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Techniques for the Evaluation of Physicochemical Quality and Bioactive Compounds in Honey

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Abstract

Honey is a concentrated aqueous solution of sugar, especially glucose and fructose, and minor amounts of dextrin, enzymes, waxes, volatile oils, organic acids, ethers, albuminoidal gum substances and minerals. Commercially available honey samples vary in quality according to various factors such as climate diversity, type of flora of the surrounding region, geographical characteristics, processing, floral supply period, and packaging and storage conditions, which can compromise the standardization and quality of the final product. The different techniques that will be presented in this chapter to assess the quality of honey are tests required by identification standards and national and international quality control or are important quality tools that can be used in the evaluation of the conditions for obtaining and processing of the honey, fraud identification and changes to and/or adulteration of the honey, ensuring the physical and chemical composition of the product and guaranteeing quality standards, directly impacting the shelf life and use and presentation of the product.

Keywords: physicochemical characterization, honey quality standards, antioxidant activity, total polyphenols, flavonoids

1. Introduction

Honey is produced by honeybees from the nectar of flowers or from secretions from the living parts of plants or from vegetable sap by sucking insects that remain on the living parts of the plant. Bees collect and transform this material with their own specific substances before

storing it and leaving it to mature in separate honeycombs [1–3]. Honey is characterized as a semi-liquid product, comprising a complex mixture of carbohydrates, especially the monosaccharides glucose and fructose; and other sugars, enzymes, lactones, wax, pigments, vitamins, amino acids, minerals, organic acids and pollen [4]. Its chemical composition varies according to the bee species, weather conditions, type of soil, physiological state of the colony, nectar source and honey maturity [5]. Its nutritional quality, which occurs due to the presence of minerals and vitamins, sensory properties, medicinal properties such as antioxidant and antiseptic activity, specific therapeutic properties, such as for the treatment of inflammatory and infectious processes, and high energy content attract many consumers [6, 7].

In Brazil, Normative Ruling No. 11 of October 20, 2000, which regulates the standardization of honey for marketing purposes, is based on European laws and approves only honey produced by bees of the *Apis* genus [3]. The physicochemical analyzes indicated by Brazilian legislation for the identity and quality of honey produced by bees from the *Apis* genus are moisture, sucrose, reducing sugars, ash, minerals, acidity, diastase activity, color and hydroxymethylfurfural (HMF) content [3]. These analyses contribute to the supervision and control of the quality of honey produced in Brazil and intended for export, and the results are compared with both Brazilian standards and those of international organizations [5, 8], see **Table 1**. Some concerns exist regarding the quality of domestically produced honey, and such tests allow the quality of imported honey to be inspected [5].

The aim of this chapter is to significantly contribute to the improvement of techniques that evaluate the quality of honey, and to propose an adjustment to the physicochemical parameters established by Brazilian law [3], adding additional analysis such as pH, formaldehyde index (mL kg^{-1}), electric conductivity (mS cm^{-1}), protein (%), total reducing sugars (%), viscosity (mPa s) and water activity. These analyzes can contribute effectively to control the quality of commercially available honey. The analysis required by existing legislation combined with

Parameters	Brazil (2000)	Mercosur (1999)	European Union (2001)
Moisture (%)	Maximum 20.0	Maximum 20.0	Maximum 20.0
Acidity (meq kg^{-1})	Maximum 50.0	Maximum 50.0	Maximum 50.0
Ash (%)	Maximum 0.6	Maximum 0.6	–
Color	Nearly colorless to dark brown	Nearly colorless to dark brown	–
HMF (mg kg^{-1})	Maximum 60.0	Maximum 60.0	Maximum 60.0
Electric conductivity ($\mu\text{S cm}^{-1}$)	–	–	Maximum 0.8
Reducing sugars (%)	Minimum 65.0	Minimum 65.0	Minimum 60.0
Saccharose (%)	Maximum 6.0	Maximum 6.0	Maximum 5.0
Diastase activity (Goethe)	Minimum 8.0	Minimum 8.0	Minimum 8.0

Source: Brazil [3]; Mercosur [9] and European Union [10].

Table 1. National and international standards for honey from *Apis mellifera* L.

the further analysis proposed by this chapter will allow important parameters for the quality of honey to be determined, such as maturity, purity, deterioration and adulteration.

2. Methods

All analyses were performed in triplicate to provide greater reliability for the results, following the methods described in the below sections.

2.1. Moisture

Water content is one of honey's most important characteristics as it influences its viscosity, specific gravity, maturity, crystallization, flavor, preservation, shelf life and palatability [11–13]. It depends on several factors such as bee species, floral source, honey harvesting time, the degree of maturity achieved in the hive (complete dehydration) and climatic factors [14].

Moisture is analyzed to determine the safety of the product, giving a quality criterion that determines the ability of the honey to remain stable and free of fermentation. A high moisture content can lead to crystallization of the product and promote the development of osmophilic microorganisms responsible for fermentation, negatively affecting its sensory characteristics and nutritional properties and reducing the shelf life of the product [15].

Method: humidity is determined according to the method described by the Atago Co [16], using the refractometric method. The method is based on the relationship between the speed of light in a vacuum and a substance through which an incidental beam of light is passed, through a honey solution [8, 16]. This device is adapted from the Abbe refractometer and features a scale, which expresses the value in brix, from which the humidity value is calculated. Three drops of honey are placed in the refractor device and, after adjusting the angle limit, the reading of the refractive index is taken directly from the scale [8]. The refractive measurement provides the dry matter content in all cases where there are pure sugar solutions. When the sugar solution is mixed with other substances, such as honey, the value found is usually very close to the total for the dry matter [17]. Therefore, to obtain the moisture from the honey, the value of the refractive index is checked with a correlation table showing the relationship between the refractive index and the moisture of the honey (**Table 1**). **Table 2** presents the result of an equation developed by Wedmore from the data of Chataway [15].

The refractive index of liquids is also temperature dependent. Generally, refractometers are regulated at 20°C [17]. If the temperature of honey is exactly 20°C, the refractive index obtained directly from **Table 1** can be applied. However, for measurement at different temperatures, the refractive index should be increased or decreased by a value of 0.00023 for each degree Celsius above or below 20°C, depending on the sample temperature. In the case of refractive index values not included in **Table 2**, the desired value can be calculated using Eq. (1).

$$y = 614 \times 60 - 400 \times x, \quad (1)$$

where y = moisture, x = refractive index.

Refractive index (20°C)	Moisture (%)	Refractive index (20°C)	Moisture (%)
1.4740	25.0	1.4865	20.0
1.4745	24.8	1.4870	19.8
1.4750	24.6	1.4875	19.6
1.4755	24.4	1.4880	19.4
1.4760	24.2	1.4885	19.2
1.4765	24.0	1.4890	19.0
1.4770	23.8	1.4895	18.8
1.4775	23.6	1.4900	18.6
1.4780	23.4	1.4905	18.4
1.4785	23.2	1.4910	18.2
1.4790	23.0	1.4915	18.0
1.4795	22.8	1.4920	17.8
1.4800	22.6	1.4925	17.6
1.4805	22.4	1.4930	17.4
1.4810	22.2	1.4935	17.2
1.4815	22.0	1.4940	17.0
1.4820	21.8	1.4946	16.8
1.4825	21.6	1.4951	16.6
1.4830	21.4	1.4956	16.4
1.4835	21.2	1.4961	16.2
1.4840	21.0	1.4966	16.0
1.4845	20.8	1.4971	15.8
1.4850	20.6	1.4976	15.6
1.4855	20.4	1.4982	15.4
1.4860	20.2	1.4987	15.2

Source: Bogdanov et al. [15] and AOAC [18].

Table 2. Determination of honey moisture from the refractive index.

2.2. pH

The pH determined refers to the hydrogen ions present in a solution of honey and can influence the formation of other components such as the production of hydroxymethylfurfural—HMF [19]. While pH analysis is useful as an auxiliary variable to estimate the quality of the product and as a parameter for evaluating total acidity, it is not directly related to free acidity due to the actions of the buffer acids and minerals present in honey [20].

The pH of honey ranges between 3.5 and 5.5 depending on its botanical source, the pH of nectar, soil or plant association, and the concentration of different acids and minerals such as calcium, sodium, potassium and other ash constituents [2, 15]. Altered values may indicate fermentation or adulteration [12, 21]. Mandibular substances added to the nectar may also change the pH of honey, a process that begins with the transport of nectar to the hive in the honey vesicle [22].

Method: pH is determined according to the method described by De Moraes and Teixeira [23]. Weigh 10 g of honey in a 100 mL beaker using an analytical balance and homogenize the sample in 75 mL of distilled water. Thereafter, using a pH meter calibrated with appropriate buffers (pH solution of 7.0 and 4.0 pH solution) for each honey sample, a direct reading is taken from the device.

2.3. Acidity

Due to the variations of some organic acids and inorganic ions such as phosphate and based on different sources of nectar, honey acidity can result from the action of the enzyme glucose oxidase produced in the hypopharyngeal glands of bees, producing gluconic acid. This enzyme remains active even during storage affecting the honey after processing due to the quantity of minerals present, and by bacteria during maturation [15, 24, 25]. Organic acids from honey represent less than 0.5% of solids, but have a considerable effect on taste [26].

Method: acidity is determined in accordance with the method described by De Moraes and Teixeira [23]. Weigh 10 g of honey in a 100 mL beaker with an analytical balance; homogenize the sample in 75 mL of distilled water; add five drops of alcoholic solution of phenolphthalein. With the aid of a pH meter and a magnetic stirrer, titration is slowly carried out with sodium hydroxide (NaOH) 0.1 N, until the solution reaches a pH of 8.5. Add 10 mL of sodium hydroxide (NaOH) 0.1 N to the sample to increase the pH to approximately 10. Titrate with hydrochloric acid (HCl) 0.1 N to slowly return the pH to 8.3. Note the volumes spent during each titration to calculate the total acidity of the sample. Acidity value is determined by Eqs. (2)–(4) and corrections of HCl and NaOH should be carried out in accordance with Eqs. (5) and (6).

$$\text{Free acidity : corrected volume of NaOH spent} \times 10, \quad (2)$$

$$\text{Lactonic acidity : (10 - corrected volume of HCl spent)} \times 10, \quad (3)$$

$$\text{Total acidity : free acidity + lactonic acidity}, \quad (4)$$

$$\text{HCl corrected} = \text{volume of HCl spent} \times \text{correction factor (fc)}, \quad (5)$$

$$\text{NaOH corrected} = \text{volume of NaOH spent} \times \text{correction factor (fc)}. \quad (6)$$

2.4. Formaldehyde content

The formaldehyde content in honey represents, predominantly, amino compounds, allowing the evaluation of peptide content, protein and amino acids [27]. This is an indicative of the presence of nitrogen in honey and is an important adulteration indicator. When low, it can suggest the presence of artificial products, while when excessively high it can show that the bees were fed hydrolyzed protein [28]. Thus, formaldehyde content can be used to prove the authenticity of honey [21].

Method: formaldehyde content is determined according to Moraes [29]. After performing the procedure for determining acidity when the pH of the sample reaches 8.3, the pH is reduced to 8.0 with two drops of 0.1 N acetic acid, and then 5 mL of 35% formalin is added to the sample. After one minute of agitation, the solution is titrated with sodium hydroxide (NaOH) 0.1 N, slowly returning the pH to 8.0. The volume of sodium hydroxide spent from the last titration is noted and the formaldehyde index is calculated in accordance with Eq. (7).

$$\text{Formaldehyde content} = \text{corrected volume of NaOH 0.1 N spent} \times 10(\text{mL kg}^{-1}). \quad (7)$$

2.5. Ash

Ash content expresses the richness of honey in mineral content [30–32]. The minerals calcium (Ca), magnesium (Mg), iron (Fe), copper (Cu), cadmium (Cd) and zinc (Zn) in the form of sulfate (SO_4^{2-}) and chloride (Cl^-) [24] are found in small amounts. Minerals influence the color of honey and are present in higher concentrations in dark honey than light-colored honey [14]. They vary depending on the floral origin, region, bee species and type of manipulation [15].

Method: the method used is proposed by Marchini et al. [8] and C.A.C. [33] and is based on the weight loss that occurs when the product is incinerated to a maximum of 550°C, resulting in the destruction of the organic matter without changing the constituents of the mineral residue or causing loss by volatilization [8]. The crucibles are identified and heated in a furnace for approximately 25 min at 300°C. They are then transferred to the desiccator for 20 min to cool down. The crucibles are weighed separately with an analytical balance and the weights recorded. Approximately 10 g of sample is weighed, and the exact weight recorded. The samples are charred on an asbestos screen using a Bunsen burner until completely carbonized. They are then incinerated in an oven, raising the temperature gradually to 600°C. Wait for 5–7 hours until incineration is complete (white to light gray color). The still hot crucibles are removed from the oven and transferred to the desiccator. After 20 min the crucibles are weighed with an analytical balance and the weight recorded. The amount of ash is determined according to Eq. (8):

$$\text{Ash}(\%) = \left[\frac{m_1 - m_2}{m_3} \right] \times 100, \quad (8)$$

where m_1 = crucible weight with ashes, m_2 = crucible weight, m_3 = sample weight (mass of honey).

2.6. Electric conductivity

Electrical conductivity is determined by the ability of ions present in a solution to conduct electrons. It has been found to assist in the determination of the botanical origin of honey, as well as correlating with ash content, pH, acidity, minerals, proteins and other substances in honey [30, 34]. Honey conductivity is a great indicator of the adulteration of honey from its original form; whether formed from nectar (with some differentiation according to species) or honeydew [2].

Method: electrical conductivity is based on the fact that salt solutions conduct an electric current between two electrodes [35]. To measure this, a conductivity meter is used. After turning on the unit and waiting for it to stabilize; wash the ampoule of the equipment with distilled water and add a 1412 $\mu\text{S}/\text{cm}$ buffer in order to calibrate the apparatus; then wait until the reading stabilizes.

Weigh 10 g of honey in a beaker on an analytical balance and transfer it to a 50 mL volumetric flask with distilled water. Take the reading as soon as the conductivity stabilizes. For each change of sample rinse the electrode with distilled water and dry it with absorbent paper.

2.7. Color

Color has a direct impact on the price of honey as it influences consumer preference and is of particular importance in the international market [8]. Variations in the color of honey are related to its floral origin, mineral content, storage and product processing, climatic factors during nectar flow and the temperature at which the honey matures in the hive [12], as well as factors such as the proportion of fructose and glucose present, nitrogen content and the instability of fructose in an acid solution [36].

Method: the evaluation of honey is based on the varying absorption of light of various wavelengths, depending upon the constituents present in the honey [19]. For the determination of color, a visible spectrophotometer is used. Select a wavelength of 560 nm; reset the tray of the machine using p.a. glycerin as a blank sample. Take the reading directly from the instrument display. Note the value and use the Pfund scale to determine the color according to range, in accordance with **Table 3**.

Color	Pfund scale (mm)*	Color range (inc)**
Water white	From 1 to 8	0.030 or less
Extra white	More than 8–17	More than 0.030–0.060
White	More than 17–34	More than 0.060–0.120
Extra light amber	More than 34–50	More than 0.120–0.188
Light amber	More than 50–85	More than 0.188–0.440
Amber	More than 85–114	More than 0.440–0.945
Dark amber	More than 114	More than 0.945

*Millimeter.

**Incidence—absorbance at 560 nm. Source: Marchini et al. [8].

Table 3. Pfund scale for determining color.

For this analysis, the honey must be liquid, without crystallization, as crystals tend to change the natural color of honey, making it lighter [8].

2.8. Hydroxymethylfurfural (HMF)

Hydroxymethylfurfural (HMF) is an intermediate product of the Maillard reaction, and is formed by the direct dehydration of sugars under acidic conditions, mainly by the decomposition of fructose during heat treatment applied to food [4, 30]. It can be a toxic compound when found in high amounts. In honey, HMF is an indicator of quality which assists in the identification of freshness when in low concentrations. Higher than permitted concentrations may mean that the product has undergone adulteration through the addition of inverted sugar (syrup), has been stored under inappropriate conditions, undergone prolonged storage, been heated, or affected by acidity, water or minerals [12, 36].

Method: the quantitative method proposed by Association of Official Analytical Chemists (AOAC) [18]. Prepare the following solutions:

Preparation of Carrez solution I: weigh 15 g of potassium ferrocyanide $K_4Fe(CN)_6 \cdot 3H_2O$ in an analytical balance; dissolve in distilled water and make up the solution in a 100 mL volumetric flask.

Preparation of Carrez solution II: weigh 30 g of zinc acetate $Zn(CH_3COO)_2 \cdot 2H_2O$ in an analytical balance; dissolve in distilled water and make up the solution in a 100 mL volumetric flask.

Preparation of the sodium bisulfite solution $NaHSO_3 \cdot 0.2\%$ (m/v): weigh 0.2 g of sodium bisulfite $NaHSO_3$ in an analytical balance; dissolve in distilled water and make up the solution in a 100 mL volumetric flask. Use this solution only on the day of preparation.

Weigh 5 g of honey in an analytical balance using a properly labeled 50 mL beaker, dissolve the sample by adding 25 mL of distilled water and then transfer it to a 50 mL volumetric flask. Add 0.5 mL of Carrez solution I and 0.5 mL of Carrez solution II and fill the volumetric flask to the meniscus with distilled water.

Add two drops of ethanol to prevent foaming. Mix the solution and filter using filter paper; discarding the first 10 mL filtered.

Label two test tubes and pipette 5 mL of the filtrate over 5 mL of distilled water in the first (sample) and 5 mL of the filtrate added to 5 mL of 0.2% sodium bisulfite solution in the second tube (blank). Shake the tubes using a vortex mixer. Measure the absorbance in a UV-vis spectrophotometer at wavelengths of 284 and 336 nm using quartz cuvettes.

Before the readings, calibrate the spectrophotometer with a blank reference for each sample evaluated. If absorbance at 284 nm exceeds 0.6 the sample is diluted with water and the blank reference with sodium bisulfite 0.2%, in the same proportions, and the reading is repeated. The HMF content in honey is calculated with Eq. (9) .

$$HMF = \frac{(A_{284} - A_{336}) \times 149.7 \times 5}{\text{Sample weight (g)}}, \quad (9)$$

where A_{284} = absorbance at 284 nm, A_{336} = absorbance at 336 nm, 149.7 = factor, 5 = theoretical value of sample weight.

2.9. Protein

Despite little being known about the proteinaceous material present in honey, and its limited occurrence, such materials can be used to detect possible adulterations in commercial products, along with water content and concentration [37]. They are also used as identification parameters for the maturity of honey [38].

Honey protein can originate either from animals or plants. Animal protein comes from the bee itself, made up of secretions from the salivary glands, along with products collected during the collection of nectar or the maturation of the honey [11], while the plant origins are the nectar and pollen collected in the field [39].

Method: Determining the level of protein in honey is based on the modification of the nitrogen of the sample into ammonium sulfate through acid digestion, distillation and the subsequent release of ammonia, which is fixed in an acidic solution and titrated. Determining the nitrogen and the conversion factor provides the crude protein result, based on the Kjeldahl method and described by Silva and Queiroz [40].

Preparation of the catalytic mixture: weigh 10 g of sodium sulfate or anhydrous potassium and 1 g of copper sulfate pentahydrate. Grind in a mortar, mix thoroughly and store in a labeled flask.

Preparation of the sample: weigh 0.5 g of the sample on vellum. Then transfer the samples to the Kjeldahl tubes and add about 2.5 g of the catalyst mixture and 7 mL of p.a. sulfuric acid.

Digestion: place the labeled tubes in a block digester and gradually increase the temperature from 50 to 50°C to 400°C and maintain for 4–6 hours.

Distillation and neutralization: turn on the unit by checking the mains voltage and open the water tap to allow circulation in the condenser, observing the amount of water in the steam generation flask, which must be above the sensor. When necessary, complete using the water linking button. Turn the dial to 7/8 of the resistance to heat the steam generator and wait for the water to boil. Dissolve the sample in the digestion tube with 10 mL of distilled water; turn off the heat; take a 125 mL Erlenmeyer flask containing 15 mL of H₂BO₃ 5% and add 5 drops of the mixed indicator—methyl red (0.1% in alcohol) and bromocresol green (0.1% in alcohol)—which is red for acidic and green for basic. Connect the digestion tube add approximately 20 mL of NaOH 50% to the hopper located above the equipment (the tap must be closed), and open the tap slowly until the sample is neutralized (becoming dark blue or dark brown). Around 15 mL was used; after neutralization is determined, close the soda tap funnel and turn on the heat button.

®*Titration:* prepare a burette with 50 mL of standard hydrochloric acid 0.01 M; titrate directly in the Erlenmeyer flask in which the distillate is placed. The end point of the titration is indicated by the solution changing color to pink. Perform the calculation according to Eq. (10).

$$\% \text{ Protein} = \frac{(V \times M \times fc \times 0.014 \times 100 \times 6.25)}{m}, \quad (10)$$

where V = volume of HCl spent in titration, M = molarity of hydrochloric acid, fc = correction factor of hydrochloric acid, 6.25 = correction factor for protein, m = sample weight.

2.10. Reducing sugars, total reducing sugars and sucrose

Sugars constitute 95% of the dry matter of honey [15], and together with water make up its main components. The monosaccharides glucose and fructose represent around 85% of the carbohydrates present in honey produced by the *Apis* genus, and are known as reducing sugars, which have the ability to reduce copper ions in an alkaline solution. Fructose has a high hygroscopicity and adds to the sweetness of honey, while glucose, due to its poor solubility, tends to influence crystallization [12]. Normally fructose is predominant as honey with high fructose rates can remain liquid for a long time, or never crystallize [2]. The disaccharides sucrose and maltose represent 10% of the sugars present in honey [41]. Sucrose represents on average 2–3% of the carbohydrates of honey from the *Apis* genus. When it exceeds this value, it indicates adulterated honey or early harvested honey, with humidity above 20% [19].

Method: This method is based on the ability of the reducing sugars glucose and fructose to reduce the copper present in a cupro-alkaline solution (Fehling's solution), characterized by the reduction of cupric ions to cuprous ions, and the oxidization of sugars into organic acids [8, 15].

Preparation of reagents: Fehling A: dissolve 34.65 g of p.a. copper sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) in distilled water; transfer it to a 1000 mL volumetric flask and complete the volume. Fehling B: dissolve 125 g of p.a. sodium hydroxide (NaOH) in 300 mL of distilled water; in the same solution dissolve 173 g of p.a. tartrate of potassium and sodium ($\text{C}_4\text{H}_4\text{KNaO}_6 \cdot 4\text{H}_2\text{O}$); complete the volume to 1000 mL and allow it to stand for 24 hours.

Standardization of Fehling's solution: weigh 0.5 g of p.a. glucose ($\text{C}_6\text{H}_{12}\text{O}_6$) pre-dried in an oven at about 70°C for 1 hour; transfer to a 100 mL volumetric flask using water. Dissolve well and adjust the volume. The standard glucose solution for the titration of the Fehling's solution should be prepared on the day of standardization. Place the standard glucose solution in the burette. Transfer 10 mL each of the Fehling Solutions A and B to a 250 mL Erlenmeyer flask using a volumetric pipette. Add 40 mL of water and heat to boiling. Trickle the standard solution without stirring until almost the end of the titration, maintaining the temperature at boiling point. Add one drop of methylene blue solution 1% and complete titration until the indicator is bleached. The time of titration should not exceed 3 min. The final titration product is around 10 mL of standard glucose solution. The result of the Fehling's solution is obtained by Eq. (11).

$$T = \frac{V \times m}{100}, \quad (11)$$

where V = volume of glucose spent in titration (mL), m = glucose mass (g).

1. *Preparation of the main sugar solution:* weigh 2 g of honey with an analytical balance in a 100 mL beaker and transfer to a 200 mL volumetric flask using distilled water.
2. *Sample preparation for titration of reducing sugars:* from the main solution (1), transfer 50 mL (mass = 0.5 g) to the 100 mL volumetric flask and complete the volume with distilled water.
3. *Sample preparation for titration of total sugars (total reducing sugars):* from the main solution (1), transfer 50 mL (mass = 0.5 g) to the 100 mL volumetric flask and add 25 mL of distilled water. Heat the bath solution at 64°C; add 10 mL of a distilled water solution plus p.a. HCl (8 mL of distilled water plus 2 mL of p.a. HCl), and leave in bath for 15 min. Allow the solution to cool until it reaches room temperature, and then add 2 drops of phenol-

phthalein indicator 1% with NaOH 5 M/L solution. At this stage, a color change from light beige to pink can be seen. The volume flask is completed to 100 mL.

Titration of reducing sugars: fill the 25 mL burette with the reducing sugar solution (2) and pipette 5 mL of Fehling A and 5 mL of Fehling B into a 250 mL Erlenmeyer flask; add 40 mL of distilled water, plus five glass beads; warm until the solution boils; titrate with approximately 14 mL of the solution in the burette; wait for the solution to return to simmering temperature for 2 min; at this stage the blue staining solution contained in the Erlenmeyer flask starts to change to a purple shade; add 5 drops of methylene blue 0.2% (bluish or purple color); heat for 2 min and begin titration by adding, drop by drop, the diluted solution of honey contained in the burette until the turning point of indicator discoloration (a blue and purple color turns into a red earth color). The amount spent in titration should be noted for further calculations.

Obs.: Total titration time should not exceed 3 min.

Titration of total reducing sugars: for this titration process use the same process as above, using a solution of total reducing sugars (3). Note the volumes spent on the three replications and calculate according to Eq. (12). For calculation of sucrose, follow Eq. (13).

$$(\%) = \frac{(100 \times 100 \times 0.05)}{0.5 \times V}, \quad (12)$$

where V = volume spent in titration, 0.05 = correction factor for Fehling's solution A and B.

$$\text{Saccharose } (\%) = (\text{RS} - \text{TRS}) \times 0.95, \quad (13)$$

where RS = reducing sugars, TRS = total reducing sugars, 0.95 = reducing factor from total reducing sugars.

2.11. Viscosity

Viscosity and the other physicochemical properties of honey depend on many factors, including composition and temperature. One of the most important factors for viscosity is water content, as viscosity generally decreases while water content increases [42]. Studies of this trait are of great importance, as the rheological models obtained are useful for identifying the rheological properties of a fluid with practical quantities such as concentration, temperature, pH and maturation index, among others. This knowledge is essential for quality control in the intermediate control in production lines and for the design of equipment and processes [43].

Method: the principle for the determination of viscosity is the torque measuring technique, based on the resistance that the fluid exerts during rotational motion[8]. Viscosity is determined by a rotary microprocessor digital bench viscometer with thermostatic bath aid.

Turn on and reset the equipment, select the specific rotor (rotor 1 or rotor 2 spindles); turn on the water bath at 25°C; place a sufficient volume of the sample in a 250 mL beaker to cover the rotor; wait for the sample to reach the set temperature. Connect the viscometer and take the reading. The standard time to perform the reading is 1 min; the percentage of the viscometer range and the rotation per minute from the equipment vary according to each sample evaluated. After 1 min of rotation, the viscosity of each sample is read directly from the viscometer timer.

2.12. Diastase activity

As honey contains enzymes in very low quantities, this activity is the result of the joint action of diastase (α - and β -amylase), alpha-glycosidase, peroxidase, lipase, invertase, glucose oxidase, catalase and acid phosphatase. These enzymes are formed from the hypopharyngeal glands of bees and nectar sources, and are also found in low proportion in pollen grains [44]. Diastase is one of the most important enzymes, and its level in honey depends on the geographical origin and botanical source. It is an indicator of product quality [45] and its function is to hydrolyze the starch molecule. It is possibly involved in pollen digestion.

Diastase activity is closely related to the structure of the honey and can be modified by denaturing performed by overheating the honey, which seriously compromises its quality [25, 46]. In addition to shelf life and heating the product, another indicator of reduced enzyme levels are honey samples from fast nectar flows, due to the accumulation of the material processed inside the hive.

Method: the principle of the method used to evaluate the diastatic index is proposed by the AOAC [18]. This technique measures the activity of alpha-amylase in honey in the presence of starch and indirectly provides information about the quality of the honey according to the degree of digestion experienced by the starch molecule over time. To carry out this analysis, some solutions should be prepared.

Preparation of iodine stock solution: weigh 22 g of p.a. potassium iodide with an analytical balance in a 250 mL beaker and add 100 mL of distilled water for the homogenization thereof. Weigh 8.8 g of p.a. iodine in an analytical balance and add the previous solution until complete homogenization. The solution is diluted and transferred to a 1 L volumetric flask and the volume completed with distilled water.

Preparation of iodine solution 0.0007 N: weigh 4 g of p.a. potassium iodide in a 100 mL beaker using an analytical balance, dissolve the solution with 30 mL of distilled water and transfer to a 100 mL volumetric flask. Add 1 mL of stock iodine solution and fill flask with distilled water.

Preparation of starch solution: weigh 2 g of anhydrous soluble starch in a 250 mL Erlenmeyer flask using an analytical balance and dilute by adding 90 mL of distilled water. Heat the solution in a heater plate and boil gently for 3 min. Keep the solution at room temperature until it cools. Transfer the flask solution to a 100 mL volumetric flask and complete the volume with distilled water (main solution).

Standardization of the starch solution: to use the starch solution in further analysis the required volume of distilled water to be added to the solution should first be determined. This allows the standard dilution of the starch solution to be set in order to obtain an absorbance reading in the spectrophotometer range from 0.760 to 660 nm.

Label two 50 mL beakers; pipette 5 mL of solution and 10 mL of distilled water into beaker 1, and 20 mL of distilled water into beaker 2. Remove 1 mL aliquots of the solution in each beaker and transfer to another labeled beaker; add 10 mL of the iodine solution 0.0007 N. Prepare five different concentrations so that the correct volume is found. Perform a reading in a spectrophotometer set to the amount of distilled water to be added to the sample, in order to make the reading in the selected absorbance range.

Starch solution used in the analysis: label a 100 mL beaker; pipette 5 mL of main solution into the beaker, add the amount of water defined in the previous step; withdraw an aliquot of 1 mL of solution from the beaker and transfer it to another labeled beaker; add 10 mL of the standard iodine solution 0.0007 N to this beaker, and perform an absorbance reading in a spectrophotometer in the 0.760 nm range. Standardize the starch solution for every new preparation.

Weigh 10 g of honey in a 250 mL beaker using an analytical balance; add 5 mL of buffer and 20 mL of distilled water, homogenize and dissolve; transfer the sample to a 50 mL volumetric flask; add 3 mL of sodium chloride solution 0.5 M; complete the volume with distilled water; pipette 10 mL of this solution into a 250 mL beaker and place it in a water bath at 40°C, wait for 15 min; pipette 5 mL of the starch solution heated to 40°C into the honey solution; mix it and remove 1 mL aliquots to an identified beaker at intervals of 5 min, then quickly add 10 mL of the iodine solution 0.0007 N and complete the volume with distilled water.

Determine the absorbance at 660 nm in a visible spectrophotometer and record the time elapsed between the mixing of the starch solution and the addition of the honey to the iodine. Take aliquots of 1 mL every 5 min to lower the absorbance value to 0.235. To determine the time the absorbance took to reach this value, plot an absorbance versus time graph. The results are expressed in the Goethe scale. The diastatic index (DI) is determined according to Eq. (14):

$$DI = \frac{300}{t}, \quad (14)$$

where t = time.

2.13. Water activity (wa)

The concept of water activity has been used to evaluate the interaction of water with other food components, as water is characterized as a major component of many foods [47]. Honey has a low water activity, a parameter which determines the available water in the food and its availability for microbial metabolism, which interferes with the microbial activity in honey. This feature gives the product microbiota stability [48], resulting in quality, preservation and longer shelf life. When there is no water available in food, the water activity measurement is equal to 0.0; however, when the sample consists entirely of pure water, then water activity is equal to 1.0 [49].

Method: the AOAC [18] method is based on the measurement of the sample dew point with internal control of the sample temperature. An infrared beam focused on a small mirror determines the precise dew point of the sample. The dew point temperature is then translated into water activity. Add 7.5 mL of honey sample to a sample capsule; close the cover on the sample chamber and wait for the vapor balance; take the reading from the display.

2.14. Total phenolics

The Folin-Ciocalteu assay was designed and standardized for the quantification of total phenols by Singleton et al. [50] and adapted by Daves [51]. The system is characterized by a mixture of sodium tungstate and sodium molybdate salts in an acid medium (hydrochloric acid and phosphoric acid), which has a yellowish color. In the presence of phenolic compounds

these salts are reduced, forming complexes (molybdenum-tungsten) and producing a bluish color. The intensity of the blue tone is proportional to the number of hydroxyl or oxidizable groups of phenolic compounds. Absorption occurs at 725 nm. Phenolics determined by Folin-Ciocalteu are often expressed as Gallic acid equivalent (GAE).

Method: total phenol concentration is determined by interpolating the absorbance of the sample based on a calibration curve constructed with standard Gallic acid, with a purity of 98%.

Preparation of Gallic acid curve: dilute 0.1531 g of Gallic acid in methanol to prepare 100 mL of an initial main solution with 1500 mgGAE/L. From this concentration obtain 10 mL of diluted solution with 0.30; 180; 330; 600; 900; 1200 and 1500 mg GAE/L. Calculate concentrations of Gallic acid equivalents (mg/L) in 10 mL of solutions prepared using Eq. (15):

$$\text{GAE(mg/L)} = (\text{mg GA/mL from main solution} \times \text{pipetted volume (mL)}) \times 100, \quad (15)$$

where GAE (mg) from the main solution (mg/mL) = 1.5 mg GAE/mL.

Pipetted volume from the main solution (mL) = 0.0, 0.1, 0.2, 1.2, 2.2, 4.0, 6.0, 8.0 and 10 mL.

Adjust the volume of solutions to 10 mL using water as solvent.

Transfer 30 μL of the diluted solutions; 2.370 μL of distilled water and 150 μL of Folin-Ciocalteu reagent to test tubes protected with aluminum foil (put distilled water in the blank sample). After 2 min, add 450 μL of sodium carbonate 15%. Close the tubes and place them in a water bath with stirring in the dark at a temperature of 37°C for 30 min. Measure the absorbance in quartz cuvettes in a spectrophotometer at a wavelength of 725 nm. Plot the Gallic acid concentration (mg/L) on the abscissa (x -axis) and the absorbance values on the ordinate (y -axis). Find the coefficient of the determined R^2 value and the corresponding linear equation, as shown in **Figure 1**. Express the results in mg GAE/L.

Preparation of initial honey solution: weigh 4 g of honey and transfer it to a 10 mL volumetric flask using distilled water as the solvent, to a honey solution concentration of 0.4 g/mL. From this honey solution, transfer 30 μL to amber test tubes or tubes protected with aluminum foil (put methanol in the blank sample); add 2.370 μL of distilled water and 150 μL of Folin-Ciocalteu reagent to test tubes protected with aluminum foil (put distilled water in the white). After 2 min, add 450 μL of sodium carbonate 15%. Close the tubes and place them in a water bath with stirring in the dark at a temperature of 37°C for 30 min. Measure the absorbance in quartz cuvettes in a spectrophotometer at a wavelength of 725 nm.

Calculation of phenolic compounds: using absorbance values (y) and the linear equation, find the x value corresponding to the total phenol content in GAE/L (1000 mL); using the total phenol values in GAE/1000 mL of the main Gallic acid solution, calculate the corresponding values in 10 mL of the honey solution used (containing 0.4 g of honey/mL). From these results, calculate the concentration of total phenols in GAE/100 g of honey. Calculate the mean and standard deviation and express the results in GAE/100 g of honey \pm deviation found.

2.15. Total flavonoids

Among the active principles present in nature, flavonoids are found in fruits, vegetables, seeds, flowers and bark, wine, cereals and food dyes. The aluminum chloride (AlCl_3) colo-

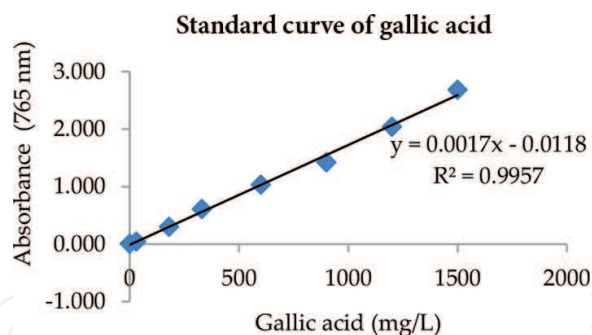


Figure 1. Standard Gallic acid curve (Gallic acid concentration \times absorbance).

rimetric method is used to obtain the limits of the flavonoid spectra. Interference from other phenolic compounds is frequently present, as the Al^{3+} cations form stable complexes with free hydroxyl groups of flavonoids. This causes the extension of the conjugated system and consequently a bath chromic shift, or in other words, a shift of the absorption maxima to a longer wavelength region, allowing quantification in a spectrophotometer at 425 nm [52].

Method: total flavonoid concentration is determined by the method of Alothman et al. [53] involving the interpolation of sample absorbance based on a calibration curve constructed with standard quercetin Sigma-Aldrich™, 95% purity.

Preparation of quercetin curve: dilute 0.5263 g of quercetin in 100 mL of methanol p.a. to prepare an initial main solution of 500 mg quercetin/L. From this concentration, obtain 10 mL of diluted solutions with 2.5, 5.0, 12.5, 25.0, 37.5, 50.0, 100.0 and 150.0 mg quercetin/L. Calculate concentrations of quercetin per liter (mg/L) of diluted solutions using Eq. (16):

$$\text{Quercetin (mg/L)} = (\text{mg quercetin/mL main solution} \times \text{pipetted volume (mL)}) \times 100, \quad (16)$$

where quercetin in the main solution (mg/mL) = 5.0 mg/mL, volume of the pipetted main solution (mL) = 0.005, 0.010, 0.025, 0.050, 0.075, 0.100, 0.200 and 0.300.

Adjust the volume of solution to 10 mL using methanol as solvent.

To obtain the curve, transfer to amber color test tubes or tubes protected with aluminum foil, 250 μ L of sample (put methanol in the blank sample); 1000 μ L of distilled water; 75 μ L $NaNO_2$ 5% in water; 600 μ L of distilled water. Shake vigorously by vortexing and measure the absorbance in quartz cuvettes at 425 nm in a spectrophotometer. Plot the quercetin concentration (mg/L) on the abscissa (x -axis) and the absorbance values on the ordinate (y -axis). Find the coefficient of the determination value R^2 and the corresponding line equation (use **Figure 2** as an example). Express the results as mg quercetin equivalent/L.

Preparation of initial honey solution: weigh 4 g of honey and transfer it to a 10 mL volumetric flask using methanol as solvent for a solution with a honey concentration of 0.4 g/mL. From this solution, transfer 250 μ L to amber test tubes or tubes protected with aluminum foil (put methanol in the white); 1000 μ L of distilled water; 75 μ L $NaNO_2$ 5% in water. After 5 min add 75 μ L $AlCl_3$ 10% in water. After 6 min, add 500 μ L $NaOH$ 1 M; 600 μ L of distilled water. Shake

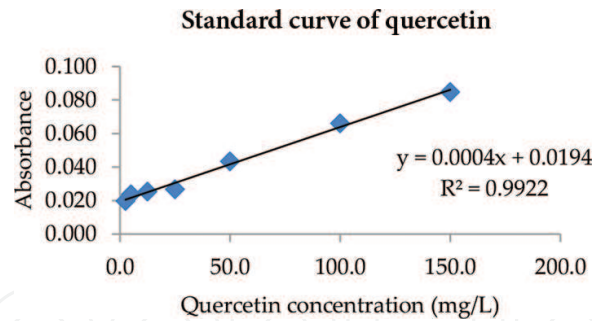


Figure 2. Standard quercetin curve (quercetin concentration \times absorbance).

vigorously by vortexing and perform an absorbance reading at 425 nm.

Calculation of total flavonoid: using absorbance values (y) and the linear equation find the x value corresponding to the total flavonoid in quercetin equivalent/L. Then, multiply the values by their respective dilutions and obtain the final QE values in mg/L. Using the total flavonoid quercetin equivalent/1000 mL of the main quercetin solution, calculate the corresponding values in 10 mL of the honey solution (containing 0.4 g of honey/mL). From these results, calculate the total flavonoid concentration in quercetin equivalent/100 g of honey. Calculate the mean and standard deviation and express the results as quercetin equivalent/100 g of honey \pm deviation found.

2.16. Ability to kidnap stable free radical 2,2-diphenyl-1-picrylhydrazyl – DPPH

Antioxidant activity is determined by the scavenging capacity of the free radical DPPH (2,2-diphenyl-1-picrylhydrazyl). The method involves reducing an alcoholic solution of purple DPPH radicals, which, upon receiving an electron or hydrogen radical, changes color from violet to yellow (diphenyl-picryl hydrazine), accompanied by a decrease in absorbance at the wavelength observed [54]. The greater or lesser capacity of the sample to reduce DPPH, or in other words to prevent oxidation, is evidenced by the percentage of DPPH remaining in the system [55]. This free radical, stable at room temperature, is reduced in the presence of an antioxidant molecule, yielding a yellow solution.

Preparation of the DPPH solution 0.06 mM: weigh 0.0023 g of DPPH (molecular weight = 4.32 g/L) and transfer to a 100 mL volumetric flask using methanol as solvent.

Preparation of the initial honey solution: weigh 8 g of honey and transfer to a 10 mL volumetric flask using methanol as solvent, to obtain a solution with a honey concentration equal to 800 mg/mL of the main solution. From this concentration, obtain 1.0 mL of the diluted solutions with 80.0, 120.0, 200.0, 400.0, 600.0 and 800.0 mg of honey/mL. Calculate honey concentrations per mL (mg/mL) of diluted solutions applying Eq. (17):

$$\text{Honey (mg/mL)} = (\text{mg honey/mL main solution} \times \text{pipetted volume (mL)}) \times 100 \quad (17)$$

where honey in the main solution (mg/mL) = 80 mg/mL.

Volume of the main solution pipetted (mL) = 0.10, 0.15, 0.25, 0.50, 0.75 and 1.00 mL.

Adjust the volume of solution to 1.0 mL using methanol as solvent.

Preparation of samples: transfer 0.2 mL of samples from each dilution to amber test tubes or tubes protected with aluminum foil, and then add 3.8 mL of DPPH 0.06 mM solution. The blank 0.2 mL of the sample is mixed with 3.8 mL of methanol so that the blank of each sample is used in the final equation. The negative control is prepared by mixing 3.8 mL of the DPPH solution 0.06 mM and 0.2 mL of methanol (neat standard).

After the preparation, the mix is shaken using a vortex mixer for 15 s and allowed to stand at room temperature in the absence of light for 30 min. Sample absorbance is measured in quartz cuvettes at 515 nm in a spectrophotometer. Results are expressed as a percentage of antioxidant activity (% AA) using Eq. (18):

$$AA (\%) = 100 - \left[\frac{(\text{Abs sample} - \text{Abs blank})}{\text{Abs control}} \right] \times 100 \quad (18)$$

where Abs_{sample} is sample absorbance; Abs_{blank} is the absorbance of the blank control and Abs_{control} is the absorbance of the negative control.

Calculation of EC₅₀: Plot the graph using the abscissa (*x*-axis) for concentrations of the tested honey (80.0, 120.0, 200.0, 400.0, 600.0 and 800.0) and the ordinate (*y*-axis) for the antioxidant activity values calculated separately for each repetition [56]. Using linear equations, compute the *x* values corresponding to the EC₅₀ value with the *y* values equal to 50, which represents the minimum concentration required to reduce the antioxidant initial concentration of DPPH by 50%, represented by the curve, as the dose-response gradient is the concentration of the compound at which 50% of the effect is observed.

Calculate the mean EC₅₀ value and standard error. The smaller the value, the higher is the antioxidant activity of the compounds present in the samples analyzed.

The completion of the analyses required under national and international law and those proposed in this chapter are required to determine the quality of honey for marketing, direct human consumption or use as a raw material for the food, cosmetics and pharmaceutical industries.

The results of sensory, physicochemical and functional properties analysis allows us to evaluate if the product meets established standards and demonstrates the features expected from good quality honey.

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