Unveiling the Impact: How Processing Malaysian Acacia Stingless Bee Honey Affects Quality and Freshness

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The quality of acacia stingless bee honey, whether raw honey (RH) or processed honey (PH 1 and PH 2), is a vital factor in determining its overall value. This study aimed to evaluate the physicochemical properties, antioxidant activity, rheological behavior, and ATR-FTIR spectroscopy of RH, PH 1, and PH 2 collected from the nectar of Malaysian acacia trees by the *Heterotrigona itama* species. The Malaysian Standard for Stingless Bee Honey and the International Codex Standard were met by standardizing the physicochemical properties, which included pH, free acidity, moisture content, insoluble matter, ash content, hydroxymethylfurfural (HMF), and sugar content. The results revealed that the HMF values in PH 1 and PH 2 exceed the maximum limits, possibly due to temperature fluctuations during the heating process. The antioxidant properties indicated that PH 1 exhibits the highest total phenolic content at 109 ± 7.109 mg GAE/g and total flavonoid content at 28.55 ± 4.173 mg QUE/g compared to RH. The rheological behavior of PH 1 and PH 2 demonstrated Newtonian flow behavior, while RH displayed dilatant behavior. Furthermore, the ATR-FTIR spectroscopy analysis revealed differences in chemical structures and specific functional groups among RH, PH 1, and PH 2. The study indicates that processing stingless bee honey significantly impacts its quality and freshness.

Keywords: Acacia; processed; stingless bee honey; physicochemical; properties

Received: September 2024; Accepted: October 2024

Stingless bees belonging to the tribe *Meliponini* (*Hymenoptera, Apidae, Apinae*) are distributed across tropical and subtropical regions of the world, where they play a significant role in both ecological balance and honey production [1, 2]. In Malaysia, *Heterotrigona itama* and *Geniotrigona thoracica* are the dominant species commercially cultivated for honey production within the stingless bee species; known locally as "kelulut" [3]. These species are integral to the growing honey market in Malaysia, where stingless bee honey (SBH) is valuable for its medicinal properties and unique flavor [4]. SBH produced in Malaysia comes from bees that forage on acacia trees *(Acacia mangium)*, leading to a distinct "acacia stingless bee honey" prized for its specific flavor profile and bioactive compounds. Honey produced by bees fed on acacia flowers gives the transparent to light yellow color of honey with a mild taste and floral fragrance. This honey also has dehumidifying, diuretic, and hemostatic properties [5]. SBH consists of carbohydrates, water, amino acids, vitamins, and minerals, and also contains phenolic and flavonoid compounds that are crucial in its antibacterial, anti-inflammatory, and antioxidant activities [6]. The composition and properties of stingless bee honey are influenced by several factors, including the species of bee, the floral origin of the nectar, and environmental conditions [7]. A previous study stated that the strong antioxidant properties of SBH can be used for antidiabetic agents and anti-cancers, and their effectiveness is better than that of *Apis mellifera* honey [8, 9].

The increasing demand for SBH has led stingless bee (SB) keepers to process the honey. However, the processing of honey can have a significant impact on its quality and freshness. Processing methods, such as heat treatment, oxidation, and fermentation, can affect the composition of honey as well as the long storage time [10]. Filtration, creaming, and pasteurization processes are often employed to enhance the shelf life and clarity of honey, but these methods may also alter the natural properties and reduce the freshness [11]. For stingless bee honey, understanding how processing affects its physicochemical and antioxidant properties is crucial for both producers and consumers. Processed honey may exhibit changes in viscosity, antioxidant capacity, and chemical structure, which can diminish the freshness and authenticity of the product [12]. Given the increasing demand for stingless bee honey in both food and medicinal markets, it is essential to understand how processing impacts the quality and characteristics of acacia SBH. Additionally,

techniques, such as ATR-FTIR spectroscopy, are vital tools for detecting changes in the chemical composition of honey, offering insights into the effects of processing and potential adulteration [13].

This study investigated the impact of processing on the quality and freshness of Malaysian acacia SBH by analyzing raw honey (RH) and two types of processed honey (PH 1 and PH 2). The study focused on physicochemical parameters (pH, free acidity, moisture content, ash content, and hydroxy-methylfurfural (HMF) levels), antioxidant properties (total phenolic (TPC) and flavonoid content (TFC)), rheological behavior, and chemical composition using Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectroscopy. By examining these factors, this research provides critical insights into how processing affects the natural qualities of acacia SBH, contributing valuable knowledge for producers, consumers, and regulatory bodies concerned with honey quality and authenticity.

EXPERIMENTAL

Chemicals and Materials

Sodium hydroxide, potassium hexacyanoferrate, zinc acetate dihydrate, sodium bisulfite, gallic acid, Folin & Ciocalteu's phenol reagent, sodium nitrite, and aluminum chloride-6-hydrate were purchased from Sigma Aldrich, USA. PH 1 (processed honey 1) was purchased from a local pharmacy in Terengganu, Malaysia, and PH 2 (processed honey 2) and RH (raw honey) were purchased from a local company in Johor Bahru, Malaysia. The processing methods of processed honeys by the manufacturers were undisclosed by the companies. All the honeys were monofloral and collected from the nectar of the acacia tree (*Acacia mangium*) by a stingless bee honey species (*Heterotrigona itama*). All materials were used as received without further purification.

Physicochemical Analysis of Honey

The physicochemical parameters (pH, free acidity, moisture content, insoluble matter, ash content, and HMF) and sugar composition of SBH (RH, PH 1, and PH 2) were investigated using the methods established by the International Honey Commission (IHC) [14] and the Codex Alimentarius Commission (CAC) [15] with certain modifications.

pH and Free Acidity

The pH and free acidity of honey were measured using the Bench Top Professional pH meter (BP3001, Trans Instruments), standardized at pH 4.0 and 7.0 by dissolving 13.5% (w/v) honey samples in distilled water. For free acidity determination, the dissolved solution was titrated with 0.1 M of sodium hydroxide (NaOH) solution until the reading reached pH 8.3. The free acidity of honey was calculated and expressed in milliequivalents acid per kilogram of honey (meq/kg) using Equation (1) below.

$$Free\ acidity = 10V \qquad \text{Eq (1)}$$

Where, V is the volume of 0.1 M NaOH used and 10 is the amount of honey sample [14].

Moisture Content

The moisture content of honey samples was analyzed using a portable digital professional hand-held refractometer (MISCO PA203X, SER. No.100-43213, USA) with automatic temperature compensated for aqueous (water-based) honey mixtures at 20℃ at a reference wavelength of 589 nm. The refractometer was equilibrated with water for 6 minutes at 20℃ before dropping honey in the refractometer. The moisture content was recorded and expressed as a percentage (%).

Insoluble Matter (IM)

The determination of IM for honey followed previously published methods [16]. A weighed filter paper (Whatman No.1 filter paper with pore size: 11 μm) was dried in an oven (100℃) for one hour and left in a desiccator to attain ambient temperature. Approximately 10% (w/v) of honey was dissolved in distilled water at 80℃ and filtered using the weighed filter paper. The honey residue left on the filter paper was dried in an oven (100℃) for 1 hour before it was cooled in the desiccator and weighed. Finally, the IM of honey was calculated and expressed in the unit of percentage by weight using Equation (2).

$$Insoluble\ matter\ (\%) = \frac{(M_2 - M_1)}{M} x\ 100 \qquad \text{Eq (2)}$$

Where, M_2 is the weight of filter paper with honey residue, M_1 is the weight of filter paper, and M is the weight of honey.

Ash Content

A weight of honey (5 g) was placed in a crucible dish before adding two drops of olive oil and heated at 300℃ until completely carbonized in a muffle furnace (Carbolite ELF 1100 Celsius, 23L). The ashing process was continued for 1 hour at a temperature of 600℃. Equation (3) was used in the determination of the ash content and expressed as ash content in (g/100 g) of honey.

$$Ash\ content\ \left(\frac{g}{100}g\right) = \frac{M_2 - M_1}{M} x\ 100 \qquad \text{Eq (3)}$$

Where, M_2 is the weight of crucible dish with ash, M_1 is the weight of crucible dish, and M is the weight of honey.

Hydroxymethylfurfural (HMF)

25 mL of distilled water was used to dissolve 5 grams of honey, and the solution was then added with 0.5 mL of each of the Carrez I and Carrez II solutions. The solution was added with distilled water until the volume of 50 mL and filtered with a filter paper (Whatman No.1, 11 µm pore size). The ratio (1:1) was used for the honey sample (honey solution: distilled water) and reference solution (honey solution: 0.2% sodium bisulfite solution) in 1 cm quartz cuvettes to measure the absorbance at 284 nm and 336 nm. The sample was diluted to a sufficiently low level for accuracy (dilution factor) by diluting it with 1 mL of distilled water and the reference solution was diluted with 1 mL of sodium bisulfite ($NaHSO_3$) solution to the same amount when the absorbance at 284 nm exceeded a value of approximately 0.6. The background absorbance at 336 nm was subtracted to determine the HMF content. The HMF content was determined using Equation (4) and expressed in milligrams per kilogram of honey.

$$HMF\ \left(\frac{mg}{kg}\right) = \frac{(A_{284-}\ A_{336})\ x\ 149.7\ x\ 5\ x\ dilution\ factor}{weight\ of\ honey\ in\ gram} \quad \text{Eq (4)}$$

Where, A_{284} is absorbance at 284 nm and A_{336} is absorbance at 336 nm.

Sugar Content

The determination of sugar content in honey followed the method established by IHC using a Shimadzu HPLC (LC - 20AD). The sugars investigated in this study included glucose, fructose, sucrose, and maltose. The HPLC system contained a refractive index (RI) detector, and the separation process used a Phenomenex column (Luna 5 µm NH2 100 Å) maintained at a constant temperature of 30°C throughout the analysis. A stainless steel analytical column (diameter 4.6 mm, length 250 mm) containing amine-modified silica gel (particle size 5 to 7 µm) was used. A syringe filter (0.45 µm) was used to filter five grams of honey dissolved in 40 mL of distilled water and 25 mL of methanol before chromatographic analysis. The calibration preparation involved dissolving a standard sugar (fructose = 2.0 g; glucose = 1.5 g; sucrose = 0.25 g; maltose = 0.15 g) in approximately 40 mL of water, and then transferred into a volumetric flask (100 mL) containing 25 ml of methanol and made up to a final volume of 100 mL with water. This standard solution remained stable for four weeks when stored in a refrigerator at 4°C. Before injection, the solution was transferred into sample vials using a syringe and a pre-mounted membrane filter. The honey sample was injected in a volume of 10 µL at a flow rate of 1.3 mL/min. The mobile phase used as a carrier which was prepared by dissolving 80% acetonitrile in ultrapure water. This freshly prepared mobile phase with an 80:20 (v/v) ratio of acetonitrile to water was filtered through a 0.45 µm PTFE membrane filter and degassed. Column clogging was prevented by using injection filters to remove impurities in the honey sample before injection. The obtained retention times were compared with standards to identify separate sugar peaks and honey samples were spiked with standards to verify chromatographic peaks. Peak quantification was determined using the average peak area with duplicate injections. The sugar content of honey was calculated using Equation (5).

$$\frac{A1\ x\ V1\ x\ m_1}{A2\ x\ V2\ x\ m_0} \quad \text{Eq (5)}$$

Where,

A_1 = Peak area or peak height of the specified sugar compound in the honey solution, measured in units of area, length, or integration

A_2 = Peak height of the specified sugar compound in the standard solution, expressed in units of area, length, or integration

V_1 = Total volume of the honey solution in milliliters (mL)

V_2 = Total volume of the standard solution in milliliters (mL)

m_1 = weight of the sugar in grams within the total volume of the standard solution (V_2)

m_0 = weight of the honey in grams

Total Phenolic Content (TPC)

Total phenolic content (TPC) in honey was determined using the Folin-Ciocalteu phenolic reagent. Briefly, 20 µL of Folin-Ciocalteu solution was added to a 96-well microplate containing 20 µL of honey solution and incubated at room temperature for 5 minutes. Then 20 µL of (10% w/v) aqueous sodium carbonate (Na_2CO_3) was added, followed by 140 µL of distilled water and measured at an absorbance of 756 nm after incubation at 30°C for 90 minutes. The standard graph was created using the gallic acid concentration series, which ranged from 250 to 3.9 µg/mL. A linear equation expressed as the concentration of total phenolic compounds in honey as µg gallic acid equivalents per mL honey (mg GAE/g).

Total Flavonoid Content (TFC)

The phenol reagent colorimetric assay was used to determine the total flavonoid content (TFC) in honey. Briefly, 55 µL of distilled water was added to a 96-well microplate containing 25 µL of the honey solution, followed by the addition of 30 µL (1.25 % w/v) of aqueous sodium nitrite ($NaNO_2$) and incubated at room temperature for 5 minutes. Then, 30 µL of (2.5 % w/v) aqueous aluminum chloride ($AlCl_3$) was added and incubated for 6 minutes at room temperature. Finally, an additional 50 µL of 1 M sodium hydroxide (NaOH) solution was added, followed by 60 µL of distilled water and measured at an absorbance of 510 nm. The standard graph was created using the quercetin concentration series, ranging from 250 to 3.9 µg/mL. A linear equation expressed as the concentration of total flavonoid compounds in

honey as µg quercetin equivalents per mL honey (mg QUE/g).

Rheological Analysis of Honey

Using a TA Instrument stationary rotary rheometer (model: DHR-2) equipped with a DIN rheological probe of concentric cylinders made of Peltier steel, the rheological properties of honey were evaluated using stationary rheology. This process involved investigating the shear stress (τ) by varying the shear rate (ϕ). Honey (15 mL) was added to the vessel and pre-sheared for one minute at 30°C at $\phi = 20\ s^{-1}$. An electronic bath with a thermostat was used to maintain the temperature at 30 ± 2 °C. The flow curves were created by cyclically changing the shear rate in ascending and descending (6 points) modes in the range of 20 to 150 sec^{-1}.The TRIOS TA Instrument software was used to collect and process the data, and the Power Law model was used to determine the rheological parameters using Equation (6) below.

$$\tau = K_c \phi^n \qquad \text{Eq (6)}$$

τ represents the shear stress (Pa), K_c is the consistency index ($Pa.sec^n$), ϕ is the shear rate (sec^{-1}), and n is the fluid behavior index (dimensionless). The graph of the relationship between shear rate and viscosity of honey samples, as well as the relationship between shear rate and stress of honey samples, were plotted.

Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR)

The infrared spectra of honey in this research were obtained by FTIR spectroscopy analysis with the ATR technique in the resolution of 4 cm^{-1} in the spectral range of 4000 cm^{-1} to 400 cm^{-1} using the Bruker INVENIO-S FTIR Spectrometer. The background spectrum against air was recorded and followed by a drop of the honey sample at the ATR crystal center. The analysis of background was intended to minimize the influence of temporal baseline shifts. The collected spectra were processed and smoothed with baseline correction and normalization using the OPUS software.

Data Analysis

All data analyses were performed in triplicate and reported as mean ± standard deviation. Statistical analysis for this study was performed by one-way analysis of variance (ANOVA) using Sigma Plot version 15.0 (Systat Software Inc., CA, USA), followed by the test of post-hoc Dunnett or analysis of differences with p values ($p < 0.05$) considered statistically significant.

RESULTS AND DISCUSSION

pH

The pH levels of the RH, PH 1, and PH 2 samples analyzed in this study complied with the Malaysian Stingless Bee Standard (pH 2.5 - 3.8) and showed acidic properties [17]. Besides, a statistically significant difference ($p < 0.05$) was observed in the pH values among the honey samples (Table 1). The pH values of RH (3.44 ± 0.015), PH 1 (3.45 ± 0.02), and PH 2 (3.51 ± 0.012) were quite similar to previous research of stingless bee species from Ecuador in the range of pH 3.08 to pH 3.58 [18]. Besides, in the semi-arid region of Brazil, the stingless bee species also showed the range of pH value of the honey as being comparable with this study, such as *Melipona fasciculata* (pH 3.4 - 3.7) and *Melipona subnitida* (pH 3.3 - 3.9) [19]. In particular, PH 2 exhibited the highest pH value compared to RH and PH 1.

The pH of honey plays a crucial role in storage and its antimicrobial properties as it impacts the texture, stability, and shelf life [20]. Organic acids, particularly gluconic acid, are primarily responsible for the low pH of honey, creating an inhospitable environment for many microorganisms that typically thrive in an optimal pH environment of 7.2 to 7.4. These acids function as natural obstacles against the presence and development of microorganisms in honey [21–23]. The low pH not only prevents the growth of microorganisms but also acts as a natural preservative, extending the shelf life of honey [24]. The variations in pH among the honey samples could be attributed to the storage condition and different concentrations or types of acids present in each honey [25].

Overall, while all honey samples in this study showed a similar range of pH values, the small variations in pH highlight the impact of natural factors and storage conditions on the chemical properties of honey. Understanding these influences can help maintain natural qualities and ensure consistent product quality of honey across different batches and sources.

Free Acidity

The free acidity (FA) in honey refers to the amount of free acid contained in the honey that can show the freshness of honey and sensitivity to unwanted fermentation. In our study, the FA values of the honey samples exceeded the maximum limit of 50 meq/kg established by the Codex Alimentarius Commission [14]. However, the free acidity value in the CAC standard was not analyzed from stingless bee honey, yet honey produced by honey bees (*Apis mellifera*). Hence, the data in this study refer to stingless bee honey that could not be directly compared to the standard due to the different species of bees. Among the samples analyzed, PH 1 exhibited the highest FA value (85.33 ± 2.082 meq/kg), followed by PH 2 (65.67 ± 2.082 meq/kg) and RH (67.33 ± 1.528 meq/kg) (Table 1). The FA in this study showed a statistically significant difference ($p < 0.05$) among the honey samples (RH, PH 1, and PH 2).

Table 1. Physicochemical analysis data of acacia stingless bee honey (RH, PH 1, and PH 2) in comparison with Malaysian Standard and International Codex Standard. The results are selected as representative data from three independent experiments. The results are expressed as mean ± STD values. *$p < 0.05$ (vs RH as control). STD: standard deviation, ND: not detected, nd: not determined, HMF: hydroxymethylfurfural.

PARAMETER	RH (Raw)	PH 1 (Processed)	PH 2 (Processed)	MALAYSIAN STANDARD STINGLESS BEE HONEY		CODEX ALIMENTARIUS COMMISSION
				RAW	PROCESSED	
pH	3.44 ± 0.015	3.45 ± 0.02	3.51 ± 0.012 *	2.5 - 3.8	2.5 - 3.8	nd
Free acidity (meq/kg)	67.33 ± 1.528	85.33 ± 2.082 *	65.67 ± 2.082	nd	nd	< 50.0
Moisture content (%)	27.73 ± 0.208	20.30 ± 0.3*	17.57 ± 0.289 *	< 35.0	< 22.0	< 20.0
Insoluble matter (%)	0.69 ± 0.031	0.69 ± 0.116	0.83 ± 0.003	nd	nd	< 0.1
Ash content (g/100 g)	0.22 ± 0.005	0.43 ± 0.069 *	0.40 ± 0.051 *	< 1.0	< 1.0	< 0.5
HMF (mg/kg)	0.06 ± 0.074	151.01 ± 4.373 *	124.64 ± 3.670 *	< 30.0	< 30.0	< 80.0
Glucose (g/100 g)	24.3	24.7	30.9	nd	nd	nd
Fructose (g/100 g)	23.1	22.9	29.9	nd	nd	nd
Sucrose (g/100 g)	ND	ND	ND	< 7.5	< 8.0	< 5.0
Maltose (g/100 g)	1.4	2.3	1.5	< 9.5	< 10.0	nd
Fructose and glucose (sum) (g/100 g)	47.4	47.6	60.8	< 85.0	< 90.0	> 60.0

Higher acidity levels, influenced by factors like sugar fermentation, geographical factors, and floral origin, can indicate potential deterioration and affect the overall quality of honey [26, 27]. Previous research stated that stingless bee honey has higher free acidity values compared to honey produced by *Apis mellifera* [28]. The free acidity in stingless bee honey can range from 5.9 meq/kg to 592 meq/kg [29]. Additionally, specific species of stingless bees produce honey with varying free acidity levels. For instance, honey from *Heterotrigona itama* has free acidity values ranging from 17.0 meq/kg to 336.2 meq/kg, while *Geniotrigona thoracica* was 95.3 meq/kg to 315.3 meq/kg. Apart from these species, *Tetragonula carbonaria* and *Tetragonula hockingsi* have exhibited higher acidity levels of 98.5 to 212.3 meq/kg and 74.1 to 202.0 meq/kg [30, 31], respectively.

The free acidity levels observed in the RH, PH 1, and PH 2 samples can be attributed to the complex balance of organic acids, inorganic esters, and ions, such as chloride, sulfate, and phosphate, that are typically present in honey [32]. Besides, the floral source and mineral composition of honey can affect the normal range of free acidity value and can be influenced by factors in the enzymatic conversion of glucose into gluconic acid. The taste of honey is influenced by its acidity, which results from the fermentation of honey sugars by sugar-tolerant bacteria and osmophilic yeasts [33]. Fructose in honey is converted into CO_2 and alcohol by yeasts, leading to the formation of acetic acid, thereby increasing the overall free acidity [22, 24, 34].

Moisture Content

The moisture content of RH (27.73 ± 0.208 %), PH 1 (20.30 ± 0.3 %), and PH 2 (17.57 ± 0.289 %) fell within the acceptable range defined by the Malaysian Standard for Stingless Bee Honey (SBH) (Table 1). There was a statistically significant difference ($p < 0.05$) in the moisture content among the honey samples. According to the standard, processed honey should have a moisture content below 22%, while raw honey should not exceed 35%. In this study, RH showed the highest moisture content due to the raw nature properties, moreover RH did not undergo any heating or processing treatments. According to previous research, untreated or unprocessed SBH shows a higher fluidity of honey texture [35]. The moisture level observed in RH contributes to the distinct sensory qualities, including its lower viscosity compared to honeybee honey [30]. In addition, there are certain

species of stingless bees from semi-arid regions of Brazil that had been reported in previous research which showed similarities in moisture content with RH in this study. The reported data showed that the moisture content of *Melipona fasciculata* was in the range of 26.4% to 27.9% and *Melipona subnitida* 27.0% [19].

The quality of honey is seen through its physical properties, like viscosity and crystallization, and it can be affected by its moisture content [36]. The existence of water works as an indicator of stability against fermentation and reflects a maturity in honey. Various factors, including climate, season, and the moisture content of the original plant nectar, can influence the moisture content of honey [37]. As a result, honey with a high moisture content can cause enhanced fermentation and change the sensory qualities. The scent and color of honey can change from their natural origins through the fermentation of sugar, including the texture and taste [34,38].

Insoluble Matter

The insoluble matter (IM) content of the honey samples analyzed exhibited the highest value in PH 2, which was 0.83 ± 0.003 %. PH 1 (0.69 ± 0.116 %) and RH (0.69 ± 0.031 %) displayed similar IM values (Table 1). No significant difference ($p > 0.05$) was observed among the honey samples. It is worth noting that the IM values of the honey samples exceeded the maximum level (0.1%) permitted by the CAC. Nevertheless, the insoluble matter value in the CAC standard is not reported from stingless bee honey, yet honey produced by honey bees (*Apis mellifera*). Therefore, the data in this study refer to SBH, which could not be directly compared to the standard due to the different species of bees. Additionally, the IM values for RH, PH 1, and PH 2 align closely with the values reported for stingless bee honey (*Heterotrigona itama*) [16].

IM value serves as an indicator of impurities such as wax, pollen, honeycomb particles, and debris, reflecting the cleanliness of the honey samples analyzed in this study. Despite exceeding the maximum limit of the CAC (0.1%), the IM values of PH 1, PH 2, and RH align closely with reported values for SBH in other studies. This suggests that the higher IM content observed in our samples may be influenced by specific characteristics of SBH production, such as variations in harvesting practices and the natural behaviors of stingless bees during honeycomb deposition. Effective manufacturing practices, including rigorous filtration, meticulous decanting, and careful processing, are crucial for mitigating IM levels and ensuring the quality and purity of stingless bee honey products [39,40].

Ash Content

The ash content of the honey samples analyzed in this study met the acceptable limits of the Malaysian Standards Stingless Bee Honey, which were not more than 1.0 g/100 g, and the CAC (not more than 0.5 g/100 g) [14, 17]. This study showed a statistically significant difference ($p < 0.05$) in ash content among the honey samples. RH showed the lowest ash content value of 0.22 ± 0.005 g/100 g compared to PH 1 (0.43 ± 0.069 g/100 g) and PH 2 (0.40 ± 0.051 g/100 g) (Table 1). The difference and high ash content among the honeys in this study could be attributed to factors such as harvesting techniques, beekeeping practices, and the material collected by the bees during foraging on various floral sources [41]. Additionally, PH 1 and PH 2 underwent processing, which could contribute to their higher ash content compared to RH, as processing and handling of honey may introduce some impurities [53].

In a previous study, ash content from 12 countries for 67 different species of stingless bees was analyzed using 522 honey samples. The results showed that *Tetrigona melanoleuca* exceeded the highest ash content among them, which was in the range of 0.01 g/100 g to 3.1 g/100 g [29]. Ash content acts as a signal for the presence of inorganic substances in honey and can be employed to identify the floral source of the honey. The type of soil in which the nectar-producing plants are grown influences the ash content. Potassium is typically the major mineral contributor to honey, with content varying between 200 and 900 ppm. Ash content is a parameter significant for assessing honey quality since ash content can detect the presence of minerals and identify irregularities, such as lack of hygiene or defects in the decanting or filtration process [34].

Hydroxymethylfurfural (HMF)

The hydroxymethylfurfural (HMF) values of three honey samples, specifically RH, PH 1, and PH 2, were examined (Table 1). The HMF values for PH 1 and PH 2 were 151.01 ± 4.373 mg/kg and 124.64 ± 3.670 mg/kg, respectively. It is important to note that both values exceeded the maximum limits set by the CAC (80 mg/kg) and the Malaysian Standard for Stingless Bee Honey (30 mg/kg) [17]. The elevated HMF levels in PH 1 and PH 2 can be attributed to temperature fluctuations occurring during the production process, as both kinds of honey underwent processing [34]. In contrast, RH, which remained in its raw state without undergoing any heating or dehumidification process, displayed the lowest HMF value of 0.06 ± 0.074 mg/kg. The data in this study showed statistically significant differences ($p < 0.05$) in the HMF values among the honey samples (RH, PH 1, and PH 2) analyzed.

The slightly higher HMF content observed in PH 1 compared to PH 2 can be attributed to the higher free acidity present in PH 1. The catalysis of free acids, leading to the dehydration of glucose and fructose, is the primary factor contributing to the formation of HMF. This process occurs during heating and results

in the generation of HMF, a heat-induced toxic compound, along with an increase in the acidity and pH values of honey [41].

Sugar Content

HPLC chromatograms were used to analyze the sugar profile of the honey samples (RH, PH 1, and PH 2) (Figure 1). The main sugars examined were fructose, glucose, sucrose, and maltose. It is worth noting that honey typically contains around 80% sugar and less than 20% water, with other compounds contributing to color and taste in smaller amounts. Figure 1 shows that glucose was the predominant monosaccharide in the honey samples, accounting for 24.3 % in RH, 24.7% in PH 1, and 30.9% in PH 2. Fructose was the second most abundant monosaccharide, with percentages of 23.1% in RH, 22.9% in PH 1, and 29.9% in PH 2. The fructose-to-glucose ratio, which ideally falls within the range of 0.9 to 1.35, was 0.93, 0.97, and 0.95 for PH 1, PH 2, and RH, respectively. These ratios indicate a higher likelihood of honey crystallization. The fructose-to-glucose ratio influences the rate of honey crystallization. Specifically, when the fructose-to-glucose ratio is below 1.0, the crystallization process is observed to occur at a faster rate. Conversely, when this ratio exceeds 1.0, the crystallization process slows down [42].

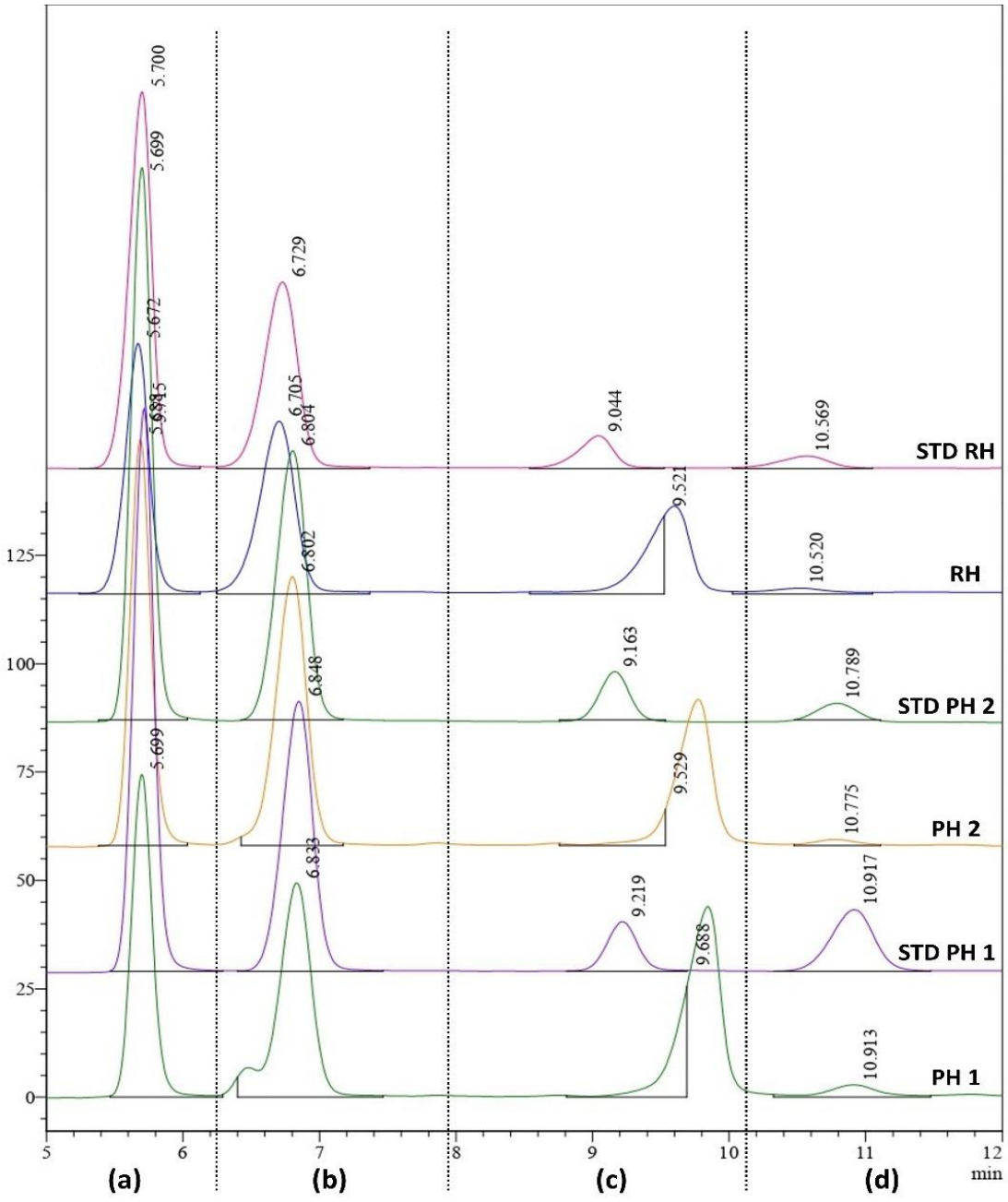

Figure 1. HPLC chromatograms for sugar profile of acacia stingless bee honey (RH, PH 1, and PH 2). The division of labeled areas (a), (b), (c), and (d) shows the retention time of fructose, glucose, sucrose, and maltose peaks, respectively, appear based on the standard sugar (STD).

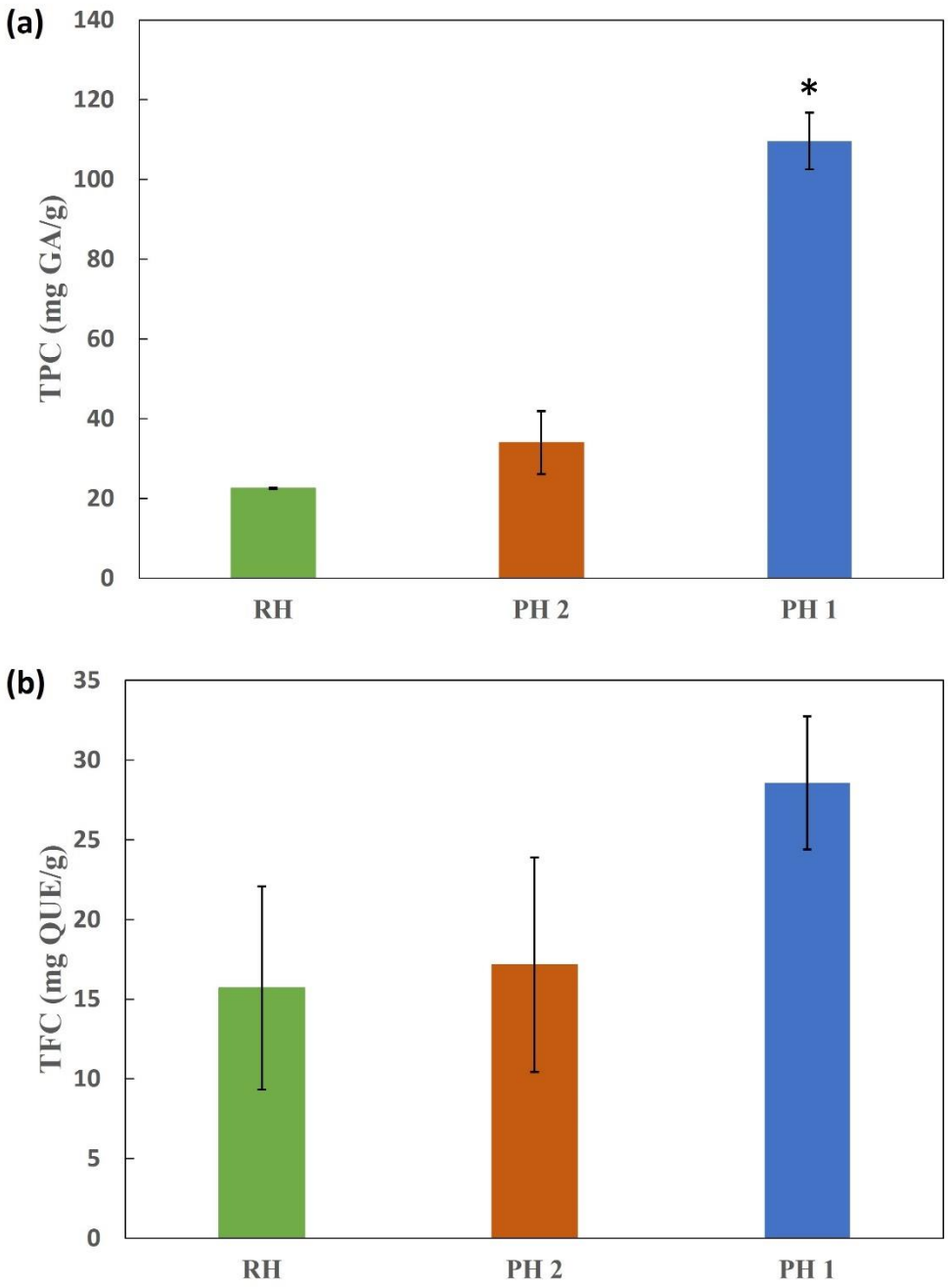

Figure 2. (a) Total phenolic content and (b) total flavonoid content of RH, PH 1, and PH 2. The graphs are selected as representative data from three independent experiments. Results are expressed as mean ± STD values. *$p < 0.05$ (vs RH as control).

Sucrose was not detected in any of the honey samples, which is consistent with its low presence in honey due to the action of the enzyme invertase [43]. Maltose content was found in all samples, with PH 1 exhibiting the highest value of 2.3 g/100 g, followed by PH 2 (1.5 g/100 g) and RH (1.4 g/100 g). It is important to note that the presence of high sucrose content in honey may indicate adulteration [44]. The sum of fructose and glucose content was highest in PH 2 (60.8 g/100 g), followed by PH 1 (47.6 g/100 g) and RH (47.4 g/100 g). PH 2 met the standard set by the CAC, which requires a minimum sum of fructose and glucose content of 60 g/100 g. However, it is noteworthy that studies have reported lower total sugar content in stingless bee honey from different regions. These variations in sugar content can be influenced by processing methods, storage conditions, botanical sources, and geographical origin, and they contribute to the differentiation of honey samples [13, 45, 46].

Total Phenolic Content (TPC)

In this study, TPC in the honey samples was investigated and revealed a statistically significant difference ($p < 0.05$) in the TPC of the honey samples

examined. Among the honey samples, PH 1 exhibited the highest TPC level, with a value of 109.68 ± 7.109 mg GAE/g. PH 2 had a TPC of 33.98 ± 7.878 mg GAE/g, while RH showed a lower TPC value of 22.54 ± 0.242 mg GAE/g (Figure 2(a)). The variation in phenolic content among the samples could be attributed to different geographical factors [47].

Generally, honey contains a range of approximately 56 to 500 mg/kg of total polyphenols, which contributes to its major antioxidant properties [48]. In our study, processed honey (PH 1 and PH 2) exhibited higher TPC values compared to raw honey (RH), which is consistent with previous research. For instance, treatments such as dehumidification and microwave techniques have been shown to significantly increase the total phenolic content in SBH by more than 43% compared to untreated honey [35].

Phenolic compounds, which are secondary metabolites found in plants, are crucial for the antioxidant activity and non-peroxide antimicrobial properties of honey. These compounds are chemically characterized by an aromatic ring bound to one or more hydroxyl groups [49]. Besides, phenolic compounds in honey come from the plants that bees visit to collect nectar and are affected by factors such as dominant flowering plants, climate, soil conditions, and geographical origin [50]. These compounds are sort of plant protectors that minimize the effects of temperature changes, light levels, water content, UV exposure, and mineral deficiencies in plants [30, 51-52].

The elevated TPC in processed honey (PH 1 and PH 2) compared to raw honey (RH) suggests that the processing methods may enhance the preservation of phenolic compounds. This increased phenolic content in processed honey could contribute to its higher antioxidant capacity and potentially greater health benefits. However, further studies are needed to fully understand how different processing techniques affect the phenolic profile and overall quality of honey.

Total Flavonoid Content (TFC)

PH 1 exhibited the highest total flavonoid content (TFC) with a value of 28.55 ± 4.173 mg QUE/g, whereas PH 2 and RH displayed slightly lower flavonoid concentrations at 17.16 ± 6.722 mg QUE/g and 15.71 ± 6.371 mg QUE/g, respectively (Figure 2(b)). Importantly, no statistically significant difference ($p > 0.05$) was observed when comparing the levels of TFC among the honey samples. Similar to phenolic acids, the flavonoid content followed a similar pattern, with the highest level observed in PH 1, followed by PH 2 and RH. This distinction can be attributed to the honey types, as PH 1 and PH 2 are processed honey, while RH is raw honey. However, it is important to highlight that the TFC values obtained in this study were lower compared to the TPC values, as phenolic acids have been identified as the primary antioxidants in honey [53].

Flavonoids are one of the antioxidant activity contributions for honey. They fall under the subclass of plant pigments that are polyphenolic compounds synthesized from the amino acid phenylalanine. Subclasses of flavonoids include catechins, anthocyanins, flavanones, flavones, flavonol glycosides, flavanone glycosides, flavonols, and isoflavones [54]. The levels of flavonoids in honey are affected by botanical and geographical factors, climate, and environmental conditions such as humidity, temperature, as well as soil composition [55, 54].

In our study, the higher TFC observed in processed honey (PH 1 and PH 2) compared to raw honey (RH) may suggest that the processing methods have a positive effect on the flavonoid content. This could enhance the antioxidant properties of processed honey. However, the lack of statistical significance in TFC among the samples indicates that while processing affects flavonoid levels, it may not significantly alter the overall flavonoid profile compared to phenolic compounds.

Rheological Properties

This study focused on analyzing the relationships of viscosity and stress against shear rate in the rheological behavior of honey (RH, PH 1, and PH 2). This analysis was carried out under controlled conditions and maintained a constant temperature of 30°C. Figure 3 (a) shows the correlation of shear rate with viscosity in PH 1, PH 2, and RH, which are explained as changes in viscosity and shear rate that were different. Among the analyzed honey samples, a consistent pattern appeared in PH 1 and PH 2, where an increase in shear rate corresponded to a decrease in viscosity. The viscosity of PH 1 and PH 2 can be seen to decrease with increasing shear rate. The graph shows that the shear-thinning behavior was observed in PH 1 and PH 2, indicating a pseudo-plastic or shear-thinning fluid. However, some infrequent substances exhibit the opposite characteristic known as shear-thickening, where increasing viscosity with increasing shear rate. The change of shear-thinning behavior to shear-thickening behavior of substances is affected by concentration and controlled by certain factors such as shape, particle size, and distribution [56].

In contrast, RH exhibited different rheological behavior compared to PH 1 and PH 2. RH showed a slight rise in viscosity when the shear rate increased, including demonstrated dilatant properties. The increasing shear rate can make the viscosity of honey able to be observed in a short time [57]. This is consistent with a claim regarding the dilatant properties of honey. RH showed rheological properties that point to a more complex, departing from the usual Newtonian liquid behavior connected to honey. The results in this study are compatible with the previous

studies that reported thixotropic and dilatant behavior in some honey varieties [58].

The distinct types of honey, like processed honey (PH 1 and PH 2) and raw honey (RH), has become a factor in the occurrence of rheological differences between them. Various treatment and filtering methods are involved in processed honey that can affect its composition and properties. Besides, moisture content becomes one of the factors that cause the variation in rheological behavior in honey. Processed honey (PH 1 and PH 2) usually undergoes a dehydration process, resulting in a lower moisture content than raw honey (RH). Moisture content has a considerable effect on the viscoelastic properties and temperature of honey, influencing its rheological behavior [59]. As a result, the lower moisture content of processed honey (PH 1 and PH 2) could explain their increased viscosity compared to RH. A previous study has demonstrated that honey exposed to severe ultrasonic heat impacts undergoes increased particle contact, resulting in a decrease in moisture content and an increase in elasticity properties [60].

The shear stress values of the honey samples (RH, PH 1, and PH 2) at various shear rates are presented in Figure 3 (b). RH, PH 1, and PH 2 exhibited an increase in shear rate with increasing stress, signifying Newtonian flow behavior. Notably, PH 2 demonstrated the highest stress level, followed by PH 1 and then RH. This discrepancy in stress levels can be attributed to variations in moisture content, with PH 2 possessing the lowest moisture content (17.57%). The lower moisture content in PH 2 tends to have higher stress values, as moisture content plays the main part in determining the rheological behavior of honey. The lower moisture content increased the viscosity and stress levels of honey. As a result, the observed trend of RH < PH 1 < PH 2 in terms of stress levels can be attributed to differences in moisture content, with PH 2 possessing the lowest moisture content among the three honey samples.

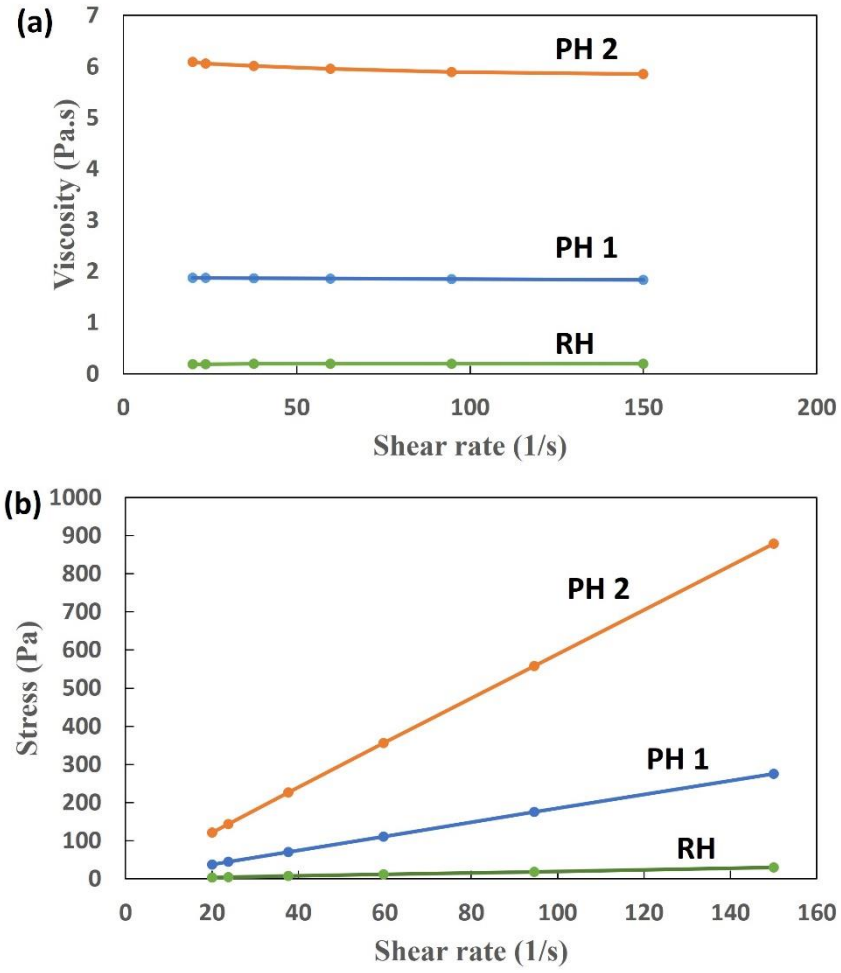

Figure 3. (a) Relationship between shear rate and viscosity and (b) relationship between shear rate and stress of RH, PH 1, and PH 2 with cyclically changing the shear rate in ascending and descending at 6-time points.

This study shows that the stress level of honey is increasingly affected by the amount of water in it, where higher moisture content influences the rheological properties of honey. The variations in stress levels between PH 1, PH 2, and RH might depend on several factors, including colloids, crystals, botanical origin, and processing conditions. Besides, the rheological behavior of honey is known as an indicator of molecular association and is affected by intermolecular interactions. Rheological parameters are further influenced by time, stress, shear rate, and temperature, all crucial considerations in manufacturing processes and equipment design [34, 61].

ATR-FTIR

ATR-FTIR spectroscopy was employed in this study to compare RH, PH 1, and PH 2 based on their spectral differences between the region of 4000 cm^{-1} to 400 cm^{-1}. The absorption peaks identified the chemical structures and specific functional groups in the honey samples. Figure 4 shows representative ATR-FTIR spectra of the three honey samples (RH, PH 1, and PH 2). The results revealed characteristic peaks and signals corresponding to different functional groups present in PH 1, PH 2, and RH. Broad peaks at 3263.20 cm^{-1} (PH 1), 3259.85 cm^{-1} (PH 2), and 3251.48 cm^{-1} (RH) indicated the presence of water, specifically the O-H stretching vibration [62]. The O-H stretching peak is commonly discovered in honey samples and has been disclosed in other studies. The peaks in the range of 2932 cm^{-1} to 2935.08 cm^{-1} were the significant signal corresponding to the C-H stretching of carboxylic acids and NH_3 stretching of free amino acids present in the honey samples [63]. These signals show the characteristics of organic acid and amino acid content that are found in honey. The peaks at around 1641 cm^{-1} were attributed to the O-H bending vibrations of water and a small number of protein molecules. These peaks were also reported in previous studies and give the overall spectrum of the honey samples (RH, PH 1, and PH 2) [45].

The spectral range around 1400 cm^{-1} to 700 cm^{-1} has been shown to be the absorption range of honey sugars [64]. This region represents the presence of monosaccharides (glucose and fructose) and the disaccharide sucrose. In particular, peaks at around 1148.13 cm^{-1} (PH 1), 1147.52 cm^{-1} (PH 2), and 1148.66 cm^{-1} (RH) are considered characteristic of sucrose. The vibrations in the 775 cm^{-1} to 920 cm^{-1} range, corresponding to C-H bending, are indicative of the presence of saccharides in all three honey samples. This region provides information about the glycosidic bonds and can indicate modifications or differences in the sugar fraction [16, 65].

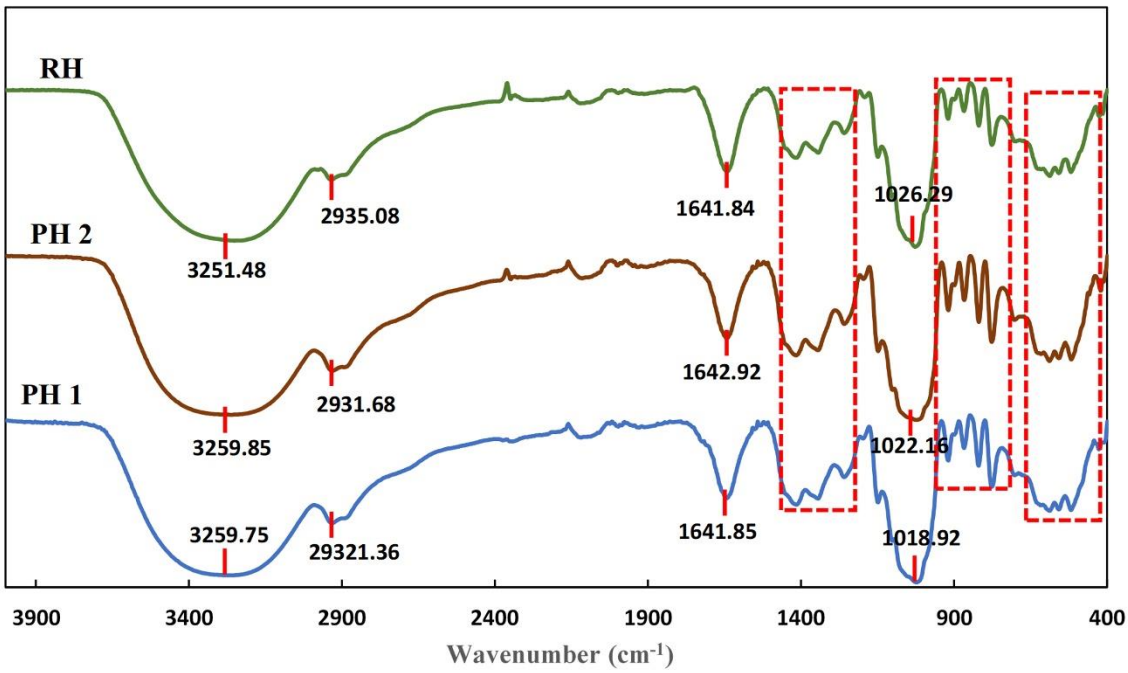

Figure 4. ATR-FTIR spectra of acacia stingless bee honey (RH, PH 1, and PH 2) in the spectral range of 4000 cm^{-1} – 400 cm^{-1}.

CONCLUSION

This study successfully analyzed the effects of processing on Malaysian Acacia Stingless Bee Honey, highlighting relative changes in physicochemical parameters, antioxidant properties, and rheological behavior among RH, PH 1, and PH 2. It was found that processing significantly affects honey quality and freshness. HMF levels in processed honey PH 1 (151.01 ± 4.373 mg/kg) and PH 2 (124.64 ± 3.670 mg/kg) exceeded safety limits, while RH had a much lower level (0.06 ± 0.074 mg/kg), likely due to temperature fluctuations during heating. The antioxidant properties showed that PH 1 displayed the highest TPC and TFC, indicating a positive effect of processing methods, like dehumidification, on these values. Additionally, the rheological analysis revealed that PH 1 and PH 2 are pseudo-plastic fluids, contrasting with the dilatant behavior of RH. ATR-FTIR spectroscopy revealed similar functional group signals among all samples. This study demonstrates that the processing of honey for commercial use impacts its quality and freshness. However, it is important to note that the processing does not introduce any trace of adulteration. Nonetheless, to address storage and logistics issues, honey must be processed to prevent the fermentation process due to yeast activity and excessive moisture content.

ACKNOWLEDGMENT

The authors wish to thank the Ministry of Higher Education for financial assistance under the Prototype Research Grant Scheme (PRGS/1/2021/STG05/UMT/02/1) and the Centre of Research and Field Services (CRaFS), Universiti Malaysia Terengganu for providing the facilities in undertaking this work.

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