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Tetragonula laeviceps honey: Biochemical composition and kinetics of 5-hydroxymethylfurfural formation

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Abstract:
The emergence of a new bee species, *Tetragonula laeviceps*, in Indonesia has attracted scientific attention that promoted further exploration. We aimed to analyze changes in the biochemical composition of *T. laeviceps* honey stored at different temperatures and to study the kinetics of hydroxymethylfurfural formation.

T. laeviceps honey was stored at 25, 50, and 80°C for 6 h. The pollen sources were identified using the melissopalynology method, followed by a biochemical analysis using UHPLC-DAD-ESI/MS. The kinetics of hydroxymethylfurfural formation were analyzed using the Arrhenius equation applied to zero-, first-, and second-order reactions.

T. laeviceps honey was multifloral (three or more pollen types, each with < 16% frequency), with dominant *Zea mays* spp. *Mays* (L.) (40.24%) and *Vigna unguiculate sesquipedalis* (L.) (22.52%). Heating at 80°C significantly ($p < 0.05$) increased phenolic acids, flavonoid acids, total phenolics, total flavonoids, and hydroxymethylfurfural, as well as significantly ($p < 0.05$) degraded diastase, invertase, glucose oxidase, and DPPH. Heating at 50°C only had a significant impact on hydroxymethylfurfural and diastase. Ferulic acid and kaempferol compounds dominated in the phenolic and flavonoid acids in all the samples. The kinetics of hydroxymethylfurfural formation followed a first-order reaction, with specific rate constants of 0.1098/h (25°C), 0.0597/h (50°C), and 0.0053/h (80°C), involving an activation energy of 69.23 KJ/mol.

This study highlights the impact of storage and heating on the chemical composition of Klanceng honey. Our findings provide practical guidance for improving honey production and storage, while enhancing the commercial value of *T. laeviceps*.

Keyword: Klanceng honey, melissopalynology, activation energy, pollen, bioactive enzymes, phenolics, flavonoids

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INTRODUCTION

The emergence of a new bee species, *Tetragonula laeviceps*, has garnered significant attention among researchers and beekeepers. This species offers an extraordinary potential for exploration, particularly regarding the active biochemical compounds found in its honey [1]. However, the available information remains very limited. This lack of data indicates an urgent need for in-depth research to uncover the full potential of *T. laeviceps* honey, as well as its possible health benefits and commercial applications.

Honey research often focuses on the impact of storage and heating, as well as the kinetics of compound degradation [2]. One of the main challenges is gaining a deep understanding of the botanical sources of honey, which can affect its chemical composition and quality. The melissopalynology method, which is used to identify the pollen sources within honey [3], offers essential insights into the variety of plants visited by bees and how this influences the characteristics of honey. Unfortunately, many previous studies do not include this method, so information about the pollen's sources is

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often overlooked. In this study, we incorporated the melissopalynology method to understand the sources of pollen in honey and the variation in its composition related to different plants visited by bees.

Previous studies have extensively reported on the composition of active compounds in various types of honey. The kinetics of hydroxymethylfurfural (HMF) formation has also been a focus of research due to its importance as an indicator of honey quality [4, 5]. However, few studies have specifically explored individual phenolic compounds and flavonoids, or the kinetics of HMF formation, in the honey from *T. laeviceps*. This information may promote further research that can enrich our understanding of the honey produced by this bee species.

A high sugar content is the main challenge facing the optimization of the analysis protocol to determine individual phenolic and flavonoid compounds in honey. It can interfere with the analysis results by masking or affecting the detection of these compounds, complicating the extraction process [6]. This makes their separation from the honey matrix difficult, affecting the accuracy and reliability of the analysis [7]. Moreover, not all phenolic and flavonoid compounds are detected in honey, especially those in low concentrations (e.g., 3,4-dihydroxybenzoic acid and rutin).

Our study aimed to analyze changes in the biochemical composition of Klanceng honey during its storage at 25 and 50°C, as well as during its heating at 80°C for 6 h. Additionally, we studied the kinetics of HMF formation in *T. laeviceps* honey. Our results can provide deep and comprehensive insights into the quality of Klanceng honey.

In addition to enriching the scientific knowledge regarding the chemical composition and quality of Klanceng honey, our study can provide a practical guidance for beekeepers in optimizing honey production and storage processes. Our findings can also lay the groundwork for further research and broader commercial application, enhancing the economic value of *T. laeviceps* in both local and global markets.

STUDY OBJECTS AND METHODS

Two kilograms of Klanceng honey was taken from a bee farm in Jambangan village (Indonesia) on March 27, 2024. The sampling area was located at S7°40'39.47232" and E111°24'15.2247". The sample was placed in a large container for homogenization. Then, it was divided into three parts.

The samples were transferred into small glass containers, marked, and sealed until the analysis. Each sample was subdivided into three parts to be analyzed over 6 h of storing at 25 (control), 50, and 80°C.

Pollen observations were conducted using the acetolysis methods [8] with an addition of 30% glycerin. The mixture was stirred, and drops of the pollen-containing solution were placed on a microscope slide (CX-23 optilab). Melissopalynological analysis followed Bandeira *et al.* [9] and involved both melissopalynological and biochemical analyses to identify the food source of the Klanceng bee (*Tetragonula laeviceps*).

The geographical origin of the honey was determined according to Budianto *et al.* [10]. 200–300 pollen grains were analyzed and identified with a frequency categorized as follows: predominant (over 45%), secondary (16–45%), important minor pollen (3–15%), and minor pollen (less than 3%). The botanical origin was inferred from the pollen and honeydew frequencies. Within a 500 m radius, the plant spread percentage was calculated as:

$$\text{Plant spread} = \frac{\text{Number of plants found}}{\text{Total number of plants}} \quad (1)$$

As for nectar-producing plants, one or two flowers were sampled to check for nectar by opening the flower and examining the liquid nectar at the flower base without measuring its volume. Extrafloral nectar was assessed by examining the liquid nectar secreted by nectar glands that develop on stems, leaves, or other plant parts.

As for pollen-producing plants, one or two flowers were sampled to examine the pollen on the anther. The pollen, usually in powder form and of yellow color, was not weighed. According to Nuraeni *et al.* [11], honey types were classified as monofloral (one pollen species with > 45% frequency), bifloral (two pollen types with > 22.25% frequency each), and multifloral (three or more pollen types with < 16% frequency each).

Phenolic compound analysis. Due to the complexity of Klanceng honey, a cleanup step was necessary to remove interfering substances before Ultimate 3000 ultra-high-performance liquid chromatography (UHPLC). This method was adapted from Wang *et al.* [12] and involved diluting about 10 g of honey with five parts of acidifier water (pH = 2, adjusted with HCL) and homogenizing it with approximately 10 g of Amberlite XAD-2 resin for 30 min using a magnetic stirrer. The mixture was then passed through a glass column (25 × 2 cm) with a PTFE tap. Phenolic compounds remained in the column, while sugars and other polar substances were washed out using 100 mL of acidified water followed by 100 mL of distilled water. Sugar removal and optimal wash volumes were checked using high-performance liquid chromatography (HPLC) with an evaporative light scattering detector (ELSD). The setup included an HPLC Surveyor Plus system with a Surveyor autosampler, a Surveyor LC Pump, and an ELSD (Alltech 3300 ELSD). Sugars were separated chromatographically using an APS-2-Hypersil amine bonded phase column (250 × 4.6 mm; 5 µm) at 30°C with an isocratic elution of acetonitrile-water (80:20) at a flow rate of 0.85 mL/min. The phenolic fraction was eluted with about 75 mL of methanol and evaporated to dryness under reduced pressure at 40°C using a Multivapor P-6 concentrator. Then, it was redissolved in 3 mL of methanol, filtered through a 0.45-µm PTFE membrane, and analyzed by UHPLC-DAD-ESI/MS for the phenolic profile.

Phenolic profile by UHPLC-DAD-ESI/MS. Experiments were conducted using an Ultimate 3000 UHPLC system with a Q Exactive™ Focus Hybrid Quadrupole-

Orbitrap mass spectrometer and a HESI probe (ThermoFisher Scientific, USA). Separation was performed on Accucore PFP columns (50 × 2.1 mm, 2.6 µm and 100 × 2.1 mm, 2.6 µm). The mobile phase consisted of water with 0.1% formic acid (solvent A) and methanol with 0.1% formic acid (solvent B). The UHPLC gradient was as follows: 0–2 min, 98–50% A; 2–8 min, 50–30% A; 8–15 min, 30–2% A; 15–17 min, 2–98% A; 17–30 min, 98% A. The flow rate was 0.3 mL/min and the injection volume was 10 µL. All the samples were filtered with a 0.45 µm PTFE membrane filter before injection. The mass spectrometer operated in the negative mode with a spray voltage of 2.5 kV and a capillary temperature of 320°C. Mass spectra were scanned in the range of 120–1000 m/z.

Phenolic acids and flavonoids were identified and quantified based on their mass spectra, accurate mass, and retention time. Collision-induced dissociation was performed for confirmation, with a collision energy of 15–60 eV. Stock standard solutions of phenolic acids, flavonoids, and trans-resveratrol (100 mg/L) were prepared in methanol and calibration curves showed good linearity ($R^2 > 0.99$). The protocol efficiency was analyzed through recovery tests, with precision assessed from replicate measurements (n = 6) of the same honey sample, and expressed as a relative standard deviation (RSD).

Total phenolic content. Phenolic compounds were analyzed using the Folin-Ciocalteu reagent and following a modified method by Nikolaeva *et al.* [13]. Briefly, 100 µL of a honey ethanolic solution (1 g honey dissolved in 10 mL of 80% ethanol) was mixed with 4 mL of deionized water and 100 µL of the Folin-Ciocalteu reagent to incubate at room temperature (25°C) for 5 min. Next, we added 300 µL of a 20% sodium carbonate solution. The mixture was homogenized and incubated in the dark for 2 h at 25°C. The absorbance was then measured at 765 nm using a UV-visible spectrophotometer. The total phenolic content was calculated by comparing the sample's absorbance to a calibration curve made with gallic acid standard (0–620 mg/L in 10% ethanol) and expressed as 1 mg gallic acid equivalents per kg honey (mg GAE/kg).

Total flavonoid content. The analysis followed a modified method from Özkök *et al.* [14]. Briefly, 500 µL of a 10% honey ethanolic solution was mixed with 450 µL of $AlCL_2$ (25 g/L), 500 µL of $NaCH_3COOH$ (100 g/L), and 4 mL of deionized water. After incubating for 15 min at 25°C, a flavonoid-aluminum complex formed, and its formation was measured at 430 nm using a UV-visible spectrophotometer. A calibration curve was prepared using quercetin (0–130 mg/L) as the standard (1 mg quercetin equivalent per kg of honey), with results reported as the mean of duplicate assays.

Diastase activity. The analysis followed the procedure established by the International Honey Commission, using a spectrophotometer at an absorbance of 620 nm. The diastase number (DN) was equivalent to diastase units/gram honey.

$$DN = 35.2 \times \text{Absorbance (620)} - 0.46 \quad (2)$$

Invertase activity. The analysis followed the procedure established by the International Honey Commission, using an EnzyChrom control sample (EIT-100_AS). The test was carried out in the pH range of 4-5, using the spectrophotometric method at 560–570 nm, with a glucose calibration standard of 0–100 µm. The incubation time ranged from 20 to 30 min.

$$\text{Invertase} = \frac{A_{\text{sample}} - A_{\text{control}}}{slope \text{T}} \quad (3)$$

where A_{sample} is the absorbance of the sample; A_{control} is the absorbance of the control; T is the incubation time.

DPPH. The analysis followed a method modified by Amor *et al.* [15] to determine DPPH. A Klanceng honey sample was dissolved in hot water (40°C) and mixed with methanol with 2 mL of DPPH (0.1 mM). Distilled water was used for the control (substitute for extract). The samples were stored in the dark at room temperature (25°C) for 60 min. The analysis was performed using a spectrophotometer with an absorbance of 517 nm.

$$\text{DPPH} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \quad (4)$$

Antioxidants in the honey sample (IC_{50}) showed a decrease in the number of DPPH radicals (50%) from the initial concentration of the sample, mg/mL.

Hydroxymethylfurfural (HMF) was determined according to the Indonesian National Standard established in line with the International Honey Commission. Its working principle was to compare the difference in absorbance at 285 and 336 nm with a comparison solution of sodium bisulfite ($NaHSO_3$). For this, a 5 g sample was mixed with 0.50 mL of a Carrez I solution, and then 0.50 mL of Carez II was added to the mixture. After that, a few drops of alcohol were added to remove the foam. Then, 5 mL of the solution was taken for analysis. The samples (g) were measured by a spectrophotometer at 285 and 336 nm.

$$\text{HMF} = \frac{(A_{285} - A_{336}) \times \text{Dilution factor}}{\text{Sample weight}} \quad (5)$$

Glucose oxidase. The activity of glucose oxidase (GO) was determined according to the method described by Hamad *et al.* [16]. and expressed in µg H_2O_2/g honey. The test was conducted using colorimetry at a wavelength of 500 nm for 5 min.

Kinetics of biochemical degradation. The coefficient of determination (R^2) and the rate of degradation constant (K_t) were calculated based on the relationship between zero-order, first-order, and second-order reactions [17]:

$$\text{Zero-order reaction: } CA = C_0 - k_0 \times t \quad (6)$$

$$\text{First-order reaction: } CA = C_0 \times e^{-k_1 \cdot t} \quad (7)$$

$$\text{Second-order reaction: } CA = (k_2 \times t + \frac{1}{C_0})^{-1} \quad (8)$$

where C_0 is the initial concentration of Klanceng honey; *CA* is the biochemical concentration after being heated for *t*; *t* – the duration of heating, h; k_0, k_1, and k_2 – the rate constants of biochemical degradation reactions at zero order, first order, and second order. The half-life ($t_{1/2}$) is the time required for the degradation of 50% of the initial concentration (C_0). Its determination was adjusted to the order of the reactions that occur, namely based on the largest R^2 value.

$$\text{Zero-order reaction: } t_{1/2} = \frac{C_0}{2k_0} \quad (9)$$

$$\text{First-order reaction: } t_{1/2} = \frac{0.693}{k_1} \quad (10)$$

$$\text{Second-order reaction: } t_{1/2} = \frac{1}{C_0 \cdot k_2} \quad (11)$$

The type of reaction order was based on the largest R^2 value. The effect of temperature (*T*) and the value of *k* were determined by the Arrhenius equation (Eq. (11)). Activation energy (*Ea*) was determined by a linear regression of the Ln*k* curve to 1/*T*.

$$\text{Ln}k = \text{Ln°K} - \left(Ea/RT\right) \quad (12)$$

where *Ea* is the activation energy for the reaction, KJ/mol; *R* is the gas constant (8.314 J/mol°K); *T* is the reaction temperature, °K; *k* is the reaction constant.

Statistical test. The ANOVA test was employed to assess the average value, range of variation, and standard deviation. Concurrently, the post-hoc Tukey HSD test was utilized to detect disparities in biochemical degradation attributed to the heating effect, with a significance level at $p < 0.05$. The correlation analysis among biochemical compounds was conducted using SPSS version 26, employing a bivariate Spearman correlation with a two-tailed approach. Furthermore, the categorization of biochemical compounds based on heating temperatures was visualized using a Chord diagram generated with Origin Pro 2024.

RESULTS AND DISCUSSION

Plant origin. The melissopalynology method was employed to identify the source of pollen in honey, offering insights into the variety of plants visited by the Klanceng bee (*Tetragonula laeviceps*). The findings from the melissopalynological examination are presented in Table 1.

Based on the analysis of pollen frequency, we identified that secondary pollen originated from two plant species, namely *Zea mays* spp. *Mays* (L.) and *Vigna unguiculate sesquipedalis* (L.). Additionally, three important plant types indicated the presence of minor pollen, while the rest had only a small amount of pollen (less than 3%). Although our observations were limited to a 500 m radius, we did not find the species *Cocos nucifera* (L.). However, we noted a pollen frequency of 1.80%, which may indicate the potential presence of this species beyond our observation radius. These findings suggest a possibility of a broader range of movement for the Klanceng bee, which is consistent with our previous study [10].

The dwarf honey bee, also known as the *T. laeviceps* bee, is one of the two species of small wild honey

Table 1 Botanical origins of the Klanceng (*Tetragonula laeviceps*) bee's diet in Magetan (Indonesia) in 2024 (melissopalynological examination)

Local name	Scientific name	Family	Pollen frequency, %	Plant spread, %	Source	
					Pollen	Nectar
Corn	*Zea mays* spp. *Mays* (L.)	Poaceae	40.24	28.72	+	–
Noodle bean	*Vigna unguiculate sesquipedalis* (L.)	Fabaceae	22.52	25.02	+	–
Papaya	*Carica papaya* (L.)	Caricaceae	14.82	11.80	+	+
Egg plant	*Solanum melongena* (L.)	Lauraceae	9.47	10.80	+	+
Mango	*Mangifera indica* (L.)	Anacardiaceae	4.23	2.98	+	+
Water apple	*Syzygium aqueum* (L.)	Myrtaceae	2.15	2.45	+	+
Rambutan	*Nephelium lappaceum* (L.)	Sapindaceae	2.10	0.78	+	+
Coconut	*Cocos nucifera* (L.)	Araceae	1.80	n.d.	+	+
Guava	*Psidium guajava* (L.)	Myrtaceae	2.67	0.62	+	+
Cottonwood	*Ceiba pentadra* (L.)	Malvaceae	n.d.	1.24	–	+
Mahogany	*Swietenia macrophylla* (L.)	Maliaceae	n.d.	0.45	–	+
Avocado	*Persea Americana* (L.)	Lauraceae	n.d.	1.82	–	+
Melinjo	*Gnetum gnemon* (L.)	Gnetaceae	n.d.	1.43	–	+
Sapodilla	*Manikara zapota* (L.)	Sapotaceae	n.d.	0.87	–	+
Sengon	*Albizzia falcata* (L.)	Mimosoidae	n.d.	0.24	–	+
Breadfruit	*Artocarpus altilis* (L.)	Moraceae	n.d.	1.22	+	+
Red chili	*Capsicum annum* (L.)	Solaneceae	n.d.	5.43	+	+
Sonokeling	*Dalbergia latifolia* (L.)	Leguminoseae	n.d.	2.13	–	+
Lamboro	*Leucaena leucocephala* (L.)	Fabaceae	n.d.	2.00	+	+

n.d. – not detected

bees found in South and Southeast Asia. Its distribution is much wider than that of its sibling species, *Apis andreniformis*. Another species, *Apis florea*, which was first identified in the late 18th century, is unique due to its distinct morphology, foraging behavior, and defense mechanisms such as generating a piping sound. *A. florea* builds an open nest and forms small colonies, making them more vulnerable to predation compared to cavity-dwelling species with a larger number of defensive workers. The distinctive characteristics of the *T. laeviceps* bee, as shown in Fig. 1, include a body length of 7–8 mm and a wing length of 8–10 mm. Worker bees have lighter-colored wings compared to guard bees. Another notable feature is that the *T. laeviceps* bees produce a substantial amount of propolis.

Optimization of the analytical protocol for determining individual phenolic compounds. Amberlite XAD 2 sorbent was chosen for its effectiveness in extracting non-polar and low-polarity compounds from aqueous samples. It is therefore suitable for selectively isolating analytes from the honey matrix. One of the main challenges in analyzing phenolic compounds in honey is a high sugar content and other impurities. To address this, the honey samples were dissolved in acidified water to denature the proteins. The resulting impurities and denatured proteins were then removed by centrifugation. The supernatant was combined with Amberlite XAD 2 resin to wash away polar substances and sugars using a glass column. The removal of sugars from the column was verified using the HPLC-ELSD method for sugar detection and quantification. This process helped determine the optimal washing volumes, which were established at 100 mL of acidified water and 100 mL of distilled water.

The details of the analytical procedures used in this method are provided in Table 2. The recovery rates for each identified phenolic compound varied, amounting to 65–75% for phenolic acids and 75–100% for flavonoids.



a b c d

Figure 1 Distinctive characteristics of *Tetragonula laeviceps* bees: (a) body length; (b) shape and color of worker bees; (c) shape and color of guard bees; and (d) shape and size of propolis in the maintenance hive

Table 2 Parameters and analytical performance of the method used for quantifying individual phenolic compounds

Compounds	Liniarity, mg/L	Correlation coefficient (R^2)	Limit of detection, mg/L	Limit of quantification, mg/L	Precision, %	Recovery, %
Gallic acid	0.5–5.0	0.9977	0.0010	0.0050	3.8500	75
3.4-Dihydroxybenzoic acid	0.5–10.0	0.9985	0.0040	0.0120	4.8500	76
p-Hydroxybenzoic acid	0.5–7.5	0.9989	0.0100	0.0500	3.3400	74
Chlorogenic acid	0.5–10.0	0.9939	0.0010	0.0060	2.8800	75
Caffeic acid	0.5–10.0	0.9993	0.0010	0.0040	4.1200	76
Syringic acid	0.5–7.5	0.9989	0.0090	0.0750	3.7500	78
p-Coumaric acid	0.5–7.5	0.9987	0.0010	0.0040	4.7400	68
Ferulic acid	0.5–10.0	0.9996	0.0030	0.0095	1.5500	73
trans-Cinnamic acid	0.5–7.5	0.9994	0.0130	0.0675	4.6200	74
Catechin	0.5–7.5	0.9981	0.0010	0.0025	4.1500	68
Epicatechin	0.5–7.5	0.9992	0.0010	0.0035	3.9200	73
Naringin	0.5–5.0	0.9967	0.0010	0.0025	3.5400	73
Rutin	0.5–10.0	0.9998	0.0030	0.0135	3.8300	90
Hesperitin	0.5–5.0	0.9994	0.0000	0.0010	3.7400	87
Myricetin	0.5–7.5	0.9987	0.0010	0.0050	4.2300	84
Quercetin	0.5–7.5	0.9986	0.0070	0.0260	3.7300	67
Kaempferol	0.5–10.0	0.9996	0.0030	0.0125	3.9400	96
Isorhamnetin	0.5–7.5	0.9985	0.0030	0.0115	1.5500	89
Apigenin	0.5–5.0	0.9977	0.0020	0.0054	2.8400	87
Pinocembrin	0.5–7.5	0.9946	0.0010	0.0053	1.2300	91
Galangin	0.5–10.0	0.9974	0.0050	0.0162	0.8400	95
Chrysin	0.5–7.5	0.9971	0.0050	0.0152	1.1200	88
Pinostrobin	0.5–5.0	0.9984	0.0010	0.0035	4.5500	94

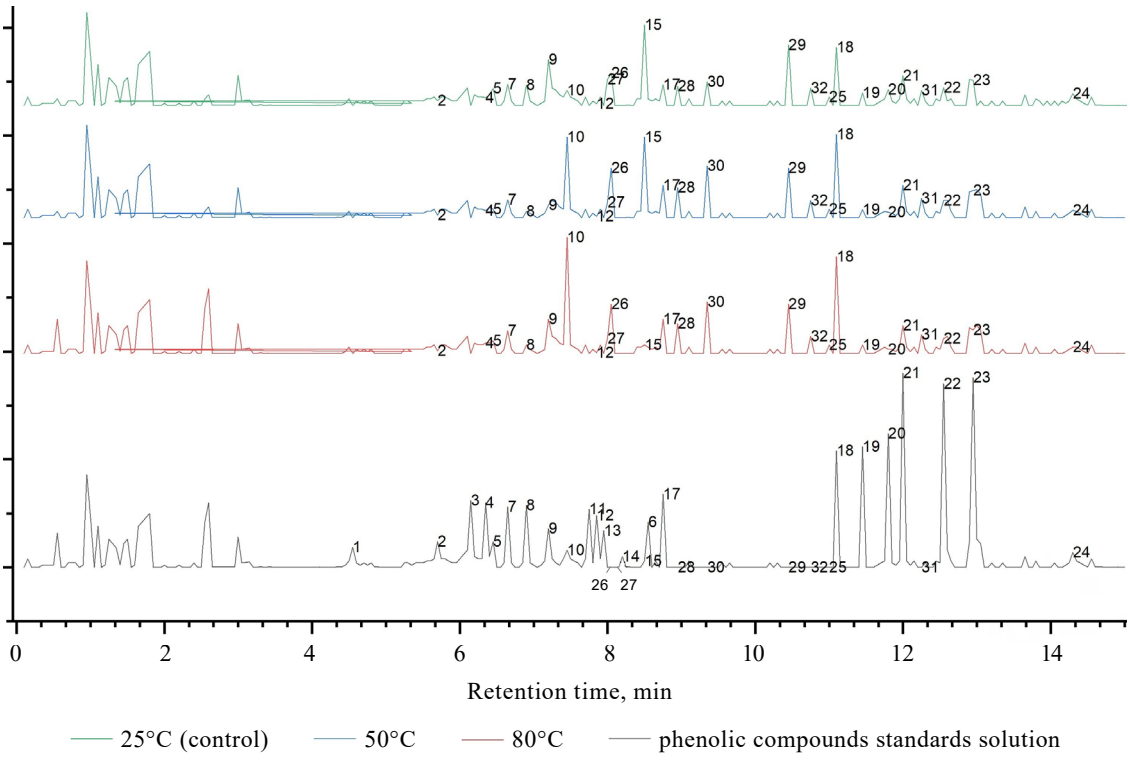
The precision values were consistently < 5%. Despite the satisfactory recovery results for most phenolic compounds, future research is recommended to implement the SPE (solid-phase extraction) procedure with commercial polymer sorbent cartridges to isolate phenolic compounds from the honey matrix, aiming to reduce the use and production of hazardous substances.

Quantification of phenolic acids and flavonoids in *Tetragonula laeviceps* honey using UHPLC-DAD-ESI/MS. We employed the UHPLC-DAD-ESI/MS technique to quantify phenolic acids and flavonoids in the honey samples following the initial isolation of the target compounds from the honey matrix. In total, 31 compounds derived from pollen, propolis, and flower nectar were identified in the honey samples. Of these, 24 compounds were quantified by comparing their retention times and MS spectra with available standards. Figure 2 illustrates a representative base peak chromatogram of a standard solution of phenolic compounds in honey heated at 25 (control), 50, and 80°C.

Out of the 24 target compounds, only 17 were succesfully identified and measured in all examined honey samples. These comprised 7 phenolic acids (chlorogenic, caffeic, 3.4-dihydroxybenzoic, *p*-hydroxybenzoic, *trans*-cinnamic, *p*-coumaric, and syringic) and 9 flavonoids (apigenin, chrysin, galangin, kaempferol, rutin, quercetin, isorhamnetin, pinocembrin, and pinostrobin). Meanwhile, catechin and epicatechin were found in very small amounts in all the samples, while gallic acid was detected in very small amounts in the experimental samples (50 and 80°C). Compounds such as naringin, hesperitin, myricetin, and *trans*-resveratrol were not identified.

In the absence of standards, the identification of additional compounds in honey extract relies on detecting deprotonated molecules, [M-H], and specific literature [18]. Utilizing the ChemSpider reference library to search for precise mass, we identified rhamnestin, abscisic acid, luteolin, pinobanskin, and pinobanskin-3-O-acetate in all the analyzed honeys. Table 3 summarizes the data for each target compound (phenolic acids, flavonoids, stilbens) and other significant compounds identified in the honey extracts.

The specific temperature regimes of 25, 50, and 80°C were chosen to simulate different storage and processing conditions that honey may undergo. These temperatures represent typical ambient storage (25°C), mild heating (50°C), and high heating (80°C), which are common in food processing and storage scenarios. The duration of 6 h allows for a significant period to observe biochemical changes, such as hydroxymethylfurfural (HMF) formation and enzyme degradation. Other temperatures and heating durations have indeed been studied in previous research, often focusing on different time scales or temperature ranges. Budianto *et al.* [10] examined the effect of heating Klanceng honey at high (70°C/7 h), medium (40°C/48 h), and low (25°C/48 h) temperatures.



1 – gallic acid; 2 – 3.4-dihydroxybenzoic acid; 3 – catechin; 4 – 4-hydroxybenzoic acid; 5 – chlorogenic acid; 6 – epicatechin; 7 – caffeic acid; 8 – siric acid; 9 – *p*-coumaricacid; 10 – ferulic acid; 11 – naringin; 12 – rutin; 13 – hesperitin; 14 – *trans*-resveratrol; 15 – *trans*-cinnamic acid; 16 – myristetin; 17 – quercetin; 18 – kaempferol; 19 – isorhamnetin; 20 – apigenin; 21 – pinocembrin; 22 – galangin; 23 – chrysin; 24 – pinostrobin; 25 – rhamnetin; 26 – abscisic acid; 27 – eriodictyol; 28 – sakuranetin; 29 – alpinetin; 30 – pinobanksin; 31 – pinobanskin-3-O-acetate; 32 – luteolin

Figure 2 UHPLC chromatogram: Relative abundance, %, of biochemical components in relation to retention time

Table 3 Quantification of phenolic acids and flavonoids in *Tetragonula laeviceps* honey using UHPLC-DAD-ESI/MS

Peak number	Compounds	RT, min	Exact mass[b] $[M-H]^-$	Accurate mass $[M-H]^-$	ppm	Mass fragments (intensity, %)	Collision energy, eV
			Phenolic acids				
1	Gallic acid[a]	4.55	1.690.131	1.690.131	0.21	124 (100)	40
2	3.4-dihydroxybenzoic acid[a]	5.70	1.530.183	1.530.183	0.12	108 (100)	40
4	*p*-hydroxybenzoic acid[a]	6.35	1.370.234	1.370.234	0.12	91 (100)	35
5	Chlorogenic acid[a]	6.45	3.530.885	3.530.885	0.31	194 (100), 85	20
7	Caffeic acid[a]	6.65	1.790.345	1.790.345	0.13	132 (100)	35
8	Syringic acid[a]	6.95	1.970.443	1.970.443	0.12	184 (100), 167, 131, 123	30
9	*p*-coumaric acid[a]	7.25	1.630.394	1.630.394	0.21	113 (100)	30
10	Ferulic acid[a]	7.45	1.930.502	1.930.502	0.21	175, 149, 136, 134 (100)	30
15	*trans*-cinnamic acid[a]	8.50	1.470.446	1.470.446	0	118 (100), 103, 62	35
			Flavonoids				
3/6	Catechin[a]/epicatechin[a]	6.00/6.55	2.890.723	2.890.723	0.22	204, 151, 125, 124,109 (100)	35
11	Naringin[a]	7.70	5.791.724	5.791.724	0.24	469, 272 (100), 151	35
12	Rutin[a]	7.80	6.091.462	6.091.462	0.22	305 (100)	35
13	Hesperitin[a]	8.00	6.091.823	6.091.823	0.14	304 (100), 272, 153	20
16	Myricetin[a]	8.75	3.170.304	3.170.304	0.31	178, 151 (100), 137	40
17	Quercetin[a]	10.00	3.010.355	3.010.355	0.12	180, 151 (100), 107	40
18	Kaempferol[a]	11.12	2.850.421	2.850.421	0.32	154 (100), 117, 93	50
19	Isorhamnetin[a]	11.50	3.150.518	3.150.518	0.62	304 (100), 256, 152, 125	40
20	Apigenin[a]	11.80	2.690.452	2.690.452	0.51	152, 118 (100), 107	50
21	Pinocembrin[a]	12.06	2.550.667	2.550.667	0.42	214, 152 (100), 107	55
22	Galangin[a]	12.58	2.690.451	2.690.451	0.54	227, 151 (100), 117	65
23	Chrysin[a]	12.93	2.530.512	2.530.512	0.41	209,182, 143 (100), 107	50
24	Pinostrobin[a]	14.34	2.690.824	2.690.824	0.32	179, 137 (100)	50
			Stilbens				
14	*trans*-resveratrol[a]	8.22	2.270.712	2.270.712	0.42	190 (100), 143	35
			Other major compounds				
25	Rhamnetin	11.02	3.150.513	3.150.513	0.23	304 (100), 165, 121	40
26	Abscisic acid	8.07	2.631.283	2.631.283	0.22	178 (100), 191	35
27	Eriodictyol	8.03	2.870.563	2.870.563	0	125 (100)	55
28	Sakuranetin	8.93	2.850.772	2.850.772	0.13	135 (100)	55
29	Alpinetin	10.42	2.690.824	2.690.824	0.22	199 (100), 133	55
30	Pinobanskin	9.32	2.710.616	2.710.616	0.22	254, 243, 165 (100), 152	55
31	Pinobanskin-3-O-acetate	12.30	3.130.723	3.130.723	0.12	2713, 254 (100)	55
32	Luteolin	10.75	2.850.407	2.850.407	0.34	214, 151, 132 (100)	50

RT – retention time; [a] – compounds that were confirmed using available standards; [b] – calculated mass of the parent ion using a free chemical database (ChemSpider)

The results showed that high-temperature heating significantly reduced enzyme activity, while two years of storage increased the HMF values to > 40 ppm. Flanjak *et al.* [19] also found similar results with sage honey under almost identical heating durations and temperatures.

According to Table 4, heating at 80°C had a significant impact ($p < 0.05$) on the phenolic acid composition in the honey samples compared to heating at 50°C. Ferulic, *p*-coumaric, and caffeic acids were the most dominant compounds. We found that under certain conditions, heating can enhance the solubility or extraction of some phenolic compounds from the honey matrix. Similar conditions were observed in jojoba honey from Iran [17], sage honey from Croatia [19], and Klanceng honey from Indonesia [10]. However, some studies showed different results, such as those on Tualang and Kelulut honey in Malaysia [20].

The increase in flavonoid compounds due to heating also showed a similar pattern. The sample heated at 80°C exhibited a significant difference ($p < 0.05$) compared to the other samples. Kaempferol, quercetin, and pinocembrin dominated in the samples stored at 25°C and heated at 50°C. After heating at 80°C for 6 h, kaempferol, pinocembrin, and chrysin dominated. Heating at 50°C for 6 h did not cause significant changes in flavonoid compounds, a finding that aligns with previous studies [21]. However, other studies showed a significant impact with longer heating durations [21, 22]. Heating can increase the solubility of flavonoids from the honey matrix [23], as heat can break down cell walls or other structures that bind flavonoids, making them easier to extract and measure [24].

HMF showed a significant increase ($p < 0.05$) in all three samples, with the highest value in the sample heated

Table 4 The effect of heating on the composition of biochemical compounds in *Tetragonula laeviceps* honey (n = 6 for each temperature)

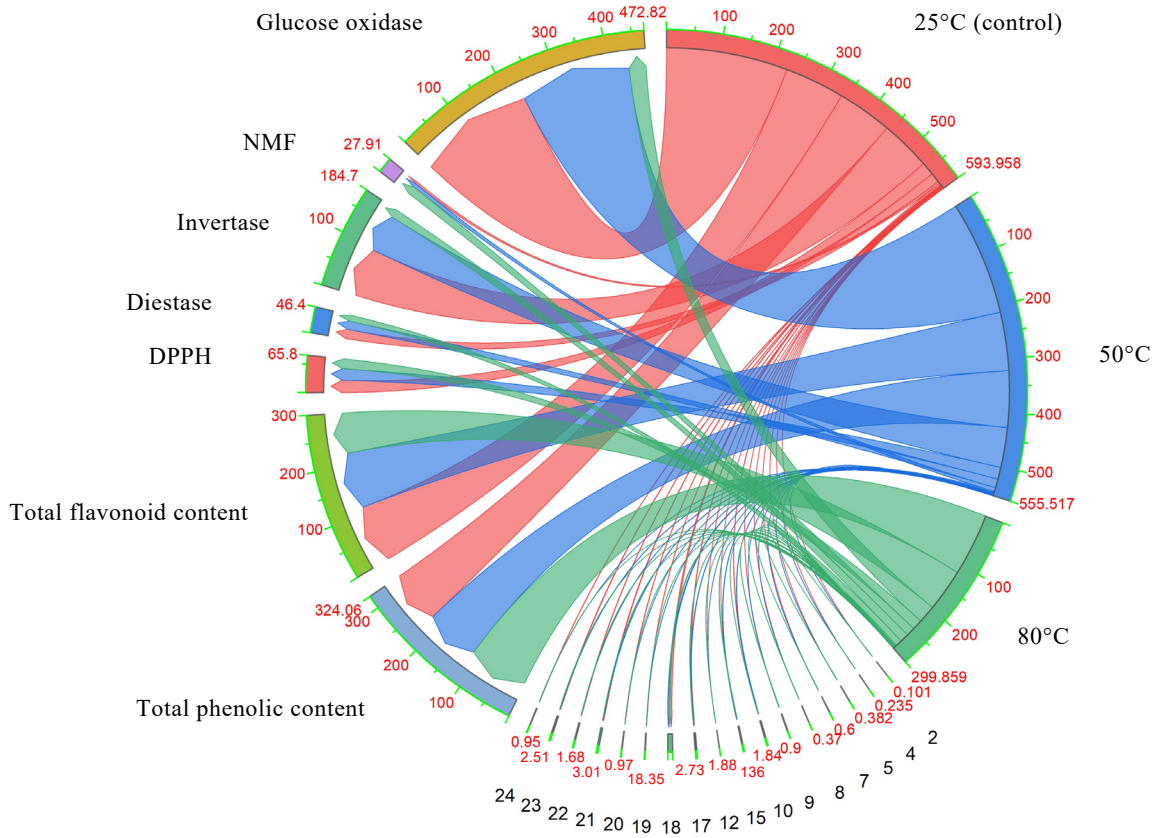
Compounds	25°C (control)	50°C	80°C
3.4-dihydroxybenzoic	0.021 ± 0.008^{b} (0.013–0.031)	0.025 ± 0.005^{b} (0.011–0.032)	0.056 ± 0.007^{a} (0.040–0.064)
p-hydroxybenzoic	0.072 ± 0.011^{b} (0.042–0.081)	0.075 ± 0.013^{b} (0.040–0.121)	0.091 ± 0.007^{a} (0.081–0.097)
Chlorogenic	0.092 ± 0.152^{b} (0.032–0.362)	0.092 ± 0.013^{b} (0.063–0.110)	0.210 ± 0.016^{a} (0.160–0.460)
Caffeic	0.162 ± 0.114^{b} (n.d.–0.252)	0.192 ± 0.022^{b} (0.162–0.266)	0.253 ± 0.015^{a} (0.210–0.412)
Syringic	0.062 ± 0.031^{b} (0.024–0.122)	0.073 ± 0.015^{b} (0.032–0.098)	0.243 ± 0.052^{a} (0.155–0.525)
p-coumaric	0.242 ± 0.110^{b} (0.102–0.514)	0.254 ± 0.052^{b} (0.105–0.367)	0.542 ± 0.157^{a} (0.218–0.675)
Ferulic	0.814 ± 0.332^{b} (0.322–1.327)	0.853 ± 0.072^{b} (0.322–1.327)	1.872 ± 0.332^{a} (1.322–2.127)
trans-cinnamic acid	0.055 ± 0.001^{b} (0.021–0.125)	0.059 ± 0.003^{b} (0.021–0.125)	0.982 ± 0.150^{a} (0.721–1.125)
∑ phenolic acids	1.520	1.623	2.536
Rutin	0.023 ± 0.010^{c} (0.011–0.042)	0.035 ± 0.006^{b} (0.021–0.075)	0.078 ± 0.005^{a} (0.051–0.095)
Quercetin	0.835 ± 0.245^{b} (0.543–1.322)	0.943 ± 0.254^{a} (0.552–1.115)	0.983 ± 0.154^{a} (0.452–1.125)
Kaempferol	2.352 ± 0.543^{b} (1.671–3.177)	2.321 ± 0.543^{b} (1.871–3.172)	3.742 ± 0.541^{a} (3.471–4.177)
Isorhamnetin	0.192 ± 0.062^{c} (0.122–0.313)	0.238 ± 0.062^{b} (0.222–0.289)	0.584 ± 0.032^{a} (0.122–0.813)
Apigenin	0.075 ± 0.022^{c} (n.d.–0.098)	0.157 ± 0.021^{b} (n.d.–0.198)	0.755 ± 0.222^{a} (n.d.-1.098)
Pinocembrin	0.731 ± 0.246^{b} (0.213–0.938)	0.945 ± 0.346^{b} (0.513–1.338)	1.405 ± 0.446^{a} (0.813–1.638)
Galangin	0.376 ± 0.125^{b} (0.115–0.534)	0.483 ± 0.146^{b} (0.213–0.738)	0.838 ± 0.246^{a} (0.513–1.338)
Chrysin	0.575 ± 0.182^{b} (0.218–0.793)	0.767 ± 0.246^{b} (0.213–0.939)	1.276 ± 0.346^{a} (0.813–1.538)
Pinostrobin	0.153 ± 0.014^{c} (n.d.–0.322)	0.234 ± 0.046^{b} (0.093–0.438)	0.665 ± 0.246^{a} (0.213–0.978)
∑ flavonoid acids	5.250	5.955	10.118
Total phenolic content, g GAE/kg	102.501 ± 0.105^{b} (97.343–105.474)	105.231 ± 0.101^{b} (97.342–113.474)	116.331 ± 0.452^{a} (101.342-123.474)
Total flavonoid content, mg QE/kg	79.092 ± 13.972^{c} (72.993–99.392)	109.201 ± 23.082^{b} (72.092–149.042)	112.271 ± 50.204^{a} (56.672–191.291)
DPPH, %	24.303 ± 2.302^{a} (18.232–36.447)	22.403 ± 4.401^{b} (15.791–26.876)	19.105± 3.207^{c} (17.982–24.764)
Diastase (DN)	18.301 ± 2.300^{a} (16.715–21.046)	16.304 ±3.303^{b} (14.923–20.097)	11.803 ±1.205^{c} (9.840–13.679)
Invertase, U/kg	94.304 ± 4.305^{a} (88.782–102.237)	74.208 ± 11.200^{a} (65.892–96.372)	16.203 ± 8.306^{b} (13.766–22.785)
HMF, mg/kg	4.321 ± 0.630^{c} (2.034–6.235)	7.305 ± 0.630^{b} (5.328–9.329)	16.302 ± 4.107^{a} (14.983–22.652)
Glucose oxidase, µg H_2O_2/g honey	226.634 ± 21.202^{a} (209.752–231.743)	212.861 ± 21.832^{a} (84.289–219.543)	33.339 ± 2.571^{b} (29.361–39.639)

The ANOVA test was followed by the Tukey HSD post hoc test with a significance level of $p < 0.05$. The same letters indicate no significant difference; DPPH – 1.1-diphenyl-2-picrylhydrazyl radical scavenging activity; HMF – 5-hydroxymethyl furan-2-carbaldehyde

at 80°C. The increase in total phenolic and total flavonoid contents did not show a significant difference at 50°C but was significantly different at 80°C. The significant increase in HMF values at 50 and 80°C indicates that HMF is highly sensitive to heating [19]. Therefore, it is an important indicator for assessing the quality of *T. laeviceps* honey and ensuring it has not been excessively heated [10]. The increase in HMF also shows the presence of cyclic aldehyde compounds obtained from the degradation of sugar compounds (Maillard reaction) [25]. HMF is formed during the acid-catalyzed dehydration of hexoses [5, 6]. Previous research recommends these parameters for heating and storage processes [26, 27].

Heating also caused a significant decrease in DPPH and diastase values in all three samples, although significant change in invertase and glucose oxidase values only occurred at 80°C. Antioxidants (DPPH) inhibit free radicals by donating electrons to oxidant compounds. These compounds participate in slowing down the damage caused by oxidation at a certain level. The decline in DPPH due to warming has been reported by many previous researchers [28–30]. Given the important role of these compounds, the researchers made DPPH a parameter to be tested. Glucose oxidase, which catalyzes the oxidation of glucose to H_2O_2 and gluconic acid, was also less sensitive to heating. Our study demonstrated that invertase is less sensitive to heating than diastase. Diastase comes from bee saliva, so the enzyme is identical to the purity of honey. The decrease in diastase activity disrupts maltose digestion and conversion of other sugars in honey. The enzyme is very sensitive to heat [5]. Storage for 6 months at 30°C reduces the concentration of diastase by half, so the researchers chose this parameter for the comparative test [31]. Invertase is an enzyme produced by the hypopharyngeal gland of bees that catalyzes the hydrolysis of sucrose into glucose and fructose. This enzyme is suspected to be sensitive to heat [10, 19]. Researchers are still debating if invertase and diastase remain effective during heating.

We used a chord diagram visualization from Origin Pro to provide a deeper understanding of the effect that heating had on the biochemical composition of *T. laeviceps* honey (Fig. 3). The diagram not only shows changes in the relative abundance of various biochemical components but also illustrates the relationships between these components that might be overlooked in tabular data representation. This visualization helps identify significant patterns and trends resulting from



2 – 3.4-dihydroxybenzoic acid; 4 – 4-hydroxybenzoic acid; 5 – chlorogenic acid; 7 – caffeic acid; 8 – siric acid; 9 – *p*-coumaricacid; 10 – ferulic acid; 12 – rutin; 15 – *trans*-cinnamic acid; 17 – quercetin; 18 – kaempferol; 19 – isorhamnetin; 20 – apigenin; 21 – pinocembrin; 22 – galangin; 23 – chrysin; 24 – pinostrobin; DPPH – 1.1-diphenyl-2-picrylhydrazyl radical scavenging activity; HMF – 5-hydroxymethyl furan-2-carbaldehyde

Figure 3 The effect of heating on biochemical compounds in *Tetragonula laeviceps* honey

heating and facilitates the interpretation of the complex effect of heating on honey.

Based on the chord diagram data, significant differences in the values of invertase and glucose oxidase were observed among the all the samples under study. Of the total 600 bands measured, the invertase reached only 20 bands in the sample heated at 80°C, 100 bands in the control sample, and 64 bands in the honey heated at 50°C. Glucose oxidase exhibited similar patterns, with only 50/600, 230/600, and 200/600 bands in the 80°C sample, control honey, and 50°C sample, respectively. These findings indicate that heating at 80°C can reduce the presence of invertase and glucose oxidase in *T. laeviceps* honey.

The Spearman correlation analysis (Table 5) showed that phenolic acids had a strong positive correlation (> 0.9) with flavonoid acids, total phenolic, total flavonoid, and HMF content. Conversely, phenolic acids showed a strong negative correlation with diastase, invertase, DPPH, and glucose oxidase. All these relationships were significant at a confidence level of $p < 0.01$. They indicated that the changes in phenolic acids consistently correlated directly with the changes in flavonoid acids, total phenolics, total flavonoids, and HMF, while correlating inversely with the changes in diastase, DPPH, invertase, and glucose oxidase. These results are consistent with previous findings [32].

Table 6 shows changes in biochemical concentrations caused by heating for 6 h. As observed, these changes affected the reaction rate constant (k) of the biochemical degradation across different reaction orders. The reaction order indicates the magnitude of the effect of reactant concentration on the rate of the reaction. The order of the reaction was determined based on the experimental data (i.e., observed changes in concentration over time), rather than being directly related to the stoichiometric coefficients of the reactants.

A zero-order reaction indicates that the reaction rate is affected by the reaction rate constant, rather than the reactant concentration. The formation rate constants for HMF showed notable variations, with the k values of 0.0189/h, 0.2618/h, and 0.6296/h at 25, 50, and 80°C, respectively.

A first-order reaction shows that the reaction rate is affected by the reactant concentration. This condition can be observed in the relationship between ln(CA) and time (t), where CA is the concentration of the reactant at time t. The increase in HMF concentration was clearly visible at 80°C (0.0053/h), 25°C (0.1098/h), and 50°C (0.0597/h).

A second-order reaction shows the relationship between the reaction rate and the square of the reactant concentration. This means that the rate is proportional

Table 5 Correlation between biochemical compounds in Klanceng honey under heat treatment

Compounds	∑ phenolic acids	∑ flavonoid acids	Total flavonoid content, mg QE/kg	Total phenolic content, mg GAE/kg	DPPH, %	Diastase (DN)	Invertase, U/kg	HMF, mg/kg	Glucose oxidase
∑ phenolic acids	1	–	–	–	–	–	–	–	–
∑ flavonoid acids	0.970**	1	–	–	–	–	–	–	–
Total flavonoid content, mg QE/kg	0.944**	0.970**	1	–	–	–	–	–	–
Total phenolic content, mg GAE/kg	0.931**	0.931**	0.960**	1	–	–	–	–	–
DPPH, %	–0.945**	–0.932**	–0.961**	–0.974**	1	–	–	–	–
Diastase (DN)	–0.944**	–0.970**	–0.974**	–0.947**	0.935**	1	–	–	–
Invertase, U/kg	–0.970**	–0.957**	–0.947**	–0.973**	0.961**	0.947**	1	–	–
HMF, mg/kg	0.892**	0.905**	0.961**	0.987**	–0.948**	–0.947**	–0.933**	1	–
Glucose oxidase	–0.906**	–0.932**	–0.987**	–0.961**	0.962**	0.935**	0.935**	–0.961**	1

** Correlation is significant at the 0.01 level (2-tailed); HMF – 5-hydroxymethyl furan-2-carbaldehyde

Table 6 Heating effects and the kinetics of hydroxymethylfurfural (HMF) formation in Klanceng honey

Temperature, °C	Time, h	HMF, mg/kg	Temperature, °C	Time, h	HMF, mg/kg	Temperature, °C	Time, h	HMF, mg/kg
25	0	3.49 ± 0.02	50	0	3.49 ± 0.02	80	0	3.49 ± 0.02
	1	3.60 ± 0.30		1	6.20 ± 0.20		1	15.00 ± 0.07
	2	3.60 ± 0.31		2	6.80 ± 0.20		2	15.60 ± 0.30
	3	3.60 ± 0.31		3	8.80 ± 0.20		3	18.00 ± 0.40
	4	3.61 ± 0.24		4	7.70 ± 0.30		4	16.70 ± 0.20
	5	3.61 ± 0.21		5	8.20 ± 0.20		5	17.30 ± 0.20
	6	3.61 ± 0.27		6	8.80 ± 0.20		6	18.00 ± 0.40

Parameter	Temperature, °C	Zero Order		First Order		Second Order		*Ea*
		k/h	R^2	*k*/h	R^2	*k*/h	R^2	KJ/mol
HMF, mg/kg	25	0.0189	0.6714	0.1098	0.9176	0.0015	0.6727	69.2332
	50	0.2618	0.9324	0.0597	0.0138	0.9939	0.8578	
	80	0.6296	0.7924	0.0053	0.0203	0.9972	0.6028	

to $[CA]^2$. Table 6 shows the linear relationship between $1/CA$ and time (t), which is characteristic of a second-order reaction. In this context, CA represents the concentration of the reactant at time t. The reaction rate constants (k) for HMF formation at 25, 50, and 80°C were found to be 0.0015/h, 0.0138/h, and 0.0203/h, respectively, indicating that the reaction proceeds faster at higher temperatures.

The activation energy (Ea) for each parameter must be determined by comparing the most considerable R^2 values in determining the reaction order. If the order reaction has been determined, then the value of k used was based on the reaction. Based on the data above, the formation of HMF follows a first-order reaction, as indicated by the highest R^2 value compared to other reaction orders.

The addition of time (t) did not significantly impact the increase in HMF compared to changes in temperature (T), which had a greater influence on HMF formation. This strengthens the findings of previous researchers [33], although some studies proved that HMF was sensitive to duration and temperature. In our study, the formation of HMF followed the first-order reaction, which is in line with previous findings [34]. Some researchers report zero-order reactions. For example, in the same sample of Kelulut honey, heating at 40 and 50°C occurred at zero order and then rose to the first order at 70°C [35]. Boussaidet *et al.* [34], who studied honey in Southern Tunisia, found that the Ea ranged from 31 to 50 KJ/mol. However, significantly higher *Ea* values of 83.07, 91.70, and 89.57 KJ/mol were recorded in multi-floral honey from Kurdistan [36]. Also, Yap and Chin [35] creported an Ea value of 125 KJ/mol in honey from stingless bees, a species similar to *T. laeviceps*.

The heating of honey during production can reduce its quality, including its antimicrobial properties and enzyme activity, while increasing HMF levels. However, heating is still necessary to meet retail standards. The use of recrystallization methods can help prevent quality degradation during the process [37].

CONCLUSION

The Klanceng bee (*Tetragonula laeviceps*), with a body length of about 7–8 mm and a wing length reaching 8–10 mm, exhibits a diverse plant visitation pattern.

Using melissopalynology, we found that this bee collects pollen from various sources, including *Zea mays* spp. *Mays* (L.) and *Vigna unguiculata sesquipedalis* (L.) Although *Cocos nucifera* (L.) was not detected within the 500 m observation radius, a pollen frequency of 1.8% suggests its potential presence beyond this range. The Klanceng bee, belonging to the Tetragonula species, plays an important role in collecting pollen and nectar from various plants.

Heating *T. laeviceps* honey at 80°C significantly ($p < 0.05$) increased the contents of phenolic acids, flavonoid acids, total phenolic content, total flavonoid content, and hydroxymethylfurfural (HMF), as well as decreased the enzymes diastase, invertase, glucose oxidase, and DPPH. However, at 50°C, there was no significant impact, except for HMF and diastase. Ferulic and kaempferol compounds dominated the phenolic and flavonoid acids in all the treatments.

The kinetics of HMF formation in Klanceng honey followed a first-order reaction, meaning that the reaction rate was affected by the concentration of the reactants. The reaction rate constant (k) for HMF formation was 0.1098/h at 25°C, 0.0597/h at 50°C, and 0.0053/h at 80°C, with an activation energy of 69.23 KJ/mol. This activation energy indicated that a significant amount of energy was required for the reaction.

In this study, we primarily focused on the biochemical aspects and HMF kinetics of Klanceng honey, excluding microbial testing, which is also crucial for assessing honey quality. To provide a comprehensive evaluation and address honey quality in its entirety, we recommend that future research incorporate microbiological analysis as well.

CONTRIBUTION

All the authors were equally involved in the research analysis and manuscript writing.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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