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Determination by Near-Infrared Spectroscopy of Perseitol Used as a Marker for the Botanical Origin of Avocado (*Persea americana* Mill.) Honey

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This paper reports the application of near-infrared (NIR) reflectance spectroscopy to determine the concentration in honey of perseitol, a sugar that is specific to avocado honey. Reference values for perseitol were obtained by high-performance liquid chromatography analysis in 109 honey samples. Although the average concentration of perseitol in honey samples was only 0.48%, accurate prediction equations were successfully developed. The regression model of modified partial least squares was superior to that of principal component regressions. Calibrations based on the first or second derivative of Log(1/*R*) were equally good ($R^2 > 0.95$). Using half of the samples for calibration and the second half for validation, the correlation between actual and predicted values of the second half was satisfactory ($R^2 = 0.87$), the slope did not differ from 1, bias was low (0.005%), and the standard error of prediction was relatively low (0.13%). It was concluded that NIRS analysis may be used to detect to what extent honeybees have harvested avocado nectar but not to authenticate avocado honey as unifloral.

KEYWORDS: Honey; NIRS; product identification; authenticity; carbohydrates; sugars

INTRODUCTION

Unifloral honeys, characterized by unique organoleptic, microscopic, and physicochemical properties, are particularly sought after, and considerable efforts are invested in their identification (*1*). Because >95% of the solids of honey are carbohydrates, investigators have attempted to associate botanical origin with sugar composition. For example, the composition of sugars in honey analysis by gas chromatography of the trimethylsilyl oxime derivatives of sugars—fructose, glucose, sucrose, and maltose—has allowed reliable classification of some, but not all, Spanish unifloral honeys (*2*). Similarly, the conclusion of a study in the United Kingdom was that honey oligosaccharide profiles, determined by HPLC, have a potentially valuable role to play in the assessment of the floral origin of honey, although it is unlikely that this procedure alone will allow unambiguous determination of all floral types (*3*).

The seven-carbon heptose sugar D-mannoheptulose and its polyol form, perseitol, are specific to the avocado (*Persea americana* Mill.) tree at all phenological stages (*4*). Perseitol is found in avocado nectar (*5*, *6*) but not in the nectar of honey plants that commonly bloom in the spring in the vicinity of avocado orchards and compete with avocado for honeybee visits, such as citrus and wild mustard flowers. Therefore, the presence of perseitol could be used as a marker for avocado honey, which would be important, because pollen analysis (melissopalinology) is not effective in predicting the contribution of avocado nectar to honey. This is because little avocado pollen is found in honey samples that exhibit the very dark color and organoleptic properties (oily taste) characteristic of avocado honey (Ohad Afik, unpublished data).

Near-infrared spectroscopy (NIRS) yields rapid (*7*) and accurate (*7*−*9*) analyses of some of the major sugars—fructose, glucose, sucrose, and maltose—but has failed in predicting the concentration in honey of minor components, such as lactone and hydroxymethylfurfural (*9*).

The aim of the present study was to establish the feasibility of determining perseitol in honey as a method to assess the authenticity of avocado honey, using reflectance NIRS.

MATERIALS AND METHODS

Samples. Mated Italian and New World Carniolan queens were introduced into queenless beehives in October 2000 ($N = 140$). In early April, at the beginning of the avocado bloom, 109 beehives of similar brood area and adult populations were selected for experiment and transferred to four avocado orchards in northern Israel. The area of

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Table 1. Glucose, Fructose, Sucrose, and Perseitol in Honey Samples: Mean (Percent) and Standard Deviation in the Calibration ($n = 55$) and Validation Sets ($n = 54$)

	glucose		fructose		disaccharides		trisaccharides		perseitol	
set	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
calibration	26.0	2.3	34.1	3.3	10.6	3.9	5.2	3.5	0.48	0.36
validation	26.3	2.1	34.5	2.9	10.2	3.7	5.0	3.4	0.48	0.35

orchards ranged from 15 to 45 ha each. All of the orchards were dominated by the Ettinger cultivar, but other cultivars, such as Pinkerton, Fuerte, and Reed, were also present. The orchards were adjacent to large citrus (mainly grapefruit) groves and fields of wildflowers (mainly Brassicaceae) that bloom simultaneously with the avocado.

After the beehives had been placed at the orchards, a second super was added to each hive, above a queen excluder, according to standard apiary methods. The honey supers were collected at the end of April, after the citrus and early-blooming cultivars (i.e. Ettinger, Pinkerton, and Fuerte) had finished blooming. The honey was extracted from each beehive separately, using a two-frame manual honey extractor, and 0.5 kg of honey was sampled from each colony for analysis. All samples were kept in sealed glass jars at room temperature until analyzed.

HPLC Analyses. Approximately 200 mg of each honey sample was solubilized in 1.5 mL of double-distilled water for 2 h at 70 °C. The sample was vortexed vigorously and filtered through a 0.2-μm nylon filter prior to HPLC analysis. Soluble sugars were separated using an Alltech 700CH carbohydrate column (300 × 6.5 mm) at 90 °C, as described by Gao et al. (*10*). The mobile phase consisted of double-distilled water at a flow rate of 0.5 mL/min, and detection was performed by differential refraction (Shimadzu RID-10A). This column effectively separates the individual hexoses glucose and fructose, in addition to the sugar alchohol perseitol. However, the column does not distinguish between individual disaccharides or individual trisaccharides, although it separates between the trisaccharides and disaccharides. Therefore, we refer to the fraction coeluting with raffinose as "trisaccharides" and the fraction coeluting with sucrose and maltose as "disaccharides" or "apparent sucrose". A standard solution of glucose, fructose, sucrose, raffinose, and perseitol was used to identify and quantify the individual sugar components in the honey samples.

NIR Analysis. Samples were heated to 40 °C for 5 min, placed in an optically flat vessel, and covered with a 1-mm path length gold diffuse reflector (NR-6543-1, Foss Tecator, Hoganas, Sweden), before scanning. Three to four milliliters of sample was needed. Spectra were scanned, from 1108 to 2492 nm, using a Foss NIRSystems 5000 reflectance apparatus (Foss Tecator). Reflectance was recorded in 2-nm steps as Log(1/*R*), where *R* represented reflectance energy.

Statistics. ISI software (*11*) was used for statistical analysis, and statistical procedures were similar to those described in ref *9*. Corrections of NIR spectra for particle size were carried out by using the Standard Normal Variance (SNV) and detrend procedure (*12*). The spectral data were analyzed to detect spectral outliers, using cutoff values of 2.5 for "T" and 4.0 for "H" (*13*, *14*). To improve resolution, the spectral data were further transformed to the first or second derivatives (*15*) of Log(1/*R*). The calibrations were performed by using the modified partial least-squares (MPLS) or the principal component regressions (PCR) procedure (*16*), using first derivatives with a subtraction gap and smoothing segment of four data points, that is, the "1,4,4,1" procedure, and second derivatives with a subtraction gap and smoothing segment of 6 points, that is, the "2,6,6,1" procedure of ISI (*11*).

The robustness of calibration and the optimum numbers of terms for calibration were tested by using the cross-validation (CV) approach. This consisted of dividing the whole set of samples into six subsets, calibrating for sugars in five subsets, while validating on the remaining sixth, for sequential six times, using a different subset for calibration and prediction every time. The standard error (SE) of CV (SECV) was calculated as the square root of the average of the squares of the five SE of prediction (SEP) values. The final calibration equation was developed with the whole set of samples, using the number of factors with the lowest SECV. In addition, robustness was assessed, by using randomly 55 samples for calibration and 54 samples for validation. The SE of validation, bias, and coefficient of correlation (R^2) between actual and calculated values were used to assess the accuracy of prediction.

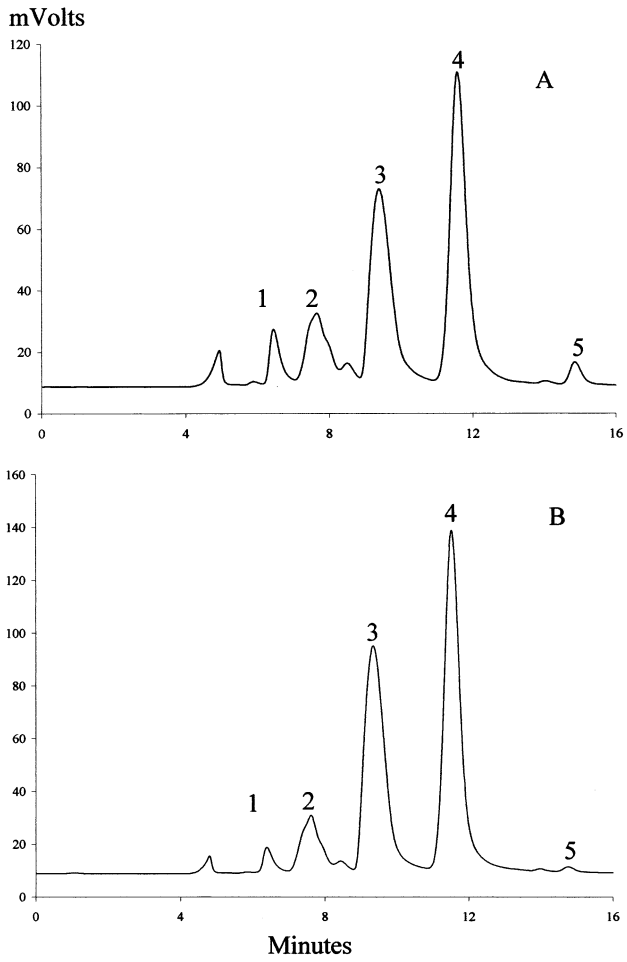

Figure 1. HPLC chromatograms of honey rich (A) or poor (B) in perseitol. Peaks: 1, trisaccharides; 2, disaccharides; 3, glucose; 4, fructose; 5, perseitol. The trisaccharide fraction (1) coeluted with the raffinose standard, and the disaccharide fraction (2) coeluted with both sucrose and maltose.

RESULTS AND DISCUSSION

Sugar Composition. The hexose sugars, glucose and fructose, accounted for ~60% of the honey analyzed, with fructose concentrations slightly higher than those of glucose (**Table 1**), similar to values reported by others (*8*, *9*). Perseitol concentration was relatively very low, ranging between 0.01 and 1.5% and averaging 0.48%. The peak of perseitol, relative to the other sugars (shown in chromatograms, **Figure 1**), was well identified. Most important, these results show that perseitol is present in honey derived from avocado nectar.

The concentrations of disaccharides and trisaccharides were higher than those often reported for honey sugars. The di- and trisaccharide composition of honeys is complex, and as many

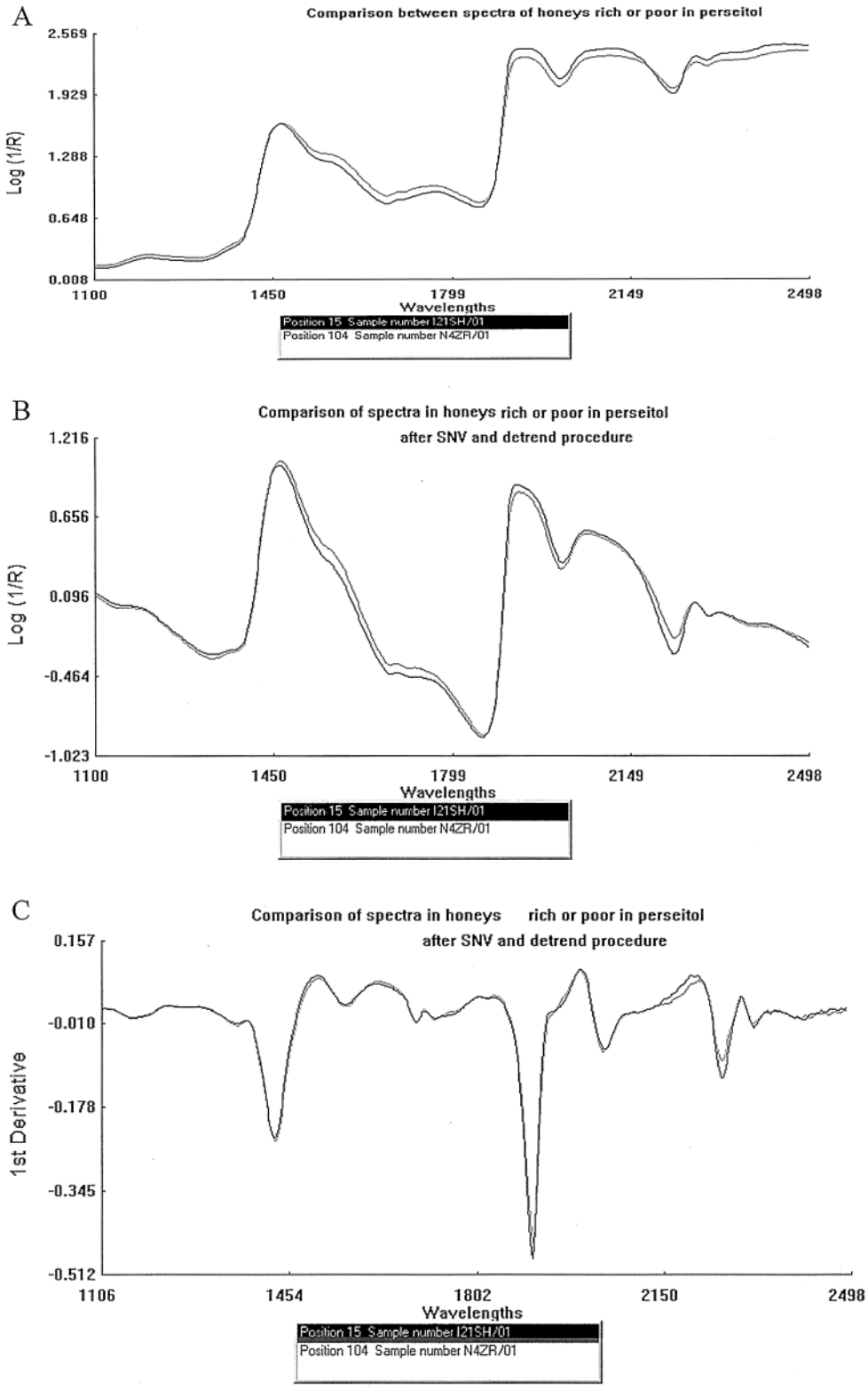

Figure 2. Untransformed NIR spectra of honeys rich (1.51%, gray line) or poor (0.01%, black line) in perseitol (A) and the same spectra after SNV and detrend (B) or first derivatizing procedures (C).

as 20 honey oligosaccharides have been identified (*17*). The trisaccharide fraction, coeluting with raffinose in our HPLC system, comprised 5% of the honey and may consist largely of erlose and raffinose. This was indicated by the loss of the trisaccharide peak in honey samples treated with invertase (not shown), indicating the presence of a terminal β-fructofuranoside. Numerous trisaccharides have been reported in honeys including high concentrations of erlose (*17*). Most, but not all, of the disaccharide peak also disappeared after invertase treatment, indicating that sucrose was a major disaccharide in the honey. However, other disaccharides were present, as indicated also by the broad disaccharide peak in the HPLC chromatogram. Because the purpose of this research was to study perseitol concentration, we did not further identify the individual oligosaccharides, and, to date, there are no reports characterizing the oligosaccharides of avocado honey in detail.

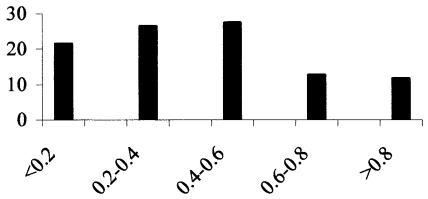

Figure 3. Distribution of perseitol concentration in honey samples (percent).

Table 2. Statistics of Optimal Calibrations, Using All Honey Samples ($n = 109$)

component	outliers withdrawn	derivative	MPLS terms	F	R^2	SEC	SECV
fructose	0	1	4	4.1	0.91	0.94	1.09
		2	2	69.4	0.88	1.07	1.12
glucose	0	1	5	37.4	0.87	0.81	1.05
		2	6	22.8	0.89	0.75	1.06
disaccharides	0	1	5	40.4	0.94	0.90	1.09
		2	6	18.7	0.98	0.81	1.11
trisaccharides	0	1	4	9.0	0.78	1.69	1.89
		2	2	28.8	0.76	1.78	1.89
perseitol	0	1	7	18.0	0.95	0.08	0.10
		2	8	60	0.97	0.06	0.10
fructose	4	1	4	24.4	0.93	0.78	0.88
	6	2	4	16.1	0.90	0.83	0.97
glucose	2	1	8	18.9	0.93	0.56	0.84
	4	2	5	12.9	0.89	0.70	0.95
disaccharides	4	1	9	29.5	0.98	0.48	0.83
	5	2	7	18.8	0.98	0.50	0.71
trisaccharides	6	1	4	8.0	0.90	1.11	1.89
	13	2	2	41.6	0.89	1.04	1.89
perseitol	5	1	7	11.5	0.95	0.07	0.09
	5	2	7	18.8	0.98	0.05	0.07

NIR Spectra. No special peak or shoulder for perseitol could be identified in the NIR spectra of honey, before mathematical transformation, after the SNV and detrend procedure or in its first derivative (**Figure 2**). No correlation >0.60 was found between particular Log(1/R) at single wavelengths and the concentration of perseitol.

Optimal Calibrations. Calibrations calculated by using PCR (not shown) were not accurate. R^2 values were 0.87, 0.75, 0.90, 0.72, and 0.68 for fructose, glucose, disaccharides, trisaccharides, and perseitol, respectively. The withdrawal of two to four outliers did not improve the accuracy of PCR calibrations: respective R^2 values were 0.89, 0.79, 0.92, 0.79, and 0.68 for fructose, glucose, disaccharides, trisaccharides, and perseitol.

The MPLS procedure yielded better results. When no outliers were withdrawn, calibrations calculated using first derivatives were better than with second derivatives for fructose and trisaccharides, but not for glucose, disaccharides, or perseitol (**Table 2**). When outliers were withdrawn from calculations, second derivatives were better for disaccharides and perseitol, but not for glucose, fructose, or trisaccharides. Working with transmittance spectra, Qiu et al. (*8*) found calibrations based on the second derivative to be more accurate for fructose, glucose, and sucrose, compared with the first derivative. In the present study, using reflectance NIR spectroscopy, first derivatives performed better for fructose, but second derivatives performed better for glucose, trisaccharides, and perseitol. The coefficients of calibration, including all 109 honey samples (without deleting outliers), were satisfactory for disaccharides and perseitol ($R^2 > 0.97$), whereas outliers had to be withdrawn to improve the calibration equations for trisaccharides. Overall, the ratio of SECV to SEC, used to estimate overfitting, as well as the number of terms in the prediction equation, were generally higher when outliers were withdrawn or when second derivatives were used. The risk of overfitting was not balanced by a dramatic improvement of R^2. Therefore, for the sake of simplicity, validations were carried out for all sugars, with the exception of trisaccharides, using only one MPLS setting, that is, the "1,4,4,1" procedure.

Table 3. Statistics of the Calibration Equations Used in the Validation Procedure, Where Half of the Samples Served for Calibration ($n = 55$) and the Remainder for Validation ($n = 54$)

component	outliers withdrawn	derivative	MPLS terms	F	R^2	SEC	SECV
fructose	0	1	4	38.2	0.95	0.77	0.94
		2	3	7.6	0.93	0.89	0.97
glucose	0	1	7	14.4	0.96	0.48	0.78
		2	6	27.2	0.94	0.56	0.88
disaccharides	0	1	5	38.2	0.90	0.52	0.85
		2	7	14.9	0.89	0.41	0.85
trisaccharides	6	2	2	258	0.89	0.78	0.91
perseitol	0	1	7	7.9	0.96	0.07	0.10
		2	7	22.1	0.96	0.07	0.13

Table 4. Mean, Bias, SEP, and R^2 of the Chemical Composition of Honey Samples in the Validation Set Predicted by NIRS ($n = 54$)

	mean	slope	bias	SEP	R^2
fructose	34.6	0.96	0.14	1.11	0.86
glucose	26.3	0.83	0.28	1.25	0.68
disaccharides	10.2	0.98	0.17	1.52	0.84
trisaccharides	4.96	1.18	–0.31	1.35	0.87
perseitol	0.48	1.1	0.005	0.13	0.87

When only 55 samples served for calibration in the calibration before the validation procedure (**Table 3**), values for R^2, SEC, and SECV were not greatly affected, compared with equations based on 109 samples.

Accuracy. Validation with independent samples, that is, samples that were not used in calibrations, is shown in **Table 4**. The coefficients of correlation between the predicted values and the reference values, that is, ~0.85, were acceptable for fructose, disaccharides, trisaccharides, and perseitol, but not for glucose. Slopes did not significantly differ from 1, except for glucose and trisaccharides. The SE values of prediction in the present study were 1.1, 1.2, 1.5, and 1.35% for fructose, glucose, and di- and trisaccharides, respectively. Similar validations, carried out in 46−58 honey samples in Spain, using similar instrumentation, yielded SE values of prediction ranging between 0.6 and 1.2% for fructose and between 0.9 and 1.5% for glucose (*9*), that is, comparable to those found in our study. However, 2−3-fold smaller SEP values were reported for fructose, glucose, and sucrose in a study conducted in Hong Kong (*8*). To discuss this discrepancy, SEP should be related to the SE of reference values, but no information is provided in the above-cited studies (*8*, *9*) on the internal SE in HPLC procedures.

The bias associated with NIRS in the present study (**Table 4**) was similar for fructose and sucrose, but higher for glucose, than reported before (*8*). The lowest accuracy, accompanied by significant bias, was found for trisaccharides. However, detailed statistics for the analysis of trisaccharide in honey by NIRS are not available in the literature, to our knowledge.

Perseitol. Despite its low content in honey, relative to other sugars (**Table 1**), perseitol was accurately identified in honey. Bias was extremely low. The absolute value for SEP (0.13%, **Table 4**) was also low. Even though successful use of NIRS in

analyses of other organic components that are of low content in feeds (~0.5%), such as tryptophan (*18*), has been reported, the concentration of perseitol was anticipated to be borderline for determination by NIRS. Therefore, the results of the present study are certainly encouraging.

The aim of the present project was to authenticate avocado honey. Even though all beehives were placed in avocado orchards at a time when avocado nectar was plentiful, it appears that perseitol content was very low in most of them (**Figure 3**). This could be expected because the attractiveness of avocado nectar to bees and the concentration of perseitol in avocado nectar are low (*6*). It also appears that the distribution of perseitol among honey samples is not normal. This implies that a beehive effect on avocado nectar harvesting exists, in agreement with the findings of Ish-Am et al. (*6*). A genetic background of honeybee colonies to collect avocado honey was shown before (*19*). Therefore, the production of avocado honey cannot be ensured by the geographic contiguity of bees with avocado trees. The question of authentication is, therefore, different for avocado honey than for other Mediterranean honeys, where contiguity infers botanical identity, such as eucalyptus honey (*2*, *20*) or sunflower honey (*2*), for which the chemical composition is needed only to prevent misuse of a controlled trademark. It is clear that avocado nectar is only one source of nectar among several others in avocado honey. Given the size of the SEP (0.13%) relative to the mean perseitol concentration (0.48%), only honeys in the highest 20% range of perseitol content may be unambiguously classified as avocado honey. Avocado honey can probably not be defined as a unifloral honey but, rather, can be defined as a honey that has been manufactured by bees foraging on avocado flowers. We suggest that NIRS analysis may be used to detect to what extent honeybees have harvested avocado nectar, but not to authenticate avocado honey as unifloral.

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