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Application of conservation and stabilization methods for shelf-life extension of *Melipona subnitida* honey

Aplicação de métodos de conservação e estabilização para prolongamento da vida útil do mel de Melipona subnitida

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ABSTRACT

Honey produced by *Melipona subnitida* has a high moisture content and is susceptible to deterioration process, requiring greater care in its conservation or aplication or conservation methods. Thus, the objective of this work was to evaluate the effect of different conservation and stabilization methods on the quality characteristics of from *Melipona subnitida*, Duke bee honey. In the end, we concluded that only treatments with heating were effective in reducing microorganisms numbers but cooling and dehumidification treatments are viable alternatives for conservation of honey in good microbiological condition.

Keywords: Cooling, Dehumidification, Pasteurization, Maturation.

RESUMO

O mel produzido por *Melipona subnitida* possui alto teor de humidade e é suscetível a processos de deterioração, necessitando de maiores cuidados na sua conservação ou aplicação ou métodos de conservação. Assim, o objetivo deste trabalho foi avaliar o efeito de diferentes métodos de conservação e estabilização nas características de qualidade do mel de *Melipona subnitida*, abelha Duke. Ao final, concluímos que apenas os tratamentos com aquecimento foram eficazes na redução

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do número de microrganismos, mas os tratamentos de resfriamento e desumidificação são alternativas viáveis para a conservação do mel em boas condições microbiológicas.

Palavras-chave: Resfriamento, Desumidificação, Pasteurização, Maturação.

Introduction

Stingless bee honeys are food products that have increasing demand in market, presenting higher prices than honey produced by honey bee (Apis genus) (Lira et al. 2014). Their antioxidant and medicinal properties contribute to the high appreciation of these honeys by consumers (Da Silva et al. 2014). Among these properties, we can cite the decrease of total cholesterol levels in blood, the increased excretion of lipids in feces and the liver and colon protection from damage (Bezerra et al. 2018).

The honey produced by *Melipona subnitida*, Duke bees (Jandaíra) has distinct characteristics from *Apis mellifera* bee honey, such as higher moisture content (Silva et al. 2018), greater acidity and lower density and sweetness (Lira et al. 2014). In Brazil there is not a legislation that regulates the physicochemical characteristics of this honey and that allows its commercialization, although there is already a legislation that recognizes it like "honey" (Brasil 2017). However, according to the international standards this product is not recognized as honey (Codex Alimentarius 2001) and is not controlled by food control authorities. The low knowledge about this product is due to the small number of researches related to it (Cardona et al. 2019).

Because of its high moisture content (23-25 g/100g), *M. subnitida* honey is susceptible to fermentation and deterioration process, requiring greater care in its conservation (Sodré et al. 2008). Post-harvest ripening is a preservation method in which the active microbiota naturally present in honey ferments the sugars, especially osmophilic yeasts of the genus *Zygosaccharomyces* (Silva et al. 2023). Dehumidification, post-harvest maturation, pasteurization and cooling are the most studied methods to maintain the physical, chemical and sensory characteristics of food for as long as possible. However, these methods can change original characteristics of honey by increasing density, non-enzymatic browning, loss of volatile compounds and crystallization, which may cause consumer's rejection, as the specific characteristics of stingless bee honey are the greatest commercial appeal of the product.

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In this way, the objective of this work was to evaluate the effect of dehumidification, post-harvest maturation, pasteurization and cooling methods on the quality characteristics of *Melipona subnitida*, Duke bee honey.

Material e methods

Sampling

A total of 1.5 L Stingless Bee (SB) honey was collected from 10 beehives of *Melipona subnitida* Duke in May 2019, and for that, a suction bomb coupled to a glass bottle previously sanitized was used. The beehives are located in Tabuleiro do Norte, Ceará, Brazil (latitude -5 ° -16 '-8 ” and longitude -38 ° -4' -25” at 24 m altitude).

In the Microbiology Laboratory of the "Instituto Federal do Ceará - Campus Limoeiro do Norte" the honey was divided into three 500-mL flasks, thus totaling three samples for study. Each sample was distributed for the application of the treatments, being three repetitions per treatment.

Two control samples, without treatment, were evaluated: Control 1 - analyzed before the methods without heating, and Control 2 - analyzed before the methods with heating. This was necessary because there was a time gap between treatments without and with heating, which changed the initial characteristics of SB honey.

The methodologies applied were based on experience and suggestion of SB honey producers.

Treatment 1: Dehumidification

The dehumidification method was carried out in domestic refrigerator, with a relative humidity of 48,77 ± 15,13 % at 2,26 ± 2,38 ºC. We distributed 80 mL of the SB honey in Petri dishes, kept them open, and monitored the total soluble solids content (ºBrix) in samples. The method finished when moisture content reached values similar to *Apis mellifera* bee honey, which is 20% (m/v) (MAPA 2000). Dehumidification treatment lasted 28 days to reach 20% moisture.

Treatment 2: Post-harvest maturation in bacteriological incubator

For post-harvest maturation, 80 mL of the SB honey were placed in Erlenmeyers closed with PVC plastic film and kept in bacteriological incubator at 35 ºC for a time necessary to finish the formation of gases, considered when the film

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remained stable, without bubbles on the plastic surface. This treatment is applied because microorganisms start to decline after stationary phase of microbial growth (Jay 2005).

Treatment 3: Cooling

The cooling method is applied because microbial growth reduces at low temperatures (Jay 2005). For this purpose, we placed 80 mL of the SB honey in Erlenmeyer flasks sealed with PVC plastic film, and kept it in domestic refrigerator for 28 days, which was the time that coincided with the end of the post-harvest maturation. The refrigerator had relative humidity of 48,77 ± 15,13 % and a temperature of 2,26 ± 2,38 ºC.

Treatment 4: Pasteurization at different temperatures

Pasteurization was carried out using 25 mL of the SB honey in test tubes, which was heated in a water bath, remaining there for 15 seconds after reaching the test temperature (60, 75 and 90 ºC), that was controlled with a digital thermometer. After pasteurization time, samples were cooled in an ice bath and analyzed.

Physicochemical evaluation

Initially, we characterized the samples through physicochemical analyzes required by the Brazilian legislation for *Apis Mellifera* bee honey (Brasil 2018), which are: reducing sugars (g /100g), non-reducing sugars (g sucrose/100 g), titratable acidity (mEq / kg), Hydroxymethylfurfural (HMF) content (mg/kg), moisture (g/100g) and microscopy (presence of pollen). The samples were also analyzed in terms of total soluble solids (ºBrix), pH and color. After each treatment, all analyzes were repeated, except for microscopic substances (pollen presence), which does not change after the treatments applied.

The total soluble solids were determined in an Abbe digital refractometer (Refractometer Optronics®) previously calibrated with distilled water, according to the AOAC methodology (AOAC 2000). The results were expressed in ºBrix.

The pH was determined in 10 g of sample diluted in 75 ml of distilled water. We used a digital pH meter (KASVI® ATC K39-0014PA) calibrated at pH 4.0, 7.0 and 10.0 using commercial buffer solutions.

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The color was measured directly in the SB honey with a spectrometer (Biospectro SP-220) at 560 nm according to the AOAC methodology (2000). Values obtained were evaluated using the Pfund scale for color classification.

The microscopic analysis, performed in triplicate, was carried out according to methodology number 7.17 of the Association of Official Analytical Chemists (AOAC 2011). The method is based on the identification and counting of pollen grains by microscopic evaluation.

Results and Discussion

Many studies about the influence of pollen and honey microbiota on stingless bees' nutrition are being developed. Bacteria of the genus *Bacillus*, yeasts of the genera *Stamerella* and *Candida* have been associated with stingless bees (Gilliam et al. 1989; Teixeira 2003; Silva et al. 2019). There is little information about affinity of fungi for stingless bees, however it is known that these microorganisms remain active in stingless bees' honey until honey's maturation, which is the time when bees close their cerumen pots, where honey is stored. However, honey harvest can take place before the end of this maturation process. The technique is already used by Brazilian meliponiculturists, especially at Northeast region of Brazil (Silva et al. 2023). For this reason, post-harvest maturation is one of conservation treatment options suggested in this study.

After characterization of SB honey, it was observed that standars applied for *Apis mellifera* bee honey cannot always be used as a comprison, mainly for the parameters reducing sugars and moisture contetnt (Table 1).

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Table 1 - Comparison of *Melipona subnitida* bee honey (SB honey) parameters with others references of the same type of honey and with *Apis Mellifera* bee honey.

References of *Melipona subnitida*					
Parameters	Values obtained	(Silva et al. 2013)	(de Almeida - Muradian et al. 2014)	(Sousa et al. 2013)	*Apis mellifera* honey legislation
Reducing sugars (g glucose/100g)	**55.08 ± 0.01**	**57.6 ± 7.62**	**54.37 ± 1.59**	**52.6 ± 2.3**	**Mín 60**
Sucrose (g/100g)	**1.17 ± 1.18**	**-**	**-**	**3.7 ± 0.5**	**Máx 5**
Titratable acidity (mEq/kg)	**31.79 ± 0.62**	**47.57 ± 12.74**	**24.87 ± 2.10**	**38.1 ± 0.4**	**Máx 50**
HMF (mg/kg)	**18.08 ± 2.41**	**13.66 ± 1.69**	**8.60 ± 0.98**	**-**	**Max 40**
Moisture	**27.00 ± 0.13**	**23.16 ± 0.65**	**24.93 ± 0.95**	**31.1 ± 1.9**	**Máx 20**
Total soluble solids (°Brix)	**73.00 ± 0.13**	**-**	**-**	**-**	**-**
pH	**2.83 ± 0.06**	**3.3 ± 0.28**	**3.90 ± 0.19**	**4.4 ± 0.3**	**-**
Color	**Light amber**	**-**	**-**	**Light amber**	**-**

Source: Codex Alimentarius (2001).

The data of SB honeys were similar to those found by Silva et al. (2013), who studied 9 samples of the same type of honey from different regions of Paraíba – Brazil. It is also similar to those found by De Almeida-Muradian, Stramm and Estevinho (2014), that studied 18 samples of Jandaíra honey from an apiary in São Paulo - Brazil, and by Sousa et al. (2013) that evaluated Jandaíra honey in Rio Grande do Norte - Brazil (Fernandes et al. 2018). In this way, we concluded that, in this case, the parameters of the honey were not influenced by the place where it was produced.

Regarding purity parameters, pollen grains were found in all evaluated plots during microscopy analyzes, in accordance with the recommendations of the Brazilian legislation, which determines that honey must contain pollen grains (MAPA 2000).

Treatments without heating

The treatments without heating were dehumidification and post-harvest maturation in a bacteriological incubator. Table 2 shows the results after the statistical analysis.

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Table 2 - Physicochemical and microbiological quality of *Melipona subnitida* bee honeys (SB honey), after conservation treatments without heating.

	Treatments			
Parameters	*Control 1	Dehumidification	Maturation	Cooling
Reducing sugars (g glucose/100g)	**55.08 c ± 0.01**	**71.08 a ± 1.87**	**62.82 b ± 1.45**	**64.71 b ± 3.95**
Sucrose (g/100g)	**1.17 a ±1.18**	**0.00 a ± 0.00**	**0.00 a ± 0.00**	**0.00 a ± 0.00**
Titratable acidity (mEq/kg)	**31.79 c ± 0.62**	**60.37 ab ± 4.46**	**67.51 a ±0.00**	**59.29 b ±4.33**
HMF (mg/kg)	**18.08 a ±2.41**	**6.18 b ±0.57**	**2.71 b ± 2.31**	**5.51 b ± 2.31**
Moisture	**27.00 a ±0.13**	**18.66 b ±0.65**	**26.63 a ±0.28**	**26.96 a ±1.3**
Total soluble solids (°Brix)	**73.00 b ± 0.13**	**81.34 a ± 0.41**	**73.37 b ± 0.28**	**73.00 b ± 0.13**
pH	**2.83 b ± 0.06**	**3.51 a ± 0.04**	**3.51 a ± 0.04**	**3.54 a ± 0.308**
Color	**0.297 a ± 0.00**	**0.460 a ± 0.16**	**0.318 a ± 0.02**	**0.232 a ± 0.00**
Molds and yeasts (log CFU/mL)	**2.53 b ± 0.22**	**3.28 b ± 0.79**	**6.18 b ± 0.07**	**2.77 b ± 0.34**
Mesophilic aerobic bacteria (log CFU/mL).	**4.01 a ± 0.73**	**3.79 a ± 0.98**	**2.82 a ± 0.28**	**2.72 a ± 0.42**

Source: Prepared by the author.
*Sample characteristics before applying treatments.

Means with same letters on the same line are not significantly different ($p \geq 0.05$) by the Tukey test.

Making a comparison with the standards established by Codex Alimentarius (2001) for *Apis mellifera* bee honey (Table 1), we can be see that the applied treatments kept SB honeys in accordance with the legal values for sucrose (max 5 g/100 g), HMF (max 40 mg/Kg) and reducing sugars (min 60 g/100g). Regarding moisture content, only the SB honey that underwent the dehumidification treatment achieved the legal standard (max 20 g/100 g), while acidity increased after all treatments, exceeding the legal requirement (max 50 mEq/kg). However Villas-Bôas and Malaspina (2005) suggest 50 g/100g as the minimum content of reducing sugars for stingless bees' honey, so, in this case all the samples analyzed would be in conformity (Villas-Boas and Malaspina 2005). Thus, Codex Alimentarius does not classify the SB honey as a quality honey, even after treatments, and therefore SB honey should be evaluated like a new honey, with its own legislation.

The dehumidified honey had the highest levels of reducing sugars, differing statistically from the others. The chilled honey and the matured one showed no statistical difference between them, whereas the control presented the lowest value

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and differed from the others. Similar behavior was observed in the values of total soluble solids, which were higher (81.34 ° Brix) in dehumidified SB honey. It can be said then that the dehumidification process increases the levels of soluble compounds in honey due to the reduction of the water content (Table 2).

When observing the titratable acidity, Control 1 presented the lowest values. The increase in acidity is justified by the development of fermenting microorganisms that guarantee the stability of honey in natural environment. These microorganisms evolve in fermentation process during storage, but in cooling treatment the microbial growth was stabilized because of the low temperature (Jay 2005; Cavia et al. 2007). The free acidity is due to the presence of organic acids, particularly gluconic acid, which are in balance with the corresponding lactones and some inorganic ions, such as phosphate or sulfate (Gomes et al. 2010). Similar results can be observed for pH, in which Control 1 had the lowest value, differing significantly ($p < 0.05$) from the treatments, and the treatments showed no difference among them regarding this parameter.

Color parameters meet the requirements of the current standard, which accepts a variation from "whitewater" to "dark amber" color (MAPA 2000), as all samples of the treatments without heating have a light amber color, with absorbance ranging from 0,232 to 0,460. The color of honeys is related to the presence of pollen, the mineral and phenolic compounds contents and varies according to the geographical origin of the beehives and the botanical varieties visited by the bees (Ramalhosa et al. 2011). Results are similar to those of Alves et (2011), that evaluating the quality of stingless bee (Jandaíra) honeys, also found the color “amber” in samples from the Brazilian states of Ceará, Piauí and Rio Grande do Norte (Alves et al. 2011).

The treatment of post-harvest maturation induces the growth of microorganisms until death (Table 2) leading to the increase acid production, which is a product of the fermentation process, and an increase in reducing sugars that are produced by the conversion of sucrose during the metabolism of microorganisms (Madigan et al. 2010).

Finally, the cooling process reduces the development of microorganisms, but do not eliminate them. In this way, there is also an increase in acidity, but less than in post-harvest maturation, and an increase in reducing sugars as a result of sucrose

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hydrolysis. De Almeida-Muradian; Stramm and Estevinho (2014), evaluating the effects of different storage conditions (room temperature, refrigeration and freezing) on the *Melipona subnitida* bee honey quality parameters, report that storage under refrigeration and freezing reduced moisture content, but maintained the acidity increase process. Similar behavior was observed in the honey stored at room temperature. These results are similar to the data found in the present study for conservation in refrigeration.

Similar results were related for Schvezov et al. (2020) in honey the yateí (*Tetragonisca fiebrigi*) after dehumidification reaching moisture 19.5 ± 0.3 g/100 g.

There are still few studies on the microbiological stabilization and dehumidification methods in honey *Melipona subnitida* bee were found in the literature, which did not allow for many comparisons.

Parallel to chemical analysis, microbiological quality responds to the real effect of maturation. Post-harvest maturation treatment showed the highest values of mold and yeast count among the conservation treatments without heating. It is important to consider that this treatment is based on stimulating the development of microorganisms until they reach the death stage in the cell cycle. The maturation process is a method that induces the fermentation process (Silva et al. 2023). However, the count of molds and yeasts and mesophilic aerobic bacteria may decrease during prolonged storage of honey (Lani et al. 2017). We concluded that a period greater than 30 days is necessary for the reduction of the cell count. None of the treatments used was able to reduce the counts as the control.

As for aerobic mesophilic bacteria count, the lowest values were associated with treatment by maturation and cooling, without statistical difference.

The preliminary results demonstrate an inefficiency of all treatments without heating in reducing microorganism count in SB honey. The post-harvest maturation should be processed in a period superior to 30 days, and the dehumidification method is an alternative for adapting the SB honey to the parameters defined by the legislation for Apis Mellifera bee honey.

Conservation treatments by pasteurization

The pasteurization treatments were carried out take into account the recommended temperature for High Temperature Short Time (HTST) for milk. This

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method involves the rapid heating of milk until 72 °C, keeping it for a few seconds (usually 15 s), and cooling it down immediately (Escuder-Vieco et al. 2018). For SB honey, three temperatures were tested: 60, 75 and 90 ºC. The results are in Table 3.

Table 3 - Physicochemical and microbiological quality of Melipona subnitida bee honeys (SB honey), after pasteurization treatments.

	Treatments			
Parameters	*Control 2	Pasteurization (60 ºC)	Pasteurization (75 ºC)	Pasteurization (90 ºC)
Reducing sugars (g glucose/100g)	**63.96 a ± 1.38**	**63.96 a ± 0.58**	**64.63 a ± 0.30**	**64.50 a ± 1.81**
Sucrose (g/100g)	**0.00 a ± 0.00**	**0.00 a ± 0.00**	**0.00 a ± 0.00**	**0.00 a ± 0.00**
Titratable acidity (mEq/kg)	**74.16 a ± 0.33**	**74.84 a ± 0.46**	**75.10 a ± 0.35**	**75.07 a ± 0.45**
HMF (mg/kg)	**84.12 a ± 3.75**	**63.22 b ± 3.71**	**46.08 c ± 2.84**	**36.79 d ± 0.62**
Moisture	**3.25 c ± 0.07**	**3.53 b ± 0.02**	**3.61 b ± 0.01**	**3.76 a ± 0.01**
Total soluble solids (°Brix)	**2.19 a ± 1.05**	**7.35 a ± 2.06**	**8.70 a ± 4.77**	**5.66 a ± 2.57**
pH	**25.84 a ± 0.33**	**25.16 a ± 0.46**	**24.90 a ± 0.35**	**24.93 a ± 0.45**
Color	**0.297 a ± 0.00**	**0.460 a ± 0.16**	**0.318 a ± 0.02**	**0.232 a ± 0.00**
Molds and yeasts (log CFU/mL)	**4.04 a ± 0.04**	**2.75 b ± 0.09**	**2.89 b ±0.14**	**2.62 b ± 0.29**
Mesophilic aerobic bacteria (log CFU/mL).	**2.22 a ± 0.00**	**2.12 a ± 0.00**	**2.58 a ± 0.90**	**2.56 a ± 0.34**

Source: Prepared by the author.

*Sample characteristics before applying treatments.

Means with same letters on the same line are not significantly different ($p \geq 0.05$) by the Tukey test.

The applied treatments kept the SB honeys in accordance with the legal values for *Apis mellifera* bee honey regarding the parameters of sucrose (max 5 g/100g), HMF (max 40 mg/Kg) and reducing sugars (min 60 g/100 g) (Codex Alimentarius 2001). With regard to moisture content, all SB honeys remained at odds with the legal parameter (min 20 g / 100 g), while acidity was reduced in treatments at 75 and 90 ºC, agreeing with the stipulated value (max 50 mEq/kg).

This behavior suggests that acidity in SB honey was composed of volatile acids, which volatilized due to the high pasteurization temperature (Araújo 2012). This behavior was also observed in pH analysis, where Control 2 showed the lowest

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results, differing statistically from the others. However, all the samples show values in accordance with the data found by other researches that studied samples of non-pasteurized stingless bee honey from Brazil (Villas-Boas and Malaspina 2005; Silva et al. 2013; de Almeida-Muradian et al. 2014). Thus, we can conclude that the pasteurization did not negatively affect SB honey samples

Although the exposure of honey to high temperatures promotes an increase in HMF content (Nozal et al. 2001), it was not found statistically significant increase in HMF levels after heating in this study. Thus, we can conclude that the temperatures applied in pasteurization did not cause harmful effects on the samples regarding the increase of HMF levels.

The moisture content was constant after the treatments, so heating treatment did not favor evaporation or concentration of SB honey.

The color in all analyzed samples agrees with the requirements of the current standards for *Apis mellifera* bee honey (MAPA 2000). However, the samples showed color ranging from “light amber” to “amber” when submitted to temperatures of 75 and 90 ºC, and showed differences in absorbance, which indicates the interference of the exposure to higher temperatures on SB honey color modification. This change is caused by the Maillard reaction, which according to Francisquini et al. (2017) is a slow reaction that occurs in food products during its prolonged storage and its exposure to heating, changing not only the color but also the aroma and flavor (Francisquini et al. 2017).

Pasteurization was efficient in controlling molds and yeasts, as Control 2 presented the highest count, while the other treatments did not differ statistically from each other. Thus, pasteurization treatment at 60 ºC caused a reduction in molds and yeasts counts by more than 1 logarithmic cycle. The pasteurization at 60 ºC was the one that least changed the physicochemical characteristics of honey.

Even though the aerobic mesophilic bacteria are sensitive to the high osmotic pressures of honey (Silva et al. 2017), the treatments used were not efficient, since none of the treatments applied promoted the reduction of these microorganisms. In this way, it is necessary a higher time/temperature binomial to guarantee its elimination.

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Conclusions

The effect of dehumidification, post-harvest maturation, pasteurization and cooling methods on the quality characteristics of honey from *Melipona subnitida*, Duke's bee demonstrated that only pasteurization reduced logarithmic cycles of molds and yeast counts. Pasteurization at 60 °C was the one that least changed original characteristics of the honey. All treatments without heating proved to be inefficient in reducing the count of microorganisms. This research showed that cooling and dehumidification treatments are viable alternatives for conservation of honey in good microbiological conditions. However, in the next research it is necessary to test other times for post-harvest maturation.

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