pollen also has forensic applications, as when materials recovered in trace amounts are compared to potential plant sources (Horrocks et al., 1998; Bryant & Jones, 2006; Mildenhall et al., 2006).

Some palynological investigations have utilised stable isotope analysis as a complement to identification of taxa at the genus and species levels. For example, carbon stable isotope analysis of pollen from soil can answer questions related to the appearance of different photosynthetic pathways (e.g. C_3 versus C_4; Amundson et al., 1997; Jahren, 2004; Descolas-Gros & Schölzel, 2007; Nelson et al., 2007). Recent studies have shown that the analysis of carbon, hydrogen and oxygen isotope ratios in pollen from modern plants can characterise the environmental conditions experienced by these plants (Loader & Hemming, 2004; Swoveland & Fall, 2009). These recent investigations using hydrogen and oxygen isotopes have focused on gymnosperm pollen. A study of the impact of temperature on angiosperm pollen by King et al. (2012) found that the carbon isotope ratios of pollen from plants grown under the same climatic conditions varied significantly and the isotope variations were not correlated with temperature.

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In addition to environmental studies, the stable isotope analysis of pollen hydrogen and oxygen atoms may also be useful for investigating provenance. This is because the hydrogen (δ^2H) and oxygen ($\delta^{18}O$) isotope values of environmental water vary spatially in a systematic and predictable manner (Bowen & Revenaugh, 2003; Bowen et al., 2007), with lower values found in high elevation, inland and cool climate regions, and higher values found in low elevation, coastal and warm climate regions. Water isotopic variations can in turn be 'recorded' by animal and plant tissues (West et al., 2006), which incorporate the isotopes of local environmental water during growth. Consequently, the hydrogen and oxygen stable isotope analysis of tissues can often be used to understand the potential regions-of-origins of the animal or plant. As examples, measured δ^2H and $\delta^{18}O$ values have aided in forensic investigations of the origin of plant-derived drugs (Booth et al., 2010; Hurley et al., 2010*a*, 2010*b*), foods (Heaton et al., 2008; Nakashita et al., 2008; Chesson et al., 2011) and beverages (Bowen et al., 2005; Chesson et al., 2010).

In order to apply pollen hydrogen and oxygen stable isotope analysis in provenance investigations of honey, pollen in sufficient quantity for analysis must first be separated from the honey. There are published methods for separating pollen granules from complex sample matrices (Amundson et al., 1997; Loader & Hemming, 2000; Jones & Bryant, 2004); at the time of pollen separation, the granules can also be prepared for observation under magnification. A pollen granule contains both vegetative and reproductive cells, which are surrounded by an inner cell wall that is comprised mostly of cellulose and commonly referred to as the intine. In turn, the intine is surrounded by a resistant outer cell wall that is comprised mainly of sporopollenin and commonly referred to as the exine (Erdtman, 1969). Since most of the distinctive, species-specific features used for pollen identification are found in the exine, pollen preparation methods typically digest the pollen cells and intine, leaving intact only the tough outer layer of pollen granules (Erdtman, 1954; Horrocks et al., 1998; Jones & Bryant, 1998).

Although gymnosperm pollen δ^2H and $\delta^{18}O$ values have been presented in at least two publications (Loader & Hemming, 2004; Swoveland & Fall, 2009), to the best of our knowledge, there has been no investigation into the impact of pollen separation and preparation methods on measured hydrogen and oxygen isotope ratios. In addition, there have been no studies on the potential usefulness of angiosperm pollen δ^2H and $\delta^{18}O$ values for climate or provenance investigations. In this study, we developed a protocol for the separation and preparation of pollen granules (as exine) from honey for hydrogen and oxygen stable isotope analysis. As part of the methods development effort, we were interested in understanding: (1) methodological challenges faced in the separation of pollen from honey and subsequent preparation of pollen for hydrogen and oxygen stable isotope analysis and (2) the isotopic variability both inherent to honey pollen and/or introduced by the separation and preparation treatment methods.

Here we present the results of a methods development effort for the separation and preparation of pollen from comb, chunk and liquid honeys for hydrogen and oxygen stable isotope analysis. We first describe the treatment processes investigated during the development effort and present data collected during methods development. We then present data that demonstrate the reproducibility of the developed pollen separation protocol. Finally, we present and discuss the measured δ^2H and $\delta^{18}O$ values of pollen separated from a variety of acquired honeys.

Material and methods

Samples used in methods development

We used three types of samples: pollen collected directly from the flowers of plants ('plant pollen'), purchased bee pollen ('bee pollen'), and pollen contained within various types of honey ('honey pollen').

Plant pollen was collected on the University of Utah campus in Salt Lake City, Utah. Sampled plants included daylily (*Hemerocallis* sp.), ragweed (*Ambrosia artemisiifolia* L.), spear saltbush (*Atriplex patula* L.) and sunflower (*Helianthus annuus* L.). Daylilies were found growing in a maintained planter. Ragweed, spear saltbush and sunflower plants were found growing together on a hillside. University-maintained sprinkler systems were nearby and we assumed all plants were regularly irrigated. Flowers from the daylily and spear saltbush plants were collected once in July 2009. Flowers from the ragweed and sunflower plants were collected on four separate occasions in July and August 2009.

Our plant pollen collection procedure was modified from a method published by the US Department of Agriculture (Jones, 2001) for the pollination of plants by hand. Blooming flowers were cut and placed in plastic zip-top bags, one bag per genus. Each bag contained flowers from multiple plants; the number of individual plants was dependent upon the size of the flowers. For example, ragweed plants had small flowers and dozens of individual plants were sampled. Fewer individual sunflower plants (4–6, on average) were sampled due to the larger size of the flowers. Plastic bags were sealed in the field and immediately returned to the laboratory. On the same day as collection,

flowers were transferred onto a piece of aluminium foil, which was loosely folded to enclose the flowers, and then placed in a drying oven (60 °C) overnight.

Dried daylily, ragweed and spear saltbush flowers were placed directly into a 100 mesh sieve and pollen was sieved onto another piece of aluminium foil. A clean metal spatula was used to open the flower heads to release pollen prior to sieving. Pollen from sunflowers was scraped from the flowers into the sieve using a clean metal spatula. Sieved pollen was visually observed under magnification for insect and plant parts. Pollen samples were stored in glass 20 ml scintillation vials at room temperature.

Bee pollen (Y. S. Organics Bee Farms, Sheridan, Illinois) was purchased from an outlet of the natural grocery Whole Foods Market in Salt Lake City, Utah, in August 2009. We additionally purchased bee pollen from a roadside stand in Hawthorne, Nevada in August 2009.

Honey pollen was separated from two comb honeys, one chunk honey and one liquid honey. The comb honey samples included clover honey from Indiana (Hunter's Honey Farm, online at www.huntershoneyfarm.com) and honey of unknown plant varietal from Georgia (Bruce's Nut-N-Honey Farm, online at www.bruceshoneyshack.com). The chunk honey was orange blossom from Florida (Walker Farms, online at www.walkerfarmshoney.com). The liquid honey was buckwheat from North Carolina (Uwharrie Mountain Honey Co.; purchased from Whole Foods Market in Salt Lake City, Utah). All honey samples were purchased in spring 2009.

Stable isotope notation and analysis

Measured hydrogen and oxygen stable isotope ratios are reported in 'delta' (δ) notation expressed in ‰, 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. $^2H/^1H$ or $^{18}O/^{16}O$) in the pollen sample and an international standard, respectively. The international standard for both hydrogen and oxygen stable isotope ratios is Vienna Standard Mean Ocean Water (VSMOW).

Samples were weighed (150 μg ± 10%) into Ag capsules, stored for at least five days under vacuum, and then analysed for hydrogen and oxygen isotope ratios. All samples were analysed in duplicate at a minimum; some samples were analysed multiple times to quantify heterogeneity. Hydrogen and oxygen stable isotope ratio measurements were made at both the Stable Isotope Facility for Environmental Research (SIRFER) at the University of Utah, Salt Lake City, Utah using a Thermo-Finnigan MAT Delta Plus XL stable isotope ratio mass spectrometer (ThermoFisher Scientific, Bremen, Germany) and at IsoForensics, Inc., Salt Lake City, Utah, using a Thermo-Finnigan MAT 253 stable isotope ratio mass spectrometer. Samples were introduced to the 1400 °C pyrolysis column of a high temperature conversion elemental analyser (TC/EA) via zero-blank autosampler (Costech Analytical, Valencia, California). The generated H_2 and CO gases were separated using a 1 m, 0.25 in (outer diameter) 5 Å molecular sieve gas chromatography column before being swept in a helium stream to the mass spectrometer.

Samples were analysed alongside a cellulose laboratory reference material with defined δ^2H and $\delta^{18}O$ values of –64‰ and +28.8‰, respectively. This cellulose reference material was used to normalise measured data via an offset correction. Analytical precision, calculated as the standard deviation (SD) of a secondary laboratory reference material (yeast, $n = 28$), was 2.2‰ for δ^2H and 0.64‰ for $\delta^{18}O$.

Statistical analyses were completed using Prism for Mac OS X (Version 5.0c; GraphPad Software Inc., La Jolla, California).

Experimental plan

Plant pollen samples were first analysed without treatment to understand isotopic variation both between different species of flowering plants and through time. One plant pollen (sunflower) and one bee pollen were analysed multiple times to investigate within sample isotopic heterogeneity. These samples had the most pollen available for analysis. In addition, the bee pollen and sunflower plant pollen was used to investigate the isotopic effect of soaking the pollen in water and subsequent drying.

To investigate the individual and collective effect of separation processes on the measured δ^2H and $\delta^{18}O$ values of pollen, we completed a multi-step study (Figure 1). This study included the two bee pollen, the two comb honeys and the liquid honey. Pollen was separated from aliquots of the two comb honeys and the liquid honey using an ethanol dilution method (Jones & Bryant, 1998, 2004). Pollen was air-dried after separation. Aliquots of both bee pollen samples were also treated to the ethanol dilution method and then air-dried. A sub-sample of each honey pollen and bee pollen was collected after ethanol dilution treatment for hydrogen and oxygen stable isotope analysis.

After collection of the sub-sample, remaining ethanol-treated material was divided. One half was suspended in 5 ml of hexane and then placed in a sonic bath for ten minutes. The suspension was centrifuged

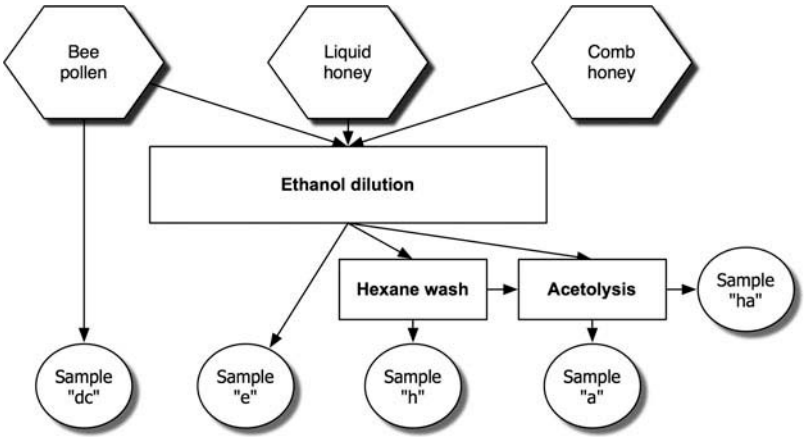

Figure 1. Flowchart presenting the order of treatment processes applied to pollen samples in a multi-step investigation of the isotopic effects of a combined pollen collection method. See text for a complete description of each treatment process.

for three minutes and the supernatant discarded; the process was repeated twice for a total of three hexane washes. The hexane-washed pellet was air dried at room temperature; a sub-sample was collected afterward for hydrogen and oxygen stable isotope analysis. The second half of the ethanol-treated material was subjected to the acetolysis method developed by Erdtman (1954) and a sub-sample was collected afterwards for analysis. A portion of the hexane-washed material was also subjected to acetolysis treatment. Finally, aliquots of both bee pollen samples were analysed untreated as treatment controls (Figure 1).

Effect of water soaking and drying treatments

Pollen samples were exposed to water and then dried multiple times in this study. To investigate the isotopic effect of drying, we soaked aliquots of store-bought bee pollen and sunflower plant pollen in local tap water ('UU-DI') for approximately three hours. Water was decanted and the wet bee and sunflower pollen samples were each divided into three 1 dram glass vials to be (a) placed in a drying oven at 60 °C overnight, (b) frozen and then placed in a freeze-dryer for 48 hours, or (c) dried at room temperature using forced air overnight.

To investigate the effect of exposure to isotopically varying water, we soaked separate aliquots of the store-bought bee pollen and sunflower plant pollen in three isotopically distinct waters for 48 hours. Experimental waters spanned a range of δ^2H and $\delta^{18}O$ values: GISP = −190‰ and −24.8‰, UU-DI = −123‰ and −16.5‰, and UU-Zero = −0.1‰ and −0.2‰, respectively. After soaking, pollen samples were air-dried at overnight at room temperature.

Application of the pollen separation and preparation method

To investigate the reproducibility of results from this pollen separation and preparation method, we subjected three separate aliquots of store-bought bee pollen, sunflower plant pollen, chunk honey and liquid honey to the complete method and compared measured δ^2H and $\delta^{18}O$ values. We then used the complete method to separate pollen from 45 previously acquired comb, chunk and liquid honeys for hydrogen and oxygen stable isotope ratio measurement. Thirty-eight of these honeys were purchased by us from online stores; see Chesson et al. (2011) and Tipple et al. (2012) for details. An additional seven samples were donated by beekeepers from Alabama, California and Pennsylvania.

Results

Isotopic variation inherent to pollen

The measured δ^2H values of pollen collected from four plant genera growing on the University of Utah campus ranged from –205‰ to –149‰ (Table I). Measured $\delta^{18}O$ values ranged from +19.8‰ to +34.2‰. The mean (± SD) δ^2H value for ragweed pollen was −205‰ (± 7‰, n = 4) and was significantly lower than the mean δ^2H value of −191‰ (± 6‰, n = 4) for sunflower pollen (unpaired t-test; t = 3.010, P = 0.024). The variances between the two genera were not significantly different. In contrast, the mean $\delta^{18}O$ value of ragweed pollen was +20.9‰ (± 1.4‰, n = 4) and was not significantly different than the mean $\delta^{18}O$ value of +19.8‰ (± 0.4‰, n = 4) for sunflower pollen. The variance of sunflower pollen samples was significantly smaller than ragweed pollen samples (P = 0.048).

Five replicate analyses of sunflower plant pollen had SDs of 0.8‰ for δ^2H and 0.2‰ for $\delta^{18}O$ values. Four replicate analyses of the store-bought bee pollen had SDs of 0.4‰ and 0.1‰ for δ^2H and $\delta^{18}O$ values, respectively. The variances between the two pollen types were not significantly different for either δ^2H or $\delta^{18}O$ values.

Table I. Results from the hydrogen and oxygen stable isotope analysis of different plant pollen collected July–August 2009.

Plant pollen	*n*	Mean	SD
Measured δ^2H value (‰)			
Daylily	1	−156	
Ragweed	4	−205	±7
Spear saltbush	1	−149	
Sunflower	4	−191	±6
Measured $\delta^{18}O$ value (‰)			
Daylily	1	+25.4	
Ragweed	4	+20.9	±1.4
Spear saltbush	1	+34.2	
Sunflower	4	+19.8	±0.4

Note: All plants were found growing on the University of Utah campus in Salt Lake City, UT. Daylily and spear saltbush plant pollen were collected only once; values represent the analysis of duplicate capsules. Ragweed and sunflower pollen were collected at four different times; values represent the analysis of duplicate capsules for each time point. Blank cells indicate no data.

The isotopic effect of drying

The isotopic difference between pollen in the three drying treatments was small for hydrogen isotope ratios (Table II), as demonstrated by the total range in δ^2H values for the bee pollen (2‰) and sunflower plant pollen (3‰). These ranges were smaller than twice the analytical precision for H isotope measurements (e.g. <4.4‰). A maximum difference of 2.3‰ for $\delta^{18}O$ values was observed between bee pollen samples in the freeze-dried and air-dried treatments (Table II). The maximum difference in $\delta^{18}O$ values between the oven-dried and freeze-dried treatments for sunflower plant pollen was 3.0‰. These ranges were larger than twice the analytical precision of ~0.6‰ for O isotope measurements. We note that all drying processes completed in the remainder of the study used air.

Table II. Results from the hydrogen and oxygen stable isotope analysis of bee pollen and sunflower plant pollen soaked in water for three hours and then dried using three different methods.

Pollen	Untreated	Oven-dried	Freeze-dried	Air-dried
Measured δ^2H value (‰)				
Bee	−111	−137	−135	−135
Sunflower	−182	−203	−205	−202
Measured $\delta^{18}O$ value (‰)				
Bee	+30.9	+28.3	+26.0	+26.8
Sunflower	+22.6	+19.8	+16.8	+18.6

Note: Samples were soaked in UU-DI water, which has measured δ^2H and $\delta^{18}O$ values of −123‰ and −16.5‰, respectively. Aliquots of the bee and sunflower plant pollen were also analysed without soaking or drying (untreated). Values presented are the means of duplicate analyses, except the sunflower pollen in the freeze-dried treatment (n = 1).

The isotopic effect of water exposure

We observed a decrease in the measured δ^2H and $\delta^{18}O$ values of bee pollen and sunflower plant pollen following a 48-hour soaking in all isotopically distinct waters used in the experiment (Table III). The difference between untreated bee pollen and bee pollen soaked in local tap water (UU-DI) was 22‰ for δ^2H values and 7.4‰ for $\delta^{18}O$ values. For sunflower pollen, the difference between untreated pollen and pollen soaked in UU-DI was 29‰ and 3.4‰ for δ^2H and $\delta^{18}O$ values, respectively. This same pattern of decrease between untreated and water-soaked pollen was observed in the drying experiment (Table II) although the length of time pollen was in water during the drying experiment was shorter (three hours) than the length of time pollen was in water during the soaking experiment (48 hours).

The cumulative effect of pollen separation and preparation processes

The analyses revealed hydrogen and oxygen element compositions and isotope ratios for pollen exposed to various stages of treatment during the pollen separation and preparation method outlined in Figure 1 (Table IV). Before treatment, the mean element composition of the bee pollen was 7% for H and 36% for O (w/w). The most treated samples of bee pollen had mean element compositions of 7% and 27% for H and O, respectively. In comparison, the most treated samples of separated honey pollen had H and O element composition ranges of 5–6% and 28–30%, respectively (Table IV).

The measured δ^2H and $\delta^{18}O$ values of the untreated bee pollen decreased after completion of all treatments (Table IV). For bee pollen #1, the decrease was 75‰ for δ^2H values and 5.8‰ for

Table III. Results from the hydrogen and oxygen stable isotope analysis of bee pollen and sunflower plant pollen soaked in three isotopically distinct waters for 48 hours.

Pollen	Untreated	In GISP	In UU-DI	In UU-Zero
Measured δ^2H value (‰)				
		(−190)	(−123)	(0)
Bee	−115	−145	−137	−137
Sunflower	−185	−211	−214	−210
Measured $\delta^{18}O$ value (‰)				
		(−24.8)	(−16.5)	(−0.2)
Bee	+29.3	+22.3	+21.9	+22.7
Sunflower	+22.0	+16.4	+18.6	+18.7

Note: Measured δ^2H and $\delta^{18}O$ values of treatment waters are given in parentheses. After soaking, samples were air-dried at room temperature overnight. Aliquots of the bee and sunflower plant pollens were also analysed without soaking and drying (untreated). Values presented are the means of duplicate analysed capsules.

Table IV. Results from the hydrogen and oxygen stable isotope analysis of different types of pollen processed using various treatments in sequence.

Pollen	Control		Ethanol dilution		Hexane wash		Acetolysis		Hexane + acetolysis	
Element composition data (%)										
	H	O	H	O	H	O	H	O	H	O
Bee #1	7	38	6	32	6	32	6	23	7	26
Bee #2	7	34	6	28	6	28	7	25	7	27
Comb honey #1			8	36	7	42	7	29	5	28
Comb honey #2			12	18	7	40	6	27	6	30
Liquid honey			7	39	6	42	6	27	6	28
Isotope ratio data (‰)										
	δ^2H	$\delta^{18}O$	δ^2H	$\delta^{18}O$	δ^2H	$\delta^{18}O$	δ^2H	$\delta^{18}O$	δ^2H	$\delta^{18}O$
Bee #1	−119	+26.1	−140	+23.8	−133	+23.6	−188	+20.3	−194	+20.3
Bee #2	−115	+29.3	−124	+25.9	−127	+25.7	−144	+21.7	−144	+21.7
Comb honey #1			−143	+25.1	−103	+26.3	−134	+21.2	−109	+18.7
Comb honey #2			−208	+31.8	−70	+34.0	−108	+25.2	−80	+27.5
Liquid honey			−88	+33.7	−67	+34.0	−131	+26.3	−115	+26.3

Note: See Figure 1 for the order of treatments and the text for a description of each treatment. Values presented are the means of duplicate analysed capsules. Blank cells denote no data could be collected.

$\delta^{18}O$ values. For bee pollen #2, the decreases were 29‰ and 7.6‰ for δ^2H and $\delta^{18}O$ values, respectively. Since it was not possible to prepare 'untreated' honey pollen, we compared the most and least treated honey pollen samples and found that measured δ^2H values of the two comb honeys increased. This increase appeared to be primarily a result of the hexane wash, which increased δ^2H values for comb honey #1 by 40‰ and for comb honey #2 by 138‰. In contrast, the measured δ^2H values of the liquid honey pollen decreased between least and most treated samples, by 27‰. The $\delta^{18}O$ values of the comb and liquid honey pollen processed with the ethanol dilution treatment decreased after being further subjected to the hexane plus acetolysis treatment (Table IV).

Reproducibility of the separation and preparation method

The measured δ^2H and $\delta^{18}O$ values of three aliquots of each of the store-bought bee, sunflower plant, chunk and liquid honey pollen processed, using the complete separation and preparation method we developed, varied (Table V). Variability in the measured δ^2H and $\delta^{18}O$ values of bee and sunflower plant pollen was low within aliquots of the same pollen; SDs were 1‰ and 0.1–0.2‰, respectively. Variability within aliquots of the same chunk and liquid honey pollen were larger, with ranges of 3–7‰ and 0.2–0.5‰ for SDs of measured δ^2H and $\delta^{18}O$ values, respectively (Table V).

Table V. Results from the hydrogen and oxygen stable isotope analysis of three aliquots of bee, sunflower plant, chunk honey and liquid honey pollen separately processed using a combined ethanol dilution, hexane wash, and acetolysis treatment.

	Measured δ^2H value (‰)		Measured $\delta^{18}O$ value (‰)	
Sample type	Mean	SD	Mean	SD
Bee pollen				
Aliquot 1	−146	±1	+24.4	±0.2
Aliquot 2	−143	±2	+24.6	±0.2
Aliquot 3	−142	±1	+24.0	±0.2
Overall	−144	±1	+24.3	±0.2
Plant pollen				
Aliquot 1	−207	±1	+18.4	±0.1
Aliquot 2	−209	±1	+18.3	±0.1
Aliquot 3	−211	±1	+18.1	±0.1
Overall	−209	±1	+18.3	±0.1
Chunk honey				
Aliquot 1	−87	±2	+28.0	±0.1
Aliquot 2	−75	±3	+28.3	±0.5
Aliquot 3	−75	±3	+28.6	±0.8
Overall	−79	±3	+28.3	±0.5
Liquid honey				
Aliquot 1	−121	±10	+29.8	±0.2
Aliquot 2	−117	±6	+30.6	±0.0
Aliquot 3	−115	±6	+31.5	±0.3
Overall	−118	±7	+30.6	±0.2

Note: See text for a complete description of the treatment that combined ethanol dilution, hexane and acetolysis processes. Values for bee and plant pollens are the means of five analysed capsules, except the $\delta^{18}O$ value of bee pollen from Aliquot 1 ($n = 4$). Values for chunk and liquid honey pollens are the mean of two analysed capsules.

Hydrogen and oxygen isotope ratios of pollen from acquired honeys

Pollen was separated from 45 comb, chunk and liquid honeys acquired from across the United States and prepared for hydrogen and oxygen stable isotope analysis using the complete method described earlier. The measured δ^2H values of the prepared honey pollen ranged from −197‰ to −80‰ (Table VI). The measured $\delta^{18}O$ values

Table VI. Results from the hydrogen and oxygen stable isotope analysis of pollen separated from 45 acquired honeys using a combined ethanol dilution, hexane wash, and acetolysis treatment method shown alongside the predicted isotopic composition of precipitation (PPT) for verified hive locations.

State	Honey varietal	Measured δ^2H value (‰)	Measured $\delta^{18}O$ value (‰)	Predicted PPT δ^2H value (‰)	Predicted PPT $\delta^{18}O$ value (‰)
Alabama	Unspecified	−88	+25.0	−31	−4.9
Alabama	Unspecified	−90	+24.1	−31	−4.9
Alabama	Wildflower	−87		−26	−4.2
California	Orange blossom	−197		−75	−9.8
California	Sage	−107	+28.9	−81	−10.4
California	Sage	−116	+28.2	−64	−8.0
California	Unspecified	−96	+26.6	−72	−9.5
California	Unspecified	−87	+28.3	−72	−9.5
California	Unspecified	−87	+28.7	−72	−9.5
California	Unspecified	−117	+29.1	−72	−9.5
California	Wildflower	−122	+27.8	−73	−9.6
California	Wildflower	−115	+27.9	−73	−9.6
California	Wildflower	−135	+25.7	−74	−9.8
Colorado	Clover	−150	+23.9	−98	−13.4
Connecticut	Unspecified	−130	+23.7	−58	−9.1
Florida	Orange blossom	−131	+27.1	−24	−4.0
Florida	Orange blossom	−88	+28.5	−21	−3.6
Florida	Sourwood	−94	+25.9	Not verified	Not verified
Florida	Rupelo	−103	+29.2	−25	−4.1
Georgia	Gallberry	−107	+27.1	−26	−4.3
Georgia	Gallberry	−117	+26.4	−27	−4.4
Georgia	Sourwood	−92	+25.5	−39	−6.2
Georgia	Unspecified	−80	+28.0	Not verified	Not verified
Georgia	Wildflower	−92	+29.2	−39	−6.2
Hawaii	Kiawe	−140	+23.7	−8	−2.4
Idaho	Unspecified	−167	+23.5	−97	−12.8
Idaho	Unspecified	−163	+23.3	−104	−13.7
Idaho	Wildflower	−137	+23.2	−97	−12.8
Iowa	Wildflower	−124	+22.8	−55	−8.0
Kentucky	Clover	−107	+24.0	−43	−6.7
Maryland	Wildflower	−98	+27.3	−50	−7.8
Massachusetts	Unspecified	−124	+23.0	Not verified	Not verified
Michigan	Wildflower	−138	+24.1	−56	−8.3
Michigan	Wildflower	−111	+22.7	−61	−9.0
Mississippi	Wildflower	−105	+21.2	−36	−5.6
New Jersey	Unspecified	−106	+27.1	Not verified	Not verified
New York	Wildflower		+22.1	Not verified	Not verified
Ohio	Wildflower	−116	+24.4	−54	−8.2
Ohio	Wildflower	−111	+23.2	−52	−7.9
Pennsylvania	Wildflower	−96	+26.4	−50	−7.9
Pennsylvania	Wildflower	−90	+21.7	Not verified	Not verified
Pennsylvania	Wildflower	−99	+25.5	−52	−8.2
Virginia	Wildflower	−97	+27.9	−49	−7.7
West Virginia	Wildflower	−97	+26.3	−50	−7.8
West Virginia	Wildflower	−106	+29.1	−50	−7.8
Mean		−113	+25.7		
SD		±24	±2.4		
Maximum		−80	+29.2		
Minimum		−197	+21.2		
Range		117	8.1		

Note: Values presented are the means of duplicate analysed capsules. Blank cells indicate no data were collected. 'State' represents the location of the hive (confirmed by seller), not necessarily the location of the seller or the store; cases where location of hive could not be verified are noted. Varietal information was provided by sellers and was not independently corroborated.

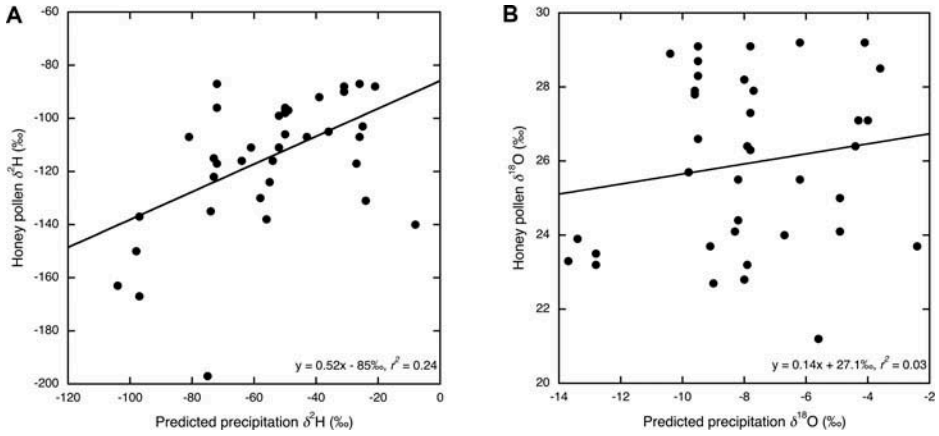

Figure 2. Bi-plots of the measured δ^2H (**A**) and $\delta^{18}O$ (**B**) values of pollen collected from honey versus the predicted δ^2H (**A**) and $\delta^{18}O$ (**B**) values of precipitation at the verified location of the hives.

ranged from +21.2‰ to +29.2‰ and were significantly positively correlated with measured δ^2H values (Pearson $r = 0.41$, $n = 42$). Using an error of 0.64‰ for values of x and 2.2‰ for values of y, the slope of the Model II (Deming) regression line describing the relationship between δ^2H and $\delta^{18}O$ values ($\delta^2H = 19.4 \times \delta^{18}O - 606.0$‰) was significantly different from a slope of zero ($P = 0.0064$).

Measured pollen δ^2H and $\delta^{18}O$ values were compared to the δ^2H and $\delta^{18}O$ values, respectively, of precipitation predicted for the verified hive locations associated with these samples [precipitation data presented in Table VI; see Chesson et al. (2011) for a description of precipitation prediction and location verification methods]. Measured pollen δ^2H values were significantly positively correlated with predicted precipitation δ^2H values (Pearson $r = 0.49$, $P = 0.0014$), although the coefficient of determination was low ($r^2 = 0.24$). There was no significant correlation between separated pollen and predicted precipitation $\delta^{18}O$ values ($P = 0.34$, $r^2 = 0.03$; Figure 2).

Discussion

The primary goal of this research effort was the development of an easy-to-implement method for separating and preparing pollen from honey for hydrogen and oxygen stable isotope analysis. Data on isotopic heterogeneity inherent to different plant and bee pollen was used to understand reporting limits for the hydrogen and oxygen stable isotope analysis of pollen separated from honey. Analytical precision for hydrogen and oxygen stable isotope ratio measurements in this work, established using the SDs of a yeast laboratory reference material, was 2.2‰ and 0.64‰, respectively. These SDs were generally smaller than the SDs observed for ragweed and sunflower plant pollen collected at multiple time points over two months: 6–7‰ for δ^2H values and 0.4–1.4‰ for $\delta^{18}O$ values (Table I). This suggests there are temporal variations in the δ^2H and $\delta^{18}O$ values of plant pollen based on environmental factors. In comparison, the SDs observed in replicate analysis of untreated plant and bee pollen from a single collection time point were only ~1‰ and ~0.2‰ for δ^2H and $\delta^{18}O$ values, respectively, reinforcing the conclusion that the observed variations in plant pollen collected over a two month period were based on environmental factors and not analytical capacities.

We observed variation in the measured δ^2H and $\delta^{18}O$ values of pollen associated with different drying treatments (oven-, freeze- and air-drying; Table II). This variation was most evident when comparing the $\delta^{18}O$ values of freeze-dried pollen to the oven-dried and air-dried pollen. The impact of variable drying methods could have a significant effect on measured δ^2H and $\delta^{18}O$ values. However, if the same drying method was used each time pollen is separated from honey, measured pollen isotope ratios should be comparable between samples. We, therefore, used a single drying process – forced air – for all drying steps in the treatment method we developed.

We found that the measured δ^2H and $\delta^{18}O$ values of pollen soaked in three isotopically distinct waters were lower after soaking. However, the decrease in measured pollen δ^2H and $\delta^{18}O$ values after soaking was generally similar among soaking treatments despite the large isotopic range among the waters used in the treatments (Table III). Considering the practical implications of this result, we note that the δ^2H and $\delta^{18}O$ values of UU-DI water are identical to values for tap water in Salt Lake City, Utah, where this methods development work was completed. The

δ^2H and $\delta^{18}O$ values of UU-Zero water are similar to those expected for tap water from the states of Texas or Florida (Bowen et al., 2007). Results from the water soaking experiment suggest that pollen could be separated from honey in either Utah or Texas and the isotopic differences of water between the two locations would not significantly impact the final measured δ^2H and $\delta^{18}O$ values of the analysed pollen.

We found variable isotopic effects caused by the different treatment methods we used to separate and prepare pollen for hydrogen and oxygen stable isotope ratio analysis (Table IV). The magnitude and direction of these effects depended in part upon pollen type. For example, the pattern observed for bee pollen was a decrease in both δ^2H and $\delta^{18}O$ values between least and most treated samples. In contrast, the δ^2H values of comb honey pollen increased between least and most treated samples. This was most evident following the hexane wash treatment. Because lipids and fats typically have low δ^2H values, this suggests wax pieces present in the pollen material collected from honey via ethanol dilution were subsequently removed with a hexane wash. This hypothesis is supported by the visual observation of pollen separated from chunk honey using the ethanol dilution method. Under magnification, we saw large pieces of material that were too irregularly shaped to be pollen granules.

The various pollen types – bee, comb and liquid honey – had nearly identical hydrogen and oxygen element compositions after receiving all treatments (Table IV). From these data, we concluded that the developed method resulted in the same type of separated material, regardless of the original sample matrix. The processing of separate aliquots of bee, plant, chunk and liquid honey pollen demonstrated that isotopic effects of each treatment were reproducible (Table V). It further demonstrated that the overall between-aliquot variation was smaller than within-aliquot variation. We concluded that the consistent application of treatment methods resulted in comparable material type and comparable measured δ^2H and $\delta^{18}O$ values.

We used our developed method to separate and prepare pollen from 45 honey samples for hydrogen and oxygen stable isotope analysis (Table VI). We observed significant isotopic variation among the measured δ^2H and $\delta^{18}O$ values of the collected pollen; these δ^2H and $\delta^{18}O$ values were significantly positively correlated. Due to the recognised impact of H and O isotopes in local environmental water on plant tissue δ^2H and $\delta^{18}O$ values (Flanagan & Ehleringer, 1991*b*), the observed positive correlation for pollen was not unexpected since there is a positive correlation between δ^2H and $\delta^{18}O$ values of meteoric water (Craig, 1961). A positive correlation has also been previously observed between the measured hydrogen and oxygen isotope ratios of liquid honey (Chesson et al., 2011). However, the only other published dataset of pollen δ^2H and $\delta^{18}O$ values, generated from the stable isotope analysis of gymnosperm pollen collected from modern trees, observed a negative correlation (Loader & Hemming, 2004). To the best of our knowledge, there are no other published datasets of gymnosperm or angiosperm pollen δ^2H and $\delta^{18}O$ values, to which we can compare our data.

While the δ^2H and $\delta^{18}O$ values of pollen extracted from the 45 honey samples were significantly correlated, the coefficient of determination for the Deming regression line fitted to the data was low ($r^2 = 0.17$). This was not expected, since we had previously observed a high coefficient of determination between the measured δ^2H and $\delta^{18}O$ values of liquid honey ($r^2 = 0.64$; Chesson et al., 2011). We had also previously observed strong correlations between the isotopic composition of predicted precipitation and liquid honey (Chesson et al., 2011) as well as beeswax (Tipple et al., 2012). In this study, however, we observed only a weak correlation between honey pollen and precipitation δ^2H values; there was no significant correlation between the $\delta^{18}O$ values of pollen and predicted precipitation. Thus, it does not appear precipitation δ^2H and $\delta^{18}O$ values are reliable predictors of honey pollen δ^2H and $\delta^{18}O$ values. There may be multiple reasons for the lack of a significant correlation between observed pollen and predicted precipitation δ^2H and $\delta^{18}O$ values; we consider two here.

First, source water from the soil is the initial factor influencing the isotopic composition of water in the plant when pollen grains are produced (Ehleringer & Dawson, 1992). In addition, plant water is greatly influenced by the local microclimate (i.e. temperature and humidity; Flanagan & Ehleringer, 1991*a*, 1991*b*). Thus, the combination of the isotopic composition of locally available water and climate should be the main drivers of pollen δ^2H and $\delta^{18}O$ values, as demonstrated for other plant tissues like leaf water and plant lipids (West et al., 2010). The isotopic composition of water available to a plant and the plant's microclimate (temperature and humidity) will vary during the growing season. The isotopic signal recorded in pollen produced by a plant early in the growing season could therefore be markedly different from the signal recorded in pollen produced by the plant later in the growing season. As evidence of this, we observed variation in the δ^2H and $\delta^{18}O$ values of ragweed and sunflower pollen collected over a two month period from the same location. Considering the pollen separated from our

purchased honey samples represented an amalgamation of multiple (unknown) production time points, there may be no clear link between that amalgamated pollen signal and the predicted precipitation δ^2H and $\delta^{18}O$ values for the growing location.

Second, the pollen we separated from our US honey samples was likely from multiple plants of the same species and even from multiple plants of different species (i.e. 'wildflower' honey). The microclimates around these plants could have differed enough to influence plant water isotope values. A study by King et al. (2012) found that pollen from multiple plants of the same species, growing under similar environmental conditions, exhibited large carbon isotopic variations. In this study, we observed that pollen collected from different flowering plant species growing in the same location had significantly different δ^2H and $\delta^{18}O$ values; plant stature and therefore microclimate for these plants was different. For example, daylily and sunflower plant pollen had a range of 40‰ for δ^2H and 5.5‰ for $\delta^{18}O$ values (Table I) and were quite different in height, suggesting that the pollen has developed under different microclimates even though plants were grown in the same macroclimate. This implies that the apparent isotopic fractionation for hydrogen and oxygen between source (local water) and product (pollen) reflects a microclimate difference. To better understand the isotopic relationship between water source and pollen, it may be more useful in the future to focus on single varietal honeys so that microclimate differences would not play a role in interpreting the seasonal patterns of pollen isotope values.

Conclusion

Our investigation into different treatments demonstrated that it is possible to separate and prepare pollen from honey for hydrogen and oxygen stable isotope analysis. Although the individual isotopic effects of different treatments included in the honey pollen separation method we developed were large, the effects were consistent between multiple preparations of separate sample aliquots. Replicate δ^2H and $\delta^{18}O$ analyses of treated pollen exhibited SDs near to or slightly higher than SDs of an isotopically homogenous reference material and we thus concluded that replicate sampling differences did not contribute to this pattern.

The measured δ^2H and $\delta^{18}O$ values of pollen samples separated from 45 honey samples acquired from across the United States were significantly and positively correlated, although the coefficient of determination of δ^2H values as a function of $\delta^{18}O$ values was lower than previously observed for liquid honey. In addition, the power of precipitation isotopic composition alone for predicting pollen δ^2H and $\delta^{18}O$ values was low, most likely because of the dominant influence of plant water on pollen isotope ratio values. For future investigations into honey sample provenance, we therefore recommend the analysis of liquid honey or beeswax components in the sample, rather than pollen analyses. Further work on the application of hydrogen and oxygen isotope ratio analysis of pollen for climate or provenance studies should focus on understanding the impacts of timing and microclimate on δ^2H and $\delta^{18}O$ values of pollen.

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References

Amundson, R., Evett, R. R., Jahren, A. H. & Bartolome, J. (1997). Stable carbon isotope composition of Poaceae pollen and its potential in paleovegetational reconstructions. *Review of Palaeobotany and Palynology*, *99*, 17–24.

Booth, A. L., Wooller, M. J., Howe, T. & Haubenstock, N. (2010). Tracing geographic and temporal trafficking patterns for marijuana in Alaska using stable isotopes (C, N, O, and H). *Forensic Science International*, *202*, 45–53.

Bowen, G. J., Ehleringer, J. R., Chesson, L. A., Stange, E. & Cerling, T. E. (2007). Stable isotope ratios of tap water in the contiguous United States. *Water Resources Research*, *43*, W03419.

Bowen, G. J. & Revenaugh, J. (2003). Interpolating the isotopic composition of modern meteoric precipitation. *Water Resources Research*, *39*, 1299–1311.

Bowen, G. J., Winter, D. A., Spero, H. J., Zierenberg, R. A., Reeder, M. D., Cerling, T. E., et al. (2005). Stable hydrogen and oxygen isotope ratios of bottled waters of the world. *Rapid Communications in Mass Spectrometry*, *19*, 3442–3450.

Bryant, V. M. & Jones, G. D. (2006). Forensic palynology: Current status of a rarely used technique in the United States of America. *Forensic Science International*, *163*, 183–197.

Chesson, L. A., Tipple, B. J., Erkkila, B. R., Cerling, T. E. & Ehleringer, J. R. (2011). B-HIVE: Beeswax hydrogen isotopes as validation of environment. Part I. Bulk honey and honeycomb stable isotope analysis. *Food Chemistry*, *125*, 576–581.

Chesson, L. A., Valenzuela, L. O., O'Grady, S. P., Cerling, T. E. & Ehleringer, J. R. (2010). Links between purchase location and the stable isotope ratios of bottled water, soda, and beer in the USA. *Journal of Agricultural and Food Chemistry*, *58*, 7311–7316.

Craig, H. (1961). Isotopic variations in meteoric waters. *Science*, *133*, 1702–1703.

Descolas-Gros, C. & Schölzel, C. (2007). Stable isotope ratios of carbon and nitrogen in pollen grains in order to characterize plant functional groups and photosynthetic pathway types. *New Phytologist*, *176*, 390–401.

Ehleringer, J. R. & Dawson, T. E. (1992). Water uptake by plants: Perspectives from stable isotope composition. *Plant Cell and Environment*, *15*, 1073–1082.

Erdtman, G. (1954). *An introduction to pollen analysis*. Waltham, MA: Chronica Botanic Company.

Erdtman, G. (1969). *Handbook of palynology: An introduction to the study of pollen grains and spores*. New York, NY: Hafner Publishing Co.

Flanagan, L. B. & Ehleringer, J. R. (1991*a*). Effects of mild water stress and diurnal changes in temperature and humidity on the stable oxygen and hydrogen isotopic composition of leaf water in *Cornus stolonifera* L. *Plant Physiology*, *97*, 298–305.

Flanagan, L. B. & Ehleringer, J. R. (1991*b*). Stable isotope composition of stem and leaf water: Applications to the study of plant water use. *Functional Ecology*, *5*, 270–277.

Gaillard, M. -J., Birks, H. J. B., Emanuelsson, U., Karlsson, S., Lageras, P. & Olausson, D. (1994). Application of modern pollen/land-use relationships to the interpretation of pollen diagrams – reconstruction of land-use history in south Sweden, 3000–0 BP. *Review of Palaeobotany and Palynology*, *82*, 47–73.

Heaton, K., Kelly, S. D., Hoogewerff, J. & Woolfe, M. (2008). Verifying the geographical origin of beef: The application of multi-element isotope and trace element analysis. *Food Chemistry*, *107*, 506–515.

Horrocks, M., Coulson, S. A. & Walsh, K. A. J. (1998). Forensic palynology: Variation in pollen content of soil surface samples. *Journal of Forensic Sciences*, *43*, 320–323.

Hurley, J. M., West, J. B. & Ehleringer, J. R. (2010*a*). Tracing retail cannabis in the United States: Geographic origin and cultivation patterns. *International Journal of Drug Policy*, *21*, 222–2228.

Hurley, J. M., West, J. B. & Ehleringer, J. R. (2010*b*). Stable isotope models to predict geographic origin and cultivation conditions of marijuana. *Science and Justice*, *50*, 86–93.

Jahren, A. H. (2004). The carbon stable isotope composition of pollen. *Review of Palaeobotany and Palynology*, *132*, 291–313.

Jones, G. D. (2001). How to collect pollen. Available from: http://pollen.usda.gov/Research_Techniques/How_to_collect_pollen.htm (accessed 27 July 2009).

Jones, G. D. & Bryant, V. M. (1998). Are all counts created equal?. In V. M. Bryant & J. Wrenn (Eds.), *New Developments in palynomorph sampling, extraction and analysis* (pp. 107–114). Dallas, TX: American Association of Statigraphic Palynologists.

Jones, G. D. & Bryant, V. M. (2004). The use of EtOH for the dilution of honey. *Grana*, *43*, 174–182.

King, D. C., Schubert, B. A. & Jahren, A. H. (2012). Practical considerations for the use of pollen $\delta^{13}C$ value as a paleoclimate indicator. *Rapid Communications in Mass Spectrometry*, *26*, 2165–2172.

Loader, N. J. & Hemming, D. L. (2000). Preparation of pollen for stable carbon isotope analyses. *Chemical Geology*, *165*, 339–344.

Loader, N. J. & Hemming, D. L. (2004). The stable isotope analysis of pollen as an indicator of terrestrial palaeoenvironmental change: A review of progress and recent developments. *Quaternary Science Reviews*, *23*, 893–900.

Mildenhall, D. C., Wiltshire, P. E. J. & Bryant, V. M. (2006). Forensic palynology: Why do it and how it works. *Forensic Science International*, *163*, 163–172.

Nakashita, R., Suzuki, Y., Akamatsu, F., Iizumi, Y., Korenaga, T. & Chikaraishi, Y. (2008). Stable carbon, nitrogen and oxygen isotope analysis as a potential tool for verifying geographical origin of beef. *Analytica Chimica Acta*, *617*, 148–152.

Nelson, D. M., Sheng Hu, F., Mikucki, J. A., Tian, J. & Pearson, A. (2007). Carbon-isotopic analysis of individual pollen grains from C_3 and C_4 grasses using a spooling-wire microcombustion interface. *Geochimica et Cosmochimica Acta*, *71*, 4005–4014.

Peyron, O., Jolly, D., Bonnefille, R., Vincens, A. & Guiot, J. (2000). Climate of East Africa 6000 ^{14}C yr B.P. as inferred from pollen data. *Quaternary Research*, *54*, 90–101.

Rudaya, N. & Li, H.-C. (2012). A new approach for reconstruction of the Holocene climate in the Mongolian Altai: The high-resolution $\delta^{13}C$ records of TOC and pollen complexes in Hoton-Nur Lake sediments. *Journal of Asian Earth Sciences*, *169*, 185–195.

Swoveland, T. & Fall, P. (2009). Analysis of deuterium from fossil pollen to reconstruct paleoclimates. *Journal of the Arizona-Nevada Academy of Science*, *41*, 87–92.

Tinner, W., Ammann, B. & Germann, P. (1996). Treeline fluctuations recorded for 12 500 years by soil profiles, pollen, and plant macrofossils in the central Swiss Alps. *Arctic, Antarctic and Alpine Research*, *28*, 131–147.

Tipple, B. J., Chesson, L. A., Erkkila, B. R., Cerling, T. E. & Ehleringer, J. R. (2012). B-HIVE: Beeswax hydrogen isotopes as validation of environment. Part II. Compound-specific hydrogen isotope analysis. *Food Chemistry*, *134*, 494–501.

Van der Knaap, W. O. & Van Leeuwen, J. F. N. (1995). Holocene vegetation succession and degradation as repsonses to climatic change and human activity in the Serra de Estrela, Portugal. *Review of Palaeobotany and Palynology*, *89*, 153–211.

West, J. B., Bowen, G. J., Cerling, T. E. & Ehleringer, J. R. (2006). Stable isotopes as one of nature's ecological recorders. *Trends in Ecology and Evolution*, *21*, 408–414.

West, J. B., Kreuzer, H. W. & Ehleringer, J. R. (2010). Approaches to plant hydrogen and oxygen isoscapes generation. In J. B. West, G. J. Bowen, T. E. Dawson & K. P. Tu (Eds.), *Isoscapes: Understanding movement, pattern, and process on Earth through isotope mapping* (pp. 161–178). Dordrecht: Springer.