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Differentiating pasture honey from eucalyptus honey based on carbon isotopic data in Uruguay

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Abstract

To avoid false declarations of geographic and botanical honey origin, traceability should be based on analytical data, which could then be processed by multivariate statistical methods. Obtaining this data, however, is costly and time consuming. Thus, it would be more convenient to acquire this information from routine trials, for example from the analysis for determination of high fructose corn syrup (HFCS) concentration in honey. The availability of a procedure of this kind in Uruguay would be useful in discriminating between honeys from grasslands to that from eucalyptus, the two main floral sources for commercial production. To this effect, honey samples (47 from pastures and 42 from eucalypts) were analyzed for $\delta^{13}\text{C}$ in both honey and its protein fraction. We identified a logistic regression model that allowed us to correctly assign 90% of the training samples, using $\delta^{13}\text{C}$ data of honey, protein fraction, and the isotopic index as variables. This model was then validated, obtaining 100% correct allocation for honeys from pasture and 90% for honeys from eucalyptus. Moreover, we found that this information could also be used to establish adulteration with HFCS based on a local stricter cut-off limit than that of -1.0‰ of the international index.

Keywords: Analytical chemistry, Food science, Food analysis

1. Introduction

Honey is widely demanded worldwide. However, for its commercialization it is necessary to assure its innocuousness and authenticity. With respect to this, European Union legislation requires that exported honey must be traceable over time (EU Directive 178/2002). This regulation is intended to prevent false geographic and botanical origin declarations, which have occurred in the past due to the strong price variation of honey according to its origin (Camina et al., 2012; Dong et al., 2016). Traceability, in general, is reported in product life cycle records (Olsen and Borit, 2013), which are clearly vulnerable to fraud. For this reason, for traceability documentation to be backed up with more reliable information, such as analytical tests or chemometric data of the product (Zhao et al., 2016; Soares et al., 2017).

Since information on the geographical and botanical origin of honey is a guarantee of the quality of the product, the ability to prove its origin is crucial for obtaining and maintaining market niches (Mohammed et al., 2018). Verification of the botanical origin of honey has been conducted historically by microscopic inspection of pollen (Louveaux et al., 1977; Jaafar et al., 2017), but this melissopalynological method requires expertise which may be costly. For this reason, efforts have been made to identify other parameters for honey that can be routinely applied in origin certification procedures. One of the most successful approaches has been the use of chemometric information (Soares et al., 2017). These variables include physicochemical properties, content of mineral (including trace) elements, concentration of phenolic, flavonoid and volatile compounds, as well as information on the intensity of honey absorption or reflectance at different wavelengths, from the visible to the near infrared spectrum, between others (Camina et al., 2012; Consonni and Cagliani., 2015; Bontempo et al., 2017; Lazarević et al., 2017). Isotopes stables also were used to discriminate between different honey floral origin, for example, Kropft, et al. (2010) and Bontempo et al. (2017) used ^{13}C and ^{15}N in addition to others variables of chemical composition. In addition, stables isotopes to be used to determine the honey authenticity as sugar adulteration (Dong et al., 2018). To identify similar honey groups, this information is then processed using multivariate statistical methods, such as discriminant analysis, logistic regression (LR), or neural network models (Camina et al., 2012). The developed model can be later used to assign a new honey sample to one of these origin groups.

In Uruguay, pasture and eucalyptus are the two major floral sources for commercial honey production (Urimpex, 2003). Although the floristic origin is normally declared when the product is marketed, this information is not always reliable, so it would be useful to have an objective protocol to corroborate this information. Corbella and Cozzolino (2005) differentiated Uruguayan honey into eucalyptus and pasture based on the use of visible (vis) and near infrared (NIR)

spectroscopy. This result was auspicious, and it is useful to classify honey from these two floral origins based on data from routine trials, such as the official analysis for determining high fructose corn syrup (HFCS) concentration, which in turn, is based only on the C isotopic composition of honey and its protein fraction. The next step would be to develop a model based on multivariate statistical methods to predict whether a sample belongs to the declared floral category. Therefore, the aim of this study was to evaluate the possibility of classifying eucalyptus and pasture honeys based only on their C isotopic composition and its protein fraction that allow apply specific criteria of authenticity to each floral type (eucalyptus and pasture) in Uruguay.

2. Materials and methods

2.1. Samples, treatment and analyses

Honey samples ($n = 89$), as well as information about floral honey origin, were provided by beekeepers from different Uruguayan regions between 2006 and 2008. All contacted beekeepers were duly accredited by Health Authorities. In terms of floral type, honey samples came either from *Eucalyptus* sp. ($n = 42$), in areas where hives were located within eucalyptus plantations, or pasture areas ($n = 47$). These pastures were composed of diverse species (more abundant: *Lotus* sp., *Trifolium pratense*, and *Trifolium repens*). Ten honey samples from each floral origin were used as a set to validate the mathematical model. In this set was done a mellisopalinologic analysis to verify botanical origin of honeys according to Louveaux et al. (1977).

All samples were filtered through a 0.10–0.15-mm sieve and then processed according to the Official Methods of Analysis AOAC 998.12 (AOAC, 1999). The protein extraction was carried out by dissolving 10 g of filtered honey in 4 mL of distilled water, followed by the addition of 2 mL of 0.67 N sulphuric acid and 2 mL of sodium tungstate 10% (w/v) to this solution. The mixture was then homogenized and heated to 80 °C until flocculation of protein fraction was visible. In cases where flocculation did not occur, 2 mL aliquots of sulphuric acid 0.67 N were successively added until flocculation occurred. The flocculated mixture was then centrifuged for 5 minutes at 1500 g and the supernatant was removed. The obtained pellet was then washed and centrifuged five times with 50 mL of distilled water and dried for 3 hours in an oven at 75 °C. The samples of filtered honey and extracted protein were placed in tin capsules and introduced into an Elemental Analyzer (EA Flash, series 1112, Milan, Italy) coupled to an Isotope Ratio Mass Spectrometer (Thermo Finnigan Delta Plus, Bremen, Germany) via a ConFlo III interface. The certified reference materials used for normalization of $\delta^{13}\text{C}$ results were IAEA-CH6 (−10.449‰) provided by International Atomic Energy Agency (IAEA) and USGS-40 (−26.39‰) obtained from United States Geological Survey (USGS).

To obtain the true $\delta^{13}\text{C}$ values of the samples were used the following equation proposed by Carter and Barwick (2012):

$$\delta^{13}\text{C}_{\text{true}(\text{sample})} = \delta^{13}\text{C}_{\text{true}(\text{RM1})} + \frac{\delta^{13}\text{C}_{\text{true}(\text{RM1})} - \delta^{13}\text{C}_{\text{true}(\text{RM2})}}{\delta^{13}\text{C}_{\text{raw}(\text{RM1})} - \delta^{13}\text{C}_{\text{raw}(\text{RM2})}} \times (\delta^{13}\text{C}_{\text{raw}(\text{sample})} - \delta^{13}\text{C}_{\text{raw}(\text{RM1})}) \quad (1)$$

Where, $\delta^{13}\text{C}_{\text{true}(\text{sample})}$ is the true value of the sample (honey, protein fraction or HFCS), $\delta^{13}\text{C}_{\text{true}(\text{RM1})}$ and $\delta^{13}\text{C}_{\text{true}(\text{RM2})}$ are the values provided by the suppliers of the RM IAEA CH6 and USGS-40 respectively, while $\delta^{13}\text{C}_{\text{raw}(\text{sample})}$, $\delta^{13}\text{C}_{\text{raw}(\text{RM1})}$ and $\delta^{13}\text{C}_{\text{raw}(\text{RM2})}$ are the measured isotopic values of the sample, the RM IAEA-CH6 and USGS-40 respectively.

The isotopic index (II) of honey was determined as the difference between the $\delta^{13}\text{C}$ values of honey and its protein fraction; when this difference was positive, the II was set as equal to zero (White and Winters, 1989).

2.2. Statistical analysis

2.2.1. Normality test

In order to test normality Shapiro-Wilk (Shapiro and Wilk, 1965) was applied.

2.2.2. Logistic regression analyses

Logistic regression is useful when predicting the value of an independent categorical variable of dichotomous response (y), based on the values of several independent quantitative and/or categorical variables (x's).

Logistic regression (LR), based on isotopic information, was used to predict the membership of honey in one of the two pre-established floral groups (eucalyptus or pasture). The output of the model represents the probability of a sample coming from two states (odds ratio). Thus, the dependent variable (y) is categorical and of binary response, admitting only two values (Lemeshow and Hosmer, 1982). In this case, when $P = 0$ the sample comes from eucalyptus, and when $P = 1$ it comes from pasture.

The advantage of applying this statistical technique is that it is not required to assume that independent regressors meet multinational criteria, neither that the covariance matrices of the two groups are equal (Lemeshow and Hosmer, 1982). The independent regressor variables included in the model were the isotopic composition of honey ($\delta^{13}\text{C}$ honey) and its protein fraction ($\delta^{13}\text{C}$ protein), as well as the previously described II. The model was then run with the rest of the samples and validated with the training set. All statistical analyses were conducted using XLStat – Base software (version 2018.2, Addinsoft SARL©).

3. Results and discussion

3.1. Carbon isotopic data for honey and protein fraction

Honey samples derived from eucalyptus and pasture presented C isotopic signatures that ranged between -27.08‰ and -23.91‰ , and which were consistent with those of plant species with C3 photosynthetic cycle (Smith and Epstein, 1971). Similarly, the C isotopic composition of the protein fraction extracted from honey ranged between -27.01‰ and -24.25‰ , in the same interval as C3 plants (Table 1). The $\delta^{13}\text{C}$ data for eucalyptus honey was in the range of the mean values reported by Roßmann et al. (1992) and Kivrak et al. (2017) for honey derived from this flora type in South America (-25.16‰) and Turkey (-26.7‰), respectively (Table 1). In contrast, our $\delta^{13}\text{C}$ data for eucalyptus honey differed from the mean value cited by Roßmann et al. (1992) as representative of South African eucalypt honey (-22.48‰). However, this difference should be expected, because this same author cited that the isotopic values of eucalyptus honey from South America and South Africa were different. The isotopic values of protein fraction from eucalyptus honey found in our study were similar to those reported by Roßmann et al. (1992) and Kivrak et al. (2017) for South American and Turkish honey, with reported means of -25.32‰ and -26.7‰ , respectively, but, as expected, the $\delta^{13}\text{C}$ values of the protein fraction from South African eucalyptus honey (-22.60‰) were also different from those from Uruguay. Uruguayan honey derived from pastures presented $\delta^{13}\text{C}$ values for both honey and protein fraction similar to those cited by White and Winters (1989) for pure clover honey from USA. These authors reported mean values for honey and protein fraction of -24.66 and -24.50‰ , respectively. Our values were also similar to those reported for honey and its protein fraction by Chen et al. (2013) of identical floral origin from China (-26.45 and -25.82‰), and by Roßmann et al. (1992) from Canada (-25.11 and -25.48‰). According to the Shapiro-Wilk test (Shapiro and Wilk, 1965), the isotopic composition of honey derived from pasture was not normally distributed ($p = 0.043$).

Table 1. ^{13}C isotopic values of honey and its protein fraction. Numbers in parentheses represent number of samples.

Matrix	Eucalyptus			Pasture		
	Mean	SD	II	Mean	SD	II
	$\delta^{13}\text{C} (\text{‰})$					
Honey	-26.48 (42)	0.32	-0.12	-25.49 (47)	0.72	-0.87
Protein	-25.56 (42)	0.37	NC	-25.60 (47)	0.67	NC

SD = standard deviation, II = isotopic index, NC = not correspond.

3.2. Logistic regression analyses

In our case, the development of the LR model included training and validation stages. The best model identified for predict the floral honey origin (from eucalyptus or pasture) had three independent variables: two were continuous (^{13}C of honey and protein fraction) and one categorical variable (II). The two categories used for this last variable were zero or negative, the same used for fraud identification in honey. Thus, this model could be used not only to differentiate between these two floral origins (dichotomous variables), but also to detect potential botanical denomination frauds. An outcome from this model of $y = 0$ would indicate that a honey sample originated from eucalyptus, while that of $y = 1$ would reveal a sample originating from meadows.

The resulting model [1] from the training stage presented a good fit. The Nagelkerke R^2 value was 0.770 and the R^2 value of Cox and Snell was 0.577. The Chi^2 value associated with the Log ratio had a probability lower than 0.0001, which indicates that the independent variables provided a significant amount of information. The Hosmer-Lemeshow statistic, whose null hypothesis (H_0) was that the current and predicted values were the same, was verified with a p-value of 0.928, while only a $p \geq 0.05$ is required to accept H_0 (Lemeshow and Hosmer, 1982).

$$\text{Prob(Honey)} = \frac{1}{1 + e^{-31.5462 - 7.6993 * \delta^{13}\text{C}_{\text{honey}} + 6.5974 * \delta^{13}\text{C}_{\text{protein}} + 2.0646 * \text{II}_{\text{Negative}}}} \quad (2)$$

A previous study by Corbella and Cozzolino (2005) could also differentiate between eucalypt and pasture honeys of Uruguay, but they used Linear Discriminant Analyses (LDA) and Discriminant Partial Least Squares. The LDA model was based on readings that encompassed the visible to the near infrared spectra of both honey types, with error rates of 8% and 25% for honey from eucalyptus and pasture, respectively. Our logistic model overall, however, was slightly more promising than that of these authors to LDA model, with corresponding error rates of 15.6% and 5.4%, respectively (Table 2). Also, the validation tests of our model with ten samples from each floral origin resulted in an error rate of 10.0% for eucalyptus honey and a perfect outcome with a 0% error rate for pasture honey (Fig. 1). These results indicate that it is possible to use this model to differentiate between honey from these two botanical origins based only of the ^{13}C composition of honey and its protein fraction. Obviously, this model could be improved by including more samples or other variables, like those derived from spectroscopic techniques or physicochemical, as in the studies of Corbella and Cozzolino (2005) and Berriel (2018a) respectively.

Table 2. Recognition ability of the logistic regression model based on the C isotopic data of honey from two floral origins.

Training set (recognition)				
Flora	Eucalyptus	Pasture	Total	% correct
Eucalyptus	27	5	32	84.4
Pasture	2	35	37	94.6
Total	29	40	69	89.9

3.3. Development of a specific criterion for authenticity control

The mean of the II minus four times its standard deviation is the cut-off value currently used to discriminate between genuine and adulterated honey with C-4 sugars, with an error probability of 1 in 25,000. In our experiment, these cut-off values were equivalent in terms of $\delta^{13}\text{C}$ to -0.12 and -0.87 ‰ for eucalyptus and pasture honey, respectively. The high II of eucalyptus honey (close to zero and less negative) originated from the $\delta^{13}\text{C}$ values of honey which were lower than those of its protein fraction. A positive difference between protein fraction and honey from honey with diverse floral origin, such as alfalfa, acacia, pine, or rhododendron, had already been reported by other authors such as [White and Winters \(1989\)](#), [Cotte et al. \(2004\)](#), [Simsek et al. \(2012\)](#) and [Bontempo et al. \(2017\)](#), respectively. In contrast, the II values of honey derived from other botanical origins are more negative, because the isotopic value of honey is higher than that of its protein fraction, similar to that observed here in pastures ([White and Winters, 1989](#)).

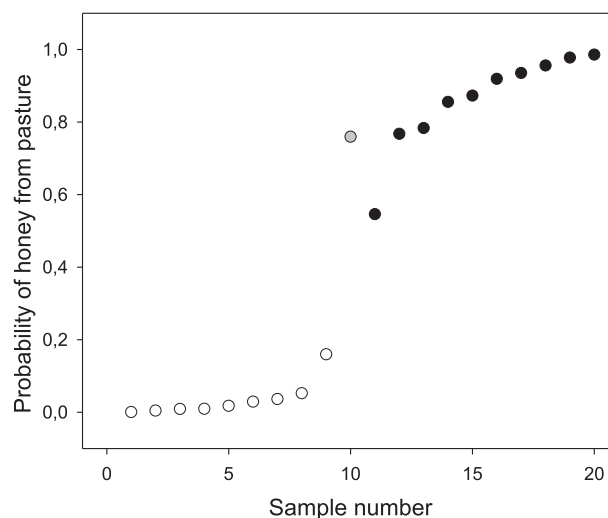


Fig. 1. Prediction ability of the logistic regression model. Open and filled symbols represent correctly classified honey samples originating from eucalypt or pasture areas, respectively. The grey symbol represents one honey sample declared as originating from a eucalypt area but classified as coming from pasture.

The two cut-off values reported in the present study were lower than the internationally established cut-off value of -1.0‰ (AOAC, 1999), and lower in eucalyptus honey but higher in pasture honey compared to the local cut-off value of -0.80‰ previously found by Berriel and Perdomo (2016) for honey from multiple botanical origins. This last cut-off value identified in Uruguay was also lower than the international limit, probably because Uruguay has a relatively homogeneous climate and is less ecologically diverse. Following the same line of reasoning, the two new specific cut-off values for eucalyptus and pasture are only valid for Uruguay. Thus, the use of these two specific cut-off values would enable an even more stringent detection of honey from eucalyptus or pasture adulterated with C-4 sugars and the uncovering of inadequate beekeeping management techniques, i.e. feeding bees excessively or too close to the harvest (Berriel, 2018b).

4. Conclusions

The results of this study are promising, since they allow differentiation of eucalyptus honey from pasture honey in Uruguay by means of a LR model based on C isotopic data from honey and its protein fraction. Besides, this data is easily available during the determination of honey adulteration with HFCS by the official method (AOAC, 1999), and this information is required for commercialization of honey production for the international market. Therefore, the additional analytical cost associated with the use of this model would be near zero. In addition, the ascertainment of the honey origin for these two floral types would allow the use of specific cut-off values at a local level, which would result in a more precise and stringent detection of honey adulteration with HFCS for these two honey types. However, it is possible that this model could be further improved by including other variables as sensory evaluation, color or quantification of volatile organic compounds specific responsible for aroma and flavor, for example.

Declarations

Author contribution statement

Verónica Berriel: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Carlos H. Perdomo: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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