Entomological Origin of Honey Discriminated by NMR Chloroform Extracts in Ecuadorian Honey

P. Vit, J. Uddin, V. Zuccato, F. Maza, E. Schievano

***Abstract*—**Honeys are produced by *Apis mellifera* and stingless bees (Meliponini) in Ecuador. We studied honey produced in beeswax combs by *Apis mellifera*, and honey produced in pots by *Geotrigona* and *Scaptotrigona* bees. Chloroform extracts of honey were obtained for fast NMR spectra. The 1D spectra were acquired at 298 K, with a 600 MHz NMR Bruker instrument, using a modified double pulsed field gradient spin echoes (DPFGSE) sequence. Signals of ^{1}H NMR spectra were integrated and used as inputs for PCA, PLS-DA analysis, and labelled sets of classes were successfully identified, enhancing the separation between the three groups of honey according to the entomological origin: *A. mellifera*, *Geotrigona* and *Scaptotrigona*. This procedure is therefore recommended for authenticity test of honey in Ecuador.

Keywords*—Apis mellifera*, honey, ^{1}H NMR, entomological origin, Meliponini.

I. Introduction

HONEY is the sweet product extracted from combs by *Apis* species and from pots by Meliponini species of bees in the world [1]. Meliponiculture is the art to keep stingless bees. "Stingleness seems rather sensational" although it does not refer to a lack of sting, but to a modified non-functional sting [2]. These bees of the Meliponini Tribe live in the tropics, and their honeys processed in cerumen pots were enjoyed before Columbus as *Apis mellifera* was introduced later [3].

In a human/animal interface study Aboriginal honey hunting in Australia is reviewed [4]. Domesticating stingless bee feral colonies in hives still needs strategies that facilitate the location of nests, and that also facilitate honey harvest [4,5].

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Networking between stingless bee keepers is an indicator of success to multiply colonies and marketing of pot-honey [5]. In a Mexican survey with Mayan stingless bees, *Melipona beecheii* showed colony loss caused by abundance of Africanized honey bee, or beekeeping experience was lower than that caused by competition for food [6]. The Brazilian large-scale survey to support and improve meliponiculture by a quantitative approach and analysis, offers a solid basis needed to increase economic profits of stingless bee products, especially pot-honey [5]. Four key roles are highlighted here to explain why Brazilian Government officers in the tropics are interested to develop the stingless bee industry by promoting: 1. Their local biodiversity, 2. Their economic importance for the role as pollinators in natural flora and commercial crops, 3. The fact that lack of stings facilitates management, 4. Easier manipulation of smaller hives than *Apis mellifera*, demanding technical skills instead of physical strength, accessible to children, women, elderly unable to care for an apiary by themselves.

Standards of pot-honey have not been created [7]. The *Codex Alimentarius Commission* international honey standards for honey are designed for *Apis mellifera* only [8]. Only the Colombian Honey Norm has included an annex with quality chemical factors of pot-honey [9]. Needs of chemical markers to authenticate the entomological origin have been approached by multivariate analysis of fructose, glucose and maltose [10], physic-chemical quality factors [11], sensory Free Choice Profile (FCP) [12], NMR and chemometrics [13], flavonoid C-glycosides [14] and O-glycosyl flavones [15], and more recently by electrophoretic patterns because different species of stingless bees produce honey with distinctive protein bands [16].

Pot-honey is known to be consumed either for family use or to reach up to 1,100% the price of *Apis mellifera* honey [17]. This great difference is a motivation to declare a false entomological origins, causing an Economically Motivated Adulteration (EMA), to be added to the four types of EMAs causing vulnerabilities in the US honey market: 1. Dilution with syrups, 2. Feeding sugar to bees during nectar offer, 3. Masking countries of origin, and 4. Antibiotic residues after excessive veterinary doses [18]. Therefore the importance to certify the entomological origin of honey.

In this work, ^{1}H NMR on chloroform extracts of honey were used to discriminate between the entomological origin of honey produced by *Apis mellifera* and pot-honey produced by the genera *Geotrigona* and *Scaptotrigona*.

II. Methods

A. Honey Samples

Ecuadorian commercial honeys from three entomological origins *Apis mellifera*, *Geotrigona* and *Scaptotrigona* were collected during field work, and kept frozen until analysis.

B. Chloroform Extract of Honey

A water:chloroform mixture (1:1) was used as extractive solvent to discard sugars −the major compounds of honey, retained in the water phase. The aroma compounds and other hydrophobic substances are separated in the organic phase. This extractive procedure yields a concentrated solution adequate for fast NMR analysis. For that purpose, portions of 6.0 ± 0.1 g honey were weighed in a 20 mL capped centrifuge tube and dissolved with 15 mL of deionized water, 15 mL of $CHCl_3$ were added and the mixture was stirred for 10 min. The biphasic mixture was then centrifuged at 10,000 rpm for 15 min at 4 °C. The lower chloroform phase was collected in a glass vial and the solvent was evaporated under a gentle stream of nitrogen. The solid residue was dissolved in 600 µL of $CDCl_3$ and placed in an NMR tube, following the scheme of NMR-based metabolomic approach previously published. Chloroform is a convenient solvent compared to dimethyl sulfoxide (DMSO) and methanol (MeOH) previously used in NMR studies of honey, because the residual chloroform signal is very sharp, in a very small region at 7.26 ppm, which does not overlap with important regions of the spectra [19].

C. ^1H NMR Spectra Acquisition

The 1D spectra were acquired at 298 K, with a 600 MHz NMR Bruker instrument, using a modified double pulsed field gradient spin echoes (DPFGSE) sequence [20]. This pulse in the DPFGSE sequence allows the removal of signals present in the (0-2 ppm) region, required to improve digitization of the weaker peaks, lower integration errors, and also better quantification of the number of resonant spins. The spectra collection, processing and analysis required thirty minutes.

C. Statistical Analysis

Signals of ^1H NMR spectra were integrated and used as inputs for PCA (Principal Component Analysis) and PLS-DA (Partial Least Squares – Discriminant Analysis) analysis [6]. PCA discriminates how one sample is different from another, and identifies the variables contributing to the variation. PLS-DA are based on similar principles as PCA, but additionally labelled sets of classes are identified, enhancing the separation between groups of observations.

III. Results

The spectra of the three entomological origins of honeys studied here (*Apis mellifera*, *Geotrigona*, and *Scaptotrigona*) were compared. The honey produced by these groups of bees share common features and have distinctive regions that merit further analysis. Expanded ^1H-NMR spectra of chloroform extracts of honey produced by these three types of bees, are compared in Fig. 1. A central region (3.5-4.5) ppm is characteristic for each bee type, because it contains the endogenous lipophilic markers, independent of floral and geographical origin. Other substances vary their concentrations.

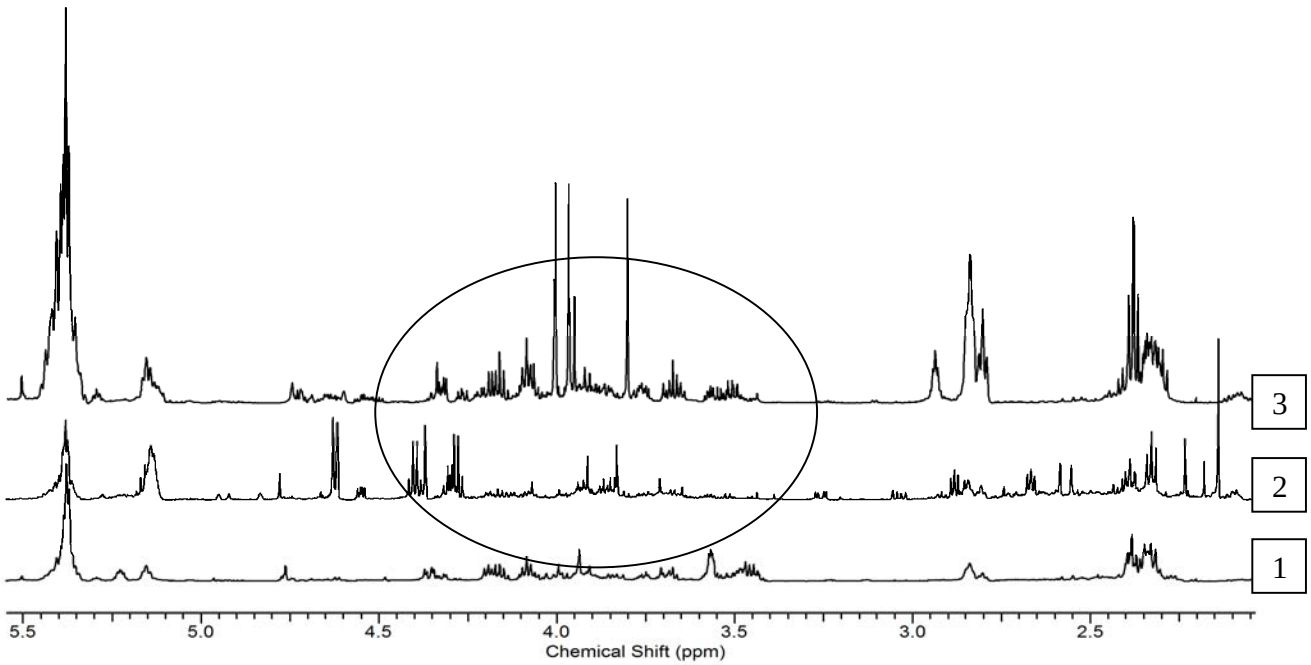

Fig. 1 Comparison in the expanded region (2.0-5.5 ppm) of ^1H-NMR spectra of chloroform extracts of honeys produced by: 1. *Apis*, 2. *Scaptotrigona*, and 3. *Geotrigona* bees in Ecuador.

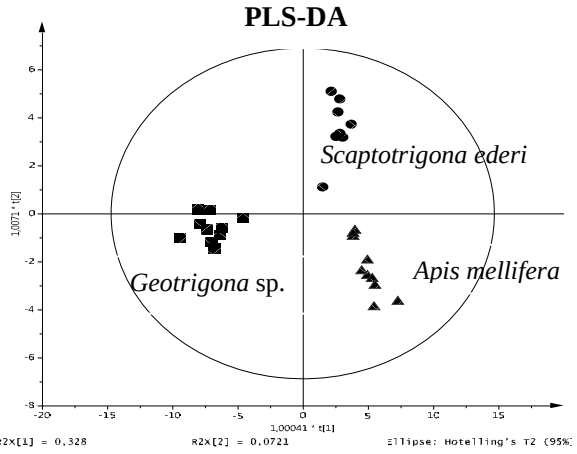

Fig. 2 Principal Component Analysis and PLS-DA on ^{1}H NMR spectra of chloroform extracts of Ecuadorian honey produced by *Apis mellifera*, and pot-honeys produced by *Geotrigona* sp. and *Scaptotrigona ederi*.

In Fig. 2 the PLS-DA discriminates the three Ecuadorian honey groups according to the entomological origin.

This graphic separation shows more similarities between *Scaptotrigona* and *Apis mellifera* honey, separated only by the second component, because *Geotrigona* honeys are separated from them by the first component.

Besides the recent NMR profiling investigation with 800 honeys originated worldwide [21], the unifloral honeys from Italy [22] and Germany [23], and the entomological and geographical origin prediction [13], this research confirms a new element for denominations of protected origins in honey: The entomological origin, so important in tropical and subtropical ecosystems with stingless bees.

The Australian cosmovision of "Sugarbag Dreaming" connects honey to the knowledge of the surrounding environment and accepted hunting practices in the Northern Territory [4]. Stingless bees, sugarbag structures and honey are conceived in their relationship to humans and other beings as an "indigenous philosophical ecology" [24]. In this way, honey is a complex matrix, and ^{1}H-NMR one complex method to perceive its chemical composition, adequate to uncover the type of bee originating each honey. Direct observation of the spectra and clusters after integrating NMR signals of honey chloroform extracts by PCA will do that. Therefore, this method is a useful reference for the official agencies and becomes an alternative method for the Ecuadorian Honey Norm [25] to test the entomological origin of Ecuadorian honey, with a biodiversity of 89 stingless bee species in the El Oro, Loja and Zamora Chinchipe provinces in the South of Ecuador [26].

IV. Conclusion

The entomological origin of the *Apis mellifera*, and the two major pot-honeys in the Ecuadorian market –*Geotrigona* and *Scaptotrigona ederi*, were successfully discriminated after PLSD-DA on ^{1}H NMR spectra of chloroform honey extracts.

Acknowledgment

To Prometeo, Senescyt, Ecuador for the scholarship to P. Vit at Universidad Técnica de Machala (UTMACH), Provincia El Oro, Ecuador. To Mrs. Elizabeth Brito Administrative Assistant from the Planning Department at UTMACH, and Mrs. Esperanza Poma Bustos, Director of Public Relations at UTMACH for their constant administrative asssitance. To 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. 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, to confirm the stingless bee genera, and for her continuous entomological support to identify species. P Vit accepted invitations extended by the Technical Committee (TC) VMD Hugo Rosero (TC President) and Eng. Maritza Farinango (TC secretar) for the revision of the Ecuadorian honey norm NTE INEN 1572 (2014-2015).

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