Authenticity of Ecuadorian Commercial Honeys

Elisabetta Schievano, Valentina Zuccato, Claudia Finotello, Patricia Vit

***Abstract*—**Control of honey frauds is needed in Ecuador to protect bee keepers and consumers because simple syrups and new syrups with eucalyptus are sold as genuine honeys. Authenticity of Ecuadorian commercial honeys was tested with a vortex emulsion consisting on one volume of honey:water (1:1) dilution, and two volumes of diethyl ether. This method allows a separation of phases in one minute to discriminate genuine honeys that form three phase and fake honeys that form two phases; 34 of the 42 honeys analyzed from five provinces of Ecuador were genuine. This was confirmed with ^{1}H NMR spectra of honey dilutions in deuterated water with an enhanced amino acid region with signals for proline, phenylalanine and tyrosine. Classic quality indicators were also tested with this method (sugars, HMF), indicators of fermentation (ethanol, acetic acid), and residues of citric acid used in the syrup manufacture. One of the honeys gave a false positive for genuine, being an admixture of genuine honey with added syrup, evident for the high sucrose. Sensory analysis was the final confirmation to recognize the honey groups studied here, namely honey produced in combs by *Apis mellifera*, fake honey, and honey produced in cerumen pots by *Geotrigona*, *Melipona*, and *Scaptotrigona*. Chloroform extractions of honey were also done to search lipophilic additives in NMR spectra. This is a valuable contribution to protect honey consumers, and to develop the beekeeping industry in Ecuador.

***Keywords*—**Fake, genuine, honey, ^{1}H NMR, Ecuador

I. Introduction

IF honey is not genuine, it is fake honey. Tropical markets are places of great biodiversity, but also of imitations of natural products such as honey. Genuine honey is stored in beeswax combs by *Apis* species and in cerumen pots by Meliponini species of bees in the world [1].

Diverse chemometric studies on honey authentication are based on physicochemical indicators [2] such as proteins [3], metals [4] and sensory [5], to mention some of them –used alone or combined.

A honey authentication test with diethyl ether, discriminates genuine honeys with three phases and fake honeys with two phases. This test and ^{1}H NMR on deuterated water honey dilutions were used to standardize key components of genuine honey produced by diverse entomological sources such as *Apis mellifera* and pot-honey produced by the genera *Geotrigona*, *Melipona* and *Scaptotrigona*, contrasted with the abundant fake honeys in the Ecuadorian market.

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II. Methods

A. Honey Samples

Forty two Ecuadorian commercial honeys from four entomological origins *Apis mellifera*, *Geotrigona*, *Melipona* and *Scaptotrigona*, plus fake honey were collected during field work in El Oro, Loja and Pastaza provinces, and kept frozen until analysis.

B. Authentication Test

Honey dilution was prepared with a fixed volume of liquid or cristallyzed honey, e.g. 1.0 mL plus the same volume of water, later 2.0 mL of diethyl ether were added and vigourosly shaked in the tube, let stand for one minute before observing the number of phases [6].

C. Deuterated Water Dilutions of Honey

For each honey sample, 200 mg of honey were dissolved in 600 µL of D_2O (Sigma-Aldrich, 99.96 atom % D, Milan, Italy), up to 1 ± 0.025 mL, 450 µL of the solution were transferred to 5 mm precision glass NMR tubes (Wilmad 535-pp). This extractive procedure yields a solution adequate for fast NMR analysis.

D. Chloroform Extraction of Honey

For each honey sample a chloroform extraction was done following a previous protocol [7] to find lipophilic additives.

E. ^{1}H NMR Spectra Acquisition

The ^{1}H NMR spectra were acquired at 298 K, with a 600 MHz NMR Bruker instrument.

F. NMR Data Processing Before Statistics

A data matrix was built, for statistical analysis if NMR spectra by considering only the sugar signals form to 3.6 ppm to 5.9 ppm excluding the segments containing water, to eliminate the variation in the water signals suppression, and the segments containing HMF resonances at 4.5 ppm. Data reduction was done by segmenting the spectra in 0.03 ppm intelligent buckets and the integral value was normalized by Total Sum Normalization. The calculations were performed using the program ACD. The obtained dataset (X matrix) was exported to Microsoft Excel and transferred into SIMCA-P+ software (v 13.0 Umetrics, Umea, Sweden).

G. Multivariate Data Analysis

The multivariate analysis was carried out onto mean-centered and unit variance (UV) scaled data through Projection to Latent Structures-Discriminant Analysis (PLS-DA). The supervised pattern recognition models Partial Projection to Latent Structures Discriminant Analysis (PLS-DA) has been chosen in order to attain classification rules for predicting the correct class. The quality of the models was described by R^2 and Q^2 values. R^2 is defined as the proportion of variance in

the data explained by the models and indicates the goodness of fit. Q^2 is defined as the proportion of variance in the data predictable by the model and indicates predictability.

III. Results

The authentication test (Fig. 1) was positive for 81% genuine honeys with three phases, of the 42 tested honeys.

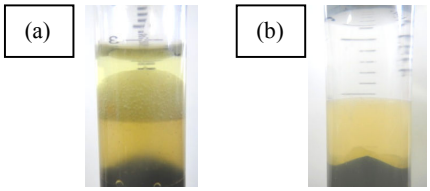

Fig. 1 Authentication test: Three phases for genuine honey (a) and two phases for fake honey (b)

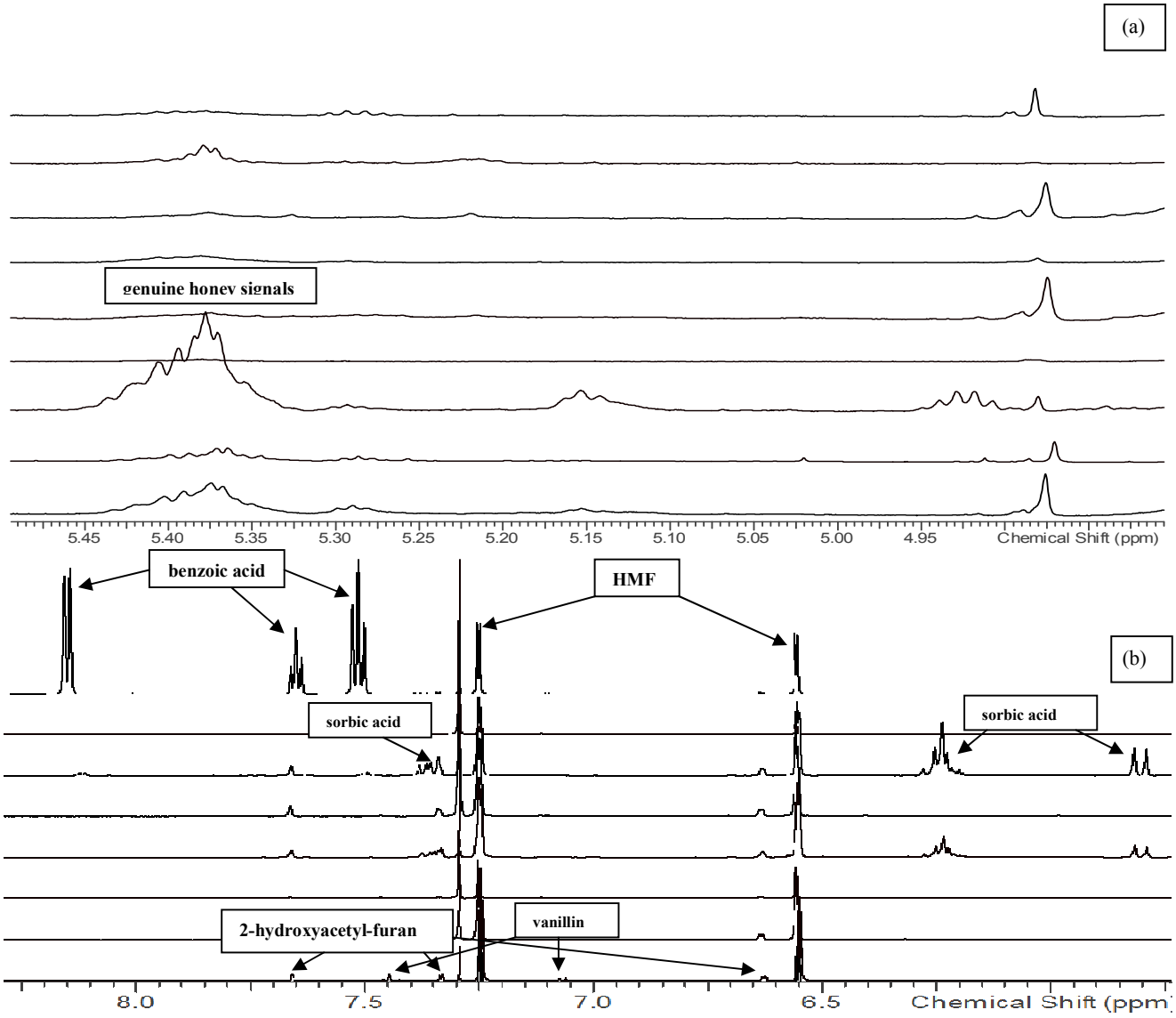

Fig. 2 Expansion of the NMR spectra of the chloroform phase of the eight false or adulterated Ecuadorian honeys: (a) Region [5.45 – 4.85 ppm], (b) Region [8.5 – 5.0]

NMR spectra of the eight false or adulterated honeys studied here (*Apis mellifera*, *Geotrigona*, *Melipona* and *Scaptotrigona*) were compared.

In Fig. 2 we show two expanded regions of the NMR spectra of the chloroform phase in eight false Ecuadorian honeys: (a) Region [5.45 – 4.85 ppm] has visible genuine honey signals (e.g. wax) indicating admixtures of syrups and honey in the lower spectra, while the upper spectra are flat as syrups; (b) Region [8.5 – 5.0] with characteristic signals of manufactured honeys with residues of benzoic acid, hydroxymethylfurfural (HMF), 2-hydroxyacetyl-furan, sorbic acid and vanillin signals.

Besides the contrast of complex spectra profiles in genuine honey compared to poorer profiles in false honeys, the admixture of genuine honey and syrup is also confirmed with the NMR spectra, because of the simultaneous presence of

natural sugars –like those of genuine honey– but a signal of excessive sucrose like in syrups.

Five chemical indicators are suggested to detect the presence of fake honey, or admixtures of fake honey with genuine honey, in Table I. First of all, compared to genuine honey, fake honey lacks aminoacids. Second, fake honeys show high HMF signals that are not seen in genuine honey. Third, Ecuadorian fake honeys use citric acid and sorbic acid as additives in their manufacturing process, and therefore these acids are detected in the NMR spectra. The sugar composition is the fourth component to differentiate genuine from fake honeys, reported as the fourth indicator [8] but sugars really represent two hallmarks: A wide spectra of natural sugars is present in genuine honey (fructose, glucose, kojibiose, maltose, melibiose, nigerose, turanose, etc.) is the fourth indicator, whereas fake honey has important signals on sucrose as a fifth indicator. An upper limit for sucrose content (lower than 5 g/100 g honey) is the standard in the Ecuadorian honey norm [9] as well as in the international Codex Alimentarius Commission [10].

TABLE I
CHEMICALS MARKERS OF HONEY DILUTED IN DEUTERATED WATER

Chemical indicators	Honey Type	
	Genuine	Fake
1. Aminoacids	Present	Absent
2. HMF	Low	High
3. Additives	Absent	Present
4. Natural sugars	Present	Absent
5. Sucrose	Low	High

The ^{1}H NMR spectra of honey dilutions in deuterated water with an enhanced aminoacid region showed signals for proline, phenylalanine and tyrosine in genuine honey, but these aminoacids were absent in false honey. The classic honey quality indicators (sugars, HMF) tested with ^{1}H NMR, confirmed that HMF content is very high in fake honeys derived from heated sucrose and syrups, compared to the low concentration up to 40 mg HMF/kg in the genuine *Apis mellifera* honey regulations [9], [10]. Sugars are also regulated compounds in honey norms, where sucrose has an upper limit of 5 g/100 g. Therefore, besides HMF, sucrose is also a target compound to detect false honey, in contrast to fructose, glucose major sugars [11], [12] and distinctive maltose in pot-honey produced by non *Melipona* stingless bees [13]. High concentrations of sucrose indicated manufactured honey. However some honeys had signals of genuine origin, with a diversity of natural minor sugars such as raffinose, turanose, nigerose, palatinose, kojibiose, others recently informed in a worldwide honey collection (erlose, isomaltose, maltose, melezitose, trehalose) of more than 800 honeys [11], and arabinose informed in a method to discriminate botanical origin in 328 honeys (manuka, multifloral, sunflower, honeydew, chestnut, acacia,orange, rape, eucalyptus) [12]. Indicators of fermentation (ethanol, acetic acid), and residues of citric acid used in the syrup manufacture, as declared in one label, in contrast to the natural citric acid reported as typical honey components [11], [12]. One of the honeys gave a false positive for genuine, being an admixture of genuine honey with added syrup, evident for the high sucrose. Sensory analysis was the final confirmation to recognize the honey groups studied here, namely honey produced in combs by *Apis mellifera*, with chatracteristic floral descriptors. Fake honeys have a major candy like odor-aroma [14]. Whereas honey produced in cerumen pots by *Geotrigona*, *Melipona*, and *Scaptotrigona*, have the entomological sensory descriptors more distinctive than the botanical origin, as previously observed for the perceptions of pot-honeys harvested in forests with the Huottuja assessors in the Venezuelan Amazon [15] and Kichwas in the Pastaza province of Ecuador.

IV. CONCLUSION

A simple method based on the number of phases after vigorous shaking of honey water dilution and diethyl ether, was complemented with NMR approach to dilucidate what are the key components of genuine and fake honeys useful in authentication routine. Five chemical indicators were suggested for that purpose because fake honeys: 1. Lack of aminoacids, 2. Show high HMF contents, 3. Keep citric acid as a marker of their human manufacture, 4. Lack of natural honey sugars, and 5. exceed the sucrose limits allowed for genuine honey.

ACKNOWLEDGMENT

To Prometeo, Senescyt, Ecuador for the grant to P. Vit at Universidad Técnica de Machala (UTMACH), Provincia El Oro. To Dr. Silvia R.M. Pedro at Universidade de São Paulo, Ribeirão Preto, Brazil, for entomological support to identify species of Meliponini. To Mrs. Elizabeth Brito Administrative Assistant from the Planning Department at UTMACH for her constant administrative asssitance. To Dr. Favian Maza, Planning Director at UTMACH, for his kind and timely solutions. To Dr. Susana Toro, Prometeo Program Manager, Quito, for her unique support along the research. To genuine honey donors of: 1. Pot-honeys: Ubertino Matamoro from Piedras Blancas; Mr. Oswaldo Ajila and Francisco Feijóo from Moromoro, Mr. Segundo Lapo from Chiriboga, Las Lajas, El Oro province; and one *Geotrigona* honey from Mr. Jorge Francisco Meza, Camarones, Manabí province; 2. *Apis mellifera* honeys: Mr. Gabriel Valencia, beekeeper from Tablazo, Esmeraldas province, Professor José Ramírez from Universidad Nacional de Loja, Loja province, and Professor Julio César Vargas, Principal of Universidad Estatal Amazónica who donated a honey from Quevedo, Los Ríos province. Also to Pichincha Beekeepers Association, to provide fake honeys currently introduced in the market. All honeys were purchased by P. Vit, except those received from meliponicultors and beekeepers. The Principal of UTMACH Eng. César Quezada Abad provided facilities for field work with the drivers Mr. Francisco Saavedra, Mr. Jairo Riofrio, Mr. Wilson Ríos, Mr. Alex Noblecilla, and Mr. William Salas. To Dr. Silvia R.M. Pedro at Universidade de São Paulo, Ribeirão Preto, Brazil, for entomological support to identify species of Meliponini. P Vit accepted invitations extended by

VMD Hugo Rosero (TC President) and Eng. Maritza Farinango (TC secretar) for the Technical Committee (TC) revision of the Ecuadorian honey norm NTE INEN 1572.

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