# Physicochemical and biological properties of honey samples from *Melipona beecheii* Bennett collected in Hopelchén, Campeche

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**ABSTRACT**

**Objective**: To determine the physicochemical and biological parameters of *Melipona beecheii* honey produced in the state of Campeche, as the basis for a future proposal for the establishment of an official Mexican standard for Melipona honey.

**Design/Methodology/Approach**: Samples of *Melipona beecheii* honey from the community of Hopelchén, Campeche, were analyzed. The results showed an acid honey with 3.89±0.13 pH, 49.85±1.74 free acidity, 68.09±2.12 total acidity, 76.95% °Brix, 21.38% humidity, 6.43±0.84 HMF, 8.58±0.25 diastase index, and 187.33±7.9 color intensity.

**Results**: The honey had a low phenolic (28.45±1.14 mgEAG/kg) and flavonoid (0.072±0.01 mg/kg) content. The low phenolic content interfered with the antioxidant activity, revealing an EC50 of 0.76. The honey showed antibacterial activity against *Staphylococcus aureus*, *Pseudomona aeruginosa*, and *Pseudomonas syringae*. A high-performance liquid chromatography (HPLC) was used to determine the chromatographic profiles of the honey; the said profiles were evaluated by chemometrics to classify the honey, according to the similarity of the chromatographic profiles.

**Study Limitations/Implications**: Despite being produced at a close distance from each other, the great variability among the small number of samples from the state of Campeche requires the characterization of the honeys by geographic zones.

**Findings/Conclusions**: The parameters determined in this study help to understand the variability in values, which is crucial for establishing quality and authenticity standards for honey. These standards will not only be applied to honey produced by the *M. beecheii* species, which is widely used in southeastern Mexico, but also to other types of honey.

**Keywords**: Honey, *Melipona beecheii*, physicochemical parameters, chemometrics.

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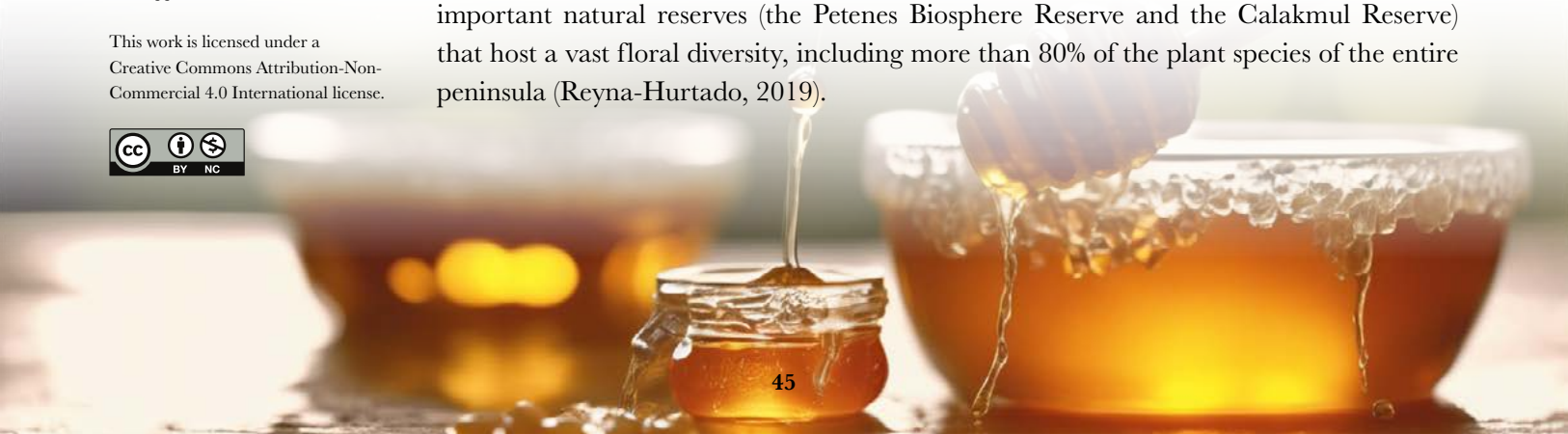
## INTRODUCTION

The distinct geology of the state of Campeche, Mexico, located in the southwestern part of the Yucatan Peninsula sets it apart from the rest of the country. It is home to two important natural reserves (the Petenes Biosphere Reserve and the Calakmul Reserve) that host a vast floral diversity, including more than 80% of the plant species of the entire peninsula (Reyna-Hurtado, 2019).



This floral diversity provides the region with different goods and services, among which the role of the melliferous and polliniferous flora in the beekeeping sector stands out (Villalobos-Zapata and Mendoza-Vega, 2016). Campeche is currently ranked as the second producer of honey nationwide (SIAP, 2021) and its honey is appreciated in the international market due to its organoleptic properties. Its composition and sensory attributes vary depending on the geographic region, climatic condition, floral origin, and type of bee (Hadju *et al.*, 2021; Santos *et al.*, 2021).

Honey production in Campeche and the Yucatan Peninsula has its origins in meliponiculture, a technique practiced by the ancient Mayans through the breeding of stingless bees, also known as meliponines (Quezada-Euán, 2018). Stingless bees have ecological, cultural, and economic importance as pollinators and as a food and medicine source (Yurrita *et al.*, 2017). There are 17 species of stingless bees in the Yucatan Peninsula (Ayala *et al.*, 2012). Eight species have been recorded in the Petenes Reserve of Campeche alone (Fernández *et al.*, 2018). However, the beekeeping practiced in the state (mainly in Calkiní, Hecelchakán, and Hopelchén) is based on the *Melipona beecheii* species, known in the Mayan language as "Xunan-Kab" or "Koolel-Kab" (Reyes-González *et al.*, 2014).

Although far less Melipona honey is produced than *Apis mellifera* honey, the demand and cost ($4,000.00-$8,000.00/liter*) for *M. beecheii* honey has increased significantly; this phenomenon has caused a recent resurgence of interest in preserving meliponiculture in countries such as Argentina, Brazil, Mexico, India, Indonesia, Thailand, Malaysia, and Australia (Zulhendri *et al.*, 2022).

A special characteristic of Melipona honey is its fermented flavor resulting from its high humidity percentage (Fernández *et al.*, 2018; Anguebes *et al.*, 2016), higher acidity and ash content (Sharin *et al.*, 2021), lower glucose and fructose composition (Pucciarelli *et al.*, 2014), and lower enzymatic activity (Nordin *et al.*, 2018). Additionally, its mineral composition is mostly made of potassium, followed by calcium, sodium, magnesium, and manganese (Biluca *et al.*, 2016).

No quality regulation exists for the commercialization of *A. mellifera* honeys, because they are not included in the Official Mexican Standards (NOM).

The FAO Codex Standard for Honey (Codex Alimentarius Commission, 2001), which establishes the definition, composition, and labelling of honey, does not cover honey produced by meliponines. This honey tends to be more acidic and have a higher water content (Vit *et al.*, 2004) and, consequently, does not comply with the parameters established in the said standard, emphasizing the importance of developing regulations for the honey produced by stingless bees.

To tackle this lack of regulation, the Kelulut (Stingless Bee) Honey - Specification (Malaysian Standard MS2683:2017) was published as the first national standard for meliponines in Malaysia. In 2019, the Secretariat of Regulation and Health Management and the Secretariat of Food and Bioeconomy of Argentina published the 17/2019 joint resolution in its Official Bulletin regarding the honey of Yateí bees (*Tetragonisca fiebrigi* Schwarz). This was the second standard proposed to regulate honey produced by meliponines in Argentina (Vit *et al.*, 2023). Consequently, the objective of this work, rather

than to carry out a regional study of a particular honey, is to determine the physicochemical and biological parameters of *Melipona beecheii* honey produced in Campeche. This research would be the basis for a future proposal to establish a NOM, similar to the Malaysian and Argentinian standards.

## MATERIALS AND METHODS

### Honey samples

The honey samples of *M. beecheii* were collected in May and July in the town of Ich ek, municipality of Hopelchén, Campeche. Six samples were obtained: five came from bee colonies located less than 500 m apart from each other and one was a commercial mixture of the honey from the five bee colonies. In the first case, the objective was to achieve homogeneity in the samples, while in the second case the honey mixture was prepared by producer. The samples were stored in plastic jars at 4 °C awaiting their analysis. After analyzing the pollen under a phase contrast microscope, the floral origin of the samples was classified as multifloral.

### Physicochemical analysis

Degrees Brix, moisture, pH, acidity, and hydroxymethylfurfural (HMF) content were determined following the NMX-F-036-NORMEX Mexican standard (2006). The Bianchi method was used to determine diastase activity, while color intensity was evaluated using the method described by Kek *et al.* (2014). All analyses were performed in triplicate. Analytical grade reagents and solvents for high-performance liquid chromatography were used. The °Brix and moisture of honey were determined by refractometry, using an RHB-92t portable refractometer. The results were set forth in °Brix and moisture percentages. The pH of the honey was determined with a Corning Pinnacle® 540 precision pH meter. The procedure established on section 8.3 of the NMX-F-036-NORMEX-2006 official standard was followed for the measurement, modifying the weight of the sample mentioned in the methodology, given the reduced quantity of honey. Free and lactonic acidity were added to calculate total acidity. The results were expressed in milliequivalents of acid per kilogram of honey (meq $kg^{-1}$).

### Hydroxymethylfurfural

The methodology established by White (1979) and Kek *et al.* (2014) was followed, processing 5.0 g of honey in 25 mL per sample. The readings were performed on a Beckman Du® 650 spectrophotometer and the results were expressed in mg $kg^{-1}$.

### Diastase activity

The Bianchi method (1990) was used to determine this variable, putting 2.0 g of honey in 2 mL of a buffer solution (17.4 g $CH_3COONa$+2.1 mL $CH_3COOH$/100 mL $H_2O$, pH 5.3). Nine serial dilutions were prepared with a 50% dilution factor, using a 1% NaCl solution as the blank. The results were reported in Gothe units.

**Color intensity**

A 50% (w/v) honey solution with warm water (45 °C) was prepared to determine this variable. The solution was filtered through a 0.45 $\mu$m MF-Millipore™ membrane, and the filtrate was analyzed using a Beckman DU® 650 UV-Vis spectrophotometer at 450 nm and 720 nm. The results were expressed in milliabsorbance units (mAU).

**Total phenols**

Total phenolic content was determined using the Folin-Ciocalteu method as modified by Kek *et al.* (2014). Measurements were taken at 765 nm. A calibration curve for gallic acid (20, 40, 60, 80, and 100 $\mu$g/mL) was developed. The results were expressed as milligrams of gallic acid equivalents (GAE) per kilogram of honey.

**Flavonoids**

Four milliliters of a 25% honey solution were mixed with 300 $\mu$L of 5% $NaNO_2$. After five minutes, 300 $\mu$L of 10% $AlCl_3$ and 2 mL of 1 M NaOH were added. Six minutes later, the solution was diluted to 10 mL with distilled water and mixed by inversion. Measurements were taken at 510 nm. Total flavonoids were quantified using a calibration curve with catechin over a concentration range (20, 40, 60, 80, and 100 $\mu$g/mL). Results were expressed as milligrams of catechin per kilogram of honey.

**Antioxidant activity**

The radical reduction method using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was performed. One gram of honey was dissolved in 1.0 mL of ethanol, creating $1\times10^{-1}$ to $1\times10^{-4}$ dilutions. Three 200-$\mu$L aliquots were taken from each dilution. Subsequently, 1.8 mL of a 0.003% DPPH solution were added to each aliquot, which was left in the dark for 30 minutes. Measurements were taken at 517 nm. Ascorbic acid ($C_6H_8O_6$) was used as a positive control and 100% ethanol was used as blank. The percentages of DPPH radical reduction were calculated using the following formula:

$$DPPH\ Reduction(\%) = 1 - \left(\left[sample\ absorbance\ /\ DPPH\ absorbance\right]\times 100\right)$$

The resulting curve was then subjected to a linear regression statistical analysis in Excel to determine the antioxidant activity of each sample in terms of its Median Effective Concentration (EC50).

**Antibacterial activity**

The antimicrobial activity bioassay was performed using the well diffusion method (Pimentel *et al.*, 2013) against suspensions of $1.5\times10^{8}$ CFU/mL of *Bacillus cereus* (ATCC 25923), *Staphylococcus aureus* (ATCC 4012), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 128), *Pseudomonas aeruginosa* (ATCC 27853), *Shigella flexneri* (ATCC 9748), and *Pseudomonas syringae* (ATCC 11043).

### HPLC analysis of phenolic acids and flavonoids

Ten g of honey were dissolved in 100 mL of distilled water. The lipophilic fraction was separated from the aqueous phase with $(C_2H_5)_2O$ three times (v/v) (2:1, 1:1, 1:1) using liquid-liquid extraction. The organic solvent was filtered through a paper filter cone with $Na_2SO_4$. Finally, the solvent was evaporated using a rotary evaporator at 37 °C and 550 psi. The extracts were diluted to a 1% concentration with HPLC-grade acetonitrile and filtered through a 0.45 $\mu$m membrane.

High-performance liquid chromatography (HPLC) analyses were performed on a Waters Alliance™ e2695 separations module with a UV detector. A Luna® HPLC column (Phenomenex) was used to separate the extract compounds with a particle size of 5.0 $\mu$m, C18, 150×4.6 mm. A gradient elution was used with mixtures of (A) formic acid and (B) acetonitrile, starting the gradient with 98% of A; 100% of B at 70 min, and 98% of A at 72 min, with a flow rate of 0.5 $\mu$L. The profiles were detected at 255 nm.

### Statistical analysis

The results of the physicochemical evaluations were expressed as mean values with standard deviations. Tukey's test was performed to compare means with a 0.05 significance level. The HPLC spectra were subjected to a chemometric analysis using a Principal Component Analysis (PCA), while Microsoft™ Excel 2010 and SIMCA 14.1 software were used to determine the hierarchical clustering. The data were preprocessed, adjusting the baseline to diminish or eliminate noise contribution. Additionally, the average of the spectra was determined.

## RESULTS AND DISCUSSION

### Degrees Brix and moisture

The percentage of °Brix in Melipona honey ranged from 76.5 to 77.25% g 100 $g^{-1}$ without significant differences between the analyzed samples (Table 1). These results are similar to those reported by Moo-Huchin *et al.* (2015) for samples from the Yucatán Peninsula (72.8 to 77.3%) and very close to the data reported by Lage *et al.* (2012) for some Melipona honeys from Brazil (68.09 to 72.12%). In general, honeys produced by stingless bees have lower °Brix values than those produced by *A. mellifera* (≥75), because stingless bee honeys have a higher water content and lower total sugar percentage (Biluca, 2016). According to the European Union, honey must meet certain sugar composition criteria to be marketed as such —*e.g.*, the sum of fructose and glucose of flower honey should not be less than 60%. Therefore, a >60% sugar in honey is considered a parameter of honey authenticity (Belay *et al.*, 2013).

Vit *et al.* (2023) used Nuclear Magnetic Resonance (NMR) to determine 41 parameters in 20 honeys from three genera of stingless bees, reporting the presence of fructose, glucose, maltose, maltotriose, raffinose, and sucrose. Their study reported a variation in total sugar content of 25.07, 54.73, and 57.75 for *Geotrigona*, *Melipona*, and *Scaptotrigona*, respectively.

Moisture content determines the amount of water present in the product and is therefore a critical quality parameter of honey. It is associated with the degree of maturity of the hive, the botanical origin of the honey sample, extraction techniques, and storage conditions

(Belay, 2013). Typically, the moisture content of Melipona honey is higher (≤30%) than in honey produced by *Apis mellifera* (≤20%) (Ramón-Sierra, 2015). The analyzed samples recorded low moisture values ranging from 21.1% to 22.3%, which fall within the maximum moisture limit of 30% for Melipona honey (Vit *et al.*, 2004) (Table 1). The results of this study are consistent with other studies that have been carried out in the Yucatán Peninsula (Moo-Huchin *et al.*, 2015; Ramón-Sierra *et al.*, 2015).

**Acidity**

Total acidity (the sum of free acidity plus lactonic acidity) is a reference parameter indicative of the fermentation state of honey. According to the bibliography, this value is highly variable within the group, as acidity corresponds to the organic acids in honey, which vary according to the floral composition and bee species (Lage *et al.*, 2012; Souza *et al.*, 2006). The honey samples evaluated in this study were acidic (pH of 3.67e±0.02 to 4.06a±0.02) (Table 1). The pH values are consistent with those reported by Dardón and Enríquez (2008) and Fonte *et al.* (2013) for *M. beecheii*. However, the samples evaluated in this study showed highly variable total acidity, ranging from 55.83±1.61 to 77.35±7.86 meq $kg^{-1}$. Since the average value is not representative, the parameter should be referred to as a range of variation between minimum and maximum values. According to the guidelines proposed for the genus Melipona by Vit *et al.* (2004), the maximum permissible total acidity should be 70.00 meq $kg^{-1}$, although some of the samples exceed this value. Flora composition may be the cause of the abovementioned variation in acidity values.

**Determination of HMF content and diastase activity**

Hydroxymethylfurfural (HMF) and diastase are the parameters most frequently used for the evaluation of honey freshness. The former is produced by the degradation of fructose and inadequate treatments of honey, with a nearly negligible presence in fresh honey, while the latter is added to honey by the bee and its activity decreases in old or heated honeys (Oddo *et al.*, 1999). Although the use of diastase as an indicator of honey quality has been questioned (White, 1994), it is still used as a reference and was evaluated in this research.

The HMF values of the honey samples ranged from 2.87 mg $kg^{-1}$ to 12.39 mg $kg^{-1}$ (Table 1). These values are lower than those previously reported by Moo-Huchin *et al.* (2015), who recorded 4 to 45.5 mg $kg^{-1}$ HMF values in honey samples collected in the

**Table 1**. Physicochemical parameters of *Melipona beecheii* honeys from Hopelchén, Campeche, Mexico.

| Collection month | Sample | Brix % | Moisture (g/100 g) | pH | Total acidity meq/kg | HMF (mg/Kg) | Diastase activity (°Gothe) |
|---|---|---|---|---|---|---|---|
| July | M1 | 77.00 | 21.20 | $3.67^{e}$±0.02 | $70.30^{ab}$±1.79 | $12.39^{a}$±3.01 | $6.50^{b}$±1.50 |
| | M2 | 77.25 | 21.20 | $4.06^{a}$±0.02 | $55.86^{b}$±1.14 | $6.51^{ab}$±0.02 | $12.00^{a}$ |
| | M3 | 76.50 | 22.30 | $3.96^{bc}$±0.01 | $77.34^{a}$±5.57 | $5.61^{ab}$±0.72 | $12.00^{a}$ |
| | M4 | 77.00 | 21.20 | $4.00^{ab}$±0.02 | $56.16^{b}$±1.19 | $2.87^{b}$ ±0.26 | $8.00^{b}$ |
| | M5 | 77.00 | 21.20 | $3.91^{c}$±0.00 | $76.90^{a}$±1.60 | $4.64^{b}$±0.95 | $5.00^{b}$ |
| March | Mixture (M6) | 77.00 | 21.20 | $3.79^{d}$±0.01 | $72.02^{a}$±1.44 | $6.59^{ab}$ ±0.09 | $8.00^{b}$ |

Different letters in the same column indicate significant differences (ANOVA, $p<0.05$).

Yucatán Peninsula. For their part, Silva *et al.* (2013) reported that 10.80 to 15.76 mg $kg^{-1}$ HMF values in nine honey samples of *Melipona subnitida* produced in Brazil. Complying with a low HMF content is important, because it guarantees the freshness of the honey purchased by consumers.

The HMF content recorded in this study suggests that the honey samples of *M. beecheii* collected in Hopelchén had been recently harvested.

Diastase is the enzyme responsible for converting starch into dextrins and sugars. The diastase number (DN) expresses, in Gothe units, the diastatic activity of honey as the number of mL of a 1% starch solution hydrolyzed by the enzyme in 1.0 g of honey. Vit *et al.* (2004) pointed out that Melipona honey exhibits low diastase activity and has a minimum diastase index on the Gothe scale (3.0). The diastase activity measurements in the samples ranged from 5.0 to 12.0 Gothe units. Therefore, the obtained data confirm that the evaluated honey was fresh, had been recently harvested, and was stored under appropriate conditions.

**Color intensity**

The color intensity of honey is a qualitative parameter that indicates the presence of pigments —which are in turn associated with the presence of antioxidants, such as carotenoids and flavonoids (Moniruzzaman *et al.*, 2013). The color intensity values of the honey ranged from 102 to 320 mAU (Table 2). Color has been related to the antioxidant activity of honey, with a higher content of phenolic compounds being reported in darker honeys (Moniruzzaman *et al.*, 2013).

**Phenolic compounds and antioxidant activity**

The total content of phenolic compounds and antioxidant activity values are presented in Table 2. The lowest total phenolic content (22.9 mg EAG $kg^{-1}$) was found in sample M4. Sample M3 showed the highest phenolic content value (37.33 mg EAG $kg^{-1}$). Samples M1 and M3 had some of the highest color intensity values, as well as a high phenolic content. However, a low correlation was recorded between these two variables ($R^2=-0.272$), similar to the correlation between color and flavonoid content ($R^2=-0.113$) and antioxidant activity ($R^2=0.304$). These results differ from those described by Can *et al.* (2015) (16.02 to 120.04 mg GAE 100 $g^{-1}$) and da Silva *et al.* (2016) (17.0 to 66.0 mg GAE $g^{-1}$).

**Table 2**. Color intensity, total phenolic and flavonoids contents, and half maximal effective concentration (EC50) of *Melipona beecheii* honey.

| Collection month | Sample | Color intensity (mUA) | Total phenolic mgEAG/Kg | Flavonoids mg Cat/Kg | EC50 |
|---|---|---|---|---|---|
| July | M1 | $320^{a}\pm2.2$ | 33.41 | 0.058 | 1.26 |
| | M2 | $111^{c}\pm5.1$ | 25.15 | 0.071 | 0.64 |
| | M3 | $302^{a}\pm16.5$ | 37.33 | 0.077 | 0.66 |
| | M4 | $102^{c}\pm11.8$ | 22.9 | 0.054 | 0.86 |
| | M5 | $127^{bc}\pm2.5$ | 28.91 | 0.089 | 0.57 |
| March | Mixture (M6) | $162^{b}\pm9.8$ | 23.01 | 0.085 | 0.61 |

The flavonoid content in the honey ranged from 0.058 to 0.088 mg catechin $kg^{-1}$; these results were similar to the findings of Muñoz *et al.* (2007), who reported a low flavonoid content (0.014-13.8 mg 100 $g^{-1}$) in a honey sample from Chile. Moniruzzaman *et al.* (2014) and other authors indicate that the geographical origin of honey affects its phenolic composition and flavonoid concentration, which in turn impact its antioxidant activity.

The antioxidant capacity of honey was determined by the DPPH assay. An EC50 between 1.261 and 0.57 mg $mL^{-1}$ was obtained; however, these values were lower than those reported by Vit *et al.* (2009), Moniruzzaman *et al.* (2014), and other authors. These results may be caused by the low color intensity, low phenolic content, and low flavonoid content, which affected the antioxidant activity.

**Antibacterial activity**

A traditional and widely spread belief about the medicinal properties of Melipona honey pertains to its antibacterial properties. Packaging *M. beecheii* honey in eye drop dispensers is not uncommon. Various authors have demonstrated the inhibitory activity of different methods against some bacterial species, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*; however, the variations among the methodologies prevented the comparison of the results (Miorin, 2003; Campeau and Patel, 2014). The well diffusion method (Pimentel *et al.*, 2013) used in this work to determine the antibacterial activity of honey was performed against six human pathogens and one phytopathogenic bacterial species (Table 3).

All samples inhibited *S. aureus* and *P. syringae*. In the case of *P. aeruginosa*, sample M3 did not show inhibitory activity. The greatest inhibitory effect of the honey was observed against *S. aureus*, impacting growth in all evaluated dilutions and recording the highest inhibition values (0.50-1.5 cm). These results are similar to the findings of Zamora and Arias (2011), who reported that the analyzed honey samples exhibited greater antibacterial activity against *S. aureus*.

Honey had very similar inhibitory results against both *P. aeruginosa* and *P. syringae*. Inhibition halos were reported only in the first dilutions (75% and 50%), with lower inhibition values than those obtained for *S. aureus*. Although the growth of *E. coli* and *S. flexneri* was not totally inhibited, a qualitative decrease in growth was observed in the samples, as the cells grew poorly on the culture medium. This effect was observed in all samples against *E. coli*, while, in the case of *S. flexneri*, the decrease in bacterial growth was observed in samples M3 to M6.

Honey did not impact *B. subtilis* and *B. cereus* at all, as no inhibition was observed in any of the evaluated dilutions. This phenomenon had been previously reported by Vermeulen *et al.* (2005) and Blaser *et al.* (2007), who mentioned not only the susceptibility of *S. aureus* to Melipona honey, but also that the main inhibitory effect on growth was found in Gram-negative bacteria.

In this regard, antibacterial validation should be conducted with reference strains deposited in international collections. Since the antagonism evaluation methodologies are diverse, the bodies that validate official protocols should specify the validation method.

**Table 3**. Antibacterial activity of honey.

| Collection month | Sample | Dilution % | Area of inhibition (cm) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | ***Bacillus cereus*** | ***Staphylococcus aureus*** | ***Bacillus subtilis*** | ***Escherichia coli*** | ***Pseudomona aeruginosa*** | ***Shigella flexneri*** | ***Pseudomonas syringae*** |
| July | M1 | 75 | 0.00 | 0.50 | 0.00 | * | 0.55 | 0.00 | 0.50 |
| | | 50 | | 0.50 | | * | 0.40 | | * |
| | | 25 | | 0.50 | | * | 0.20 | | * |
| | | 10 | | 0.40 | | 0.00 | 0.10 | | 0.00 |
| | M2 | 75 | 0.00 | 0.60 | 0.00 | * | 0.75 | 0.00 | 0.50 |
| | | 50 | | 0.60 | | * | 0.55 | | 0.40 |
| | | 25 | | 0.60 | | 0.00 | 0.40 | | 0.00 |
| | | 10 | | 0.30 | | 0.00 | 0.00 | | 0.00 |
| | M3 | 75 | 0.00 | 0.85 | * | * | * | * | 0.85 |
| | | 50 | | 0.80 | 0.00 | * | * | | 0.65 |
| | | 25 | | 0.50 | 0.00 | * | * | | 0.00 |
| | | 10 | | 0.00 | 0.00 | * | * | | 0.00 |
| | M4 | 75 | 0.00 | 1.50 | 0.00 | * | 0.80 | * | 1.50 |
| | | 50 | | 1.50 | | * | 0.80 | | 0.70 |
| | | 25 | | 1.00 | | * | 0.00 | | 0.70 |
| | | 10 | | 0.70 | | * | 0.00 | | 0.00 |
| | M5 | 75 | 0.00 | 1.30 | 0.00 | * | 0.80 | * | 0.80 |
| | | 50 | | 0.90 | | * | 0.70 | | 0.40 |
| | | 25 | | 0.90 | | * | 0.70 | | 0.00 |
| | | 10 | | 0.60 | | * | 0 | | 0.00 |
| March | Mixture (M6) | 75 | 0.00 | 1.50 | 0.00 | * | 0.50 | * | 0.50 |
| | | 50 | | 1.50 | | * | 0.00 | | 0.50 |
| | | 25 | | 1.40 | | * | 0.00 | | 0.00 |
| | | 10 | | 0.80 | | * | 0.00 | | 0.00 |

The area of inhibition (cm) is shown after a 24-h incubation.
(*) Decreased concentration of bacterial growth.

## Chemometric Analysis of Chromatographic Profiles by HPLC

Before performing chemometric analyses, a series of pretreatments had to be conducted on the raw chromatographic profiles, since baseline variations and retention time negatively affect the analysis results. The airPLS function was used to correct baseline deviations.

A principal component analysis (PCA) was applied to analyze the chromatographic data from the six honey samples, in order to understand similarities in the distribution and abundance of the chromatographic peaks.

The first principal component (PC1) was strongly associated with the variables of samples M5 and M6, accounting for 60% of the variance. The second principal component (PC2) explained 20% of the variance and was mainly associated with the variables of samples M1, M2, M3, and M4. The cumulative variance of these principal components amounted to 80% (Figure 1).

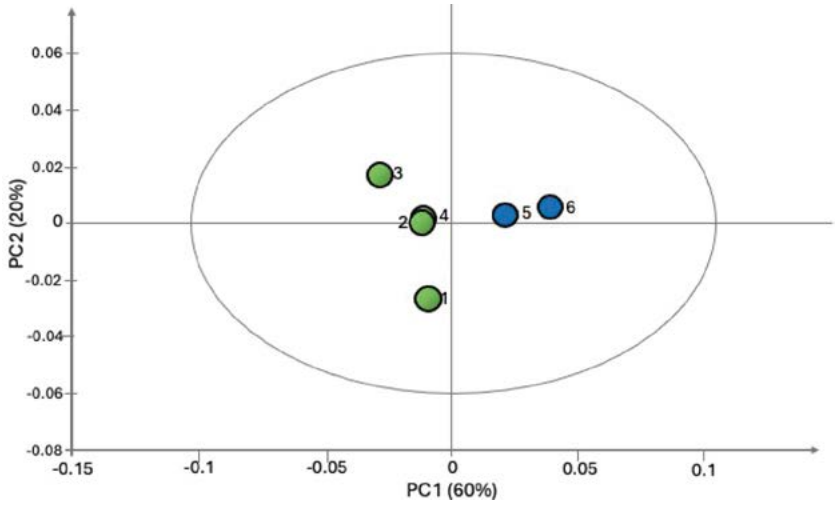



**Figure 1**. Result of the Principal Component Analysis (PCA) applied to the six honey samples.

A loading plot analysis was performed to determine the components related to the previous clustering. This type of analysis indicates the influence of a given component on the different chromatographic profiles. PC1 is the component at $t_R$ 30 min, which influences the separation from the other samples, with higher concentrations in M5 and M6. The slight separation between them is attributed to the component at $t_R$ 17.1 min, which is more abundant in M5. In contrast, the component at $t_R$ 22.6 min influences grouping (b) and the component at $t_R$ 29.0 min is estimated to be involved in the separation observed for sample M3 within its group. Meanwhile, the component at $t_R$ 11.6 min separates sample M1 within PC2. Finally, the components at $t_R$ 29.0, 24.7, and 23.7 min are correlated variables —*i.e.*, they are shared by all samples in PC2 (Figure 2). Another way to determine the similarity of the chromatographic profiles was the use of a hierarchical clustering analysis, calculating and comparing the similarity between samples. The resulting dendrogram enables the visualization of the distances between samples, forming groups based on their similarity.

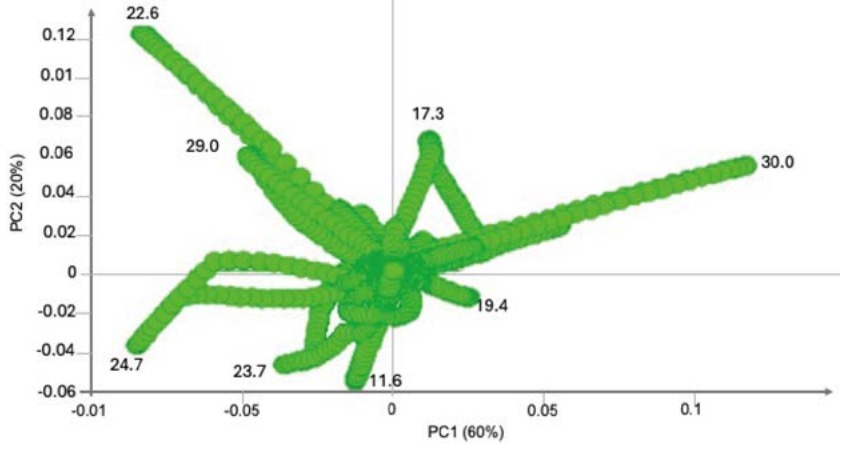
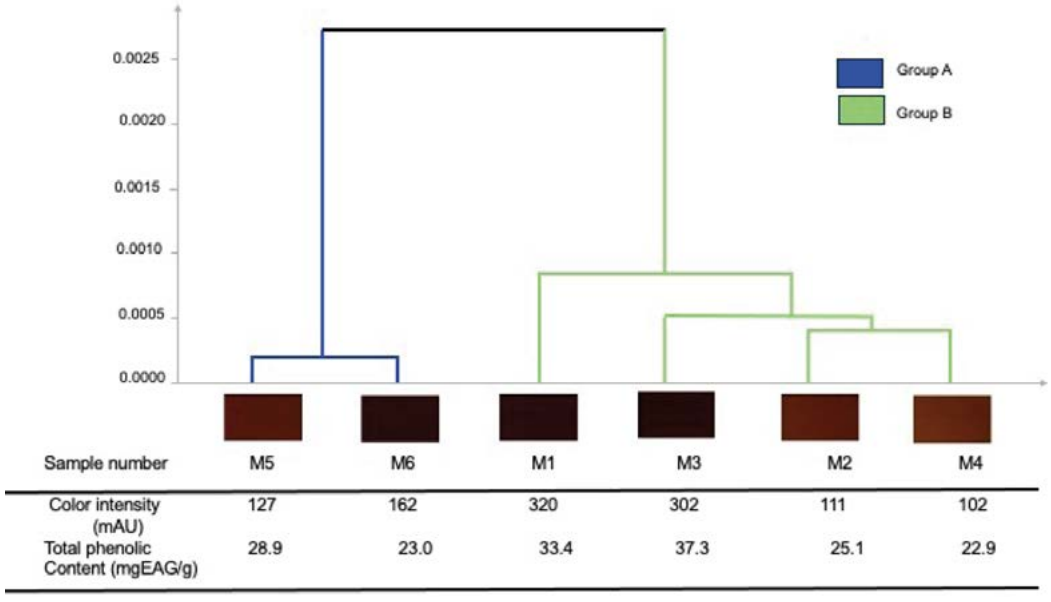



**Figure 2**. Principal Component Analysis of the resulting chromatographic profiles.

The dendrogram obtained in this study consisted of two groups: A (M5 and M6) and B (M1, which shares greater similarity with M3, and to a lesser extent with M2 and M4). This classification was correlated with the color intensity and phenolic content of the honeys. Group A comprised samples with intermediate levels of phenolic compounds and color intensity, while Group B divides the samples into those with higher and lower phenolic content and color intensity, respectively (Figure 3).




| Sample number | M5 | M6 | M1 | M3 | M2 | M4 |
|---|---|---|---|---|---|---|
| Color intensity (mAU) | 127 | 162 | 320 | 302 | 111 | 102 |
| Total phenolic Content (mgEAG/g) | 28.9 | 23.0 | 33.4 | 37.3 | 25.1 | 22.9 |

**Figure 3**. Color intensity of six honey samples with the same geographical origin.

Although no compound was identified, the principal component analysis indicates a variability among the small number of samples analyzed from Campeche, despite the proximity of collection sites. This result suggests the need to characterize honey according to its geographic region.

## CONCLUSIONS

The advancements shown can contribute to the establishment of a legal and standardization framework (NOM: Official Mexican Standard) for Melipona honey. Some methodologies, such as the determination of other compounds by HPLC, are not currently applicable, given the lack of sufficient data about the diversity of the components of this type of honey. Its incorporation is, therefore, a pending matter. The therapeutic, antimicrobial, and antioxidant biological characteristic of Melipona honey are confirmed. The chemometric analysis of the chromatographic profiles (in this case, for *M. beecheii*) confirms the diverse botanical origins that can be found in the same geographic location.

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