

Effect of Storage Time on the Quality of Stingless Bee (*Tetragonula biroi* Friese) Honey

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Stingless bee honey (SBH) has a unique sour taste and distinct flavor compared to honey from *Apis* species. The demand for SBH has increased in the Philippines because of its known therapeutic properties. Even if it is sold immediately after harvest, it is crucial to know its shelf-life in anticipation of large-volume production shortly. Thus, this study was conducted to determine if storage for 5 mo at room temperature will alter its physicochemical characteristics. The average values for the pH level, moisture content, HMF content, reducing sugar content, and total sugar content during the 5-mo storage period were 3.30 ± 0.02 , $26.95 \pm 0.07\%$, 2.40 ± 0.55 mg/kg, $46.08 \pm 0.43\%$, and $46.78 \pm 1.51\%$, respectively. The values for each parameter did not change significantly during the duration of the study. This indicates that SBH can remain stable for at least a 5-mo period.

Keywords: 5-hydroxymethylfurfural, honey, physicochemical analysis, shelf life, *Tetragonula biroi* Friese

An economic analysis conducted by Locsin and co-authors (2021) showed that the propagation of stingless bees in the Philippines is feasible due to the abundant populations of this species, resistance to pests and diseases, and ability to forage more floral resources. The Philippines has 12 species of stingless bees, seven of which are unique species (Cervancia *et al.* 2023). Among the species of stingless bees present in the country, *Tetragonula biroi* Friese is the major producer of honey, pollen, and propolis, which are known to have various medicinal properties (Locsin *et al.* 2021). Desamero and co-authors (2017) found out that honey produced by *T. biroi* is a potential neuroprotective agent against ischemic stroke.

An important property of honey is its stability and resistance to spoilage over long periods of time. This is mainly due to the stomach enzyme deposited by bees, which – when combined with nectar – yields gluconic acid and hydrogen peroxide by-products, thereby increasing the acidity and antibacterial properties of honey. However, raw honey is still susceptible to spoilage upon fermentation (McHugh 2017). The same physicochemical properties of stingless bee honey (SBH) that make it unique from honey from the bees of the genus *Apis* are also responsible for its potentially faster rate of quality deterioration and proneness to fermentation (Čadež *et al.* 2015). Generally, fermentation in honey is a process of chemical reactions catalyzed by microorganisms that cause a change in taste, texture, aroma, and quality of honey (Čadež *et al.* 2015).

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Most food items often benefit from fermentation such that it is essential for their processing and preservation. Examples are sauerkraut, miso, cheese, and tempeh. Honey, however, is an exception.

The high moisture content in SBH – which is influenced by various environmental factors such as processing methods, internal hive weather and humidity conditions, and the variety of nectar sources – provides a favorable condition for the growth and reproduction of various microorganisms that trigger fermentation in honey, thereby leading to its spoilage (Čadež *et al.* 2015; Hassan *et al.* 2021). One such microorganism that can withstand the high-sugar environment in honey is the osmophilic yeast, which grows on honey when given enough moisture, which SBH is relatively rich in compared to honey from the genus *Apis* (Čadež *et al.* 2015). Further adding to this is the hygroscopicity of honey, which allows it to absorb moisture from the surrounding atmosphere, which in turn further increases the moisture content of honey.

This study aims to determine if storage for 5 mo at room temperature will alter the physicochemical properties of SBH. This will be useful in the formulation of quality standards for SBH and boosting its marketability.

The honey samples were collected from three independent hive colonies of stingless bees located in Sayo Nora Bee Farm, Majayjay, Laguna, the Philippines (14.169146, 121.448465). Honey samples were extracted by pressing the honey pots through a filter to exclude extraneous matter. The honey was then filtered for a second time and transferred into glass jars (approximately 60 g honey/jar), which were sterilized using UPANG Plus Sterilizer and Dryer. The filtered honey samples were pooled and subdivided into 15 glass jars (three glass jars for each month). Raw filtered honey was used for the experiment such that the samples were not subjected to any form of heat treatment and were subjected to the study immediately after harvest. The method used for collecting honey was based on the protocol of the University of the Philippines Los Baños (UPLB) Bee Program.

The effect of storage time was monitored by measuring the quality parameters over a period. Measurements were taken first at the beginning of the experiment (at Day 0) and then once every month. The study was conducted for 5 mo, wherein three replicates of honey samples were used and each replicate was analyzed using three trials. The honey sample used in this study is a composite from three independent hives. Honey being sold in the market is usually a composite from several hives and not just from one hive.

The pH of the raw SBH was determined based on the method of Chutong and co-authors (2016). In a beaker, 5 g of honey sample was dissolved in 37.5 mL of distilled

water. The solution was stirred, and the pH electrode was immersed to obtain the pH value. The pH meter (WalkLAB Professional pH meter HP9010) was calibrated at pH 4.0 and 7.0 prior to analysis. This procedure is in accordance with the methods set by the International Honey Commission (IHC 2009) for pH determination.

A few drops of honey samples were spread evenly on a handheld refractometer (Aichose) to determine the moisture content according to the AOAC (2005) Official Method 969.38.

The HMF content of the honey samples was determined using the White method, as described in the AOAC Official Method 980.23. In a 50-mL beaker, 5 g of honey was dissolved with 25.0 mL of water. This solution was transferred quantitatively into a 50-mL volumetric flask, where 0.5 mL of Carrez solution I [3.75 g of potassium ferrocyanide ($K_2Fe(CN)_6 \cdot 3H_2O$) diluted in 25 mL distilled water] and II [7.5 g of zinc acetate ($Zn(CH_3COOH)_2 \cdot 2H_2O$) diluted in 25 mL distilled water] were added subsequently. The solution was mixed in between the addition of Carrez solution I and II and was filled up to mark with water afterward. The resulting solution was poured through a filter paper. The filtrate was collected but with the first 10 mL rejected. Then, 5.0 mL of the filtrate was pipetted into two separate test tubes, wherein 5.0 mL of water was added to the first test tube, whereas 5.0 mL of 0.2% sodium bisulfite solution was added to the second test tube and thoroughly mixed. This served as the sample and reference solution, respectively.

In the span of 1 h, the absorbance of the sample solution against the reference solution in a 10-mm quartz cuvette was measured at 284 nm and 336 nm (Shimadzu UV-mini 1240).

The following equation was used to calculate the HMF content:

$$HMF \text{ in mg/kg} = \frac{(A_{284} - A_{336})(F_{cct\text{or}})(5)(D)}{W}$$

where:

A_{284} = absorbance at 284 nm

A_{336} = absorbance at 336 nm

D = dilution factor, in case dilution is necessary

W = weight in g of the honey

$$Factor = 149.7 = \left(\frac{126}{16830} \times \frac{1000}{10} \times \frac{100}{5} \right)$$

where:

126 = molecular weight of HMF

16830 = molar absorption coefficient of HMF at 284 nm

1000 = mg/g

10 = c/L

100 = g honey reported
5 = nominal test portion weight

The free-reducing sugars of the honey samples were determined using the 3,5-dinitrosalicylic acid (DNS) method with minor modifications (Trinh *et al.* 2022). First, the DNS reagent was prepared according to the method of Gonçalves (2010) by dissolving 0.5 g of DNS in 25 mL of distilled water at 80 °C. Upon cooling to room temperature, 10 mL of 2 N NaOH will be added. This was prepared by dissolving 2 g of solid NaOH in 25 mL of distilled water. Afterward, 15 g of potassium sodium tartrate ($\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$) was added, and the solution was mixed well. The resulting solution was then diluted to 50 mL with distilled water.

For the DNS test proper, each 0.5 g honey sample was weighed and dissolved in 20 mL of water in a beaker. The solution was transferred quantitatively to a 50-mL volumetric flask and filled up to mark. Filtration was performed next, followed by dilution of 2 mL of the filtrate with 10 mL. From this solution, 2 mL was pipetted into a test tube, and 1 mL of DNS solution was added to it. This was kept in a thermostatic bath at 90 °C until a reddish-brown color appeared. Once the solution had cooled to room temperature, 7 mL of distilled water was added. The absorbance was determined using a UV-Vis spectrophotometer (Shimadzu UV-mini1240) at 540 nm. A calibration curve using glucose with a concentration scale of 0, 200, 400, 600, 800, and 1000 ppm was created. The reducing sugar content of the honey samples was determined using the following equation:

$$\% \text{ reducing sugar} = \frac{(C)(V)(DF)(1/1000)}{(W)(1000)} \times 100$$

where:

C = concentration from the calibration curve (ppm)
V = volume of the solution (mL)
DF = dilution factor
1/1000 = conversion factor to cancel out the volume unit
1000 = conversion factor to cancel out the mass unit
W = weight of sample (g)

The total sugars of the honey samples were determined based on the phenol-sulfuric acid method (Trinh *et al.* 2022). A 0.5 g honey sample was weighed and dissolved in 20 mL of water in a beaker. The solution was transferred quantitatively to a 100-mL volumetric flask and filled up to mark. The solution was filtered then 1 mL of the filtrate was pipetted into another 100-mL volumetric flask and filled up to mark. From this solution, 1 mL was pipetted into a test tube, where 1 mL of 5% phenol solution was added to it and mixed well. Then, 5 mL of concentrated H_2SO_4 was added. The reaction mixture was allowed to

cool before the absorbance was determined using a UV-Vis spectrophotometer (Shimadzu UV-mini1240) at 490 nm. A calibration curve using glucose with a concentration scale of 0, 10, 20, 30, 40, and 50 ppm was also created. The total sugar content of the honey samples was determined using the following equation:

$$\text{Total sugar content (\%)} = \frac{(C)(V)(DF)(1/1000)}{(W)(1000)} \times 100$$

where:

C = concentration from the calibration curve (ppm);
V = volume of the solution (mL)
DF = dilution factor
1/1000 = conversion factor to cancel out the volume unit
1000 = conversion factor to cancel out the mass unit
W = weight of sample (g)

The data obtained were expressed as mean \pm standard deviation (s.d.). The mean values for the physicochemical properties of honey samples at different months were subjected to one-way analysis of variance (ANOVA) using Microsoft Excel (2016). The confidence levels to be considered statistically significant will be $p < 0.05$ for a difference between means of 95% (Kędzierska-Matyssek *et al.* 2016).

The effect of storage time on SBH stored at room temperature for 5 mo was investigated by monitoring changes in various physicochemical properties such as pH, moisture content, HMF content, and sugar content. The atmospheric conditions for the duration of the study included a room temperature of 26.5–30.5 °C, a relative humidity of 64.5–72.0%, and an atmospheric pressure of 748.0 mmHg. The results of the physicochemical properties of SBH stored for 5 mo are summarized in Table 1.

The pH values obtained had values ranging from 3.28 ± 0.00 to 3.32 ± 0.01 (Table 1). Based on previous studies by Rozman and co-authors (2022), these values fall within the acceptable range set by the Department of Malaysian Standards (2.5–3.8). Statistical analysis using one-way ANOVA showed no significant difference ($p > 0.05$) with respect to time.

The pH value of honey is typically influenced by the amounts of acids such as amino acids, phenolic acids, and organic acids (Amin *et al.* 2018) and minerals like potassium, calcium, copper, manganese, iron, and phosphorus (Esa *et al.* 2022) present in the honey, which is consequently a function of various climatic and geographic factors. Since the pH level of the honey samples did not change significantly, this implies that the honey samples were stable during the 5-mo storage period because fermentation did not occur.

Table 1. Physicochemical properties of SBH stored for 5 mo.

Month	pH*	Moisture content* (%)	HMF content* (mg/kg)	Reducing sugar* (%)	Total sugar* (%)
November	3.28 ± 0.00	27.00 ± 0.00	2.96 ± 1.96	45.65 ± 1.28	46.65 ± 5.54
December	3.31 ± 0.02	26.83 ± 0.26	2.08 ± 0.72	46.36 ± 0.82	46.25 ± 2.79
January	3.31 ± 0.01	27.00 ± 0.00	2.55 ± 0.98	46.11 ± 0.99	49.42 ± 2.04
February	3.32 ± 0.01	27.00 ± 0.00	2.78 ± 1.80	46.61 ± 3.68	45.79 ± 5.80
March	3.28 ± 0.01	26.92 ± 0.20	1.62 ± 0.82	45.64 ± 0.79	45.84 ± 0.78
Average	3.30 ± 0.02	26.95 ± 0.07	2.40 ± 0.55	46.08 ± 0.43	46.78 ± 1.51

*Values expressed as mean ± std. dev. (n = 9)

*Values are not significantly different based on one-way ANOVA ($p > 0.05$)

The moisture content values of the honey samples ranged from 26.83 ± 0.26 to 27.00 ± 0.00 (Table 1). The moisture content is the honey parameter that determines the capability of honey to resist spoilage and remain stable (Cruz *et al.* 2021). Although the values obtained are higher than the 20% maximum moisture content level set by the Codex Alimentarius Standards, Chua and co-authors (2012) argue that tropical honey may contain a moisture content greater than the limits specified in the European Honey Legislation and Codex Alimentarius Standards. This is attributed to the hygroscopicity of honey, which allows it to absorb excess moisture in the environment. Hence, honey from countries in tropical regions is more susceptible to higher moisture content due to the greater amounts of rainfall and higher humidity.

A considerable volume of research works about honey showed a significant positive correlation between moisture content and water activity (Serin *et al.* 2018; van Boekel 2023). Water activity refers to the free and adsorbed water, whereas the moisture content also includes bound water, which is less available for microbial growth, as well as enzymatic and chemical reactions (Bradley 2010). Since honey is composed mostly of sugars which bind with water, the water activity of honey is usually low. Additionally, the low pH condition of honey further inhibits the growth of spoilage microorganisms.

The moisture content of the honey samples was coherent with the honey quality limits from tropical countries such as those of the Indonesian National Standard as stated in SNI 8664-2018 (maximum at 27.5%) and the Department of Malaysian Standards (maximum at 35%). The moisture content was also observed to have no significant change with respect to time based on one-way ANOVA ($p < 0.05$). This is indicative of the stability of SBH in storage despite having high levels of moisture. The ability of SBH to resist quality degradation may be attributed to its high levels of polyphenols, which have been consistently linked to the antibacterial activity of honey against a wide range of bacteria and fungi (Sousa *et al.* 2016).

HMF is the intermediate product of the acid-catalysis of hexose and the dehydration of fructose in the Maillard reaction. It is, therefore, a criterion related to the freshness or aging of honey. Fresh honey often has low HMF levels that rarely exceed 10 mg/kg. Values above this may signify potential overheating or adulteration of the honey samples (Cardona *et al.* 2019).

The HMF content measured for the SBH samples ranged from $1.62 \text{ mg/kg} \pm 0.82$ to $2.96 \text{ mg/kg} \pm 1.96$ (Table 1). The low level of HMF detected in the honey samples is indicative of the freshness of the samples based on the standards by Cardona and co-authors (2019). Furthermore, the HMF values obtained adhere to both the SBH quality standards by the Department of Malaysian Standards (maximum at 30 mg/kg) and the Indonesian National Standard, as stated in SNI 8664-2018 (maximum at 50%).

Statistical analysis also shows no significant difference based using one-way ANOVA ($p > 0.05$). This suggests that the samples have remained fresh based on their low and unchanged HMF content, which they have maintained in terms of quality and thereby resisting degradation over the course of 5 mo of storage.

The reducing sugar content is another quality standard for honey with the SNI 8664-2018 requiring a minimum of 55% for SBH (Saputra *et al.* 2021). As shown in Table 1, the reducing sugar levels of SBH during 5 mo of storage were lower than the minimum set by the SNI 8664-2018, ranging from only $45.65 \pm 1.28\%$ to $46.61 \pm 3.68\%$.

However, studies from other authors show that the minimum reducing sugar content of SBH may vary depending on the bee species and origin. It may go as low as 25% for *H. itama* in Malaysia (Kek *et al.* 2017) and as high as 75.9% for *Frieseomelitta varia* in Brazil (Duarte *et al.* 2018). In addition, data from the study by Braghini and co-authors (2021) suggested a minimum value of 50% reducing sugar content for SBH, but some exceptions were also noted for some values found to be close to 40%.

Variations in the reducing sugar content are believed to be due to high moisture content or the presence of other sugars that have yet to be studied (Braghini *et al.* 2021). It may also be a function of humidity, which affects the nectar production of feed source plants. According to Adalina and co-authors (2020), the sugar content of honey is inversely proportional to humidity. In the 5-mo storage period, marked differences were not observed for the reducing sugar content ($p > 0.05$). This suggests that the quality of the SBH in this study was preserved and the honey remained stable against fermentation, which would otherwise have decreased the sugar content of the honey.

The total sugar content of the SBH samples in storage ranged from $45.79 \pm 5.80\%$ to $49.42 \pm 2.04\%$ (Table 1). A study by Chuttong and co-authors (2016) showed that the total sugar content of SBH from Thailand is 51%. Moreover, analysis of fresh *T. laeviceps-pagdeni* honey also from Thailand showed that the total sugar content is 71.3% (Chuttong *et al.* 2015). These results were higher compared to the report of Belina-Aldemita and co-authors (2022) from the Philippines (34.90–39.72%).

Similar to the low reducing sugar content, the moisture-rich composition of SBH may be liable for the low total sugar content. Since the total sugar content is composed of both reducing sugars and non-reducing sugars, it is possible that the low reducing sugar content may have consequently lowered the total sugar content as well.

Despite the low sugar content, statistical analysis using one-way ANOVA showed that the mean total sugar measurements have no significant difference ($p > 0.05$) with respect to time. This implies that the honey samples have remained stable and resisted quality degradation.

Fermentation is typically prompted by a high moisture content as this allows various microorganisms, such as yeast cells, to thrive. Sugar is decomposed by yeast cells into acetic acid, carbon dioxide, and water. The increase in the acidity of honey would consequently favor the formation of HMF through acid catalysis or the Maillard reaction. Therefore, fermentation may manifest as an increase in the moisture content and HMF content, as well as a decrease in sugar content and pH level. However, since these changes were not observed, it may be implied that fermentation did not occur and the raw honey samples may be considered stable throughout the duration of the 5-mo storage period.

According to de Almeida-Muradian and co-authors (2013), the high moisture content of SBH may allow certain yeasts to grow. However, it can resist spoilage by fermentation due to the polyphenols incorporated in the honey processing of bees in the hive. The low pH level may also prevent the growth of microorganisms, which cannot tolerate the acidic medium.

CONCLUSION

From the results of the study, the values obtained for the different physicochemical parameters for honey quality were also all in accordance with different studies conducted on SBH in tropical regions, as well as the standards set by the Indonesian National Standard SNI 8664-2018 and the Department of Malaysian Standards. Based on statistical analysis using one-way ANOVA, it can be concluded that the pH level, moisture content, HMF, reducing sugar, and total reducing sugar of SBH did not vary significantly with respect to time. This stability can be attributed to the high acidity of the honey and high polyphenol content, which prevent the growth of various microorganisms that can catalyze the quality degradation of honey. Thus, the quality of raw SBH was maintained and has remained stable in 5 mo of storage in room temperature conditions.

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