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Microbiological and physicochemical characterization of organic and nonorganic honey sold in São Paulo State

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Abstract

This study assesses the microbiological and physicochemical quality of organic and nonorganic honey sold in São Paulo State, Brazil. A total of 26 samples were analyzed, 10 samples of organic honey (OH) and 16 of non-organic honey (NOH) sold in São Paulo State, Brazil. Physicochemical analyses revealed notable variations in moisture, acidity, and hydroxymethylfurfural (HMF) levels between OH and NOH. The average moisture content was 17.74 ± 1.47 g/100 g for OH and 18.12 ± 1.73 g/100 g for NOH. Regarding acidity, OH had an average of 26.90 ± 14.58 mEq/kg, while NOH had 34.32 ± 11.22 mEq/kg. Regarding HMF, OH had an average of 23.40 ± 38.70 mg/kg and NOH had 35.82 ± 39.94 mg/kg. Samples showing deviations from legal standards indicate potential processing or storage issues. The Fiehe test indicated possible fraud in one OH sample and four NOH samples. The Lund test showed no precipitation in any samples, suggesting adulteration in all of them. Diastatic enzyme research revealed the absence of enzymes in three samples, indicating adulteration or inadequate processing. The mean counts of mesophiles were 23 CFU/g for OH and 55.33 CFU/g for NOH, while the counts of molds and yeasts were 126.5 CFU/g for OH and 2289 CFU/g for NOH, exceeding recommended limits and raising concerns about product safety. These findings underscore the need for rigorous quality control measures throughout honey production to ensure safety, purity, and authenticity.

Keywords: adulteration; contamination; purity.

Practical Application: Physicochemical and microbiological alterations underscore the need for quality control and safety in both organic and nonorganic honey.

1 INTRODUCTION

Honey is a food product produced by honeybees, from flower nectar or secretions from living parts of plants or excretions of plant-sucking insects found on living parts of plants, which bees collect, transform, combine with their specific substances, store, and allow to mature in the combs of the hive (Brasil, 2000; 2017). Organic honey (OH) can be defined as a product free from undesirable chemical and biological contaminations, such as antibiotics, pesticides, and agrochemicals. Unlike nonorganic honey (NOH), this product is not directly controlled by beekeepers, but by the bees themselves, which seek the best floral sources, which can be from native areas or organic agriculture (Pereira et al., 2020).

Honey can be contaminated by the microbiota of the bees themselves, as well as other factors related to a lack of hygiene during handling, extraction, and processing of honey (Lopes, 2008). However, it is also common to find variations in the physicochemical composition of honey that may be caused by climatic conditions, flowering, maturation stage, bee species, processing, and storage (Alves, 2008).

The legislation for honey (Brasil, 2000) establishes quality control parameters for the product, indicating the analyses and methods to be employed. Thus, through physicochemical and microbiological tests, it is possible to detect irregularities and perform quality control on the product before it is commercialized.

The moisture content of honey determines its ability to remain stable and resist deterioration from yeast fermentation (International Honey Commission, 2002). Acidity is considered an important antimicrobial factor, providing greater stability to the product regarding microorganism development (Silva et al., 2023). The diastase enzyme test assesses enzymatic activity in honey, and its absence indicates adulteration. The Lund reaction is a qualitative method that indicates the presence of albuminoids, proteins naturally present in honey; their absence indicates fraud, while the Fiehe reaction with resorcinol in the acidic medium can highlight the presence of substances such as hydroxymethylfurfural (HMF) produced during honey overheating or the addition of sugar syrups (Mendes et al., 2009; Santos et al., 2011).

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Fermentation can occur when molds and yeasts are present, which results from the consumption of sugars by the yeasts, producing by-products that alter the natural taste and aroma of the product (Hooper, 1976). The presence of coliforms indicates the poor microbiological quality of the food in terms of shelf life or safety, due to the presence of foodborne pathogens; therefore, these microorganisms are used to assess food safety (Sant'Ana, 2003). Mesophilic aerobic bacteria provide information about the hygienic–sanitary characteristics of the processing and storage of the product.

This study aimed to evaluate the microbiological and physicochemical quality of organic and nonorganic honey sold in the state of São Paulo, Brazil.

2 MATERIALS AND METHODS

2.1 Honey samples

The samples of OH and NOH were sent to the Food Inspection Laboratory of the Department of Animal Production and Preventive Veterinary Medicine, Universidade Estadual Paulista "Júlio de Mesquita Filho," School of Veterinary Medicine and Animal Science, Botucatu.

A total of 26 samples, of which 10 were of OH (without identification of the floral source) and 16 samples of NOH (wildflower, eucalyptus, and orange blossom) were subjected to microbiological and physicochemical analyses.

2.2 Microbiological analyses

In total, 25 g of sample were weighed on an analytical balance into a sterile flask, and 225 mL of 0.8% saline solution was then added (1:1). For the preparation of the sequential dilutions, a 1 mL aliquot was taken from the first dilution and added to a test tube containing 9 mL of 0.1% saline solution until 1:100 dilution.

2.2.1 Enumeration of mesophilic aerobic bacteria, molds, and yeasts

For the enumeration of mesophilic aerobic bacteria, Petrifilm AC was used, and for the enumeration of molds and yeasts, Petrifilm YM followed the manufacturer's recommendations (3M Company, 2024a; 2024b).

2.2.2 Total coliforms and thermotolerant coliforms

Three sets of test tubes containing lauryl sulfate tryptose (LST) broth and an inverted Durham tube were inoculated with sample dilutions and incubated in a culture oven at 35°C for up to 48 h. Positive tubes, which showed gas production in the Durham tube, were simultaneously subcultured into brilliant green bile broth 2% and EC broth, and then incubated for up to 48 h, respectively, at 35°C and a water bath at 45°C. Positive tubes were observed, and the results were evaluated using the most probable number table, expressed as MPN/g (Eaton et al., 2005).

2.3 Physicochemical analyses

2.3.1 Moisture content by the refractometric method

The honey moisture content measurement was conducted using the refractometric method according to the procedures outlined in ABNT NBR 15714-2 (ABNT, 2009) using the table-top refractometer manufactured by Bausch & Lomb® (USA). A drop of honey was applied, and adjustments were made to align the point of intersection of the lines within the observation field. Refractive index correction was carried out based on the ambient temperature, with 0.00023 subtracted or added for each degree below or above 20°C, respectively.

2.3.2 Determination of the acidity

The determination of honey acidity adhered to the protocols outlined in ABNT NBR 15714-6 (ABNT, 2016a). Specifically, 10 g of honey were measured into a 250 mL beaker, to which 75 mL of distilled water and 10 drops of 1% phenolphthalein alcoholic solution were added. Titration was then carried out using 0.1 N sodium hydroxide solution until the solution turned pink. It was applied in the Equation 1:

$$mEq/kg = V \times fc \times 10 \quad (1)$$

where:

V: volume spent on the titration;

FC: 0.1 N NaOH solution correction factor;

10: mass (g) used in the titration.

2.3.3 Determination of HMF (Fiehe test)

A total of 5 g of the sample were measured into a 50 mL glass beaker, followed by the addition of 5 mL of ethyl ether. The mixture was vigorously shaken using a glass rod, and the ethereal layer was then transferred into a porcelain dish and left to evaporate. Subsequently, 0.5 mL of 1% resorcinol hydrochloric solution was added. The appearance of a vivid red color indicated the presence of commercial glucose or overheated honey (Instituto Adolfo Lutz, 2008).

2.3.4 HMF spectrophotometric method (Winkler)

A total of 10 g of honey were dissolved in 20 mL of water and then transferred to a 50-mL volumetric flask. From this solution, 2 mL was taken and mixed with 5.0 mL of p-toluidine solution in two separate test tubes. In the first tube, 1 mL of distilled water (reference solution) was added, while in the second tube, 1 mL of 0.5% barbituric acid solution (sample) was added. The absorbance of these solutions at 550 nm was determined using the Equation 2:

$$HMF\ (mg/kg) = 190 \times Absorbance/cuvette\ thickness\ (1\ cm) \quad (2)$$

where:

the number 190: the dilution factor and extinction coefficient (Zappala et al., 2005).

2.3.5 Lund test

A total of 2 g of the sample were weighed into a 50-mL beaker and then transferred to a 50 mL graduated conical tube with the aid of 20 mL of pure water. Following this, 5 mL of 0.5% tannic acid solution was added. Pure water was further added until the volume reached 40 mL. The conical tube was then capped, shaken, and allowed to stand for 24 h. In the presence of pure honey, a precipitate formed at the bottom of the tube within the range of 0.6–3 mL. However, in the presence of adulterated honey, either no precipitate was formed or it did not exceed the maximum volume within the specified interval (Instituto Adolfo Lutz, 2008).

2.3.6 Diastatic yeast research

A total of 10 g of the sample were weighed into a 50 mL test tube, followed by the addition of 20 mL of pure water and thorough mixing. Subsequently, 10 mL of this solution was transferred to a test tube, to which 1 mL of 1% soluble starch solution was added. The mixture was shaken and placed in a water bath at 45°C ± 2°C for 1 h. Afterward, the tube was removed from the bath, and 1 mL of iodine solution was added. A blank test was conducted without heating. The colors obtained were then compared. In the presence of the diastase enzyme, an olive green or brown color would appear. Conversely, a blue color would manifest in the absence of these enzymes (ABNT, 2016b; Instituto Adolfo Lutz, 2008).

2.4 Statistical analysis

The values of moisture, acidity, and HMF assays obtained from the samples were conducted in duplicates and statistically analyzed by ANOVA using a completely randomized design, supplemented with Tukey's test for mean comparison. Absolute frequencies (AF), relative frequencies (RF), and relative frequencies in percentage (RF%) were calculated for qualitative physicochemical assays (Lund, Fiehe, and diastase). For microbiological analyses, the nonparametric Kruskal–Wallis method was used. All statistical analyses were performed at a significance level of 5%.

3 RESULTS AND DISCUSSION

3.1 Physicochemical analyses

The Tukey's test at a 5% probability level shows that the means obtained for moisture, acidity, and HMF exhibited significant differences ($p > 0.05$) (Table 1).

The high moisture content in honey may be due to the environment in which it is produced. If the relative humidity of the air is 60%, honey with 18.3 g/100 g moisture tends to absorb water due to its hygroscopic nature; if it is lower than 60%, honey with 18.3 g/100 g moisture tends to lose water to the environment (Crane, 1999). The ideal moisture content in honey is from 16.8 to 17%, ensuring that the product is not at risk of fermentation and can be stored for longer consumption. Above 21% moisture content, honey is prone to fermentation (Wiese, 2005).

The legislation establishes a reference value of 20 g/100 g for moisture content. Above this value, there is a risk of fermentation, leading to a shorter shelf life of the product and rendering it unsuitable for consumption. The moisture values found were mostly within the established standards, except for only one sample of NOH that was outside the standards.

Only one sample of NOH exceeded 50 mEq/kg (Table 2), meaning it was outside the legal limits (Brasil, 2000). According to Koblitz (2008), samples that have undergone the intentional addition of acidulants or sugars obtained by acid hydrolysis may present acidity values above those established by legislation. Silva et al. (2018) found all samples within the limits, with a minimum acidity of 8.78 mEq/kg and a maximum of 26.53 mEq/kg.

Notably, five samples were found to be outside the legal limits (Brasil, 2000), with one from OH and four from NOH (Table 2). Sodré et al. (2007) observed that 20% of the honey samples analyzed had values above the permitted limit by the current regulation. The results of HMF by Schlabitz et al. (2010) ranged from 1.73 to 30.85 mg/kg, all within the standards established by legislation.

The qualitative detection of HMF is based on the Fiehe reaction. The addition of commercial glucose or the overheating of honey results in a reddish coloration. This compound formed due to the presence of resorcinol in an acidic medium is indicated as a positive sample (Instituto Adolfo Lutz, 2008). The Fiehe test should be negative (Brasil, 2000). The Lund and

Table 1. Statistical analysis (ANOVA) supplemented with Tukey's test at a 5% significance level and coefficient of variation (CV) and p-values. Mean (±) standard deviation of moisture values (g/100 g), acidity (mEq/kg), and HMF (mg/kg) of organic honey (OH) and nonorganic honey (NOH) sold in São Paulo State.

Test	Sampling	N	Mean ± SD	CV (%)	p-value
Moisture (g/100 g)	OH	10	17.74 ± 1.47 a[1]	9.06	> 0.05
	NOH	16	18.12 ± 1.73 a[1]		
Acidity (mEq/kg)	OH	10	26.90 ± 14.58 a[1]	39.99	> 0.05
	MOH	16	34.32 ± 11.22 a[1]		
Hydroxymethylfurfural (mg/kg)	OH	10	23.40 ± 38.70 a[1]	127.18	> 0.05
	MOH	16	35.82 ± 39.94 a[1]		
Total		26			

[1]Lowercase letters in the same column indicate that there is no statistically significant difference ($p < 0.05$) when comparing different types of honey (organic and nonorganic).

Fiehe tests are considered complementary qualitative analyses that can indicate honey adulteration or poor storage.

The Lund reaction is based on the precipitation of albuminoid substances by tannic acid. The Lund test should be positive, indicating pure honey, when precipitation occurs in the range of 0.6–3 mL (Brasil, 2000). It was observed that in the Lund reaction, the samples did not form precipitates; therefore, all honey samples can be classified as adulterated (Table 3). In the Fiehe reaction, 5 out of 26 samples (1 organic and 4 non-organic) were positive (Table 3), indicating the addition of commercial glucose or overheating of the honey. According to Ribeiro and Starikoff (2018), it was observed that in the Lund reaction, 9.09% of the samples did not form a precipitate, characterizing adulterated honey samples. In the Fiehe reaction, 18.18% of the samples were positive, indicating the addition of commercial glucose or overheating of the honey.

Diastase is an enzyme relatively sensitive to heat; its total or partial absence of activity indicates overheating, long-term storage under poor temperature conditions, or even adulteration (Scripcă & Amariei, 2021). Out of the 26 samples analyzed, three showed the absence of diastatic enzymes (Table 3), indicating adulteration. Melo et al. (2016) showed that five samples showed the presence of diastatic enzymes, while another five samples showed an indication of adulteration, 50% of the samples are outside the legislation requirements regarding this parameter.

3.2 Microbiological analyses

The presence of molds and yeasts in honey can occur naturally, but their proliferation is favored by specific conditions of humidity and temperature, which can lead to the fermentation of the product. This fermentation is triggered by the action of osmophilic yeasts on the sugars present in honey, resulting in the production of alcohol and carbon dioxide, which can alter the taste and quality of honey (Góis et al., 2013).

The results obtained demonstrated that the mean standard count of molds and yeasts in all samples exceeded the maximum limit of 1.0 x 10^2 CFU/g (Table 4) established by Brazilian legislation (Brasil, 2001). Specifically, the mean for OH was 1.26 x 10^2 CFU/g, while for NOH it was 2.29 x 10^2 CFU/g. Previous studies, such as David et al. (2017), reported mold and yeast counts below 3.0 CFU/g in all analyzed samples. In samples of

Table 2. Absolute frequency (AF), relative frequency (RF), and relative frequency in percentage (RF%) of quantitative assays (moisture, acidity, and HMF) according to the allowed values for organic honey (OH) and nonorganic honey (NOH) sold in São Paulo State.

Test	Sampling	Result	AF	RF	RF (%)
Moisture (g/100 g)	OH (n = 10)	> 20	0/26	0.00	0
		≤ 20	10/26	0.38	38
	NOH (n = 16)	> 20	1/26	0.04	4
		≤ 20	15/26	0.58	58
Acidity (mEq/kg)	OH (n = 10)	> 50	0/26	0.0	0
		≤ 50	10/26	0.38	38
	NOH (n = 16)	> 50	1/26	0.04	4
		≤ 50	15/26	0.58	58
Hydroxymethylfurfural (mg/kg)	OH (n = 10)	> 60	1/26	0.04	4
		≤ 60	9/26	0.35	35
	NOH (n = 16)	> 60	4/26	0.15	15
		≤ 60	12/26	0.46	46
Total		26	1	1.00	100

Table 3. Absolute frequency (AF), relative frequency (RF), and relative frequency in percentage (RF%) of qualitative assays for organic honey (OH) and non-organic honey (NOH) sold in São Paulo State.

Test	Sample	Result	AF	RF	RF (%)
Fiehe test	MO (n = 10)	Positive	1/26	0.04	4
		Negative	9/26	0.35	35
	MNO (n = 16)	Positive	4/26	0.15	15
		Negative	12/26	0.46	46
Lund test	MO (n = 10)	Positive	0/26	0.0	0
		Negative	10/26	0.38	38
	MNO (n = 16)	Positive	0/26	0.0	0
		Negative	16/26	0.62	62
Diastatic yeast research	MO (n = 10)	Presence	9/26	0.35	35
		Absence	1/26	0.04	4
	MNO (n = 16)	Presence	14/26	0.54	54
		Absence	2/26	0.07	7
Total		26	1	1.00	100

honey from the northwest region of Rio Grande do Sul, Ludwig et al. (2018) found counts ranging from 33 CFU/g to 7.5 x 10^3 CFU/g. Therefore, in this study, there is no significant difference in mold and yeast count between the two types of honey (Table 4) because the p-value (0.65072) was greater than the significance level of 0.05.

The count of mesophilic aerobic bacteria is an important indicator of the sanitary quality of foods. In this study, for OH, the mean mesophilic aerobic microorganism count is 23 CFU/g. For NOH, the mean is 55.33 CFU/g, indicating that NOH has a higher mesophilic aerobic microorganism count than OH. According to Franco and Landgraf (2005), counts exceeding 106 CFU/g indicate significant contamination. Although Caldas et al. (2020) detected mesophilic aerobic bacteria in 40% of the samples analyzed, the counts were low. However, the presence of mesophilic aerobic microorganisms may be related to the high moisture content present in some samples.

Based on the results of the Kruskal–Wallis test for mesophilic aerobic count (Table 5), there is not enough evidence to conclude that there is a significant difference in mesophilic count between OH and NOH sold in the state of São Paulo, as the p-value is greater than the significance level of 5%.

It is important to note that both Brazilian and international legislation do not require mandatory microbiological analyses for honey, but recommend the adoption of good manufacturing practices (GMP) during the processing of the product. Ther fore, the results obtained in the microbiological analyses of the samples indicate the need for greater quality control throughout the honey production chain to ensure its safety and quality.

4 CONCLUSION

The physicochemical analyses revealed significant differences in moisture, acidity, and HMF content between OH and NOH. While most samples met the established standards, a few exceptions were noted, particularly in terms of acidity and HMF levels. These deviations could indicate potential issues with processing or storage, emphasizing the importance of adherence to quality control measures. Qualitative tests for adulteration, such as the Fiehe and Lund tests, showed mixed results, with some samples indicating possible adulteration through the presence of commercial glucose or overheating. Additionally, the absence of diastatic enzymes in some samples further suggests potential adulteration or improper processing.

Microbiological analyses revealed varying levels of mesophilic bacteria, mold, and yeast counts, with some samples exceeding recommended limits. While microbiological standards for honey are not mandatory under current legislation, the presence of high microbial counts raises concerns about product safety and quality, highlighting the need for improved hygiene practices during honey processing and storage.

Overall, these findings underscore the importance of stringent quality control measures throughout the honey production process to ensure the safety, purity, and authenticity of the final product.

Table 4. Nonparametric Kruskal–Wallis method with 5% significance for the analysis of mold and yeast count (CFU/g) for organic honey (OH) and nonorganic honey (NOH) sold in São Paulo State.

Test	OH	NOH
N	10	16
Mean	126.5	2,289
Standard deviation	124.99	5,584
Median	120	70
Minimum	15	10
Maximum	365	17,000
Degrees of freedom (DF)	1	
Chi-square (X^2)	0.20499	
p-value	0.65072	
Coefficient of variation (%)	30.6	

Table 5. Nonparametric Kruskal–Wallis method with 5% significance for the analysis of mesophilic aerobic count (CFU/g) for organic honey (OH) and non-organic honey (NOH) sold in São Paulo State.

Test	OH (CFU/g)	NOH (CFU/g)
N	10	16
Mean	23	55.33
Standard deviation	20.03	69.07
Median	10	20
Minimum	10	10
Maximum	70	190
Degrees of freedom (DF)	1	
Chi-square (X^2)	1.7387	
p-value	0.1873	
Coefficient of variation (%)	13.05	

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